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Microbial communities inhabiting oil-contaminated soils from two major oilfields in Northern China: Implications for active petroleum-degrading capacity

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Although oilfields harbor a wide diversity of microorganisms with various metabolic potentials, our current knowledge about oil-degrading bacteria is limited because the vast majority of oil-degrading bacteria remain uncultured. In the present study, microbial communities in nine oil-contaminated soils collected from Daqing and Changqing, two of the largest oil fields in China, were characterized through high-throughput sequencing of 16S rRNA genes. Bacteria related to the phyla *Proteobacteria* and *Actinobacteria* were dominant in four and three samples, respectively. At the genus level, *Alkanindiges*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium*, and *Rhodococcus* were frequently detected in nine soil samples. Many of the dominant genera were phylogenetically related to the known oil-degrading species. The correlation between physiochemical parameters within the microbial communities was also investigated. Canonical correspondence analysis revealed that soil moisture, nitrate, TOC, and pH had an important impact in shaping the microbial communities of the hydrocarbon-contaminated soil. This study provided an in-depth analysis of microbial communities in oil-contaminated soil and useful information for future bioremediation of oil contamination.

Keywords: oil-degrading bacteria, bioremediation, microbial community analysis, Illumina sequencing

Introduction

Oilfields harbor a wide diversity of microorganisms with various metabolic potentials, such as sulfate-reducing, sulfide-oxidizing, iron-reducing, nitrate-reducing, and fermentative bacteria and archaea (Semple and Westlake, 1987; Voordouw *et al.*, 1996; Hubert and Voordouw, 2007; Silva *et al.*, 2013). The bacteria capable of degrading petroleum components are of great interest due to their capabilities to clean up oil contamination. Crude oil is a complex mixture composed of aromatic and non-aromatic hydrocarbons and n-alkanes, many of which are toxic and hazardous (Salanitro *et al.,* 1997). These organic compounds could act as electron donors for the indigenous microbial communities and thus be degraded via microbial metabolism. Therefore, an investigation of the microbial community, especially the microorganisms that are able to degrade toxic and hazardous petroleum compounds, is of great interest for the cleanup of crude oil pollution. Indeed, the microbial community and oil-degrading bacteria have been extensively studied using both culture-dependent and -independent methods (Atlas, 1981; Röling *et al.*, 2004; Head *et al.*, 2006; Margesin *et al.*, 2007; Yakimov *et al.*, 2007; Luo *et al.*, 2009; Sun *et al.*, 2010). Kostka *et al.* (2011) isolated 24 bacterial strains from beach sands contaminated by deep-water horizon oil spills. All of these isolates were confirmed as oil-degrading bacteria clustered within the genera *Alcanivorax*, *Marinobacter*, *Pseudomonas*, and *Acinetobacter*. More recently, numerous bacteria responsible for biodegradation of petroleum components have been identified. For example, *Desulfosporosinus* spp. was frequently encountered in oil fields (Kaster *et al.*, 2007; Mayumi *et al.*, 2011) and was identified as a biodegrader of such oil components as BTX (benzene, toluene, and xylene) (Winderl *et al.*, 2010; Sun and Cupples, 2012; Sun *et al.*, 2014a, 2014b). In addition to oil-degrading bacteria, oil-degrading functional genes have been characterized in oil-ontaminated environments. For example, Liang *et al.* (2010) investigated the functional gene involved in contaminant biodegradation from five oil-contaminated fields in China. Their results indicated that the functional genes responsible for organic contaminant degradation were most abundant in all of the samples. In all, investigation of the oil-degrading microbial community may provide useful information for the bioremediation of oil-related pollution.

