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PAH BIODEGRADATION IN SOIL-WATER SUSPENSIONS CONTAMINATED WITH WASTE OIL

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Abstract : Polycyclic aromatic hydrocarbon (PAH) biodegradation patterns were measured for soils that were freshly spiked and field aged with waste oil. The maximum attainable bioaugmentation efficiencies for the two freshly spiked soils were different. This was attributed to the dissimilarity in soil type and total expandable clay mineral (ECM) content. The soil rich in ECM (4.0%) achieved a lower PAH biodegradation (37%) compared to 57% PAH reduction in the soil containing 2.2% ECM. Bioaugmentation decreased the PAH content from 3,217 to 2,260 mg PAH/kg soil for more than 30 years field aged soil system, whereas the level was reduced from 6,158 to 3,928 mg PAH/kg soil for the freshly spiked soil system. For the field aged soil, biostimulation efficiency was comparable to that obtained for bioaugmentation.

Key Words : Bioaugmentation, Biostimulation, Freshly spiked, Field aged, Polycyclic aromatic hydrocarbons

INTRODUCTION

Wherever petroleum is drilled, stored, handled, refined, transported or transferred, there is the potential for spills. As a result, petroleum and its residues are some of the most common natural and anthropogenic contaminants in terrestrial, as well as aquatic, environments.[1] The primary constituents of petroleum are hydrocarbons ranging in size from one carbon atom to large compounds containing >200 carbons. The remaining compounds contain substituted elements, particularly nitrogen, sulfur, and oxygen.[2,3] Polycyclic aromatic hydrocarbons (PAHs), straight-chain alkanes, branched alkanes, cyclic alkanes, mononuclear aromatics, and resins asphalthenes are the primary categories associated with petroleum products. PAHs pose the greatest environmental concern over the other categories since they (1) are highly carcinogenic and toxic; (2) have a high occurrence of contamination; and (3) are recalcitrant to biodegradation.[4~6]

Despite many laboratory studies to bioremediate PAHs in a simulated field environment, real *in situ* bioremediation field applications have not been as successful. This is attributed to inherent characteristics of lab-scale studies. For instance, most lab studies were conducted (1) using spiked PAHs as the sole contaminant; (2) inoculating only with commercially available bacteria; and/or (3) without accounting for compound aging and soil properties.[5,7~11] Most contaminated sites contain more than just PAHs,

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therefore implementing only pure, commercially available strains will not facilitate the biodegradation of all contaminants. A change in soil properties (i.e., increase in soil organic matter or clay minerals) can increase contaminant sorption, thereby decreasing bioavailability. The extent of sorption-desorption is often exacerbated by contaminant weathering.

Therefore, this study was carried out to garner a better understanding of PAH bioremediation effectiveness in regards to different sorbents (i.e., dissimilar soils) as well as contaminant aging. To meet this goal, three interconnected objectives were evaluated: (1) evaluation of the impact of expandable clay minerals (ECM, smectite and vermiculite) and soil organic matter (SOM) on bioremediation efficiencies; (2) investigation of compound aging on the achievable extent of degradation by comparing a soil that was freshly spiked and field aged; and (3) determination of the effectiveness of biostimulation versus bioaugmentation for a field aged soil. Herein, biostimulation refers to the addition of nutrients and terminal electron acceptor for stimulating indigenous microbes. Bioaugmentation is defined as the addition of foreign/enhanced bacteria, nutrients, and a terminal electron acceptor.

MATERIALS AND METHODS

Approach

A step-wise PAH biodegradation experiment was conducted. The first experimental set was used to evaluate the intertwined nature of soil properties. Free phase waste oil that originated from the same process facility as the field aged soil was spiked to two clean soils with different ECM and SOM contents. Herein waste oil refers to 'spent' cutting oil previously used in petroleum drilling operations. EcoPetrol facility reports verified that the primary constituents in the spent cutting oil have been the same since 1970. Therefore, the spiked soil had the same primary contaminant categories/fractions (PAHs, aliphatics, asphaltenes, etc) of waste oil as those in the field-aged soil. The field-aged soil was also used to isolate the bacteria enrichment culture for bioaugmentation experiments. The second experimental set evaluated the aging effect by comparing bioaugmentation results for the freshly spiked and field aged soils. The last set of experiments evaluated biostimulation versus bioaugmentation for the field aged soil.

