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Full Length Research Paper

Effects of asphalt on the enzymatic activity and bacterial community in soil

Xin Yu[1]*, Yu-hong Wang[2] and Di Wu[1]

[1]College of Civil Engineering and Transportation, Hohai University, Xikang Road 1#, Nanjing, Jiangsu P.R. 210098, China.
[2]Department of Civil and Structural Engineering, the Hong Kong Polytechnic University, Hung Hom, Hong Kong, P.R. China.

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Asphalt is an important material widely used in the coating of surfaces such as roads, roofs, linings of water basins and pipes, but it can be potentially hazardous to human and environment because certain substances exist in asphalt, including polycyclic aromatic hydrocarbons. However, it is unknown about the effect of asphalt on soil. The aim of this study is to examine the effects of asphalt on the physiochemical parameters, the enzymatic activity, and the copy number of 16s rRNA bacteria and archaea in soil. Freshly collected garden oils were amended with asphalt of various amounts after 1, 15 and 26 days, respectively. Asphalt application increased the total organic carbon, decreased the total phosphorus and pH values in soil while total nitrogen was not altered, suggesting asphalt can alter the nutrient composition. The copy number of 16s rRNA of bacteria increased in soil with addition of asphalt but that of archaea decreased. The activity of catalase, dehydrogenase, urease, sucrose, cellulase and polyphenol oxidase increased in soil modified with asphalt. Principal components analysis of these parameters resulted in three major components. Urease, sucrose, cellulose, bacteria, dehydrogenase, dehydrogenase, total organic carbon and total nitrogen weighed heavily in component 1, suggesting that the increase of organic carbon affect the enzymatic activity significantly. Although the asphalt may contain much toxic toxicity, asphalt can potentially improve the fertilization of the soil.

Key words: Soil, asphalt, 16s rRNA, principal components analysis, bacteria.

INTRODUCTION

Asphalt (or bitumen) is a highly viscous liquid or semi-solid material, which is mainly produced from crude oil refinery process and is also present in some natural deposits. Asphalt composition can be divided into four generic groups: saturates, aromatics, resins, and asphaltenes (Rasoulzadeh et al., 2011). Asphalt has been widely used in the coating of surfaces such as roads, roofs, linings of water basins and pipes (Brandt et al., 2001). However, certain substances including polycyclic aromatic hydrocarbons (PAHs) in the asphalt were found to be potentially harmful for human and environment. These substances can be released into environment by leaching; in several countries, limits have been set for their releases, for example in surface water and/or drinking water (Brandt et al., 2001). Consequently, measures have been taken to obtain modified bitumen and to decrease polycyclic aromatic hydrocarbons (PAH) emissions and simultaneously improve bitumen performance grade (Rasoulzadeh et al., 2011). However, asphalt with PAHs can be retained in soil. For example, Sadler et al. (1999) found that PAH concentration in soil, below the asphalt cover of an electric trolly bus depot, was in the order of 1–10 mg/kg. Brantley and Townsend (1999) reported that the concentration of nine of the 16 (Environmental Protection Agent PAHs in the leaching from reclaimed asphalt pavement were above the detection limit that ranged from 0.25 to 5 mg/L.

*Corresponding author. E-mail: yuxin2009@hhu.edu.cn. Tel: +86-25-83786353.

Therefore, effects of bitumen and its toxic components in the soil should be studied further.

There is a huge diversity of organisms belonging to different taxonomic and physiologic groups that interact at different levels within the community in soil biota (Lopes et al., 2011). In this biota, soil microorganisms constitute a source and are the driving force behind many soil processes, including the transformation of organic matter, nutrient release, the transformation of C, N, P and S, the degradation of xenobiotic organic compounds, the formation of soil physical structure and the enhanced nutrient uptake of plant (Chen et al., 2010; Lopes et al., 2011). For these reasons, the importance of microorganisms is unquestionable in the maintenance of quality and productivity of agricultural soils. Biologically and biochemically mediated processes in soils are of the utmost importance to ecosystem functions (Tejada et al., 2011). The responsiveness of microorganisms to environmental factors suggests that disturbances imposed by agricultural treatments may lead to alterations in the composition and activity of soil microbiota and, therefore, may affect soil quality (Shibahara and Inubushi, 1997; Lopes et al., 2011). Many biological parameters, including soil enzymes, have been used to assess soil quality and health as affected by agricultural practices (Gianfreda et al., 2005; Truu et al., 2008; García-Ruiz et al., 2009) and been used as potential soil quality indicators because they are sensitive to ecological stress and land management practices (Tejada, 2009). It has been shown that enzymes may react to changes in soil management more quickly than other variables and therefore may be useful as early indicators of biological changes (Zabaloy et al., 2008).

