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Environmental impact statement:

The effects of biostimulation procedures including regulating C:N:P ratio, applying surfactants and electron acceptors on the degradation of crude oil contaminants and the evolution of microbial community were examined simultaneously so to provide an ecological insight into the biostimulation. The degradation of polar compounds of crude oil contaminants was characterized by negative-ion ESI FT-ICR MS, showing a biased increase in the relative abundance of naphthenic acids. The real-time PCR shown the promoted proliferation of bacteria, in which *Gammaproteobacteria* increased most significantly. The proliferation of fungi was, however, inhibited by the accumulation of the degradation products. An enriched abundance of *alkB* gene was observed during the degradation of saturated hydrocarbons. Above information helps to understand and optimize the biostimulation process.

ARTICLE

Kinetic and multidimensional profiling of accelerated degradation of oil sludge by biostimulation

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Biostimulation, which employs nutrients to enhance the proliferation of indigenous microorganisms and therefore the degradation of contaminants, is an effective tool for treatment of oil-contaminated soil. However, the evolution of microbial ecology, which responds directly to stimulation procedures and intrinsically determines the degradation of oil contaminants, has rarely been explored, particularly in the context of biostimulation. In this study, the effects of biostimulation procedures including regulation of the C:N:P ratio, as well as application of surfactants and electron acceptors on the degradation of crude oil contaminants and evolution of the microbial community were examined simultaneously to provide ecological insight into the biostimulation. Real-time PCR showed that biostimulation promoted proliferation of bacteria, with *Gammaproteobacteria* showing the greatest increase. However, the proliferation of fungi was inhibited by accumulation of the degradation products. The degradation of polar compounds of crude oil contaminants was characterized by negative-ion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (negative-ion ESI FT-ICR MS), showing a biased increase in the relative abundance of naphthenic acids. Principal component analysis (PCA) showed that different species in oil sludge have different degrading rate during biostimulation. The addition of fertilizer with surfactant and electron acceptor stimulated profoundly the indigenous microorganism with N₁, O₁ and O₂ species as substrates while those with O₃ and O₄ species were little affected. Enriched abundance of the *alkB* gene was observed during the degradation of saturated hydrocarbons. Monitoring of the kinetics of the microbial community, functional genes and degradation offers a comprehensive view to understanding and optimization of the biostimulation process.

Introduction

Oil sludge, which is often generated during exploitation, transportation and refining processes, is a recalcitrant mixture containing a large amount of oily mud, sand and water. Total petroleum hydrocarbons (TPHs), which primarily consists of alkanes, aromatics, nitrogen-, sulfur-, and oxygen- containing compounds, are the most deleterious components in oil sludge. Many components of oil sludge have been identified as priority pollutants by the USEPA¹. Furthermore, oil sludge undergoes several aging and weathering processes that eventually lead to higher average molecular weights and shifts in TPHs fraction ratios.

Bioavailability might be the limiting factor controlling the biodegradation of such compounds.

Vasudevan *et al.* reported accelerated degradation of oil sludge in response to the addition of bulking agent or inorganic nutrients². Soliman *et al.* achieved significant removal of total petroleum hydrocarbon from Egyptian soil polluted with oily sludge using biostimulation and bioaugmentation³. Mohan *et al.* compared the efficiency of bioaugmentation, biostimulation and co-substrate addition of oil sludge using a slurry reactor and found that a combination of the three methods gave the highest degradation⁴. In their studies of bioremediation of petroleum contaminated soil, Liu *et al.* established a consortium composed of *Enterobacter cloacae*

and *Cunninghamella echinulata*, which showed synergy in both the growth and metabolism of petroleum hydrocarbons. The application of wheat straw as a stimulant in addition to inoculation of the above-mentioned consortium further improved degradation of petroleum contaminants⁵. Based on the above experimental studies, the poor bioavailability of oil sludge requires the input of nutrients to stimulate the proliferation of indigenous microorganisms and thus enable subsequent degradation of oil sludge. Crivelaro *et al.* examined the effect of vinasse as a stimulant for the biodegradation of oil sludge⁶. Wang *et al.* observed enhanced growth of two species of bacteria in response to the addition of bulking that suppressed the proliferation of other types of bacteria⁷. Rahman *et al.* confirmed the positive effects on the bioremediation of *n*-alkane in petroleum sludge⁸ in response to the addition of a bacterial consortium, rhamnolipid biosurfactant and NPK solution.

The mixed and complex nature of oil sludge requires involvement of complex microbial consortia in degradation of oil sludge contaminated soil. Understanding the microbial kinetics in such degradation is essential to optimization of the biodegradation process. Such an understanding could be realized with the aid of modern molecular biological tools. In our previous study, we monitored the seeding and proliferation of the inoculant and the resulting microbial community by denaturing gradient gel electrophoresis (DGGE) analysis of the V3 zone of 16S rDNA⁹ and evaluated the restoration of taxonomic and functional genes after bioaugmentation of petroleum contaminated soil using a cloning library of taxonomic genes (16S rRNA gene for bacteria and 18S rRNA gene for eukaryotes) and functional genes (*nifH*, *amoA* and *narG*)¹⁰. Conversely, the degradation spectrum of the oil sludge can also be monitored by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). In practice, negative-ion ESI FT-ICR MS has been used to resolve and identify acids and neutral heteroatom compounds in crude oil¹¹⁻¹⁹. Over 3000 acidic compounds with different elemental compositions have been identified using negative-ions from heavy crude oil in South America²⁰. In this study, we used these tools to establish a kinetic and multidimensional view of the degradation of oil sludge in which variations in microbial community, functional genes and the oil residue spectrum are displayed simultaneously.

In the present study, biostimulation of oil sludge sampled from Karamay Oilfield (Xinjiang, China) was conducted and variations in the content and composition of oily sludge were analyzed by GC-MS and negative-ion ESI FT-ICR MS, which is further analysed by PCA method. Additionally, the changes in soil 16S rRNA (bacterial), 18S rRNA (fungal), bacterial flora and the *alkB* gene, which represent alkane-degrading bacterial communities, were monitored by real time PCR. Microbial ecological insight was thus established to account for the differential performance of the biostimulation procedures.

Materials and Methods

Materials

Oil sludge was collected from the Karamay Oilfield, Xinjiang, China and sieved through a 2.0 mm diameter sifter prior to experiments. Karamay Oilfield is in Xinjiang and is one important oil field in China. The oil sludge generated during exploitation and refining represents a serious environmental problem. This is the driving force for the present study. The detailed characteristics of oil sludge are given as Table S1 in ESI. All chemicals were of analytical grade unless otherwise noted.

