Pollution Study 2022, 3(1), 2003.
https://doi.org/10.54517/ps.v3i1.2003

Article

Investigation into the enhancement of microbial remediation of petroleum-contaminated soil through chemical oxidation techniques

Kewei Xu[1], Lei Gu[1,*], Xuying Zheng[1], Biao Wang[2,3], Peng Guo[2,3]

[1] Wuxi Research Institute of Petroleum Geology, Petroleum Exploration and Production Research Institute, Petroleum Exploration and Production Research Institute, China Petroleum and Chemical Corporation, Wuxi 214126, Jiangsu, China
[2] Institute of Petroleum Engineering Technology of Sinopec Jiangsu Oilfield Company, China Petroleum and Chemical Corporation, Yangzhou 225009, Jiangsu, China
[3] Research Center of Oil and Gas Microbial Engineering of Jiangsu, Yangzhou 225009, Jiangsu, China
*** Corresponding author:** Lei Gu, syky@sinopec.com

CITATION

Xu K, Gu L, Zheng X, et al. Investigation into the enhancement of microbial remediation of petroleum-contaminated soil through chemical oxidation techniques. Pollution Study. 2022; 3(1): 2003. https://doi.org/10.54517/ps.v3i1.2003

ARTICLE INFO

Received: 9 October 2022
Accepted: 29 October 2022
Available online: 13 November 2022

Abstract: This research utilized a bioreactor approach for the remediation of petroleum-contaminated soil, enhancing the process with chemical oxidation to investigate its efficacy. Additionally, the study employed the BIOLOG ECO board and high-throughput sequencing techniques to delve into the mechanisms behind the microbial community's response. The findings indicated that after 240 days of bioremediation, the treatments involving standard bioremediation (NP) and bioremediation enhanced with oxidants (NP_O) reduced the soil's total petroleum hydrocarbons from an initial 30,649 $mg \cdot g^{-1}$ to 5889 $mg \cdot g^{-1}$ and 2351 $mg \cdot g^{-1}$, respectively. The soil concentration of petroleum hydrocarbons following oxidation-enhanced bioremediation was found to be below the national risk control threshold (GB 36600-2018). Further analysis using BIOLOG ECO micropore tests and high-throughput sequencing revealed that microbial activity in the oxidant-treated soil was promptly rejuvenated. The study identified potential bacterial markers for petroleum hydrocarbon degradation in the treatment with chemical oxidation-enhanced bioremediation, including *Genus Microbacterium*, *paracoccus*, *pseudomonas*, *stenotrophomonas*, and *Porticoccaceae*_C1.B045.

Keywords: petroleum-contaminated soil; chemical oxidation; bioremediation; 16S rDNA; bacterial community

As an important energy and chemical raw material, petroleum enters the soil environment during its exploitation, transportation, processing and use, resulting in serious soil pollution [1]. When oil enters the soil, it will destroy the soil structure and reduce the available soil nutrients such as nitrogen and phosphorus. As the main pollutants in petroleum, petroleum hydrocarbons mainly include alkanes and aromatic hydrocarbons. These pollutants can enter organisms through volatilization or food chain enrichment, and pose a serious threat to the ecological environment and human health. Therefore, oil contaminated soil needs to be repaired urgently.

The commonly used remediation methods for petroleum contaminated soil mainly include physical, chemical and biological remediation. Bioremediation is a low-cost and environmentally friendly sustainable remediation technology [2]. This technology uses microbial metabolism to achieve the goal of degrading pollutants [3]. However, at the later stage of bioremediation, refractory pollutants, such as high ring aromatic hydrocarbons, long-chain alkanes, and toxic oxygenated intermediates will inhibit microbial activity, affect microbial metabolism, and thus reduce the remediation efficiency [3,4].

