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Diagnosing bioremediation of crude oil-contaminated soil and related geochemical processes at the field scale through microbial community and functional genes

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Abstract

Purpose: Bioremediation is widely considered the most desirable procedure for remediation of oil-contaminated soil. Few studies have focused on the relationships among microbial community, functional genes of biodegradation, and geochemical processes during field bioremediation, which provide crucial information for bioremediation.

Methods: In the current study, the microbial community and functional genes related to hydrocarbon and nitrogen metabolism, combined with the soil physico-chemical properties, were used to diagnose a set of bioremediation experiments, including bioaugmentation, biostimulation, and phytoremediation, at the field scale.

Result: The results showed that the added nutrients stimulated a variety of microorganisms, including hydrocarbon degradation bacteria and nitrogen metabolism microorganisms. The functional genes reflected the possibility of aerobic denitrification in the field, which may be helpful in biodegradation. Biostimulation was found to be the most suitable of the studied bioremediation methods in the field.

Conclusion: We offer a feasible approach to obtain useful bioremediation information and assist with the development of appropriate remediation procedures. The findings improve our knowledge of the interactions between microorganisms and edaphic parameters.

Keywords: Microbial community, Functional genes, Diagnose, Bioremediation, Oil-contaminated soil

Introduction

Oil contamination in water and soil is a worldwide environmental problem (Lu et al. 2014), posing a huge threat to human health and natural ecosystems (Chen et al. 2015). Compared with physical and chemical remediation, bioremediation is regarded as the optimal method for remediation of oil-contaminated soil because it is inexpensive, efficient, and applies environmentally

friendly processes (Adetutu et al. 2015). The successful application of bioremediation techniques, such as bio-augmentation, biostimulation, and phytoremediation, for remediating oil spills was reported in numerous studies (Adams et al. 2015; Cai et al. 2016; Mrozik and Piotrowska-Seget 2010; Yavari et al. 2015). Field-scale bioremediation works were also conducted in some oil-contaminated fields, and the obtained results were satisfactory. Most of them were ex situ methods, such as biopiles and prepared beds (Álvarez et al. 2017; Gomez and Sartaj 2013; Gomez and Sartaj 2014; Jørgensen et al. 2000), which are always time-consuming and expensive (Farhadian et al. 2008), and therefore unsuitable for mass soil. In situ bioremediation is widely suitable and

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has been used for years (Han et al. 2008; Menendez-Vega et al. 2007; Mishra et al. 2001; Pizarro-Tobías et al. 2015; Zhang et al. 2017). However, few studies monitored microorganisms, so the status of degradation microorganisms in the soil could not be determined (Wu et al. 2016).

Whether bioremediation is successful mainly depends on the biodegrading microorganisms (Wu et al. 2016). These microorganisms may be affected by other microorganisms and added nutrition. An understanding of the activities of biodegrading microorganisms and the relationships between microorganisms and environmental conditions is essential for the development of appropriate remediation procedures (Boopathy 2000; Wu et al. 2017; Xue et al. 2015).

For this reason, many studies focused on the microbial community associated with oil-contaminated soil. In the early years, studies focused on the changes in microflora before and after soil contamination or during the natural attenuation process (Leys et al. 2004; Mason et al. 2014; Röling et al. 2004; Viñas et al. 2005). More recently, the microbial community and hydrocarbon-degradation-related genes have been monitored during oil bioremediation in laboratory studies (Shahi et al. 2016; Wu et al. 2016; Wu et al. 2017). On the field scale, Pizarro-Tobías et al. (2015) assessed bioremediation and rhizoremediation technologies in an oil-polluted site in Spain and monitored the variations in microbial communities and activity.

As nutrients can stimulate the metabolism of oil-degrading microorganisms, and microbial communities and activities may vary, lacking nutrients is one of restricting factors for bioremediation (Liang et al. 2011; Wu et al. 2016). However, nutrients may affect other microorganisms as well as the oil-degrading microorganisms and affect the geochemical processes. For example, ammonium as the nitrogen source may also stimulate the growth of nitrifying bacteria and enhance the nitrification process. Whether the other stimulated microorganisms can promote oil biodegradation remains unclear. Knowledge is limited about the relationships between the various geochemical processes and hydrocarbon degradation on the field scale.

To diagnose the relationship between hydrocarbon degradation and related geochemical processes on the field-scale, a set of bioremediation experiments, including bioaugmentation, biostimulation, and phytoremediation, were conducted at an oil well site. We obtained and analyzed some information about the microbial community and functional genes related to hydrocarbon and nitrogen metabolism, combined with the soil physico-chemical properties. Alfalfa was seeded at the beginning of phytoremediation, but the alfalfa did not survive, and the area was occupied by Bermuda grass

that was actually applied in the phytoremediation process. Based on the field experiment, we determined (1) the relationships among hydrocarbon degradation, microbial community, functional genes, and nitrogen cycle during bioremediation, and (2) the oil degradation efficiency in the set of bioremediation experiments on the field-scale. The mechanisms of hydrocarbon biodegradation and nitrogen transformation at the field-scale were identified.

Materials and methods
Site description
The field to be remediated was part of an abandoned oil well site located in Puyang county, Henan province, China (Fig. 1). The shallow stratum of the area was formed by the alluvial deposits of the Yellow River. The stratum is sand interbedded with clay. The shallow aquifer is mainly composed of silt and fine sand in Holocene (Q_4) and Epipleistocene (Q_3) strata. The upper aeration zone is mainly sandy loam soil.

The oil well was abandoned in 2000 after a short period of operation due to low productivity. A blowout occurred when the well was drilled, and as a result, the dispersed oil heavily contaminated 3000 m^2 of farmland soil. Surface oil deposits remain scattered around the well. An area (1×4 m) with similar soil concentrations (approximately 2 g kg^{-1} of total hydrocarbons) was designated as the study area.

Bioremediation process
The study area was divided into four blocks: blank control (Ctrl), bioaugmentation (BA), biostimulation (BS), and phytoremediation (Phyto). Each block was a soil cuboid that was 80 long, 80 cm wide, and 30 cm deep (Fig. 1). According to the dry bulk density of soil (1.56 g cm^{-1}), there was about 300 kg dry soil in each block. The 5-cm-deep surface-layer soil covering the contaminated soil was removed. Then, the soil in each block was homogenized with plowing to even the oil concentration in the soil. Ctrl was treated as the blank to which no remediation procedure was applied. The BS block was sprinkled with nutrients dissolved in deionized water. The BA block was sprinkled with nutrients and oil-degrading microorganism, *Arthrobacter*, which was previously isolated from the field soil (Ning et al. 2013), and inoculated in Luria-Bertani (LB) medium at 28 °C until reaching a density of 10^{10} colony forming units (CFU)/mL. LB plate counts were used to estimate numbers of microorganisms (Mueller et al. 1991). The expected final density in the soil was 10^8 CFU/g soil. The Phyto block was treated the same as the BA block plus alfalfa seeds. The optimal ratio of carbon:nitrogen:phosphorous (C:N:P) is approximately 100:15:1 for

