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## Selective enrichment of a pyrene degrader population and enhanced pyrene degradation in Bermuda grass rhizosphere

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**Abstract** Rhizosphere soil has a more diverse and active microbial community compared to nonvegetated soil. Consequently, the rhizosphere pyrene degrader population (PDP) and pyrene degradation may be enhanced compared to nonvegetated bulk soil (NVB). The objectives of this growth chamber study were to compare (1) Bermuda grass (*Cynodon dactylon* cv. Guymon) growth in pyrene-contaminated and noncontaminated soils and (2) pyrene degradation and PDP among NVB, Bermuda grass bulk (BB), and Bermuda grass rhizosphere soil (BR). Soils were amended with pyrene at 0 and 500 mg kg<sup>-1</sup>, seeded with Bermuda grass, and thinned to two plants per pot 14 days after planting (DAP). Pyrene degradation was evaluated over 63 days. The PDP was enumerated via a most probable number (MPN) procedure at 63 DAP. Bermuda grass root growth was more sensitive to pyrene contamination than shoot growth.

Pyrene degradation followed first-order kinetics. Pyrene degradation was significantly greater in BR compared to BB and NVB with rate constants of 0.082, 0.050, and 0.052 day<sup>-1</sup>, respectively. The PDPs were 8.01, 7.30, and 6.83 log<sub>10</sub> MPN g<sup>-1</sup> dry soil for BR, BB, and NVB, respectively. The largest PDP was in soil with the most rapid pyrene degradation. These results indicate that Bermuda grass can grow in pyrene-contaminated soil and enhance pyrene degradation through a rhizosphere effect.

**Keywords** Pyrene · Phytoremediation · Rhizosphere · *Cynodon dactylon* · Degradation population · Biodegradation

### Introduction

Some polycyclic aromatic hydrocarbons (PAHs) are toxic, recalcitrant soil pollutants (Sims and Overcash 1983) that are by-products of fossil fuel combustion and industrial processes (Lijinsky 1991). Soil PAH contamination arises primarily from aerial fallout, industrial or sewage effluent leakage, and petroleum product disposal (Giger and Blumer 1974; LaFlamme and Hites 1978). Many PAHs are known carcinogens and/or mutagens making soil reclamation a priority (Miller and Miller 1981).

The environmental fate of PAHs in soils has been investigated. The PAHs composed of four or more benzene rings are strongly adsorbed to soil colloids, relatively insoluble in water, and rarely leach (Edwards 1983; Knox et al. 1993). Volatilization and plant uptake are minimal due to low vapor pressures and rapid adsorption (Reilley et al. 1996; Sims and Overcash 1983; Edwards 1983). Consequently, their environmental fate is governed primarily by colloidal adsorption and microbiological degradation (Cerniglia 1992; Reilley et al. 1996).

Phytoremediation may expedite the reclamation of PAH-contaminated soil through a rhizosphere effect (Anderson et al. 1993). The rhizosphere is a carbon-enriched soil zone under the direct influence of plant roots (Curl and Truelove 1986). Enhanced microbial numbers and activity have been reported in PAH-contaminated rhizosphere soil compared

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to nonvegetated soil (Banks et al. 1999; Lee and Banks 1993; Miya and Firestone 2000; Nichols et al. 1997; Reilley et al. 1996; Schwab and Banks 1995). Greater PAH dissipation has been reported in vegetated soil compared to nonvegetated soil (Aprill and Sims 1990; Liste and Alexander 2000; Miya and Firestone 2000; Schwab and Banks 1995). Conversely, other studies have shown that pyrene degradation was not increased by vegetation (Lalande et al. 2003; Olexa et al. 2000). Given the conflicting results, a research project was designed to compare Bermuda grass growth in contaminated and noncontaminated soils while evaluating pyrene degradation and the pyrene degrader population (PDP) among Bermuda grass rhizosphere (BR), Bermuda grass bulk soil (BB), and nonvegetated bulk soil (NVB).

