

PHYTOREMEDIATION OF ALKYLATED POLYCYCLIC AROMATIC HYDROCARBONS IN A CRUDE OIL-CONTAMINATED SOIL

PAUL M. WHITE, JR.¹, DUANE C. WOLF^{2,*}, GREGORY J. THOMA³,
and CHARLES M. REYNOLDS⁴

¹Kansas State University, Department of Agronomy, Manhattan, KS 66506; ²University of Arkansas, Department of Crop, Soil, & Environmental Sciences, Fayetteville, AR 72701; ³University of Arkansas, Department of Chemical Engineering, Fayetteville, AR 72701; ⁴Cold Regions Research and Engineering Laboratory, 72 Lyme Road, Hanover, NH 03755

(*author for correspondence, e-mail: dwolf@uark.edu, Tel.: 479-575-5739, Fax: 479-575-7465)

(Received 27 March 2005; accepted 11 August 2005)

Abstract. Phytoremediation uses plants and their associated microorganisms in conjunction with agronomic techniques to remove or degrade environmental contaminants. The objective of the field study was to evaluate the effect of vegetation establishment plus fertilizer addition on the biodegradation of alkylated polycyclic aromatic hydrocarbons in a crude oil-contaminated soil. Four replications of the following treatments were used: non-vegetated non-fertilized control; fescue (*Lolium arundinaceum* Schreb.) – ryegrass (*Lolium multiflorum* L.) mixture + fertilizer; or bermudagrass (*Cynodon dactylon* (L.) Pers.) – fescue mixture + fertilizer. Vegetation was successfully established at the site that had an initial total petroleum hydrocarbon (TPH) concentration of 9,175 mg/kg. While alkylated two-ring naphthalenes were degraded in all treatments equally, there was greater degradation of the larger three-ring alkylated phenanthrenes-anthracenes and dibenzothiophenes in the vegetated fertilized plots compared to the non-vegetated non-fertilized plots. In this field study, an increase in rhizosphere soil volume associated with increased root length along with nutrient additions resulted in increased total bacterial, fungal, and polycyclic aromatic hydrocarbon (PAH) degrader numbers that most likely resulted in increased biodegradation of the more recalcitrant alkylated polycyclic aromatic hydrocarbon compounds in the crude oil-contaminated soil.

Keywords: biodegradation, natural attenuation, phytotransformation, rhizosphere enhanced remediation, total petroleum hydrocarbons

1. Introduction

Throughout the industrial world, petroleum is the primary source of fuel. As with any large scale industrial process, petroleum production can lead to contamination of soil and groundwater. Major causes of crude oil-contaminated soil include leaking storage tanks and pipelines, land disposal of petroleum waste, and accidental or intentional spills (Bossert and Bartha, 1984). Regulatory agencies require that these sites be cleaned up or the responsible parties can face substantial penalties.

Phytoremediation is the use of plants and their associated soil microorganisms, soil amendments, and agronomic techniques to remove or render harmless

environmental contaminants (Cunningham *et al.*, 1996). Increased biodegradation of organic contaminants occurs in the rhizosphere, the zone of soil directly adjacent to and under the direct influence of plant roots (Frick *et al.*, 1999). Interactions in the rhizosphere can stimulate contaminant degradation by enhancing soil physical, chemical, and biological properties. Phytoremediation is relatively non-invasive and provides a low-cost remedial option well suited to many sites. Rock and Sayre (1998) estimated phytoremediation clean up costs of \$162/m³ petroleum-contaminated soil compared to \$810/m³ for excavation and incineration.

For successful phytoremediation, both plants and microbes must survive and grow in crude oil-contaminated soil. In addition to C, heterotrophic microorganisms require inorganic nutrients to degrade organic contaminants (Walworth *et al.*, 1997). Generally, N is the growth limiting nutrient and, therefore, is needed in the highest concentration (Alexander, 1994). Organic and inorganic N amendments resulted in increased plant biomass production and greater reductions of TPH (White *et al.*, 2003). Appropriate agronomic practices such as tillage and lime additions can also be used to improve soil physical and chemical conditions to enhance plant and microbial growth.

Grass species have been suggested as effective plants for phytoremediating petroleum-contaminated soils (Aprill and Sims, 1990; Schwab and Banks, 1994). Grasses have fibrous root systems, resulting in large root length and surface area per unit volume of surface soil. The fibrous roots would provide a larger surface for colonization by soil microorganisms than a taproot (Anderson *et al.*, 1993) and allow for greater interaction between the rhizosphere microbial community and the contaminant (Schwab and Banks, 1994). Nichols *et al.* (1997) found that bacteria populations in general, and hydrocarbon-degrader populations specifically, were stimulated by the growth of alfalfa (*Medicago sativa L.*) and alpine bluegrass (*Poa alpina L.*) in soil and by the addition of hexadecane, phenanthrene, pyrene, benzoic acid, and *cis*-decahydronaphthalene to the soil. The specific plants chosen for a phytoremediation site will be influenced by factors such as climate and moisture regime. In order to extend the time of phytoremediation, both warm- and cool-season plant species could be employed.

