Tracking alterations of alkyl side chains of N1 species in heavy crude oil after anaerobic biodegradation with negative-ion electrospray ionization coupled with high-field Fourier transform ion cyclotron resonance mass spectrometry

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Tracking Alterations on Alkyl Side Chains of N_1-species in Heavy Crude Oil After Anaerobic Biodegradation with Negative-Ion ESI FT-ICR MS

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Abstract

Rationale

Heteroatoms are relatively abundant and believed to be bio-resistant in heavy crude oils. However, few studies have focused on biodegradation of these heteroatomic compounds.

Methods

The heteroatoms, especially N_1-species, in a blank crude oil and three treated oils co-incubated in the anaerobic sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB) and fermentative consortia (FC) cultures were detected by negative-ion electrospray ionization (ESI) coupled to high-field Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS).

Results

The relative abundance of N_1-species in the three treated oils decreased, while the relative abundance of O_2-species increased. Remarkably, the relative abundances of N_1-species with low carbon number increased and those with higher carbon number decreased.

Conclusion

These results revealed that the anaerobic biodegradations of heavy crude oil occurred. With the direct evidences, the degradations of alkyl side chains of N_1-species by those anaerobic microbes could be deduced.

Keywords: heteroatoms, anaerobic biodegradation, FT-ICR MS, N_1-species, alkyl side chains

Crude oils are compositionally complex organic mixtures consisting of hydrocarbons, heteroatomic compounds, as well as a small percentage of metals[1]. Heteroatomic compounds being composed of N-, S-, and O-containing species make up a relatively small portion of crude oil, less than 15%, but make a great contribution to physical and chemical properties of crude oils. These heteroatoms are typically found in the high boiling fractions which raise a number of problems including plugging of oil wells, residue deposition in pipelines, and increasing amounts of distillation residues[2].

Microbial natural degradation the petroleum compounds, such as alkanes and aromatic hydrocarbons, usually focus on environmental aspects[3,4]. The neutral nitrogen compounds and acid NSO compounds in crude oils experienced subsurface anaerobic biodegradation were thoroughly analyzed[5]. Another scenario, during microbial enhanced oil recovery (MEOR), with the features of economic and ecological advantages, could also alter the crude oil compounds by cultured microbes with injected nutritions[6,7]. A major mechanism of microbial processes to enhanced oil recovery of heavy oils involves *in situ* conversion of long-chain compounds to short-chain ones by petroleum-degrading microorganisms[8]. Hydrocarbons ranging from C15-40 are susceptible to biodegradation[4,9,10]. However, biodegradations of polar compounds during MEOR process were not clearly confined.

The relative susceptibility of polar compounds to microbial biodegradation was seldom examined because of that heteroatom-containing compounds are more resistant to microbial catabolism than that to alkanes[4,11]. However, toward the degradation of crude oil coupled to reduction of different non-oxygen terminal electron acceptors, such as nitrate and sulfate, compounds with low carbons atoms have been found to be more recalcitrant than mid- to high-molecular weight alkanes[12-14]. Therefore, the present study on the relative susceptibility of heteroatomic compounds to anaerobic microbial alteration would be a meaningful attempt to discover the microbial degradation of petroleum compounds with high molecular weight.

Electrospray ionization (ESI) coupled to high-field Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) technique has served as a powerful composition analysis tool for detailed characterization of complex mixtures in crude oils[15-18]. Moreover, nitrogen compounds, having a tendency to exist in higher relative abundance in the high boiling points fractions, have been resolved and identified based on negative-ion ESI FT-ICR MS

examinations[19-21].

At present, the level of assessing the susceptibility of hydrocarbons to biodegradation performs only on individual hydrocarbon classes. This way is particularly suited for examining the underlying physiology and metabolism of hydrocarbon degraders by tracing the structure and quantity changes of the individual substrate. However, during a field trail of MEOR, it is impossible to trace alterations of every individual compound due to the facts that crude oils are complex mixtures and the compounds could be attacked by various kinds of microorganisms. Therefore, tracing one class compound seemed to be the practicable way to carried out our investigation.