Soil Origins, Characterization and Contaminant Spiking

Clean soil samples were obtained from two distinctly different areas. The Ohio (OH) soil was collected at a depth of 0.9 to 1.5 m from an area representative of the predominant soil type in northeastern Ohio, USA. The Colombia (CO) soil originated from a non-contaminated area in Santander, Colombia, South America. Neither PAHs nor petroleum-derived contaminants were detected in the two clean soils. The field aged CO soil was sampled from a site in Santander used for disposing the cutting oil from drilling processes. The site was adjacent to the clean CO soil sampling point. Once collected, the soils were stored in sealed containers and placed in a dark, dry area at room temperature to prevent the occurrence of photooxidation reactions. After characterizing the soils, only the soil fraction with a particle size < 2 mm was used for the bioremediation tests.

The soil characterization resulted in a soil classification of a sandy loam for the clean OH soil and a sandy clay loam for the clean CO soil as shown in Table 1. The field aged CO soil was assumed to have the same soil classification that the clean CO soil had, since it was sampled from the same region and depth. It had been aged with waste oil more than 30 years. There was a periodic introduction of waste oil to the site, however the primary contaminant classifications remained the same. The total petroleum hydrocarbon (TPH) was measured to be 6.52%wt. Nitrogen and phosphorous concentrations were 2,214 and 10 mg/kg, respectively. In general, phosphorus biological requirement is approximately one-sixth of

Table 1. Characteristics of the field aged and clean soil samples

Analysis		Field aged CO soil	Clean CO soil	Clean OH soil
pH[a]	(in 0.01M $CaCl_2$)	6.9	3.6	6.5
Moisture content[a]	(%wt)	2.57	2.24	5.23
Total organic carbon[a]	(%wt)	10.50	1.77	0.83
Soil organic matter[a]	(%wt)	19.24	3.54	1.84
Cation exchange capacity[a]	(meq/100g)	6.47	4.24	9.18
Soil classification	(by USDA)	Sandy clay loam	Sandy clay loam	Sandy loam
Soil fractions (%wt)	Sand	58.1	57.3	72.3
	Clay	20.1	20.2	11.1
	Silt	22.5	22.5	16.6
Clay Mineralogy (%wt)[b]	Smectite	0	0	5
	Vermiculite	20	20	15
	Kaolinite	49	49	10
	Chlorite	8	8	12
	Illite	3	3	38
	Interstratified	15	15	10
	Quartz	5	5	10

[a]Methods from Page et al. (1992)[12]; [b]X-ray diffraction method; [c]values are means of triplicate samples

nitrogen[13] and an optimum nutrient content for an oil degradation is C:N:P=100:10:1[14], indicating a necessity of nutrient amendments for the current bioremediation experiment.

Waste oil was initially diluted in acetone (50:50, v/v) to obtain a more homogenous mixing with the clean soils. After the (waste oil+acetone) mixture was spiked to the clean soils (25:75, v/wt), the acetone was evaporated at room temperature. For ensuring a homogeneous distribution of waste oil, the spiked soils were liberally mixed with a clean spatula during the acetone evaporation. A greater amount of acetone or a more destructive solvent would have been necessary to achieve initial concentrations closer to that of the field aged soil. However, this would have greatly altered the soils sorption-desorption capacity and subsequent biodegradation.

Bacteria Source, Isolation, and Maintenance

The indigenous consortium was isolated from the field aged CO soil following standard subculturing procedures. Specifically, seven grams of the field aged CO soil, 0.1 g of phenanthrene (PHE), 0.1 g of pyrene (PYR), and 200 mL of an inorganic nutrient solution were added to 250 mL Erlenmeyer flasks that were shaken at 28 ± 2 °C on a Lab Line shaker operating at 125 rpm. The inorganic nutrient solution consisted of 1.33 (in g/L) KH_2PO_4, 2.67 K_2HPO_4, 1NH_4Cl, 2Na_2SO_4, 2KNO_3, 0.05 $FeSO_4$, 7H_2O and 0.2 $MgSO_4 7H_2O$. One milliliter of trace metal solution containing (in g/L) 3.7 $CaCl_2 2H_2O$, 2.5 H_3BO_3, 0.87 $MnCl_2$, 0.65 $FeCl_3$, 0.44 $ZnCl_2$, 0.29 $Na_2Mo_4 2H_2O$, 0.01 $CoCl_2$ and 0.001 $CuCl_2$ was added to make the final nutrient solution. After five weekly transfers (10% volume), the solution was cultured on agar plates coated with PHE and PYR in order to obtain the (PHE+PYR)- degrading consortium. PAH coating was achieved by dissolving 0.1g each PAH in 10 mL acetone and spraying PAH in acetone on the solidified agar surface. Acetone was allowed to evaporate at room temperature. Successive plating was repeated once a week over five weeks. The plating step transferred five visually different colonies that could utilize PYR and PHE as the sole carbon sources. After the isolation, the culture was maintained for the biodegradation experiments, by weekly transfers of 33% of volume to 100 mL of fresh nutrient solution containing 10 mg PHE and 10 mg PYR in crystalline form. The