Biostimulation protocols

Five biostimulation protocols, denoted as A to E, were used to treat oil sludge. All treatments consisted of 200g of oil sludge in a 1L flask.

A (Control): Oil sludge was sterilized and 0.3% (w/w) sodium azide was added to suppress microbial contamination.

B (Natural attenuation): Oil sludge was not sterilized and no chemicals or microbes were added.

C (Fertilizer): (NH₃)₂SO₄ (0.67% w/w) and Na₂HPO₄ (0.52% w/w) with a C:N:P ratio of 100:1.25:1 were added.

D (Fertilizer and surfactant): (NH₃)₂SO₄ (0.67% w/w) and Na₂HPO₄ (0.52% w/w) with a C:N:P ratio of 100:1.25:1 and Tween 80 (0.5% w/w) were added.

E (Electron acceptor): (NH₃)₂SO₄ (0.67% w/w) and Na₂HPO₄ (0.52% w/w) with a C:N:P ratio of 100:1.25:1 were added together with Tween 80 (0.5% w/w), FeCl₃·6H₂O (100 µg/kg) and NaNO₃ (100 µg/kg).

Water was also added to each sample to give a terminal water weight percentage of 200% (w/w). All treatments were prepared in triplicate.

Bioremediation was carried out in a shaker at 175 rpm and 30°C. During bioremediation, 5g of soil were taken and stored at -20°C every 7 days until molecular analysis.

Assays

TPHs content in soil: The content of total petroleum hydrocarbons (TPHs) in soil samples was determined using the USEPA recommended procedure²¹. Furthermore, the crude oil sample was subjected to Saturates/Aromatics/Resins/Asphaltenes fraction analysis (SARA fractionation) according to the Chinese Standard Analytical Method for the Petroleum and Natural Gas Industry (Standard No. SY/T 5119-2008).

GC-MS analysis: Saturated and aromatic hydrocarbon components were analyzed by gas chromatography/mass spectrometry (GC-MS, DSQ Thermo Fisher Scientific Co., Ltd., China) using an AB-5MS column (30 m × 0.25 mm × 0.25 µm). The analytical procedure was as follows: the temperature was increased from 60°C to 150°C at 10°C/min, and then from 150°C to 300°C at a rate of 3°C/min. During analysis, the carrier gas was helium and the split ratio was

20:1. The interface temperature for mass spectrometry was 250°C, giving a determined molecular weight of 35 to 650 Da.

Negative-ion ESI FT-ICR MS analysis: Samples collected at different times during bioremediation were dissolved into toluene to form solutions with a final concentration of 10 mg/mL for negative ESI FT-ICR MS analysis. Next, 20 mL of the solution was diluted with 1.0 mL of toluene/methanol (1:3, v/v) solution and then uniformly mixed with 15 µL of 35% (w/w) of NH₄OH to facilitate deprotonation of acidic compounds in ESI. Analysis was conducted on an Apex-ultra FT-ICR MS (Bruker Daltonics, USA) equipped

with a 9.4T superconducting magnet. The operating parameters of the negative ESI FT-ICR MS were applied according to previous studies^{19, 20, 22}. Peaks with a m/z between 150 and 1000 Da and signal intensity greater than five times the standard deviation of the baseline noise (SNR >5) were collected. Kendrick high resolution mass spectrometry analysis was then used to convert the measured masses from the IUPAC mass scale to the Kendrick mass scale. The peaks displayed upon ESI FT-ICR MS were identified by calculating their corresponding accurate molecular weight. Assignment of the molecular formula of compounds was limited to 100 ¹²C, 3 ¹⁴N, 9

Table 1 Primers and RT-PCR conditions

Primers	Sequence (5'-3')	Thermal condition
Total bacteria		
926F	AAACTCAAAGGAATTGACGG	95 °C for 1min, one cycle, 95 °C for 15s, 61.5 °C for 30s, 72 °C for 30s, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
1062R	CTCACRRACGAGCTGAC	
<i>Alphaproteobacteria</i>		
Eub338	ACTCCTACGGGAGGCAGCAG	95 °C for 15min, one cycle, 95 °C for 15s, 55 °C for 30s, 72 °C for 30s, 80 °C for 30s, 35cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
Alf685	TCTACGRATTTACCCYCTAC	
<i>Betaproteobacteria</i>		
Eub338	ACTCCTACGGGAGGCAGCAG	95 °C for 15min, one cycle, 95 °C for 15s, 55 °C for 30s, 72 °C for 30s, 80 °C for 30s, 35cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
Bet680	TCACTGCTACACGYG	
<i>Gammaproteobacteria</i>		
1080rF	TCGTCAGCTCGTGTGTGA	95 °C for 1min, one cycle, 95 °C for 15s, 61.5 °C for 30s, 72 °C for 30s, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
r1202R	CGTAAAGGGCCATGATG	
<i>Actinobacteria</i>		
Act920F3	TACGGCCGCAAGGCTA	95 °C for 1min, one cycle, 95 °C for 15s, 61.5 °C for 30s, 72 °C for 30s, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
Act1200R	TCRTCCCCACCTTCCTCCG	
<i>Firmicutes</i>		
928F-Firm	TGAAACTYAAAGGAATTGACG	95 °C for 1min, one cycle, 95 °C for 15s, 61.5 °C for 30s, 72 °C for 30s, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
1040FirmR	ACCATGCACCACCTGTC	
<i>Bacteroidetes</i>		
798cfbF	CRAACAGGATTAGATACCCT	95 °C for 1min, one cycle, 95 °C for 15s, 61.5 °C for 30s, 72 °C for 30s, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
Cfb967R	GGTAAAGTTCCCTCGCGTAT	
<i>Planctomycetes</i>		
Plancto352f	GGCTGCAGTCGAGRATCT	95 °C for 15min, one cycle, 95 °C for 15s, 60 °C for 30s, 72 °C for 30s, 80 °C for 30s, 35cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
Plancto920r	TGTGTGAGCCCCCGTCAA	
<i>alkB</i>		
alkBFd	AACTACMTCGARCAYTACGG	95 °C for 1min, one cycle, 95 °C for 20s, 50 °C for 30s, 72 °C for 45s, 35 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
alkBRd	TGAMGATGTGGTYRCTGTTCC	
Total fungi		
FR1	AICCATTCATTCGGTAIT	95 °C for 10min, one cycle, 95 °C for 15s, 50 °C for 30s, 70 °C for 1min, 40 cycles, 95 °C for 15s, 60 °C for 1min, 95 °C for 15s
FF390	CGATAACGAACGAGACCT	

¹⁶O, 2 ³²S, 2 ¹³C and 200 ¹H atoms due to the limitations of the method. Compounds containing the same number of heteroatoms were categorized into the same class. In addition, compounds in the same class were further classified according to the number of rings and double bonds.