Petroleum bitumen can be degraded in an abiotic way under natural conditions with the help of various external factors such as atmospheric oxygen, oxygen dissolved in rainwaters/percolating waters and in the action of aerobic and anaerobic micro-organisms (Charrié-Duhaut et al., 2000). In fact, bacteria commonly inhabit subsurface oil reservoirs and even in naturally occurring terrestrial oil seeps and natural asphalts that are comprised of highly recalcitrant petroleum hydrocarbons (Kim and Crowley, 2007). By analyzing the 16S rRNA gene sequences and DNA encoding aromatic ring hydroxylating dioxygenases from two tar pits differing in chemical composition, Kim and Crowley, (2007) revealed a wide range of phylogenetic groups within the *Archaea* and *Bacteria* domains, in which individual taxonomic clusters were composed of sets of closely related species within novel genera and families. Rhizosphere microflora of plants has been used for the phytoremediation of bitumen-contaminated soil (Muratova et al., 2003). However, to our best known, little work has been done on the effects of asphalt on enzymatic activity and bacterial community in soil. The aim of the research as presented in this paper was to study the effects of asphalt on the physio-chemical properties of soil, to investigate alterations in the number of bacteria and archeae, and to assess the changes in enzymatic activities in soil amended with asphalt.

MATERIALS AND METHODS

Soil sampling and processing

Surface soil samples (0–20 cm) were collected from a garden in the campus of Hohai University, Nanjing, China. All stones, visible roots and fauna were removed from the soil samples. Asphalt was dissolved in bitumen emulsifier and added to 1000 g of soil samples. Each sample contains about 33, 66, 99 and 122 g of asphalt was named as N1, N2, N3 and N4, respectively. Subsamples were employed to determine soil physiochemical properties using standard methods recommended by the Chinese Society of Soil Science (Lu, 1999) and the remaining samples were used for soil incubation experiment.

Total organic carbon (TOC) was determined by using the liquiTOC II (Elementar). pH of soil was measured by detecting the homogenate of samples and ddH_2O at the ratio of 1:10 (w/v) using a digital pH meter. Kjeldahl nitrogen (TN, total nitrogen) in the samples of soil was determined using the Kjeldhal method. Total phosphorus (TP) and organic matter (OM) were measured according to Jin and Tu (1990).

Analysis of soil enzyme activities

Urease activity was measured as described by McGarity and Myers (1967). Five grams of soil were placed in a 50 mL Erlenmeyer flask, with 1 mL toluene being added subsequently. The contents were allowed to stand for approximately 15 min until the toluene had completely penetrated the soil. After adding a 20 mL potassium citrate-citric acid buffer (pH 6.7) and a 10 mL of 10% urea solution to the mixtures, the flasks were stoppered, shaken and then incubated at 37°C for 24 h. Urea solution (dissolved in distilled water) were used as control and the control experiment was conducted simultaneously. The ammonia released by hydrolysis of urea was determined on the filtrate by the colorimetric indophenol blue method. The unit of urease activity was reported as mg NH_4^+-N released/(kg dry soil·24 h).

Pyrogallic acid is the substrate of polyphenol oxidase (PPO) and can be canalized into purpurigallin by PPO. The mixture containing 1 g soil and 10 mL of 1% pyrogallic acid was incubated at 30°C. After adding 4 mL disodium hydrogen phosphate-citric acid buffer (pH 4.5) to the mixture for 2 h incubation, the products purpurigallin was extracted by ether. It was then measured by a spectrophotometer at 430 nm. Polyphenol oxidase activity was expressed as mg purpurigauin/(g dry soil·2 h) (Zhan et al., 2010).

Catalase activity was determined by $KMnO_4$ titration method with H_2O_2 as substrate. The mixture containing 2 g of soil, 40 mL distilled water and 5 mL of 0.3% H_2O_2 were well shaken for 20 min at 25°C. After filtration, 5 mL of 1.5 mol/L H_2SO_4 was added to 10 mL extract and the remaining H_2O_2 was measured by 0.1 mol/L $KMnO_4$. Catalase activity was expressed in mL 0.1 mol/L $KMnO_4$ g^{-1} dry soil·20 min^{-1}.

