Pyrene Mineralization by *Mycobacterium* sp. Strain KMS in a Barley Rhizosphere

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To determine whether the soil *Mycobacterium* isolate KMS would mineralize pyrene under rhizosphere conditions, a microcosm system was established to collect radioactive carbon dioxide released from the labeled polycyclic aromatic hydrocarbon. Microcosms were designed as sealed, flow-through systems that allowed the growth of plants. Experiments were conducted to evaluate mineralization of ^{14}C-labeled pyrene in a sand amended with the polycyclic aromatic hydrocarbons degrading *Mycobacterium* isolate KMS, barley plants, or barley plants with roots colonized by isolate KMS. Mineralization was quantified by collecting the $^{14}CO_2$ produced from ^{14}C-labeled pyrene at intervals during the 10-d incubation period. Roots and foliar tissues were examined for ^{14}C incorporation. Mass balances for microcosms were determined through combustion of sand samples and collection and quantification of $^{14}CO_2$ evolved from radiolabeled pyrene. No pyrene mineralization was observed in the sterile control systems. Greater release of $^{14}CO_2$ was observed in the system with barley colonized by KMS than in microcosms containing just the bacterium inoculum or sterile barley plants. These findings suggest that phytostimulation of polycyclic aromatic hydrocarbons mineralization could be applied in remediation schemes.

INTEREST IN THE BIODEGRADATION of polycyclic aromatic hydrocarbons (PAHs) is prompted by their ubiquitous presence, worldwide distribution, and potentially deleterious effects on human health (Guerin, 1999; Habe and Omori, 2003; Krutz et al., 2005). PAHs constitute a broad class of organic compounds that are generally described as organic compounds with two or more aromatic rings in various structural configurations. The effective technologies available for the remediation of PAH-contaminated sites include biodegradation (Kanaly and Harayama, 2000; Keith and Telliard, 1979; Menzie et al., 1992). Certain bacteria have the enzymatic machinery to degrade low- and high-molecular-weight PAHs (Dean-Ross and Cerniglia, 1996; Derz et al., 2004; Habe and Omori, 2003; Kanaly and Harayama, 2000; Miller et al., 2004).

Mycobacterium isolate KMS is a PAH-degrading bacterium isolated from soils contaminated with wood preservatives where land treatments for bioremediation were successful (Miller et al., 2004). The KMS strain mineralizes the multi-ring structure benzo[a]pyrene and smaller ring structures in laboratory-culture conditions (Miller et al., 2004), which makes it a valuable candidate for biodegradation because PAHs generally occur as a complex mixture, not as single compounds (Frysinger et al., 2003). Child et al. (2007) showed that strain KMS strongly colonizes barley plant roots and grows well on root wash materials. The presence of root wash materials did not prevent the mineralization of pyrene by the KMS strain (Child et al., 2007).

Studies of PAH degradation involving plants have been conducted with a variety of species in a range of soils with assorted rhizospheric microbial communities (Table 1). The effect of plants on PAH degradation varies with plant type and environmental conditions. In several studies, phytoremediation of PAHs is shown to be effective, with higher mineralization rates in rhizosphere soil than in unvegetated soil (Banks et al., 2003; Chen et al., 2003; Ferro et al., 1994; Kim et al., 2004; Nedunuri et al., 2000; Olson and Fletcher, 1999). Among the plants' many possible contributions to higher biodegradation rates is the promotion of higher, actively metabolizing microbial loads (Karthikeyan and Kulakow, 2003; Yan-zheng and Li-zhong, 2005; Yoshitomi and Shann, 2001). Paul and Clark (1989) showed that microbial populations and activities associated with roots are as much as 100 times that of the bulk soil.

Other studies have shown that a more active rhizosphere population does not always correspond with higher PAH degradation rates. Lalande et al. (2003) found that in field conditions pyrene

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Abbreviations: LB, Luria broth; PAH, polycyclic aromatic hydrocarbons.