Real-Time PCR: DNA was extracted from 0.25 g soil samples using a Power Soil[®] DNA isolation kit (MoBio Laboratories, USA). During the bioremediation processes, the copy numbers of 16S rRNA for bacteria and bacterial flora (*Actinobacteria*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*,

Gammaproteobacteria, *Bacteroidetes* and *Planctomycetes*), 18S rRNA for fungal, and the *alkB* gene was monitored using the real-time PCR. Briefly, real-time PCR was performed in a 20 µL reaction volume consisting of 10 µL of 2×SYBR Green Mix (Toyobo, Japan), 0.8 µL of 10 µM of each primer, 2 µL DNA template and 6.4 µL ddH₂O. The PCR amplification primers and amplification conditions are shown in Table 1.

Statistical analysis: The parameters tests were expressed as means ± standard error. The values were subjected to standard one-way

ANOVA with 95% confidence limits ($P \leq 0.05$) using SPSS17.0 statistical analysis.

Results and discussion

Degradation of TPHs under different bioremediation treatments

Degradation of the TPHs treated using the five bioremediation protocols is summarized in Figure 1.

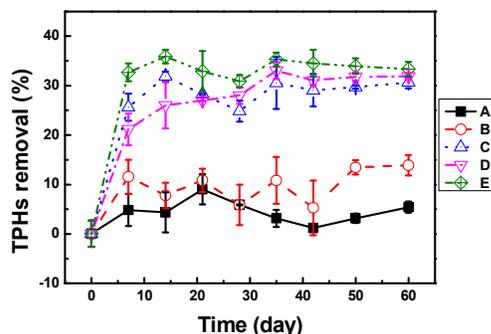


Figure 1. Percentage of TPHs removal after different treatments. A: Control; B: Natural attenuation; C: Addition of $(\text{NH}_3)_2\text{SO}_4$ and Na_2HPO_4 with a C:N:P ratio of 100:1.25:1; D: C in conjunction with 0.5% Tween 80; E: D with the addition of an electron acceptor.

For A, in which soil was sterilized and sodium azide was added to inhibit microbial proliferation, the TPHs content remained unchanged during the 60-day treatment period. For B, in which water was added to sustain the natural attenuation, the removal of TPHs reached 13.9% after 60 days. For C, D and E, in which exogenous nutrients were applied, TPHs removal reached 30.7%, 31.9% and 33.4%, respectively, which was two times higher than that obtained by natural attenuation. The improved TPHs removal in case D relative to case C indicated the effectiveness of the applied surfactant at improving the bioavailability of petroleum. The further improved TPH removal shown in case E relative to case D and C suggest the effectiveness of the added electron acceptors that facilitated oxidation of the petroleum contaminants. It is noted here that the C:N:P ratio was optimized in a pre-experiment as shown in Figure S1, indicating the optimal C:N:P ratio is 100:1.25:1 for removal of TPH. Thus in all following experiments, the C:N:P ratio was fixed as 100:1.25:1.

Degradation of different fractions of TPHs during biostimulation (Case E)

Due to the complexity of oil sludge, it is necessary to monitor changes in the components of oil sludge during biostimulation. In this study, components of oil sludge were divided into four types, asphaltenes, resins, aromatics and saturates. The contents of these components were monitored and then subjected to SARA fractionation analysis by Silica Gel-Alumina Column Chromatography as shown in Figure 2. Here, only SARA analysis of Case E is shown.

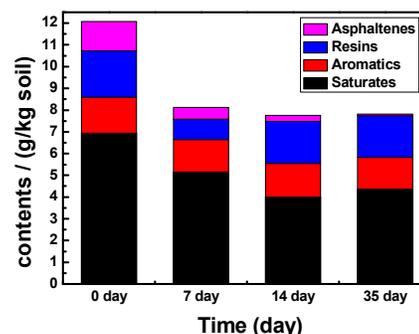


Figure 2. SARA analysis of degradation products during biostimulation (Case E). The standard deviation of data is within $\pm 6.8\%$. For clarity, the error bar is not drawn.

As shown in Figure 2, the initial contents of saturated, aromatic, resin and asphaltene hydrocarbons of the untreated oil sludge were 57.6%, 13.7%, 17.6% and 11.1%, respectively (day 0). After 35 days of bioremediation, the contents of the above components were 55.7%, 19.0%, 24.5% and 0.9%, respectively.

Based on the results shown in Figure 2, the saturated hydrocarbons, which were the richest components in the crude oil, were degraded most rapidly during the initial stage (Day 0 to Day 7) relative to the other components. The degradation of the resin and asphaltene was more obvious after 7 days. Interestingly, a minor increase in the content of saturated hydrocarbons was observed at Day 35 relative to Day 14. We believe that this could be attributed to conversions of other petroleum components during the degradation, as shown in Figure S2 (ESI). The oil content in resins increased to 24.45% at day 35 day, possibly due to the accumulation of naphthenic acids during biodegradation, as described below.

FT-ICR MS analysis

Characterization of the N/S/O compositions during biostimulation.

The TPHs in the contaminated soil were extracted and analyzed by negative ESI FT-ICR MS as described in the experimental section. The mass spectra of samples taken at day 0, 7, 14 and 35 are shown in Figure S2. More than 10,000 peaks ($\text{SNR} > 5$) between m/z 150–1000 were detected for each sample.

The relative abundance of the heteroatom compounds was defined as the signal intensity (peak height) of a peak divided by the total intensity of all detected peaks in the mass spectrum. The relative abundance of N/O classes after treatment by biostimulation for Case E are shown in Figure 3.

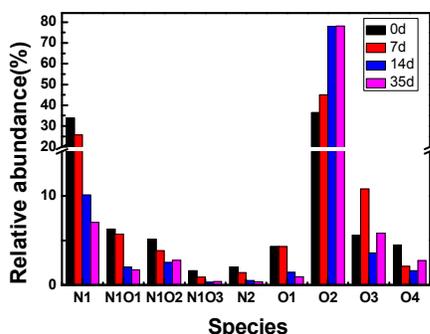


Figure 3. Relative abundance of species with heteroatom (N/O) after biostimulation (Case E)

As shown in Figure 3, O₂ species (36.5%) were the main compounds in the crude oil samples (Day 0), and the relative abundance of O₂ species continuously increased, reaching 82.3%