Dehydrogenase activity was determined by colorimetric method (Ohlinger, 1996). Five grams of soil samples were added in 5 ml of substrate (2, 3, 5-triphenyltetrazolium chloride). Concentration of substrate varied with the type of soil and a 0.1–2% TTC (2, 3, 5-triphenyltetrazolium chloride) solution (w/v) was prepared in tris-buffer. Five milliliter tris-buffer with substrate was added in the control experiment, and then incubated at 30°C in darkness because trizolium dyes are light sensitive. After incubation for 24 h, triphenyl formazan was formed and was extracted with 25 ml acetone and measured spectrophotometrically at 546 nm. Concentration of 1 g Triphenyl formazan (TPF) in the filtrate was determined from calibration standards (mmol INTF g^{-1} h^{-1}).

Sucrase activity was measured by Hoffmann–Seegerer method (Zhou 1988). Ten grams of soil was combined with 10 ml 20% sucrose solution in pH 5.5 buffer and shaken vigorously in a 100 ml flask at 37°C for 24 h. After the addition of 0.2 mol L^{-1} Na_2SO_4, the content of sucrose was measured by using starch indicator. Reagent blanks were produced for each sample by adding toluene to soil subsamples prior to reagent additions to inhibit sucrase activity. The difference in content of sucrose between reagent blanks and samples was taken as the sucrase activity (ml (10 g soil)$^{-1}$ (24 h)$^{-1}$). Soil cellulase activity was determined by hydrolysis of carboxymethylcellulose (CMC) according to the method described by Pancholy and Rice (1973). All determinations of enzymatic activity were performed in triplicates, and all values reported are averages of the three trials performed on cold-dried soil (-65°C).

DNA extraction and real-time quantitative PCR (qPCR) reactions

DNA was extracted from sediments by using the UltraClean Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. In addition, the primer sets Arc787f (5'-ATTAGATACCCSBGTAGTCC-3')/Arc1059r (5'-GCCATGCACCWCCTCT-3') and BAC338F (5'-ACT CCT ACG GGA GGC AGC AG-3'.)/BAC518R (5'-ATT ACC GCG GCT GCT GG-3') were used to quantify copy number of 16S RNA of the Archaea and Bacteria, respectively. A 50 µL of reaction solutions were prepared with 25 µL real-time PCR iQ SYBR Green SMX reagents (Biorad), 1 µL of each primer (final concentration, 500 nM), 2 µL template DNA and 22 µL PCR-grade water. Real-time PCR was performed by using a MiniOpticon Real-Time PCR instrument with CFX manager software (Biorad). The three-step amplification protocol was as follows: initial denaturation for 5 min at 95.0°C followed by denaturation for 45 cycles of 10 s at 94°C, annealing at 55.0°C for 30 s (Plate Read) and extension at 72.0°C for 30 s. To evaluate the specific of the primers, the melt curve of the products was made immediately from 50 to 95°C at the increase of 0.5°C for 0:05 (plate read) after the end of cycle.

Statistical analysis of the data

All the data were analyzed by ANOVA and the significance of the differences among samples were identified using Tukey's post hoc tests and were assumed when $p<0.05$. Principal component analysis (PCA) methods in SPSS 13.0 were used to analyze the physicochemical parameters based on the correlation analysis among parameters.

RESULTS

Effects of asphalt application on the physiochemical prosperities of soil

Compared with the controls, TOC content increased significantly in soil amended with asphalts. The TOC content is increased with the increase of asphalt. A little alteration was detected in TOC content at 1day as compared with that at 15 and 26 days (Figure 1A).

However, no significant alteration in TN content was detected among treatments (Figure 1B). As shown in Figure 1C, total phosphorus decreased significantly in N4 as compared with control after application of asphalt for 1, 15 and 26 days. Alteration in pH values (Figure 1D) showed a trend similar to the total phosphorus. These might be because that there was lower pH values and total phosphorus in asphalt.