Chemical oxidation remediation is a technical method to rapidly degrade pollutants by using chemical oxidants (such as Fenton reagent, potassium permanganate, sodium persulfate, etc.) [5]. During the oxidation of total petroleum hydrocarbons (TPHS) and other organic pollutants, chemical oxidants can degrade long-chain alkanes into short-chain alkanes without being affected by soil biological toxicity, so as to remove pollutants. It is expensive to use chemical oxidation completely for remediation, and it will cause secondary pollution to the soil. However, the use of an appropriate amount of chemical oxidants to strengthen bioremediation can overcome the limitations of the above single repair technology and effectively improve the repair efficiency [6–8]. Sodium persulfate has the advantages of high solubility, good stability, wide range of pH adaptation and recovery of soil microbial activity after remediation. It has become the main chemical oxidant to strengthen bioremediation [9]. For example, Luo et al. [10] have shown that oxidant enhanced bioremediation can effectively improve the removal efficiency of organic pollutants in soil.

In this study, a bio reactor was used to remediate an oil contaminated soil in Jiangsu oil field, and sodium persulfate was used to oxidize the oil contaminated soil in the later stage of remediation, so as to study the effect of further strengthening bioremediation. The relationship between the removal effect of TPHS by different treatments and the changes of microbial activity and community structure was explored to provide a theoretical basis for chemical oxidation enhanced bioremediation of petroleum contaminated soil.

1. Materials and methods

1.1. Instruments and reagents

Instruments: GC-FID (Agilent 7890b, USA), rotary evaporator, microplate reader, etc.

Reagent: sodium persulfate ($Na_2S_2O_8$) is analytical reagent; hexane (C_6H_{14}) and dichloromethane (CH_2Cl_2) are chromatographically pure; anhydrous sodium sulfate (analytical purity, activation at 400 ℃ for 6 h), florisil (60~100 mesh, for chromatography; activation at 400 ℃ for 6 h).

1.2. Test soil

The oil contaminated soil is taken from Jiangsu oil field, with TPHS content of 30,649 $mg \cdot kg^{-1}$ and pH of 8.01.

1.3. Experimental design

Bioremediation adopts bio reactor. Biological reactor is 160 L. In the composting process, 12% of the sludge was added with pig manure and 3% with straw. The specific operations are as follows: add pig manure and straw into the oil sludge, fully mix them evenly, wrap the compost barrel with thermal insulation film, connect the leakage liquid outlet pipe at the bottom, add water to adjust the initial moisture content of the pile to about 50%, and regularly turn the pile for ventilation and add water. Insert the thermometer into the reactor and record the temperature

every day. In order to avoid uneven distribution of pollutants in the sludge, multi-point sampling and mixing method was adopted for sampling, and samples were taken at the 0, 40, 80, 120, 160 and 240 days of composting. Take 50 g samples each time, freeze-dry the collected samples, grind them and sift them through 60 mesh sieve. Use GC-FID to measure the TPHS content, and detect the soil microbial activity, community structure and diversity in the 240th day. The treatment is as follows:

(1) Control: 70 kg sludge;

(2) Bioremediation (NP, bioremediation treatment with nitrogen and phosphorus added):

70 kg oil sludge + 8.4 kg pig manure +2.1 kg straw

(3) Oxidant enhanced bioremediation (NP_O, oxidant enhanced on the basis of NP):

70 kg oil sludge + 8.4 kg pig manure +2.1 kg straw + oxidant

50 mmol/kg sodium persulfate and 10 mmol/kg ferrous sulfate were added to the oxidant on the 160th day of composting, and samples were taken to measure the concentration of TPHS after full stirring.

1.4. Extraction and detection of TPHS

Weigh 2G of freeze-dried soil sample (60 mesh), use dichloromethane as extractant, and extract petroleum hydrocarbons in 54 ℃ water bath by Soxhlet extraction method for 16~18 h. After the extracted liquid is evaporated and concentrated, it is purified by a glass sand core chromatographic column containing 4 g floric silica and 1 g anhydrous sodium sulfate. The purified petroleum hydrocarbon is eluted by chromatographic pure hexane. After the purified eluent is concentrated to 2 mL, it is detected by GC-FID. The gas chromatographic conditions are as follows: the national environmental protection standard of the people's Republic of China—Determination of petroleum hydrocarbon in soil and stratification (c10~c40)—gas chromatography (HJ 1021–2019).