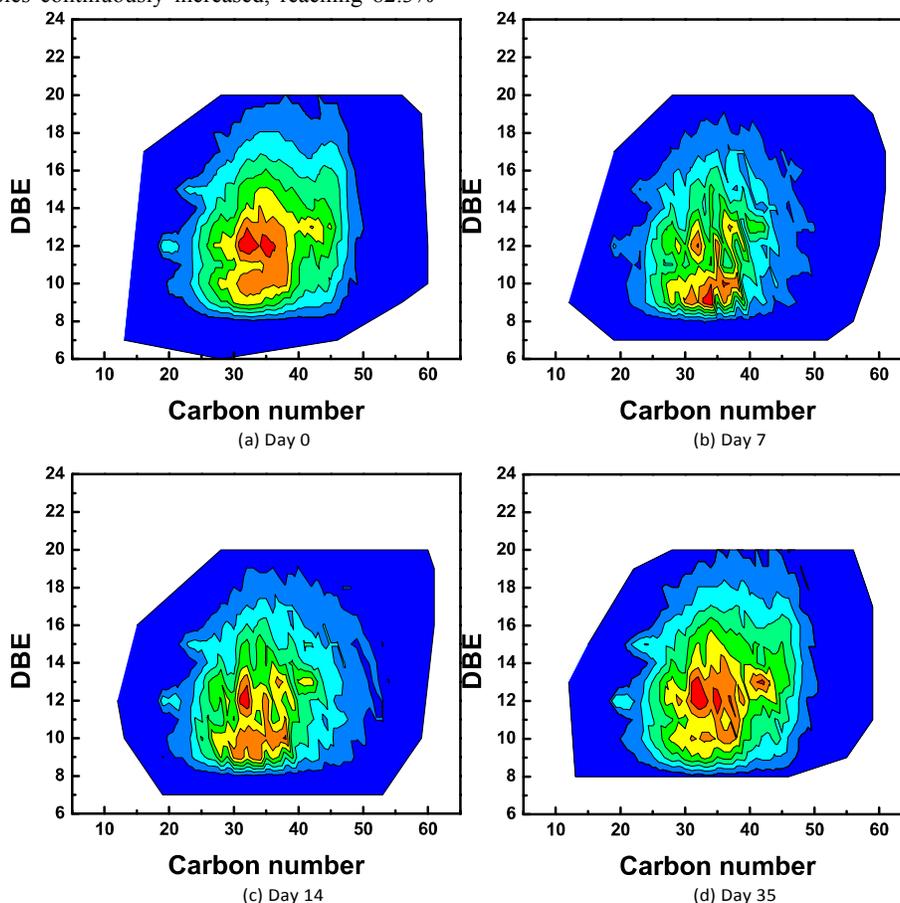


Figure 4. Changes in N₁ species as a function of carbon number and DBE in response to biostimulation (Case E). Color of spectra indicate the percentage of specific compounds occupying total species.

The N₁ species in the crude oil were most abundant, with DBE values of 9–15 and carbon numbers ranging from 25 to 40 as shown in Figure 4(a). These species included carbazole-like compounds (DBE=9), benzocarbazole-like compounds (DBE=12) and dibenzocarbazoles compounds (DBE=15). Implementation of

on Day 35. However, species N₁ gradually decreased from 36.5% to 7.06%. NO₁, NO₂, NO₃ and N₂ species showed the pattern of change. These results suggested the biased uptake of petroleum components by microorganisms. Surprisingly, S species compounds were not detected in the four samples.

Changes in N species compounds

N species compounds in the crude oil primarily exist in the form of azoles, carbazoles and indoles. N₁, N₁O₁, N₁O₂, N₁O₃ and N₂ species were detected in the samples. Because the major species are N₁ species, changes in the relative abundance map with double bond equivalent (DBE) and carbon number for N₁ species are shown in Figure 4.

biostimulation (Case E) led to a gradual decrease of the species with a DBE of 9, indicating the degradation of carbazole-like compounds (Figure 4(b-d)). However, species with DBE values of 12 and 15 gradually increased, indicating benzocarbazole-like compounds and dibenzocarbazoles compounds do not easily

degrade. Additionally, species with DBE values below 7 were completely degraded after 35 days. It should be emphasized here that the majority of N_1 species in crude oil sludge are carbazole-like compounds, accounting for about 30% of the total (Figure 4(a)). Thus, the effectiveness of degradation of these species can decrease the total amount of N_1 species, which is consistent with results shown in Figure 3.

Changes in O_1 species compounds

The relative isoabundance of O_1 species is shown in Figure 5.

Figure 5(a) shows the contour map of O_1 species of untreated oil sludge. The DBE value mainly ranged from 1 to 10 and the carbon number primarily ranged from 25 to 45. The O_1 species with DBE values of 4, 5, 6 and 7 were more abundant than those of other species. Once the DBE is 4, the major components are alkylphenols. During biostimulation treatment (Case E), the abundance of species composed of DBEs of 4–6 and carbon numbers below 40 gradually decreased. This was because alkylphenols (DBE=4) are intermediate metabolites in the degradation pathway of petroleum hydrocarbon. Interestingly, O_1 species composed of a DBE of 2 or 5 and a carbon number of 40 showed little degradation.