Table 1. Phytoremediation studies examining polycyclic aromatic hydrocarbons (PAH) degradation.

Growth matrix	Plant species	Rhizosphere microflora	PAH type	Plant effect on PAH remediation rates	Reference
Soil	mulberry	ND†	mixture	increased	Olson and Fletcher, 1999
Soil	alfalfa, barley, tall fescue, orchard grass	ND	anthracene	increased	Kim et al., 2004
Soil	tall fescue, switchgrass	ND	pyrene	increased	Chen et al., 2003
Root wash	kou, milo, oat, orange, willow, and poplar trees	*Pseudomonas putida*	phenanthrene	decreased	Rentz et al., 2004
Soil	ryegrass, radish, spinach	ND	pyrene and phenanthrene	increased	Yan-zheng and Li-zhong, 2005
Sand	barmultra	*P. fluorescens*	naphthalene	increased	Kuiper et al., 2001
Peat-sand mixture	barley and rape	*P. fluorescens, aureofaciens*	phenanthrene	increased	Anokhina et al., 2004
Earth–sand mixture	white mustard	*Sphingomonas yanoikuyae*	phenanthrene	increased	Hynes et al. 2004
Soil	oat, radish, pine	ND	pyrene	increased	Liste and Alexander, 2000
Soil	legumes and grasses	ND	pyrene	no change	Chekol and Vough, 2004
Estuarine sediment	salt marsh plants	*Pseudomonas, Paenibacillus, nocardioforms*	mixture	ND	Daane et al., 2001
Soil	tall fescue, ryegrass, clover	ND	mixture	increased	Parrish et al., 2004
Soil	ryegrass	ND	pyrene	decreased	Lalande et al., 2003
Soil	bermuda grass	ND	pyrene	increased	Krutz et al., 2005

† Not determined.

degradation rates were higher in nonvegetated soils than in vegetated soils. Chekol and Vough (2004) observed that elevated microbial counts in PAH-contaminated rhizosphere soil did not increase remediation rates above nonrhizosphere soil with lower microbial counts. Rentz et al. (2004) observed repression of phenanthrene degradation by *Pseudomonas putida* in the presence of root extracts. Genes encoding enzymes involved in PAH breakdown were downregulated or were not expressed due to metabolic inhibition caused by the presence of more easily used compounds exuded from plants. Metabolic inhibition has been shown to affect PAH degradation rates in other systems as well (Bouchez et al., 1995; Keuth and Rehm, 1991).

The majority of PAH phytoremediation studies have been conducted under field conditions where the microbial community has not been characterized or controlled. Few studies have measured PAH remediation rates in gnotobiotic conditions with a specific plant/microbe combination. A controlled rhizospheric environment is imperative to ensure that responses are due to treatments alone and not to artifacts from suboptimal growth conditions or interfering microbes.

Although soil provides a growth medium most similar to field conditions, there are several disadvantages of using soil as a growth matrix in phytoremediation studies. Soil is difficult to sterilize, and structural and geochemical changes occur from sterilization procedures (Henry et al., 2006). In addition, the humic content of soils is difficult to determine and has been shown to change the bioavailability of PAHs (Laor et al., 1999).

Other solid substrates that provide growth conditions similar to field conditions are often used for studies with axenic and gnotobiotic cultures (Henry et al., 2006). Sand has been used as a growth medium for plant culture studies because it is easily sterilized and has fewer reactive surfaces than soil or clay (Henry et al., 2006). Sand also is convenient for use in PAH

degradation studies because there is no existing humic acid.

In this study we wished to determine whether KMS isolate mineralized PAHs in a rhizosphere environment. Thus, a suitable microcosm system allowing for gnotobiotic conditions, plant growth, and quantification of PAH mineralization rates was designed. We also report that mineralization of the PAH pyrene is higher in microcosms with barley roots colonized by *Mycobacterium* isolate KMS than in microcosms with just the bacterium or barley alone. Barley (*Hordeum vulgare*) was chosen as the host plant for these studies because we had demonstrated that among nine other grasses chosen it was among the best for stand survival in PAH- and salt-contaminated soils (Sims et al., unpublished data).