Oil fields are major sources of terrestrial crude oil contamination. In the oilfields with active drilling and oil production activities, a large amount of oil waste was produced and contaminated surface water, groundwater aquifers, and soil in the surrounding areas (O'Rourke and Connolly, 2003; Kharaka *et al.*, 2005; Osborn *et al.*, 2011). Bioremediation of oil contamination is an important process to remediate oil contaminations. Because the factors (e.g., indigenous mic-

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robial communities, geochemical and nutrient environments) that are closely related to efficiency of bioremediation can be site dependent and a lack of proper understanding may lead to failures in bioremediation, there is a need to increase the biological knowledge of this process. A comprehensive understanding of the microbial community, especially the oil-degrading microorganisms, will lead to an improved ability to design and optimize bioremediation processes. In this study, we selected the two largest oilfields in China as models to study how *in situ* oil contamination stimulates the oil-degrading bacteria in terrestrial environments. We utilized high-throughput sequencing to profile microbial communities in these soil samples, enabling deeper sequencing depth and better understanding of the communities than would be obtained by studies using classical molecular techniques (e.g., 16S rRNA clone libraries, DGGE and T-RFLP). This study aims to offer novel knowledge regarding the microbial diversity and composition in the contaminated soil of oil fields and contribute to the bioremediation of oil spills.

Materials and Methods

Site information and sampling strategy

Soil samples were obtained from oil-contaminated soil in two large oilfields from different geographic regions of Northern China. Daqing (DQ) oilfield was located in Northeast China and Changqing (CQ) oilfields in Northwest China. Both regions have a temperate continental monsoon climate. Contaminated soils (2-10 cm beneath the surface) were collected by skimming the upper 2 to 3 cm of soil adjacent to crude oil pumping wells, in which contamination had occurred for 23 years in the DQ oil field and eight years in the CQ oil field. Sampling occurred in May 2013 for the DQ oil field and June 2013 for the CQ oil field. Three samples were taken from the CQ oilfield and six samples were taken from the DQ oilfield. A map showing the location of the DQ and CQ oilfields and the sampling sites is shown in Fig. 1.

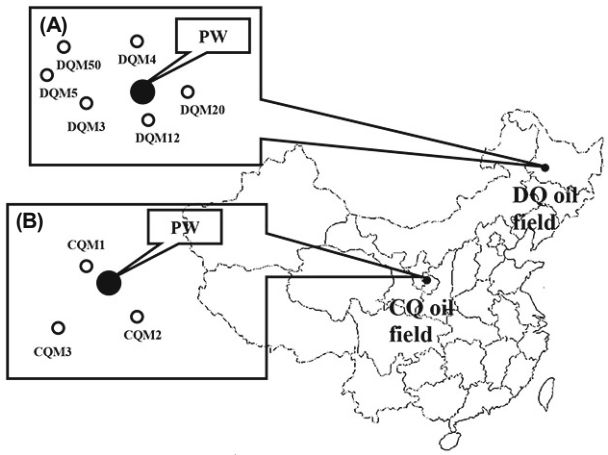

Fig. 1. Map showing the locations of the Daqing (DQ) and Changqing (CQ) oilfields. The enlarged map shows the details of the sampling points in the two oilfields.PW stands for pumping well. Pumping well in the DQ oil field has been in operation for 23 years and that in the CQ oil field has been in operation for nine years. The daily production is three tons for the DQ pumping well and two tons for the CQ pumping well. The distances between each sample location with PW are as follows: CQM1 (20 m); CQM2 (45 m); CQM3 (85 m); DQM4 (20 m); DQM12 (15 m); DQM20 (30 m); DQM3 (40 m); DQM50 (65 m); DQM5 (70 m).

Chemical analysis

Soil samples from each site were homogenized by thorough mixing and then stored in a refrigerator at 4°C for further processing. Then, ten grams of soil were air-dried for 48 h and passed through a 2-mm sieve to remove leaves, plant roots, and gravel. Five-gram dry soil samples were put into a 150-ml Erlenmeyer flask and mixed with 25 ml distilled water (1:5 soil-water ratio). The mixture was left to equilibrate for 20 min after shaking for 5 min. The pH was measured using a calibrated HACH HQ30d pH meter (HACH). The supernatant was filtered with a 0.45-μm membrane. Sulfate and nitrate concentrations were determined using the Ion Chromatography System (DIONEX ICS-1500). Soil TOC and TN were determined by the elemental analyzer (Vario EL/micro cube). Soil moisture was determined by sampling 5 g of soil and then drying it at 105°C for 24 h to achieve a constant weight. The differences in soil weight before and after drying were used to determine soil moisture.