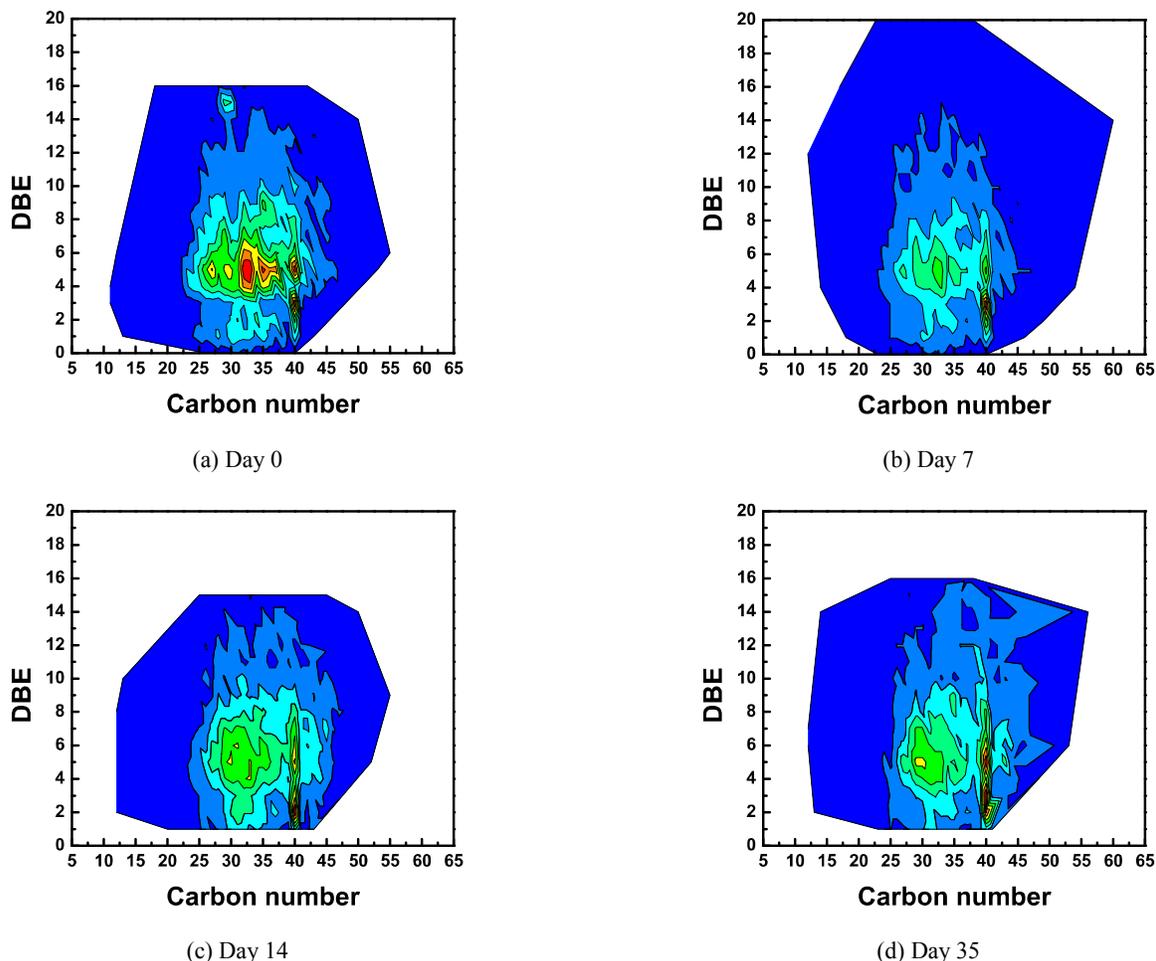


Figure 5. Changes in O_1 species as a function of carbon number and DBE in response to biostimulation (Case E). Color of spectra indicate the percentage of specific compounds occupying total species.

Microbial degradation of petroleum hydrocarbons involves a number of oxidation processes; therefore, the distribution, types and relative abundance of oxygen-containing compounds provide strong evidence of the biodegradation pathway. A higher relative abundance of O_2 species is associated with more complete biodegradation. Accordingly, the relative abundance of O_2 species

represented the biodegradability of the petroleum hydrocarbons. The distribution of O_2 species in different samples is shown in Figure 6.

Compounds with DBE=1 and carbon number=16 and 18, which correspond to C_{16} and C_{18} fatty acids, respectively, were not

considered because they were usually contaminants during negative ESI analysis. In the crude oil (Day 0 sample), O_2 species with DBE=1–6 and carbon numbers of 20–40 had a high relative abundance. Once subjected to remediation, fatty acids (DBE=1) were gradually degraded and the relative abundance of compounds with DBE=2, 3 and 4 increased significantly. Compounds with DBE=3 had the highest relative abundance, followed by those with

DBE=2 in the Day 35 sample. In addition, the process of biodegradation was accompanied by the enrichment of naphthenic acids with DBE=2 and 3. Three accumulated regions then gradually formed between C_{20} – C_{30} , C_{30} – C_{40} and C_{40} – C_{45} .

Relative isoabundance plots of DBE versus carbon number for O_3 species of four different samples are shown in Fig. 7.

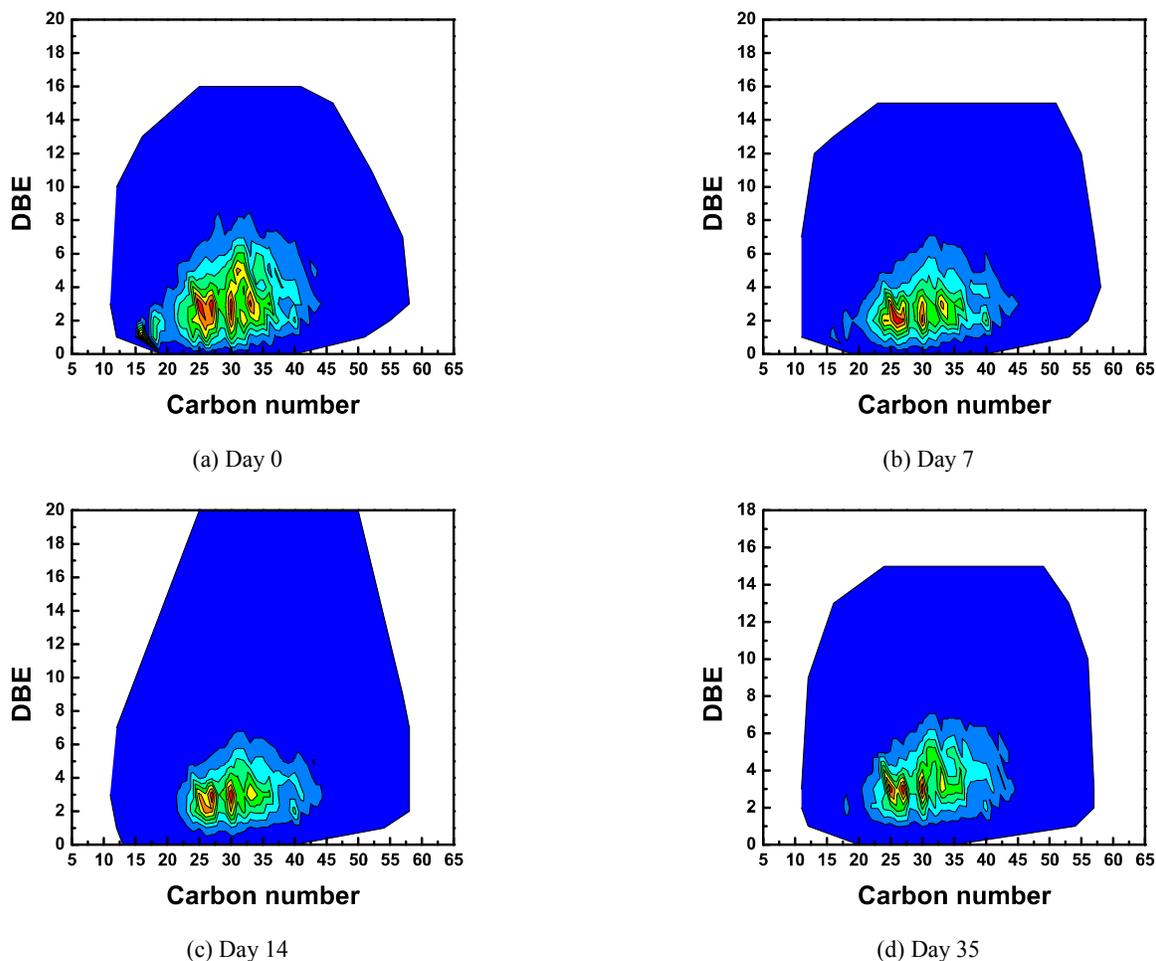


Figure 6. Changes in O_2 species as a function of carbon number and DBE in response to biostimulation (Case E). Color of spectra indicate the percentage of specific compounds occupying total species.

