

# Isotope fractionation ( $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ ) and microbial community response in degradation of petroleum hydrocarbons by biostimulation in contaminated soil

Heng Liu

Xi'an University of Architecture and Technology

Manli Wu (✉ [wumanli1974@hotmail.com](mailto:wumanli1974@hotmail.com))

Xi'an University of Architecture and Technology School of Environmental and Municipal Engineering

Xiqian Guo

Xi'an University of Architecture and Technology

Huan Gao

Xi'an University of Architecture and Technology

Yinrui Xu

Xi'an University of Architecture and Technology

---

## Research Article

**Keywords:** Petroleum-contaminated soil, Microbial community, Carbon, Nitrogen, Isotope effect

**Posted Date:** May 3rd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-183754/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

This study investigated the isotope effects of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  and microbial response during biodegradation of hydrocarbons by biostimulation with nitrate or compost in the petroleum-contaminated soil. Compost and  $\text{KNO}_3$  amendments promoted the total petroleum hydrocarbon (TPH) removal accompanied by a significant increase of *Actinobacteria* and *Firmicutes* phyla. Soil alpha diversity decreased after 90 days of biostimulation. An inverse significant carbon isotope effect ( $\epsilon_{\text{C}} = 16.6 \pm 0.8\text{‰}$ ) and strong significant nitrogen isotope effect ( $\epsilon_{\text{N}} = -24.20 \pm 9.54\text{‰}$ ) were shown by the  $\text{KNO}_3$  supplementation. For compost amendment, significant carbon and nitrogen isotope effect were  $\epsilon_{\text{C}} = 38.8 \pm 1.1\text{‰}$  and  $\epsilon_{\text{N}} = -79.49 \pm 16.41\text{‰}$ , respectively. A clear difference of the carbon and nitrogen stable isotope fractionation was evident by  $\text{KNO}_3$  or compost amendment, which indicated the mechanisms of petroleum degradation by adding compost or  $\text{KNO}_3$  are different.

## 1. Introduction

Petroleum is a mixture of various of hydrocarbons including aliphatic and aromatics fractions. The exploitation, transportation and application of crude oil and petroleum products may cause petroleum to seep into the soil which changed physical and chemical properties of soil and led to great damage to the ecological environment around the world (Wang et al., 2016; Shahi et al., 2016a; Cho et al., 2019; Mahmoud and Bagy, 2019). Alexander et al. (2010) have confirmed that some small molecular weight hydrocarbons can be biodegraded gradually under natural conditions, while the high molecular weight hydrocarbons may stay in the soil for a long time. Due to the slow degradation of petroleum hydrocarbons in the process of natural attenuation, it is particularly important to stimulate the activity of autochthonous microbial consortia using various amendments, which provides an efficient biostimulation strategy with less disturbance to the soil micro-ecosystem.

Biostimulation, as a promising remediation strategy by enhancing activities of the native microorganisms in soil, has been widely concerned and achieved remarkable achievements in the past few decades (Meynet et al. 2012; Karthick et al. 2019; Onotasamiderhi et al., 2019; Xu et al., 2019). It is generally believed that the activities of indigenous microorganisms are limited by oxygen, water, nitrogen, phosphorus, and other nutrients (Schjønning et al., 2011; Ali et al., 2016). Remediation of soil by supplementing of stimulators such as nitrogen, phosphorus, compost and bulking agent is a common and effective method for contaminant removal. Among all the amendments, inorganic salts containing nitrogen and phosphorus are widely applied in remediation of oil contaminated soil (Abed et al., 2015; Shahi et al., 2016b; Wu et al., 2019). In addition, compost amendments have shown good effects in stimulating indigenous microorganisms to degrade petroleum hydrocarbons, and were considered to have great development potential in the future due to the characteristic of recycling waste (Dadrasnia et al., 2016; Huang et al., 2019).

The stable isotope technology, originated from the application in geology and geochemistry, has played an important role in illustrating the carbon and nitrogen isotope fractionation effects in biodegradation of organic pollutants (Fan et al., 2019; Fiorentino et al., 2019). Carbon isotope fractionation occurred when the lighter carbon atom ( $^{12}\text{C}$ ) was involved in the cleavage of C-H chemical bond earlier than those of heavier carbon ( $^{13}\text{C}$ ) during degradation of hydrocarbon compounds. Since petroleum mainly consisted of saturated and aromatics hydrocarbons and asphaltenes, the carbon isotope fractionation effects were inconsistent among different fractions of petroleum hydrocarbons with various molecular weights and structures, which can distinguish the natural attenuation of crude oil and petroleum product during weathering process (Wang and Fingas, 1995). Li et al. (2018) revealed that the carbon isotope fractionation of polycyclic aromatic hydrocarbons (PAHs) can be used to distinguish the degradation degree of PAHs in crude oil and fuel oil. For nitrogen isotope, more attention is focused on the fractionation effect of nitrogen in nitrates which are widely used as inorganic nitrogen source. Hu et al. (2020) compared the nitrogen isotope fractionation effects of three types of nitrates between the field constructed wetland and the laboratory simulated denitrification process and concluded the organisms in the wetland had a greater influence on the  $^{15}\text{N}$  isotope fractionation than that in the laboratory simulation.

There were significant differences in carbon isotope fractionation effects for various types of hydrocarbons when environmental conditions such as oxygen, moisture and nutrition levels changed (Tyler et al., 1994). Previous study illustrated the  $^{13}\text{C}$  enrichment factor for aromatic hydrocarbons was 1.3 greater in aerobic than in anaerobic conditions, which was caused by the specific enzymes involved in the biodegradation of benzene, toluene, and xylene (Meckenstock et al., 2004). Although the influences of temperature, soil properties, and the contents of soil organic matter on carbon isotope effects of organic pollutants have been well elucidated by many research, few studies have been carried out to investigate the carbon and nitrogen isotope effects during biostimulation remediation of oil contaminated soil presently.

In this study, three bioremediation methods including enhancing soil humidity, adding inorganic salt compounds and organic compost, were applied to promote petroleum hydrocarbon degradation in a aged pollution soil. The degradation rates of petroleum hydrocarbon, microbial community structures, and isotope effects of  $^{13}\text{C}$  and  $^{15}\text{N}$  during remediation were investigated simultaneously. This work provided the intrinsic relevance between petroleum hydrocarbons removal and the effects of imported nutrition in soil.