Polycyclic aromatic hydrocarbons (PAH) can be a toxic recalcitrant portion of crude oil. While they represent less than 2% of the bulk composition of typical oil, they constitute a long term concern for many sites (Douglas *et al.*, 1996). Many of these aromatic compounds contain alkyl functional groups covalently bonded to a C in an aromatic ring. For example, C1-phenanthrene designates a phenanthrene molecule with one functional group, such as a methyl or ethyl group (Kinghorn, 1983). Barakat *et al.* (2001) listed groups of PAH compounds such as naphthalenes, phenanthrenes, anthracenes, dibenzothiophenes, fluoranthenes, pyrenes, and chrysenes that were present in oil and each group consisted of a parent compound and a homologous series of alkylated compounds.

The objective of the field study was to evaluate the effect of vegetation establishment plus fertilizer addition on the biodegradation of alkylated polycyclic aromatic hydrocarbons and microbial population dynamics in a crude oil-contaminated soil three years after a spill occurred.

2. Materials and Methods

2.1. PLOT ESTABLISHMENT

A field study was initiated at an oil storage/separation facility near El Dorado, AR, which was vandalized in 1997, resulting in crude oil-contamination of the surrounding soil. To reduce contaminant spatial variability at the study site, three microplots were established in each plot. Random soil samples were collected throughout the study area and thoroughly mixed using a cement mixer. Two kg of the homogenized crude oil-contaminated soil was placed in each of 12 10-cm × 15-cm soil “socks” made from a polypropylene mesh material with 1 × 2-mm openings. The cylindrical microplots were then placed in the soil at a depth of 0 to 15 cm in each of the 4 control plots (4 replications × 3 sample times). An additional 24 socks containing 2-kg of the homogenized crude oil-contaminated soil with appropriate fertilizer and lime additions were placed in the 8 vegetation plus fertilizer treatments (4 replications × 3 sample times × 2 vegetation types). Prior to fertilizer or lime addition, four contaminated soil samples were analyzed for the initial characterization of soil chemical properties and contaminant levels and served as the time 0 samples. Contaminant levels, root growth, and soil chemical and biological properties were analyzed by excavating one randomly selected microplot from each of the 12 plots at 6 (July 2000), 17 (May 2001), and 21 (September 2001) mo after plot establishment.

2.2. VEGETATION ESTABLISHMENT

The study evaluated 3 treatment systems: non-vegetated non-fertilized control; fescue (*Lolium arundinaceum* Schreb. (KY31))-ryegrass (*Lolium multiflorum* L. (Marshall)) mixture + fertilizer; or bermudagrass (*Cynodon dactylon* L. Pers. (Alicia))-fescue mixture + fertilizer. Fescue and ryegrass, cool-season grasses, were established from seed and bermudagrass, a warm-season grass, was sprigged. The experimental design was established to be in concert with the U.S.E.P.A. “Remediation Technology Development Forum (RTDF) Phytoremediation Action Team (www.rtdf.org).

Root growth was analyzed by removing a 5-cm diameter × 0 to 15-cm deep core from the microplots 6, 17, and 21 mo after plot establishment. The roots were removed from the soil core, washed, and stained with 0.1 g methylene blue/L 10% ethanol (95%) (v/v). Stained roots were digitized using an Epson model LA1600

scanner and analyzed for root length and surface area using Regent WinRhizo[®] digital imaging software.

2.3. SOIL ANALYSES

The soil at the study site was a Sacul fine sandy loam (clayey, mixed, thermic, Aquic Hapludult). Prior to fertilizer or lime addition, plant available nutrients in the soil from the four composite samples were extracted with Mehlich 3 solution and levels determined by inductively coupled plasma spectroscopy, total C and N with a Leco CN 2000[®], and pH and EC by electrode (1:1 and 1:2 soil:water extract, respectively) (Donahue, 1992). Initial soil nutrient levels were not adequate for optimum plant growth with Mehlich 3 extractable P, K, Ca, and Mg levels of 5, 44, 351, and 44 mg/kg, respectively. The pH and %N were 5.5 and 0.05%, respectively. Inorganic fertilizer (13-13-13) and dolomitic lime at rates of 1,600 and 1,450 kg/ha, respectively, were applied to the vegetated fertilized treatments at the initiation of the experiment. Vegetated fertilized plots received additional applications of 320 kg 33-0-0/ha after each sampling at 6, 17, and 21 mo. All fertilizer and lime was purchased at a local farm supply center.

For soil biological analyses, 0 to 15 cm soil samples from the microplots were aseptically collected, placed in sterile containers, and transported on ice to the laboratory. Ten-fold serial dilutions were prepared and total bacterial and fungal numbers were determined using 0.1X tryptic soy agar (TSA) and Martin's medium, respectively (Zuberer, 1994). The most probable number (MPN) of PAH degrading microbes was enumerated using a 96-well microtiter plate procedure in which a mineral salts liquid medium was amended with a mixture of phenanthrene, dibenzothiophene, anthracene, and fluorene as the C-source (Haines *et al.*, 1996; Wrenn and Venosa, 1996).