DNA extraction, PCR amplification, and Illumina sequencing

Genomic DNA was extracted from soil samples using the FastDNA® spin kit (MP Bio) following the manufacturer's protocol. The DNA concentration was then determined using a NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Scientific). The V4 region of 16S rRNA genes was amplified using the 515f/806r primer set (Caporaso *et al.*, 2011). 16S rRNA tag-encoded ultra-high-throughput sequencing was carried out on the Illumina MiSeq platform at Novogene. Sequences were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) software and UPARSE pipeline (Caporaso *et al.*, 2010). Default settings ($r = 3$, $p = 0.75$ total read length; $q = 3$; $n = 0$) for Illumina processing in QIIME were used to filter the raw reads and obtain high quality reads by denoising and removing chimeras, and then the UPARSE pipeline was used to assign taxonomy to 97% similarity via RDP classifier (Wang *et al.*, 2007). Species richness was indicated with Chao1 and Shannon indices for nine libraries (Schloss *et al.*, 2009). The reads have been deposited into the NCBI short reads archive database (SRX731327).

Canonical correspondence analysis and cluster analysis

Canonical correspondence analysis (CCA) was performed to evaluate which chemical properties had the most significant influence on the microbial community structure. The significant correlations of the physiochemical parameters were examined by a Monte Carlo permutation. The triplot was generated by CANOCO 4.5 and the figure was generated by CanoDraw 4.0 (Biometrics Wageningen). Geochemical parameters and relative abundances of dominant genera were used for cluster analysis. The "clustsig" package in R was used to perform the cluster analysis. Unweighted Pair Group Method with Arithmetic mean (UPGMA) was performed to compare geochemical parameters in different samples. Actual-Bray-Curtis was used to compare dominant genera in different samples.

Table 1. Physicochemical properties and estimated OTUs of the oil-contaminated soil samples from the Daqing (DQ) and Changqing (CQ) oilfields

Soil	TOC (g/kg)	Moisture (%)	pH	SO_4^{-2} (mg/L)	NO_3^- (mg/L)	OTUs
CQM1	648.7	1.5	8.16	10.34	2.74	1677
CQM2	957.6	1.9	8.3	7.38	2.15	1471
CQM3	1492.6	2.28	8.8	9.47	3.26	2098
DQM3	35.0	26.73	9.23	20.19	2.39	1557
DQM4	82.5	16.45	9.44	3.7	1.37	1885
DQM5	65.4	13.44	9.5	35.9	1.69	1899
DQM12	34.4	17.89	9.67	28.78	3.95	1937
DQM50	88.3	14.56	10.03	15.8	6.06	2232
DQM200	79.8	24.69	8.84	3.35	3.52	2482

Results

Physiochemical analysis

TOC, soil moisture, pH, and nitrate and sulfate levels showed variation between the two geographically distant sites (Table 1). Samples from the CQ oilfield showed higher concentrations of TOC than did samples from the DQ oilfield, indicating a higher organic loading (oil contamination) in CQ than in DQ. Taking into account that oil drilling is the major activity in the sampling site, we proposed that the major components of TOC would be the petroleum components. Therefore, the CQ oil field samples might be more contaminated by petroleum than the DQ oil field samples. Nevertheless, the DQ oilfield samples showed higher soil moisture than did samples from the CQ oilfield, and DQM3 and DQM200 had the highest soil moisture among all examined soil samples. All soil samples were alkali, with pH values greater than 8. Among them, DQM50 had the highest pH value of 10.03, followed by DQM12 with a pH value of 9.67. In general, samples from the DQ oilfield had a higher pH than those from the CQ oilfield. All samples showed very low concentrations of nitrate, ranging from 1.37 mg/L to 6.06 mg/L. DQM5 had the highest concentration of sulfate, 35.90 mg/L, while sulfate concentrations in DQM4 and DQM200 were below 4 mg/L.

