HHS Public Access

Author manuscript

Biodegradation. Author manuscript; available in PMC 2016 February 13.

Published in final edited form as:

Biodegradation. 2012 July ; 23(4): 621–633. doi:10.1007/s10532-012-9538-9.

Long-term simulation of in situ biostimulation of polycyclic aromatic hydrocarbon-contaminated soil

Stephen D. Richardson,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. Solutions-IES, Inc., Raleigh, NC, USA

Maiysha D. Jones,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

David R. Singleton, and
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Michael D. Aitken
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Stephen D. Richardson: RichardsonS@solutions-ies.com

Abstract

A continuous-flow column study was conducted to evaluate the long-term effects of in situ biostimulation on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil from a manufactured gas plant site. Simulated groundwater amended with oxygen and inorganic nutrients was introduced into one column, while a second column receiving unamended groundwater served as a control. PAH and dissolved oxygen (DO) concentrations, as well as microbial community profiles, were monitored along the column length immediately before and at selected intervals up to 534 days after biostimulation commenced. Biostimulation resulted in significantly greater PAH removal than in the control condition (73% of total measured PAHs vs. 34%, respectively), with dissolution accounting for a minor amount of the total mass loss (~6%) in both columns. Dissolution was most significant for naphthalene, acenaphthene, and fluorene, accounting for >20% of the total mass removed for each. A known group of PAH-degrading bacteria, ‘Pyrene Group 2’ (PG2), was identified as a dominant member of the microbial community and responded favorably to biostimulation. Spatial and temporal variations in soil PAH concentration and PG2 abundance were strongly correlated to DO advancement, although there appeared to be transport of PG2 organisms ahead of the oxygen front. At an estimated oxygen demand of 6.2 mg O_2/g dry

Correspondence to: Stephen D. Richardson, RichardsonS@solutions-ies.com.

Present Address:
M. D. Jones, Proctor & Gamble, Cincinnati, OH, USA

Electronic supplementary material The online version of this article (doi:10.1007/s10532-012-9538-9) contains supplementary material, which is available to authorized users.

soil and a porewater velocity of 0.8 m/day, it took between 374 and 466 days for oxygen breakthrough from the 1-m soil bed in the biostimulated column. This study demonstrated that the presence of oxygen was the limiting factor in PAH removal, as opposed to the abundance and/or activity of PAH-degrading bacteria once oxygen reached a previously anoxic zone.

Keywords

In situ biostimulation; Polycyclic aromatic hydrocarbons; Manufactured gas plant site; Oxygen; Soil columns

Introduction

Soil and sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) are complex systems with multiple compound-, microbial-, and matrix-specific factors that can ultimately impact the success of bioremediation strategies. For bioremediation to be effective, an adequate supply of bioavailable carbon (PAHs and other substrates), macro- and micro-nutrients, electron acceptors, and a well-established contaminant-degrading community must be present in the subsurface (Eriksson et al. 2000). However, PAH-contaminated field sites such as former manufactured gas plant (MGP) sites are frequently nutrient- and oxygen-limited (Madsen et al. 1996). PAHs also partition into various domains within the soil matrix that limit their bioavailability, including soil organic matter (SOM), pitch, black carbon such as coal, coke, and soot, and nonaqueous-phase liquids such as oil or tar (Khalil et al. 2006; Luthy et al. 1997). These limitations lead to reduced PAH biodegradation and continued long-term dissolution of PAHs in the saturated subsurface (Madsen et al. 1996; Murarka et al. 1992).