Fig. 1 Remediation site location
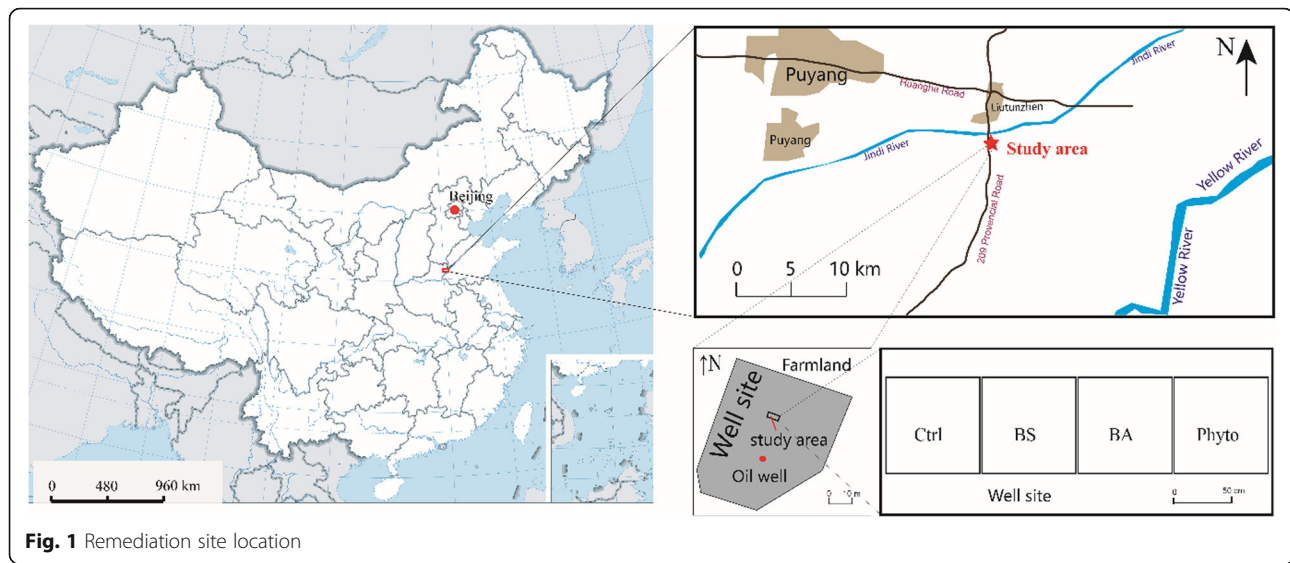
biodegradation (Graham et al. 1999). However, as a high salt content in soil may hinder bioremediation, nutrients were added across several separate occasions when watering. In the first fertilization, ammonium sulfate $((NH_4)_2SO_4$, 0.43 g/kg soil) and monopotassium dihydrogen phosphate $(KH_2PO_4$, 0.067 g/kg soil) were added. The nutrients were added corresponding to the oil concentration of 0.67 g/kg of hydrocarbons and according to the optimal C:N:P ratio. The nutrient solution was added again in the BA, BS, and Phyto blocks after 2 weeks. Purified water was added irregularly to the BA, BS, and Phyto blocks to ensure that the water content of the soil was approximately 15% of the soil mass. The Ctrl block was not treated. The lowest and highest soil surface temperatures were monitored every day by maximum-minimum thermometers during the remediation process. When the daily lowest temperature was below 15 °C, the soil surface was covered with polyethylene ground film used for farms to promote microorganism activities. The soil in the BA and BS blocks was turned over twice after every sampling.

The alfalfa in the Phyto block died 2 days after sprouting. The alfalfa was then replanted. Shading and moisture-retention measurements were taken to promote alfalfa growth in this block. However, no alfalfa plants survived for more than a few days. Instead, the block was occupied by the surrounding weed, Bermuda grass, after 2 weeks. Bermuda grass may be helpful for the remediation of oil-contaminated soil (Razmjoo and Adavi 2012). Therefore, it was allowed to follow its own course in this block.

Sampling and physico-chemical analyses

A five-point sampling, coning, and quartering method was used to collect samples; thus, a mixed sample was collected in each block at each time. About 50 g soil for each sample was collected from 15 cm below the surface using a sterile knife and placed in aluminum boxes previously sterilized at high temperature. The collected samples were immediately stored in a cooler with an ice pack and then transferred to the laboratory and stored at − 70 °C until analysis. These samples were used for the extraction of genomic DNA for the microbiological and functional gene analysis. Another soil subsample was stored in automatically sealing plastic bags for analysis of physical and chemical characteristics. Samples were collected on days 0, 3, 7, 11, 16, 22, 28, 34, 47, 62, 74, and 125 of the remediation process in Ctrl, BA, and BS blocks. Samples were only collected on days 0, 3, 7, 11, 62, 74, and 125 in the Phyto block because there was grass growing after the 11th day, and the sampling would have destroyed the grass. The samples were also collected on the site and its neighboring farmland on day 0 and 1 year prior to the start of the experiment. The oil content in the soil samples was determined using supersonic extraction and ultraviolet-visible spectrophotometric methods. Trichloromethane was used as the oil extraction solvent, and oil was extracted supersonically for 15 min twice. Then, the extraction solvent was evaporated at 65 °C, and the remnant was dissolved by petroleum ether. An ultraviolet-visible spectrophotometer was used to determine oil content at a 225-nm wavelength (Li 1999). The water content, pH, electrical conductivity, and nitrate, nitrite, ammonium, and chloridion contents in the soil samples were measured as previously described (Lu 2000). Each parameter was measured twice. The samples collected on days 0, 3, 11, 47, and 125 of the experimental blocks and on farmland neighboring the contaminated field this year (F_ty) and 1 year ago (F_oya), and in the contaminated field 1 year

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ago (Ctrl_oya) were chosen to analyze the DNA information, including the functional genes and microbial communities.

DNA extraction and gene quantification

DNA was extracted from the soils using the E.Z.N.A™ Mag-Bind Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) in accordance with the manufacturer's protocol.

To quantify the distribution of anammox bacteria (AB), ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), denitrifying bacteria, dissimilatory nitrite-reducing bacteria, and hydrocarbon-degrading bacteria, the corresponding functional genes, including *amx* (corresponds to AB), *amoA* (corresponds to AOA), *amoA* (corresponds to AOB), *nosz* (corresponds to denitrifying bacteria), *nap* (corresponds to dissimilatory nitrite-reducing bacteria), *alkB* (corresponds to alkane-degrading bacteria), and *alcvx* (corresponds to a hydrocarbon-degrading bacteria genus *Alcanivorax*) were quantified with a StepOne™ Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) based on SYBR® Premix Ex Taq™ (Tli RNase H Plus) (TaKaRa, Dalian, China). SYBR Green and absolute quantification methods were used to quantify the target genes. The acceptable R^2 values of standard curves of each gene were greater than 0.99. The primer sequences, annealing temperatures, amplified fragment size, and targets are listed in Table 1, and standard

plasmids were prepared according to previous studies (Kostka et al. 2011; Shu et al. 2016; Sun et al. 2012). To validate the reproducibility, each reaction was performed in duplicate.