## 2. Materials And Methods

### 2.1. Soil and compost

Oil-polluted soil samples were collected from the north of Shaanxi province, one of the main oil exploration areas in China, and the soil had a long pollution history over 5 years. According to previous

Loading [MathJax]/jax/output/CommonHTML/jax.js

methods (Wu et al., 2019), the samples obtained from the topsoil of contaminated site were mixed evenly and then packed in sterilization bags for storage at low temperature and transported to the lab. Results of soil properties determination are shown in Table 1. The decayed organic fertilizer used in the study was made from pig manure: rice husks with a dry mass ratio of 1: 2 and 5.0% charcoal slag heap. The organic compost properties were as follows: N, 20400 mg kg<sup>-1</sup>; P, 6500 mg kg<sup>-1</sup>; K, 36200 mg kg<sup>-1</sup>; Na, 3810 mg kg<sup>-1</sup>; Ca, 21300 mg kg<sup>-1</sup>; Mg, 2420 mg kg<sup>-1</sup>; Al, 1330 mg kg<sup>-1</sup>; S, 1575 mg kg<sup>-1</sup>; Cu, 400 mg kg<sup>-1</sup>; Fe, 1615 mg kg<sup>-1</sup>; Mn, 284 mg kg<sup>-1</sup>.

Table 1  
Physical and chemical characteristics of the soil

Main characteristics	Values
TPHa (mg kg <sup>-1</sup> )	24166 ± 40.46
Moisture content (%)	6.34 ± 0.05
pH	8.01 ± 0.03
Available phosphorus(mg kg <sup>-1</sup> )	13.79 ± 0.11
Total nitrogen(mg kg <sup>-1</sup> )	1005 ± 12.21
Ammonia nitrogen(mg kg <sup>-1</sup> )	8.21 ± 0.47
Nitrate nitrogen (mg kg <sup>-1</sup> )	20.13 ± 0.54
<sup>a</sup> TPH: Total Petroleum Hydrocarbon.	

## 2.2. Biostimulation

Three treatments included: (1) CK: addition of 100 mL deionized water to 0.8 kg soil to increase soil moisture content by 15%; (2) MS: amendment of 11.8 g KNO<sub>3</sub> to adjust the ratio of C:N to 100:10 in the 0.8 kg soil; (3) SC: addition of 11.8 g compost to 0.8 kg soil. All the biostimulation remediation experiments were carried out in flowerpots and made in triplicate at room temperature for 90 days. In addition, the soil was agitated with sterilized glass rods every three days so that the remediation could be performed under oxic conditions.

## 2.3 Determination of TPH, saturate and aromatic fractions

Determination of TPH, saturate and aromatic fractions in soils was based on the methods of Wu et al. (2020). In brief, 1 g of soil was added to 15 mL mixed solvent of n-hexane and methylene chloride with the volume ratio of 1:1 in a weighing bottle of known weight, and the petroleum hydrocarbons in soil were extracted for 10 min by an Ultrasonic Processor (JY96-IIN, Ningbo Science Biotechnology CO. LTD, China). Then, the mixture was centrifuged at 8000 r min<sup>-1</sup> for 10min. The extraction and centrifugation processes were repeated three times and mixed to obtain total petroleum hydrocarbon (TPH) from the

Loading [MathJax]/jax/output/CommonHTML/jax.js

and total petroleum hydrocarbon were determined by

gravimetric method according to the difference in the weight of the weighing bottle (Mishra et al., 2001). The saturate and aromatic fractions of TPH were obtained according to the description by Wu et al. (2020). Briefly, 5 mL of n-hexane was added to the TPH and transferred to the Super Flash Alumina Neutral columns (SF 15–24 g, 20.8 × 112 mm, Agilent Technologies), which was repeated three times and then placed in a fume hood to obtain saturate components. The aromatic components were obtained by adding benzene to the remainders in the Super Flash Alumina Neutral columns, subsequently. The saturate and aromatic fractions were respectively quantified gravimetrically (Gao et al., 2019).

## 2.4 Genomic DNA extraction and Illumine sequencing data analysis

Extraction, integration and quantitative analysis of total DNA of the bacterial community in the soils were performed by Power Soil DNA extraction kit (MoBio Laboratories, USA), agarose gel electrophoresis and Qubit® 2.0 DNA detection kit (Life, USA), respectively. The primers 341F (CCCTACACGACGCTCTTCCGATCTGCCTACGGGNGGCWGCAG) and 805R (GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC) were applied for PCR amplification (Qu et al., 2016). The PCR products were purified by 0.6 times of magnetic beads and sequenced by Sangon Biotech Co., Ltd. (ftp://ftp.sangon.com:21148). Prinseq software (version 0.20.4) and Usearch (version 5.2.236) were used for revising the initial data. The OTUs and diversity of microbial community were analyzed by RDP classifier (version 2.12) and Mothur (version 1.30.1), respectively.

## 2.5 Soil total carbon and nitrogen contents, carbon and nitrogen stable isotopes detection

The contents of total carbon and nitrogen, the values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in soils were determined by elemental analyzer (Vario PYRO cube, Elementar Co., Hanau, Germany) and isotope ratio mass spectrometer (IsoPrime IRMS, Elementar Co., Hanau, Germany), respectively. In detail, 20 mg soil was air-dried, ground and enclosed in tin foil. Then, they were fully burned at a high temperature of 1120°C and the contents of elements and isotopes were obtained based on the  $\text{CO}_2$  and  $\text{NO}_x$  generated. Throughout the measurement process, pure carbon dioxide and nitrogen were used as reference gases, and helium was used as carrier gas. In addition, isotopic data were calculated according to the following equations:

$$\delta^{13}\text{C} = (R_{\text{sample}} / R_{\text{Vienna Pee Dee Belemnite}} - 1) \times 1000 \quad (1)$$

$$\delta^{15}\text{N} = (R_{\text{sample}} / R_{\text{N}_2 - \text{atm}} - 1) \times 1000 \quad (2)$$

in which R meant ratio of heavier isotope ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) to lighter isotope ( $^{12}\text{C}$ ,  $^{14}\text{N}$ ). Besides, Vienna Pee Dee Belemnite and  $\text{N}_2$  were defined as the standard with a R value of 0.

$$\Delta^{13}C = \delta^{13}C_t - \delta^{13}C_0 = \epsilon_c \times \ln f \quad (3)$$

where  $\delta^{13}C_0$  and  $\delta^{13}C_t$  are carbon isotope ratios at the beginning and at t days of the experiment, respectively. The letter  $f$  represented the ratio of the total carbon content at t days to the initial total carbon content in the soils. Similarly, nitrogen isotope enrichment factors ( $\epsilon_N$ ) can be obtained using the following equation:

$$\Delta^{15}N = \delta^{15}N_t - \delta^{15}N_0 = \epsilon_N \times \ln f \quad (4)$$

## 2.6 Data analysis

All the data representing the physical and chemical properties of soil appeared in the form of mean  $\pm$  standard deviation (SD) based on three replicated experiments. Origin software (version 9.0, China) was used to analyze carbon and nitrogen isotope effects in soils during 90 days of remediation. The difference among biostimulation treatments were significant for p values < 0.05.

## 3. Results And Discussion

### 3.1 Effects of biostimulation on TPH, saturates and aromatics degradation

The degradation tendency of petroleum hydrocarbons during 90 days of biostimulation remediation is shown in Fig. 1. Addition of deionized water, mineral nutrition and compost reduced the petroleum hydrocarbon contents from 24166 to 21438, 18480 and 18232 mg kg<sup>-1</sup> soil, which represented 11.29%, 23.53% and 24.55% of aged hydrocarbons removal, respectively (Fig. 1(a)).