Active bioremediation strategies such as biostimulation can be used to supply nutrients, oxygen, and other amendments to the subsurface to enhance indigenous microbial activity and contaminant biodegradation (Bamforth and Singleton 2005; Borchert et al. 1995; Mohan et al. 2006). The benefits of adding oxygen and/or nutrients on PAH biodegradation have been well-documented in laboratory studies of former MGP and wood-preserving site soils (Breedveld and Sparrevik 2000; Eriksson et al. 2000; Li et al. 2005; Liebeg and Cutright 1999; Lundstedt et al. 2003; Talley et al. 2002); however, only a few studies have focused on the direct effects of biostimulation on the indigenous microbial community and PAH-degrading bacteria (Ringelberg et al. 2001; Viñas et al. 2005). Viñas et al. (2005) used denaturing gradient gel electrophoresis (DGGE) and most-probable-number analyses to detect distinct shifts in microbial diversity and an increase in PAH-degrading bacteria, respectively, along with a concurrent decline in individual PAH concentrations after 200 days of nutrient amendment and aeration in slurried creosote-contaminated soil. Likewise, Ringelberg et al. (2001) correlated a reduction in PAH concentrations with an increase in microbial community biomass [using ester-linked phospholipid fatty acid (PLFA) analysis] and genes encoding known PAH-degrading enzymes in bioreactor-treated MGP sediment. Although ex situ, continuously mixed slurry systems can improve PAH bioavailability and the distribution of oxygen and/or nutrients, the costs and space requirements associated with soil excavation can preclude their use at many field sites. Alternatively, in situ techniques

are often less energy intensive and more cost effective than conventional ex situ treatments. Numerous column studies have been conducted to investigate in situ PAH biodegradation, dissolution, and bioavailability, as well as transport of PAH-degrading bacteria (Breedveld and Karlsen 2000; Bodour et al. 2003; Chi and Amy 2004; Kim et al. 2008; Lahlou et al. 2000; Millette et al. 1998; Tiehm et al. 1997; Wehrer and Totsche 2005; Zhang et al. 2008); however, artificially contaminated soils and/or cultured PAH-degrading bacteria were used in most of these studies.

To our knowledge, no study has investigated the long-term efficacy of biostimulation for PAH removal and its simultaneous effect on the indigenous microbial community and PAH-degrading bacteria in field-contaminated MGP soil under in situ conditions. We operated two continuous-flow columns packed with soil from a former MGP site to monitor concurrent changes in soil- and aqueous-phase PAH concentrations and abundances of indigenous PAH-degrading bacteria for over 500 days; one column was subjected to continuous biostimulation and the other served as a control. Monitoring of dissolved oxygen (DO) over the column length permitted spatial and temporal correlation of soil PAH concentration and PAH-degrader abundance to DO advancement over time. Overall oxygen demand and transport of desorbed PAHs in the column effluents were also quantified.

Materials and methods

Chemicals

Dichloromethane (>99.5%), acetone (>99.5%), acetonitrile (>99.9%), and sodium sulfate (>99%) were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Anthracene-d_{10} (98%) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Soil

Contaminated soil was collected from a former MGP site in Salisbury, NC, USA. The soil was sieved through a 10-mm wire screen, mixed with sterile 40/50 grade silica sand (Unimin Corporation, Le Sueur, MN) at a 50:50 ratio (dry weight), and stored at 4°C prior to column packing. Addition of the silica sand was necessary to maintain low-pressure flow during long-term column operation; preliminary column studies with the source material alone yielded very high inlet pressures (>100 psi). Sand addition had minimal impact on the indigenous soil microbial community as evaluated by DGGE (data not shown). In subsequent discussion, the final packing material is referred to as “column soil”.

The column soil contained 83% sand, 14% silt, and 3% clay, with total organic matter of 8.3% and extractable organic matter of 0.64%; physical properties of the column soil are summarized in Richardson et al. (2011). Total PAH concentration [sum of 13 of the 16 U.S. Environmental Protection Agency (USEP-A)-regulated PAHs] was 295 ± 65 mg/kg dry soil (n = 33), with phenanthrene comprising 44% of the total PAH mass (Table 1).

Simulated groundwater

Simulated groundwater was prepared based on historical ion concentrations of groundwater in the region of the MGP site (Groves 1976). The solution contained 1.83 g $CaCl_2 \cdot H_2O$, 1.01

g $MgSO_4 \cdot 7H_2O$, 2.19 g $NaHCO_3$, 1 mL of an 88 mg/L KCl solution, and 1 mL of 1 N H_2SO_4 in 20 L of sterile-filtered reagent water. Simulated groundwater for the biostimulated column was supplemented with 1 mL of a nutrient stock solution containing 59.4 g/L of NH_4NO_3 and 29.2 g/L of K_2HPO_4, yielding final nitrogen and phosphorus concentrations of 1.0 and 0.3 mg/L, respectively. This feed solution for the biostimulated column was continuously sparged with pure oxygen (99.6%, Air Gas National Welders). For the control column, unamended groundwater was equilibrated with the atmosphere and therefore was air-saturated when it entered the column. New batches of ground-water were prepared weekly to minimize the potential for bacterial growth in the feed solutions.