Real-time PCR detection of the bacteria and archaea in soil with asphalt application

To study the effects of adding asphalt on bacteria and archaea, real-time PCR was performed to examine the copy number of 16S rRNA in the treatments with addition of asphalt. As shown in Figure 2A, the copy number of bacterial 16S rRNA increased significantly in statistic level ($p<0.05$) in treatments N1-N4 at 15 days and N1-N3 at 26 days. However, the 16s rRNA copy number of archaea decreased after addition of asphalt in treatments N2, N3 and N4 at 1, 13 and 26 days (Figure 2B).The results indicated that the addition of asphalt promotes the growth of bacteria but inhibited that of archaea.

Effects of asphalt application on the enzymatic activities in soil

As shown in Figure 3A, catalase activity increased significantly in soil amended with asphalt compared with Con at 1, 15 and 26 days. In N1, N2, N3 and N4, 1.88, 1.88, 3.56 and 3.71 times of Con were detected, respectively, at 15 days. Dehydrogenase activity increased with the increase of asphalt at 15 days while reached a highest value in N3 at 26 days (Figure 3B).

Urease activity did not change significantly among treatments at 1 day (Figure 4A). Compared with the Con, the increase of urease activity ranged from 25 to 85% and from 25 to 128%, respectively at 15 and 26 days. Compared with the Con, a significant increase of sucrose activity were detected in treatment N4 at 1 day as well as in treatments N1, N2, N3 and N4 at 15 and 26 days (Figure 4B). Figure 4C shows that cellulase activity increased in treatment N1, N2, N3 and N4 at 15 and 26 days. By 26 days, it increased by 60, 140, 160 and 180% of control values, respectively, at N1, N2, N3 and N4. Polyphenol oxidase is a tetramer which contains four atoms of copper per molecule, and binding sites for two aromatic compounds and oxygen. The increase of polyphenol oxidase was detected in treatments N2, N3 and N4 significantly at 15 days, while no significant alterations among treatments were observed at 1 and 26 days (Figure 4D).

Relationship among urease, catalase and polyphenol oxidase

Three components were extracted and the total variances of component 1, 2 and 3 were 63, 15 and 11%, respectively. As shown in Table 1 and, urease, sucrose,

