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Environmental Toxicology and Chemistry, Vol. 25, No. 2, pp. 386–391, 2006

Printed in the USA
0730-7268/06 $12.00 + .00

# EFFECT OF SIMULATED RHIZODEPOSITION ON THE RELATIVE ABUNDANCE OF POLYNUCLEAR AROMATIC HYDROCARBON CATABOLIC GENES IN A CONTAMINATED SOIL

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(*Received* 16 *May* 2005; *Accepted* 2 *September* 2005)

**Abstract**—Microcosms were used to investigate whether soil exposure to mulberry root extracts (rich in phenolic compounds) select for bacteria that degrade polynuclear aromatic hydrocarbons (PAHs). Unlike previous studies with freshly spiked soil, the present experiments were conducted with soils aged for 518 d with [$^{14}$C]phenanthrene to decrease bioavailability and avoid exaggerating the selective pressure exerted by PAHs relative to the rhizosphere effect. Microcosms simulating contaminated planted soil were exposed to carbon at 20 mg/L/week of mulberry root extract for 211 d to simulate rhizodeposition. Contaminated bulk soils microcosms were amended with a C-free mineral medium to discern the effect of rhizodeposition. Uncontaminated soil controls also were exposed to similar dose regimes. Real-time quantitative polymerase chain reaction was used to enumerate total bacteria and PAH degraders harboring the genes *nahAc* (coding for naphthalene dioxygenase), *todC1* (coding for toluene/benzene/chlorobenzene dioxygenase), *bmoA* (coding for hydroxylating monooxygenases), and *dmpN* (coding for phenol hydroxylase). Exposure to root extracts enhanced the growth of total bacteria and PAH degraders in both contaminated and uncontaminated rhizosphere microcosms. The relative abundance of PAH-degrader gene copies (as a fraction of the total bacteria) was similar for different treatments, suggesting that the root extracts did not select for PAH degraders. Overall, these results suggest that rhizodeposition from phenolic releasers contributes to the fortuitous (but not selective) proliferation of PAH degraders, which may enhance phytoremediation.

**Keywords**—Oxygenases Polynuclear aromatic hydrocarbons Phytoremediation Real-time polymerase chain reaction

## INTRODUCTION

Phytoremediation has great potential for cleaning up root-accessible soils contaminated with polynuclear aromatic hydrocarbons (PAHs) [1–4]. In addition to stabilizing soils and improving aeration, plants can stimulate microbial activity and biochemical transformations in the rhizosphere through the release of root turnover and exudates [5]. Plants release 7 to 27% of the total plant mass annually as rhizodeposition [6]. The amount and composition of organic carbon released into the rhizosphere, however, varies between plants. This might create a nutritional bias in the rhizosphere of some plants that offers a selective advantage for microorganisms with certain desirable catabolic properties. Thus, plants that release copious amounts of phenolics might encourage growth of aromatic hydrocarbon–degrading microorganisms.

Because microbial aromatic dioxygenases generally exhibit relaxed substrate specificity [7], the proliferation of phenolic-degrading bacteria could lead to enhanced PAH biodegradation by way of cooxidative processes [8]. This concept, called rhizosphere metabolomics, has been exploited previously to enhance colonization of plant roots with desired bacteria. Oger et al. [9] showed that genetic modification of the lotus plant to enhance production of two opines in its root exudates resulted in a larger number of opine degraders within the rhizosphere community. Narasimhan et al. [10] used this principle to enhance selectively the colonization of a flavonoid-releasing *Arabidopsis* sp. by a polychlorinated biphenyl (PCB)–degrading *Pseudomonas* sp. that uses phenylpropanoid (a flavonoid) as a carbon source. Superior colonization and growth of this PCB-degrading strain in the rhizosphere resulted in 90% removal of the PCBs from contaminated sand.