Compost or KNO<sub>3</sub> amendment was more effective for stimulating the activities of indigenous microorganisms than that of deionized water. In our previous studies, hydrocarbons removal rates were 16% and 18% after 10 and 8 weeks of remediation by 20% soil moisture contents, respectively (Wu et al., 2019, 2017). Nwankwegu et al. (2016) reported the organic compost as the source of nutrients and bulking agent promoted 93.31  $\pm$  3.98% of diesel oil degradation, which was more effective than nitrogen and phosphorus fertilizers (71.36  $\pm$  5.60% hydrocarbons degradation rate) amendments in the diesel contaminated soil for eight weeks of incubation. In this study, removal rates of petroleum hydrocarbons were lower than the those of the previous study. The reason for this may be that the aged hydrocarbons are difficult to degrade by soil microorganisms in the loessal soil.

The saturate hydrocarbon fractions in the untreated oil-contaminated soil was 16394  $\pm$  160 mg kg<sup>-1</sup>, and they decreased to 15509  $\pm$  124, 13597  $\pm$  136 and 9076  $\pm$  148 mg kg<sup>-1</sup> in the CK, MS and SC treatments after 90 days of biostimulation, representing removal rates of 3.25%, 17.8% and 45.1%, respectively

and from 4554 mg kg<sup>-1</sup> in the untreated soil to 3607  $\pm$  130,

1942 ± 143 and 3507 ± 151 mg kg<sup>-1</sup> in the CK, MS and SC treatments, representing degradation rates of 20.8%, 57.4% and 23.0%, respectively. Thus, KNO<sub>3</sub> amendment was more effective for removing aromatics while compost addition could degrade more the saturates.

### 3.2 Influence of biostimulation on soil bacterial community

Table 2 shows that the number of OTUs based on the 97% similarity threshold were CK (5531) > MS (5317) > SC (5025) in the soil microcosms after 90 days of bioremediation. The ACE index were 10849, 10018 and 9824 and Chao 1 index were 8954, 8176 and 7785 in the CK, MS and SC soil samples, respectively, which indicated that enhancing soil moisture was beneficial to the survival of various of soil microorganisms, while KNO<sub>3</sub> and compost supplementation could reduce the richness of soil microorganisms. It can also be concluded from Table 2 that the soil microbial community rehabilitated by deionized water addition was the most evenly distribution because the ranking of the Simpson index was CK (0.0026) < MS (0.0301) < SC (0.0405). The inorganic nitrogen fertilizer and compost had great interference on the uniformity distribution of microbial community. Nwankwegu et al. (2016) confirmed that it was a failure to evaluate the degradation potential of diesel oil by the increase of total heterotrophic bacterial in the soil, because only specific hydrocarbon utilizing microorganisms were related to the removal rate of diesel oil. Our study revealed that although KNO<sub>3</sub> and compost reduced soil microbial species and the community distribution uniformity, the existence of functional hydrocarbon degrading bacteria may be enhanced which could lead to higher petroleum hydrocarbons degradation in the MS and SC treatments.

Table 2  
Bacterial taxonomy and diversity indices in the CK, MS and SC remediation soil.

soil samples	CK	MS	SC
sequencing analysis			
OTUs num	5531	5317	5025
Diversity indices			
Shannon index	7.16	6.14	5.22
Simpson index	0.0026	0.0301	0.0405
Ace index	10849	10018	9824
Chao1 index	8954	8176	7785

Figure 2 reflects the compositions of microbial community in soils in terms of dominant phyla, classes and genera. *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Planctomycetes* phyla were dominant in all three treatments but the relative abundance changed in different treatments (Fig. 2(a)). The relative abundance of the *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Planctomycetes* phyla in the CK treatment were 25.21%, 23.40%, 16.96% and 9.08%, respectively. In the MS and SC treatments, the relative abundance of the *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Planctomycetes* phyla were 23.10% and 23.10% respectively, which were not significantly different

from that in the CK treatment. Compared to the CK treatment, the relative abundance of *Actinobacteria*, *Acidobacteria* and *Planctomycetes* phyla changed obviously in the MS and SC treatments with the values of 40.0%-31.5%, 15.0%-8.21% and 4.20%-4.21, respectively. In addition, percentage distribution of *Firmicutes* phylum in the SC treatment (27.3%) was the most, which had a significant increase compared with the CK (1.68%) and MS (7.08%) treatments. The dominant classes affiliated to phyla of *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Planctomycetes* were *Alphaproteobacteria*, *Actinobacteria*, *Acidobacteria\_Gp7* and *Planctomycetia*, respectively. Compost and KNO<sub>3</sub> addition had minor effect on the microbial community at the class level (Fig. 1(b)-(e)). *Firmicutes*, *Proteobacteria*, *Acidobacteria* and *Actinobacteria* phyla have proven to be important in mitigating the long-chain alkane, PAHs and other types of hydrocarbons pollution in the soil environment (Zhang et al., 2012; Wu et al., 2019; Wu et al., 2020). Wolf et al. (2019) found that soil polluted by pyrene substantially increased the microorganisms belonging to *Firmicutes* phylum in the clay and sandy loam soils. In addition, phyla of *Actinobacteria* increased while *Acidobacteria* and *Proteobacteria* decreased in the soils remediated by amendments of KNO<sub>3</sub> or sterilized nutrition, indicating that the hydrocarbon degradation may be attributed to the increase of *Actinobacteria* phylum which could utilize petroleum hydrocarbon as their energy source for cellular metabolic activity.

The influences of the biostimulation treatments on the genus level were great (Fig. 1(f)). *Steroidobacter*, *Intrasporangium*, *Blastopirellula*, *Saccharibacteria\_genera\_incertae\_sedis*, *Parcubacteria\_genera\_incertae\_sedis*, *Aquisphaera* and *Rhodococcus* were new genera appeared in the MS and SC treatments compared with the CK soil. Specially, *Intrasporangium* also became a second dominant genus (5.6%) in the SC treatment, which may be related to hydrocarbons degradation. Genera of *Solirubrobacter*, *Spartobacteria*, *incertae\_sedis* and *WPS-1\_genera\_incertae\_sedis* with the relative abundance more than 1% in the CK treatment that was not appeared in the MS and SC treatments. *Gp6* was the most dominant genus in the CK (6.4%), while the relative abundance of *Gemmata* was the greatest in the MS (3.21%) and SC (5.90%) treatments. Therefore, it can be concluded that the compositions of indigenous flora in oil-contaminated soil was influenced by nutrition amendments (Seklemova et al., 2001), which may be related to the higher hydrocarbon removal rates in the MS and SC treatments.