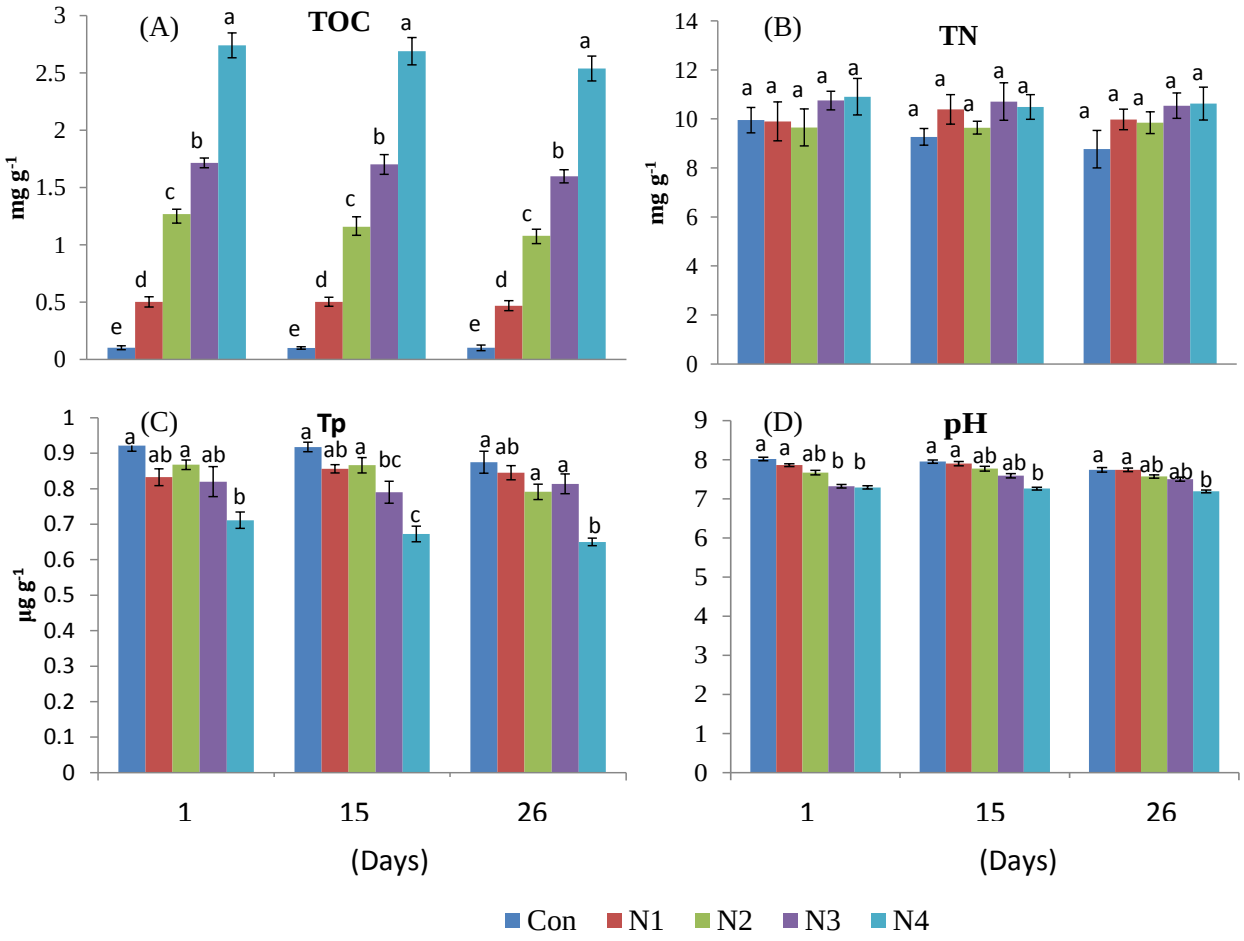

Figure 1. Alterations of total organic carbon (TOC; A), total nitrogen (TN; B), total phosphorus (TP; C) and pH (D) in soil contemned with asphalts. (Different upper-case letters indicated the difference in statistics ($p<0.05$).

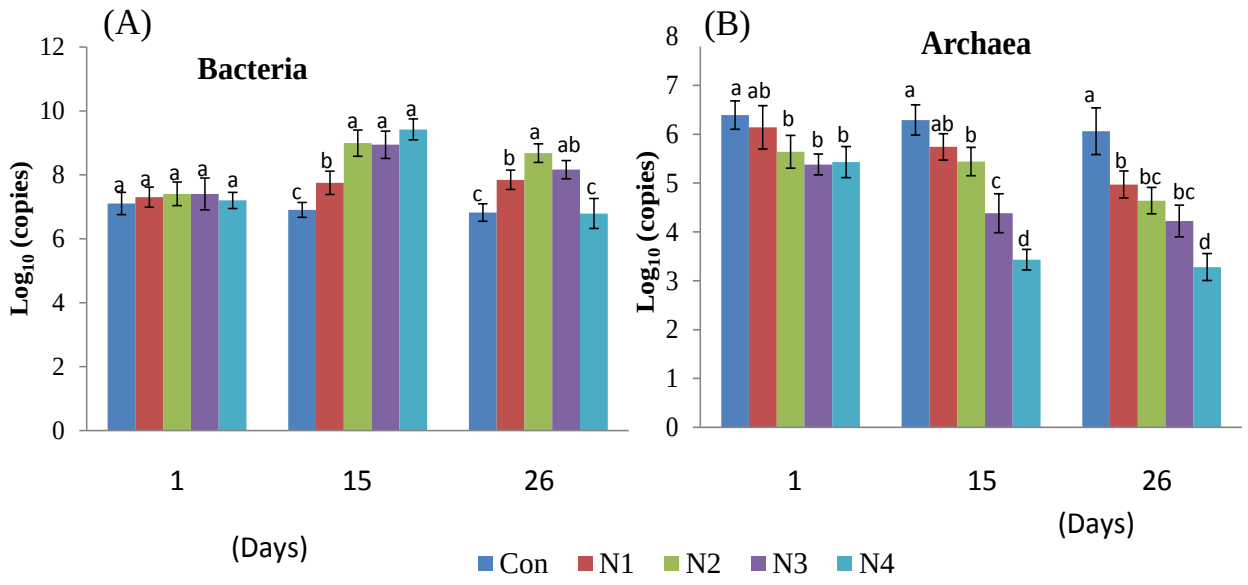

Figure 2. Alterations in 16S rRNA copy numbers of bacteria (A) and archaea (B) in soil amended with asphalt for 1, 15 and 26 days.