Materials and Methods

Cultures

The mycobacterium cultures, inoculated from stocks stored at −70°C in 15% glycerol, were grown in 50 mL Luria broth (LB) medium in 200 mL flasks, shaking at 220 rpm at 25°C. Four-day-old cultures, late log-phase cells, were used for seed inoculation.

Experimental Setup

Microcosms were used to evaluate the fate of pyrene in a sand environment. Sand and plants, raised from seeds, were housed in Magenta boxes (Magenta, Chicago, IL). ^{14}C-pyrene–amended sand was planted or inoculated with one of the following: (i) sterile barley seedlings, (ii) *Mycobacterium* isolate KMS cells, or (iii) sterile barley seedlings inoculated with the mycobacterium. A sterile, uninoculated, pyrene-amended microcosm was run as a control. The three treatments and uninoculated control were arranged in a four-block, randomized box design. Fifty milliliters of sterile, deionized water was added to each microcosm, which was then placed in a clear,
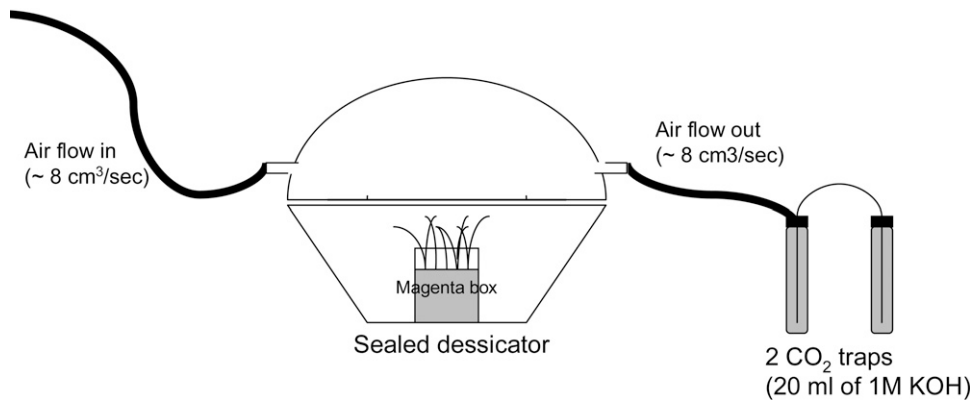
Fig. 1. Schematic of the microcosms used to examine mineralization by barley colonized with *Mycobacterium* isolate KMS.

sealed, flow-through system. Each desiccator housed one Magenta box. The air in the system was purged daily, and $^{14}CO_2$ evolved emissions were collected.

Microcosm Design

A sealed flow-through system was constructed using 190-mm diameter by 260-mm high desiccator with a translucent polystyrene bottom and polycarbonate top (Scienceware, Pequannock, NJ) (Fig. 1). Desiccators were sterilized by autoclaving before starting the experiment. Air flow-through was achieved with an in-valve and an out-valve on the desiccator. The two sections of the desiccator were sealed using Parafilm and eight 1.25-in binder clips. The containers were left sealed for the duration of the 10-d experiments. Ambient air was pumped through the system for 4 h in every 24-h period at a velocity of 8 cm^3 s^{-1}, as regulated by a needle valve. This flow rate for 4 h was shown to be sufficient for complete air turnover and $^{14}CO_2$ removal from the system by separate studies where $^{14}CO_2$ was added into the sealed system and subsequently removed and trapped with 1 M KOH traps. Nalgene (Rochester, NY) clear plastic tubing was used for air inflow and out-flow and was sterilized by autoclaving before use. A section of the air inlet tube was packed with cotton to remove airborne microbial contaminants at low air flow rates (Owen and Johnson, 1955). Air outflow was passed through a series of two 40-mL glass VOA vials in series, each containing 40 mL 1 M KOH to trap evolved $^{14}CO_2$. The photoperiod was set at 24 h because the response of barley to photoperiod is not significant for early phases of growth (Saarikko and Carter, 1996). Temperature was maintained at 22 ± 1°C. Two 100 W soft white light bulbs were used as the energy source.