2.4. TPH AND PAH ANALYSES

Soil samples collected at the initiation of the study and from microplots at 6, 17, and 21 mo after plot establishment were extracted with hexane:methylene chloride (1:1 v/v) by accelerated soxhlet following modified EPA Method 3541. Total Petroleum Hydrocarbon (TPH) levels in the extract were analyzed by gas chromatography with flame ionization detection (GC/FID) following modified EPA Method 8015 using a Hewlett Packard (HP) 5890 GC with a DB-5 30 m × 0.32 mm ID × 0.25 μm film column. One μL of sample was injected and the injector and detector temperatures were 300 and 325 °C, respectively. The temperature program was as follows: Initial temperature 35 °C (hold 5 min), rate 4 °C/min to 320 °C (hold 15 min), total run time 91 min. The PAH concentrations and the biomarker hopane (C₃₀ 17α(H),21β(H)-hopane) present in the extract were measured using gas chromatography with mass spectroscopy by EPA Method 8270 using an HP 6890 GC connected to a 5973 Mass Selective Detector in selected ion monitoring mode with

a DB-5MS 30 m \times 0.25 mm ID \times 0.25 μ m film column. Two μ L of sample was injected and the injector and detector temperatures were 300 and 290 $^{\circ}$ C, respectively. The temperature program was as follows: Initial temperature 40 $^{\circ}$ C (hold 1 min), rate 6 $^{\circ}$ C/min to 300 $^{\circ}$ C (hold 16 min), total run time 60 min (U.S. EPA, 1998).

2.5. STATISTICS

The study was analyzed as a split plot in which the whole plot structure was a randomized complete block design with four replications and treatment as the factor. The split plot factor was time. Data were subjected to ANOVA and means separated by the LSD by SAS, version 8 (SAS Institute, Cary, N.C.).

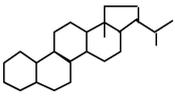
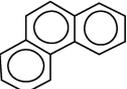
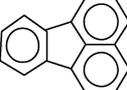
3. Results and Discussion

3.1. POLYCYCLIC AROMATIC HYDROCARBON LEVELS

Biodegradation of PAHs should (i) decrease with increasing molecule size and (ii) decrease within a homologous series with increasing number of alkylations (Kennicutt, 1988). Five PAH groups prevalent in the crude oil-contaminated soil were evaluated: naphthalenes, phenanthrenes-anthracenes, dibenzothiophenes, fluoranthenes-pyrenes, and chrysenes (Table I). The phenanthrenes and anthracenes were combined as were fluoranthenes and pyrenes due to similarity of the chemical structures. The initial PAH levels in the oil-contaminated soil reflect the 3-yr weathering period that occurred from the time of the spill until the field study was initiated (Table I). Across the three treatments following the 21-month field study, the mean concentrations of naphthalene, phenanthrene, anthracene, dibenzothiophene, fluoranthene, pyrene, and chrysenes were 3, 1, 0, 0, 2, 2, 68 μ g/kg, respectively. To reduce sample heterogeneity in the crude oil-contaminated soil, samples were extensively mixed prior to placing the soil in the microplots. We also used a biomarker, C₃₀ 17 α (H),21 β (H)-hopane, as an internal standard to normalize PAH values (Frontera-Suau *et al.*, 2002; Prince *et al.*, 1994; Teal *et al.*, 1992; Venosa *et al.*, 1997). The PAH data were normalized against the corresponding hopane value obtained for that specific sample, to obtain a PAH to hopane ratio. There was not a significant change ($P = 0.05$) in hopane concentrations for the three treatments during the study.

Naphthalene, a two-ring structure, should be more easily degraded by soil microorganisms than larger PAHs and was 7 μ g/kg in the weathered crude oil-contaminated soil at initiation of the study (Table I). Naphthalene and alkylated-naphthalene levels in the vegetated fertilized treatments were not significantly different ($P = 0.05$) from the values in the control plots at any sample time. Such findings might be expected for relatively volatile and labile compounds. The

TABLE I
Initial soil TPH and PAH levels observed at the phytoremediation field site.

| Parameter | Units | Mean±Std Dev | | Chemical Structure |
|---|------------|--------------|------------------|---|
| TPH (GC/FID) | mg/kg | 9,175 ± 866 | | |
| N/A | | | | |
| C ₃₀ 17 α (H),21 β (H)-hopane | μ g/kg | 1,700 ± 216 | |  |
| Naphthalene | μ g/kg | 7 ± 2 | | |
| C1-Naphthalenes* | μ g/kg | 12 ± 9 | | |
| C2-Naphthalenes | μ g/kg | 138 ± 22 | naphthalene |  |
| C3-Naphthalenes | μ g/kg | 735 ± 90 | | |
| C4-Naphthalenes | μ g/kg | 2,350 ± 480 | | |
| Phenanthrene | μ g/kg | 0 ± na | phenanthrene |  |
| Anthracene | μ g/kg | 0 ± na | | |
| C1-Phenanthrenes-Anthracenes | μ g/kg | 220 ± 34 | | |
| C2-Phenanthrenes-Anthracenes | μ g/kg | 1,225 ± 126 | | |
| C3-Phenanthrenes-Anthracenes | μ g/kg | 2,900 ± 216 | anthracene |  |
| C4-Phenanthrenes-Anthracenes | μ g/kg | 3,000 ± 294 | | |
| Dibenzothiophene | μ g/kg | 7 ± 14 | dibenzothiophene |  |
| C1-Dibenzothiophenes | μ g/kg | 528 ± 127 | | |
| C2-Dibenzothiophenes | μ g/kg | 3275 ± 499 | | |
| C3-Dibenzothiophenes | μ g/kg | 7450 ± 1159 | | |
| Fluoranthene | μ g/kg | 0 ± na | fluoranthene |  |
| Pyrene | μ g/kg | 9 ± 17 | | |
| C1-Fluoranthenes-Pyrenes | μ g/kg | 190 ± 27 | | |
| C2-Fluoranthenes-Pyrenes | μ g/kg | 673 ± 73 | | |
| C3-Fluoranthenes-Pyrenes | μ g/kg | 943 ± 144 | pyrene |  |
| Chrysene | μ g/kg | 35 ± 70 | | |
| C1-Chrysenes | μ g/kg | 260 ± 177 | | |
| C2-Chrysenes | μ g/kg | 665 ± 446 | chrysene |  |
| C3-Chrysenes | μ g/kg | 925 ± 624 | | |
| C4-Chrysenes | μ g/kg | 925 ± 618 | | |

*C number indicates alkylation of parent compound.

hopane-normalized concentrations of C1-, C2-, C3-, and C4-naphthalenes, significantly decreased during the first 6 mo of the field study (Table II). By 17 mo, the levels of all alkylated-naphthalene compounds were less than or equal to the detection limit of 6 μ g/kg (data not shown).