Microbial community sequencing

The DNA was PCR amplified using 25 cycles with the Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC) primers (Herlemann et al. 2011). The PCR products were purified and then adjusted to 10–20 ng of DNA. Metagenomic sequencing was conducted on the MiSeq sequencing platform at Sangon Biotech Co., Ltd. (Shanghai, China) (Hatta et al. 2016). The sequence reads were trimmed, optimized, subsampled, aligned, and clustered into operational taxonomic units (OTUs) (Liu et al. 2017). The representative sequences were annotated with the RDP-classifier 2.2 (QIIME) (Lan et al. 2012). The species diversity index was calculated according to the α diversity analysis to evaluate the species diversity in the soil samples (Korenblum et al. 2012). The community composition of each sample was determined to the genus level.

Statistical analysis

The relationships between the samples and the microbial community at the gene level were revealed by principal component analysis (PCA), which was used to cluster the distribution of microbial community composition

Table 1 Primers used for qPCR and thermal programs in this study

Function	Target gene	Sequence (5'-3') of parimer pairs	Annealing (℃)	Acmplicon size (bp)	Thermal program
Total bacteria	16S rRNA	341F: CCTACGGGAGGCAGCAG	60	200	30s at 94 ℃, 40 cycles of 10 s at 94 ℃, 30 s at annealing temperture, and 40 s at 72 ℃
		518R: ATTACCGCGGCTGCTGG			
Anammox bacteria	*Amx*	Amx809f: GCCGTAAACGATGGGCACT	60	282	
		Amx1066r: AACGTCTCACGACA CGAGCTG			
Ammonia-oxidizing archaea (AOA)	*amoA (AOA)*	amoAF: STAATGGTCTGGCTTAGACG	53	635	
		amoAR: GCGGCCATCCATCTGTATGT			
Acmmonia-oxidizing bacteria (AOB)	*amoA (AOB)*	amoAF: GGGGTTTCTACTGGTGGT	60	491	
		amoAR: CCCCTCKGSAAAGCCTTCTTC			
Denitrifying bacteria	*nosz*	nosZ1F: WCSYTGTTCMTCGACAGCCAG	63	251	
		nosZ1R: ATGTCGATCARC TGVKCRTTYTC			
Dissimilarity nitrite reducing bacteria	*napA*	napA3F: CCCAATGCTCGCCACTG	60	130	
		napA3R: CATGTTKGAGCCCCACAG			
Alkane-degrading bacteria	*alkB*	alkBf :AAYACNGCNCAYGARCTN GGNCAYAA	55	550	
		alkBr:GCRTGRTGRTCNGARTGNCGYTG			
Alcanivorax	16S rRNA	Alcvx464F: GAGTACTTGACGTTACCT ACAG	60	220	
		Alcvx675R: ACCGGAAATTCCACCTC			

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(Tong et al. 2019). The abundances of functional genes and physicochemical parameters were set as environmental factors (Liu et al. 2016). The relationships between functional genes and environmental factors, including the ammonium, nitrite, nitrate, and oil contents were determined by redundancy analysis (RDA). Both PCA and RDA were conducted using the CANOCO4.5 software (Microcomputer Power, Ithaca, NY, USA) (Chen et al. 2013). Cluster analysis was conducted based on the weighted UniFrac distance to

compare the microbial communities in the different soil samples (Lozupone and Knight 2005).

Results and discussion

Bioremediation and nitrogen transformation characteristics

The variations in the oil contents of the soil are shown in Fig. 2a. Although the curves varied under the heterogeneity of the oil distribution, after 125 days of remediation, the oil content in the BA and BS blocks decreased
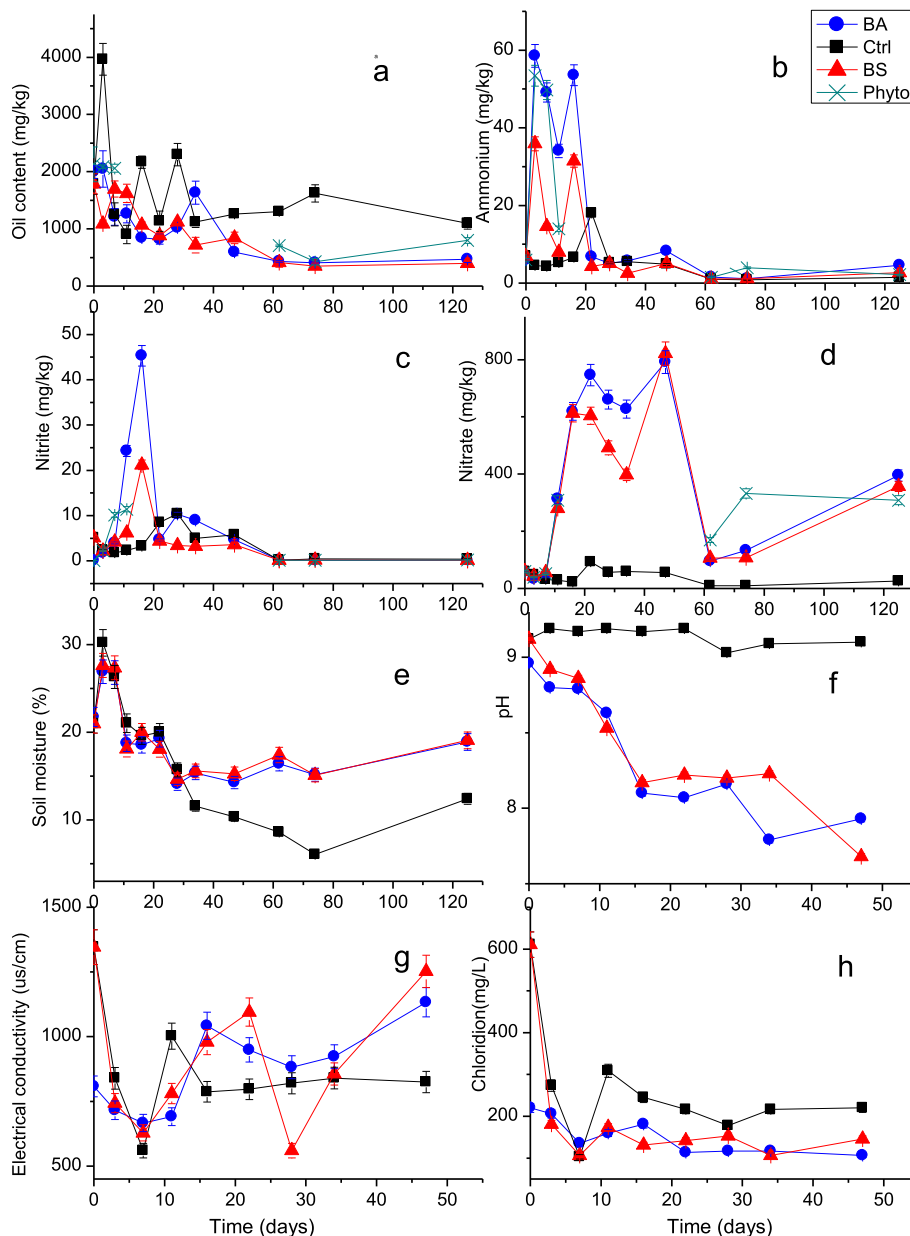
Fig. 2 Variations in **a** oil content, **b–d** nitrogen concentration, and **e–h** other physical and chemical parameters. A five-point sampling, coning, and quartering method was used to collect samples, thus a mixed sample was collected in each block at each time. Error bars present the standard deviation (SD) of test results (*n* = 2)

