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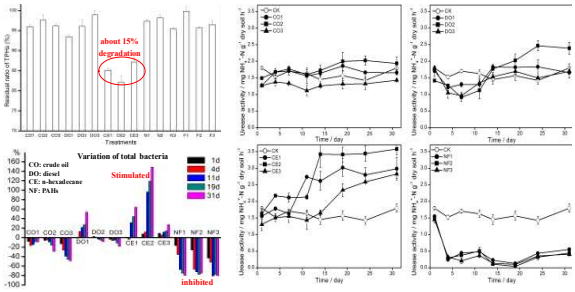

Monitoring micro-ecology of soil being contaminated by different PHCs through quantification of relevant genes and enzymatic activities.

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ARTICLE

Ecological and enzymatic responses to petroleum contaminations

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The changes in microbial ecology interpreted from taxonomic and functional genes and biological functions represented by urease and dehydrogenase activities were monitored to soil being contaminated by different petroleum hydrocarbons including crude oil, diesel, *n*-hexadecane and poly-aromatic hydrocarbons (PAHs). It was shown that the presence of *n*-hexadecane distinctly stimulated the activity of indigenous microorganisms, especially alkane degrading bacteria, and led to over 20% degradation of *n*-hexadecane within one month while no obvious degradation of the other three kinds of petroleum hydrocarbons. The simulation effect was most remarkable in the soil spiked with a medium concentration (2500 mg·kg^{-1} dry soil) of *n*-hexadecane. On the contrary, the presence of PAHs completely inhibited above-mentioned bioactivities of the soil. The content of PAH degrading bacteria, however, increased over 10-fold, indicating the selection effect of PAHs on soil bacteria. The impacts of diesel and crude oil on the microbial ecology and biological functions varied significantly with their concentration. The disclosure of the ecological and enzymatic responses is also helpful to soil bioremediation.

Environmental impact

The understanding of the impacts of different hydrocarbons on soil function and microbial community is essential to both the assessment of contamination and the subsequent remediation. The established results are obtained through the comparison of contaminated soil against the normal soil but are limited to those exposed to petroleum hydrocarbons for a long time. This study performed a real time monitoring of soil being contaminated by different petroleum hydrocarbons through the amounts of taxonomic and functional genes and the urease and dehydrogenase activities. The parallel display of microbial ecology and the biological function of soil are helpful to the design and implementation of soil bioremediation.

Introduction

While the oil remains one of the major energy and chemical resources, the worldwide exploitation, refining, transportation and employment of petroleum and petroleum products make petroleum hydrocarbons (PHCs) widespread contaminants in this globe. Crude petroleum consists of linear and branched chain alkanes, cycloalkanes and aromatics as dominant constituents, and also small amounts of nitrogen-, oxygen- and sulfur- containing compounds, such as pyridine, phenol and thioether, respectively.[1] Some components, mainly polycyclic aromatic hydrocarbons are hazardous xenobiotics,[2] showing potential teratogenic, carcinogenic and mutagenic activities,[3] and threaten human health when accumulating in crops.

Microbes in soil are crucial to ecosystem functioning, like decomposition, biomass transfer and biogeochemical cycling (for instance, nitrogen cycling).[4] Degradation of contaminants is one of the most important functions performed by microorganisms. The addition of exogenous microbes with degrading capacities has been proposed as an acclaimed method (called 'bioaugmentation') for the remediation of petroleum contaminated soils.[5-7] On the other hand, soil microorganisms are the best indicators of soil contamination because of their highly sensitive to environmental perturbation.[8] Soil enzyme activities and microbial community structure and diversity are the two most important aspects to evaluate the soil microorganisms and their biological functions and thus being applied to assess the contamination by hydrocarbon contaminants and the evaluation of remediation practices.[3,9 - 14] Recent advances in molecular biological techniques have enabled rapid profiling of microbial ecosystems at genetic level.

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PCR-DGGE and gene clone library were applied by Wu to assess the biodiversity of bacteria, fungi and microorganisms involved in nitrogen cycle in crude oil contaminated soils before and after bioremediation.[12, 15] While many of these techniques were applied to obtain qualitative biodiversity data through the comparison of contaminated soil against the normal soil, [16 - 18] the 'real-time' tracking and quantification of functional microbes during the contamination processes were rarely reported.

Real-time PCR or quantitative PCR (Q-PCR) enables the quantification of taxonomic and functional gene copy numbers of samples from soil. Real-time PCR-based analyses can combine traditional end-point PCR with fluorescent detection technologies to record the accumulation of fluorescent signal in real time during each cycle of the PCR amplification. The proportionality between the fluorescent signal and the starting concentration of template enables the quantification of gene numbers. Real-time PCR has been shown to be a highly reproducible and sensitive method to quantitatively monitor changes of taxonomic and functional genes under multifarious experimental and field environments.[19-22]

The objective of this study is to monitor the dynamic changes in soil microbial ecology in terms of taxonomic and functional bacterial numbers by real-time PCR of relevant genes and, simultaneously, the soil dehydrogenase and urease activities of the soil being contaminated by different petroleum hydrocarbons. These ecological and enzymatic responses are expected to assist the assessment, design and implementation of soil bioremediation.

Materials and methods

Soil treatment and sampling

Soils used for simulated systems were collected from a parterre (0-20 cm down from the surface) in Tsinghua University (Beijing, China, 116.34 °E 40.01 °N). The physicochemical properties were determined at the Beijing Academy of Agricultural and Forestry Sciences. The results are presented in Table 1. The soils were first sieved by a 2 mm diameter sifter, and then divided evenly into 13 samples, each of which was placed in a ϕ15 cm × 20 cm cylindrical container to form a 10 cm-depth microcosm. Container 1 with natural soil was used as an uncontaminated control (using "CK" as an abbreviation). Soils in container 2, 3 and 4 were artificially contaminated by crude oil with content of 2,000, 7,500 and 12,500 mg·kg^{-1} dry soil, denoted as CO1, CO2 and CO3, respectively. Soils in container 5, 6 and 7 were contaminated by commercial 0# diesel oil with content of 1,500, 7,000 and 10,000 mg·kg^{-1} dry soil, denoted as DO1, DO2 and DO3, respectively. Soils in container 8, 9 and 10 were contaminated by *n*-hexadecane with content of 800, 2,500 and 6,000 mg·kg^{-1} dry soil, denoted as CE1, CE2 and CE3, respectively. Soils in container 11, 12, and 13, denoted as NF1, NF2 and NF3, were contaminated by the mixture of naphthalene and phenanthrene, in which the contents of naphthalene were 400, 2,000 and 4,000 mg·kg^{-1} dry soil and those of phenanthrene were 800, 2,500 and 6,000 mg·kg^{-1} dry soil, respectively. Above-mentioned starting concentrations of petroleum components were chosen according to the peak concentration of crude oil determined in our previous field practice, which reached 15,000 mg·kg^{-1} dry soil, and the average fractionation composition of crude oil. All contaminated soils and their uncontaminated control were incubated at 25 ℃ with soil moisture maintained at 60% of capillary water capacity. At the end of 1, 4, 7, 11, 14, 19, 24 and 31 days, 20 g of soil samples were collected from 10 points at the superstratum and substratum from each treatment. Each sample was separated by two parts, one of 5 g was stored at -20 ℃ until DNA extraction, and the other of 15 g was stored at 4 ℃ for total petroleum hydrocarbons (TPHs) and enzyme activities measurement. All above-mentioned treatments were triplicated.

