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# Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population and activity determination

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## HIGHLIGHTS

- Biostimulation and bioaugmentation achieved 48% and 58% hydrocarbon degradation.
- Hydrocarbon degradation rate correlated with degrader population increase.
- The degradation rate correlated with degrader activity increase in Biolog assay.
- MPN and Biolog were efficient methods for assessing hydrocarbon degradation rate.

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## ABSTRACT

Bioremediation of hydrocarbon degradation in petroleum-polluted soil is carried out by various microorganisms. However, little information is available for the relationships between hydrocarbon degradation rates in petroleum-contaminated soil and microbial population and activity in laboratory assay. In a microcosm study, degradation rate and efficiency of total petroleum hydrocarbons (TPH), alkanes, and polycyclic aromatic hydrocarbons (PAH) in a petroleum-contaminated soil were determined using an infrared photometer oil content analyzer and a gas chromatography mass spectrometry (GC-MS). Also, the populations of TPH, alkane, and PAH degraders were enumerated by a modified most probable number (MPN) procedure, and the hydrocarbon degrading activities of these degraders were determined by the Biolog (MT2) MicroPlates assay. Results showed linear correlations between the TPH and alkane degradation rates and the population and activity increases of TPH and alkane degraders, but no correlation was observed between the PAH degradation rates and the PAH population and activity increases. Petroleum hydrocarbon degrading microbial population measured by MPN was significantly correlated with metabolic activity in the Biolog assay. The results suggest that the MPN procedure and the Biolog assay are efficient methods for assessing the rates of TPH and alkane, but not PAH, bioremediation in oil-contaminated soil in laboratory.



## 1. Introduction

Petroleum spills often occur by accidents during pumping, transportation, and refining. Petroleum is a complex mixture including aliphatic and aromatic hydrocarbons, which are known to have negative effects on human health and the environment. Thus, the US Environmental Protection Agency (U.S. EPA, 1986) considers these hydrocarbons as priority environmental pollutants.

Bioremediation has been considered an efficient, cost-effective, and environmentally sound method to degrade hydrocarbons in petroleum-contaminated soils (Salanitro and Dorn, 1997; Liebeg and Cutright, 1999; Margesin and Schinner, 2001; Zhang et al., 2008; Chiu et al., 2009; Wu et al., 2013; Kharusi et al., 2016; Ma

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et al., 2016). This method includes bioaugmentation and biostimulation (Lladó et al., 2012; Wu et al., 2016). Bioaugmentation is a technology of inoculating exogenous microorganisms into the contaminated soil to degrade hydrocarbons (Wu et al., 2013), and biostimulation of supplying nutrients to the soil to stimulate the hydrocarbon degrading capacity of the indigenous microorganisms (Sanscartier et al., 2009 Chang et al., 2013; Wu et al., 2016).

Some studies have reported the petroleum hydrocarbon degradation efficiency being associated with the petroleum hydrocarbon degrader population (Krutz et al., 2005; Kauppi et al., 2011; Taccari et al., 2012; Wu et al., 2016). However, information about relationships between the rate of hydrocarbon degradation in oil-contaminated soil during bioremediation and hydrocarbon degrader population and activity as measured in laboratory is limited (Lors et al., 2010). The most probable number (MPN) is a popular method for counting microbial populations in laboratory (Wu et al., 2013), and the Biolog assay is a common method for identifying microorganisms and measuring the carbon metabolic activity (Dos Santos et al., 2002; Kadali et al., 2012). These two methods should be effective and efficient for investigating these relationships.

We conducted a microcosm study to evaluate the effects of bioaugmentation and biostimulation in oil-contaminated soil on hydrocarbon degradation rate and hydrocarbon degrading microbial populations. We also measured the hydrocarbon degrading activity of microorganisms from the soil by Biolog assay using three self-defined carbon sources (standard petroleum hydrocarbons, n-hexadecane, and polycyclic aromatic hydrocarbons). The objectives were (1) to assess the correlations between hydrocarbon degradation rate in oil-contaminated soil and abundance of hydrocarbon degrading microbial populations measured by the MPN procedure, and microbial hydrocarbon degrading activity in the Biolog assay and (2) to explore relationships between the degrader populations measured and their activities in the Biolog assay.

## 2. Materials and methods

### 2.1. Soil for the microcosm study

Petroleum-contaminated soil was collected in Shaanxi province, China. The collecting site in the oil field was described by Wu et al. (2016), but the specific sampling spots were different for these two studies. Selected chemical and microbiological properties of the soil for this study are listed in Table 1.