Experimental design

Details of the column design are presented in the Supporting Information (Figure S1). Briefly, each column was 110 cm long, 10.2 cm in diameter, and constructed of stainless steel. Each column contained a 100-cm bed of column soil underlain by a 5-cm bed of clean sand. The columns were operated for ~780 days in a constant-temperature room at 20°C, receiving a continuous supply of simulated groundwater in a downward flow direction. A flowrate of 2.1 L/day (corresponding to a porewater velocity [v] of 0.8 m/day) was maintained for ~630 days and subsequently reduced to 1.4 L/day (v = 0.5 m/day) to stabilize rising inlet pressures. Three soil sampling ports (Ports A, B, and C) were positioned 30, 55, and 80 cm below the top of each column, respectively, along with nine additional smaller ports for monitoring porewater DO concentrations. Both columns were operated under control conditions for 250 days (‘equilibration phase’) to allow for bed consolidation, initial DO advancement, and stabilization of inlet pressures before biostimulation was initiated in one of the columns. The end of the equilibration phase is defined as t = 0, from which all subsequent time points are referenced.

Soil samples were collected from the surface of the soil bed and Ports A, B, and C of the biostimulated and control columns at six time points: t = 0, 31, 93, 184, 380, and 534 days after initiation of biostimulation. During each sampling event, approximately 170 g (dry wt.) and 50 g (dry wt.) of column soil were collected from the surface of the soil bed and each sample port, respectively, using dedicated, sterilized, stainless-steel core sampling devices, for extraction of PAHs and microbial DNA. Sampling accounted for the removal of approximately 15% of the initial soil mass from each column over the duration of the study.

PAH analyses

Soil samples for PAH analysis were collected in 30-mL glass centrifuge vials, stored at 4°C, and extracted the following day using the extraction and quantification method outlined in Richardson et al. (2011), except 3 g (wet wt.) aliquots of column soil were extracted and 5 g of sodium sulfate was added to each extraction vial. Of the 16 EPA-regulated PAHs, acenaphthylene was not detected using this quantification method; dibenz[*a*,*h*]anthracene and indeno[1,2,3-*c*,*d*]pyrene were detected at concentrations near their respective method detection limit and are excluded from subsequent analyses. The total mass of PAHs remaining in each column after treatment was calculated by linear interpolation of final PAH concentrations (t = 534 days) between sample locations and summing the resulting PAH

masses from each column section. PAH concentrations at Port C were assumed to be representative of the soil between the port and the bottom of the soil bed.

Aqueous-phase PAH concentrations were monitored in the column effluents over time. Samples were collected in 1-L acid-washed glass vessels and extracted using 47-mm C18 solid phase extraction (SPE) disks (Empore™, 3 M Inc., St. Paul, MN) according to EPA Method 3535 (USEPA 1996). Prior to extraction, an internal standard (0.1 mL of 100 mg/l anthracene-d_{10} in acetonitrile) was added to each sample to measure the recovery efficiency of the extraction procedure. A 0.7 μm pore-size glass-membrane pre-filter (Whatman® GF/F, Sigma-Aldrich, St. Louis, MO) was placed above the C18 disk to retain any suspended solids in the sample. After filtration of the 1-L effluent samples, the C18 disks and pre-filters were placed in separate 30-mL glass centrifuge vials with Teflon-lined caps and extracted with dichloromethane and acetone (10 mL each) per the PAH extraction method outlined in Richardson et al. (2011), except sodium sulfate and glass beads were not added and the combined extracts were brought up to 100 mL with acetonitrile. PAHs were not detected in the pre-filter extracts for all sample locations and time points.