Determination of total petroleum hydrocarbons

The total petroleum hydrocarbons in soil samples were extracted according to the procedure recommended by U.S. Environmental Protection Agency (EPA).[23] A sample of 2 g dry soil and 25 mL of hexane/acetone mixture (1:1 v/v) were mixed and subjected to microwave extraction in a microwave accelerated solvent extraction system (CEM Corporation, USA) at 120 ℃ for 30 min. After filtration, the residue was washed with 10 mL hexane/acetone mixture and filtrated again. The filtrate obtained in above two steps was collected together. THPs were quantified by a gas chromatograph with a flame ionization detector (GC-FID, SHIMADZU, Japan) equipped with a ϕ 0.53 mm × 30 m capillary column (Ultra Alloy DX30, Frontier Lab, Japan). The operation program of the GC-FID for the TPH analysis was started with both injector and detector temperatures of 300 ℃. Oven temperature was programmed from initial 40 ℃ (held for 2 min) to 290 ℃ in the speed of 15 ℃·min^{-1} and then kept at 290 ℃ for 5 min.

Determination of soil enzyme activities

Urease (Ure) activity was determined by a modified method according to literature[13]. Briefly, 2 g of soil sample was moistened with 600 μL toluene for 15 min in 50 mL centrifuge tubes, followed by adding 2 mL of 10% urea and 4 mL of citrate buffer (pH 6.7). Then the samples were incubated at 37 ℃ in a 145 r·min^{-1} shaker for 24 h, diluted to 20 mL with preheated water (37 ℃) and oscillated thoroughly and subjected to an immediate filtration. The filtrate of 500 μL was transferred into a 25 mL volumetric flask containing 5 mL distilled water; 2 mL sodium phenate and 1.5 mL sodium hypochlorite with the active chlorine of 0.9% were added. The flask was left for 20 min and then diluted to the given volume. The absorbance at 578 nm of the blue-colour solution was determined and the urease activity was expressed as mg NH_4^+-N · g^{-1} dry soil · h^{-1}. All samples were treated in triplicate.

The assay of dehydrogenase (Deh) activity was developed according to literature[9]. Briefly, 1g of soil sample was mixed with 2 mL of 0.1 M glucose solution and 2 mL of 0.5% 2,3,4-triphenyltetrazolium chloride (TTC) solution, and incubated at

Table 1 Physicochemical properties of soil samples

Soil texture (%)			Organic matter	Total nitrogen	Ammonium nitrogen	Nitrate nitrogen	Available phosphorus	Soil pH	Conductivity
sand	silt	clay	(g/kg)	(g/kg)	(mg/kg)	(mg/kg)	(mg/kg)		(μS/cm)
27.8	55.7	16.5	20.9	1.56	6.88	18.1	13.4	7.89	106

37 ℃ in a 145 r·min^{-1} shaker for 16 h. The reaction product, reduced triphenylformanzan (TPF), was extracted with acetone. After filtration against MS® PTFE Syringe Filter, the absorbance at 485 nm was determined and the dehydrogenase activity was expressed as μg TPF · g^{-1} dry soil. All samples were triplicated.

Extraction of total soil DNA

DNA extraction from 0.25 g of the soil samples was performed using PowerSoil® DNA Isolation Kit (MoBio Laboratories, USA). The extracted total DNA was then subjected to electrophoresis on 0.8% agarose gel, after which it was stored at -20 ℃ until further PCR amplification and real-time PCR analysis.

Primers and conventional PCR

PCR primers for analysis of taxonomic and functional genes were selected according to literatures.[24 - 29] The taxon-specific primer pairs were used to amplify fragments of 16S rRNA genes in γ-Proteobacteria, Actinobacteria and Bacteroidetes. The functional gene primer pairs were used to amplify fragments of genes encoding the key enzymes in nitrogen fixation (*nifH*), denitrification (*narG*), ammonia oxidation (*amoA*), alkane degradation (*alkB*), and naphthalene degradation (*nah*). All primer pairs were listed in Table 2.

Conventional PCR was performed in 25 μL reaction volume containing 12.5 μL of 2 × PCR Master Mix (TAKARA, Japan), 0.4 μM of each primer, and 1 μL of template DNA. The amplifications of taxonomic genes were performed with the following temperature profiles: (i) an initial denaturing step of 5 min at 95 ℃; (ii) 30 cycles, with one cycle consisting of 95 ℃ for 15 s, 61.5 ℃ for 15 s and 72 ℃ for 30 s; and (iii) a final extension step of 10 min at 72 ℃. The PCR protocols for functional genes were the same as in the literatures. All experiments included negative controls without template DNA. PCR products were detected by 1.5% agarose gels.

Standard curves

The PCR products were purified using a TIANQuick Midi Purification Kit (TIANGEN, China), ligated with the pMD19-T cloning vector (TAKARA, Japan) and transformed into DH5α (TIANGEN, China) competent cell. Transformants were selected on LB agar plates containing ampicillin (final concentration of 50 μg·mL^{-1}), 4 μL of 200 mg·mL^{-1} IPTG and 40 μL of 20 mg·mL^{-1} X-gal. Positive clones confirmed by colony PCR were inoculated into LB liquid medium with ampicillin (50 μg·mL^{-1}), and incubated at 37 ℃, 145 r·min^{-1} for 12 h. The plasmid DNA was extracted using a Plasmid Miniprep Kit (Biomiga, USA). The concentrations of plasmids were determined by a NANODROP 1000 Spectrophotometer (Thermo Scientific, Rockwood, TN, USA). Ten-fold serial dilution was conducted to construct standard plasmid DNA with 10^3 to 10^8 gene copies·μL^{-1}. Standard curves for the taxonomic and functional genes were generated by plotting the threshold cycle (*Ct*) values versus the logarithmic values of gene copy numbers.

Table 2 Primer pairs used in this research.