### 3.3 Carbon and nitrogen depletion

The contents of carbon and nitrogen elements in the contaminated soils were detected by elemental analyzer. The soil total carbon content was  $32327 \pm 30 \text{ mg kg}^{-1}$  in the initial oil-contaminated soil (Table 3), and TPH content was determined by ultrasonic extraction and gravimetric method with the value of  $24166 \pm 40.46 \text{ mg kg}^{-1}$  (Table 1). According to the description of Margesin et al. (2007), the proportion of carbon content of TPH was  $20541 \text{ mg kg}^{-1}$  which derived from 85% TPH concentration. Thus soil organic carbon in polluted soil was  $11786 \text{ mg kg}^{-1}$  which are calculated by subtracting the carbon proportion of TPH from soil total carbon.

Table 3

Soil total carbon (STC) and soil total nitrogen (STN) contents,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in the CK, MS and SC remediation soil.

Treatments	Time (days)	STC (mg/kg)	STN (mg/kg)	C/N	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
CK	0d	32327 ± 30	1762 ± 8.2	10.97 ± 0.2	-25.16 ± 0.3	5.79 ± 0.2
	15d	31876 ± 25	1751 ± 7.1	10.10 ± 0.1	-26.95 ± 0.1	5.81 ± 0.1
	30d	31369 ± 24	1747 ± 9.2	9.37 ± 0.2	-26.84 ± 0.5	5.85 ± 0.1
	45d	29987 ± 28	1730 ± 10.1	10.01 ± 0.3	-27.01 ± 0.2	5.92 ± 0.3
	60d	30128 ± 32	1715 ± 13.5	10.40 ± 0.2	-26.81 ± 0.1	6.01 ± 0.1
	90d	30102 ± 29	1698 ± 6.5	10.10 ± 0.1	-27.18 ± 0.4	6.21 ± 0.2
MS	0d	32327 ± 27	1919 ± 5.4	10.07 ± 0.1	-25.16 ± 0.2	8.13 ± 0.2
	15d	29158 ± 24	1768 ± 5.2	10.02 ± 0.1	-26.90 ± 0.1	12.59 ± 0.2
	30d	30003 ± 18	1731 ± 5.1	10.43 ± 0.1	-26.71 ± 0.1	12.71 ± 0.1
	45d	27905 ± 30	1718 ± 5.3	8.67 ± 0.1	-27.04 ± 0.1	13.04 ± 0.1
	60d	27189 ± 26	1701 ± 5.2	8.88 ± 0.2	-27.47 ± 0.2	13.68 ± 0.1
	90d	27227 ± 21	1693 ± 4.9	8.73 ± 0.1	-28.01 ± 0.1	13.74 ± 0.2
SC	0d	32327 ± 24	1847 ± 10.2	10.46 ± 0.1	-25.16 ± 0.5	6.25 ± 0.2
	15d	32130 ± 30	1763 ± 12.1	8.73 ± 0.4	-26.13 ± 0.2	7.45 ± 0.1
	30d	32482 ± 32	1726 ± 10.5	8.76 ± 0.1	-26.16 ± 0.3	8.06 ± 0.1
	45d	31024 ± 16	1715 ± 8.3	8.67 ± 0.1	-28.18 ± 0.2	8.86 ± 0.1
	60d	29153 ± 26	1696 ± 6.4	8.72 ± 0.2	-29.81 ± 0.4	10.21 ± 0.1
	90d	28595 ± 16	1690 ± 7.9	8.63 ± 0.3	-29.92 ± 0.2	10.48 ± 0.2

Two kinds of method including alkaline potassium persulfate digestion-UV spectrophotometry and elemental analyzer were respectively used to determine soil total nitrogen. The content of soil total nitrogen (STN) was  $1005 \pm 12.21 \text{ mg kg}^{-1}$  (Table 1) by using UV spectrophotometry and  $1762 \pm 8.2 \text{ mg kg}^{-1}$  (Table 3) by elemental analyzer. One possible reason for an obvious difference between them was that there were some special nitrogen forms in the polluted soil which can't be digested by alkaline potassium persulfate.

The contents of soil total carbon (STC) obviously decreased with time in the MS and SC treatments compared with the CK treatment. This result was consistent with the TPH degradation trends in our study

$\text{KNO}_3$  and compost treated soil samples (MS and SC samples) had lower C:N ratios on the 90th days of bioremediation than earlier days (Table 3), while there was less reduction of the C:N ratio in the deionized water-treated soil. Previous literatures reported that soil microorganisms were suitable for living at a nutritional level with a C:N ratio of 100:10 (Prescott et al., 2002). In this study, the C:N ratios had been maintained at about 10:1 in the CK treatment, and these ratios were close to 10 in the first 30 days during the bioremediation and then decreased to about 8 in the MS treatment, while the C:N ratios were reduced from about 10 to 8 on the 15th day in the SC treatment. Additionally, the TPH content was reduced in all three treatments, which was the main reason for the reduction of soil total carbon and the C:N ratios. The reason for the reduced C:N ratios in the SC and MS treatment may be that the degradation rates of TPH by the amendment of  $\text{KNO}_3$  or compost were faster than that in the CK treatments.

### 3.4 Carbon and nitrogen isotope effect

The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of soil samples detected by stable isotope ratio mass spectrometer are shown in Table 3. According to Equations 3 and 4, carbon isotope enrichment factors respectively were  $\epsilon_c = 28.3 \pm 0.3\text{‰}$ ,  $\epsilon_c = 16.6 \pm 0.8\text{‰}$  and  $\epsilon_c = 38.8 \pm 1.1\text{‰}$  in the CK, MS and SC treatments after 90 days of remediation (Fig. 3). Previous study have illustrated that the carbon isotope of petroleum hydrocarbons has almost no fractionation effect in the process of petroleum hydrocarbon biodegradation, which means that the carbon isotope enrichment factor is not shift by hydrocarbon degradation (Aggarwal and Hinchee, 1991). During the bioremediation, the  $\delta^{13}\text{C}$  values of inorganic carbon in loessal soil was closely originated from the decomposition of organic matter (Ehleringer et al., 2000; Bouchard et al., 2008) and soil total carbon isotope enrichment was the result of the transformation of soil total carbons including inorganic and organic fractions. A significant changes in  $\delta^{13}\text{C}$  of soil total carbons can be used as an indicator of the petroleum hydrocarbon degradation in the contaminated soil. Figure 1(b) shows  $\text{KNO}_3$  supplement was more effective for removing the aromatics while compost addition were beneficial to degrade the saturates. Thus the significant difference of carbon enrichment factors of  $\epsilon_c = 16.6 \pm 0.8\text{‰}$  in the MS and  $\epsilon_c = 38.8 \pm 1.1\text{‰}$  in the SC treatments maybe caused by the degradation of aromatic and saturated hydrocarbons, respectively.