Microbial degradation of the nitrogen compounds usually focuses on the degradation of the nonbasic nitrogen compounds, particularly carbazole and its alkyl derivatives, because they represent the majority of the total nitrogen and because the basic nitrogen compounds can be readily extracted if desired. So in this paper, we focused on tracking alterations of neutral nitrogen compounds during microbial treatments in three anaerobic enrichment cultures of sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB), fermentative consortia (FC) that are stated to be the three major respiratory types of anaerobic biodegradation of crude oils[22]. The objective of this study is to add valuable information on anaerobic biodegradation of heteroatoms-containing compound in heavy crude oil.

Materials and methods

sample collection

About 10 L Water-oil sample and 2 L crude oil sample were collected directly from the wellhead of the production well of T6191 in the Middle Block No. 6 of Xinjiang Oil Field in Karamay city located at north-west of China, and filled fully into sterile plastic bottles and transported to the laboratory as fast as possible for inoculation. The crude oil with a viscosity of approximate 110 mPa·s was identified as heavy oil. Table 1 displayed mass fractions of saturate and aromatic compounds, resins and asphaltenes, and those were determined according to Chinese Standard Analytical Method for Petroleum and Natural Gas Industry (SY/T 5119-2008, details in supplementary materials).

Media preparation

A modified basal seawater medium containing per liter 10 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.25 g KCl, 0.6 g KBr, 0.5 g KH_2PO_4 and 1 mL resazurin of 1% (w/v) was used in this study. Three stock solutions of 1 M $NaHCO_3$, trace elements and vitamin solution[23] were sterilized through filtration. For SRB enrichment, the modified basal seawater medium was supplemented per liter with 4 g Na_2SO_4, 1 g Na-acetate, 1 g yeast extract. For NRB enrichment, the basal seawater medium was supplemented per liter with 3 g KNO_3, 1 g Na-acetate, 1 g yeast extract. And for FC enrichment, the basal seawater medium was supplemented per liter with 4 g peptone and 10 g yeast extract. After Autoclaved, 30 mL 1 M $NaHCO_3$, 1 mL trace elements and 1 mL vitamin solution were added (per liter) and pH was adjusted to 7.0-7.2. 100 mL of medium was dispensed into 200 mL stoppered serum bottles and flushed with N_2-CO_2 (80/20,v/v)[23].

Inoculum preparation

In this study, the water-oil samples collected from the production well of T6191 was used as inoculum. In order to ensure the presence of petroleum degradation microbes, microbial community inhabiting in the sample was detected based on 16S rDNA clone library technique[24]. Results revealed that *Desulfocapsa* sp. was predominant at 66.4%, followed by uncultured Cytophaga-Flavobacter-Bacteroidetes (CFB) group (9.1%), *Syntrophus* sp. (3.8%), *Spirochaetes* sp. (3%), *Acrobacter* sp. (2.3%) and *Denitrovibrio* sp. (1.5%). Most of the detected microbes were reported to be capable of anaerobic degradation petroleum[14,24,25]. The detail description of microbial analysis was in the supplementary materials (Fig. S1).

According to microbial analysis, each bottle of prepared enrichment medium was inoculated with 5% (v/v) produced water and 5 mL crude oil and cultivated at 25□ representing the *in situ* temperature of the oil reservoir for 120 days. A blank experiment without inoculum was also incubated. Each culture was conducted in triplicate.

Oil sample preparation

After incubation, the enrichment cultures were transferred to centrifuge tubes, and then centrifuged at 10,000 g for 10 min. The anaerobic treated oil sample in the above layer was collected for negative ion ESI FT-ICR MS analysis[15]. Each oil sample was diluted with toluene to produce a 10 mg/mL mixture. Each mixed solution was prepared by dissolving 20

μL into 1mL toluene/methanol (1:1, v/v) solution, redistilled twice. The raw heavy crude oil sample was prepared following the same procedure as the control sample. Except the steel piston of 10 μL Hamilton syringe, all utensils for handling and transfer solvents were glassware.

ESI FT-ICR MS analysis.