Plant Growth: Matrix Preparation and Spiking

Industrial quartz sand (35 mm) (Unimin Corp., Emmett, ID) was used as the solid growth matrix. Sand (200 g) was placed into 400 cm^3 Magenta boxes (99 mm high by 68 mm wide). The boxes of sand were sterilized at 121°C for 40 min at 103 kPa pressure. After storing at room temperature for 24 h to allow fungal and bacterial spore germination, the boxes were sterilized again at 121°C for 40 min. A mixture (20 mg) of ^{14}C-labeled and unlabeled pyrene dissolved in 155 µL ethanol was added to

the 200 g sand. Total disintegrations per second per Magenta box and 200 g sand were 14 340 000 ± 276 000. The sand was mixed with a sterilized metal spatula, and the ethanol was allowed to evaporate. After the evaporation step, the Magenta boxes were sealed and placed in the dark at 20°C. The pyrene-amended sands were aged for 14 days. Upon completion of the aging process, the sand was mixed again using a sterilized metal spatula, sealed, and placed in the dark at 20°C until use.

Barley Seed Sterilization

Barley seeds were processed to remove endogenous surface microbes and microbial endophytes (Bishop et al., 1997). Seeds were immersed in 30% hydrogen peroxide for 5 min and washed with sterile water for 3 min, followed by three 1-min washes with sterile water to remove any remaining hydrogen peroxide. The seeds were heat treated by suspension in sterile water at 50°C for 30 min. After the heat treatment step, the seeds were surface sterilized again with hydrogen peroxide and washed with water. These seeds were plated on LB agar plates and incubated at 22°C for up to 48 h to permit germination. Only seedlings growing without signs of microbial contamination were used in the studies.

Barley Seedling Inoculum

Cells of *Mycobacterium* sp. strain KMS were harvested during log-phase growth in LB liquid medium after 4 d, washed twice in sterile water, and suspended in sterile water to a final cell density of approximately 10^8 cfu/ml. Seedlings were inoculated by submersion in the inoculum for 30 s. To determine the number of mycobacterium cells adhering to each seedling, inoculated barley seedlings were submersed in 1 mL sterile water and vortexed for 30 s. Serial dilutions of the water fractions onto LB agar medium were performed, and cfu ml^{-1} of cells were ascertained after growth at 24°C for 7 d.

Seedling Planting and Microcosm Inoculation

Ten seedlings per microcosm, inoculated or uninoculated with the mycobacterium, were planted into the sterile sand. Microcosms amended only with bacteria were inoculated with 10 10-µL aliquots of the 10^8 cfu ml^{-1} cell suspension used in seedling inoculation. The number of bacteria in the 10-µL aliquot volume corresponded to the bacterial cell count from inoculated seeds. The 10-µL aliquots were added equidistant from one another at the same depth and pattern that the seeds were planted. Each microcosm housed one Magenta box containing sterile barley seedlings, KMS cells, or barley seedlings inoculated with isolate KMS. No amendments were added to the sand for control conditions.

¹⁴CO₂ Collection

Two ¹⁴CO₂ traps (containing 20 mL of 1 M KOH) were sampled daily throughout the study. After each daily aeration period, a 1-mL aliquot of the KOH ¹⁴CO₂ trap solution was added to 6 mL of Ready Gel scintillation cocktail (Beckman Coulter, Fullerton, CA) and was analyzed by liquid scintillation counting in a Beckman LS 5000 machine. One milliliter of fresh 1 M KOH was added to the ¹⁴CO₂ trap to compensate for the 1-mL aliquot removal.