The  $\delta^{15}\text{N}$  value of soil total nitrogen was variable in three different treatments during 90 days of incubation. And the nitrogen isotope effects ( $\epsilon_N$ ) were respectively  $\epsilon_N = -7.23 \pm 4.08\text{‰}$ ,  $\epsilon_N = -24.20 \pm 9.54\text{‰}$  and  $\epsilon_N = -79.49 \pm 16.41\text{‰}$  in the CK, MS and SC treatments at the end of the bioremediation. Previous study reported that humidity had a significant effect on the transformation of nitrogen in the soil through the mineralization of organic nitrogen by microorganisms, and promoted the metabolic activities of petroleum hydrocarbon degrading bacteria (Jia et al., 2019; Liu et al., 2020). Additionally, nitrogen interactive priming effect between the amendments and soil could greatly enhanced by input of exogenous nitrogen (Fiorentino et al., 2019). Therefore, it can be inferred that nitrogen isotope effects occurred during the bioremediation are attributed to both mineralization of organic nitrogen by indigenous microorganism and utilization of nitrogen for hydrocarbons degradation after addition of the deionized water,  $\text{KNO}_3$  and compost in the oil-contaminated soil.

To further investigate the enrichment trend of carbon and nitrogen isotopes, carbon and nitrogen dual-isotope relationship in the three different treatments are shown in Fig. 4. The carbon and nitrogen dual stable isotopes showed clear differences due to the different hydrocarbons degradation and nitrogen utilization mechanisms by microbial population among the three treatments (Fig. 4). A inverse significant carbon isotope effect ( $\epsilon_c = 28.3 \pm 0.3\text{‰}$ ) and a normal significant nitrogen isotope effect ( $\epsilon_N = -7.23 \pm 4.08\text{‰}$ ) in the CK treatment. On the other hand, a inverse significant carbon isotope effect ( $\epsilon_c = 16.6 \pm 0.8\text{‰}$ ) and a strong significant nitrogen isotope effect ( $\epsilon_N = -24.20 \pm 9.54\text{‰}$ ) in the MS treatment, and a significant carbon isotope effect ( $\epsilon_c = 38.8 \pm 1.1\text{‰}$ ) as well as a strong significant nitrogen isotope effect ( $\epsilon_N = -79.49 \pm 16.41\text{‰}$ ) in the SC treatment. Thus, carbon isotope enrichment factors are inverse significant while nitrogen isotope enrichment factors are normal significant in all three treatments. This difference was caused by different degradation mechanisms of petroleum hydrocarbon pollutants.

Additionally, the nitrogen isotope effects based on the  $\delta^{15}\text{N}$  of  $\text{KNO}_3$  (1.008‰) and compost (5.3‰) made the enrichment factors variable in the MS ( $\epsilon_N = -24.20 \pm 9.54\text{‰}$ ) and SC ( $\epsilon_N = -79.49 \pm 16.41\text{‰}$ ) treatments, respectively. The nitrogen enrichment factors in the SC treatment was the largest, followed by the MS treatment, and the CK treatment was the smallest (Fig. 3a), which was consistent with the degradation rate of the TPH in the three treatments, implying that petroleum hydrocarbons were degraded accompanied by the utilization and transformation of nitrogen. The ranking of carbon isotope enrichment factors among the three treatments was the SC > CK > MS, which was inconsistent with the degradation rate of TPH, indicating that the enrichment of carbon isotope in the soils may be significantly affected by the different fractions of petroleum degradation and inorganic carbon transformation in loessal soil.

In this study, carbon and nitrogen isotopes showed different enrichment effects after introducing various of stimulants to the soil. Microbial diversity and uniformity also changed by different treatments. The isotope enrichment effects are related to the change of the soil microbial community structure or the soil microbial biomass by the amendments (Ma et al., 2020). It is necessary to investigate soil microbial biomass for understanding isotope enrichment mechanisms during bioremediation of oil-contaminated soil in future studies.

## 4. Conclusions

Biostimulation strategy has proven to be effective for mitigating hydrocarbons pollution in soil, but little is known about the carbon and nitrogen isotope effects during bioremediation. The value of C/N decreased accompanied with the petroleum hydrocarbons biodegradation during remediation by supplement of  $\text{KNO}_3$  and compost. Isotope enrichments are one of the important characteristics for biodegradation of petroleum hydrocarbons which are different in polluted soils remediated by various of biostimulants. Our results provided a new perspective to investigate the biodegradation mechanisms of petroleum hydrocarbons during bioremediation of petroleum contaminated soil.

## Declarations

Loading [MathJax]/jax/output/CommonHTML/jax.js

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.52070154); (No. 21577109).

## References

1. Abed RMM, Al-Kharusi S, Al-Hinai M (2015) Effect of biostimulation, temperature and salinity on respiration activities and bacterial community composition in an oil polluted desert soil. *Int Biodeter Biodegr* 98:43–52
2. Alexander M (2000) Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environ Sci Technol* 34:4259–4265
3. Ali H, Mohammad HF, Mahin S (2016) The effect of soil type on the bioremediation of petroleum contaminated soils. *J Environ Manage* 180:197–201
4. Aggarwal PK, Hinchee RE (1991) Monitoring in situ biodegradation of hydrocarbons by using stable carbon isotopes. *Environ Sci Technol* 25:1178–1180
5. Bouchard D, Hunkeler D, Höhener P (2008) Carbon isotope fractionation during aerobic biodegradation of n-alkanes and aromatic compounds in unsaturated sand. *Org Geochem* 39:23–33
6. Cho E, Park M, Hur M, Kang GY, Kim S (2019) Molecular-level investigation of soils contaminated by oil spilled during the Gulf War. *J Hazard Mater* 373:271–277
7. Dadrasnia A, Usman MM, Wei KSC, Velappan RD, Jamali H, Mohebbali N, Ismail S (2016) Native soil bacterial isolate in malaysia exhibit promising supplements on degrading organic pollutants. *Process Saf Environ* 100:264–271
8. Ehleringer JR, Buchmann N, Flanagan LB (2000) Carbon isotope ratios in belowground carbon cycle process. *Ecol Appl* 10:412–422
9. Fiorentino N, Sánchez-Monedero MA, Lehmann J, Enders A, Fagnano M, Cayuela ML (2019) Interactive priming of soil N transformations from combining biochar and urea inputs: A  $^{15}\text{N}$  isotope tracer study. *Soil Biol Biochem* 31:166–175
10. Fan FL, Yu B, Wang B, George TS, Yin HQ, Xu DY, Li DC, Song AL (2019) Microbial mechanisms of the contrast residue decomposition and priming effect in soils with different organic and chemical fertilization histories. *Soil Biol Biochem* 135:213–221
11. Gao S, Liang JD, Teng TT, Zhang M (2019) Petroleum contamination evaluation and bacterial community distribution in a historic oilfield located in loess plateau in China. *Appl Soil Ecol* 136:30–42
12. Hu YM, Jin ZF, Hu QY, Hu J, Ni CH, Li FL (2020) Using stable isotopes to identify nitrogen transformations and estimate denitrification in a semi-constructed wetland. *Sci Total Environ*. <https://doi.org/10.1016/j.scitotenv.2020.137628>