1.5. Analysis of microbial activity by carbon utilization method (Biolog method)

In this study, BIOLOG ECO board was used to measure the metabolic activity of microbial communities in each treatment soil. Weigh a fresh soil sample equivalent to 5 g of dry soil, put it in 50 mL of 0.85% NaCl sterile solution, shake it in a 30 ℃ shaking incubator at the speed of 150 R·min^{-1} for 30 min, and after standing for 15 min, dilute the supernatant 100 times. Add diluent 150 into each hole of BIOLOG ECO board μ 50. Incubate in a 30 ℃ incubator for 7 days, and determine the absorbance at 590 nm in the microplate reader every 24 h. The average well color development option (awcd) of 31 holes is used to characterize the average activity of microorganisms. The calculation formula is as follows:

$$\mathrm{AWCD}=\frac{\sum_{i=1}^{31}(C_i-R)}{31}$$

where: is C_i the absorbance value of hole *I*; *R* is the absorbance value of the control hole.

1.6. High throughput sequencing

Weigh 0.5 g of fresh soil sample and use soil DNA Extraction Kit (fast DNA® Spin kit for soil, mp biomedicals, USA), extract soil DNA according to the instructions provided in the kit. The soil DNA samples were sent to Shanghai Meiji Biomedical Technology Co., ltd., China. The 16S rRNA gene of the bacteria was amplified and purified by PCR using primer 515f (5'-gtgccagcmgc cgcgg-3') and 907r (5'-ccgtcaattcmttragtt-3'). The library was constructed by using the PCR products with uniform quality and sequenced on the Illumina miseq platform. After noise reduction, the measured sequences are divided into operational taxonomic units (otus) according to 97% similarity, and the classification information of otus is compared by Silva database. According to the minimum sequence number of 27,876 in this study sample, the microbial community diversity index, including Shannon, chao, invsimpson and ACE, is calculated by using the extracted data. Principal component analysis (PCA) was used to study the similarity and difference of bacterial community composition among and within each treatment. Lefse software was used for linear discriminant analysis (LDA) to study the bacteria with significant enrichment in each treatment.

1.7. Data processing

The data were analyzed by variance and plotted by R, and the differences among the treatments were compared by Duncan multiple methods ($p < 0.05$).

2. Results

2.1. Changes of TPHS during soil remediation

Bioremediation can significantly reduce the content of TPHS in soil, as shown in **Figure 1**. After 240 days of repair, the content of TPHS in soil treated with NP and NP_O decreased from 30,649 mg·kg^{-1} to 5889 mg·kg^{-1} and 2351 mg·kg^{-1}, respectively. All meet the industrial land standard of 4500 mg·kg^{-1}, and in NP_O treatment, chemical oxidation can further enhance the removal of TPHS in soil.

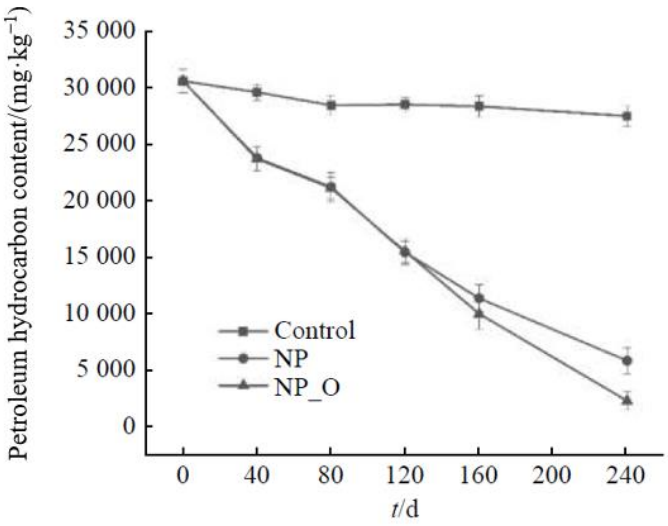

Figure 1. Residual TPHS in soils in each treatment.

2.2. Changes in microbial activity and quantity during soil remediation

Figure 2 shows that compared with the control group, the bioremediation treatment group (NP, NP_O) significantly enhanced the metabolic activity of the microbial community. Moreover, compared with NP treatment group, the metabolic activity of microorganisms in group NP_O was hardly affected by oxidants.