Oxygen monitoring

A FOXY fiber optic oxygen sensor system (Ocean Optics, Dunedin, FL) was used to measure DO concentrations in the porewater of the biostimulated and control columns. The DO sensor was inserted through a sterile hypodermic needle that was used to pierce the septum on each DO port, and it was rinsed with ethanol between measurements. The oxygen demand of the column soil was calculated using oxygen breakthrough curves at DO ports where oxygen saturation corresponding to the introduced gas (oxygen or air) had been established. Specifically, the total oxygen mass advancing beyond a given port was subtracted from the oxygen mass influx and divided by the mass of dry soil above the selected port.

Molecular analyses

DNA extraction was performed on triplicate 0.5–0.7 g (wet wt.) aliquots of soil collected from each sample location and time point using the procedure outlined in Richardson et al. (2011). The mass of DNA in the replicate extracts was measured using a NanoDrop ND-3300 Fluorospectrometer (Thermo Scientific; Waltham, MA) and Quant-iT Picogreen dsDNA Kit (Invitrogen; Carlsbad, CA). Since DNA masses were similar among replicates, the triplicate extracts were pooled for subsequent molecular analyses. DGGE and quantitative PCR (qPCR) were conducted as previously described in Richardson et al. (2011). The abundance of 16S rRNA genes from dominant naphthalene-, phenanthrene-, and pyrene-degrading bacteria, previously identified in the Salisbury MGP soil by stable-isotope probing (SIP), were measured using qPCR primer sets presented in Jones et al. (2011). Screening for SIP-identified groups was first tested in DNA extracts from Port A samples at select time points (t = 0, 93, 380, and 534 days). If at least one cycle threshold (C_t) value for a given group-specific primer was within the linear range of the respective standard curve, duplicate qPCR runs were conducted for all sample locations and time points. However, if the C_t values for a given group fell outside the linear range over all the time points, the SIP-identified group was considered below detection.

Statistical analyses

The nonparametric Spearman rank statistical test (JMP® 7.0.1, SAS Institute Inc., Cary, NC) was used to identify pairwise correlations between three experimental variables: DO concentration, individual soil PAH concentration, and 16S rRNA gene copy number of SIP-identified PAH-degrading bacterial groups (Jones et al. 2011). Spearman rank order analysis tests if any significant monotonic relationship (either increasing or decreasing) exists between two variables and reports a coefficient (r_S) between −1 (perfectly negative correlation) and +1 (perfectly positive correlation). To account for the small number of data points ($n = 6$) in the data sets for PAH and bacterial abundance, coefficients corresponding to p-values <0.15 were considered to be significant. DO concentrations measured closest in time to each soil sampling event were used for comparison to PAH and bacterial abundance time-series data.

Outlier tests were performed on replicate soil extracts that contained any PAH concentration well above the respective initial concentration in the column soil (Table 1). Suspect values greater than the upper quartile of the initial column soil concentration by more than three times the interquartile range (i.e., the difference between the upper and lower quartiles) were deemed to be outliers and were excluded from the data set; 20 out of 216 replicates were identified as outliers. Other statistical analyses [i.e., Student t-test and, Tukey–Kramer Honestly Significant Difference (HSD) test] were performed using JMP® 7.0.1 (SAS Institute Inc., Cary, NC).

Results and discussion

DO profile

After the 8-month equilibration phase ($t = 0$), DO fronts in the biostimulated and control columns were similar, extending 15–20 cm below the top of the column; DO concentrations were ≤ 0.1 mg/L in the remainder of the columns (Fig. 1). Once oxygen-saturated groundwater was introduced to the biostimulated column, DO concentrations in that column rose sharply and advanced at a greater rate than in the control column, achieving breakthrough between 374 and 466 days (not shown in Fig. 1). The DO front in the control column did not advance beyond 45 cm from the top of the column over the entire course of the study. Based on the DO penetration data, the oxygen demand of the column soil was estimated to be 6.2 mg O_2/g dry soil in the biostimulated column and 4.9 mg/g in the control column. The theoretical oxygen demand associated with oxidation of the 13 detectable PAHs accounted for 14% of the total oxygen demand exhibited in the biostimulated column soil.