## Materials and methods

The Ap horizon of a Roxana fine sandy loam (coarse-silty, mixed, nonacid, thermic Typic Udifluvents), with no known prior exposure to PAHs, was passed through a 2-mm sieve. Gravimetric water content was determined by drying samples at 105°C for 24 h (McInnes et al. 1994). Particle size analysis determined with the hydrometer method (Bouyoucos 1953) was 51% sand, 46% silt, and 3% clay. Plant nutrient soil concentrations extracted with Mehlich 3 extractant (Mehlich 1984) and measured by inductively coupled plasma spectrometry (Soltanpour et al. 1996) were P 54 mg kg<sup>-1</sup>, K 86 mg kg<sup>-1</sup>, Ca 508 mg kg<sup>-1</sup>, and Mg 124 mg kg<sup>-1</sup>. Soil pH (1:1) was 6.1 (Thomas 1996), and organic matter was 0.5% as measured by the Walkley–Black procedure (Nelson and Sommers 1982).

Treatments were established by packing the equivalent of 1.3 kg dry soil in 1-l standard plastic pots to a bulk density of 1.25 Mg m<sup>-3</sup>. Pots were covered with Saran Wrap and preincubated in the growth chamber for 2 weeks. All incubations were at a constant 25°C with 12-h day/night light cycles. Soil water content was adjusted to and maintained at -0.033 MPa (15% water). Pyrene was added to soils at 0 and 500 mg kg<sup>-1</sup> by fortifying 125-g subsamples with 0.65 g pyrene dissolved in 50 ml acetone. Noncontaminated treatments received an equal volume of acetone without pyrene. Acetone was evaporated from the fortified subsamples for 24 h before combining and mixing subsamples with the appropriate soil treatments (Brinch et al. 2002). Vegetated treatments were planted with 20 Bermuda grass seeds and then thinned 14 DAP to two plants per pot.

Samples were collected 14 through 63 DAP on a 7-day interval. Shoot and root measurements were conducted at each sampling period. Root fresh weight was measured, and the sample was subsequently split to determine root dry weight and length (Tennant 1975). Shoot and root dry weights were determined by oven drying at 60°C to a constant weight. Total root length was calculated using the dry weight ratio.

Nonvegetated bulk and BB soil samples were collected at all sampling periods. Bermuda grass rhizosphere soil was collected as described by Angle et al. (1996) starting 35

DAP. Prior to 35 days, sufficient BR for analysis could not be collected due to the limited development of the rhizosphere. All soil samples collected for pyrene analysis were stored at 4°C until analysis. Biological analyses were conducted at the time of sample collection.

Each soil sample was extracted four times following modified EPA methods 3500 and 3550 protocols (U.S. EPA 1996). Five milliliters of hexane was combined with 3.0 g crushed Na<sub>2</sub>SO<sub>4</sub> and 3 g soil. The suspension was vortexed, sonicated for 24 h, and subsequently centrifuged for 10 min. The supernatants were combined and passed through a glass wool/Na<sub>2</sub>SO<sub>4</sub> filter, evaporated to dryness, and brought to volume with acetonitrile. An aliquot was removed for high-performance liquid chromatography (HPLC) analysis on a 15 cm×4.6-mm (5-μm) LC-PAH column (Supelco, Inc., Bellefonte, PA) with a modular system composed of a Shimadzu SCL-10A system controller, SIL-10A auto-sampler, LC-10AT HPLC pump, and SPD-10AV UV-VIS variable wavelength detector set at 254 nm (Shimadzu Scientific Instruments, Inc., Kyoto, Japan). The mobile phase was 7:3 CH<sub>3</sub>CN/H<sub>2</sub>O (v/v) with a flow rate of 1.5 ml min<sup>-1</sup> and an injection volume of 50 μl. The limit of pyrene quantification was 1 mg kg<sup>-1</sup>. Samples were corrected for a 92±3% recovery.

The PDPs were enumerated 63 DAP by a modified MPN procedure described by Wrenn and Venosa (1996). A 40-ml *n*-pentane solution containing 10 mg ml<sup>-1</sup> pyrene was filter sterilized with a Millex-gs 0.22-μm filter unit. A 10-μl aliquot of the *n*-pentane solution was added to sterile flat bottom microplate wells containing 270 μl of Bushnell–Haas (BH) medium (Bushnell and Haas 1941). For each rhizosphere sample, fresh roots with attached rhizosphere soil were placed in 99-ml dilution bottles for the 10<sup>-2</sup> dilution. Vegetated and nonvegetated bulk soil dilutions were performed by adding 1 g moist soil to the 10<sup>-2</sup> dilution. A tenfold serial dilution was made with phosphate buffer solution (Greenburg et al. 1992). For each dilution, 30 μl was pipetted into five separate microplate wells. Positive and negative controls were included for the procedure. Microplates were placed in partially sealed Zip-locke bags and incubated at 28°C for 21 days. Positive wells were identified with iodinitrotetrazolium violet (Haines et al. 1996). Each MPN was determined from the appropriate table (Cochran 1950) and expressed as log<sub>10</sub> MPN g<sup>-1</sup> dry soil.