from 2000 to approximately 500 mg kg^{-1}, which is a level similar to that in the soil sampled in the nearby farmland (F_oya and F_ty). The oil contents in the Phyto and Ctrl blocks also decreased but with a lower decrease rate. The oil-removal efficiencies were approximately 78%, 77%, 60%, and 46% in the BA, BS, Phyto, and Ctrl blocks, respectively. The BA and BS treatments yielded the same degradation efficiency. This suggests that the added microorganism, *Arthrobacter*, played a negligible role, which is consistent with some studies (Wu et al. 2016; Yu et al. 2005). However, some other studies found that bioaugmentation with exogenous bacteria may enhance the degradation (Bento et al. 2005; Bidja Abena et al. 2019; Roy et al. 2018). The contradictory results may be caused by the different soil characteristics, different strains and their concentrations, and different experimental conditions. The saline-alkaline soil and changing temperature and moisture of the field experiment may have been unsuitable for the survival of our added microorganism.

The differences between the oil-removal in BA (or BS) and Ctrl blocks were approximately equal to the calculated oil consumed by the added nutrient stimulated microorganisms according to the optimal C:N:P ratio (100:15:1). These findings suggest that the added C:N:P ratio was suitable for the field. Another factor, aeration by plowing, may have increased the difference in oil-removal efficiencies between BA (BS) blocks and the control block, as there was no additional plowing other than the blending at the beginning. The Phyto block was also not turned over for the growth of grass and had the lowest oil-removal efficiency.

The oil content in the Ctrl block dramatically fluctuated, which may have been due to the heterogeneity of the oil content in the field and the soil not being turned over as much as in the BA and BS blocks. The decreasing oil content in the block may have been caused by natural attenuation, which was mainly due to microorganisms (Agnello et al. 2016). The initial turning over the soil may supply oxygen.

The forms of nitrogen dramatically changed once ammonium were added to the oil-contaminated soil. The ammonium (Fig. 2b) concentration decreased, while that of nitrate (Fig. 2d) increased from about 50 to 300 mg/L during the first 10 days. The nitrate concentrations peaked (about 600 mg/L) on days 16 and 22 in the BS and BA blocks, respectively, and peaked again (about 800 mg/L) on day 47 in both blocks. After day 47, the nitrate concentrations sharply decreased and reached a low point (about 100 mg/L) on day 62, gradually increasing thereafter. Nitrite (Fig. 2c) is formed during the process, and its concentration peaked on day 16 in both the BS and BA blocks. Ammonium and nitrite were gradually

eliminated in the final days of the experiment. The nitrogen concentration, including that in ammonium, nitrate, and nitrite, in the BA block was greater than in the BS block for the culture medium containing nitrogen. The Phyto block was similar to the BA block. Figure 2 also suggests that some nitrogen was imported into this block through nitrogen fixation by microorganisms and other processes, such as soil organic matter mineralization (Peoples et al. 1995). This fixed nitrogen can be used as a nitrogen source for hydrocarbon biodegradation. This result may also be explained by the volatilization of the hydrocarbons and photolysis (Fine et al. 1997), which were promoted on summer days by the high temperature and strong light intensity.
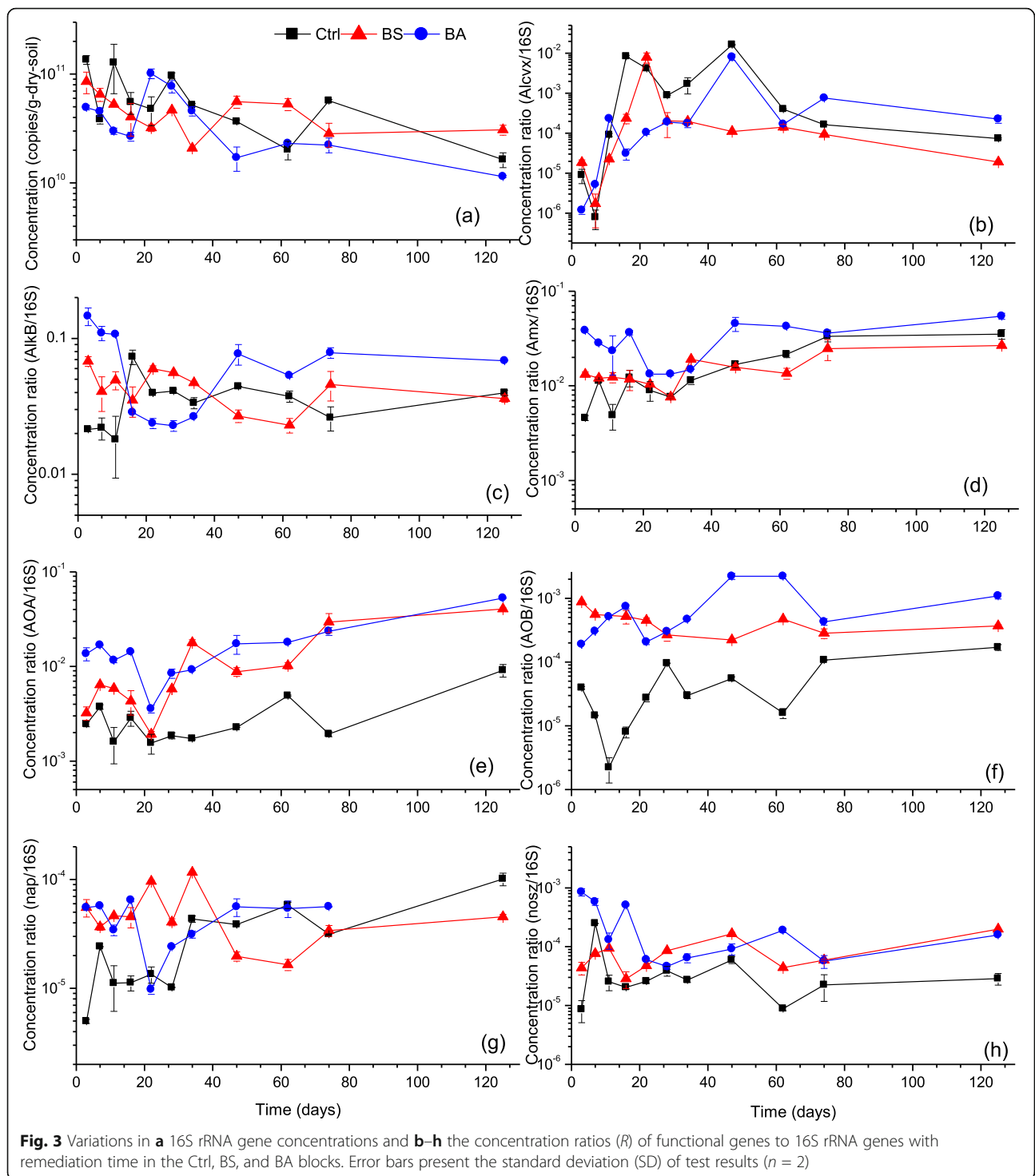
The moisture content (Fig. 2e) in the BA and BS blocks was maintained at approximately 15% after day 28, while the moisture content in the Ctrl block was below 15% during that period. The pH (Fig. 2f) dramatically decreased in the BA and BS blocks due to the acidity of the nutrient solution, whereas that of the Ctrl block remained the same. The amplitudes of the variations in the electrical conductivity (Fig. 2g) and chloride ion concentration (Fig. 2h) were small in all the blocks, except in the initial samples.