Soil PAH concentration

After 534 days, 73% of the total PAH mass in the biostimulated column was removed (by dissolution, biodegradation, and sample loss) as compared to 34% in the control column (Fig. 2). The majority of PAH mass removed during biostimulation consisted of three- and four-ring PAHs (86 and 66%, respectively), although 41% of the five-ring PAH mass was also removed (Table 2). In the control column, total mass removals for three-, four- and five-ring PAHs were 37, 33, and 29%, respectively (Supporting Information, Table S1).

PAH mass loss due to sampling ranged from 4 to 23% for all PAHs in both columns (Tables 2, S1).

Time-series graphs of soil PAH concentration for all sample locations in both columns are summarized in the Supporting Information (Figures S2–S5), with selected PAHs from Port A presented in Fig. 3. The greatest reduction in total PAH concentration occurred at Port A of the biostimulated column (80%), where three-, four-, and five-ring PAH concentrations were significantly lower ($p < 0.05$) than their respective initial column soil values after 534 days. Significant reductions ($p < 0.05$) in all three-ring PAHs, fluoranthene, and pyrene were observed after only 31 days of biostimulation (Fig. 3 and Supporting Information, Figure S3). Chrysene, benzo[*a*]anthracene, and the five-ring PAHs required longer treatment times (up to 184 and 380 days, respectively) before concentrations significantly declined; no significant change in benzo[*g*,*h*,*i*]perylene concentration was observed. In addition, concentrations of all three-ring PAHs, fluoranthene, and pyrene at Port A of the biostimulated column approached a threshold after their initial rapid decline (Fig. 3, Supporting Information, Figure S3). This biphasic pattern in PAH removal is commonly reported in field-contaminated soils and reflects variations in PAH bioavailability over time within various soil domains (Luthy et al. 1997). The decreased rate of PAH removal associated with the threshold region is indicative of mass transfer limitations (Alexander 2000; Madsen et al. 1996).

In the control column, total PAH concentration at Port A decreased by 37%, with three-ring PAHs comprising the majority of the total PAH reduction. Only acenaphthene and fluorene declined significantly ($p < 0.05$) during early time points (within 31 and 93 days, respectively), while significant reductions of other three-ring and all four-ring PAHs were not observed until the last sampling event (534 days). Five- and six-ring PAH concentrations at Port A of the control column were not significantly different ($p > 0.05$) than respective initial column soil values over the course of the study (Fig. 3, Supporting Information, Figure S3).

PAH removal at Ports B and C of the biostimulated column was lower than at Port A (73 and 55%, respectively), and significant reductions ($p < 0.05$) in concentration were limited to the three- and four-ring PAHs (Supporting Information, Figures S4 and S5). At Ports B and C of the control column, concentrations for all but two PAHs (acenaphthene and anthracene at Port C) were not significantly different than initial column soil values (Supporting Information, Figures S4 and S5). PAH concentrations in the surface soil were highly variable for both columns, possibly due to soil heterogeneity resulting from the larger soil mass removed at each sampling event relative to the soil sampling ports (Supporting Information, Figure S2).

Aqueous-phase PAH concentrations

Effluent PAH concentrations were monitored over time to estimate the contribution of PAH dissolution to overall PAH removal in each column. Naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene were detected in the effluent samples, while higher molecular-weight PAHs remained below detection in the effluent, consistent

with other studies on PAH transport from MGP soils (Enell et al. 2004; Gustavson and Harkin 2000; Reemtsma and Mehrtens 1997; Zamfirescu and Grathwohl 2001).

Time-series graphs of aqueous-phase PAH concentrations are provided in the Supporting Information (Figure S6), with selected PAHs presented in Fig. 4. Of the detectable PAHs, phenanthrene was the most abundant in the column effluents during the equilibration phase. Aqueous-phase PAH concentrations gradually decreased in the column effluents over time, with naphthalene and fluorene concentrations below detection after 534 days. Although PAH concentrations in the biostimulated column effluent were somewhat lower than in the effluent from the control column at later time points, the total PAH mass leached from each column was similar (6.5 and 6.2%, respectively) over the course of the study (Fig. 2). Dissolution accounted for removal of >20% of naphthalene, acenaphthene, and fluorene in both columns. Overall, PAH dissolution generally decreased with increasing molecular weight of the compound for both columns. The lower extent of naphthalene dissolution is likely due to its weathered state in the original MGP soil and subsequent volatilization during soil processing, leaving largely the less desorbable fraction in the soil when it was packed into the column.