Previous studies have reported higher concentrations of PAH degraders in the rhizosphere than in unplanted (bulk) soils (Table 1). It is unclear, however, whether the increase in PAH degraders was the result of an overall increase in microbial population in the rhizosphere or if certain rhizosphere factors (e.g., phenolic substrates) selected for PAH degraders. Furthermore, most of these studies were performed using soils freshly spiked with PAHs [1,2,11]. In such experiments, the contaminant may exert an exaggerated selective pressure relative to the rhizosphere effect, because aging at most contaminated sites, including natural cycling processes such as wet–dry and freeze–thaw [12], decreases PAH bioavailability and their ability to support the growth of specific degraders. Thus, it is important to determine if the phenolic-releasing rhizosphere can either influence or maintain a large PAH-degrading population in aged contaminated soils. In particular, a need exists for data regarding plant-induced genotypic changes to improve our understanding of microbial–plant interactions that influence the efficacy of phytoremediation.

The present study addresses the effect of phenolic-rich rhizodeposition on the concentration and relative abundance of PAH degraders in aged contaminated soil.

## MATERIALS AND METHODS

### *Collection and characterization of root extracts*

Mulberry root extracts were used in the present studies, because previous characterization studies showed that the ex-

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Table 1. Reported values for hydrocarbon degraders and total bacteria in contaminated and uncontaminated soils

| Soils | Plant | Rhizosphere to bulk soil ratio | | Hydrocarbon degraders to total bacteria ratio | | Reference |
|---|---|---|---|---|---|---|
| | | Total bacteria | Hydrocarbon degraders | Rhizosphere | Bulk soil | |
| Freshly spiked soils | | | | | | |
| Benzo[*a*]pyrene | Tall fescue | 10 | | | | [4] |
| Phenanthrene | Oat | 2 | 5.5 | 1.2 | 0.13 | [2] |
| Fuel oil | Maize | 15.8 | | | | |
| Uncontaminated soil | Maize | | | 0.0177 | | [30] |
| 3- to 6-ring polynuclear aromatic hydrocarbons | Ryegrass | ND[a] | 15 | ND | ND | [31] |
| Hydrocarbon mixture | Ryegrass | 16.7 | ND | ND | ND | |
| Uncontaminated | Ryegrass | 10 | ND | ND | ND | [11] |
| Hydrocarbon mixture | Alfalfa | 2.3 | 8.7 | 1 | 0.1 | |
| | Bluegrass | 1 | 2 | 0.3 | 0.1 | |
| Uncontaminated | Alfalfa | 9.1 | 8.1 | 0.6 | 0.1 | |
| | Bluegrass | 2.6 | 3.5 | 0.1 | 0 | [27] |
| Uncontaminated[b] | Poplar | 3.4 | 5.0 | $1.3 \times 10^{-6}$ | $1.1 \times 10^{-6}$ | [32] |
| Aged soils | | | | | | |
| 3- to 6-ring polynuclear aromatic hydrocarbons[c] | Ryegrass | 66 | 4.6 | 0.2 | 0.15 | [31] |
| Creosote | Fescue | ND | 2.6 | ND | ND | [33] |
| Phenanthrene[d] | Mulberry root extract | 1.9 | 1.7 | $1.7 \times 10^{-3}$ | $1.9 \times 10^{-3}$ | Present study |
| Uncontaminated | Mulberry root extract | 1.7 | 1.7 | $8.5 \times 10^{-4}$ | $8.6 \times 10^{-4}$ | Present study |

[a] ND = not determined.
[b] Benzene, toluene, and/or xylene degraders.
[c] Aged for 180 d.
[d] Aged for 518 d.

tracts not only were good growth substrates but also contained high concentrations of phenolics (28% of total organic carbon) [13]. Root extracts were collected as described previously [13]. Briefly, dark, fine roots (<1 mm), which are representative of the root turnover material that predominates in rhizodeposition [5], were used as the source of the root extract. The roots were homogenized in distilled water (Biospec Products, Bartlesville, OK, USA), centrifuged (13,000 rpm), and filter-sterilized (0.22 μm) before storage at 4°C. Root extracts were diluted with mineral medium before use to preclude potential inorganic nutrient limitation.