Microbial community composition in two oil fields

From each oil-contaminated sample, 9,051–21,859 high-quality reads were retrieved. Using a 97% sequence similarity cut-off, the number of OTUs in each soil microbial community was 1471-2482 (Table 1). Based on OTU abundances, one soil sample from the DQ oilfield (DQM200) had the richest diversity, followed by a sample from the CQ oilfield (CQM3). In this study, eight bacterial (Fig. 2A) and archaeal phyla (Fig. 2B) were identified in all of the samples, including *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Crenarchaeota* (Archaea), *Planctomycetes*, *Firmicutes*, *Verrucomicrobia*, and *Chloroflexi*. It is noteworthy that archaeal reads only accounted for 9.25% of the total reads, indicating a relatively small amount of archaea occurring in these contaminated soils. Based on the average relative abundance, *Proteobacteria* was the most dominant phylum, with an average relative abundance of 24.69% in nine samples, followed by *Actinobacteria*, with an average relative abundance of 19.79%. *Proteobacteria* was the most abundant phylum in four samples, including CQM2, DQM3, DQM4, and DQM50. *Proteobacteria* mainly consisted of two classes, *Gammaproteobacteria* and *Alphaproteobacteria*. *Actinobacteria* was the most abundant phylum in CQM1, CQM3, and DQM5. *Acidobacteria* was the most abundant phylum in DQM200, and *Verrucomicrobia* was the most abundant phylum in DQM12.

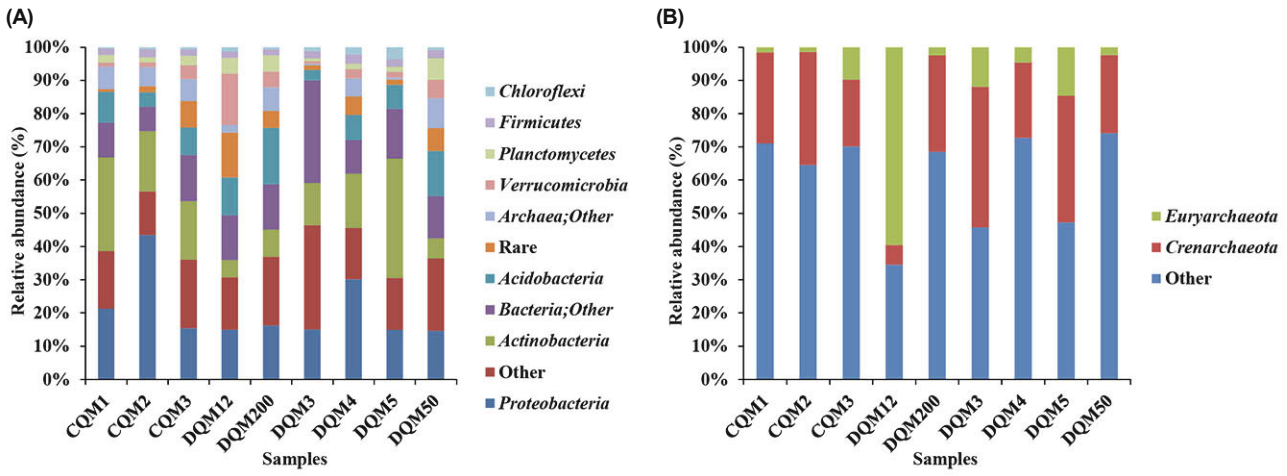

Fig. 2. Taxonomic classification of bacterial reads (A) and archaeal reads (B) retrieved from nine oil-contaminated samples at the phylum level via 16S rRNA Illumina sequencing.