Target	Name	Sequence	Length
16S rDNA[24]			
Universal	926f	AAACTCAAAKGAATTGACGG	154 bp
	1062r	CTCACRRCACGAGCTGAC	
γ-Proteobacteria	r1080f	TCGTCAGCTCGTGTYGTGA	138 bp
	r1020r	CGTAAGGGCCATGATG	
Actinobacteria	act-f	TACGGCCGCAAGGCTA	299 bp
	act-r	TCRTCCCCACCTTCCTCCG	
Bacteroidetes	bact-f	CRAACAGGATTAGATACCCT	188 bp
	bact-r	GGTAAGGTTCCTCGCGTAT	
nifH[25]	nifH-f	TGCGAYCCSAARGCBGACTC	360 bp
	nifH-r	ATSGCCATCATYTCRCCGGA	
amoA[26]	amoA-f	GGGGTTTCTACTGGTGGT	491 bp
	amoA-r	CCCCTCKGSAAAGCCTTCTTC	
narG[27]	narG-f	TAYGTSGGGCAGGARAAACTG	110 bp
	narG-r	CGTAGAAGAAGCTGGTGCTGT	
alkB[28]	alkB-f	AACTACMTCGARCAYTACGG	100 bp
	alkB-r	TGAMGATGTGGTYRCTGTTCC	
nah[29]	nah-f	CAAAARCACCTGATTYATGG	377 bp
	nah-r	AYRCGRGSGACTTCTTTCAA	

Real-time PCR assays

Real-time PCR was performed in 20 μL reaction volume containing 10 μL of 2 × SYBR Green PCR Mix (TOYOBO, Japan), 0.2 μM of each primer, and 1 μL of template DNA on a 7300 Real Time PCR System (ABI, USA). The real-time PCR protocols were the same as conventional PCR, except for the following procedures: (i) 40 cycles were implemented in the second step, and (ii) the melting curve step with 15 s at 95 ℃, 1 min at 60 ℃ and 15 s at 95 ℃ instead of the final extension step was carried out. Data collection took place during the extension step of the reaction. All reactions were conducted in triplicate.

Statistical analysis

The mean value and standard deviation of three replicates of the total petroleum hydrocarbons, soil enzyme activities, and taxonomic and functional gene numbers were obtained. The mean values were compared by one-way analysis of variance at a level of $P \leq 0.05$. All statistical analyses were conducted using SPSS 19.0.

Results and discussion

Degradation of TPHs

Figure 1 shows the residual TPHs concentration in soil after 31-day degradation. Considering the volatilities of petroleum hydrocarbons, the natural evaporation is subtracted from results. It is seen from Figure 1 that the degradation of TPHs in soils contaminated by crude oil (CO1-CO3), diesel oil (DO1-DO3) and polycyclic aromatic hydrocarbons (PAHs) was marginal compared to that in *n*-hexadecane contaminated soils (CE1-CE3), in which the degradation reached 15% to 20%.

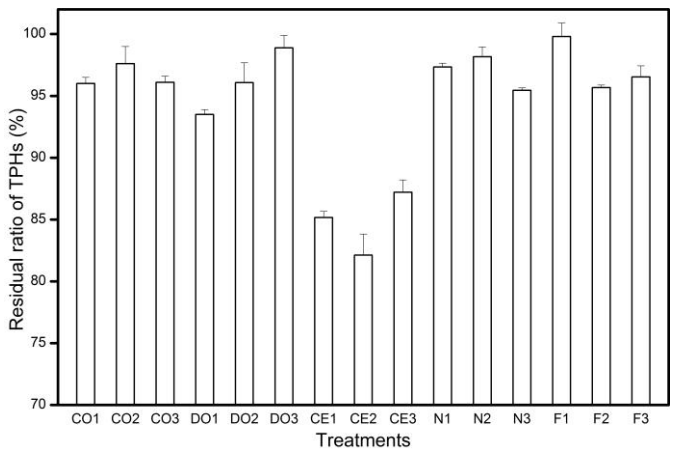
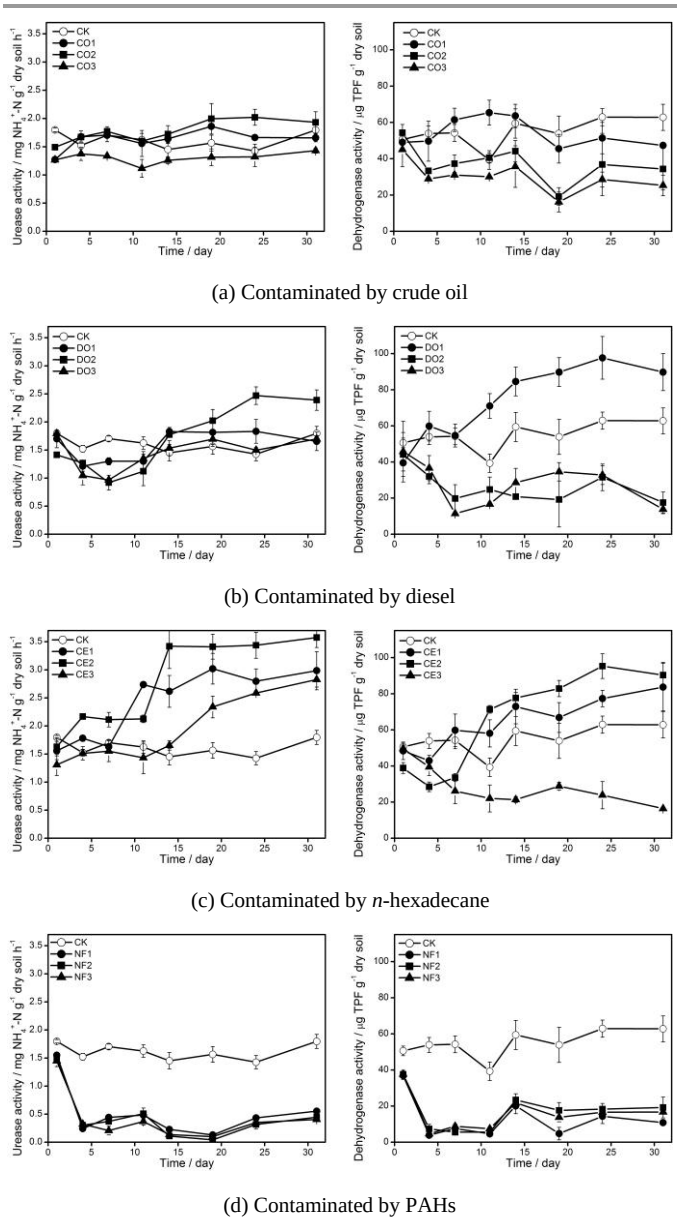


Fig. 1 The ratio of residual of different TPHs after 31 days incubation

Soil enzyme activities

The activities of urease and dehydrogenase in above mentioned soil samples were monitored and shown in Figure 2.