*Soil characteristics and aging with phenanthrene*

Because contaminants in freshly spiked soils tend to behave very differently from those that are subject to long-term exposure to soil, the experiments in the present study were carried out with aged soils. Uncontaminated soils (silty loam to silty clay loam alluvium, organic carbon fraction [$f_{oc}$] = 0.039; Minnesota Valley Testing Labs, Nevada, IA, USA) were spiked in the laboratory with [9-$^{14}$C]-labeled phenanthrene (300 mg/kg soil and 10,000 disintegrations per minute [dpm]/g soil) using hexane as the solvent carrier. The soils were then dried and stored in sealed containers in the dark at room temperature for 518 d before the experiment. Recovery of the $^{14}$C label in the soil (87.8 ± 0.5%) was measured by combustion after the aging process using an OX600 R.J. Harvey biological oxidizer (Hillsdale, NJ, USA). Uncontaminated controls (uncontaminated rhizosphere and uncontaminated bulk microcosms) were prepared using uncontaminated soils that had been stored for 518 d in the same manner as the contaminated soils were prepared.

*Microcosm setup*

Microcosms used to investigate the effects of rhizodeposition on the fate of [$^{14}$C]phenanthrene [14] also were used to determine the effects of rhizodeposition on the concentration and relative abundance of PAH degraders. These microcosms were prepared using 100 ml of serum bottles (nine replicates initially) containing 30 g of [$^{14}$C]phenanthrene-contaminated soil (300,000 dpm/reactor) and 100 ml of Hunter's mineral medium [15] (bulk soil microcosms). Microcosms simulating soils exposed to rhizodeposition were amended on a weekly basis with 2 ml of mulberry root extract (carbon, 1,000 mg/L), resulting in a microcosm final carbon concentration of 20 mg/L. Sterile controls (poisoned rhizosphere and sterile bulk microcosms) were prepared as described above except for the addition of $HgCl_2$ (50 mg/L) and $NaN_3$ (0.13 g/L). Every week, 2 ml of the supernatant in the rhizosphere microcosms (contaminated and uncontaminated) were replaced with root extract to stimulate intermittent additions of root-derived substrates in the rhizosphere environment. For microcosms simulating bulk soils, the supernatant sample was replaced with 2 ml of Hunter's mineral medium.

*DNA extraction*

Microcosms were killed at 74 d (within the bacterial exponential-growth phase) to investigate the effect of simulated rhizodeposition on the size of the total PAH-degrading and total bacteria population. The DNA was extracted from soil samples with the MoBio Power Soil DNA isolation kit (Carlsbad, CA, USA) according to the manufacturer's protocols. A bead-beating device (Model Mini Beadbeater-8; Biospec, Bartlesville, OK, USA) was used for soil lysis. The concentration and purity of the DNA were measured based on the ratio of absorbance at 260 nm for DNA and 280 nm for protein using an Amersham Biosciences spectrophotometer (Model Ultraspec 2100 Pro; Piscataway, NJ, USA). A 50-μl soil DNA sample was collected in a 2-ml Eppendorf vial and stored at

Table 2. Primers and probe sequences used in real-time quantitive polymerase chain reaction

| Target | Forward primer |
|---|---|
| Phenol hydroxylase (*dmpN*)[b] | 5′-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC-3′ |
| Toluene dioxygenase (*todC1*)[b] | 5′-ACCGATGA(A/G)GA(C/T)CTGTACC-3′ |
| Toluene monooxygenase (*bmoA*)[b] | 5′-T(C/T)TC(A/C/G)AGCAT(A/C/T)CA(A/G)AC(A/C/G)GA(C/T)GA-3′ |
| Toluene monooxygenase (*bmoA*)[b] | 5′-TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG-3′ |
| Naphthalene dioxygenase (*nahAc*)[b] | 5′-CAAAA(A/G)CACCTGATT(C/T)ATGG-3′ |
| Bacteria[c] | 5′-CGGTGAATACGTTCYCGG-3′ |
| Bacteriophage λ[d] | 5′-ACGCCACGCGGGATG-3′ |