The treatment main effect was significant for the more resistant phenanthrenes-anthracenes, as the vegetated fertilized plots had lower C2-, C3-, and C4-phenanthrenes-anthracenes than the non-vegetated non-fertilized control plots

TABLE II

Hopane normalized C_x-naphthalene, -phenanthrene-anthracene, -dibenzothiophene, -fluoranthene-pyrene, and -chrysene concentrations at four sample times in the crude oil-contaminated soil. There was a significant ($P = 0.05$) main effect for time, so the values are presented as means for the three treatments.

| Alkylated PAH | Sampling Time (mo) | | | | LSD |
|---|--------------------|----------|----------|---------|-------|
| | 0 | 6 | 17 | 21 | |
| ----- $\mu\text{g compound}/\mu\text{g hopane}$ ----- | | | | | |
| Naphthalene | | | | | |
| C1-Naphthalenes* | 0.007 a** | 0.000 b | 0.002 b | 0.002 b | 0.002 |
| C2-Naphthalenes | 0.081 a | 0.014 b | 0.000 b | 0.000 b | 0.016 |
| C3-Naphthalenes | 0.434 a | 0.092 b | 0.001 c | 0.000 c | 0.071 |
| C4-Naphthalenes | 1.374 a | 0.357 b | 0.000 c | 0.000 c | 0.212 |
| Phenanthrene/Anthracene | | | | | |
| C1-Phenanthrenes-Anthracenes | 0.129 a | 0.020 b | 0.000 b | 0.000 b | 0.023 |
| C2-Phenanthrenes-Anthracenes | 0.727 a | 0.226 b | 0.048 c | 0.006 c | 0.113 |
| C3-Phenanthrenes-Anthracenes | 1.724 a | 0.761 b | 0.336 c | 0.135 d | 0.186 |
| C4-Phenanthrenes-Anthracenes | 1.780 a | 0.829 b | 0.592 c | 0.230 d | 0.133 |
| Dibenzothiophene | | | | | |
| C1-Dibenzothiophenes | 0.308 a | 0.076 b | 0.000 c | 0.002 c | 0.050 |
| C2-Dibenzothiophenes | 1.930 a | 0.672 b | 0.133 c | 0.027 c | 0.309 |
| C3-Dibenzothiophenes | 4.389 a | 1.981 b | 0.709 c | 0.298 c | 0.609 |
| Fluoranthene/Pyrene | | | | | |
| C1-Fluoranthenes-Pyrenes | 0.113 a | 0.080 b | 0.073 b | 0.004 c | 0.031 |
| C2-Fluoranthenes-Pyrenes | 0.401 a | 0.254 b | 0.229 b | 0.210 b | 0.045 |
| C3-Fluoranthenes-Pyrenes | 0.566 a | 0.374 b | 0.333 b | 0.316 b | 0.074 |
| Chrysene | | | | | |
| C1-Chrysenes | 0.154 a | 0.141 a | 0.100 b | 0.076 b | 0.041 |
| C2-Chrysenes | 0.396 ab | 0.443 a | 0.305 bc | 0.238 c | 0.097 |
| C3-Chrysenes | 0.548 a | 0.432 ab | 0.451 ab | 0.390 b | 0.136 |
| C4-Chrysenes | 0.552 a | 0.418 ab | 0.376 b | 0.367 b | 0.138 |

*C number indicates alkylation of parent compound.

**For a given alkylated PAH, means in a row followed by the same letter are not significantly different ($P = 0.05$)

(Figure 1). The more labile C1-phenanthrenes-anthracenes levels were not different and the findings were similar to the data for the two-ringed naphthalene compounds. The two vegetated treatments were not different from each other. Miya and Firestone (2001) also found increased phenanthrene biodegradation rates in soil planted to slender oat (*Avena barbata* Pott ex Link) as compared to a non-vegetated control. In soil amended with a mixture of eight PAHs, ranging from 3 to

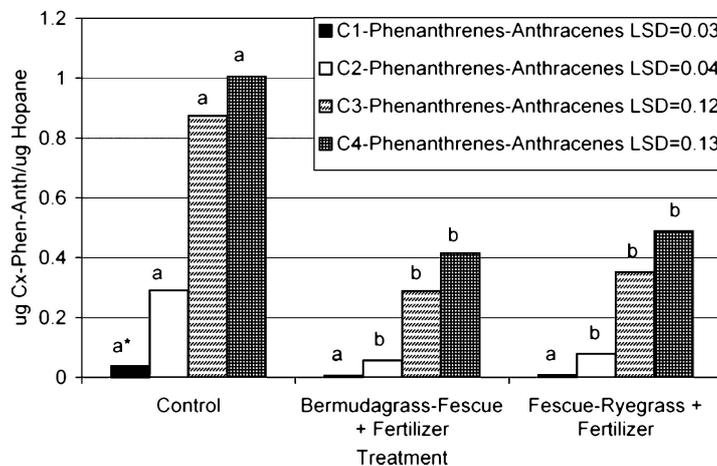


Figure 1. Hopane normalized C1-, C2-, C3-, and C4-phenanthrene-anthracene levels in the vegetated fertilized and non-vegetated non-fertilized plots of the phytoremediation field study. There was a significant ($P = 0.05$) treatment main effect, so the values presented are means for the 0, 6, 17, and 21 month samplings. *Bars for a given alkylation with the same letter are not significantly different at the $P = 0.05$ level.