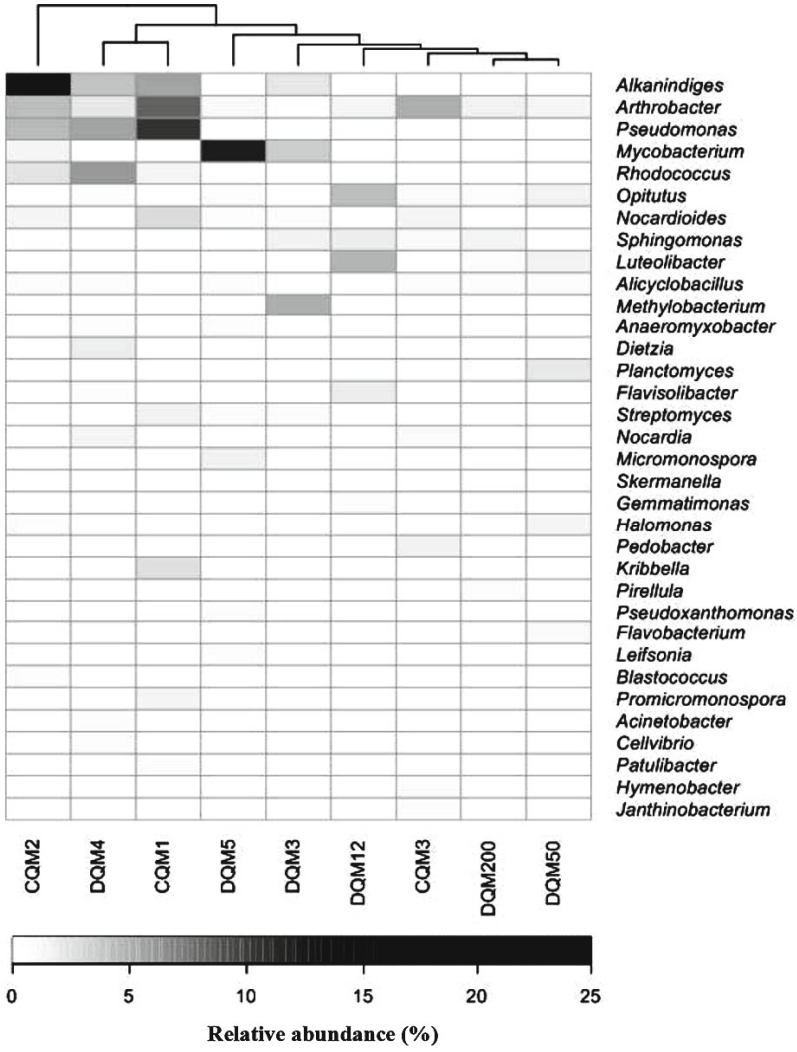

Fig. 3. Heatmap analysis of the distribution of dominant phylotypes (with a relative abundance >1% in at least one soil sample) in the nine samples. The double hierarchical dendrogram shows the microbial distribution of the nine samples. The relative percentage values for the microbial genera are depicted by the color intensity. The legend can be found at the bottom of the figure.

A heatmap (Fig. 3) shows the frequently detected bacterial genera in the oil-contaminated samples. At the genus level, the variations of microbial communities were more evident. An abundance of the genera *Alkanindiges*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium*, *Rhodococcus*, and *Methylobacterium* (with a relative abundance greater than 5% in at

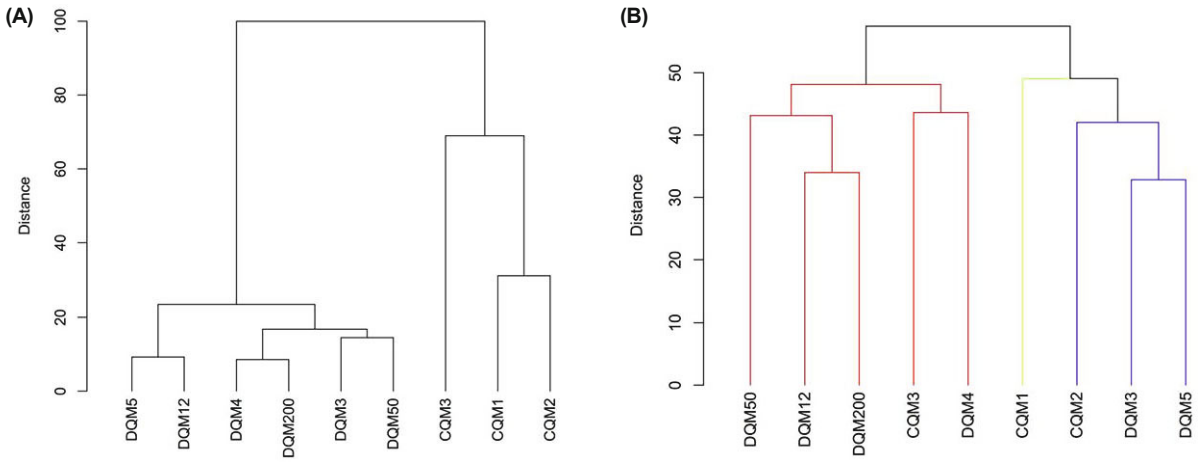

Fig. 4. Cluster analysis showing the comparison between geochemical parameters of the different sampling sites (A) and dominant genera (B). In Fig. 4B, different colors indicate different statistically significant groups (P<0.05).

least one sample) was observed. Other genera, such as *Pedobacter*, *Nocardioides*, *Sphingomonas*, *Opitutus*, *Micromonospora*, and *Planctomyces*, were ranked among the top three genera in at least one soil sample.