[a] The reporter dye used was FAM (6-carboxyfluorescein) or TET (tetrachloro-6-carboxyfluorescein), and the quencher dye was either TAMRA (6-carboxytetramethylrhodamine) or BlackHole Quencher-1 (Integrated DNA Technologies, Coralville, IA, USA). SYBR-Green was from Applied Biosystems, Foster City, CA, USA.
[b] The primers were designed by Baldwin et al. [17].
[c] The primers and probe were developed by Suzuki et al. [34].
[d] The primers and probe were used by Beller et al. [16].

75°C (Model Isotemp Basic; Fisher Scientific, Pittsburgh, PA, USA).

*Polymerase chain reaction analysis*

Real-time quantitative polymerase chain reaction (RTQ-PCR) was used with primers targeting aromatic oxygenase genes to quantify microbial genotypic shifts resulting from simulated plant-rich rhizodeposition. The concentration of total bacteria was measured using the universal primers BACT1369F and PROK1492R [16]. The concentration of PAH degraders was measured using primers for genes coding for naphthalene dioxygenase (*nahAc*) subfamily N.2.A, ring hydroxylating monooxygenases (*bmoA*) subfamilies R.2 and R.3, phenol hydroxylase (*dmpN*) subfamily R.1, and toluene dioxygenase (*todC1*) subfamily D.2.C [17] (Table 2). All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA, USA). The PCR mixtures contained 1× SYBR-Green or Taqman buffer (Applied Biosystems, Foster City, CA, USA), 500 ηM forward and reverse primers, 250 ηM probe, and sterile DNase-free water to make up a final volume of 25 μl. The PCR reactions were performed using a Sequence Detector (Model ABI 7500; Applied Biosystems) with the following temperature conditions: 50°C for 2 min, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The number of gene copies in each sample was estimated based on the following equation:

$$\text{Gene copy}/\mu\text{l} = [(\mu\text{g DNA}/\mu\text{l})/(4.6 \times 10^6 \text{ bp/genome})] \times (9.1257 \times 10^{14} \text{ bp}/\mu\text{g DNA}) \times (1 \text{ gene/genome})$$

assuming that the approximate size of the bacterial genome used as the standard in the calibration curves was $4.6 \times 10^6$ bp ($\sim 9.12576 \times 10^{14}$ bp/μg DNA), which is equivalent to the size of the *Escherichia coli* genome [18], and just one gene copy per genome. The primer set designed using the DNA sequences of specific strains (i.e., *Pseudomonas putida* strain G7 [*nahAc*], *P. putida* strain F1 [*todC1*], *P. putida* strain CF600 [*dmpN*], and *P. aeruginosa* strain JI104 [*bmoA*]) was assumed to be representative of all other bacteria containing the catabolic gene. These assumptions also were used to quantify bacteria but not bacteriophage λ, because in this case, the solutions contained DNA fragments of a length identical to those used in the standards. Calibration curves (10 to $10^8$ gene copies/g soil) were prepared for all genes under consideration, yielding $r^2$ values of 0.99 or greater.