5 rings, the degradation of all PAHs was greater in the ryegrass rhizospheric than non-rhizospheric soil (Binet *et al.*, 2000). Dissipation of 3- and 4-ring PAHs was shown to be increased in soil amended with artificial root exudates and NH_4NO_3 compared to the unamended control (Joner *et al.*, 2002).

The time main effect was also significant for the three-ringed alkylated phenanthrenes-anthracenes (Table II). The C1-phenanthrenes-anthracenes decreased to $\leq 0.02 \mu\text{g PAH}/\mu\text{g hopane}$ by 6 mo, and the C2-phenanthrenes-anthracenes decreased to $\leq 0.05 \mu\text{g PAH}/\mu\text{g hopane}$ by 17 mo. The C3- and C4-phenanthrenes-anthracenes levels decreased at each sample time, but were still present at low levels at 21 mo. This progression supports Kennicutt's (1988) hypothesis that biodegradation rates should decrease with increasing alkylation of the parent compound.

The treatment main effect was significant for the C2- and C3-dibenzothiophenes, where the vegetated fertilized plots had lower levels than the non-vegetated non-fertilized plots (Figure 2). Time significantly affected the C1-, C2-, and C3-dibenzothiophenes levels, which decreased from 0 to 6 mo, and from 6 to 17 mo (Table II). While there was not a significant treatment effect for the alkylated two-ringed naphthalenes, C1-phenanthrene-anthracenes, or C1-dibenzothiophenes, there was an impact of phytoremediation management on the more complex phenanthrenes-anthracenes and dibenzothiophenes.

A significant treatment effect ($P = 0.05$) was observed for the four-ringed C1-fluoranthenes-pyrenes, with values of 0.044, 0.055, and 0.071 $\mu\text{g C1-fluoranthene-pyrene}/\mu\text{g hopane}$ for bermudagrass, fescue, and control treatments, respectively.

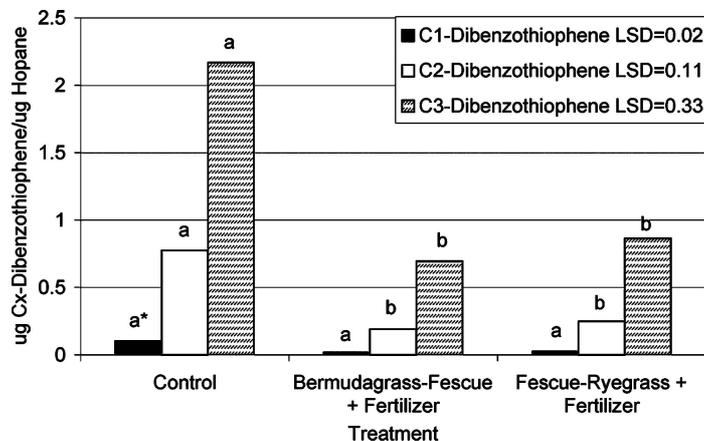


Figure 2. Hopane normalized C1-, C2-, and C3-dibenzothiophene levels in the vegetated fertilized and non-vegetated non-fertilized plots of the phytoremediation field study. There was a significant ($P = 0.05$) treatment main effect, so the values presented are means for the 0, 6, 17, and 21 month samplings. *Bars for a given alkylation with the same letter are not significantly different at the $P = 0.05$ level.

However, there was no treatment effect on C2- or C3-fluoranthene-pyrene levels. There was also a significant ($P = 0.05$) effect of time on the C1-, C2-, and C3-fluoranthenes-pyrenes with lower levels by 6 mo, which were further decreased at 21 mo for the C1-fluoranthenes-pyrenes, but not for the C2- or C3-fluoranthenes-pyrenes (Table II).

For the larger four-ringed alkylated chrysenes, the treatment main effect was not significant. The time main effect was significant for each of the four alkylated compounds, but none of the 0 mo were different from 6 mo nor were the 17 mo different from the 21 mo concentrations (Table II). Sufficient substrates in the phenanthrenes-anthracenes group may have delayed the degradation of larger, more complex molecules, as microbes maximize their C metabolism efficiency by preferentially using less resistant compounds (Carmichael and Pfaender, 1997). Reduction of biodegradable 2- and 3-ring PAH compounds could also reduce the potential for co-metabolism of the recalcitrant 4-ring PAHs (Huesemann *et al.*, 2002). The two- and three-ring compounds were degraded to very low levels suggesting bioavailability was not a limiting factor for biodegradation. Toxicity of the PAHs or the alkylated PAHs was not evaluated in the present study. Bioassays have been shown to be sensitive indicators to evaluate the quality of petroleum-contaminated soil (Plaza *et al.*, 2005).