13. Huang YJ, Pan H, Wang QL, Ge YY, Liu WX, Christie P (2019) Enrichment of the soil microbial community in the bioremediation of a petroleum-contaminated soil amended with rice straw or sawdust. *Chemosphere* 224:265–271
14. Karthick A, Roy B, Chattopadhyay P (2019) A review on the application of chemical surfactant and surfactant foam for remediation of petroleum oil contaminated soil. *J Environ Manage* 243:187–205
15. Li XJ, Zhao Q, Wang X, Li Y, Zhou QX (2018) Surfactants selectively reallocated the bacterial distribution in soil bioelectrochemical remediation of petroleum hydrocarbons. *J Hazard Mater* 344: 23–32. <http://doi.org/10.1016/j.jhazmat.2017.09.050>
16. Li Y, Liu Y, Jiang DW, Xu JX, Zhao XD, Hou YC (2018) Effects of weathering process on the stable carbon isotope compositions of polycyclic aromatic hydrocarbons of fuel oils and crude oils. *Mar Pollut Bull* 133:852–860
17. Liu H, Gao H, Wu ML, Ma C, Wu JL, Ye XQ (2020) Distribution characteristics of bacterial communities and hydrocarbon degradation dynamics during the remediation of petroleum-contaminated soil by enhancing moisture content. <https://doi.org/10.1007/s00248-019-01476-7>
18. Ma QY, Wen Y, Wang DY, Sun XD, Hill PW, Macdonald A, Chadwick DR, Wu LH, Jones DL (2020) Farmyard manure applications stimulate soil carbon and nitrogen cycling by boosting microbial biomass rather than changing its community composition. *Soil Biol Biochem* 144 <https://doi.org/10.1016/j.soilbio.2020.107760>
19. Mahmoud AE, Bagy MMK (2019) Microbial degradation of petroleum hydrocarbons. *Bioresour Technol* 223:277–286
20. Margesin R, Mmerle MH, Tschlerko D (2007) Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: effects of hydrocarbon concentration, fertilizers, and incubation time. *Microb Ecol* 53:259–269
21. Meckenstock RU, Morasch B, Griebler C, Richnow HH (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. *J Contam Hydrol* 75:215–255
22. Meynet P, Hale SE, Davenport RJ, Cornelissen G, Breedveld GD, Werner D (2012) Effect of activated carbon amendment on bacterial community structure and functions in a PAH impacted urban soil. *Environ Sci Technol* 46:5057–5066
23. Mishra S, Jyot J, Kuhad RC, Lal B (2001) In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Curr Microbiol* 43:328–335
24. Nwankwegu AS, Orji MU, Onwosi CO (2016) Studies on organic and in-organic biostimulants in bioremediation of diesel-contaminated arable soil. *Chemosphere* 162:148–156
25. Onotasamiderhi TI, Paola M, Russell JD, David W (2019) Impacts of activated carbon amendments, added from the start or after five months, on the microbiology and outcomes of crude oil bioremediation in soil. *Int Biodeter Biodegr* 142:1–10
26. Prescott H (2002) *Klein Microbiology* (fifth ed.), Mcgraw-hill
27. Qu YY, Zhang XW, Shen WL, Ma Q, You SN, Pei XF, Li SZ, Ma F, Zhou JT (2016) Illumina MiSeq [Loading \[MathJax\]/jax/output/CommonHTML/jax.js](#) of single-walled carbon nanotubes on microbial communities

- of wastewater treatment systems. *Bioresour Technol* 211:209–215
28. Jia J, Bai JH, Gao HF, Wang W, Yin S, Wang DW, Han L (2019) Effects of salinity and moisture on sediment net nitrogen mineralization in salt marshes of a Chinese estuary. *Chemosphere* 228:174–182
  29. Seklemova E, Pavlova A, Kovacheva K (2001) Biostimulation based bioremediation of diesel fuel: field demonstration. *Biodegradation* 12:311–316
  30. Schjønning P, Thomsen IK, Petersen SO, Kristensen K, Christensen BT (2011) Relating soil microbial activity to water content and tillage-induced differences in soil structure. *Geoderma* 163:256–264
  31. Shahi A, Aydin S, Ince B, Ince O (2016a) Evaluation of microbial population and functional genes during the bioremediation of petroleum-contaminated soil as an effective monitoring approach. *Ecotox Environ Safe* 125:153–160
  32. Shahi A, Aydin S, Ince B, Ince O (2016b) Reconstruction of bacterial community structure and variation for enhanced petroleum hydrocarbons degradation through biostimulation of oil contaminated soil. *Chem Eng J* 306:60–66
  33. Tyler SC, Crill PM, Brailsford GW (1994)  $^{13}\text{C}/^{12}\text{C}$  fractionation of methane during oxidation in a temperate forested soil. *Geochim Cosmochim Acta* 58:1625–1633
  34. Wang SY, Kuo YC, Hong A, Chang YM, Kao CM (2016) Bioremediation of diesel and lubricant oil-contaminated soils using enhanced landfarming system. *Chemosphere* 164:558–567
  35. Wolf DC, Cryder Z, Gan J (2019) Soil bacterial community dynamics following surfactant addition and bioaugmentation in pyrene-contaminated soils. *Chemosphere* 231:93–102
  36. Wang Z, Fingas M (1995) Differentiation of the source of spilled oil and monitoring of the oil weathering process using gas chromatography–mass spectrometry. *J Chromatogr A* 712(2):321–343
  37. Wu ML, Wu JL, Zhang XH, Ye XQ (2019) Effect of bioaugmentation and biostimulation on hydrocarbon degradation and microbial community composition in petroleum-contaminated loessal soil. *Chemosphere* <https://doi.org/10.1016/j.chemosphere.2019.124456>
  38. Wu ML, Li W, Warren AD, Ye XQ, Chen LM (2017) Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination. *Chemosphere* 169:124–130
  39. Wu ML, Ma Ch, Wang D, Liu H, Zhu CC, Xu HN (2020) Nutrient drip irrigation for refractory hydrocarbon removal and microbial community shift in a historically petroleum-contaminated soil. *Sci Total Environ*. <http://doi.org/10.1016/j.scitotenv.2019.136331>
  40. Xu JL, Zhao MH, Wang R, Du J, Zhang QJ (2019) Efficiently dedicated oxidation of long-chain crude oil in the soil by inactive SOM-Fe. *Chem Eng J*. <https://doi.org/10.1016/j.cej.2019.121913>
  41. Zhang DC, Mörtelmaier C, Margesin R (2012) Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil. *Sci Total Environ* 421–422:184–196