The experimental design was a randomized complete block with three replications and a 4×8 factorial treatment structure having four pyrene–Bermuda grass combinations including 0.0 mg kg<sup>-1</sup> BR, 500 mg kg<sup>-1</sup> BR, 0.0 mg kg<sup>-1</sup> BB, 500 mg kg<sup>-1</sup> BB, and eight sampling times. The natural logarithm of pyrene concentration was regressed on time with a first-order kinetics model. The initial 14-day lag period was not considered in the data analysis. Analysis of covariance determined if slopes differed among BR, BB, and NVB. The PDPs were analyzed by analysis of variance. Means were separated with Fisher's LSD. Statistical significance was defined as *p* values ≤0.05. All statistical analyses were carried out with SAS version 6.12 (SAS Institute, Cary, NC).

## Results and discussion

Bermuda grass roots were more sensitive to pyrene than shoots. Pyrene reduced root length at  $\geq 28$  days, root dry weight at  $\geq 42$  days, and shoot dry weight at 63 days (Table 1). These trends remained evident throughout the study. Similar trends are reported for alfalfa grown in pyrene- and anthracene-contaminated soil (Reilley et al. 1996). Shoots may be less sensitive to PAH contamination compared to roots since pyrene translocation is minimal (Pradhan et al. 1998; Reilley et al. 1996; Sims and Overcash 1983; Edwards 1983).

Pyrene degradation exhibited a 14-day lag phase (Fig. 1) that is not uncommon since acclimation of the degrader population depends on soil, contaminant concentration, temperature, aeration status, and other undefined factors (Alexander 1994; Lalande et al. 2003; Olexa et al. 2000). An adaptation period is often necessary before indigenous soil microorganisms can effectively degrade the added pyrene (Binet et al. 2000). A reduced adaptation period for pyrene mineralization and a higher pyrene degradation rate were reported for ryegrass-planted systems compared to unplanted systems (Ferro et al. 1999). The first-order rate

constant ( $k$ ) was greater in rhizosphere soil (BR) compared to BB or NVB (Table 2). The rate constants were not different between BB and NVB suggesting that enhanced pyrene dissipation in vegetated system was attributed to the rhizosphere. Greater contaminant dissipation in the rhizosphere compared to nonvegetated soils has been reported for pesticides (Anderson et al. 1994; Anderson and Coats 1995; Marchand et al. 2002; Perkovich et al. 1996) and PAHs (Miya and Firestone 2000). Plant root exudates have been associated with enhanced rhizosphere contaminant dissipation (Burken and Schnoor 1996; Miya and Firestone 2001; Nichols et al. 1997; Siciliano and Germida 1998; Yoshitomi and Shann 2001). Root exudates may facilitate the cometabolic transformation of recalcitrant compounds (Hsu and Bartha 1979). Cometabolism was the suggested mechanism for enhanced  $^{14}\text{C}$ -pyrene mineralization in alfalfa rhizosphere soil supplemented with organic acids (Schwab and Banks 1995). Another explanation for enhanced rhizosphere degradation is selective degrader population enrichment.

Selective enrichment of a degrader population in rhizosphere soil compared to bulk soil has been reported for hydrocarbons (Miya and Firestone 2000; Nichols et al.