Functional genes diagnosis

The 16S rRNA concentration and the ratio of functional genes to 16S rRNA are shown in Fig. 3. This figure shows that in most cases, nitrogen cycle-related genes, *amoA* (AOA), *amoA* (AOB), and *nosZ* were more abundant in the BA and BS blocks than in the Ctrl block. However, the hydrocarbon-degrading bacterial genes were not all the same. The variations in the *alcvx* gene in the Ctrl, BA, and BS blocks were almost parallel. A*lkB* was more abundant in BA and BS than that in Ctrl during the first 11 days, but in the following days, *alkB* in Ctrl was more abundant than in BA or BS. The 16S rRNA gene concentrations in the BA and BS blocks both decreased during the first 11 days but increased after nutrients were added. The concentrations then either decreased or remained the same concentrations. The 16S rRNA gene concentrations in the Ctrl block exhibited a decreasing trend.

The relationship between the functional genes and environmental factors is shown in the RDA plot (Fig. 4). All the genes except *alcvx* had close relationships with the ammonium and oil contents.

The nitrogen concentration variation and formation, as well as the functional genes, indicate that microorganisms related to nitrogen transformation remained active in the oil-contaminated soil. This finding is consistent with that of previous studies (Xu et al. 1995). Figure 4 shows that nitrogen transformation

Fig. 3 Variations in **a** 16S rRNA gene concentrations and **b**–**h** the concentration ratios (*R*) of functional genes to 16S rRNA genes with remediation time in the Ctrl, BS, and BA blocks. Error bars present the standard deviation (SD) of test results ($n = 2$)

bacteria had a close relationship with hydrocarbon degradation.

Chloride ions (Cl^-) cannot be metabolized by microorganisms or plants, adsorbed by soil particles or easily formed by precipitation, and are often used as a tracer of soil water movement (White and Broadley 2001). Except for the initial raining days, the chloride ion concentrations

(Fig. 2h) in the BA and BS blocks were almost constant throughout the remediation process. This finding indicated that the added nitrogen had not seeped into the lower soil. Therefore, the decrease in inorganic nitrogen, including that in ammonium, nitrite, and nitrate, was mainly caused by the volatilization of ammonium and microbial mediation. Nitrification caused a dramatic

Fig. 4 RDA biplot analysis of the functional genes and environmental variables
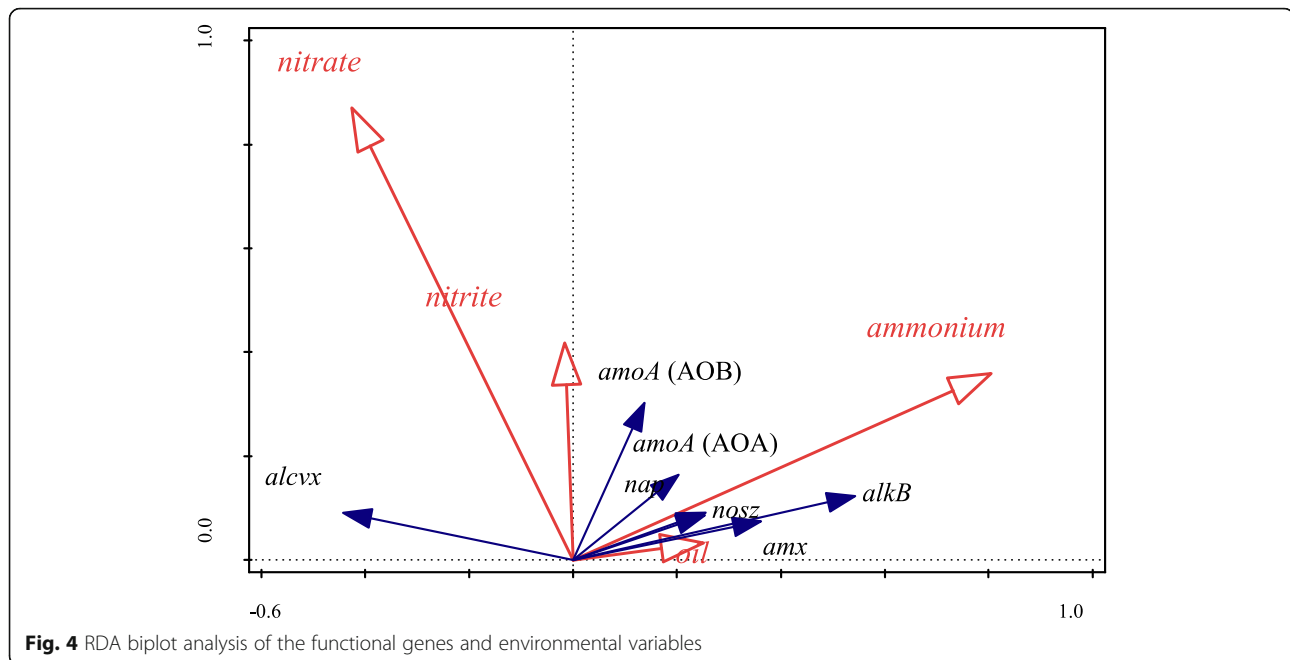
decrease in ammonium and an increase in nitrate after the nutrients were added (Wde and Kowalchuk 2001). The nitrate concentrations dramatically decreased during the days after nutrients were added, which may have been due to the denitrification and assimilation of microorganisms (Cabrera et al. 2016). The variations in related functional genes were consistent with the nitrogen variations.