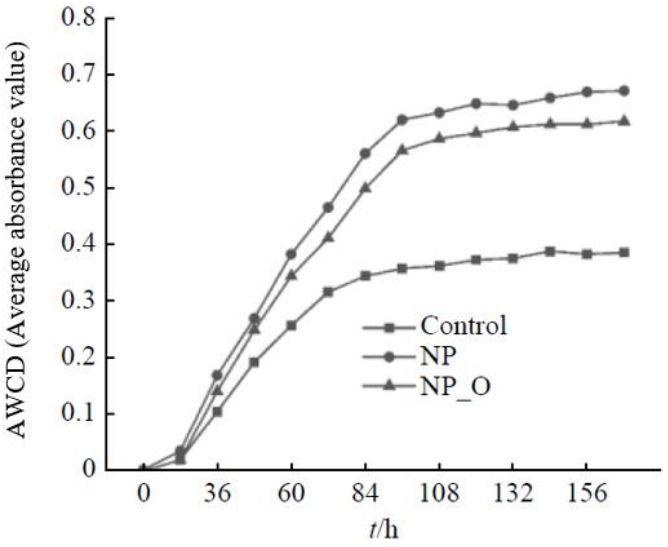

Figure 2. Variation in AWCD of petroleum contaminated soil under different treatments after 240 days.

2.3. Changes in bacterial community structure and diversity during soil remediation

2.3.1. Variation of bacterial community diversity

The results of principal coordinate analysis (pcoa) are shown in **Figure 3**. The bacterial community structure within each treatment was similar, and the bacterial community structure among each treatment was significantly different. Chemical oxidation treatment (NP_O) and non oxidation treatment (control and NP) were

located at the negative and positive ends of pcoa1 axis respectively; the bio reactor treatment group (NP, NP_O) and control (control) were separated by pcoa2 axis and located at the positive end and negative end respectively.

The soil bacterial diversity index in each treatment is shown in Table 1. The results of alpha diversity index showed that compared with the control treatment, bioremediation treatment (NP, np_o) significantly reduced the bacterial diversity in soil, and chemical oxidation enhanced bioremediation treatment (np_o) further reduced the bacterial diversity in soil.

Table 1. Diversity index of the bacterial community in each treatments.

	Richness	**Chao**	**Shannon**	**Invsimpson**
Control	958 ± 38	1124 ± 64	4.67 ± 0.06	36.3 ± 3.24
NP	709 ± 26	974 ± 58	3.75 ± 0.09	15.15 ± 1.29
NP_ O	248 ± 10	324 ± 53	3.31 ± 0.11	12.36 ± 1.30

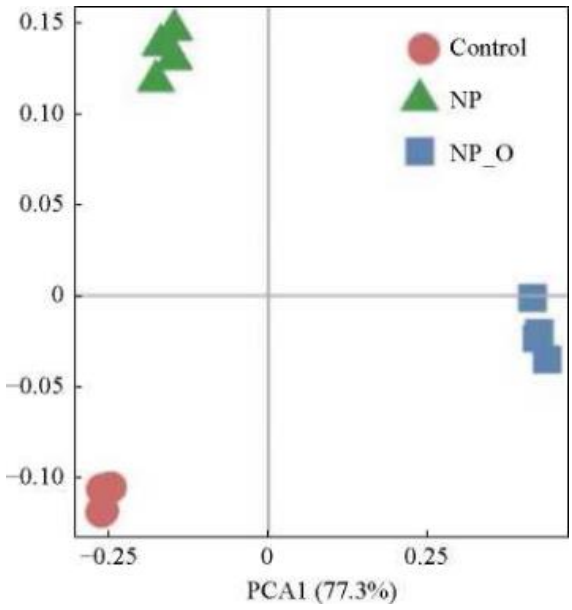

Figure 3. Pcoa analysis of soil bacterial community in each treatment.