¹⁴C Sand Analysis and ¹⁴C Plant Tissue Analysis

Concentrations of ¹⁴C in the sand and plant materials were determined using combustion, trapping, and scintillation counting. At the conclusion of the study, any plant materials were removed, and the sand was mixed with a sterile metal spatula. Triplicate samples of 0.5 g were combusted at 900°C in a Model OX-600 Combustor (R.J. Harvey, Hillsdale, NJ). The evolved ¹⁴CO₂ was collected in a solution of 50% Ready Gel/40% methanol/10% monoethanol amine and directly analyzed by scintillation counting. The total activity within the sand compartment was determined by multiplying the activity per unit mass by the total mass of each sand compartment.

For plant analysis, roots and shoots/leaves were collected, combusted at 900°C, collected, and analyzed as described previously. All available plant tissues were combusted, giving the total activity within the plant tissues.

To determine whether ¹⁴C-labeled plant materials had taken up pyrene or pyrene metabolites from the soil or had fixed previously released ¹⁴CO₂, a control microcosm was run with continuous air flow to limit ¹⁴CO₂ accumulation and fixation by the plants. Seedlings were inoculated with M. KMS and planted as outlined previously. Plant tissues were analyzed as described previously. The control value was subtracted from test plant tissue disintegrations per minute readings, and the difference was ascribed to pyrene mineralization and subsequent ¹⁴CO₂ fixation.

Statistical Analysis

A randomized box design was used in ANOVA, and comparisons of means computations were done in JMP Version 5.0.1 for Windows (SAS, Cary, NC). The Tukey honestly significant difference test was used to identify significant differences among means when the ANOVA indicated that differences existed. Differences between means were considered to be statistically significant at $p \leq 0.05$.

Results and Discussion

¹⁴CO₂ Collection

Mineralization of pyrene from the microcosms is shown in Fig. 2. Little to no pyrene mineralization was measured in the sterile, uninoculated microcosms and in microcosms where noninoculated barley was grown. Microcosms inoculated with the bacterium alone showed a slow, steady rate of mineralization over the 10-d period. The highest mineralization took place in microcosms supporting barley seedlings with roots
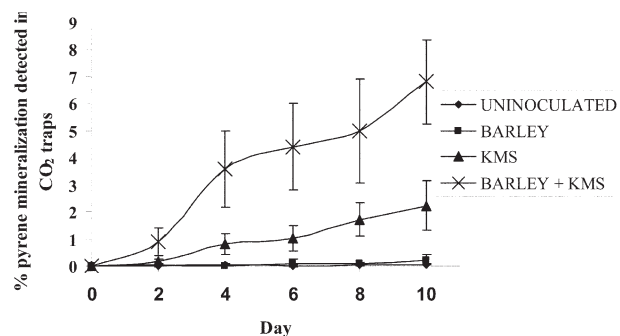
Fig. 2. Pyrene mineralization in microcosms containing barley, the *Mycobacterium* sp. strain KMS or barley with roots colonized by the mycobacterium cells. Data are the mean of three independent experiments for each microcosm ± SD.

colonized by the mycobacterium. These mineralization rates from the colonized plants were more than double those observed in the presence of bacteria alone. Root colonization by strain KMS had no observable effect on plant growth characteristics or on plant mass (data not shown). Pyrene exhibited no visible adverse effects on the growth of barley in this study.