Mass analysis of all oil samples was performed with a Bruker apex-ultra FT-ICR mass spectrometer, which equipped with a 9.4 T superconducting magnet. Through a syringe pump, each prepared sample was injected at a flow rate of 180 μL/h into an Apollo II electrospray source. For negative-ion generation, the operating conditions included emitter voltage, 4.0 kV; capillary column introduce voltage, 4.5 kV; capillary column end voltage, -320 V. Under a 2.4 V of directed current (DC) voltage and 400 Vp-p of radio-frequency (RF) amplitude, ions were accumulated externally in a hexapole ion trap for 0.1 s. All of the ions passed through a single quadrupole, accumulated for 4 s in an argon-filled hexapole collision pool that was operated at at 5 MHz and 400 Vp-p of RF amplitude. The delay was set to 1.2 ms for ions to transfer from the collision pool to an ICR cell. The mass range was set at m/z 200-700 Da for excited ions by RF excitation attenuated at 13 dB. The data size was set to 2 M, and 128 scan FT-ICR data sets were co-added to enhance the signal-to-noise ration and dynamic range[16,26].

Mass calibration and data processing

A calibration of mass range from m/z 200 Da to 700 Da was carried out for ESI FT-ICR MS. Alkyl carbazole series were used as standards. Mass values with FT-ICR mass spectral magnitude greater than 5 times the standard deviation of the baseline noise were converted to Kendrick mass and exported to a data set. A custom software described in Shi's paper at year 2010 was used for data processing[14]. A two-mass scale-expanded segment close the most abundant peak in the spectrum was selected for all measured mass, and then each peak was identified in detail. The reference peaks containing at least one of those heteroatomic species were arbitrarily picked. Within a set ±0.001 Kendrick mass defect (KMD) tolerance, each class species and its isotopes with different DBEs and carbon numbers were detected[15].

Results

Distribution alterations of heteroatom class species

The negative ion ESI FT-ICR MS broadband spectra (200-700 Da) of the raw heavy crude oil and the three treated oils collected from SRB, NRB and FC enrichment cultures were obtained (Fig.S2 in supplementary materials). The mass distributions of treated oils were found to be different in comparison to the original oil. For each oil sample more than 4900 peaks at 200-700 Da were detected, of which approximately 3000 were assigned with the molecular formulas by exact masses. As the mass resolving power (m/Δm 50%) was insufficient to resolve all of the heteroatomic species present in oil samples, the mainly focused class species derived from the negative ion ESI FT-ICR MS spectra of the oil samples were N_1, NO, NO_2, O_1 and O_2.

The relative abundances of each class heteroatomic species for the crude oil and the three treated oils are presented in Figure 1. Relative abundance is defined as the magnitude of each peak divided by the sum of magnitudes of all identified peaks, excluding the isotopic peaks in the mass spectrum. N_1-species were the predominant class species in the negative ion ESI FT-ICR mass spectra of the raw heavy crude oil sample and the three treated oil samples. The relative abundance of N_1-species slide from 73.2% in the raw crude oil to 68.5%, 65.1% and 67.2% in the SRB, NRB and FB treated oil samples, respectively. While, the relative abundances of another dominant class, O_2-species, in treated oils appeared to increase to 25.9%, 30.1% and 28.2% in the SRB, NRB and FB treated oil samples, respectively, from 20.8% in the raw heavy crude oil sample. In summary, in the heavy crude oil samples after co-incubated with three major anaerobic respiratory microbes, the relative abundance of N_1-species decrease, while the relative abundance of O_2-species increase.