Accounting for PAH losses due to sampling and dissolution, biodegradation appeared to be the primary mechanism of mass removal for all PAHs except naphthalene in the biostimulated column (Table 2). In the control column, however, dissolution accounted for greater mass loss of naphthalene, acenaphthene, and fluorene than did biodegradation (Supporting Information, Table S1).

Effect of DO on PAH removal

The reduction of soil PAH concentrations in the biostimulated column soil corresponded well with advancement of the DO front with depth. At Port A, significant negative correlations ($r_s < -0.71$; $p < 0.15$) were identified for most PAHs while, at Ports B and C, significant negative correlations ($r_s < -0.76$; $p < 0.1$) were limited to three- and four-ring PAHs (Table 3). In the control column, negative correlations between DO and PAH concentrations were also identified at Port A; however, r_s values were not significant for most PAHs (data not shown). Rank order analyses were not performed on data for Ports B and C for the control column because oxygen was not detected at these depths. Overall, these results suggest a strong correlation between the presence of oxygen in the porewater and reductions in PAH concentrations in the column soil. Other column studies have reported similar spatial distribution of contaminants with respect to oxygen presence/absence after short-term biosparging (Adams and Reddy 2003; Talley et al. 2004).

Microbial abundance and diversity

Profiles of total bacterial 16S rRNA genes were obtained by DGGE analysis of DNA extracts from the biostimulated and control columns to identify any gross differences in the overall microbial community over time (Fig. 5). Immediately before biostimulation ($t = 0$), community profiles appeared more diverse in the surface soil of both columns (Fig. 5; lane 1), each containing more prominent bands than respective communities at Ports A–C (Fig. 5; lanes 8, 15, and 22, respectively). After biostimulation, the surface soil community remained

similar over time in both columns, with few differences in the intensity or position of prominent bands (Fig. 5; lanes 2–6). However, clear shifts in community profiles were observed at Ports A–C in both columns at various time points. In the biostimulated column, a number of new bands that were prominent in the surface soil community appeared at Ports A, B, and C after 31, 93, and 184 days, respectively (Fig. 5a; lanes 9, 17, and 25). More subtle shifts in the control column communities occurred after 31 days at all sample ports (Fig. 5b, lanes 9, 16, and 23).

A number of bacterial groups capable of degrading naphthalene, phenanthrene, and pyrene in the column soil were identified by SIP in an earlier companion study (Jones et al. 2011) including *Variovorax* (naphthalene), *Acidovorax* (phenanthrene), and “Pyrene Group 2” (PG2; pyrene). Of these bacterial groups, PG2 was a significant component of the column soil community; all other tested SIP-identified PAH degraders were below the qPCR quantification limits (~3×10^5 16S rRNA genes/g soil). PG2 has been previously identified as a dominant member of the bacterial community in soil from a different MGP site (Singleton et al. 2005, 2007).

Prior to biostimulation, the quantity of 16S rRNA genes of PG2 organisms was greatest in the surface soil of both columns, with concentrations of $3.7 \pm 1.2 \times 10^7$ in the biostimulated column and $3.4 \pm 0.4 \times 10^7$ genes/g in the control column (Fig. 6). Concentrations were lower at Port A and were near or below detection at Ports B and C in both columns. Total bacterial abundance was also greatest in the surface soil prior to biostimulation (~2×10^8 genes/g), up to an order of magnitude higher than in samples collected from Ports A, B, and C (Fig. 6).