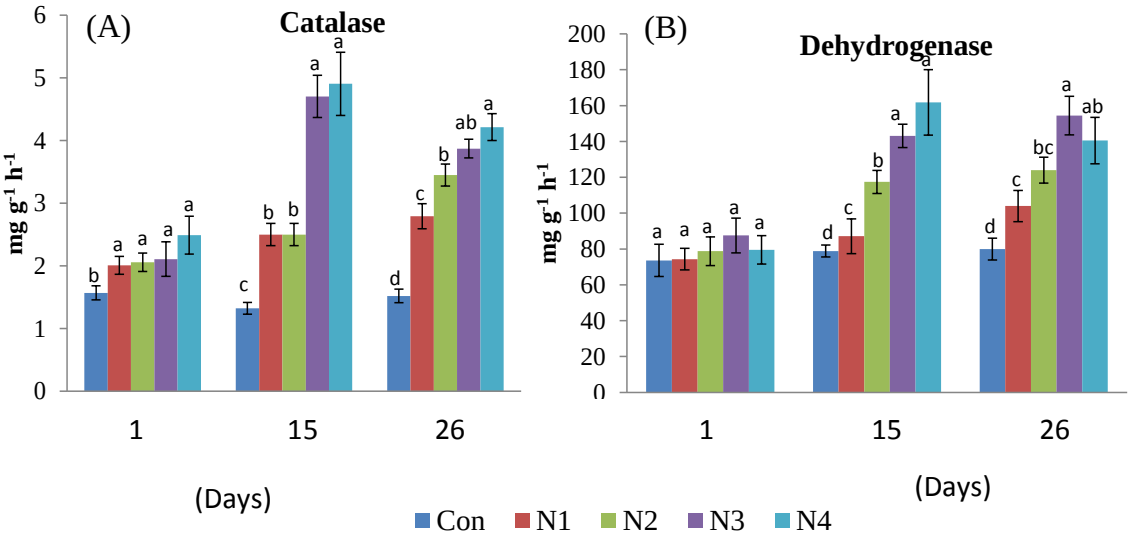

Figure 3. Alterations in activities of catalase (A) and dehydrogenase (B) in soil amended with asphalt for 1, 15 and 26 days.