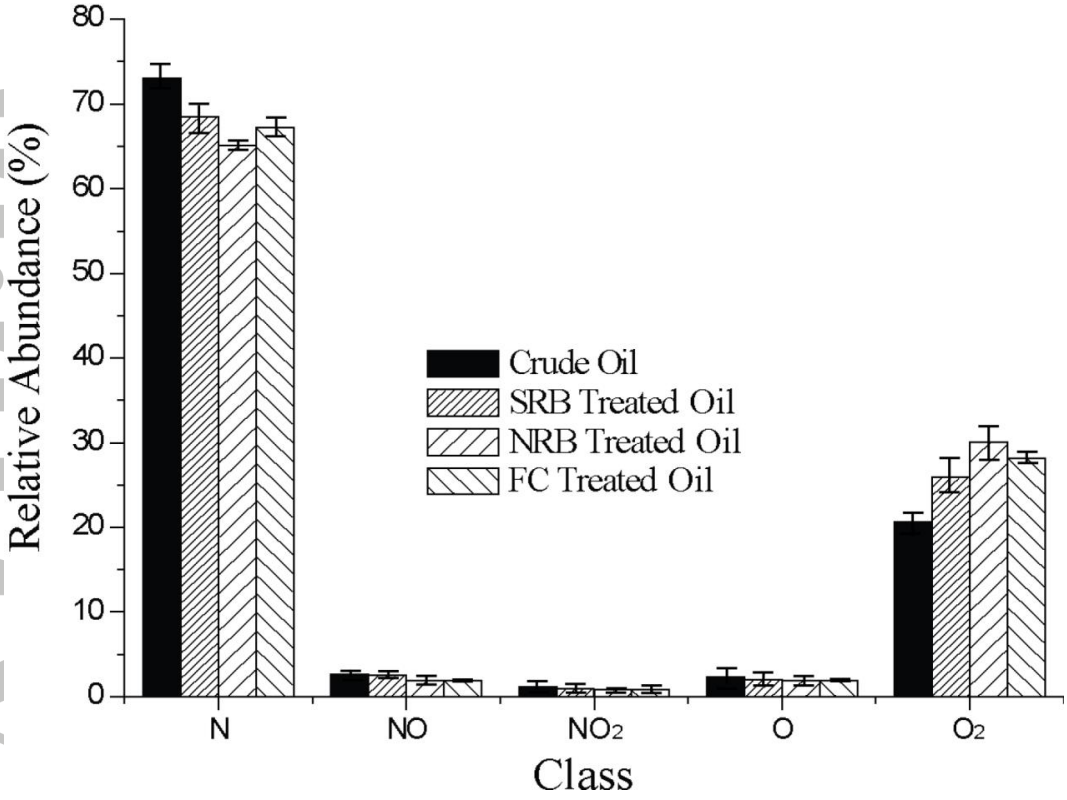

Figure 1 The relative abundances of heteroatomic species in the raw heavy crude oil and three oil samples co-incubated with SRB, NRB and FC enrichment cultures. The results were averaged from triplicate cultures.

Distribution alterations of DBE versus Carbon Number of N_1-species

The iso-abundance maps with dot-size-coded plots, double-bond equivalent (DBE) values and carbon numbers for the N_1 class species in a blank crude oil and three treated oils were shown in Figure 2. Comparison of the range in both DBE and carbon number distribution clearly illustrates the similarity among the crude oil and three treated oils. The N_1-species in all oil samples were distributed over a wide range of DBE values of 6−21 and carbon numbers of 15−47, while centered at DBE of 9−13 and carbon number of 23−32. There seemed to be not significant changes on the spread of DBE and carbon number distributions of N_1 class species after the treatments using three typical anaerobic microbes based on the iso-abundance maps. However, a more meticulous analysis was conducted to identify the subtle alterations.