Nested PCR [19] reactions were performed on a Biometra thermocycler (Model T-gradient; Goettingen, Germany) with the following temperature conditions: 50°C for 2 min, followed by 95°C for 10 min and then 30 cycles at 95°C for 15 s and 55°C for 1 min, with a final extension at 70°C for 10 min. The PCR mixtures contained 1× Taqman buffer, 500 ηM of each universal bacterial primer BACT1369F and PROK1492R, 2 μl of the sample DNA, and sterile DNase-free water to make up a final volume of 25 μl. The amplified PCR product (2 μl) was then mixed with 1× Taqman buffer, 500 ηM of the forward and reverse primers coding for oxygenase genes (Table 2), and sterile DNAase-free water to make up a final volume of 25 μl. Temperature cycle was set as described above. The PCR products (10 μl) were visualized by electrophoresis (Model 200/2.0; Bio-Rad, Hercules, CA, USA) on an agarose gel (1%) prepared with Tris acetate–ethylenediaminetetra-acetic acid buffer and stained with ethidium bromide (0.0001%).

Although the recovery for the internal standard was low (0.79% ± 0.05% for contaminated and 0.25 ± 0.01% for uncontaminated soil samples), it was highly consistent within treatments, which permitted dependable comparative analysis. Similarly low DNA recoveries are commonly reported [20,21] and probably are caused by the binding of sample impurities during DNA extraction (e.g., humic acids), which interfere with the activity of Taqman polymerase during RTQ-PCR analysis [22].

To understand the effect of root extracts on the microbial community, the results were reported as concentration of PAH degraders (i.e., bacteria harboring oxygenase genes), total bacteria, ratio of rhizosphere PAH degraders to bulk soil PAH degraders (R to S ratio), and ratio of PAH degraders to total bacteria in a given soil. The bacterial concentration initially present in the soils before PAH addition was $1.1 \times 10^7$ cells/g soil.

## RESULTS AND DISCUSSION

A parallel study on how phenolic-rich rhizodeposition affects the fate of PAHs in aged soils [14] showed higher phenanthrene mineralization in the rhizosphere (57.7% ± 0.9%) than in bulk soil microcosms (53.2% ± 0.7%; $p < 0.05$) (Fig. 1). Based on pure-culture studies with the bioluminescent reporter *P. putida* strain HK44 and the same mulberry root extracts, we ruled out that enhanced phenanthrene degradation was caused by enhanced catabolic enzyme induction at the individual cell level [13]. Whereas some phenolics released by plants (e.g., methyl- and acetyl-salicylate *l*-carvone and *p*-cymene) can induce oxygenases with relaxed substrate specificity [13,23], labile substrates in the root extracts interfere

Table 2. Extended

| Reverse primer | Probe[a] |
|---|---|
| 5′-CGCCAGAACCA(C/T)TT(A/G)TC-3′ | SYBR-Green |
| 5′-CTTCGGTC(A/C)AGTAGCTGGTG-3′ | SYBR-Green |
| 5′-TT(A/G/T)TCG(A/G)T(A/G)AT(C/G/T)AC(A/G)TCCCA-3′ | SYBR-Green |
| 5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA-3′ | SYBR-Green |
| 5′-A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCAA-3′ | SYBR-Green |
| 5′-GGWTACCTTGTTACGACTT-3′ | FAM-5′-CTTGTACACACCGCCCGTC-3′-BHQ |
| 5′-AGAGACACGAAACGCCGTTC-3′ | TET-5′-ACCTGTGGCATTTGTGCTGCCG-3′-TAMRA |

with the expression of genes that code for such catabolic enzymes [13]. This suggests that the beneficial effect of phenolic-rich rhizodeposition is mainly caused by promoting the proliferation of PAH degraders. Specifically, enhanced growth of competent genotypes could compensate for the interference that labile substrates exert on the expression of catabolic genes, which is conducive to faster biodegradation [13]. To our knowledge, however, no previous studies have characterized indigenous genotypic shifts in aged contaminated soil exposed to phenolic-rich rhizodeposition. This information is important to support plant-selection criteria for PAH phytoremediation applications.