microbes were grown with PHE and PYR as sole carbon sources for more than 16 months, prior to initiating the biodegradation experiments.

Biodegradation Experiment

For this study, biostimulation is defined as the addition of nutrients and terminal electron acceptor (TEA). Bioaugmentation is the addition of bacteria, nutrients, and TEA. In order to verify microbial degradation, parallel control reactors containing a biocide (200 mg/L of NaN_3) were also prepared in duplicate. The 'sterile' control reactors (i.e., received NaN_3) were amended with nutrients to maintain the same initial ionic strength (which could affect PAH desorption) as the biostimulation and bioaugmentation reactors. Natural attenuation controls (i.e., water addition) were not used since a previous study documented the inability of the indigenous species to biodegrade the contaminants when nutrients and a TEA were not supplied.[15]

For each treatment, 2 g of each soil (freshly spiked or field aged) were added to 40 mL duplicate reactors. Next, 20 mL of mineral medium solution and 75 mg H_2O_2/(L mineral medium) as the TEA were added to each reactor. The mineral medium was comprised of 5 mM $CaCl_2$ with the following salts (in g/L): 0.053 KH_2PO_4; 0.1068 K_2HPO_4; 2.0 NH_4Cl; 2.0 Na_2SO_4; 1.0 KNO_3; and 0.2 $MgSO_47H_2O$. As mentioned earlier, 200 mg/L of biocide was added to the control reactors. For the bioaugmentation reactors, a fully acclimated (PYR+PHE)-degrading consortium was harvested by centrifugation at 1,070 g for 20 minutes, washed prior to inoculation. The washing step was repeated three times to ensure the removal of any remaining PHE and PYR in the growth media. The bacteria were resuspended in 0.55% NaCl solution and 10 ml added to each reactor at the concentration of 1.6×10^6 colony forming unit (CFU)/mL. The microbial activity of the soil prior to the amendments was $<10^2$ CFU/mL, therefore the final cell density in the reactors after the inoculum was 1.6×10^6 CFU/mL.[16] Once all the constituents had been added, the reactors were placed in a Lab Line shaker operating at 28 $\pm$ 2 °C and 125 rpm.

For the field aged CO soil, biostimulation was conducted by adding the nutrient solution alone. Since the consortium used was isolated from this field aged soil, it was hypothesized that indigenous microorganisms could be revived via the nutrient amendment and utilize the contaminants. The results were compared with those from bioaugmentation to determine if treatment time was sufficient for reviving the bacteria. A concurrent study had determined that microbial activity would drop sharply after 21 days if nutrient/TEA amendments were not supplied.[16] Therefore, after 21 days all of the reactors were refreshed by allowing the contents to settle, decanting the supernatant, and adding 20 ml of the mineral medium/TEA described above. The refreshment cycle followed the general recommended nutrient loading rate.[13] Following this refreshment, experiments were continued for another 21 days. Therefore, each reactor contained 2 g soil and 20 mL mineral medium (i.e., suspended solid density of 67 mg/mL) for a total duration of 42 days.

Microbial growth was monitored during bioremediation on agar plates containing 0.3% (w/v) tryptic soy broth, 1.5% agar and the inorganic medium. The agar plates were coated the PYR and PHE to enable the enumeration of the PAH degraders. Sampling was conducted after a vigorous hand shaking of the reactors.

Chemical Analysis

Duplicate reactors from the biodegradation experiment were periodically sacrificed, centrifuged at 1,070 g for 20 minutes, and the supernatants were carefully decanted. The remaining solid fractions were transferred to petri dishes and dried. The PAH concentration in both liquid and soil fractions were analyzed by GC. For the analysis of the supernatants, liquid-liquid extraction was employed using hexane at a ratio of 60% hexane, 40% supernatant.