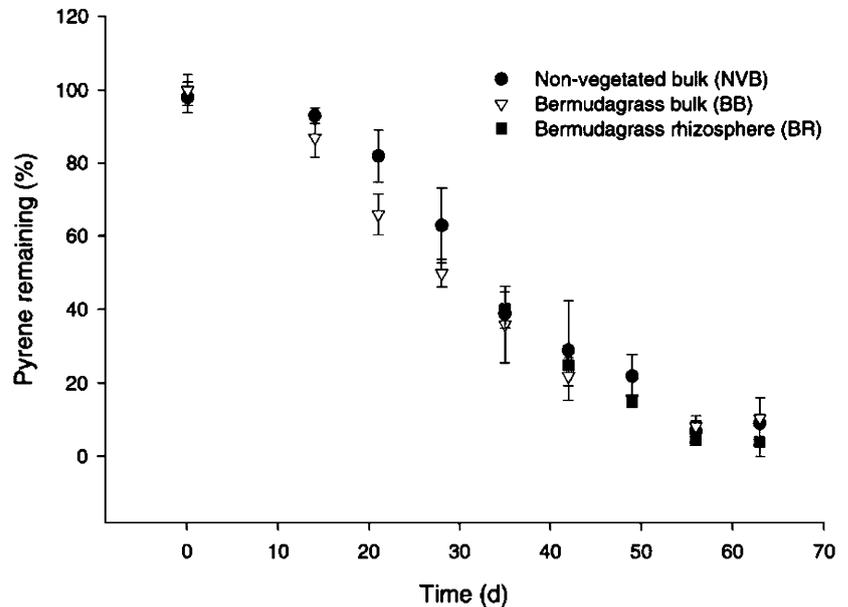
**Table 1** Bermuda grass shoot and root growth in pyrene-contaminated and noncontaminated soils

Parameter	Treatment	14 days	21 days	28 days	35 days	42 days	49 days	56 days	63 days
Shoot dry weight (g pot <sup>-1</sup> )	Noncontaminated	0.003a	ND <sup>a</sup>	0.144a	ND	1.850a	2.483a	2.930a	4.10a
	Contaminated	0.001a	0.002	0.019a	0.113	0.926a	1.570a	2.564a	2.230b
Root dry weight (g pot <sup>-1</sup> )	Noncontaminated	0.000a	ND	0.021a	ND	1.188a	2.101a	2.925a	3.456a
	Contaminated	0.000a	0.000	0.002a	0.017	0.1666b	0.651b	0.933b	1.652b
Root length (cm pot <sup>-1</sup> )	Noncontaminated	11.2a	ND	523.5a	ND	11,376.7a	9,744.2a	17,455.6a	10,755.3a
	Contaminated	6.8a	7.1	55.8b	295.8	1,226.7b	2,226.5b	8,440.5b	4,162.3b

Initial pyrene concentration for contaminated treatments was 500 mg kg<sup>-1</sup>. Treatment means followed by different letters are significantly different at  $p < 0.05$  for each parameter and time

<sup>a</sup>Data not collected

**Fig. 1** Percent pyrene remaining over time in Bermuda grass rhizosphere (BR), Bermuda grass bulk (BB), and nonvegetated bulk (NVB) soil. The symbol for Bermuda grass rhizosphere soil does not appear until 35 days due to insufficient rhizosphere development. Bars indicate  $\pm 1$  standard error



**Table 2** First-order rate constant ( $k$ ), standard error,  $r^2$ , and  $T_{1/2}$  for Bermuda grass rhizosphere (BR), Bermuda grass bulk (BB), and nonvegetated bulk (NVB) soil

Treatment	Rate constant ( $k$ ) (day <sup>-1</sup> )	Standard error (day <sup>-1</sup> )	$r^2$	$T_{1/2}$ <sup>a</sup> (day)
Bermuda grass rhizosphere (BR)	0.082a	0.011	0.76	8.4
Bermuda grass bulk (BB)	0.050b	0.005	0.89	13.9
Nonvegetated bulk (NVB)	0.052b	0.005	0.82	13.3

Rate constants followed by different letters are significantly different at  $p < 0.05$

<sup>a</sup> $T_{1/2} = 0.693/(k)$  and does not include the 14-day lag phase that was observed

1997) and pesticides (Sandmann and Loos 1984). In this study, the Bermuda grass rhizosphere PDP was selectively enriched compared to both NVB and BB (Fig. 2). Pyrene degrader populations were 8.01, 7.30, and 6.83 log<sub>10</sub> MPN g<sup>-1</sup> dry soil for BR, BB, and NVB, respectively. Noncontaminated treatments had PDP less than the minimum detectable number of 3.09 log<sub>10</sub> MPN g<sup>-1</sup> dry soil. In a silt loam soil amended with 2,000 mg pyrene kg<sup>-1</sup> soil, total bacterial or fungal numbers were not affected by pyrene contamination, but pyrene degrader numbers increased from less than the undetectable level of 2.77 to 7.09 log<sub>10</sub> degraders g<sup>-1</sup> soil (Gentry et al. 2003).