In addition, soil geochemical profiles and dominant genera were grouped by cluster analysis. As indicated by cluster analysis, geochemical profiles were site-specific, with those from the CQ oilfield grouped together and those from the DQ oilfield formed another group (Fig. 4A). At the genus level, microbial communities derived from DQM4, DQM12, DQM50, and DQM200 grouped together (Fig. 4B). However, microbial communities from DQM3 and DQM5, along with CQM2, grouped together. Three microbial communities from the CQ oilfield were distributed into three different groups. These observations indicated the geographic difference could shape microbial communities, but this is not the only factor. Moreover, the microbial community composition would be structured by *in situ* geochemical factors, as indicated by the community similarity between samples taken from different oil fields.

Relationship between microbial community and the environment

In the current study, CCA was conducted to evaluate the correlations between the dominant genera (>1%) and selected geochemical parameters of soil samples (pH, SO_4^{-2}, NO_3^-, TOC, annual average precipitation and temperature, and moisture). CCA results, as summarized in the triplot (Fig. 5), showed a relationship between environmental variables and microbial communities. The overall microbial communities were significantly linked to soil moisture, nitrate levels, pH, annual average temperature and precipitation, and TOC. CCA ordination showed that microbial community compositions were also associated with specific environmental conditions. For example, *Alkanindiges*, *Arthrobacter*, *Pseudomonas*, and *Nocardioides* correlated positively with higher TOC concentrations. *Alicyclobacillus*, *Acinetobacter*, and *Sphingomonas* were positively linked to soil moisture. *Dietzia* were correlated with sulfate concentrations. *Methylobacterium*, *Rhodococcus*, and *Mycobacterium* were positively associated with nitrate concentrations.

Discussion

In the current study, we used Illumina sequencing to characterize the indigenous microbial communities in the topsoil from two large oilfields in Northern China. The pollution by petroleum components may stimulate the growth of some oil-degrading microorganisms and make these microorganisms the dominant taxa (Harayama *et al.*, 2004). Therefore, we hypothesized that many dominant microorganisms might be correlated with petroleum biodegradation. Based on this hypothesis, we paid more attention to dominant phylotypes in each sample, and we investigated their potential role in the biodegradation of petroleum components and interactions with native geochemical environments.

Correlation between environmental parameters and bacterial community

pH appears to be one of the most important environmental parameters among the factors tested in the present study. It was reported that pH has significant effects on the overall microbial communities in various environments (Fierer and Jackson, 2006; Lauber *et al.*, 2009). Any significant deviation of pH would impose stress on single-celled organisms because the intracellular pH of most microorganisms is usually within 1 pH unit of neutral (Fierer and Jackson, 2006). Several environmental parameters, such as nutrient availability and cationic metal solubility, are often correlated with soil pH (Brady and Weil, 1996). Therefore, changes in pH may affect the distribution of these factors and drive the shift in microbial community composition.

In the present study, soil moisture was strongly linked to microbial community variance. Soil moisture varied significantly in two oilfields. All samples from the DQ oilfield demonstrated higher soil moisture than did the CQ samples. CCA results demonstrated that soil moisture might play an important role in shaping the indigenous microbial communities. Based on CCA analysis, we hypothesize that low moisture content might be a limiting factor in biodegradation. Increasing soil moisture may increase the bioavailability of the oil hydrocarbons and other nutrients as well as bacterial activities, and finally increase the performance of