Plant ¹⁴C Labeling

¹⁴C levels in barley plants grown in pyrene-amended microcosms in continuous air-flow conditions were assayed to determine plant uptake of pyrene and/or partial degradation products of pyrene and the incorporation of any released ¹⁴C-CO₂ by photosynthesis. Figure 3 compares levels of ¹⁴C label in barley tissues from microcosms with discontinuous air flow and microcosms with continuous air turnover. The ¹⁴C accumulated in barley roots grown in discontinuous air-flow conditions showed values above the ¹⁴C levels in the growth matrix, indicating possible uptake of low amounts of pyrene or partial degradation products. ¹⁴C concentrations in barley leaves grown in discontinuous air-flow conditions showed ¹⁴C levels in plant tissues that were higher than those measured in continuous air-flow conditions, indicating fixation of ¹⁴C-labeled CO₂ through photosynthesis and transportation of metabolites into the root tissues.

Total Mineralization

Mineralization detected from the ¹⁴CO₂ traps does not reflect total mineralization because some of the label was likely assimilated into the plants through photosynthesis. We estimated the photosynthetically trapped ¹⁴C levels by subtracting the label in barley tissues from plants grown in continuous air-flow conditions from ¹⁴C levels of plants grown in the discontinuous air flow microcosms. Figure 4 shows the estimated percent total mineralization (trap plus estimated plant photosynthetic assimilation) for all four microcosm conditions. The plants with roots colonized by cells of the KMS isolate mineralized pyrene to a greater extent than observed from microcosms with just the bacterium or uninoculated barley. The level of mineralization from the colonized barley was greater than the sum of the mineralizations from the microcosms containing barley plus the bacterial inoculum.
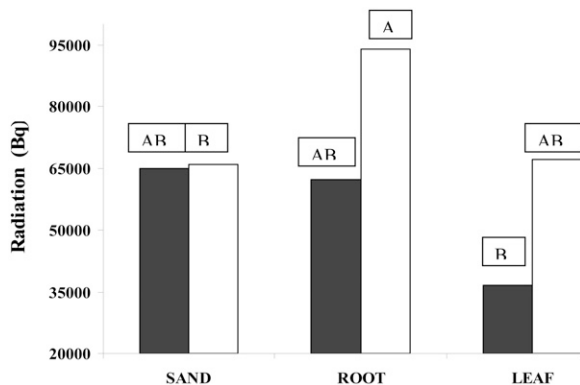
Fig. 3. [14]C levels in sand, roots, and leaf tissues of barley from 10-d-old microcosms under continuous or discontinuous air flow conditions. Data are the mean of three independent experiments ± SD.

Mass Balance

Mass balance analysis (Table 2) showed recovery values of 87 to 101% of total disintegrations per second. Label was measured separately in barley leaves and roots. Differences between microcosms in ^{14}C pyrene concentrations in the sand matrix were nondiscernible.

The successful mineralization observed with the mycobacterium-colonized barley suggests that the process could be used in bioremediation. Seed inoculation followed by colonization of the root throughout its length would effectively disperse the active bacteria into contaminated soil layers. However, field soils have complex rhizosphere communities where there is extensive competition between microbes, and these interactions could influence root colonization by the PAH-degrading mycobacterium cells and PAH degradation rates. We found (Child et al., 2007) that root colonization by the KMS strain was maintained in the presence of another root colonizer, *Pseudomonas putida* KT2440 (Ramos-Gonzalez et al., 2005). Future studies with other plant genera and complex native soils are needed to determine the commercial potential for phytostimulation with rhizospheres containing the PAH-degrading mycobacteria.

We speculate that the presence of low ^{14}C label in the plants reflects the small fraction of labeled pyrene that was soluble in water and, thus, available to the plants (Salt et al.,

Table 2. Mass balance and partitioning of ^{14}C from labeled pyrene from the microcosm studies after 10 d. Values represent percent of total Bq added to the sand in each microcosm at the beginning of the studies. Radiolabeled pyrene remaining in sand and in plant tissues was measured through combustion, trapping, and scintillation counting. Data shown are the mean of three independent experiments ± SD.