The concentrations of the GC-detectable PAHs in the soil phase were determined by Soxhlet

extraction with methylene chloride for 15 hours in accordance with EPA method 3540. The extract was then evaporated using a Buchi rotavapor (model R-114) at 40°C. The cellulose thimble containing the extracted soil was dried at 48 °C for 4 hours. After drying, the thimble and extraction flasks were reweighed to determine both the percent of compound(s) in the soil and the extraction efficiency (i.e., compound's recovery rate). The residual contained in the extraction flask was redissolved in 20 mL of trichloroethylene for subsequent analysis via gas chromatography (GC).

The GC used was a Shimadzu 14A equipped with a Rtx-5 capillary column (30 m × 0.32 mm, 0.25 μm, crossbond 5% diphenyl-95% dimethyl polysiloxane) and a flame ionization detector. The method was comprised of an initial column temperature of 35°C for the first 4 min, a 10°C/min ramp to 325°C, which was then held for 4 min. The temperatures of injector and detector were both 325°C. Hydrogen was used as the carrier gas. A previous study found that the compound recovery efficiency through the Soxhlet extraction and GC analysis was within 98.6 ± 1.5%.[17]

The GC was operated with a PAH standard solution (Supelco 48905-U) to obtain reference areas of known PAH concentrations. The retention time and reference factors were then used to quantitatively determine the concentration of PAHs present in the samples. Based on the GC parameters, benzo(a)anthracene and chrysene, benzo(b)fluoranthene and benzo(k)fluoranthene, ideno(1,2,3-c,d)pyrene and dibenz(a,h)anthracene were measured at the same detection times of 23.3 min, 25.7 min, and 28.4 min, respectively. However, no additional effort was given to further separate these compounds since they were not present in the field aged and freshly spiked soils.

RESULTS AND DISCUSSION

Characterization of PAHs in Field Aged and Freshly Spiked Soils

As outlined in the Materials and Methods section, each reactor was centrifuged to separate the two phases prior to analysis. The aqueous phase was then analyzed to assess the presence (and subsequent biodegradation) of PAHs that had desorbed from the soil. The soil phase was analyzed to determine concentration of sorbed PAHs, microbial activity, and extent of biodegradation. GC analysis was unable to detect any PAHs in the aqueous phase. This could be attributed to either the compounds' concentration being below the detection limit (0.01 μg/mL) or to the absence of compounds due to a high aqueous biodegradation. Therefore, data presentation was based on soil phase concentrations. Table 2 shows the distribution of GC-detectable PAHs in the free phase waste oil. Ecopetrol (Colombia, SA) determined that the waste oil also contained 19.1 % (w/w) saturated alipahtics (C_{10} to nC_{36}), 4.8% resins and 0.6% ashphaltenes. However these two contaminant classifications were not tracked during the biodegradation study due to limited availability of analytical equipment. Table 2 also contains the distribution of the field aged CO soil and the freshly spiked CO and OH soils. As shown, PAHs with molecular weights greater than PYR were not detected in the soils. Among the nine PAHs, three-ring PAHs such as acenaphthalene, acenaphthene, fluorene, PHE, and anthracene were present at total of 70w/w%, followed by 30w/w% for the four-ring PAHs such as fluoranthene and PYR. It should be noted that the freshly spiked soils had twice the initial PAH concentration than the field aged soil. It was believed that the field aged CO soil would not exhibit significant PAH biodegradation even when bioaugmented. The field-aged soil had been subjected to over 30 years weathering, therefore the bioavailability of the PAHs would be limited.[18~20] Soils containing compounds with limited bioavailability are often classified as persistent, or resistant to bioremediation.

Total GC-detectable PAH Biodegradation

Parallel control reactors had abiotic losses in

Table 2. Distribution of PAHs in free-phase waste oil, field aged CO soil and freshly spiked CO and OH soils