2.3.2. Bacterial community classification

The results of relative abundance at the phylum and genus levels of bacterial communities are shown in **Figure 4**. Chemical oxidation enhanced bioremediation significantly reduced the diversity of bacterial communities at the phylum level. In each treatment, the relative abundance of Proteus was the highest in the control group, np and NP_O was 68.35%, 67.22% and 76.35%, among which gammaproteobacteria was the most dominant, and its relative abundance increased significantly in bioremediation treatment. Chemical oxidation enhanced bioremediation improved the relative abundance of Bacteroidetes.

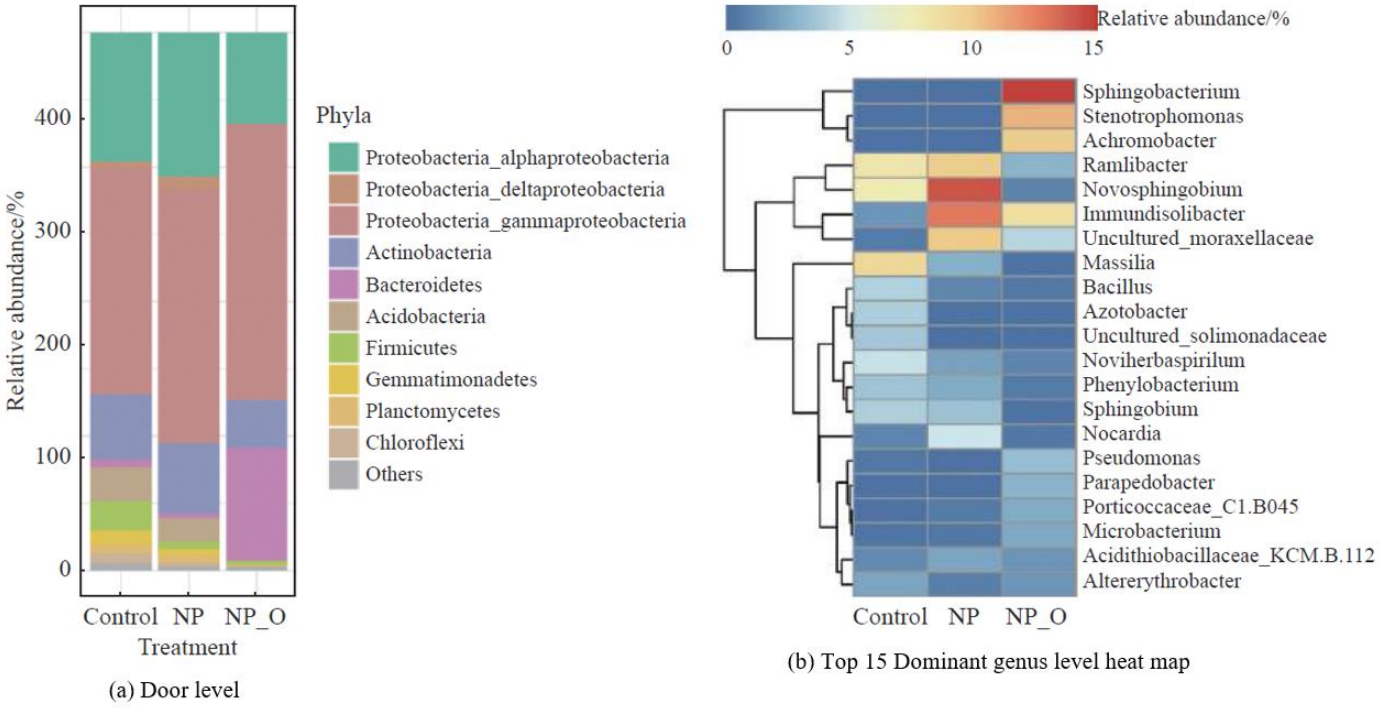

Figure 4. Relative abundance of bacterial community in each treatment.