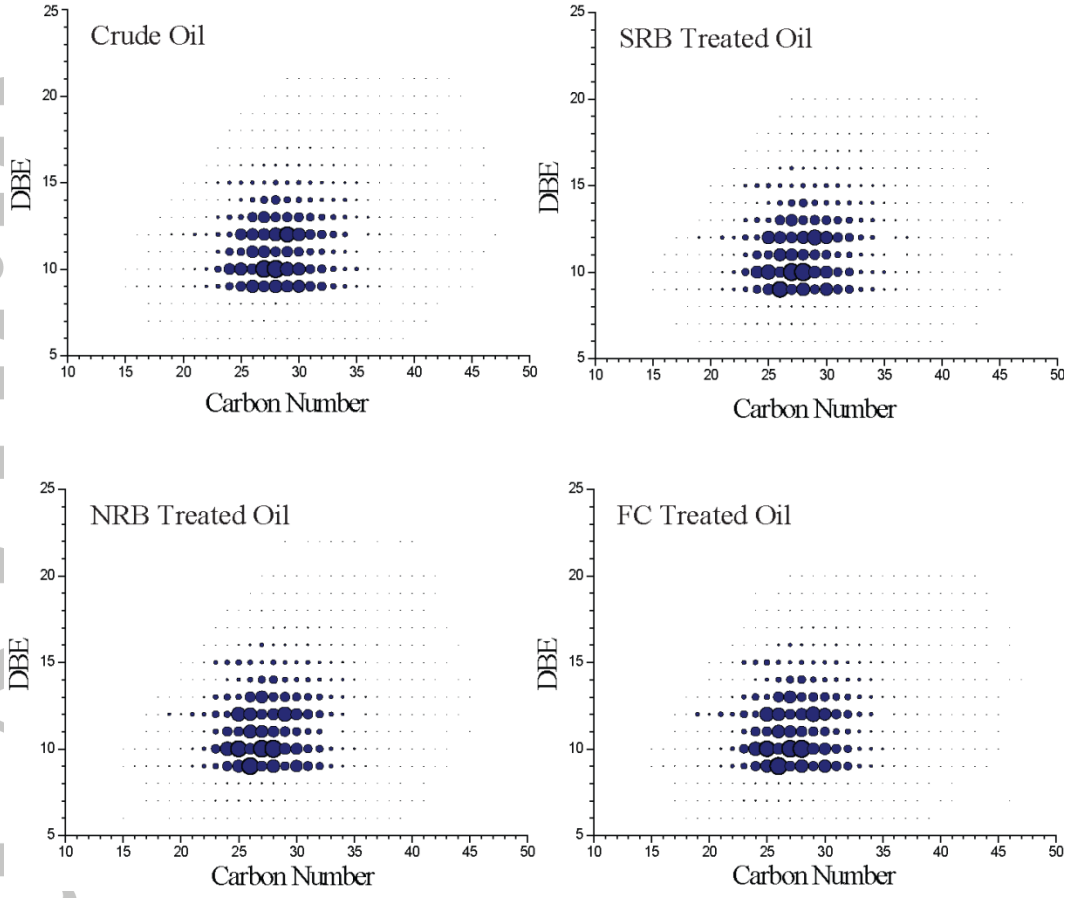

Figure 2 Iso-abundance plots of DBE values vs carbon number of N_1-species in a blank crude oil and three oil samples collected from SRB, NRB and FC enrichment cultures. The size of circles showed the relative abundance of N_1-species.

Relative abundance alterations of N_1-species with each carbon number

The carbon numbers of N_1-species with same DBE indicate the pattern of alkyl distribution. Compounds with higher carbon numbers are those having longer alkyl side chains and/or multi-substituted alkyl side homologues. Figure 3 and 4 show the carbon number distributions for dominant N_1-species with DBE of 9, 10, 11, 12 and 13. N_1-species with DBE value 9 are most likely carbazoles. From the Figure 3A, the relative abundances for each carbon number match well between the crude oil and its treated oil samples, and compounds with 23-32 carbon numbers were dominant. However, the relative abundances of N_1-species (DBE value 9) with carbon number below 26 in the three treated oils increased, while, those with carbon number over 26 decreased. The relative abundance of N_1-species with DBE value of 9 and carbon number of 26 (carbazoles with 14 additional methylene groups)

appeared to increased most. Similarly, N_1-species with DBE value of 12 are most likely benzocarbazoles that have an additional ring of benzene compared with those with DBE value of 9. From the Figure 4A, the relative abundance of N_1-species with DBE value of 12 and carbon number of 26 was also the point of the turn. N_1-species with DBE value of 10, 11 and 13 are most likely carbazoles with additional cyclic alkane rings[26]. From the Figure 3B, 3C and 4B, the dominant compounds matched well between the crude oil and its treated oils, and the apparent alterations were that the relative abundances of N_1-species with carbon number below 28 in the three treated oils increased, while, those with carbon number over 28 decreased. While in the Figure 4B, the relative abundances for each carbon number after co-incubated with SRB appeared slighter alterations than those of NRB and FC. Even so, the relative abundance of N_1-species with DBE value of 13 and carbon number of below 27 increased and those with carbon number over 27 decreased after treatments. Exceptionally, the relative abundances of N_1-species with carbon number of 29 of treated oils of SRB and NRB were higher than that in crude oil.

One significant finding was that the proportion of low carbon number N_1-species to high carbon number ones increased after co-incubation in three major anaerobic microbial enrichment cultures.