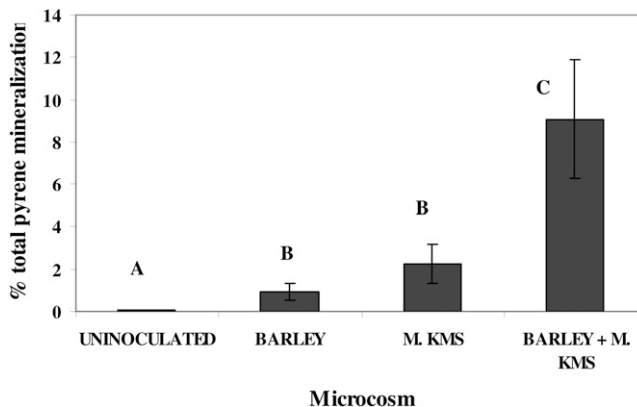| [14]C partitioning	Noninoculated microcosm	Barley microcosm	*Mycobacterium* microcosm	Barley + *Mycobacterium* microcosm
Sand	94.6 ± 5.5	90.3 ± 8.7	85.1 ± 9.3	91.7 ± 2.0
Leaf tissue	–	0.4 ± 0.2	–	1.5 ± 1.0
Root tissue	–	0.4 ± 0.0	–	1.0 ± 0.2
[14]CO_2 traps	0.1 ± 0.2	0.2 ± 0.2	2.2 ± .9	6.8 ± 1.6
% Recovery	94.7 ± 5.5	91.3 ± 8.6	87.3 ± 9.1	101.0 ± 3.3

Fig. 4. Total pyrene mineralization in microcosms containing sand, barley, the bacterial strain KMS, or barley grown with roots colonized by *Mycobacterium* sp. strain KMS. Data combine the [14]CO_2 trap data shown in Fig. 2 plus the ^{14}C estimated to be incorporated into the plant tissues from fixation of evolved [14]CO_2. This estimated [14]CO_2 fixation by photosynthesis was obtained by subtracting ^{14}C levels detected in plant tissues in a continuous-flow environment from ^{14}C levels detected in discontinuous air flow microcosms. Data are the mean of three independent experiments ± SD.

1998). Additionally, label could arise from incorporation of released [14]CO_2 by photosynthesis. This low rate of pyrene mineralization in microcosms with sterilized barley seedlings agreed with findings for other plants (Anokhina et al., 2004; Chen et al., 2003; Gao and Zhu, 2004; Liste and Alexander, 2000; Salt et al., 1998; Xu et al., 2005). Gao and Zhu (2005) observed that, for 12 plant species tested, of the total plant-enhanced loss of pyrene from soils, only 0.23% was due to the uptake and degradation of pyrene by plants.

Conclusions

This study is the first analysis to our knowledge of mineralization of a higher-ring PAH, pyrene, with a defined rhizosphere containing a defined bacterial isolate, the *Mycobacterium* sp. strain KMS, colonizing barley roots. In other studies with defined microbes, isolates of *Pseudomonas* and *Sphingomonas* were demonstrated to affect the degradation of the three-membered ring PAH, phenanthrene, when in association with plant roots (Table 1).

The microcosm system permitted mineralization to be measured successfully. The system allowed for plant growth under gnotobiotic conditions, stringent control of air throughput, and effective capture of volatilized radiolabeled compounds. This system provided a simple, portable, inexpensive, and reusable design that was effective for bench-scale, short-term phytoremediation experiments.

At the end of the 10-d experiment, harvest of the barley plants showed that the roots had traveled throughout the sand matrix The roots likely improved oxygen diffusion to the mycobacterium cells, enhancing the aerobic transformation of the PAH (Grady et al.,1989:Kanaly and Harayama, 2000, Khan et al., 2001, Liang et al., 2006). Plating of the roots of plants grown from mycobacterium-inoculated seedlings demonstrated that strain KMS maintained root tip colonization as the root grows (Child et al., 2007). We propose that the bacteria would have been dispersed throughout the entire sand matrix as they traveled with the roots.

Acknowledgement

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