Contaminants	Distribution, % (w/w)			
	Free-phase oil	Aged CO soil	Spiked CO soil	Spiked OH soil
Two rings:				
Naphthalene	2.9	0	2.9	2.6
Subtotal	2.9	0	2.9	2.6
Three rings:				
Acenaphthalene	9.2	7.6	9.4	8.4
Acenaphthene	17.0	13.1	17.4	16.6
Fluorene	16.5	15.9	16.5	17.8
Phenanthrene	15.9	17.4	16.5	16.4
Anthracene	9.4	15.2	8.5	9.8
Subtotal	66.0	69.2	68.2	69.0
Four rings:				
Fluoranthene	16.7	16.2	16.5	16.2
Pyrene	12.2	14.6	12.4	12.2
Subtotal	28.9	30.8	28.9	28.4
Total GC-detectable PAHs	100	100 (3,217 mg/kg)	100 (6,158 mg/kg)	100 (6,419 mg/kg)

the range of 9-12% which occurred during the first few days of the experiment. These initial losses for the freshly spiked CO and OH soils were primarily attributed to the naphthalene disappearance for both systems. Prior to the experiments, the freshly spiked CO and OH soils had 180 and 168 mg naphthalene/kg soil, respectively. By the second day naphthalene concentrations were below detection limits. Acenaphthalene and acenaphthene also exhibited volatilization losses during the first few days. However their abiotic losses were at a lesser degree than that of naphthalene. Following this phenomenon, the parallel control systems kept a relatively constant PAH concentration during the experiment.

When reactors were bioaugmented, PAH biodegradation was greater for the freshly spiked OH soil than the freshly spiked CO soil (Table 3). The total PAH concentration had been reduced by 53% by day 21 for the freshly spiked OH soil, whereas the maximum PAH biodegradation achieved only 37% by day 23 for the freshly spiked CO soil. After this time, the degradation rate appeared to slow due possibly to the formation of smaller molecular weight PAHs as degradation byproducts from the transformation of more complex higher molecular weight PAHs or due possibly to the potential toxicity produced by other degradation products.[5] By the 42nd day, total PAH concentrations (including the degradation byproducts) were reduced by 44% and 36% of the initial concentrations for the freshly spiked OH and CO soils, respectively. The overall degradation efficiencies (i.e., percent difference between initial and final concentrations) for the freshly spiked soils by day 42 were not statistically different in terms of total PAHs (Table 4). However, the initial and final concentrations were statistically significant for both soils. There were also differences between the degradation of the individual PAHs.

The phenomenological difference in individual PAH degradation efficiencies is a direct indication of the influence of soil type on bioremediation. For example, the application of fertilizer for hydrocarbon bioremediation was more effective for the sandy sediment microcosm than for the mineral sediment microcosm.[21] The OH soil was characterized to have 72.3% of sand, whereas the CO soil had 57.5% sand. As previously mentioned, the net ECM content was 2.2% and 4.0% for the OH soil and CO soil, respectively. Along with the soil type, this different ECM amount was believed to affect the PAH biodegradation effectiveness in this study. It has been reported that when the

Table 3. Soil-phase GC extractable PAHs (in mg/kg soil) in bioaugmented reactors of freshly spiked OH and CO soils.* Data are means of combined duplicates. Standards deviations are within ±5%

PAHs	Soils	Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 23	Day 28	Day 35	Day 42
Naphthalene	OH	168	0	0	0	0	0	0	0	0	0
	CO	180	89	116	0	0	0	0	0	0	0
Acenaphthalene	OH	540	368	280	241	299	233	207	268	338	296
	CO	576	467	465	540	364	370	525	382	326	356
Acenaphthene	OH	1068	756	634	499	545	493	490	568	597	574
	CO	1070	940	836	809	761	715	544	725	595	597
Fluorene	OH	1143	824	704	575	599	554	596	594	676	660
	CO	1013	1014	915	842	870	739	553	832	687	635
Phenanthrene	OH	1050	770	673	538	553	531	579	580	619	586
	CO	1015	905	874	802	822	688	588	768	685	639
Anthracene	OH	628	400	383	327	330	298	312	370	356	393
	CO	526	515	502	599	463	427	623	399	511	454
Fluoranthene	OH	1042	758	721	499	532	485	529	560	621	582
	CO	1013	838	823	728	744	645	533	704	663	648
Pyrene	OH	780	655	557	437	461	447	458	497	508	501
	CO	765	696	715	657	658	603	594	642	628	599
Total	OH	6419	4531	4232	3116	3319	3041	3171	3437	3714	3592
	CO	6158	5464	5246	4977	4682	4187	3960	4452	4095	3928

*: After 21 days all reactors were refreshed by allowing the contents to settle, decanting the supernatant, and adding 20 ml of the mineral medium/TEA as described in the text.