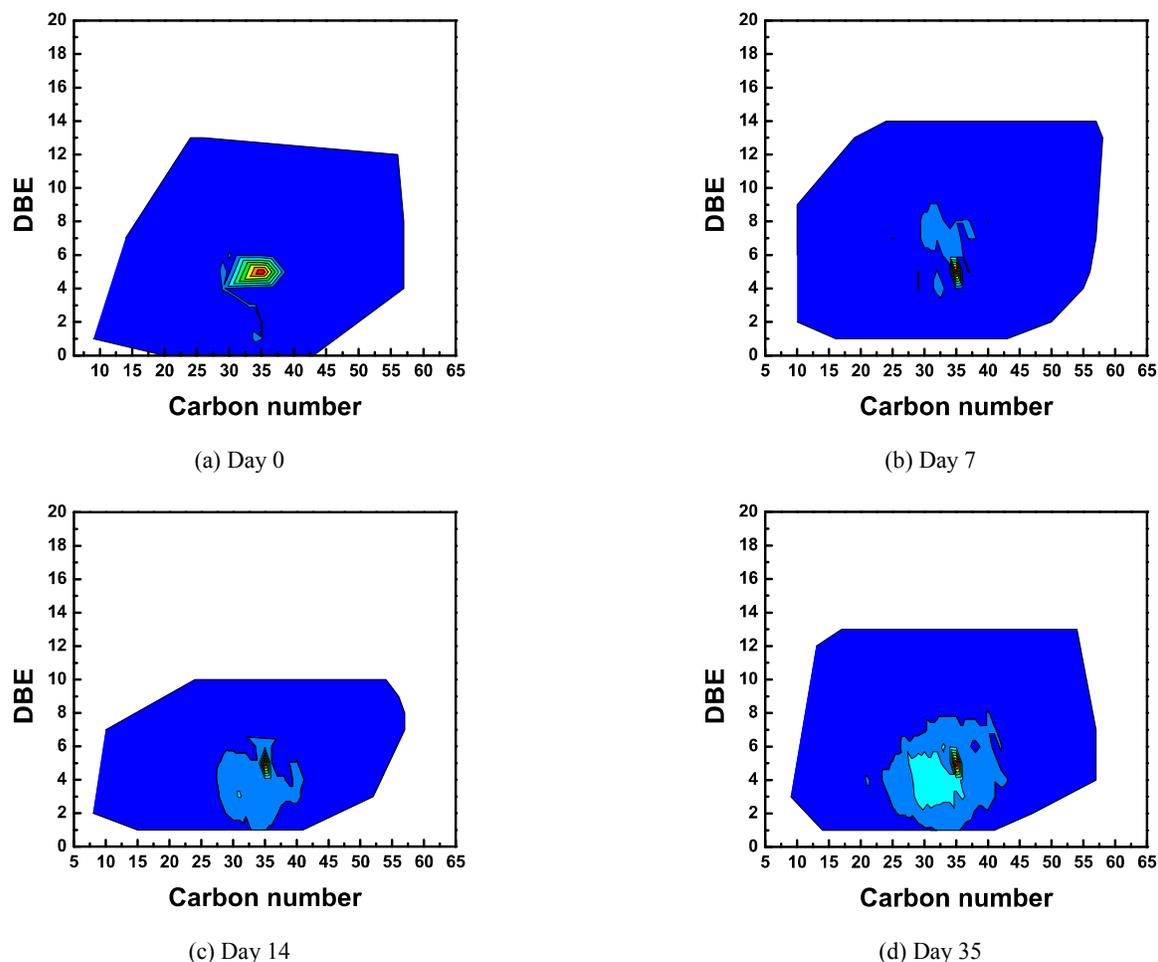


Figure 7. Changes in O_3 species as a function of carbon number and DBE in response to biostimulation (Case E). Color of spectra indicate the percentage of specific compounds occupying total species.

During biodegradation, the relative abundance of O_3 species was highest in the sample taken on day 14. Biodegradation enhanced the relative abundance of compounds with $DBE=2-7$. The compounds with $DBE=6-13$ and carbon number=32-37 were converted from other compounds on Day 35. It was shown that the hydroxylation of naphthenic acids (NAs) formed O_3 species. Furthermore, $C_{35}H_{61}O_3$ had the highest relative abundance, reaching 0.31%, 0.30%, 0.14% and 0.11% in the samples collected on day 0, 7, 14 and 35, respectively. The relative isoabundance

plots of DBE versus carbon number for O_4 species of the four different samples are shown in Figure. 9. O_4 species were mainly distributed in the range of $DBE=0-10$ and carbon atoms=15-55. The relative abundance with $DBE=2-5$ and carbon atoms=25-45 was specifically enhanced in the bioremediation process. When compared with the O_2 species, the toxicity of O_4 species was lower. Accordingly, the toxicity can be reduced by biological oxidation of naphthenic acid.