Most microorganisms, including hydrocarbon-degrading bacteria, consistently use ammonium as their optimal nitrogen source (Hasinger et al. 2012; Kern et al. 2017). Figure 4 shows that all the genes except the *alcvx* gene had close relationships with ammonium. Nitrification is a natural process that has been considered for bioremediation for many years (Sayavedra-Soto et al. 2010). The nitrification gene *amoA* is positively correlated with oil content, and *alkB* is crucial in hydrocarbon degradation (Viggor et al. 2015). Nitrate can be used as an electron acceptor, and hydrocarbons can be used as electron donors during denitrification (Lueders 2017). Therefore, the process of denitrification can be regarded as the hydrocarbon degradation process in oil-contaminated soil. The genes involved in denitrification, including *nap* and *nosz*, had positive correlations with oil content and *alkB* gene expression. Consequently, these processes of nitrogen transformation are conducive to oil degradation. The qPCR of the *alcvx* gene targeted the genus *Alcanivorax*, which contains alkane-degrading species (Barbato et al. 2015). *Alcanivorax* always correlates with a high nitrate level (Mulla et al. 2017), which is consistent with our findings.

Microbial community diagnosis

The coverage of this sequencing was greater than 0.97, which suggests that the results of the sequencing were reliable. The abundance distributions of microorganisms are shown in Fig. 5.

The sum of *Pseudomonas*, *Acinetobacter*, and unclassified bacteria accounted for more than half of the total bacteria in all the samples. The abundance of *Pseudomonas* was greater than that of any other bacteria. The added bacteria *Arthrobacter*, isolated from the field soil and used for bioaugmentation, was only present in the F_ty, BA_11d, and BS_47d samples, and the relative abundances were only 0.03%, 0.01%, and 0.01%, respectively. The percentages of unclassified bacteria in the contaminated soils were greater than those in the uncontaminated soils, whereas *Pseudomonas* exhibited the opposite trend. Samples F_oya and F_ty, which were collected from uncontaminated farmland in different years, had similar abundance distributions. In contrast, the abundance distributions in Ctrl_oya and Ctrl_0 samples, which were collected from the same contaminated site but in different years, were less similar.

The cluster analysis (Fig. 6) also shows similar results. The early remediation stage samples (days 3 and 11), untreated contaminated soils (Ctrl _0 and Ctrl _oya), Ctrl _125d, and farmland samples were grouped together. Except for Ctrl _125d, the later remediation stage samples (days 47 and 125) were grouped together. The samples collected at the same time from the BS and BA blocks had similar microflora.

The microbial community structure of Ctrl _125d was similar to that of the uncontaminated soils. This finding
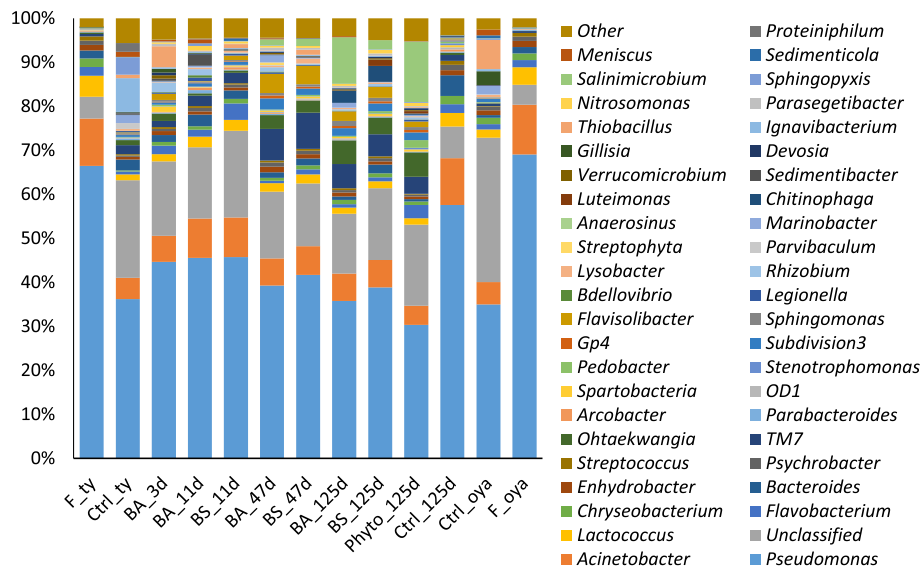
Fig. 5 Distributions of bacteria in the 13 samples at the genus level. Taxa were represented at > 0.5% frequency in at least one sample. "Others" refers to the taxa with a maximum abundance of < 0.5% in any sample. Unclassified refers to the sequences that could not be assigned to known bacterial genus. F_ty stands for the sample collected from a farmland neighbouring the contaminated field this year. BA_3d, BA_11d, BA_47d, and BA_125d represent the samples collected after 3, 11, 47, and 125 days, respectively, from the beginning of the remediation process in the BA block. BS_11d, BS_47d, and BS_125d represent the samples collected after 11, 47, and 125 days, respectively, from the beginning of the remediation process in the BS block. Phyto_125d represents the sample collected 125 days after the beginning of the remediation process in the Phyto block. Ctrl_oya is a sample that was collected in the contaminated site 1 year ago. F_oya is the sample collected from a farmland neighbouring the contaminated field 1 year ago
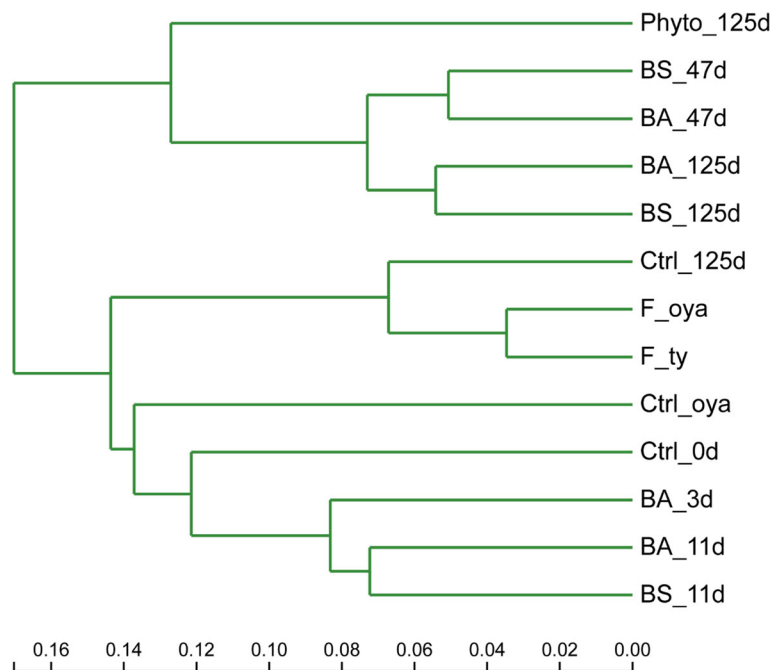
Fig. 6 Cluster analysis of the samples based on the weighted UniFrac distance and 16S rRNA genes

suggests that this sample experienced similar environmental conditions to those in the uncontaminated soils. The sample was collected at the surface of this block, which was constantly exposed to the air and sun and received no nutrient additions, which was similar to the uncontaminated soils. Nutrient and other environmental factors, such as moisture and aeration, strongly affect microorganism communities (Jungmann et al. 2015).