3.2. SOIL MICROBIAL NUMBERS

Root exudates consisting of sugars, vitamins, amino acids, organic acids, and nucleotides (Rovina and McDougall, 1967), coupled with convective flow of nutrient

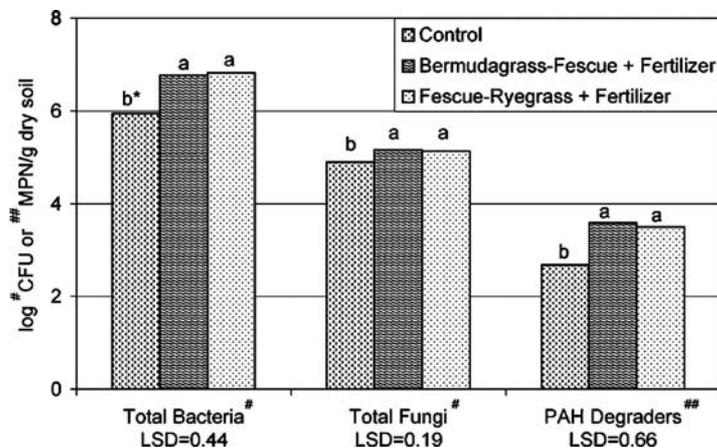


Figure 3. Bacteria, fungi and PAH degrader numbers in the vegetated fertilized and non-vegetated non-fertilized plots of the phytoremediation field study. There was a significant treatment main effect, so the values presented are means for the 6, 17, and 21 month samplings. *Bars for a given organism with the same letter are not significantly different at the $P = 0.05$ level for bacteria and fungi and $P = 0.10$ for PAH degraders.

solutes into the rhizosphere can lead to increased microbial numbers and activity (Reynolds *et al.*, 1999). Total bacterial numbers were significantly lower in the non-vegetated non-fertilized plots than in the bermudagrass-fescue + fertilizer and fescue-ryegrass + fertilizer treatments (Figure 3). The two vegetated fertilized treatments were not different. Similar results were obtained by Hutchinson *et al.* (2001) who found that tall fescue and bermudagrass growth and inorganic fertilizer additions increased TSA culturable bacteria isolated from a petroleum sludge-contaminated soil. Lower total fungal numbers were also found in the non-vegetated non-fertilized treatment compared to the bermudagrass-fescue + fertilizer and fescue-ryegrass + fertilizer plots.

More complex aromatic compounds may be metabolized by more specialized microbes. Increasing the number of PAH degraders is important to achieve more complete levels of crude oil decomposition. The PAH degrader numbers followed the same pattern as the bacteria and fungi, but were significant at $P = 0.10$ (Figure 3). The PAH degraders comprised approximately 50% of the total bacterial numbers.

Plants can release phenolic compounds (Liste and Alexander, 1999) that can act as PAH analogs and could enhance the rhizosphere effect by increasing the PAH degraders in the vegetated fertilized plots. While Townsend *et al.* (2000) found PAH degrader levels increased 3 to 4 orders of magnitude after the addition of oil, they did not observe a difference between fertilized or control plots. Also, Gentry *et al.* (2003) found that the number of pyrene degraders was significantly greater in soil amended with pyrene compared to unamended soil. In soil amended with

500 mg pyrene/kg, Krutz *et al.* (2005) reported pyrene degrader numbers of 8.01 \log_{10} MPN/g in bermudagrass rhizosphere compared to <3.09 in non-contaminated bulk soil. The number of PAH degraders was shown to be significantly higher in the ryegrass rhizosphere of soil spiked with eight PAHs and aged for 6 mo (Binet *et al.*, 2000). Oil addition increased PAH degrader numbers in a freshwater wetland compared to an unamended control, but nutrient addition did not increase numbers (Haines *et al.*, 2002).

3.3. ROOT GROWTH

While initial vegetation establishment at the field site was successful, early plant growth was reduced due to drought conditions. There was a significant increase in root length over time with values of 28, 125, and 124 km/m³ soil at 6, 17, and 21 mo, respectively. The values at 17 and 21 mo were not different and there was no difference between the two vegetated fertilized treatments (data not shown).

The increases in root growth would increase the volume of rhizosphere soil, which could positively impact microbial numbers and activity, resulting in enhanced contaminant biodegradation rates. As expected, root length and surface area were highly correlated ($R = 0.98$). Additionally, root length was negatively correlated to the concentration of C2-, C3-, and C4-phenanthrene/anthracene compounds and C2- and C3-dibenzothiophenes ($R = -0.60$ to -0.71). These results suggest that increasing root length resulted in increased contaminant degradation.

A conceptual model presented by Thoma *et al.* (2003a, b) showed that growing plant roots and their associated microbial community increased biodegradation of crude oil contaminants. Banks *et al.* (2003a) also concluded that stimulation of microorganisms in the rhizosphere increased microbial numbers and activity resulting in enhanced TPH degradation compared to unvegetated controls. There were also differences noted in the metabolic diversity of microbial communities in vegetated and unvegetated petroleum-contaminated soil (Banks *et al.*, 2003b).

4. Conclusions

Phytoremediation of crude oil-contaminated soils can be a viable option where plants can grow and the contaminants are present in the plant root zone. Results from the field study show that three years after an oil spill, phytoremediation management of contaminated sites through vegetation establishment plus fertilizer addition led to a reduction of crude oil contaminants. In addition, it was shown that vegetation plus fertilizer were most important in reduction of the more recalcitrant fractions. While there was not a significant treatment effect for the alkylated two-ringed naphthalenes, C1-phenanthrene-anthracenes, or C1-dibenzothiophenes, there was enhanced degradation of the more complex alkylated phenanthrenes-anthracenes and dibenzothiophenes attributable to phytoremediation.