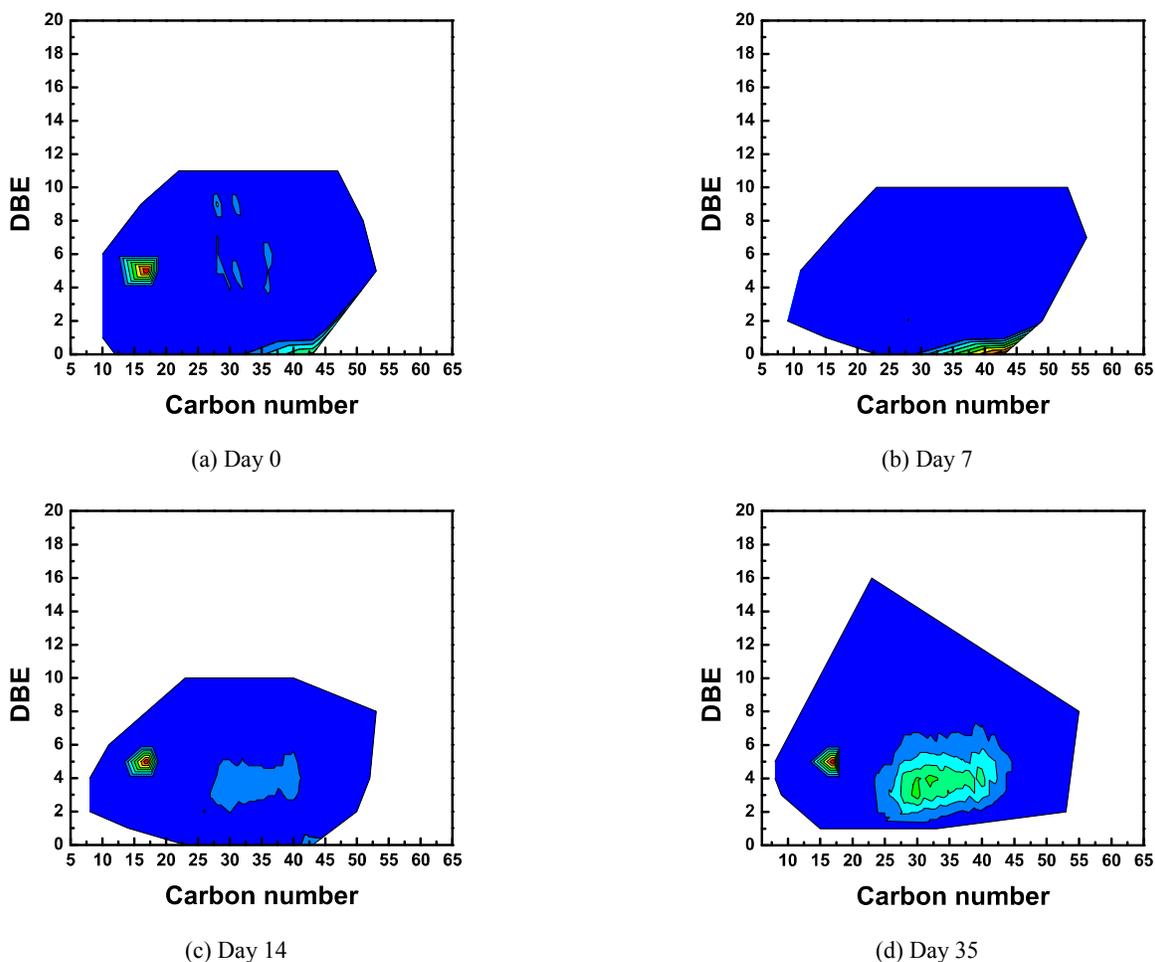


Figure 8. Changes in O_4 species as a function of carbon number and DBE in response to biostimulation (Case E). Colour of spectra indicate the percentage of specific compounds occupying total species.

Principal component analysis (PCA) of NO species detected by FI-ICR MS

PCA is linearly reduced dimension method and is widely used in multivariate statistical analysis for generating a global interpretation of data, as shown in Figure 4 to 8. Biplots of the score and loading parameters according to PCA results were shown in Figure 9(a), in which the score plot shows the distribution of samples treated with different time by biostimulation (Case E), while the loading plot presents different species of samples. It is shown in Figure 9 that the samples (day 0, day 7, day 14, and day 35) can be separated into three groups by the PCA coordinates. Group 1 contained sample of day 0 and day 7, group 2 contained sample of day 14 and group 3 contained sample of day 35. Thus degradation of crude oil in oil sludge undergoes slow degradation (from day 0 to day 7), quick degradation (from day 7 to day 35, with possible maximum at day 14) and very slow degradation (after day 35). Combined with gene analysis in Figure 10, it is concluded that when fertilizers in together with surfactant and electron acceptor were added into oil sludge, indigenous microorganism is firstly “activated”, leading to a significant degradation of N_1 , O_1 and O_2 , as shown in Figure 9. This is consistent with previous

isoabundance plots shown in Figure 4, Figure 5 and Figure 6. Once major degradable species in oil sludge were exhausted, the degrading rate slows down, giving a very slow degradation at day 35. In other words, addition of degrading microorganism or chemical degradation is requested, for a complete degradation of oil sludge.

In addition, PCA analysis was applied to all detected species in FT-ICR MS. Here PCA analysis of N_1 species and O_3 species, who are most easily and hardly degradable, was choose as example in Figure 9(b) and (c), respectively. It is shown in Figure 9(b) that most N_1 species have highest degrading rate at day 7, and most species with carbon number below 50 and DBE below 18 are easy for degradation by indigenous microorganism. This further indicates that most N_1 species are degradable, which is consistent with results shown in Figure 3 and Figure 4. It is shown in Figure 9(c) that for O_3 species, only species with OBE below 10 and carbon number below 30 are degradable. The maximum degrading rate is at day 14, indicating indigenous microorganisms have to spend more time to realize degradation of O_3 species.

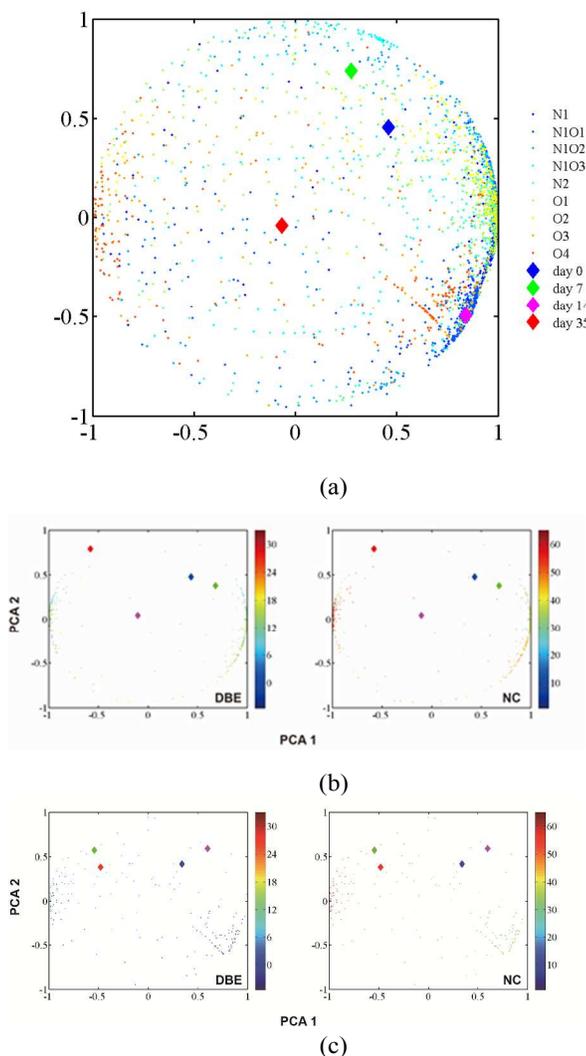


Figure 9. PCA analysis. (a) Major species classes at different treating time. Points with different colours indicate different species. Diamond with blue is projection of untreated oil sludge (day 0); Diamonds with green, purple and red are projection of oil sludge with different treating time; (b) PCA analysis of N_1 species. Color from cold to warm indicates DBE and NC from low value to high one; (c) PCA analysis of O_3 species. Color from cold to warm indicates DBE and NC from low value to high one

The evolution of microbial ecology

Changes in microbial flora and functional genes during bioremediation are shown in Figure 10.