**Table 1**
Selected chemical and microbiological properties of the soil used for the microcosm study.

| Main characteristics | Values |
|---|---|
| TPH[a] (mg $kg^{-1}$) | 20200 ± 170 |
| Alkane (mg $kg^{-1}$) | 15400 ± 130 |
| Aromatics (mg $kg^{-1}$) | 2180 ± 20 |
| pH | 8.4 ± 0.3 |
| Total carbon (g $kg^{-1}$) | 765 ± 80 |
| Total nitrogen (mg $kg^{-1}$) | 921 ± 40 |
| Total phosphorus (mg $kg^{-1}$) | 160 ± 20 |
| Total bacterial numbers (cells $g^{-1}$) | $2.3 \pm 0.4 \times 10^7$ |
| TPH degraders (MPN[b] $g^{-1}$) | $1.7 \pm 0.4 \times 10^5$ |
| Alkane degraders (MPN $g^{-1}$) | $5.0 \pm 0.6 \times 10^4$ |
| PAH[c] degrader population (MPN $g^{-1}$) | ND[d] |

[a] TPH: Total Petroleum Hydrocarbon.
[b] MPN: Most Probably Number.
[c] Polycyclic aromatic hydrocarbon.
[d] ND: Not Detected.

### 2.2. Experiment design for the microcosm study

Detailed information about the experimental design was previously provided by Wu et al. (2016), but treatments were modified in this study as presented here in brief. Four treatments included (1) CK: untreated dried soil; (2) WHC: 20% moisture in soil; (3) BA: bioaugmentation with a hydrocarbon-degrading consortium composed of *Pseudomonas stutzeri* GQ-4 strain KF453954, *Pseudomonas* SZ-2 strain KF453956, and *Bacillus* SQ−2 strain KF453961, and 20% soil moisture content; (4) BS: biostimulation with $(NH_4)_2SO_4$ and $KH_2PO_4$ and 20% soil moisture content.

### 2.3. Total petroleum hydrocarbon (TPH), alkane, and PAH analysis

The TPH was determined as previously described in Wu et al. (2016). Alkanes and PAHs were separated using the Super Flash Alumina Neutral columns (SF 15−24 g, 20.8 × 112 mm, Agilent Technologies). Alkane and PAH solutions were obtained by eluting the columns with hexane and dichloromethane, respectively. The concentrations of alkane and PAH in the solutions were analyzed using gas chromatography/mass spectrometry (GC-MS) (Agilent Technologies, Palo Alto, CA).

For alkane and PAH analysis, the separation column was a HP-5-MS column (30 m × 25 mm × 0.25 μm; Agilent Technologies), and helium was carrier gas at flow rates of 1.0 and 1.2 mL $min^{-1}$, respectively. Temperature programming for alkane was an initial 5 min at 65 °C, and then heating at a rate of 10 °C $min^{-1}$ to 300 °C (holding for 2 min). Programming for PAH was initial 1 min at 50 °C, heating at 15 °C $min^{-1}$ to 170 °C (holding for 2 min), followed by heating at 8 °C $min^{-1}$ to 210 °C, and finally at 3 °C $min^{-1}$ to 300 °C (holding for 5 min). For alkanes, the injector and detector temperatures were 300 °C and 230 °C, respectively. The selected ion monitoring mode was used, and injection volume was 1 μL in the splitless mode. For PAH analysis, the multiple reaction monitoring mode was used, and injection volume was 10 μL.

### 2.4. Determination of TPH-, alkane- and PAH-degrading microbial populations

The TPH-, alkane- and PAH-degrading microbial populations in the microcosm soils were determined by a modified most probable number (MPN) procedure as described by Wu et al. (2016). For determination of TPH-degrading microbial population, standard petroleum hydrocarbons were chosen as the only carbon source. For determination of alkane- and PAH-degrading microbial populations, n-hexadecane and a mixture containing three PAHs were chosen as the only carbon source, respectively.

### 2.5. Determination of hydrocarbon degrading activity in the Biolog assay

Biolog (MT2) MicroPlates (Biolog, Hayward, CA) were used to evaluate the hydrocarbon degrading activity of microorganisms from the microcosm soil. The carbon sources supplied to the Biolog plates were standard petroleum hydrocarbons for TPH-degraders, n-hexadecane for alkane-degraders, and PAH solution (0.4 g anthracene, 0.4 g phenanthrene, and 0.2 g pyrene in 1000 mL dichloromethane) for PAH-degraders. Experimental procedures followed Taha et al. (2015). Degrading activity was expressed as average well color development (AWCD) (Garland and Mills, 1991).

### 2.6. Statistical analysis

All experiments were conducted with three replications. The experimental results are presented as the mean ± one standard