Table 4. Statistical differences (one-way ANOVA tests at α = 0.05) between each treatment combinations at day 42, and between the initial and final (day 42) concentrations of total GC-detectable PAHs

System		Treatment	Statistical difference	
			Comparison of final ΣPAHs between two treatments	Comparison of ΣPAHs between initial and final stages of a treatment
Freshly spiked	Control [a]	OH CO	NO [d] (i.e., $p>0.05$)	YES [e] YES
	OH	Control Bioaug	NO	YES YES
	CO	Control Bioaug	YES (i.e., $p<0.05$)	YES YES
	Bioaug [b]	OH CO	NO	YES YES
Field aged	CO	Control Bioaug.	NO	YES YES
		Control Biostim [c]	NO	YES YES
		Bioaug Biostim	NO	YES YES

[a] Control reactor: nutrient solution + biocide (200 mg NaN_3 /L).
[b] Bioaugmented reactor: nutrient solution + bacteria inoculation.
[c] Biostimulated reactor: nutrient solution only.
[d] For example, (PAHs in the OH and CO control reactors analyzed after 42-day experiment were not statistically different.
[e] For example, in the OH control reactor, (PAHs after 42-day experiment was significantly decreased in comparison to those at the beginning of experiment.

total organic carbon (TOC) content is <0.1 wt% in natural solids, sorption to mineral surfaces may contribute significantly to the overall uptake of hydrophobic organic compounds by soils and sediments.[22,23] In a study on PAH sorption to humic acid-mineral complexes, Onken and Traina (1997) found that experimental values of partitioning coefficient normalized to the TOC content were higher than those predicted from octanol-water partitioning coefficient.[24] Their results indicated that PAH partitioning to the SOM was not the sole interaction. In other words, a significant amount of sorption to mineral surfaces occurred simultaneously with partitioning to the SOM. This was verified by a concurrent sorption-desorption study that utilized both the OH and CO soils as well as a soil from New Mexico (8.4% OM, 4.46% TOC and 1.67% ECM). The sorption affinity was in the order of CO>>OH>New Mexico soils. Desorption was in the order of New Mexico>> OH>CO.[25] The New Mexico soil, which had the most OM and least amount of expandable clays, bound the least amount of PAHs and exhibited the greatest desorption. Since the ECM expands upon wetting it can provide an internal surface area for PAH binding as high as 570 to 700 m^2/g.[26] For instance, the permanent negative charge of smectite can form a bridge between the clay and SOM fraction of soils, thus providing another sorption site.[27]

A previous biodegradation experiment using the same soils examined here further corroborated this phenomenon. The total biodegraded PHE when freshly spiked at 100 mg/kg in the OH soil and CO soil was 95% and 90%, respectively, at the end of a 32-day experiment. Furthermore, the total PYR biodegradation was 78% for the OH soil and 65% for the CO soil. As with this study, the CO soils did not obtain as high bioremediation efficiencies as the OH soil due to a stronger sorption to the ECM.[28]

Meanwhile, bioaugmentation yielded only a 30% maximum PAH biodegradation for the field aged CO soil by day 23 (Figure 1a). By the end of the experiment, the formation of degradation

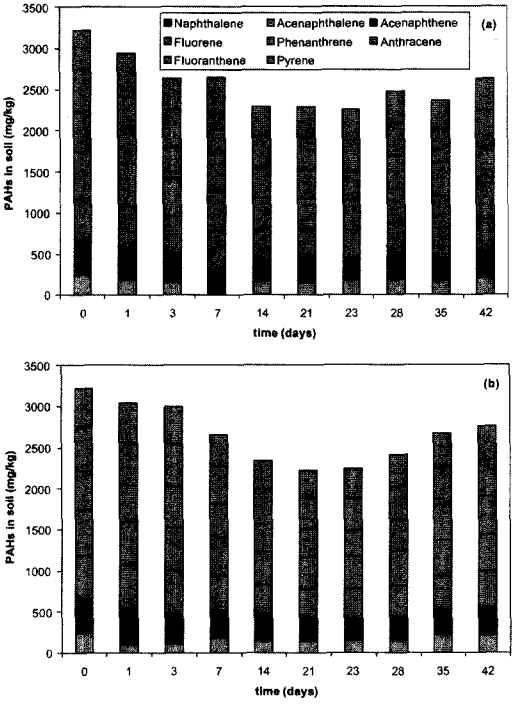

Figure 1. Soil-phase GC detectable PAHs in the field aged CO soils: bioaugmented system (a) and biostimulated system (b). The legend for Figure 1(b) is the same as for Figure 1(a). Data are means of combined duplicates. Standards deviations are within ±5%. After 21 days all reactors were refreshed by allowing the contents to settle, decanting the supernatant, and adding 20 ml of the mineral medium/TEA as described in the text.

byproducts, and their subsequent degradation, resulted in an overall, 42-day degradation efficiency of 18%. Although the two CO freshly spiked and field aged soil systems had similar maximum biodegradation efficiencies, compound aging created a significant difference in terms of the total PAH reduction. This was evident by the individual PAHs having different concentrations (comparing Table 3 and Figure 1).