The degradation pattern was 2-ring > 3-ring > 4-ring and decreased with increased alkylation of larger ringed structures. This progression of degradation was increased with addition of plants and fertilizer. The increased root growth and microbial levels, including PAH degrader numbers, appeared to be important factors influencing the degradation rates.

Acknowledgments

The authors thank K.J. Davis, W.D. Kirkpatrick, and L.J. Krutz for their assistance. This research is supported in part by the Integrated Petroleum Environmental Consortium (IPEC); U.S. Army Research Office (ARO) contract/grant number DACA89-97-K-005/DAAG55-98-4-0379; and the Army Environmental Quality Technology Program, work unit EC-B06 BT25 "Biodegradation Processes of Explosives/Organics Using Cold Adapted Soil Systems."

References

- Alexander, M.: 1994, *Biodegradation and bioremediation*. Academic Press, Inc., San Diego, CA.
- Anderson, T. A., Guthrie, E. A. and Walton, B. T.: 1993, 'Bioremediation in the rhizosphere: Plant roots and associated microbes clean contaminated soil', *Environ. Sci. Technol.* **27**, 2630–2636.
- Aprill, W. and Sims, R. C.: 1990, 'Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil', *Chemosphere* **20**, 253–265.
- Banks, M. K., Kulakow, P., Schwab, A. P., Chen, Z. and Rathbone, K.: 2003a, 'Degradation of crude oil in the rhizosphere of *Sorghum bicolor*', *Int. J. Phytorem.* **5**, 225–234.
- Banks, M. K., Mallede, H., and Rathbone, K.: 2003b, 'Rhizosphere microbial characterization in petroleum-contaminated soil', *Soil Sed. Contam.* **12**, 371–385.
- Barakat, A. O., Qian, Y., Kim, M. and Kennicutt, M. C.: 2001, 'Chemical characterization of naturally weathered oil residues in arid terrestrial environment in Al-Alamein, Egypt', *Environ. Int.* **27**, 291–310.
- Binet, P., Portal, J. M. and Leyval, C.: 2000, 'Dissipation of 3-6 ring polycyclic aromatic hydrocarbons in the rhizosphere of ryegrass', *Soil Biol. Biochem.* **32**, 2011–2017.
- Bossert, I. and Bartha, R.: 1984, 'The fate of petroleum in soil ecosystems', in: R. Atlas (ed.) *Petroleum Microbiology*, Macmillan Publ. Co., New York. pp. 435–473.
- Carmichael, L. M. and Pfaender, F. K.: 1997, 'The effect of inorganic and organic supplements on the microbial degradation of phenanthrene and pyrene in soils', *Biodegradation* **8**, 1–13.
- Cunningham, S. D., Anderson, T. A., Schwab, A. P. and Hsu, F. C.: 1996, 'Phytoremediation of soils contaminated with organic pollutants', *Adv. Agron.* **56**, 55–114.
- Donahue, S. J. (ed.): 1992, *Reference soil and media diagnostic procedures for the southern region on the United States*. Southern Coop. Ser. Bull. 374. Virginia Agric. Exp. Stn., Blacksburg, VA.
- Douglas, G. S., Bence, A. E., Prince, R. C., McMillen, S. J. and Butler, E. L.: 1996, 'Environmental stability of selected petroleum hydrocarbon source and weathering ratios', *Environ. Sci. Technol.* **30**, 2332–2339.
- Frick, C. M., Farrell, R. E. and Germida, J. J.: 1999, *Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites*, Pet. Tech. All. Can., Calgary, AB.