As shown in Figure 2(a), the presence of crude oil with concentrations ranged from 2,000 to 25,000 mg·kg^{-1} showed no effect on the urease activity, even after 31 days. On the other hand, the presence of crude oil at concentration exceeding 7,500 mg·kg^{-1} led to a reduction of dehydrogenase activity. The presence of crude oil of low concentration, *i.e.*, 2,000 mg·kg^{-1}, also showed no effect on dehydrogenase activity. For diesel-contaminated soil, as shown in Figure 2(b), the presence of diesel with concentration of 1,500 mg·kg^{-1} did not altered urease activity. A higher diesel content, *e.g.* 7,000 mg·kg^{-1}, however, led to firstly a reduction and, after 15 days, an increase in the urease activity. The increase in the dehydrogenase activity in the presence of diesel with low concentration, *i.e.*, 1,500 mg·kg^{-1}, indicated the stimulation effect on indigenous microorganism. Once the diesel concentration reached 7,000 and 15,000 mg·kg^{-1}, however, inhibition effect took place. Figure 2(c) shows the effect of *n*-hexadecane on urease and dehydrogenase activity. Here the presence of *n*-hexadecane from 800 to 2,500 mg·kg^{-1} enhanced both urease and dehydrogenase activities. In the presence of high concentration of *n*-hexadecane (6,000 mg·kg^{-1}), however, urease activity was still stimulated while dehydrogenase activity was inhibited. Compared with crude oil and diesel, *n*-hexadecane is preferred by indigenous microorganism. This can



(a) Contaminated by crude oil

(b) Contaminated by diesel

(c) Contaminated by *n*-hexadecane

(d) Contaminated by PAHs

Figure 2 Enzyme activity of contaminated soil (left: Ure; right: Deh)

be used for screening petroleum-degradable microorganism or as simulant for biostimulation. As shown in Figure 3(d), the presence of PAHs inhibited the activity of urease (by 80% within 4 days) and dehydrogenase, indicating that PAHs are harmful to indigenous microorganisms, as reported elsewhere.[30]

As a kind of amidase, urease transforms organic nitrogen to ammonium nitrogen by catalysing the hydrolyzation of amido bond in organic substance, thus improves nitrogen nutritional status in soil. The inhibition of urease activity by crude petroleum has been reported elsewhere.[13,31,32] Margesin[33] found that soil urease activity was stimulated by diesel while inhibited by PAHs. The results in this study further indicated a correlation between the type and concentration of pollutant and its effect on urease activity. The stimulating effect shown by *n*-hexadecane might be ascribed to the tolerance of urease-related

indigenous microorganisms and, moreover, the carbon and energy resource provided by *n*-hexadecane. Due to the high susceptibility to PAHs and high concentration of petroleum, the urease activity may be used as a biochemical indicator of heavy petroleum contamination.[34,35]

Dehydrogenase is a key enzyme involved in microbial metabolism, through which we can intuitively understand the metabolic activity of soil microorganisms. Diversified results about the impact of petroleum on soil dehydrogenase activity are available up to date. Wyszkowska and Wyszkowski[11] showed that petroleum contamination inhibited dehydrogenase activity. Megharaj[36] showed that inhibition of dehydrogenase activity was greater in soil polluted with petroleum hydrocarbons of moderate extent than at a high content. In contrast, the positive impact of petroleum and diesel on soil dehydrogenase activity are also reported.[3,9,10] This discrepancy might be ascribed to the different experimental conditions. Our results showed that the alteration of dehydrogenase activity caused by petroleum pollution varied with the type of petroleum hydrocarbons, which agrees with that obtained by Frankenberger *et al.*.[37] The stimulation of soil dehydrogenase activity in low concentration of diesel and *n*-hexadecane contaminated soils indicates that they been easily adapted and utilized by indigenous microorganisms thus enhanced microbial metabolic activities. On the contrary, as the representatives of PAHs, naphthalene and phenanthrene were highly toxic and refractory, resulting in the inhibition of microbial metabolism and the decrease of dehydrogenase activity.

Profiling of taxonomic genes during the degradation process

The quantification results of soil total bacteria, γ-Proteobacteria, Actinobacteria, and Bacteroides by real-time PCR of the 16S rRNA gene were given in Fig.3.

As shown by Figure 3a that the pollution by crude oil of low concentration did not inhibit the proliferation of bacteria but high concentration crude oil did. Simulating effects appeared in the presence diesel of low concentration, while a significantly profound simulating effect was observed in the case of *n*-hexadecane of moderate concentration (CE2) (Fig.3a). As the representatives of soil bacteria, γ-Proteobacteria, Actinobacteria and Bacteroides also showed positive response to *n*-hexadecane. Crude and diesel oil exhibited similar influence on γ-Proteobacteria and Actinobacteria: displaying stimulating effect at low concentration while inhibiting effect at high concentration (Fig.3b&c). Interestingly, the presence of diesel of low concentration (DO1) gives the highest amount of γ-Proteobacteria, revealing the possible connection between γ-Proteobacteria and soil urease activity (Fig.2a). However, Bacteroides was inhibited by crude and diesel regardless of their concentration (Fig.3d).