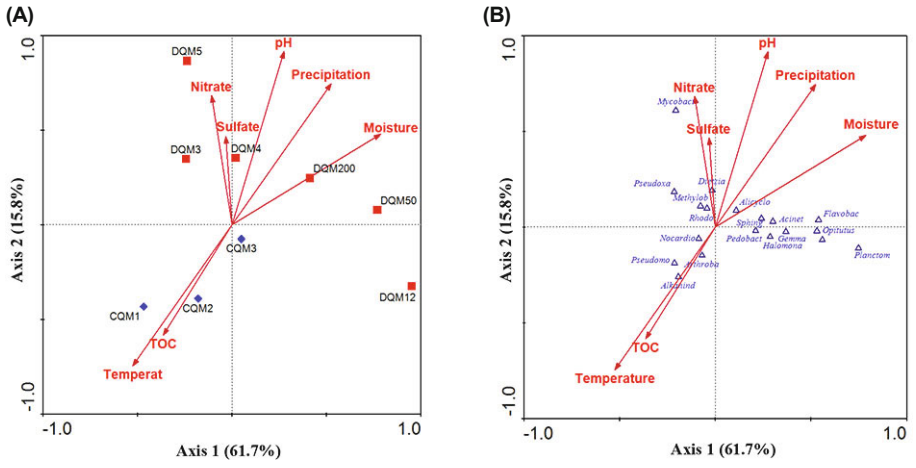

Fig. 5. Canonical correspondence analysis (CCA) of 16S rRNA gene data and geochemical parameters. Arrows indicate the direction and magnitude of geochemical parameters associated with bacterial community structures. (A) Relationship between geochemical parameters with sampling sites. (B) Relationship between geochemical parameters with important genera. Abbreviations: *Alkanind*, *Alkanindiges*; *Pseudomo*, *Pseudomonas*; *Arthroba*, *Arthrobacter*; *Nocardio*, *Nocardioides*; *Halomona*, *Halomonas*; *Pedobact*, *Pedobacter*; *Planctom*, *Planctomyces*; *Sphing*, *Sphingomonas*; *Gemma*, *Gemmatimonas*; *Flavobac*, *Flavobacterium*; *Acinet*, *Acinetobacter*; *Alicyclo*, *Alicyclobacillus*; *Rhodo*, *Rhodococcus*; *Methylob*, *Methylobacterium*.

bioremediation. In addition, other environmental factors, such as nitrate levels and TOC, were strongly linked to the overall microbial communities.

CCA also tested the influence of environmental parameters on specific bacterial phylotypes. TOC concentrations have a positive effect on the members of the genera *Alkanindiges*, *Arthrobacter*, *Pseudomonas*, and *Nocardioides*. The background TOC concentrations of DQ and CQ oilfield are 2.31 g/kg and 2.05 g/kg, respectively. This remarkable difference of TOC in contaminated and uncontaminated soils indicated TOC would be an appropriate indicator of oil pollutant and a major part of TOC could be attributed to a crude oil component in oil-contaminated soils. Therefore, these four genera may have a potential link with oil degradation. In accordance with our CCA analysis, all of these genera contain some species that are able to degrade crude oil (Rosenberg *et al.*, 1979; Bogan *et al.*, 2003; Hasanuzzaman *et al.*, 2004; Schippers *et al.*, 2005). Nitrate concentrations and soil moisture were positively associated with many genera, including some known oil biodegraders, such as *Dietzia*, *Rhodococcus*, *Sphingomonas*, and *Acinetobacter*. One implication of the CCA analysis was that an increase of nutrients, such as nitrate and soil moisture, might stimulate the growth of oil-degrading bacteria and enhance bioremediation performance.

Potential oil-degrading bacteria

Most of the dominant genera could be described as aerobes, which is reflective of the fact that the microbial communities may be in contact with permeated oxygen. Based on the average relative abundance, *Alkanindiges*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium*, and *Rhodococcus* were ranked among the top five genera in nine soil samples. It is interesting that all of these top five genera contain some species that is able to degrade hydrocarbons (Rosenberg *et al.*, 1979; Sorkhoh *et al.*, 1990; Kelley and Cerniglia, 1995; Bogan *et al.*, 2003; Hasanuzzaman *et al.*, 2004).