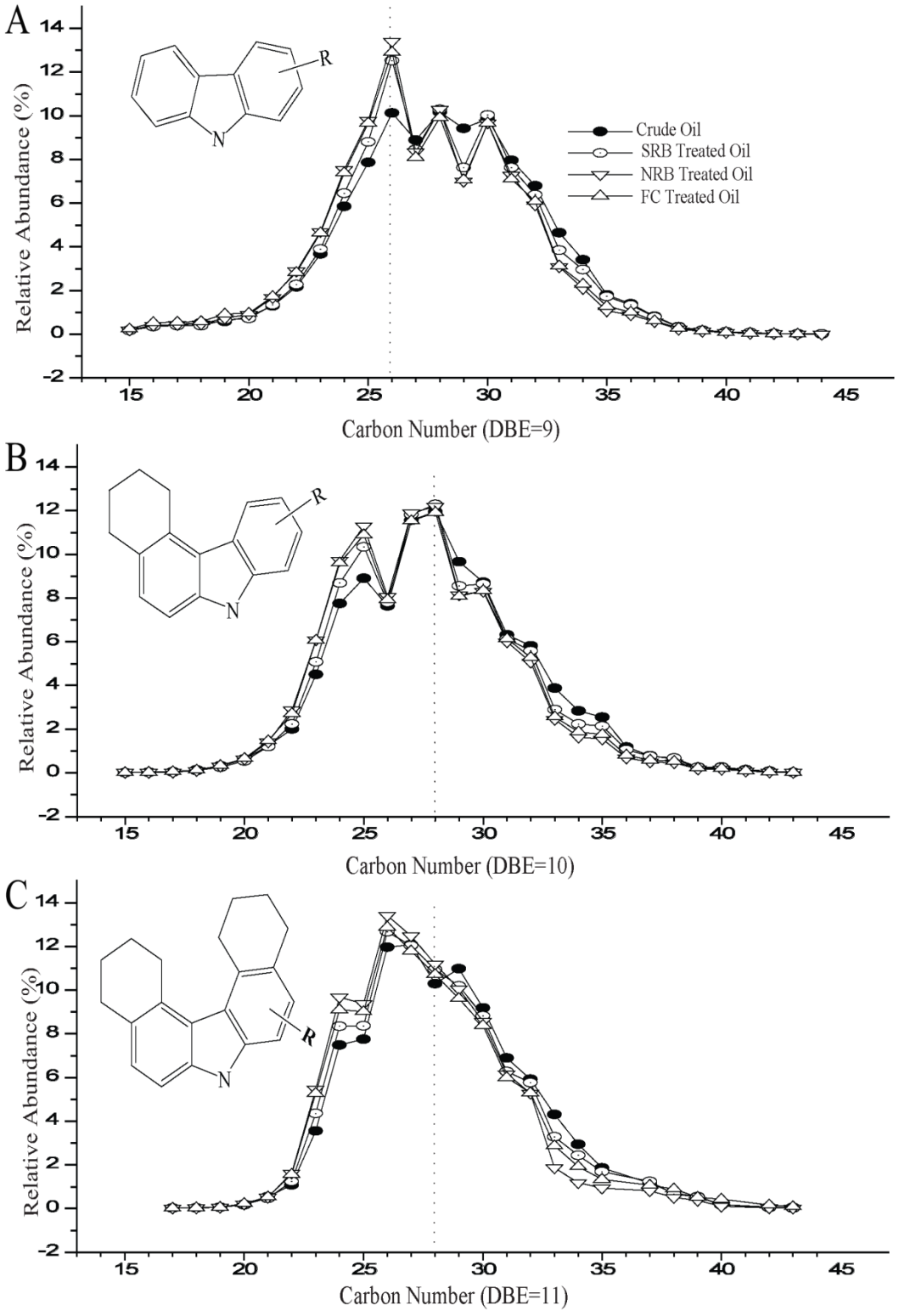

Figure 3 Carbon number distributions for the dominant N_1-species with DBE of 9, 10 and 11. The results were presented as averages from the triplicate cultures.

