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Contributions of ryegrass, lignin and rhamnolipid to polycyclic aromatic hydrocarbon dissipation in an arable soil

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ABSTRACT

Bioremediation of polycyclic aromatic hydrocarbon (PAH)-contaminated soil is often limited by inadequate microbial activity and/or low PAH bioavailability. A pot experiment was performed with an aged contaminated arable soil to explore the remediation potential of ryegrass and lignin, which are believed to improve microbial degradation, as well as the biosurfactant rhamnolipid. On average, the concentration of 15 priority PAHs was reduced by 41.7% in the combined ryegrass, lignin and rhamnolipid treatment after 90 days. In contrast, there was no reduction in PAH concentration when each treatment was used alone. The rhamnolipid was beneficial for successful remediation, as shown by the lack of PAH transformation in all non-rhamnolipid treatments. The total amount of PAHs that accumulated in ryegrass biomass was less than 0.1% of the initial amount in the pot. When the theoretical estimate of plant uptake was considered, it suggested that rhizoremediation rather than direct uptake contributed to PAH dissipation. High-throughput sequencing analysis demonstrated that lignin addition substantially changed the fungal and bacterial communities; however, there was no indication that lignin selected for known bacterial PAH degraders. Nevertheless, a [^{14}C]benz(a)anthracene-spiked microcosm experiment showed that lignin amendment led to enhanced PAH mineralization and nonextractable residue formation. Taken together, these findings highlight the importance of selecting bioremediation treatments that can simultaneously stimulate microbial activity and increase PAH bioavailability to achieve remediation effectively. Treatments incorporating rhizoremediation, biostimulation and biosurfactant addition hold promise for detoxifying aged PAH-contaminated agricultural soil.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have two or more fused benzene rings and are predominantly formed during incomplete combustion of mineral fuels and biomass. The most recent estimate indicates that more than 520 × 10^3 tons of the 16 US Environment Protection Agency (USEPA) priority PAHs are released into the environment each year globally (Zhang and Tao, 2009), which may cause significant soil pollution through atmospheric deposition. PAHs are detected in soils around the world (Wilcke, 2007) and have been identified as major organic pollutants in agricultural soils of China (Lu et al., 2015). Thus, these chemicals pose potential human health risks via the food chain. As such, remediation measures should be taken for PAH-polluted agricultural soils.

Bioremediation is a promising approach to detoxifying PAH-contaminated soil through biological processes (Gan et al., 2009). Many bacteria can use PAHs as carbon and energy sources, resulting in their mineralization; however, PAHs with ≥4 rings (high molecular weight PAHs, HMW PAHs) are relatively resistant to bacterial degradation because of their high hydrophobicity and stability (Bamforth and Singleton, 2005). Compared to bacteria, fungi have different mechanisms for transforming PAHs, such as extracellular ligninolytic enzymes and intracellular cytochrome P450 monooxygenases (Harms et al., 2011), and mounting evidence suggests that fungi are a potential resource for the clean-up of HMW PAH-polluted soil (Aranda, 2016).

A series of strategies have been developed to enhance microbial degradation of PAHs. Biostimulation, the addition of a bulking agent to stimulate soil microbes, is among the most common *in situ* treatments of

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agricultural soil. For example, successful reduction of HMW PAHs was achieved by the addition of lignin-rich materials (Lladó et al., 2013), because ligninolytic enzymes, such as LiP, MnP and laccase, have exceptional capacities for PAH transformation and may contribute to soil PAH dissipation through co-metabolic mechanisms (Wu et al., 2008b). Phytoremediation is another green technology for remediation of contaminated soil. Among others, ryegrass has a vast root system and has been widely used in bioremediation (Gan et al., 2009). Plants may enhance PAH dissipation by direct uptake (phytoextraction) or through rhizospheric processes (rhizoremediation) (Alkorta and Garbisu, 2001). Stimulation of rhizosphere microbes by root exudates tends to be the primary mechanism of rhizoremediation, as shown by a comprehensive analysis of rhizospheric microbial degraders (Bourceret et al., 2015). Nevertheless, the contribution of plant uptake has rarely been assessed (Gao and Zhu, 2004).

One crucial issue affecting biological transformation is the availability of pollutants. Surfactants are often used to increase the water solubility of hydrophobic organic pollutants, enhancing plant uptake (Zhu and Zhang, 2008) and improving microbial degradation (Fernando Bautista et al., 2009). Compared to synthetic surfactants that could be toxic to microorganisms, biosurfactants appear to be a safe alternative due to their biodegradability and biocompatibility (Ławniczak et al., 2013). Rhamnolipid is a biosurfactant produced by *Pseudomonas aeruginosa* and has shown potential in improving PAH bioavailability, thus is useful in the remediation of aged contaminated soil (Mulligan, 2005).

In practice, remediation strategies are often combined for optimal pollutant removal. For example, surfactants coupled with biostimulation or phytoremediation combined with microbial inoculation are often more effective in remediation of aged polluted soil than any measure in isolation (Johnson et al., 2004; Lladó et al., 2015). In this study, we assessed the bioremediation of a long-term polluted arable soil using three strategies, namely phytoremediation (ryegrass), biostimulation (lignin addition) and biosurfactant enhancement (rhamnolipid addition). Soil PAH dissipation and plant uptake were monitored. Also, quantitative PCR and high-throughput sequencing were used to detect shifts in the soil bacterial and fungal communities. A second microcosm experiment was performed to examine the effects of lignin and rhamnolipid on freshly spiked ^{14}C-benz(a)anthracene in the same arable soil. Both mineralization and soil fractionation of the ^{14}C-PAH were determined. The aims of this study were therefore (1) to evaluate the combined strategies in terms of soil detoxification, (2) to estimate the contributions of ryegrass, lignin and rhamnolipid to PAH dissipation, and (3) to explore the mechanisms underlying the PAH dissipation.