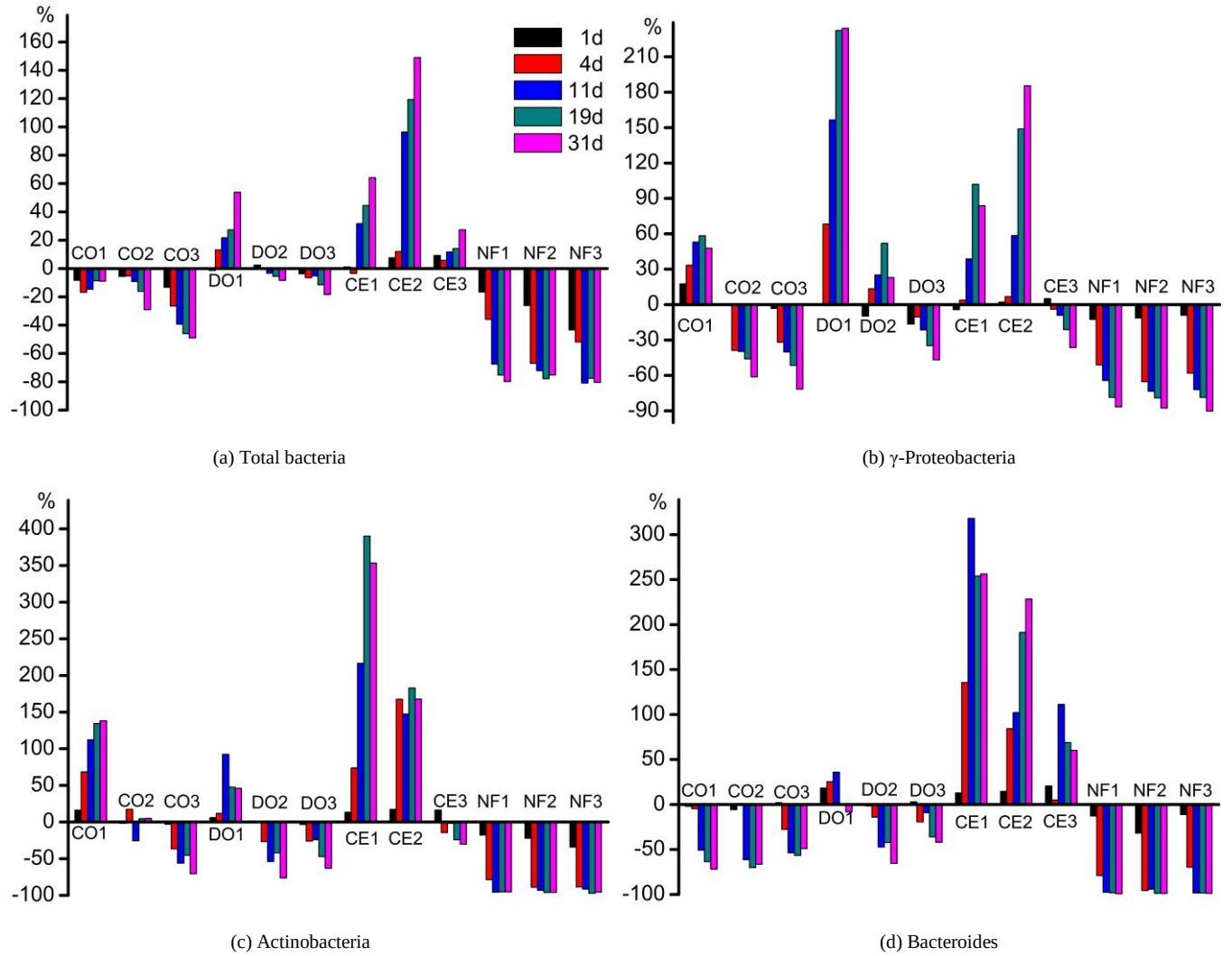
Many high taxonomic levels like phylum and class of bacteria show 'ecological coherence',[38-40] thus usually have different response to environmental turbulence,[41,42] resulting in the change of bacterial community structure. Wu has reported that the pollution with 4,000 mg·kg^{-1} of crude petroleum altered the community structure of soil bacteria, in which the amount of Proteobacteria and Actinobacteria increased while Bacteroides decreased.[15] This is consistent with the results shown in Fig.5a, the case of light pollution. It has been reported that presence of petroleum altered the microbial community structure but had no effect on the total amount of bacteria.[43,44] However, our study showed that the total amount of bacteria increased once low concentration of diesel and *n*-hexadecane presents (Fig.3a), indicating the high bioavailability of lightweight petroleum and *n*-alkane.

Profiling of functional genes during biodegradation

The real-time PCR quantification of soil functional bacteria involved in nitrogen fixation, denitrification, ammoxidation, alkane degradation, and naphthalene degradation are shown in Fig.4.

As shown in Fig.4a and b, the increase in crude oil concentration led to a reduction in the amount of nitrogen fixing bacteria (NFB) and ammonia oxidizing bacteria (AOB). The presence of low concentration of diesel didn't reduced NFB population and, however, increased AOB population. The presence of high concentration diesel gave slight inhibition to NFB but not AOB. The amount of NFB and AOB significantly increased in the presence of *n*-hexadecane of appropriate concentrations. As for denitrifying bacteria (DNB), as shown in Figure 4(c), the presence of crude oil, diesel and *n*-hexadecane exhibited stimulating influence in all concentrations. The population of alkane degrading bacteria (ADB) was dramatically increased in soils contaminated with *n*-hexadecane, diesel and low concentration of crude oil (Fig.4d). The only exception (CO3) might due to the longer adaptive phase of ADB in severe polluted soil. Nevertheless, the content of ADB in soil total bacteria increased as others (Fig.5b). The largest population of ADB was found in the presence of moderate *n*-hexadecane, which was consistent with the results of soil TPHs (Fig.1) and dehydrogenase activity (Fig.2b). Though the severe inhibition of the soil total bacterial activity was, PAHs exhibited a selection effect on potential PAH degrading bacteria (PDB), *i.e.*, the content of PDB in PAH contaminated soils increased about one order of magnitude (Fig.5b). Similar selection effect, though less significant, was also found in the presence of crude oil.

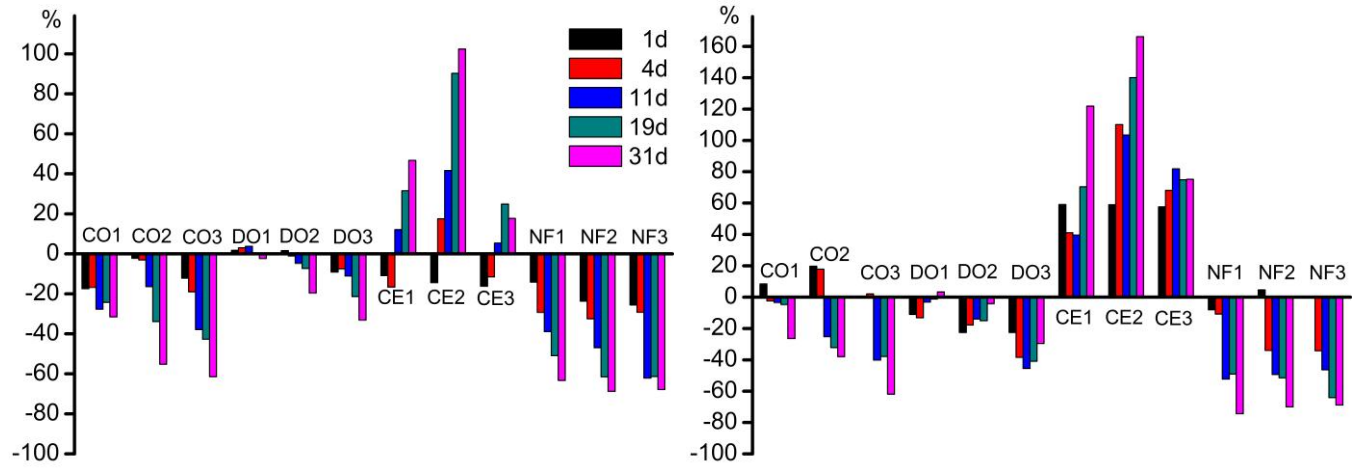
Nitrogen cycle, containing nitrogen assimilation, ammoniation, nitrogen fixation, nitrification and denitrification, is one of the most important cycles in biosphere. Urease is one of the enzymes involved in ammoniation. The key enzymes involved in the latter three functional reactions are dinitrogenase, ammonia oxidase and nitrate reductase, encoded by *nifH*, *amoA* and *narG* genes, respectively. The population of bacteria involved in nitrogen cycle reflects nitrogen nutritional status in soil. The presence of NFB and AOB is beneficial for the accumulation of available nitrogen, while the presence of DNB is the contrary. It has been observed that the pollution of petroleum hydrocarbons altered the population of NFB.[45,46] Musat *et al.*[47] observed that nitrogen fixation was not stimulated by petroleum. The present study showed that the