- Frontera-Suau, R., Bost, F. D., McDonald, T. J. and Morris, P. J.: 2002, 'Aerobic biodegradation of hopanes and other biomarkers by crude oil-degrading enrichment cultures', *Environ. Sci. Technol.*, **36**, 4585–4592.
- Gentry, T. J., Wolf, D. C., Reynolds, C. M. and Fuhrmann, J. J.: 2003, 'Pyrene and phenanthrene influence on soil microbial populations', *Bioremed. J.* **7**, 53–68.
- Haines, J. R., Herrmann, R., Lee, K., Cobanli, S. and Blaise, C.: 2002, 'Microbial population analysis as a measure of ecosystem restoration', *Bioremed. J.* **6**, 283–296.
- Haines, J. R., Wrenn, B. A., Holder, E. L., Strohmeier, K. L., Herrington, R. T. and Venosa, A.D.: 1996, 'Measurement of hydrocarbon-degrading microbial populations by a 96-well most-probable-number procedure', *J. Ind. Microbiol.* **16**, 36–41.
- Huesemann, M. H., Hausmann, T. S., and Fortman, T. J.: 2002, 'Microbial factors rather than bioavailability limit the rate and extent of PAH biodegradation in aged crude oil contaminated model soils', *Bioremed. J.* **6**, 321–336.
- Hutchinson, S. L., Banks, M. K. and Schwab, A. P.: 2001, 'Phytoremediation of aged petroleum sludge: Effect of inorganic fertilizer', *J. Environ. Qual.* **30**, 395–403.
- Joner, E. J., Corgie, S. C., Amellal, N. and Leyval, C.: 2002, 'Nutritional constraints to degradation of polycyclic aromatic hydrocarbons in a simulated rhizosphere', *Soil Biol. Biochem.* **34**, 859–864.
- Kennicutt, M. C.: 1988, 'The effect of biodegradation on crude oil bulk and molecular composition', *Oil Chem. Pollut.* **4**, 89–112.
- Kinghorn, F.: 1983, *An introduction to the physics and chemistry of petroleum*, John Wiley & Sons, Ltd., New York.
- Krutz, L. J., Beyroudy, C. A., Gentry, T. J., Wolf, D. C., and Reynolds, C. M.: 2005. 'Selective enrichment of a pyrene degrader population and enhanced pyrene degradation in bermudagrass rhizosphere', *Biol. Fert. Soils* **41**, 359–364.
- Liste, H. H. and Alexander, M.: 1999, 'Rapid screening of plants promoting phenanthrene degradation', *J. Environ. Qual.* **28**, 1376–1377.
- Miya, R. K. and Firestone, M. K.: 2001, 'Enhanced phenanthrene biodegradation in soil by slender oat root exudates and root debris', *J. Environ. Qual.* **30**, 1911–1918.
- Nichols, T. D., Wolf, D. C., Rogers, H. B., Beyroudy, C. A. and Reynolds, C. M.: 1997, 'Rhizosphere microbial populations in contaminated soils', *Water Air Soil Pollut.* **95**, 165–178.
- Plaza, G., Nalecz-Jawecki, G., Ulfig, K., and Brigmon, R. L.: 2005, 'The application of bioassays as indicators or petroleum-contaminated soil remediation', *Chemosphere* **59**, 289–296.
- Prince, R. C., Elmendorf, D. L., Lute, J. R., Hsu, C. S., Haith, C. E., Senius, J. D., Dechert, G. J., Douglas, G. S. and Butler, E. L.: 1994, '17i(H),21k(H)-Hopane as a conserved internal marker for estimating the biodegradation of crude oil', *Environ. Sci. Technol.* **28**, 142–145.
- Reynolds, C. M., Wolf, D. C., Gentry, T. J., Perry, L. B., Pidgeon, C. S., Koenen, B. A., Rogers, H. B. and Beyroudy, C. A.: 1999, 'Plant enhancement of indigenous soil micro-organisms: A low-cost treatment of contaminated soils', *Polar Rec.* **35**, 33–40.
- Rock, S. A. and Sayre, P. G.: 1998, 'Phytoremediation of hazardous wastes: Potential regulatory acceptability', *Remediation* **8**, 5–17.
- Rovina, A. D. and McDougall, B. M.: 1967, 'Microbiological and biochemical aspects of the rhizosphere', in: A. D. McClaren and G. H. Peterson (eds.), *Soil Biochemistry*, Marcel Dekker, Inc., New York. pp. 417–463.
- Schwab, A. P. and Banks, M. K.: 1994, 'Biologically mediated dissipation of polyaromatic hydrocarbons in the root zone', in: T. A. Anderson and J. R. Coats (eds.) *Bioremediation through rhizosphere technology*, ACS Symp. Ser. 563. Am. Chem. Soc., Washington, D.C. pp. 132–141.
- Teal, J. M., Farrington, J. W., Burns, K. A., Stegeman, J. J., Tripp, B. W., Woodin, B. and Phinney C.: 1992, 'The West Falmouth oil spill after 20 years: Fate of fuel oil compounds and effects on animals', *Mar. Pollut. Bull.* **24**, 607–614.

- Thoma, G. J., Lam, T. B. and Wolf, D. C.: 2003a, 'Mathematical modeling of phytoremediation of oil-contaminated soil: Model development', *Int. J. Phytorem.* **5**, 41–55.
- Thoma, G. J., Lam, T. B. and Wolf, D. C.: 2003b, 'Mathematical modeling of phytoremediation of oil-contaminated soil: Sensitivity analysis', *Int. J. Phytorem.* **5**, 125–136.
- Townsend, R. T., Bonner, J. S. and Autenrieth, R. L.: 2000, 'Microbial dynamics during bioremediation of a crude oil-contaminated coastal wetland', *Bioremed. J.* **4**, 203–218.
- U. S. EPA: 1998, 'SW-846 online test methods for evaluating solid wastes physical/chemical methods', [Online]. Available at: <http://www.epa.gov/epaoswer/hazwaste/test/main.htm> (verified 5 January 2005).
- Venosa, A. D., Suidan, M. T., King, D. and Wrenn, B. A.: 1997, 'Use of hopane as a conservative biomarker for monitoring the bioremediation effectiveness of crude oil contaminating a sandy beach', *J. Ind. Microbiol. Biotechnol.* **18**, 131–139.
- Walworth, J. L., Woolard, C. R., Braddock, J. F. and Reynolds, C. M.: 1997, 'Enhancement and inhibition of soil petroleum biodegradation through the use of fertilizer nitrogen: An approach to determining optimum levels', *J. Soil Cont.* **6**, 465–480.
- White, Jr., P. M., Wolf, D. C., Thoma, G. J. and Reynolds, C. M.: 2003, 'Influence of organic and inorganic soil amendments on plant growth in crude oil-contaminated soil', *Int. J. Phytoremed.* **5**, 381–397.
- Wrenn, B. A. and Venosa, A. D.: 1996, 'Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure', *Can. J. Microbiol.* **42**, 252–258.
- Zuberer, D. A.: 1994, 'Recovery and enumeration of viable bacteria', in: R. W. Weaver (ed.) *Methods of soil analysis. Part 2. Soil Sci. Soc. Am.* **5**, 119–144.