2. Materials and methods

2.1. Soil

The arable soil used in the study was collected on 7 Sep. 2015 near a smelting plant in Nanjing, Jiangsu Province, China (31°53′48″N, 118°36′59″E). At the time of sampling, the farmland was used for maize cultivation. The soil is a sandy loam, with a pH of 7.1, 12.7 g kg^{-1} of total carbon, 1.3 g kg^{-1} of total nitrogen, 0.57 g kg^{-1} of total phosphorus, and 19.3 g kg^{-1} of potassium (as K_2O). The bulk density of the soil was 1.14 g cm^{-3}. The total amount of 15 USEPA priority PAHs (excluding acenaphthylene) in this arable soil was 8.59 mg kg^{-1}, consisting of 11.0% 3-ring, 47.1% 4-ring, 28.7% 5-ring and 13.0% 6-ring PAHs. Specifically, the concentration of benz(a)anthracene in the soil was 0.85 mg kg^{-1}. The soil was air-dried, sieved (5 mm), homogenized and stored at room temperature in the dark.

2.2. Pot experiment

For the pot experiment, 2.0 kg of air-dried soil was added to each plastic pot of 20-cm diameter, and the soil moisture was adjusted to 60% water holding capacity (WHC) at the beginning of the incubation. Seven treatments, including an unamended control, were established in four replicates as shown in Table 1. For each planted pot, 20 ryegrass (*Lolium multiflorum* Lam) plantlets from the germination of seeds on moist perlite for 7 days were transplanted. Alkali lignin (Sigma-Aldrich) was spiked into the soil at a concentration of 1% (w/w) and well mixed. Prior to the moisture adjustment on day 0 and 30, 125 ml of 2 g l^{-1} rhamnolipid (90% mixture of mono- and di-rhamnolipid, critical micelle concentration (CMC) 50 mg l^{-1}, Zijin Biotech, Huzhou, China) solution was slowly poured on the soil surface, giving a final concentration of 125 mg kg^{-1} soil. During the incubation, the pots were irrigated every 2–3 days to keep the soil moisture.

Table 1
Experimental treatments in the pot experiment.

Treatment	Ryegrass (P)	Lignin (L)	Rhamnolipid (R)
Control	–	–	–
P	+	–	–
L	–	+	–
R	–	–	+
PL	+	+	–
PR	+	–	+
PLR	+	+	+

After a 90-day incubation in a greenhouse, a composite sample composed of five soil cores (approximately 15 cm in depth) was collected from each pot with an auger sampler. A 50-g subsample was stored at −20 °C for molecular analysis, and the remaining sample was air-dried, sieved and stored at 4 °C until PAH analysis. Ryegrass roots and shoots were separately harvested, rinsed, dried and homogenized prior to PAH determination.

2.3. PAH analysis

PAHs in soil and ryegrass were extracted and determined as previously described with minor modifications (Wu et al., 2016b). Briefly, 10.0 g of soil or 1.0 g of plant sample was spiked with 0.4 μg 1-fluoropyrene dissolved in acetone as an internal standard, and was extracted on a Soxhlet apparatus with dichloromethane for 24 h. Prior to the ultra-fast liquid chromatography (UFLC-20 system, Shimadzu, Kyoto, Japan) analysis, the extracts were concentrated and purified with activated silica gel. Fifteen of the 16 priority PAHs (excluding acenaphthylene) were determined with a reversed phase C18 column (Shim-pack XR-ODSII, Kyoto, Japan). All concentrations are presented based on a soil dry weight. The toxic equivalency factors (TEFs) of PAHs were obtained from the literature (Nisbet and LaGoy, 1992).

2.4. DNA extraction and quantitative PCR

Soil DNA was extracted from about 0.5 g of sample with a FastDNA Spin Kit for Soil (MP Biomedicals, OH) following the manufacturer's instructions. The quality and quantity of DNA were assessed with a NanoDrop 1000 spectrophotometer (Thermo, DE) and by electrophoresis. Tenfold-diluted DNA was used in all downstream analyses to avoid the inhibition of co-extracted soil contaminants.

Bacterial 16S rRNA, fungal 18S rRNA and bacterial PAH-ring hydroxylating dioxygenase (PAH-RHDα) genes were enumerated by quantitative PCR (qPCR) with the primer sets 515f/907r (Stahl and Amann, 1991; Muyzer et al., 1995), nu-SSU-0817-5′/nu-SSU-1196-3′ (Borneman and Hartin, 2000) and GP-F/GP-R (Cébron et al., 2008), respectively. QPCR was performed on a CFX96 instrument (Bio-Rad) based on SYBR Green chemistry. Triplicate reactions were run for each sample, and the qPCR was performed as described previously (Wu et al., 2015). All qPCR standards were generated by cloning the respective gene fragments into the plasmid pEASY-T1 (Transgen Biotech,