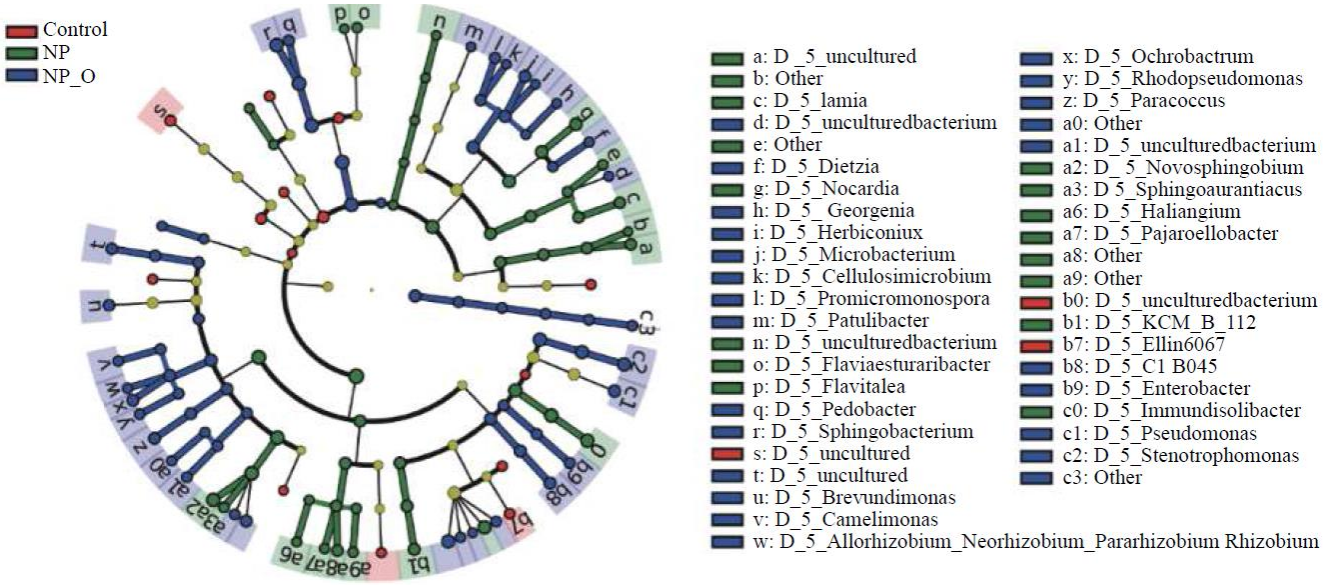

Figure 5. The community difference map of bacteria in soils of different treatment groups identified by linear discriminant analysis (LDA).

At the genus level, the dominant genera and the marker genera in each treatment were analyzed by heat map and lefse. As shown in **Figure 4b** and **Figure 5**. In the lefse diagram, the circles from the center to the outside represent the levels of phyla, class, order, family and genus, and the size of the dots represents the relative abundance of microorganisms. Red (control), cyan (NP) and blue (NP_O) dots represent microbial markers in the corresponding color group. The results show that NP_ The main relative abundance of O treatment is high, and the bacterial markers are *Microbacterium*, *paracoccus*, *pseudomonas*, *stenotrophomonas*,

*porticoccaceae*_C1.B045, etc. Where NP_O treated *Microbacterium* (2.47%), *paracocus* (2.98%), *pseudomonas* (3.51%), *stenotrophomonas* (11.98%) and *porticoccaceae*. The relative abundance of C1.B045 (2.64%) was higher than that of other treatment groups.

3. Discussion

Microbial remediation of oil contaminated soil is low cost and environmentally friendly, which is conducive to sustainable development. However, in the later stage of bioremediation, the long-chain alkanes in petroleum pollutants are not easy to be degraded by microorganisms and remain in the soil. The toxic intermediates produced during the degradation of pollutants will inhibit the microbial activity, resulting in the decline of degradation efficiency and the extension of remediation cycle in the later stage of bioremediation. The research shows that chemical oxidation can effectively destroy long-chain alkanes and rapidly degrade organic pollutants and their intermediates. The use of appropriate oxidants can quickly restore the activity of soil microorganisms and significantly reduce the content of soil TPHS [11]. In this study, chemical oxidation was used to strengthen the bioremediation of petroleum contaminated soil by bio reactor. The changes of microbial community structure during the remediation process were analyzed to explore the remediation effect and mechanism. The results showed that after 240 days of bioremediation by bio reactor (NP, NP_O), the TPHS in soil were divided into 5889 $mg \cdot kg^{-1}$ and 2351 $mg \cdot kg^{-1}$ respectively, among them, the content of petroleum hydrocarbons in the soil after chemical oxidation enhanced bioremediation is lower than the standard (4500 $mg \cdot kg^{-1}$) in the standard for management and control of soil pollution risks of construction land for soil environmental quality (Trial) (GB 36600-2018).