Temporal trends in PG2 abundance in the surface soil and at Port A of the biostimulated column were generally consistent with those observed for the total bacterial community (Fig. 6a). Concentrations of PG2 organisms in the surface soil gradually decreased with time, whereas at Port A, PG2 organisms increased by 1.2-log over the first 93 days and subsequently decreased by an order of magnitude during the remainder of the study. In the control column, the abundance of PG2 organisms remained steady in the surface soil and increased by 1.3-log over time at Port A (Fig. 6b). These trends in PG2 abundance corresponded well with the consumption of oxygen demand in the upper region of the biostimulated column. Based on an average oxygen demand of 6.2 mg O_2/g soil, oxygen demand of the soil above Port A was exhausted after ~200 days, which coincides with the initial decline in PG2 concentration after 184 days (Fig. 6). Likewise, the gradual decrease in PG2 abundance in the surface soil might reflect a lack of available carbon to sustain growth (Bouchez et al. 1995; Volkering et al. 1992). In separate desorption experiments, the residual PAHs were found to be significantly less available in the biostimulated soil than in the control at Port A after 534 days (Richardson and Aitken 2011).

The most significant changes in PG2 organisms, however, were observed in the lower section of both columns (Ports B and C) where the abundance of PG2 organisms increased by at least two orders of magnitude by day 534 (Fig. 6). These observed increases in PG2 abundance occurred ahead of the advancing oxygen fronts for the respective columns, suggesting that bacterial transport from the upper region of each column may contribute to

PG2 establishment with depth, particularly in the control column where the lower region was anoxic over the course of the study. Alternatively, oxygen introduced from extrusion of soil samples at these ports may have stimulated PG2 growth between sampling events; however, this seems unlikely since a similar rise in PG2 abundance was not observed at Port C of the biostimulated column during the early sampling events. Anaerobic metabolism of other available substrates or biodegradation intermediates by PG2 bacteria is also a potential explanation for their detection at greater depths, although we would have expected substantial amounts of these organisms to be present during the equilibration phase if this were the case. Nevertheless, no representative of PG2 has yet been isolated in pure culture and, therefore, its physiology remains unknown.

Spearman rank analyses also identified a number of significant correlations between PG2 abundance and individual PAH concentrations in both columns (Supporting Information, Table S3). In the biostimulated column, increases in PG2 abundance were negatively correlated to PAH concentration at Ports A, B and C, with significant correlations ($r_s < -0.77$, $p < 0.1$) identified for three- and four-ring PAHs at Ports B and C. Significant correlations were identified at Port C in the control column for most PAHs (except naphthalene); however, these results were influenced by the unexpected drop in PAH concentration during the fifth sampling event (380 days) (Supporting Information, Figure S5). Ringelberg et al. (2001) also identified significant relationships (using Spearman rank analyses) between individual PAH concentrations and PAH-degrading bacteria in bioreactor-treated sediment. Specifically, reductions in three-ring PAHs correlated well with variations in abundance of PLFA-defined genera and genes encoding PAH-degrading enzymes over the three-month treatment period.

Phenanthrene and pyrene have been previously identified as growth substrates for PG2 organisms (Singleton et al. 2005, 2007) and recent SIP analyses of the original MGP soil have also implicated PG2 in the degradation of fluoranthene and benz[*a*]anthracene (Jones et al. 2011). Although Spearman rank analyses support a relationship between PG2 abundance and PAH concentrations, the exact role of PG2 organisms in the degradation of any single PAH cannot be directly inferred from the available data. Along with PG2 organisms, other PAH-degrading bacteria in the mixed column soil community may also be capable of growth on a given PAH.

Conclusions

In this study, soil PAH concentrations and spatial and temporal variations in the quantity of a group of bacteria known to be PAH degraders were strongly correlated to oxygen advancement over time, which underlines the importance of maintaining oxygenated conditions for PAH biodegradation. Bacterial transport was also implicated as a factor in the establishment of PAH-degrading bacteria ahead of the oxygen front. The potential for migration of PAH-degrading bacteria and their rapid response to oxygen are important findings relevant to the biodegradation of mobilized PAHs within zones of subsurface contamination.