As noted earlier, the OH and CO soils had different fractions of organic matter and clay. Although previous sorption-desorption studies focused on only pyrene and phenanthrene, the other PAHs would also preferentially bind to one of the soil constituents based on their physi-

cochemical characteristics. The ease of sorption and desorption will directly impact bioavailability and thus, the maximum degradation efficiencies. As mentioned previously, the initial PAH concentration was 6,158 mg/kg for the freshly spiked CO soil and 3,217 mg/kg for the field aged CO soil. Therefore, the extent of biodegradation was 2,231 mg/kg (i.e., 36%) for the freshly spiked CO soil and 590 mg/kg (18%) for the field aged soil. This indicates that the actual PAH bioremediation for the field aged soil was only half that of the freshly spiked soil.

Reduced bioavailability with an increase in compound aging has been a well-defined phenomenon, presumably due to elevated mass transfer limitations in the soil with time.[10,11,29~32] It should be noted that even though only 18% total PAH biodegradation was achieved, bioremediation of the field aged soil through bioaugmentation was still promising. Within 23 days, 965 mg/kg was biodegraded. By the 42nd day, the net reduction appeared to have been reduced to 590 mg/kg due possibly to the formation of degradation byproducts as mentioned previously. The field aged soil also contained 19.1% saturated aliphatics (96.9 mg/kg). Considering that these constituents would be degraded more easily than PAHs and that the PAHs would be more tightly sorbed than the freshly spiked systems, these amounts were significant from a phenomenological standpoint. A statistical comparison of the bioaugmented freshly spiked and field aged soils was not conducted since the field aged soil had too many confounding variables (auxiliary carbon sources, differences in initial concentrations, etc.). However, if the confounding variables could be eliminated, the net overall PAH reduction of 2,231 versus 590 mg/kg would have been significant.

Biostimulation results for the field aged CO soil showed a statistically similar PAH biodegradation efficiency to that for the bioaugmentation (Figure 1b, Table 4). Again, efficiency could not be the only evaluation tool since these two systems were different in terms of the biodegradation rate and microbial growth. Compared to the early-stage biodegradation for the bioaugmented field aged CO system, the biostimulated system did not depict a noticeable PAH reduction until day seven. In conjunction with this phenomenon, microbial growth was not substantial prior to day seven. According to Vogel,[33] in cases where contaminant toxicity or a lack of appropriate indigenous microorganisms are important, bioaugmentation provides certain advantages over biostimulation. However, the biostimulation effectiveness for this study was indicative of the bioremediation potential for the field aged CO soil. Successful bioremediation of petroleum aged soils by indigenous soil microorganisms in conjunction with nutrient amendments were also documented.[29,34]

Continued microbial activity requires periodic nutrient and terminal electron acceptor supplements.[15,34] After refreshing the system (day 21), the fraction of the total GC-detectable PAHs was increased in all systems. However, the total PAH concentrations were still lower than the initial concentrations (Table 3 and Figure 1). This was due to the normal metabolic pathways exhibited by PAHs. In other words, the increased PAHs were from the transformation of more complex PAHs and/or other higher molecular weight compounds present in the waste oil into smaller molecular weight compounds that were detectable by the GC.[5,36] Since a similar trend was not present in the control reactors, the change could not have been due to a change in ionic strength by the nutrient refreshment.

The generation of the degradation byproducts was not immediate since the lower molecular weight PAH is not the first degradation intermediate. Under aerobic conditions, microbial attack occurs at one of the fused rings, with the primary intermediate from dioxygenase reactions being a cis-dihydrodiol. This is followed by the generation of a lower molecular weight PAH containing a similar parent structure (i.e., dibenz (a,h) anthracene → anthracene → benzene →

catechol).[13,37] The presence of other compounds that were not detectable by the GC method implemented (i.e, chrysene-d12, benzo(g.h.i) perylene, perylene, etc.) could also contribute to the increase in lower molecular weight PAHs, thereby resulting in an increase in measurable PAHs.