In order to further reveal the mechanism of chemical oxidation enhanced bioremediation, this study found that compared with NP. After 80 days of O treatment, the removal amount of soil TPHS was further increased, but there was no significant difference in microbial metabolic activity. It indicates that adding an appropriate amount of oxidant should be oil loving in the process of soil remediation. In addition, the research data of Hou et al. also proved that the microbial activity of these bacteria can be quickly restored and the petroleum hydrocarbons can be quickly removed. This is consistent with the results reported by Zhang et al. [12].

In order to further explore the relationship between bacterial community and petroleum hydrocarbon degradation, high-throughput sequencing was used to analyze the structure and diversity of bacterial community. PCA shows that the main controlling factor affecting the change of community structure is chemical oxidation. The free radicals produced are highly oxidizing, and even cause damage to the cell structure, leading to the failure of microbial activity [13], thus reducing the richness of the microbial community structure and the diversity of the microbial community (see **Table 1**). Analysis of microbial community structure at phylum level showed that NP_O The relative abundance of actinomycetes decreased and that of Bacteroidetes increased in o treatment. At the gate level, compared with the control, the relative abundance of gammaproteobacteria in the bio reactor treatment group

(NP, NP_O) increased significantly, and the removal rate of petroleum hydrocarbons in this treatment group was the highest. Previous studies have shown that gammaproteobacteria is the main functional group of bacteria degrading petroleum hydrocarbons in petroleum contaminated soil [14]. Although the bioremediation treatment group (NP, NP_O) has reduced the diversity of microbial communities, it has nothing to do with the diversity of dominant degradation microbial communities for pollutant removal, and is mainly related to the proliferation of relevant functional bacteria in the soil, which is consistent with the previous research results of the research group, that is, the removal of petroleum hydrocarbons is related to the enrichment of certain types of functional bacteria [15].

The results of LDA analysis show that at the generic level, microbacterium, paracocus, pseudomonas, stenotrophomonas and porticoccaceae C1.B045 is NP_O major bacterial markers treated; studies have shown that Arthrobacter and paenarthrobacter have the ability to degrade petroleum. For example, Bai et al. [16] have shown that Pseudomonas and Stenotrophomonas can effectively degrade hydrocarbon pollutants. Xu et al. [17] found that throbacter is the main oil loving genus in oil contaminated soil. In addition, the research data of Hou et al. [15] also proved that these bacteria are the main bacterial groups for repairing petroleum hydrocarbon pollutants. It indicates that the degradation of petroleum hydrocarbons is related to the enrichment of these two bacteria.

4. Conclusion

NP and NP_O treatment reduced TPHS in soil from 30,649 $mg \cdot g^{-1}$ to 4389 $mg \cdot g^{-1}$ and 1351 $mg \cdot g^{-1}$ respectively, and the concentration of petroleum hydrocarbons in the repaired soil was lower than the national risk control value (GB 36600-2018). The results of BIOLOG ECO micropore analysis and high-throughput sequencing further showed that the microbial activity in the soil treated with oxidants recovered rapidly, and in the pre oxidation combined with *biostimulation remediation*, *microbacterium*, *paracocus*, *pseudomonas*, *stenotrophomonas* and *porticoccaceae*_C1.B045 as the main bacterial marker, most of them have the ability to degrade petroleum hydrocarbons. The results showed the feasibility of enhanced microbial remediation by chemical oxidation, and provided a theoretical basis for the practical application of petroleum hydrocarbon contaminated soil remediation in the future.

Funding: National Natural Science Foundation of China (41202241); Sinopec foundation foresight project (P19003); Supported by national key R & D plan (2020YFC1808802).

Conflict of interest: The authors declare no conflict of interest.

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