# Figures

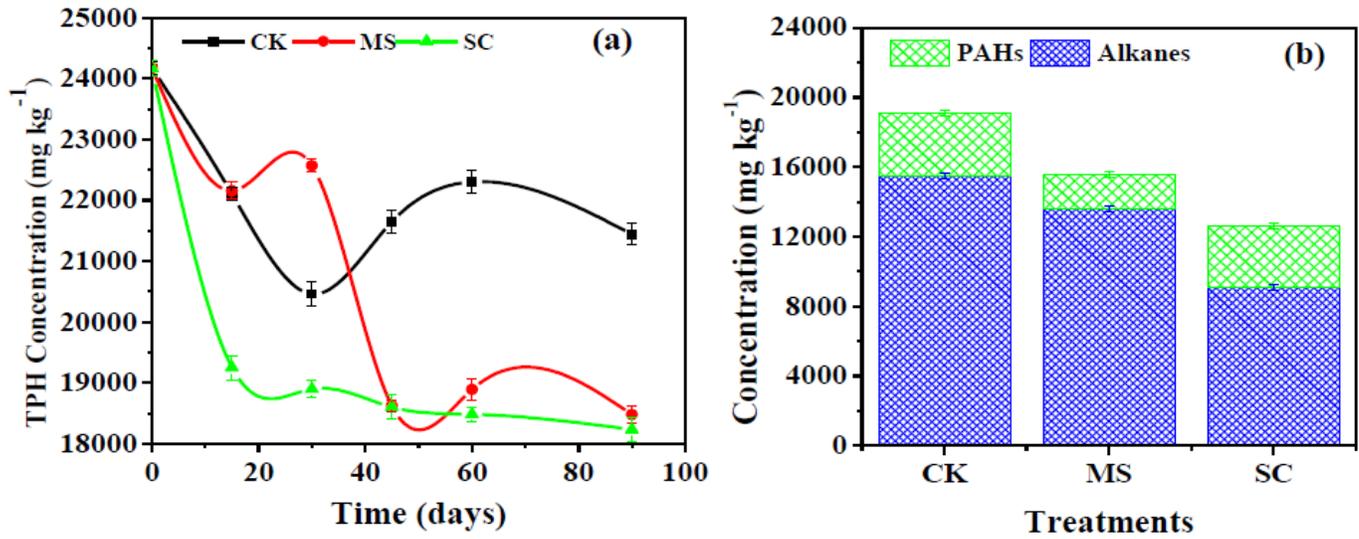
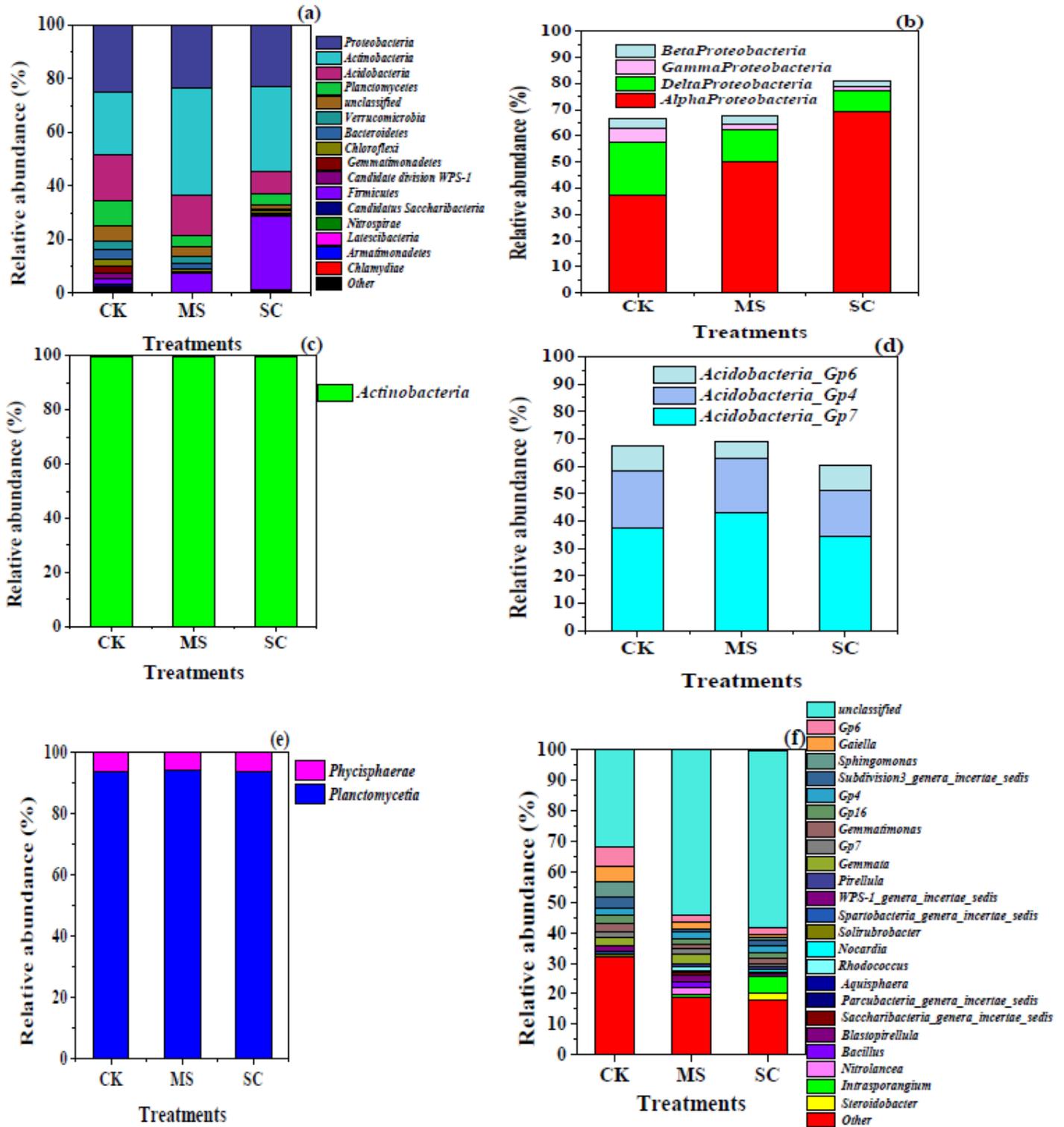