During the remediation processes, the variation characteristics of microbial communities, as well as the *amoA* (AOA), *amoA* (AOB), and *nosZ* genes in the BA and BS blocks were similar. In addition, except for the various nitrogen concentrations in the early remediation stage, the soils collected in the BA and BS blocks at the same time had similar nitrogen concentrations, but different physical and chemical parameters. This result suggests that the added strain played a negligible role in oil degradation and the transformation of nitrogen, which is consistent with previous research (Wu et al. 2016). Although the added, *Arthrobacter* strain was initially isolated from this contaminated field, they could barely survive alongside the existing indigenous bacteria.

Therefore, regardless of the strain added, there were no differences between the BA and BS blocks in terms of treatment effects; both had similar parameters and microbial communities.

The Phyto block, whose soil was turned over only once and had the same added nutrients as the BA and BS blocks, demonstrated characteristics similar to those in the BA and BS blocks rather than those in the Ctrl block according to samples collected on the last day. We speculate that the nutrients affected the microbial activity more than the tillage. We observed some differences between the Phyto and BA (or BS) blocks due to the undisturbed soil and the growth of Bermuda grass in the Phyto block, and plant growth in the plot may also play on a crucial role in microbial community evolution.

The PCA of the samples and microorganisms (at the genus level) is presented in Fig. 7. The sample points can be divided into the following groups: the pre-remediation-stage group (group 1), the earlier-stage group (group 2), middle- and later stage group (group 3), and uncontaminated plus Ctrl_125d sample group (group 4). The *Meniscus*, *Ignavibacterium*,
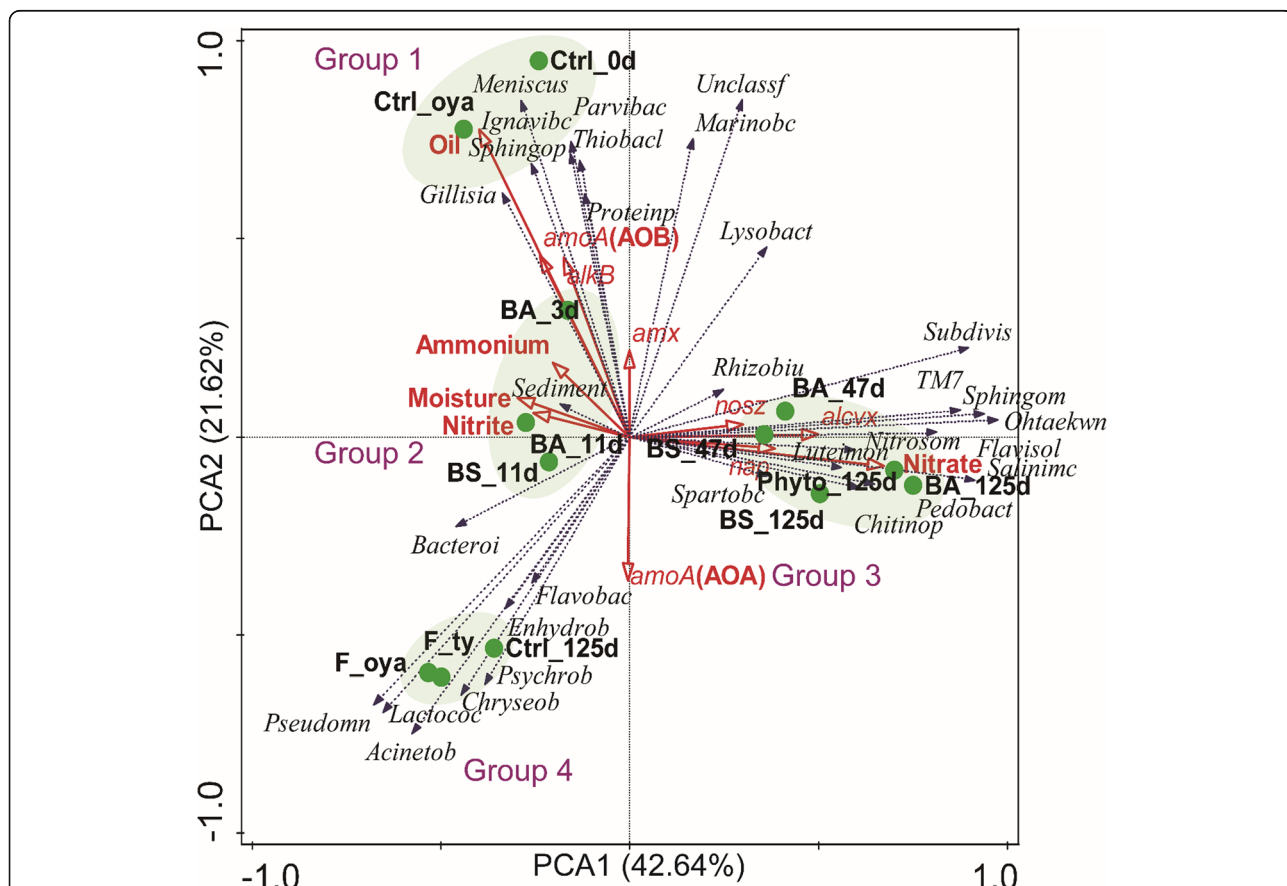
Fig. 7 PCA biplot analysis of samples and microorganisms (at the genus level). The green points represent the samples. The black rays with solid arrows represent the microorganism species. The red rays with open arrows represent the environmental factors, including the nitrogen and functional genes

Thiobacillus, *Gillisia*, *Sphingopyxis*, and *Parvibaculum* bacteria were included in the first group samples. The third group corresponded to the *Ohtaekwangia*, *Salinimicrobium*, TM7, *Sphingomonas*, *Nitrosomonas*, *Flavisolibacter*, *Luteimonas*, *Pedobacter*, *Chitinophaga*, and *Spartobacteria* bacteria. The samples in the last group had close relationships with the *Lactococcus*, *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Chryseobacterium*, and *Enhydrobacter* bacteria.

Those in the first group of samples (Fig. 7) were related to variations in hydrocarbon degradation and the oxidization of reduced sulfur compounds, which are present in crude oil. *Thiobacillus* can oxidize the reduced sulfur compounds to sulfate (Pronk et al. 1990). The abundance of *Thiobacillus* was reduced in the later samples because the added nutrients contained sulfur, which affects the oxidization of sulfur compounds. *Meniscus* and *Gillisia* are associated with hydrocarbon degradation (Guibert et al. 2012). *Ignavibacterium*, a strictly anaerobic chemoheterotroph with versatile metabolic characteristics (Sun et al. 2015), can use aniline (Sun et al. 2015) and hydrocarbons (Salam et al. 2017). *Sphingopyxis*, a crude-oil-degrading marine bacterium (Kim et al. 2014), can degrade aromatic hydrocarbons (Rodriguezr et al. 2015) associated with ammonia assimilation (Williams et al. 2009). *Parvibaculum* can oxidize alkane (Rosario-Passapera et al. 2012), linear alkylbenzene sulfonate (Schleheck et al. 2011), and polycyclic aromatic hydrocarbons (PAHs) (Lai et al. 2011). This capability is in line with the functional genes (*alkB* and *amoA* (AOB)) and environmental factors (oil and ammonium contents) shown in Fig. 7.