Alkanindiges showed very high relative abundances in CQM1 (5.48%) and CQM2 (24.86%). In the DQ oilfield, this phylotype also showed very high abundances in three samples. *Alkanindiges*is a recently discovered genus, belonging to the family *Moraxellaceae* within the phylum *Gammaproteobacteria*. *Alkanindiges* spp. has been frequently detected in oil-contaminated soil (Bogan *et al.*, 2003; Kasai *et al.*, 2005; Chang *et al.*, 2010), indicating that *Alkanindiges* may exhibit the capacity to carry out oil degradation. It has been reported that *Alkanindiges* contained many species that are able to degrade the major components of crude oil (Bogan *et al.*, 2003; Klein *et al.*, 2007). For example, *Alkanindiges illinoisensis* is an anaerobic squalane-degrading bacterium isolated from oilfield soils (Bogan *et al.*, 2003). This genus also contained obligate alkane degraders in activated sludge systems (Klein *et al.*, 2007) and bacteria metabolizing a wide variety of hydrocarbons, including poly aromatic hydrocarbons (PAHs), alkane and squalane (Ron and Rosenberg, 2010). The enrichment of *Alkanindiges* species in our oil-contaminated soil samples indicated that they might play an important role in *in situ* oil bioremediation. Another dominant genus identified in this study, *Pseudomonas* populations, has been commonly encountered in oil-contaminated environments. *Pseudomonas* contained a group of metabolic versatile bacteria that are able to degrade various petroleum hydrocarbons (Atlas, 1981). For instance, *Pseudomonas aeruginosa*, isolated from a petroleum-contaminated soil sample from Northeast India, was able to utilize crude petroleum hydrocarbons as a sole source of carbon and energy (Das and Mukherjee, 2007). Some *Pseudomonas* strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways were able to degrade petroleum hydrocarbons (Whyte *et al.*, 1997). The dominance of *Pseudomonas* in both the DQ and CQ oil fields suggests this group of bacteria may be ubiquitous in oil-contaminated environments.

Two phylotypes, *Arthrobacter* and *Mycobacterium*, belonging to the phylum *Actinobacteria* were frequently detected in the current study. *Arthrobacter* was highly enriched in all samples taken from the CQ oilfield, but showed less abundance in the DQ oil field samples. *Arthrobacter* spp. are notable for their hydrocarbon biodegradation capabilities. Dating back to 1967, Stevenson found that *Arthrobacter* spp. was able to utilize aromatic hydrocarbons as acarbon source (Stevenson, 1967). Later, strains of *Arthrobacter* were found to be able to degrade a wide variety of chemical compounds, including polychlorinated biphenyl (Gilbert and Crowley, 1997), p-nitrophenol (PNP) (Jain *et al.*, 1994), 4-chlorobenzoic acid (Marks *et al.*, 1984), phenanthrene (Keuth and Rehm, 1991), and fluorine (Casellas *et al.*, 1997). Another dominant genus, *Mycobacterium*, identified in this work, was characterized as degraders of different PAHs, such as phenanthrene, fluorene, fluoranthene, and pyrene (Boldrin *et al.*, 1993). For example, *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site, were able to degrade pyrene, benz[a]anthracene, andbenzo[a]pyrene (Schneider *et al.*, 1996).

Bacteria relating to the genera *Luteolibacter*, *Methylobacterium*, and *Rhodococcus* exhibited high abundance (relative abundance >5%) in only one sample. This observation indicated that these genera might not be ubiquitous phylotypes in oil-contaminated environments. *Luteolibacter* spp. have not commonly been detected in oilfields. Relatives of this genus were isolated from rhizosphere (Johansen *et al.*, 2005), a methamidophos-manufacturing factory (Wang *et al.*, 2011), and human blood (Kämpfer *et al.*, 2009). *Methylobacterium* was proposed as a genus of facultatively methylotrophic bacteria (Chistoserdova *et al.*, 2003). The enrichment of *Luteolibacter* and *Methylobacterium* may be not be directly correlated with oil degradation. *Rhodococcus* consisted of numerous hydrocarbon-degrading bacteria (Wilson and Jones, 1993; Haritash and Kaushik, 2009). Due to some unknown reasons, the *Rhodococcus*-related bacteria were only enriched in a limited number of samples.

Conclusion

In this study, we used high-throughput sequencing to characterize the microbial community composition in nine oil-contaminated soil samples from two large oilfields in China. *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* were the three most abundant bacterial groups in oil-contaminated soil. A number of known oil-degrading bacteria were iden-

tified in these soils, such as *Alkanindiges*, *Arthrobacter*, and *Mycobacterium*, indicating a dynamic turnover of petroleum components in these contaminated soils. CCA analysis indicated that increasing soil moisture and nitrate concentrations in oil-contaminated sites might stimulate the growth of oil-degrading bacteria.

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