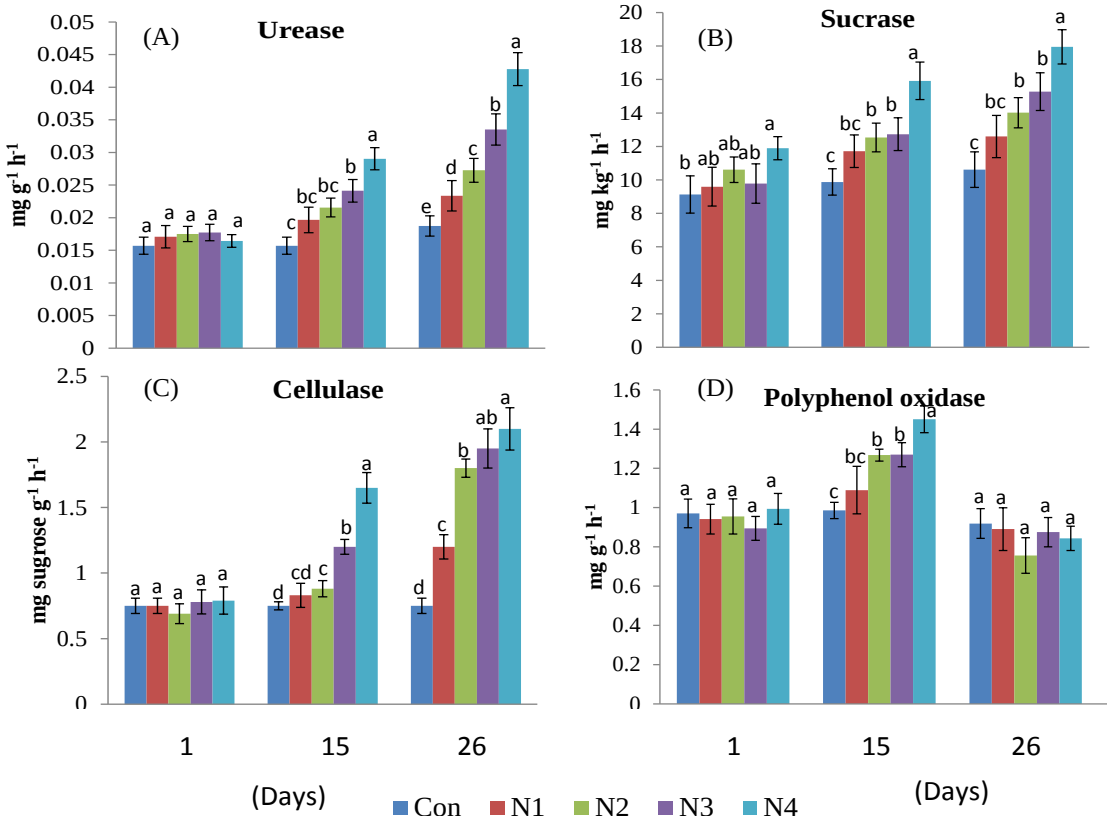

Figure 4. Alterations in activities of rease (A), sucrose (B), cellulose (C) and polyphenol oxidase (D) in soil amended with asphalt for 1, 15 and 26 days.

cellulose, bacteria, dehydrogenase, dehydrogenase TOC and TN weighed heavily in component 1, while bacteria and PPO in component 2.

DISCUSSION

It is well known that asphalt is an oil-based non-

Table 1. Principal component matrix of these parameters.

Parameter	Component		
	1	2	3
Urease	0.869	-0.180	0.390
Sucrase	0.923	0.000	0.243
Cellulase	0.871	-0.155	0.431
Catalase	0.947	0.197	0.057
Bacteria	0.546	0.743	0.067
archae	-0.906	0.220	-0.058
Dehydrogenase	0.913	0.262	0.257
PPO	0.282	0.821	-0.318
TOC	0.817	-0.127	-0.530
TN	0.567	0.254	-0.342
Tp	-0.806	0.366	0.344
pH	-0.764	0.418	0.423

crystalline solid or viscous substance, soluble in carbon disulfide and composed primarily of highly condensed polycyclic aromatic hydrocarbons (Jahromi and Khodaii, 2009). This can explain the increase of total organic carbon with the increase of asphalt in soil (Figure 1). Overall, the TOC content in treatment N2, N3 and N4 decreased slightly at 26 days as compared with 1 day, though the decrease is not statistically significant. The total nitrogen did not alter but the total phosphorus decreased, suggesting that the asphalt contains a level of total nitrogen similar to the soil while the level of phosphorus is lower than that soil. Recent report shows that bitumen's composition is similar to residues of highly weathered crude oils and refined petroleum products found in marine and freshwater ecosystems 20 to 30 years after spills, and it is bioaccessible and biodegradation takes place under a wide variety of environmental conditions (Armstrong et al., 1997; Thomas et al., 2001; Ramos-Padrón et al., 2011). Potter and Duval, (2001) found that Cerro Negro bitumen was degradated by a consortium of marine benthic microorganisms. In the present study, the increase of bacteria and enzyme activity may be attributed to the biological decomposition of the asphalt in soil. The decreased pH in soil after adding asphalt demonstrates that asphalt with a lower level of pH value and can be used to remediate alkali soil.

The study of multiple biological and biochemical properties is often suggested because they are very responsive, act as indicators of soil disturbance, and provide immediate and precise information on small changes occurring in soil (Hueso et al., 2011). It has been indicated that soil microbes could adapt themselves in accordance with the environmental change by conversion of proliferation and metabolism each other (Marshall et al., 2000). Soil microbial activity is regarded as one of the most important parameters of soil microorganisms and has been suggested as an indicator of soil health (Barros et al., 2007; Brussaard et al., 2007). In the present study, the number of bacteria increased significantly in soil in response to the addition of asphalt; however, it decreased back to the control values in N4 compared with control at 26 days. This may be ascribed to their adaption mechanism. For example, environmental change between environmental demands for survival and the individual's capacity to adapt to these changes, namely imbalance, is defined as stress, and such responses are needed in adapting to demands of ever-changing circumstances (Marshall et al., 2000). Recent report shows that microbial communities responsible for carbon and sulfur cycling in light of microbial activity and biogeochemical measurements in an active oil sand tailings pond, which receives and stores the solid and liquid waste from bitumen extraction (Ramos-Padrón et al., 2011). They also found that high sulfate concentrations in ponds can affect the activities of several archaea, for example the numbers of methanogens. Consequently, the decrease of archaea concentration can be partially ascribed to the increased sulfate in this study, since the sulfate existed in asphalt and methanogens exist in the soil (Ramos-Padrón et al., 2011).