The second group was a transient group. The samples collected in the early days were distributed in this region. These days, adequate ammonium, moisture, and nitrite were transformed from ammonium. Except for *sedimen*, there was no specific bacterium in the group.

The third group (Fig. 7) corresponded to bacteria associated with nitrogen transformation and easily degradable hydrocarbon compounds as well as some bacteria that are sensitive to oil. *Ohtaekwangia*, a nitrifying organism, can oxidize ammonium to nitrate (Rodríguez-Caballero et al. 2017) and can metabolize easily degradable organic matter (Li et al. 2014). *Salinimicrobium* could barely survive in the highly contaminated soil because it is sensitive to petroleum hydrocarbons. As a result, these bacteria can be used as oil contamination indicators (Wang 2011). The BA125, Phyto125, and BS125 samples, which were collected in the final sampling, were teeming with these types of bacteria, which indicate that the oil content had sufficiently decreased in these treated blocks. TM7 and *Spartobacteria* genera *incertae sedis* are associated with hydrocarbon degradation (Huang and Li 2014; Salam et al. 2017; Song et al.

2015). *Sphingomonas* strains are capable of metabolizing polycyclic aromatic hydrocarbons under low nutrient conditions (Ye et al. 2006; Zylstra and Kim 1997). *Nitrosomonas*, a nitrifier organism, can oxidize ammonium to nitrate (Suzuki et al. 1974) and can nitrify and denitrify at the same time when grown under oxygen limitation (Bock et al. 1995). The *Flavisolibacter* level has significant positive relationships with a number of denitrification genes (Huang et al. 2016). *Luteimonas* has the ability to reduce nitrates to nitrogen (Young et al. 2007), *Pedobacter* has the ability to degrade oil hydrocarbons (Margesin et al. 2003; Zhang et al. 2010), and *Chitinophaga* has the ability to reduce nitrate (Kim and Jung 2007). These processes were also consistent with the functional genes and environmental factors present.

The samples in the last group had close relationships with bacteria that had few or negative correlations with oil and nitrogen. *Pseudomonas* and *Acinetobacter* occupied larger proportions than any other bacteria in all the samples, especially in the fourth group. *Pseudomonas* is a genus of aerobic bacteria that contains 191 validly described species. This genus includes human pathogens, plant pathogens, soil bacteria, and plant growth-promoting bacteria (EUZéBY 1997) that can also degrade hydrocarbons (O'Mahony et al. 2006). The high abundance of *Pseudomonas* in uncontaminated soil may have been caused by the crops and the lack of influence of oil. *Acinetobacter* (Doughari et al. 2011) and *Chryseobacterium* (Bernardet et al. 2015) are widely distributed in nature and commonly occur in soil and water, but oil and anaerobic environments may harm them. *Lactococcus* produces a single product, lactic acid, as the major or only product of glucose fermentation (Crisan and Jay 2005), and glucose may exist in crops not contaminated by crude oil. *Psychrobacter* is oxidase-positive, with a strictly oxidative metabolism, and they are moderately halotolerant (Bozal et al. 2003). In consequence, the presence of salt in the contaminated soil may have affected their survival.

Relationships between oil biodegradation and nitrogen transformation

The above discussion suggests that the process of oil biodegradation is always accompanied by nitrogen transformations, such as denitrification or nitrification. Denitrification is one of the important hydrocarbon biodegradation processes under anaerobic conditions (Hutchins et al. 1991). The study field was under aerobic conditions because there was adequate available oxygen from the frequently turned over soil, and the ammonium was transformed into nitrite and nitrate rapidly through nitrification. Therefore, the denitrification in the soil could be identified as aerobic denitrification. Previous studies showed aerobic denitrifying bacteria degrade

hydrocarbons (Kwapisz et al. 2008) but they lacked field data. This study may supply the data to support this finding.

Studies showed that the petroleum hydrocarbon may slow down nitrification (Chang and Weaver 1997; Deni and Penninckx 1999), but no report has shown that oil biodegradation is hindered by the presence of ammonium, even if both of the processes consume oxygen and may compete for the limited oxygen. Some studies showed that a few pure cultured nitrifying bacteria can oxidize a wide variety of hydrocarbon substrates through the action of ammonia monooxygenase, the key enzyme for nitrification (Chang et al. 2002; Deni and Penninckx 1999). Our results show that nitrification has no negative effect on oil biodegradation. Although *alkB* and *amoA* had a positive relationship, abundances of both in the oil biodegradation and nitrification microorganisms were promoted by ammonium. Thus, future research should consider whether nitrification has a positive effect on oil biodegradation in the field.

Our findings showed that the added ammonium promoted the oil biodegradation. There are two potential reasons for this finding. Firstly, ammonium supplied the nitrogen source for the biodegradation microorganisms. Secondly, ammonium may transform into nitrate by nitrification, and the nitrate may serve as the electron acceptor for biodegradation through denitrification.

Practical application suggestion

The current study and many previous studies revealed that contaminated oil exploit sites are always accompanied by saline and alkaline stress (Liu et al. 2019; Liu et al. 2014; Wang et al. 2012). Therefore, the added alien creatures, including microorganisms and plants, struggle to survive in the saline-alkaline environment. Cultivating biodegradation microorganisms and plants is laborious and time-consuming. Therefore, bioaugmentation and phytoremediation methods are not recommended. From this study, biostimulation with ammonium and phosphate could be a good choice for site remediation in practical applications.

Conclusions

The nutrients added to oil-contaminated soil stimulated a variety of microorganisms, including hydrocarbon-degrading bacteria and nitrogen metabolizing microorganisms. The functional genes in nitrogen metabolism, such as *amx*, *amoA*, *nap*, and *nos*, showed a positive correlation with the alkane monooxygenase gene *alkB*. Thus, aerobic denitrification may occur in the field, which could be helpful for oil biodegradation. Phytoremediation and bioaugmentation nearly failed in the field, potentially because the environment was unsuitable for the survival of added bacterial strains, *Arthrobacter*, and

alfalfa. Biostimulation was found to be the most suitable bioremediation method in the field and could have wide suitability because no new organisms are introduced to the contaminated soil. Our findings improve our knowledge about the interactions between microorganisms and edaphic parameters and offer a feasible approach to obtain useful bioremediation information and assist in the development of appropriate remediation procedures.

Ethics approval and consent to participate
Not applicable.

Authors' contributions
Pingping Cai carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Zhuo Ning designed the study and carried out the experiments. Yaci Liu and Ze He participated in the experiments and data analyses. Manlan Niu and Jiansheng Shi participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no conflict of interest.

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