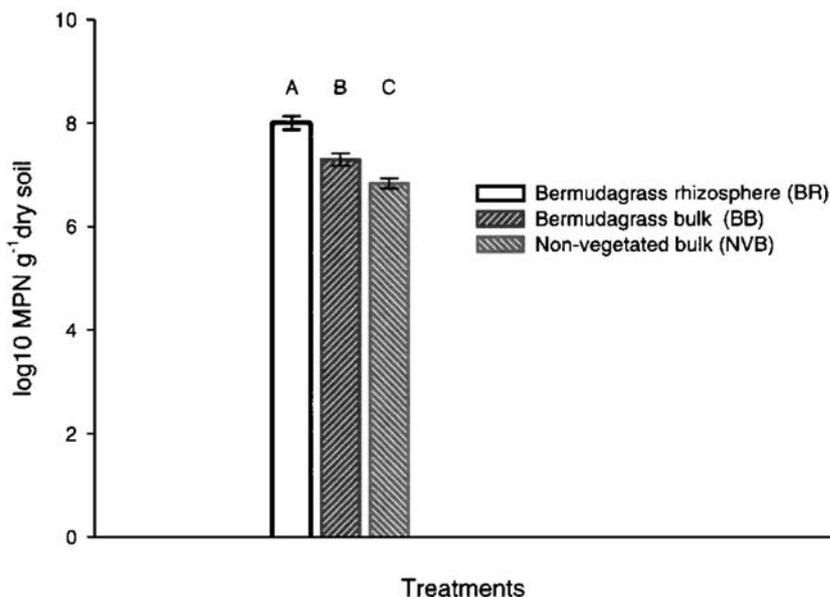
Plant root exudates, specifically phenolic analogs, may facilitate the selective enrichment of a degrader population (Nichols et al. 1997; Sandmann and Loos 1984). Laboratory studies have demonstrated that polychlorinated biphenyl (PCB) analogs enhance PCB-degrading bacterial populations by selectively improving their growth over non-PCB degrading microbes (Bedard et al. 1987). Data indicate that rhizosphere degrader enrichment is not a prerequisite for

enhanced biodegradation (Miya and Firestone 2001). However, in this study, the largest PDP was associated with the largest pyrene degradation rate constant. This trend has been reported for other contaminants (Jayachandran et al. 1998) and is logical since the probability for contaminant and contaminant–degrader contact is proportional to degrader population (Holden and Firestone 1997).

The pyrene degrader MPN method estimates microbial numbers that have the potential to degrade pyrene but does not provide information on the actual pyrene degradation activity of the microorganisms. The MPN method can underestimate PDP and does not account for cometabolism by microbial consortia (Johnsen et al. 2002). The inconsistency between the PDP (Fig. 2) and the first-order pyrene degradation rate constants (Table 2) could reflect a shift in the microbial community structure of the degraders that was not evident in the MPN determinations (Parrish et al. 2004). In the BR, there was a significant increase in pyrene degrader numbers and activity that was reflected in a more rapid degradation of pyrene. In the BB and NVB soils, the differences in PDP could be related to possible rhizosphere degraders inadvertently being included in the BB sample. In the NVB, there was only bulk soil and no roots that could have served as a possible source of rhizosphere PDPs. Additionally, the MPN determinations were conducted following the 63-day incubation and only reflect the numbers at that specific time.

Bermuda grass root growth is more sensitive to pyrene contamination than shoot growth. However, Bermuda grass was established from seed and grew in pyrene-contaminated soil. Moreover, both pyrene degradation and pyrene degrader populations were greater in Bermuda grass rhizosphere soil compared to bulk soil. The pyrene degrader population was largest in soil with the most rapid pyrene degradation rate. These results indicate that the Bermuda grass rhizosphere can be important in stimulating pyrene degradation.

**Fig. 2** Pyrene degrader populations in pyrene-amended soil at 63 days after planting for Bermuda grass rhizosphere (BR), Bermuda grass bulk (BB), and nonvegetated bulk (NVB) soil. Means followed by different letters are significantly different at  $p < 0.05$ . Bars indicate  $\pm 1$  standard error



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