Microbial community in soil is involved in numerous ecosystem functions like nutrient cycling and organic matter decomposition (Hueso et al., 2011), and it is a more-reactive component of a terrestrial ecosystem to external stress than plants and animals (Panikov, 1999). Soil microorganisms synthesize and secrete extracellular enzymes, which constitute an important part of the soil matrix. Enzymes play an important role in soil nutrient cycles; consequently, factors influencing soil microbial activities will affect the production of the enzymes which control nutrient availability and soil fertility (Hueso et al., 2011). Catalases use a two-electron transfer mechanism

to split hydrogen peroxide into molecular oxygen and water and scavenge H_2O_2 and protect cells from damage caused by reactive oxygen species (Guwy et al., 1999).

The increased catalase activity was detected in soil containing asphalt, suggesting that the increase of H_2O_2 may be induced by hazardous substances in asphalt (Barreira et al., 2007). Dehydrogenase could be used as an index for the total oxidative activities of the cell (Singh and Kumar, 2007). The increased dehydrogenase activity in this study suggests that asphalt increases the overall microbial activity, since dehydrogenase activity in soil could be used as measurement for overall microbial activity (Singh et al., 2008).

Urease is one of the most commonly assayed soil enzymes because it greatly influences the transformation and fate of an important fertilizer – urea (Qin et al., 2010). Urease in soil is involved in the hydrolysis of C–N bonds of some amides and urea (Madejón et al., 2001) and is more sensitive to pollution (Bååth 1989). Consequently, soil urease activity has attracted a considerable attention due to the increased use of urea as a fertilizer to increase the soil productivity. In this study, the increased urease was detected in soil with addition of asphalt, indicating that certain substances existed in asphalt can be useful to improve the nitrogen cycle in soil.

Sucrase is an extracellular enzyme that catalyzes the hydrolysis of sucrose into glucose and fructose, while cellulase is a carbohydrate-hydrolyzing enzyme that catalyzes the hydrolysis of cellulose. The increased activity of sucrose and cellulose may be ascribed to the increased total organic carbon and also suggest that soil fertility increased in soil amended with asphalt (Ge et al., 2009). Polyphenol oxidase catalyzes the o-hydroxylation of monophenols to o-diphenols (phenol molecules containing two hydroxyl substituents). Polyphenol oxidase is an enzyme activated and induced by aromatic compounds like PAHs (Zhou, 1987). Therefore, the increase of PPO activity may be induced by the increase of PHAs in soil amended with asphalt, since the activity of polyphenol oxidase was positively correlated with PAHs concentration (Chen et al., 2004). However, the decrease of PPO activity may be inhibited by the inhibitors (Girelli et al., 2004).

Microbes in soil are a complex ecosystem and play vital roles in soil fertility through their roles in nutrient cycling and organic matter decomposition (Wainwright, 1978; Wang et al., 2010). The microbial structure can also affected by the environmental factors such as TOC, pH and nutrient elements. Therefore, investigating effects of asphalt on the soil microbial activities and other related properties proves useful in risk assessment (Wang et al., 2010). PCA is a multivariate statistical technique that can simplify large data sets and allow reducing the number of variables to a smaller set of orthogonal factors for easier interpretation. As shown in Table 1, the urease, sucrose, cellulose, 16S rRNA of bacteria, dehydrogenase, dehydrogenase TOC and TN weigh heavily in component 1, indicating that these parameters were positively related to the addition of asphalt. The increased enzyme activities may be ascribed to the increased number of bacteria or the activation of the expression of these genes in bacteria. Recent report shows that the bioremediation of bitumen-contaminated soil was almost equally successful with and without the use of plants (Muratova et al., 2003).

In conclusion, asphalt application altered the nutrient conditions and physiochemical properties by increasing the total organic carbon and decreasing the total phosphorus and pH values. The copy number of 16S rRNA of bacteria increased in soil with the addition of asphalt but that of archaea decreased. In addition, the activity of catalase, dehydrogenase, urease, sucrose, cellulase and polyphenol oxidase increased in soil, suggesting that the asphalt application enhanced the activity of soil enzymes. The results showed that asphalt can potentially improve the fertilization of the soil; however, potential toxic substances existing in asphalt remain to be further investigated.

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