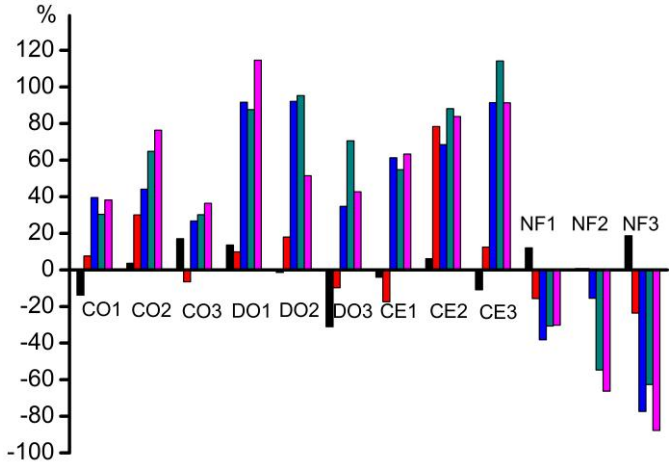
(a) Total bacteria (b) γ-Proteobacteria

(c) Actinobacteria (d) Bacteroides

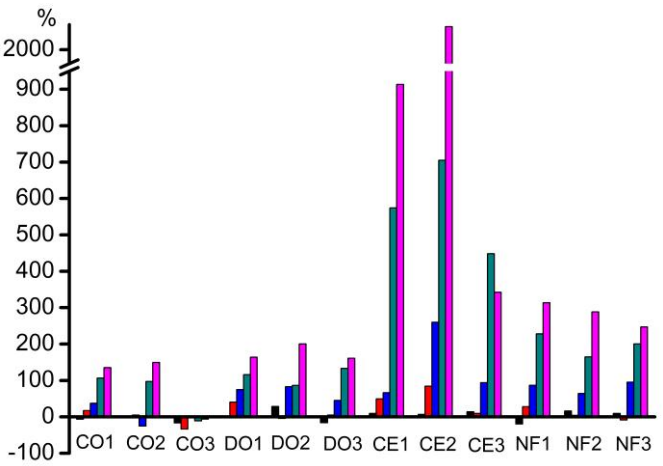
Fig. 3 Quantification of 16S rRNA genes. The percentage variation of gene copy numbers in soils with petroleum hydrocarbons is determined using that in control as the benchmark. Error bars were not shown for the clarity of figures.



(a) *nifH* gene (b) *amoA* gene

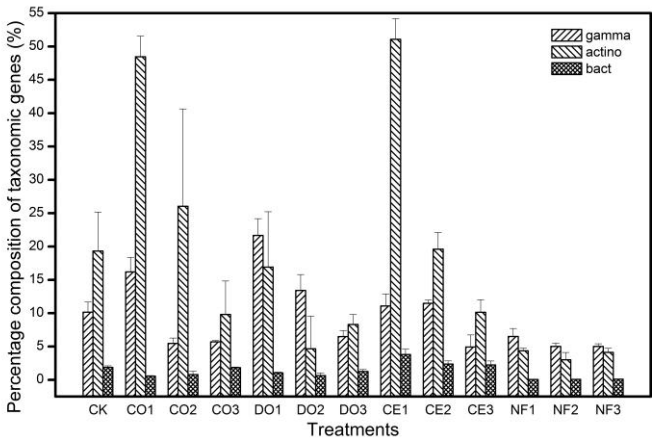


(c) *narG* gene

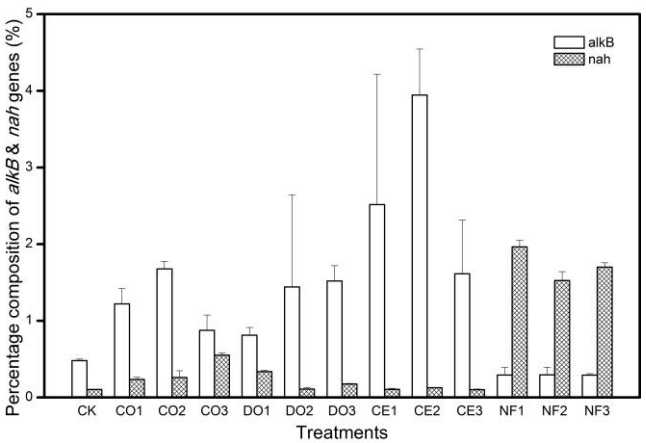


(d) *alkB*/*nah* gene

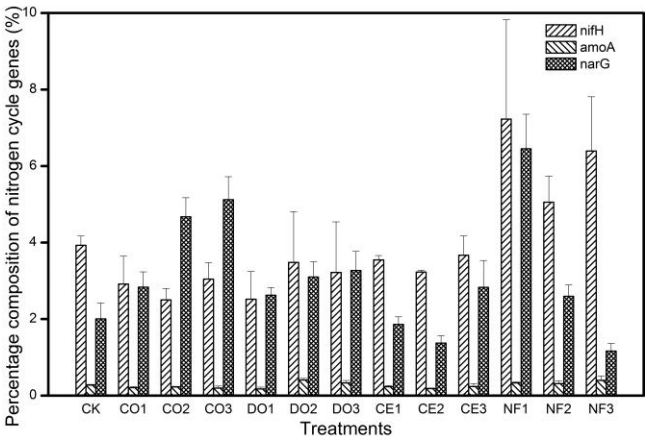
Fig. 4 Quantification of functional genes (*alkB* gene was detected in CK, CO, DO and CE treatments, *nah* gene was detected in CK and NF treatments). The percentage variation of gene copy numbers in soils with petroleum hydrocarbons is determined using that in control as the benchmark. Error bars were not shown for the clarity of figures.