Following 211 d of simulated rhizodeposition, contaminated rhizosphere microcosms had higher total bacterial concentrations than the contaminated bulk microcosms ($8.9 \pm 0.64 \times 10^6$ vs $4.7 \pm 0.45 \times 10^6$ copies/g soil) (Fig. 2). Similarly, uncontaminated rhizopshere microcosms had higher total bacterial concentrations compared to those in the uncontaminated bulk controls ($2.3 \pm 0.15 \times 10^7$ vs $1.6 \pm 0.31 \times 10^7$ copies/g soil). These differences were statistically significant at the 95% confidence level. The highest bacterial concentration was measured in the uncontaminated rhizosphere microcosms. It is unclear why the total concentration of bacteria was higher in the uncontaminated than in the contaminated microcosms. Whether the accumulation of phenanthrene metabolites (e.g., phenol and quinones) inhibited bacterial growth (e.g., by affecting energy transduction across the cell membranes) [24,25] was not investigated. Previous work, however, demonstrated that accumulation of such PAH metabolites (>10 ppm) inhibited pure cultures of *Pseudomonas* strains (M. Eriksson, Royal Institute of Technology, KHT, Stockholm, Sweden, unpublished data).

Theoretically, assuming that a (decay-corrected) net cell yield coefficient for mulberry root extracts of 0.33 mg cell dry weight/g total organic carbon [13] and considering that the rhizosphere microcosms were fed an average of 20 mg/L of carbon every 7 d for 74 d (equivalent to a one-time carbon dose of 211 mg/L/reactor), the rhizosphere would have supported a microbial population of $5 \times 10^7$ cells/g soil, which was within the same order of magnitude observed in our experiments ($\sim 10^7$ copies/g soil).

The PAH-degrader population was estimated by targeting different oxygenase genes (Table 2). Neither naphthalene dioxygenase (*nahAc*) nor toluene monooxygenase (*bmoA*) were detected by RTQ-PCR in our samples (detection limit, $\sim 10^2$ copies/g soil). The detection of these genes using nested PCR methods as described elsewhere [19], however, suggests that these genes were present at very low copy numbers.

We recognize that the primers used in the present study may not account for all the genes coding for aromatic hydrocarbon oxygenases (therefore underestimating the concentration of PAH-degrading bacteria). Nonetheless, these results (exhibiting relatively low coefficients of variation) suffice to evaluate relative genotypic shifts in microbial populations exposed to rhizodeposition.

The higher mineralization of PAH observed for the rhizosphere microcosms (Fig. 1) is consistent with the significantly higher total oxygenase copy numbers found in these samples ($2.6 \times 10^4$ copies/g soil) versus those found in contaminated bulk soil microcosms ($1.4 \times 10^4$ copies/g soil; $p <$

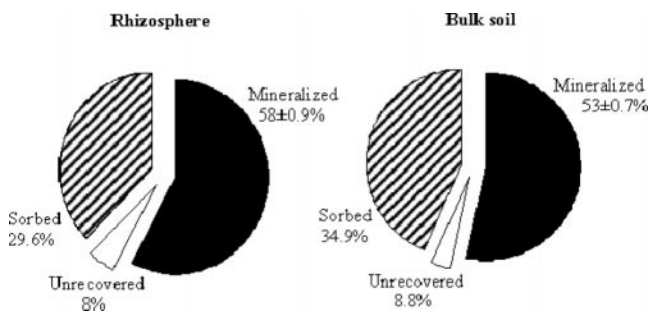


Fig. 1. Fate of [$^{14}$C]phenanthrene (300 mg/kg soil, 10,000 disintegrations per min [dpm]/g soil) after 211 d of incubation.