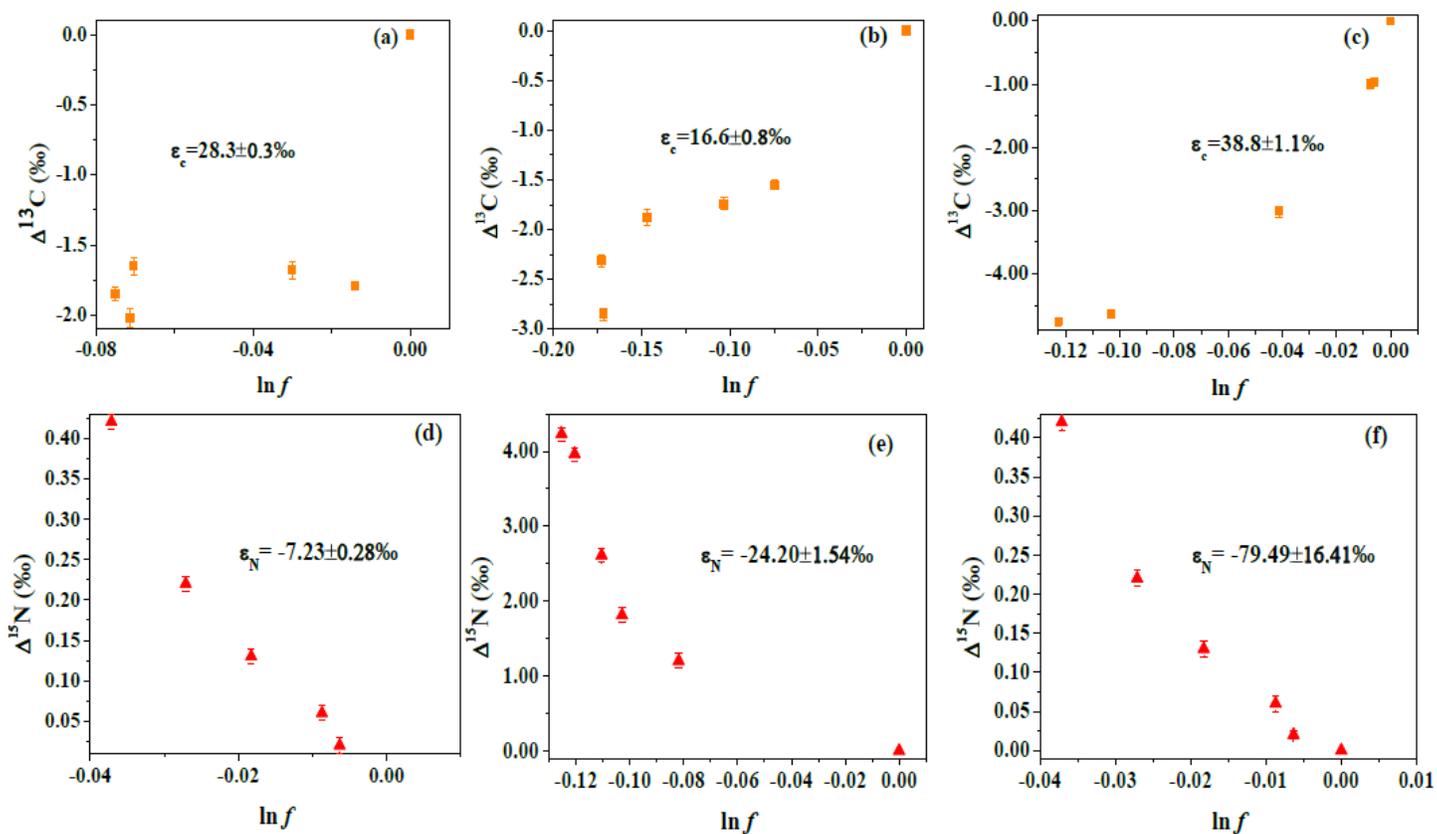
Figure 1

Performance of TPH concentration in soil after adding deionized water (CK), KNO<sub>3</sub> (MS) and compost (SC) (a); Concentration of aromatics and saturates in different treatments after 90 days incubation. Errors bars indicate  $\pm$ SD of triplicate samples.



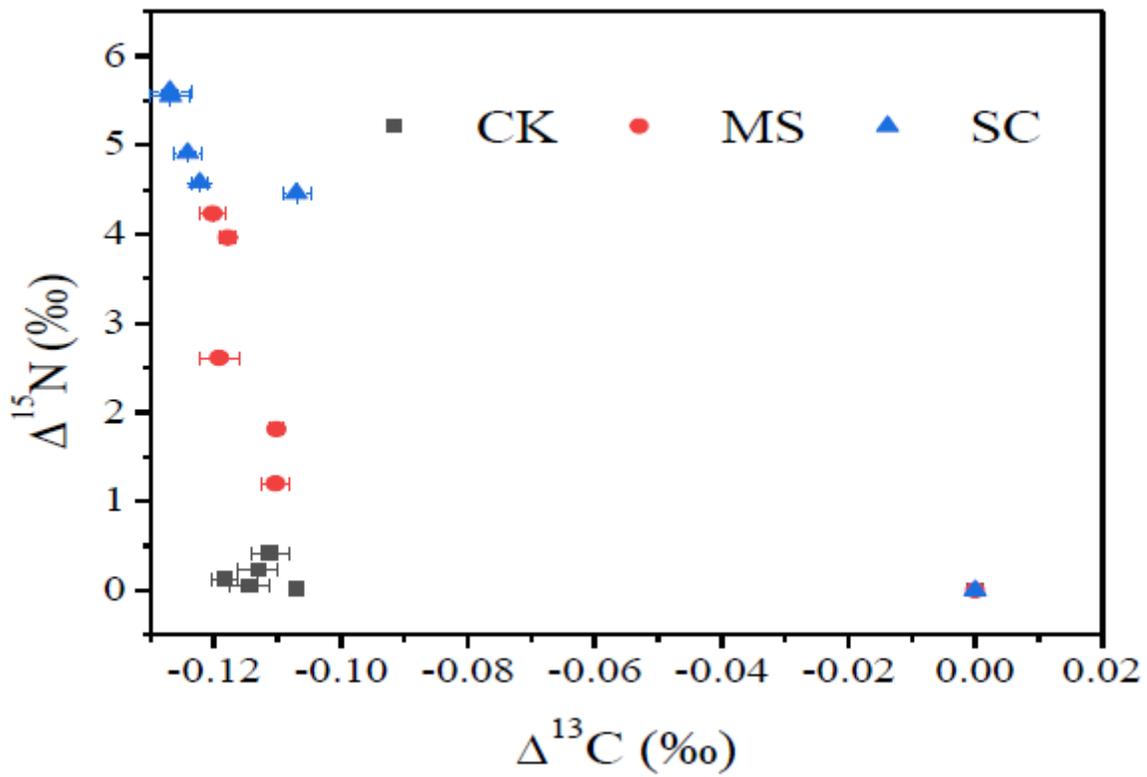
**Figure 2**

Composition of microbial communities in soil after adding deionized water (CK), KNO<sub>3</sub> (MS) and compost (SC). The most abundant bacterial phyla (a); the class level distribution of top four dominant phyla of Proteobacteria (b); Actinobacteria (c); Acidobacteria (d); Planctomycetes (e); The most abundant bacterial genera (f).



**Figure 3**

Carbon isotope enrichment in soil after adding deionized water (a),  $\text{KNO}_3$  (b) and compost (c). Nitrogen isotope enrichment in soil after adding deionized water (d),  $\text{KNO}_3$  (e) and compost (f). Errors bars indicate  $\pm$ SD of triplicate samples.



**Figure 4**

Carbon and nitrogen dual-isotope plots in all three different biostimulation remediation: adding deionized water (CK), KNO<sub>3</sub> (MS) and compost (SC). Errors bars indicate  $\pm$ SD of triplicate samples.