(a) Taxonomic genes



(b) *alkB* and *nah* genes



(c) Nitrogen cycle genes

Fig. 5 Percentage of (a) taxonomic genes, (b) *alkB* and *nah* genes and (c) nitrogen cycle genes among total amount of 16S rRNA gene at the end of the experiment (31st day)

percentage of NFB population decreased slightly in *n*-hexadecane, diesel and crude oil contaminated soils (Fig.5c), similar to those reported elsewhere.[48] However, the absolute number of NFB in soils spiked with *n*-hexadecane, especially at the medium concentration, increased significantly, which might provide extra available nitrogen for soil microorganisms. The population of NFB in PAHs contaminated soils exhibited a significant decrease. Similar results was reported by Knowles and Wishart who found that exposure to a light PAHs caused strong inhibition of nitrogen fixation.[49] The concentration of AOB, which are usually classified as chemoautotrophs, was two to three orders of magnitudes lower than total bacterial in oilfields,[50,51] indicating that AOB was not the key population of bacterial community in petroleum contaminated soils. This

might accounts for its independence to petroleum contamination. However, this is not in consistent with that reported by dos Santos *et al*. who found that AOB content declined with the increase in crude oil concentration.[52] The stimulating effect for DNB might be related to the living mode of DNB. The addition of petroleum hydrocarbons resulted in the diminution of soil porosity and the formation of anoxic microcosms, thus stimulated the rate of denitrification.

The amount of potential alkane degrading bacteria and PAH degrading bacteria have been determined by real-time PCR using *alkB* and *nah* genes which encode the alkane hydroxylase and naphthalene dioxygenase, respectively, as functional markers. The results showed that in a short period (1 month) after the addition of petroleum, the content of ADB (PDB) among total bacteria rose from 0.5% (0.1%) to 4% (2%) (Fig.5b). Similar enrichments in indigenous degrading microorganisms by the selective pressure exerted by petroleum hydrocarbons has been reported[53-57] whether they are low or high toxic. In soil samples being contaminated over a long period, the content of degrading microorganisms reaches 90%.[58] Among all, the population of ADB in *n*-hexadecane contaminated soils was the largest, indicating its highest biodegradability. Though *nah* gene was employed to be a functional marker to study PAH degrading bacteria in this work, it is noted that the degrading enzyme system is extremely complex and thus more related genes should be detected in order to have a comprehensive understanding of PAH degraders.[59-62]

Conclusions

A simulated petroleum contamination system consisting of 12 samples with 4 different kinds of petroleum hydrocarbons in different concentrations was established. The quantification of taxonomic and functional genes through real-time PCR as well as the determination of soil urease and dehydrogenase activities was performed to evaluate the impact of petroleum contamination on soil microbial ecology and biological function. The results showed that the presence of *n*-hexadecane, especially at a medium concentration, stimulated the amount of total bacteria and ADB and increased the activities of soil urease and dehydrogenase. Among the four kinds of petroleum hydrocarbons, only *n*-hexadecane exhibited a significant biodegradation, revealing the low toxicity and high biodegradability of *n*-hexadecane. Crude and diesel oil didn't alter the total amount of bacteria but stimulated ADB and dehydrogenase activity. The stimulating effect became weaker when the concentration of petroleum increased. Urease activity was inhibited by crude oil and diesel except for that of low concentration. As the representatives of high toxic PAH, naphthalene and phenanthrene strongly inhibited the amount of bacteria and soil enzymes. As to the taxonomic and functional bacterial community in soil, it is found that the petroleum contamination inhibited nitrogen fixation while stimulated denitrification. However, the content of AOB in total bacteria was independent to petroleum contamination.

The results above indicated that the impact of petroleum on soil microbial ecology varied with the type and concentration of petroleum hydrocarbons. A comprehensive profile of soil microbial ecology through the combination of gene quantification and enzyme activity assay, as attempted by the present study, is helpful to the assessment, design and implementation of soil bioremediation.

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References

1 A. G. Marshall and R. P. Rodgers, *Proceedings of the National Academy of Sciences*, 2008, **105**, 18090-18095.
2 M. R. Gray, D. K. Banerjee, P. M. Fedorak, A. Hashimoto, J. H. Masliyah and M. A. Pickard, *Applied Microbiology and Biotechnology*, 1994, **40**, 933-940.
3 J. Wyszkowska, M. Kucharski and J.Kucharski, *Journal of Environmental Studies*, 2006, **15**, 501-506.
4 P. G. Falkowski, T. Fenchel and E. F. Delong, *Science*, 2008, **320**, 1034-1039.
5 K. Zhang, X. F. Hua, H. L. Han, J. Wang, C. C. Miao, Y. Y. Xu, Z. D. Huang, H. Zhang, J. M. Yang, W. B. Jin, Y. M. Liu and Z. Liu, *Chemosphere*, 2008, **73**, 1387-1392.
6 K. Zhang, Y. Y. Xu, X. F. Hua, H. L. Han, J. N. Wang, J. Wang, Y. M. Liu and Z. Liu, *Biochemical Engineering Journal*, 2008, **41**, 251-257.
7 T. J. Gentry, C. Rensing and I. L. Pepper, *Critical Reviews in Environmental Science and Technology*, 2004, **74**, 63-67.
8 V. Andreoni, L. Cavalca, M.A. Rao, G. Nocerino, S.Bernasconi, E. Dell'Amico, M. Colombo and L. Gianfreda, *Chemosphere*, 2004, **57**, 401-412.
9 I. G. Byun, H. U. Nam, S. K. Song, I.S. Hwang, T.H. Lee and T. J. Park, *Korean Journal of Chemical Engineering*, 2005, **22**, 917-921.
10 A. Saviozzi, R. Cardelli and M. Cozzolino, *Compost Science & Utilization*, 2009, **17**, 55-60.
11 J. Wyszkowska and M. Wyszkowski, *Journal of Toxicology and Environmental Health, Part A*, 2010, **73**, 1202-1210.
12 Z. Wu, H. Dong, L. Zou, D. Lu and Z. Liu, *Applied Biochemistry and Biotechnology*, 2011, **164**, 1071-1082.
13 H. Guo, J. Yao, M. Cai, Y. Qian, Y. Guo, H. H. Richnow, R. E. Blake, S. Doni and B. Ceccanti, *Chemosphere*, 2012, **87**, 1273-1280.
14 T. Bahrampour and V. S. Moghanlo, *International Journal of Agriculture: Research and Review*, 2012, **2**, 671-679.
15 Z. Wu, L. Zuo, D. Lu and Z. Liu, *Journal of Environmental Monitoring*, 2011, **13**, 2904-2913.
16 V. Marc, S. Jordi and J. Mar á, *Applied and Environmental Microbiology*, 2005, **71**, 7008-7018.
17 M. Coppotelli, A. Ibarrolaza, M. T. Del Panno and I. S. Morelli, *Microbial Ecology*, 2008, **55**, 173-183.