As shown in Figure 10(a), the bacterial population, denoted as 16S rRNA copies, increased from 2×10^{10} to 1×10^{11} within 7 days, where it was maintained for the remainder of the experiment under Case E. These findings indicate that indigenous bacteria are stimulated by nutrients, surfactants and electron acceptors, favoring bioremediation. As shown in Figure 10(b), the population of fungi, denoted as 18S rRNA copies, decreased significantly from 4.5×10^8 to 0.5×10^8 within 35 days, indicating that metabolites secreted from bacteria during biodegradation of oil sludge might be toxic to fungi. It should be noted that the populations of both

bacteria and fungi in non-contaminated soil are very low as shown in Figure 10 (Column C). This is because the local soil near the pollution site contains almost no plants. As a result, contamination by soil sludge provides organic matter, which stimulates endogenous microorganisms, resulting in higher accumulation of bacteria and fungi in oil-contaminated soil than unpolluted soil.

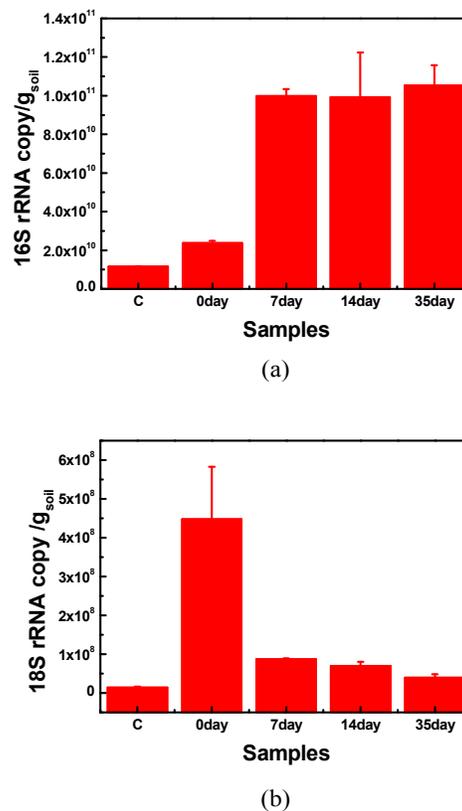


Figure 10. Changes in population of bacteria and fungi during biostimulation (Case E). (a) Bacterial 16S rRNA; (b) Fungi 18S rRNA.

The composition of bacteria is shown in Figure 11.

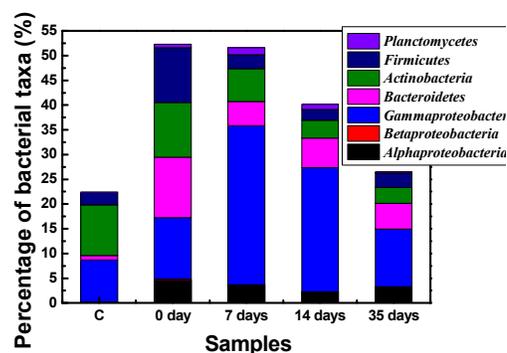


Figure 11. Change in bacterial taxa during biostimulation (Case E). The standard deviation of data is within $\pm 1.9\%$. For clarity, the error bar is not drawn.

As shown in Figure 11, contamination led to an increase in the abundance of several bacterial phyla/classes, including

Alphaproteobacteria, *Gammaproteobacteria*, *Bacteroidetes*, *Acidobacteria* and *Firmicutes*. Biostimulation improved the growth of *Gammaproteobacteria* to a large extent, with the abundance reaching 32.16% in the 7-day sample and then gradually decreasing. These findings indicate that *Gammaproteobacteria* may play an important role in the biodegradation of petroleum hydrocarbons. *Planctomycetes* showed a similar change as *Gammaproteobacteria*, while the abundance of *Betaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* showed no obvious change in response to the bioremediation process. These observations are consistent with those reported by Roling *et al* and Sininghe Damste *et al*.^{23,24}

We also measured changes in the copy number of *alkB* gene, which encodes alkane monooxygenase for the catalysis of alkane degradation under aerobic conditions (Figure 12). The *alkB* gene has been found in various bacteria, including *Pseudomonas*, *Rhodococcus*, *Alcanivorax* and *Xanthobacter*.

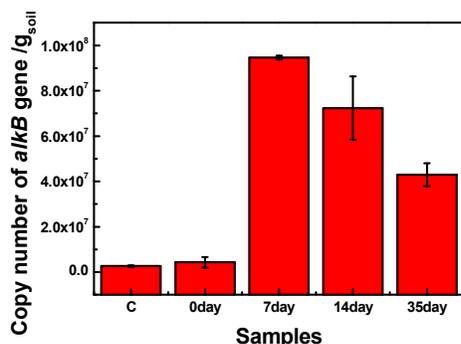


Figure 12. Dynamic changes in the *alkB* gene during bioremediation

As shown in Figure 12, the copy number of *alkB* genes in the day 7 sample reached $9.46 \times 10^7 g_{soil}^{-1}$, then increased by 22-fold compared to that shown in the sample taken on the first day. Biostimulation significantly increased the relative abundance of the *alkB* gene, which is responsible for the degradation of saturated hydrocarbons during bioremediation. Therefore, we can monitor changes in the *alkB* gene to reflect the degradation of saturated hydrocarbons.

Conclusions

This study demonstrated the effectiveness of biostimulation for the treatment of high oil content sludge, in which variation of the *alkB* gene is closely related to the degradation of saturated hydrocarbons. Naphthenic acids accumulated and the relative abundance of N species compounds was obviously decreased in response to biostimulation. Principal component analysis showed that different species in oil sludge have different degrading rate during biostimulation. The addition of fertilizer with surfactant and electron acceptor stimulated profoundly the indigenous microorganism with N_1 , O_1 and O_2 species as substrates while those with O_3 and O_4 species were little affected. The accumulation of toxic compounds inhibited

the growth of microorganisms, particularly fungal species. The kinetic input from the microbial society, the functional gene, and the chemical spectrum of degraded species offered a comprehensive view of the oil sludge degradation. The results presented herein will enable the design and optimization of biostimulation processes.

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Notes and references

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