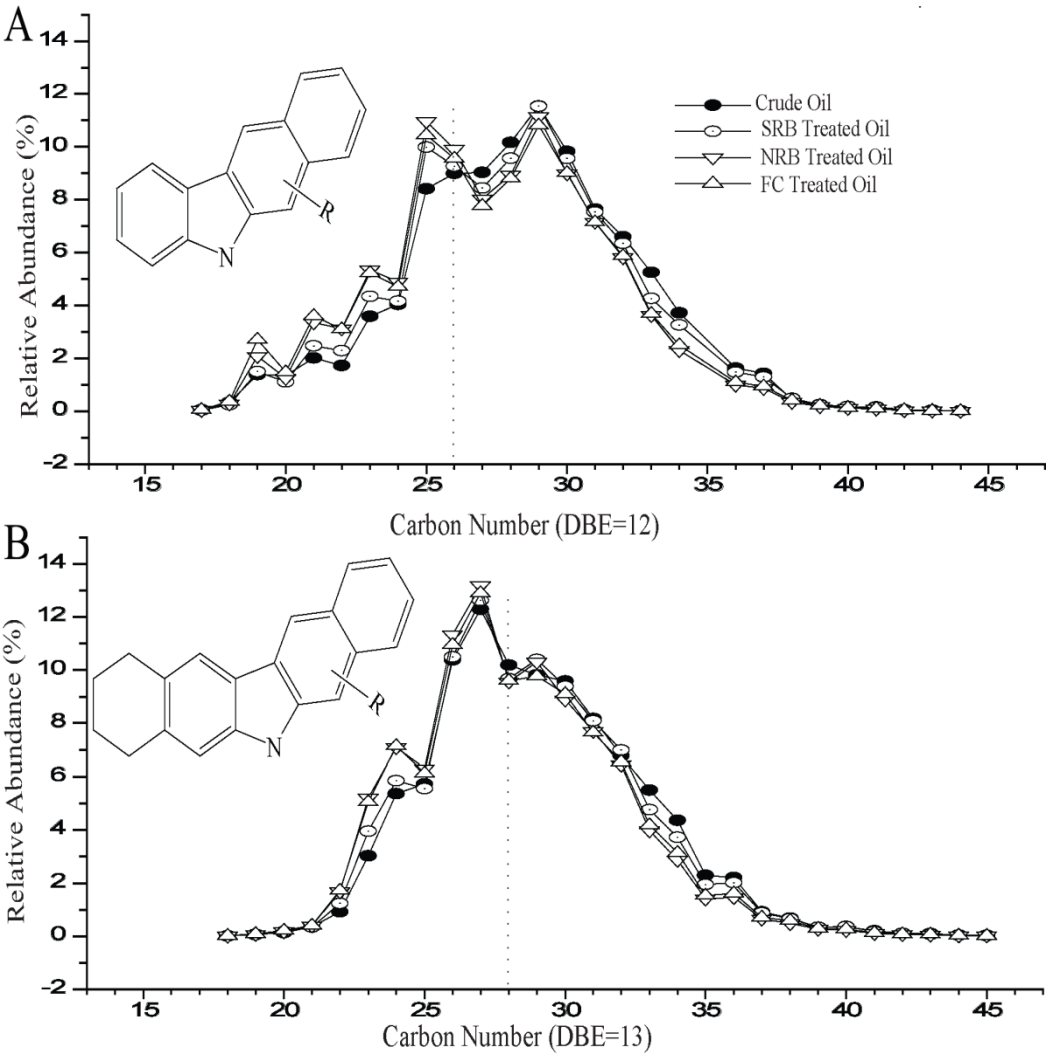

Figure 4 Carbon number distributions for the dominant N_1-species with DBE values of 12 and 13.

Discussion

Biodegradation occurrence based on alterations of class distribution

Biodegradation undoubtedly alters the compound class distributions of crude oil[5]. In this study, the relative abundances of O_2-species (dominated at DBE of 1−3 and carbon number of 21−29 in Figure S3) showed increase after the three major anaerobic microbial treatments, which was similar to previous studies that the increase of O_2-species is more predominant in the heavily to severely biodegraded oils [1,5]. Specially, in order to guarantee the detected compound classes were similar kinds of molecules, all oil samples were subjected to the negative ion ESI FT-ICR-MS analysis under same operating procedures and conditions.

O_2-species detected are predominately carboxylic acids because those species are selectively ionized in negative ion ESI[16,26]. As previously stated, acids are important intermediates in the degradation of complex organic matters, and could also be intermediates in the anaerobic degradation of hydrocarbons[25,27,28]. In this study, each compound in the heavy crude oil potentially might be attacked by various degraders. The saturated and aromatic compounds, major potential substrates for anaerobic microbes to produce acids, represented 67.7% in the crude oil under study (Table 1).