18 E. Katsivela, E. R. B. Moore, D. Maroukli, C. Strompl, D. Pieper and N. Kalogerakis, *Biodegradation*, 2005, **16**, 169-180.
19 G. Harms, A. C. Layton, H. M. Dionisi, I. R. Gregory, V. M. Hawkins, K. G. Robinson and G. S. Sayler, *Environmental Science & Technology*, 2003, **37**, 343-351.
20 J. C. López-Gutiérrez, S. Henry, S. Hallet, F. Martin-Laurent, G. Catroux and L. Philippot, *Journal of Microbiological Methods*, 2004, **57**, 399-407.
21 A. Cébron, M. P. Norini, T. Beguiristain and C. Leyval, *Journal of Microbiological Methods*, 2008, **73**, 148-159.
22 L. Mårtensson, B. Díez, I. Wartiainen, W. Zheng, R. El-Shehawy and U. Rasmussen, *Plant and Soil*, 2009, **325**, 207-218.
23 U. S. E .P .A, *Hazaedous Waste Test Methods*, Method 3546: Microwawe Extraction, U. S., 2007.
24 T. Bacchetti De Gregoris, N. Aldred, A. S. Clareand J. Grant Burgess, *Journal of Microbiological Methods*, 2011, **86**, 351-356.
25 Rösch, A. Mergel and H. Bothe, *Applied and Environmental Microbiology*, 2002, **68**, 3818-3829.
26 J. H. Rotthauwe, K. P. Witzel and W. Liesack, *Applied and Environmental Microbiology*, 1997, **63**, 4704-4712.
27 L. Philippot, S. Piutti, F. Martin-Laurent S. Hallet and J. C. Germon, *Applied and Environmental Microbiology*, 2002, **68**, 6121-6128.
28 S. M. Powell, S. H. Ferguson, J. P. Bowman and I. Snape, *Microbial Ecology*, 2006, **52**, 523-532.
29 R. Baldwin, C. H. Nakatsu and L. Nies, *Applied and Environmental Microbiology*, 2003, **69**, 3350-3358.
30 I. C. Eom, C. Rast, A. M. Veber and P. Vassuer, *Ecotoxicology and Environmental Safety*, 2007, **67**, 190-205.
31 W. T. Frankenberger, *Bulletin of Environmental Contamination and Toxicology*, 1988, **40**, 66-68.
32 J. Wyszkowska and J. Kucharski, *Polish Journal of Environmental Studies*, 2000, **9**, 479-486.
33 R. Margesin, G. Walder and F. Schinner, *Acta Biotechnologica*, 2000, **20**, 313-333.
34 G. F. Antonious, *Journal of Environmental Science and Health, Part B*, 2003, **38**, 479-488.
35 H. Li, Y. Zhang, I. Kravchenko, H. Xu and C. Zhang, *Journal of Environmental Sciences*, 2007, **19**, 1003-1013.
36 M. Megharaj, I. Singleton, N. C. McClure and R. Naidu, *Archives of Environmental Contamination and Toxicology*, 2000, **38**, 439-445.
37 W. T. Frankenberger and J. B. Johanson, *Journal of Environmental Quality*, 1982, **11**, 602-607.
38 N. Fierer, M. A. Bradford and R. B. Jackson, *Ecology*, 2007, **88**, 1354-1364.
39 L. Philippot, D. Bru, N. P. A. Saby J. Cuhel, D. Arrouays, M. Simek and S. Hallin, *Environmental Microbiology*, 2009, **11**, 3096-3104.
40 L. Philippot, S. G. E. Andersson, T. J. Battin J. I. Prosser, J. P. Schimel, W. B. Whitman and S. Hallin, *Nature Reviews Microbiology*, 2010, **8**, 523-529.
41 S. C. Long, C. M. Aelion, D. C. Dobbins and F. K. Pfaender, *Microbial Ecology*, 1995, **30**, 297-307.
42 J. Joynt, M. Bischoff, R. Turco, A. Konopka and C. H. Nakatsu, *Microbial Ecology*, 2006, **51**, 209-219.
43 J. O. Ka, Z. Yu and W. W. Mohn, *Microbial Ecology*, 2001, **42**, 267-273.
44 A. Maruyama, H. Ishiwata, K. Kitamura, M. Sunamura, T. Fujita, M. Matsuo and T. Higashihara, *Microbial Ecology*, 2003, **46**, 442-453.
45 J. R. Haines, R. M. Atlas, R. P. Griffiths and R. Y. Morita, *Applied and Environmental Microbiology*, 1981, **41**, 412-421.
46 I. M. Head, D. M. Jones and W. F. M. Röling, *Nature Reviews Microbiology*, 2006, **4**, 173-182.
47 F. Musat, J. Harder and F. Widdel, *Environmental Microbiology*, 2006, **8**, 1834-1843.
48 A. Jordan, P. J. McNicol and M. R. Marshall, *Canadian Journal of Microbiology*, 1978, **24**, 643-649.
49 R. Knowles and C. Wishart, *Environmental Pollution (1970)*, 1977, **13**, 133-149.
50 A. Agrawal and B. Lal, *FEMS Microbiology Ecology*, 2009, **69**, 301-312.
51 Gittel, K. B. Sørensen, T. L. Skovhus, K. Ingvorsen and A. Schramn, *Applied and Environmental Microbiology*, 2009, **75**, 7086-7096.
52 A. F. dos Santos, E. L. S. Marques, E. Gross, S. S. Souza, J. C. T. Dias, M. Brendel and R. P. Rezende, *Genetics and Molecular Research*, 2012, **11**, 190-201.
53 M. A. Ramsay, R. P. J. Swannell, W. A. Shipton, N. C. Duck and R. T. Hill, *Marine Pollution Bulletin*, 2000, **41**, 413-419.
54 S. Harayama, Y. Kasai, A. Hara, *Current Opinion in Biotechnology*, 2004, **15**, 205-214.
55 M. Viñas, J. Sabaté, M. J. Espuny and A. M. Solanas, *Applied and Environmental Microbiology*, 2005, **71**, 7008-7018.
56 M. Uyttebroek, A. Spoden, J. J. Ortega-Calvo, K. Wouters, P. Wattiau, L. Bastiaens and D. Springael, *Journal of Environmental Quality*, 2007, **36**, 1403.
57 Rojo, *Environmental Microbiology*, 2009, **11**, 2477-2490.
58 R. S. Burlage, G. S. Sayler and F. Larimer, *Journal of Bacteriology*, 1990, **172**, 4749-4757.
59 R. W. Eaton and P. J. Chapman, *Journal of Bacteriology*, 1992, **174**, 7542-7554.
60 S. L. Fuenmayor, M. Wild, A. L. Boyes and P. A. Williams, *Journal of Bacteriology*, 1998, **180**, 2522-2530.
61 A. A. Khan, R. F. Wang, W. W. Cao, D. R. Doerge, D. Wennerstrom and C. E. Cerniglia, *Applied and Environmental Microbiology*, 2001, **67**, 3577-3585.
62 H. Habe and T. Omori, *Bioscience, Biotechnology, and Biochemistry*, 2003, **67**, 225-243.