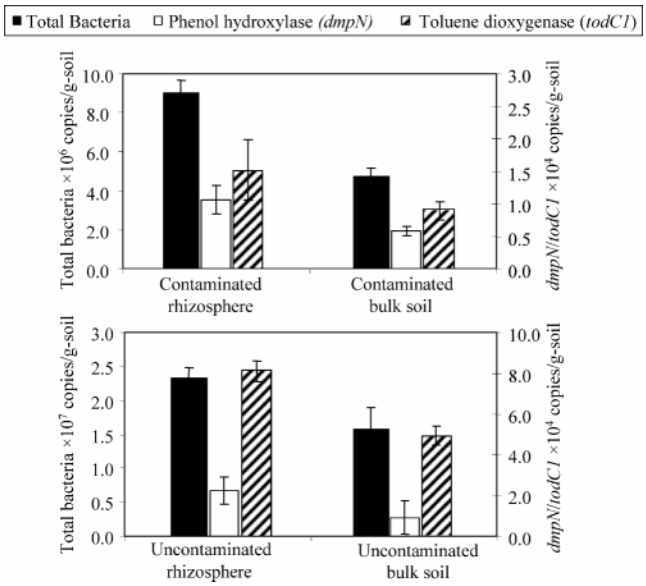


Fig. 2. Real-time quantitative polymerase chain reaction (RTQ-PCR) analysis of soil samples from contaminated (**A**) and uncontaminated (**B**) rhizosphere and bulk soils (sacrificed after 74 d). The concentrations of total bacteria and phenanthrene (polynuclear aromatic hydrocarbon) degraders harboring *dmpN* (coding for phenol hydroxylase) or *todC1* (coding for toluene/benzene/chlorobenzene dioxygenase) genes were statistically higher ($p < 0.05$) in rhizosphere than in bulk soil microcosms for both contaminated (**A**) and uncontaminated (**B**) treatments. Error bars depict one standard deviation from triplicate microcosms.

0.05). This suggests that the enhanced PAH mineralization was a direct outcome of the increase in PAH-degrader concentration in simulated rhizosphere soils. Specifically, intermittent addition of phenolic-rich root extracts encouraged the growth of total bacteria and PAH degraders, with nearly twofold increases in rhizosphere microbial concentration relative to bulk soils for both contaminated and uncontaminated microcosms (R to S ratio) (Table 1).

The amount of organic carbon released by rhizodeposition (2–27%, which includes exudation of soluble plant products plus root turnover from sloughing and cell death) varies between plants but generally is estimated to be between 10 and 100 mg/g root material [26]. This availability of organic matter in the rhizosphere has been reported to increase the concentration of total bacteria (by 4- to 100-fold) and PAH-degrader populations (by 1- to 17-fold) compared to those in bulk soils [2,11,27–29]. The higher ratio of total bacteria in the rhizosphere to bulk soil (R to S ratio) observed in other studies (Table 1) suggests that we could have obtained a more pronounced rhizosphere effect by increasing the dosage of carbon. The organic carbon dosage used here to simulate rhizodeposition (20 mg/L/week) is on the low end of total organic carbon concentrations reported for root exudation, which typically range from 50 to 400 mg/L [2,8]. Our relatively low dose was selected to ensure root extract availability over the 30-week duration of the experiment.

The ratio of PAH degraders to total bacteria was similar for the contaminated (~0.2%) and uncontaminated (~0.1%) treatments. Previous studies have reported the proliferation of PAH degraders in the rhizosphere environment (Table 1). Unlike previous studies that did not rely on molecular tools, our present results show that growth of specific PAH degraders was not preferential. Therefore, neither simulated rhizodeposition nor PAH contamination exerted a significant selective pressure for PAH degraders.

In summary, the present study addressed the effect of rhizodeposition on the size and relative abundance of the microbial population associated with PAH biodegradation. Plant-derived compounds stimulated the growth of specific PAH degraders similar to the extent that they stimulated the growth of total bacteria. This suggests that rhizodeposition encourages the fortuitous growth of microorganisms harboring the catabolic potential (e.g., oxygenase enzymes) for PAH biodegradation without exerting selective pressure. Nevertheless, such plant-induced genotypic shifts could enhance overall removal of PAHs in aged contaminated soil during phytoremediation.

*Acknowledgement*—The present study was supported by the National Science Foundation. We thank Loring F. Nies and Brett D. Sutton (School of Civil Engineering, Purdue University).

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