Total Microbial Growth

Figure 2 shows the trend of the total heterotrophic bacteria (THB) population that served as the PAH degraders during the experiments. ield aged CO system compared to that for the freshly spiked systems. Consequently, the substantial population growth in this soil system was attributed to more easily biodegradable contaminants such as aliphatics.

When biostimulated, the field aged CO soil also showed a significant microbial growth with the maximum population of 6×10^{10} CFU/g soil at day 14. However, the growth peak appeared later than that of the bioaugmented system. This change in the growth pattern was attributed to lag phase associated with "reviving" the indigenous microorganisms and facilitating the population shift for degrading the PAHs.

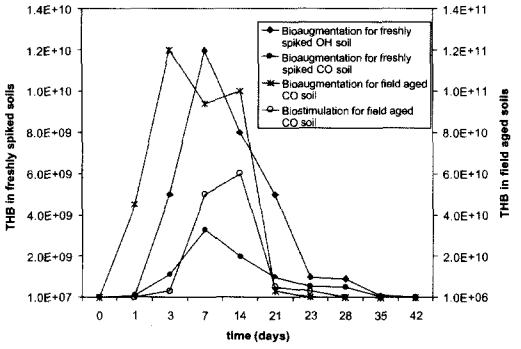

Figure 2. Total heterotrophic bacteria (THB) growth for the three soils (in CFU/g). After 21 days all reactors were refreshed by allowing the contents to settle, decanting the supernatant, and adding 20 ml of the mineral medium/TEA as described in the text. The initial number of THB at Day 0 was 1.6×10^{7} for the bioaugmented systems, whereas $<1.0\times10^{3}$ for the biostimulated system. THB population was greater than the initial numbers for each system during the experiment. Exception was found at Day 42 for bioaugmented field aged CO soil system which had 1.0×10^{6} at Day 42.

Between the two freshly spiked soil systems, a greater THB growth occurred for the OH soil. Since the same experimental conditions were used for both soils, the difference in THB growth should correlate to the PAH biodegradation trend. Previously, the achieved PAH biodegradation was 53% for the OH soil and 37% for the CO soil. Furthermore, the PAH biodegradation was faster for the OH soil than for the CO soil. As evident in Figure 2, the bacterial growth patterns did correlate with the extent and rate of PAH biodegradation between the two freshly spiked soils. Since the biodegradation of unidentified compounds in the freshly spiked and field aged soil systems were significantly different, the THB trend was attributed to a different biodegradability and bioavailability of all TPHs (tracked and non-tracked). This was supported by the lack of an increase in THB after the day 21. If carbon availability (i.e., contaminant availability) were not an issue, the microbial activity would not have decreased after receiving the nutrient/TEA supplement.

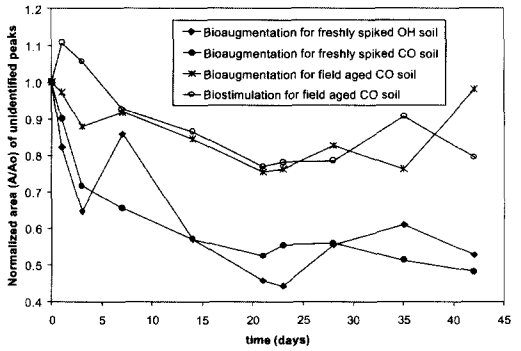

Figure 3. Normalized area for GC-detectable unidentified peaks. After 21 days all reactors were refreshed by allowing the contents to settle, decanting the supernatant, and adding 20 ml of the mineral medium/TEA as described in the text.

CONCLUSIONS

The difference in the achievable PAH biodegradation amount between the two spiked soils was attributed to the differences in soil characteristics. A smaller amount of the total GC-detectable PAHs were biodegraded in the ECM-rich CO soil. In addition, as the compound(s) aged in the soil, they became more persistent. In the same CO soil, a 37% (6,158 to 3,959 mg/kg) maximum PAH biodegradation was achieved for the freshly spiked system, whereas the field aged soil depicted a 30% (3,217 to 2,260 mg/kg) decrease. The effectiveness of biostimulation for the field aged CO soil was comparable to that of bioaugmentation, thereby depicting a potentially effective treatment by a simple nutrient amendment.

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