N_1-species selectively ionized by negative ion electrospray are dominated by carbazoles and its derivatives. Aerobic carbazole degradation by a variety of carbazoles degrading microorganisms has been well investigated over the last two decades. These different carbazole degraders follow similar degradation pathways, and the first step is catalysis by carbazole dioxygenase, which converts carbazole to 2'-aminobiphenyl-2,3-diol[29-32], while, the literature about anaerobic microbial biodegradation of carbazole was rare. Nonetheless, the carbazole biodegradation under anaerobic conditions could be somehow possible.

In this study, it is impossible to trace alterations of every compound that could be attacked by anaerobic microbes. However, with the direct evidences detected in this study, the production of acids, the consumption of N_1-species and the alterations of class distribution, those could demonstrate the heavy crude oil anaerobic biodegradation occurrence.

Biodegradation of alkyl side chains of N_1-species

From the iso-abundance maps of N_1-species in the blank crude oil and three treated oils, little change was observed in the distributions of dot-size-coded plots (double-bond equivalent (DBE) values versus carbon numbers). However, when we examined the carbon number distributions for N_1-species with same DBE, obvious alterations appeared. The most apparent was that the relative abundances of low carbon number N_1-species increased, while the relative abundances of higher carbon number ones decreased, which suggested that the long alkyl side-chains might attacked by three major anaerobic microbes. Similarity, it was reported that the reduced degree of alkylation in all DBEs of N_1 compounds in oils subjected to greater microbial activity[5]. And they suggested that degrading enzyme can attack long side-chains as if they were alkanes. Although the consideration of crude oil biodegradation

under anaerobic conditions was negligible until the early 1980s[3], two mechanisms for the anaerobic oxidation of n-alkanes were proven. One was the activation of the sub-terminal (C-2) carbon atom followed by addition to fumarate[33]. The other mechanism was carboxylation with inorganic carbon at C-3[25]. Regularly, for N_1-species with DBE of 9 and 12, the relative abundances of compounds with carbon number over 26 decreased; and for N_1-species with DBE of 10, 11 and 13, the relative abundances of compounds with carbon number over 28 decreased. The occurrence of the trends might relate to the anaerobic biodegradation of side-chains, but how microbes attack those side-chains was still unclear. The identification of the underlying mechanisms requires further in-depth research.

In addition, three different types of microbial communities with different kinds of terminal electron acceptors were enriched in this study. The available results showed similar patterns of the alterations of N_1-species in the three anaerobic microbes enrichment cultures co-incubated with heavy crude oil, which indicated that biodegradation of the crude oil in the three anaerobic microbes enrichment cultures co-incubation might followed the same metabolism mechanisms.

Conclusion

In this study, we demonstrated that N_1-species in heavy crude oil could be degraded by the common anaerobic microbes through detection technology of negative ion ESI FT-ICR MS. Under the three classic anaerobic conditions, the increase of the relative abundances of O_2-species was a typical signal of the biodegradation occurrence. Furthermore, the relative abundances of low carbon number N_1-species increased, while the relative abundances of mid- to high carbon number ones decreased, suggesting that anaerobic microbial alteration occurred in the N_1-species compounds with long alkyl side-chains. This was the direct evidence for the anaerobic biodegradation of long alkyl side-chains in heteroatomic compounds. As the complex of the heavy crude oil and the less referenced article, the mechanisms involved in anaerobic biodegradation heteroatomic species in heavy crude oil was still unclear, further in-depth researches are required.

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Author contributions

S. Su, F. Zhang, and Y. She designed the experiments. S. Su and H. Dong performed the experiments. G. Yu and D. Hou contributed to manuscript preparation. S. Su and H. Dong wrote the manuscript. Q Shi, I. M. Banat, Z. Wang and Y. Gu contributed to experiment results discussions and comments on the manuscript.

Additional information

Supplementary information accompanies this paper can be available at https://link.springer.com/journal/216.

Competing interests

The authors declare no competing interests.

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Table 1 Mass fractions of saturate and aromatic compounds, resins and asphaltenes in the crude oil of T6191.

Fractions	Saturate compounds	Aromatic compounds	Resins	Asphaltenes
Abundance in % (w/w)	41.4	26.3	22.4	9.9