Even though substantial PAH degradation was achieved in this study as a result of continuous biostimulation, time scales for significant PAH removal were generally long, greater than 380 days for some four- and five-ring PAHs. Significant reductions in the six-ring benzo[*g*,*h*,*i*]perylene were not observed even after 534 days of biostimulation. Despite the presence of an established PAH-degrading community and available oxygen and nutrients, PAH concentrations appeared to approach a threshold over time, reflecting limitations in PAH mass transfer and bioavailability. Oxygen transport was also governed by the oxygen demand of the column soil, resulting in slow DO penetration into deeper regions of the column. It is clear from these results that careful consideration of matrix-specific factors such as oxygen demand, PAH bioavailability, and the quantity of PAH-degrading bacteria is essential for the successful design and implementation of in situ bioremediation strategies in weathered contaminated soils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Randall Goodman and Glenn Walters for their help in the design and construction of the columns. We also thank Dr. Wei Sun and Joe Rigdon of the UNC Department of Biostatistics for assistance with statistical analyses. This work was supported by the National Institute of Environmental Health Sciences (Grant No. 5 P42 ES005948).

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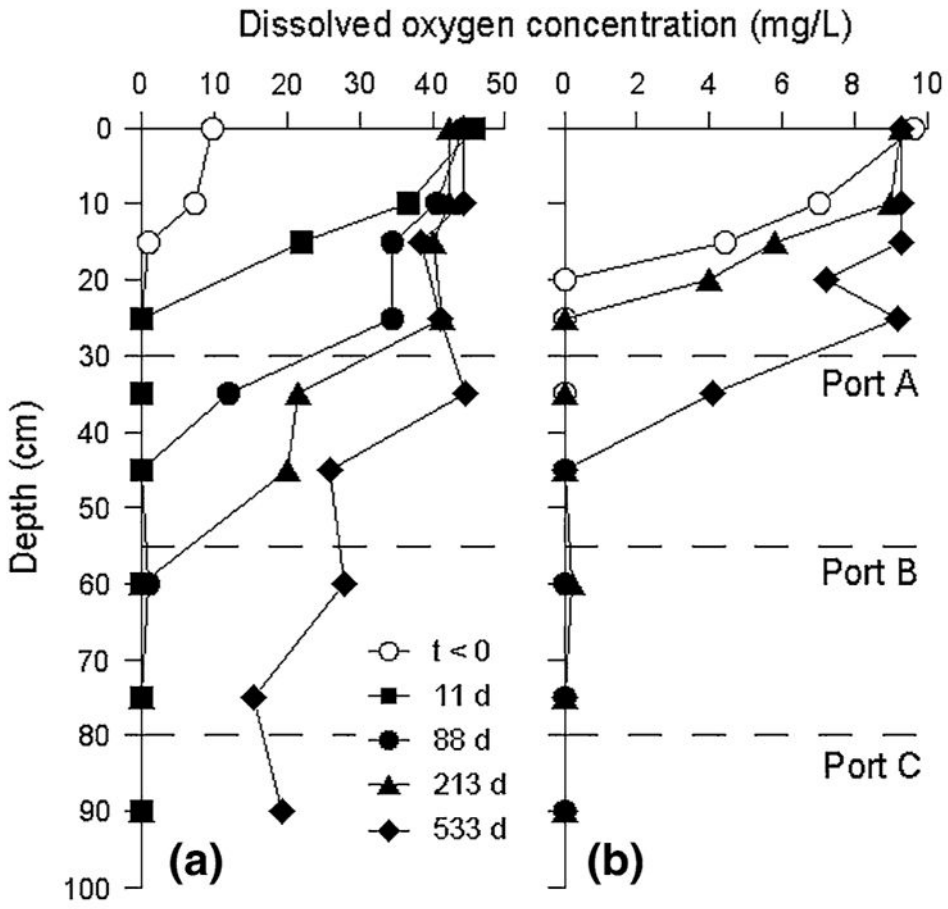

Fig. 1.

DO profiles of the biostimulated (**a**) and control (**b**) columns over time. Oxygen measurements at the end of the equilibration phase ($t < 0$) are denoted with *open circles*. Oxygen concentrations at the *top* of each *column* (depth = 0) were measured in the feed groundwater reservoir. Data for time points other than those shown are omitted for clarity. *Dashed lines* indicate the location of the soil sample ports. Note the different scales on the *x*-axes for the two *panels*

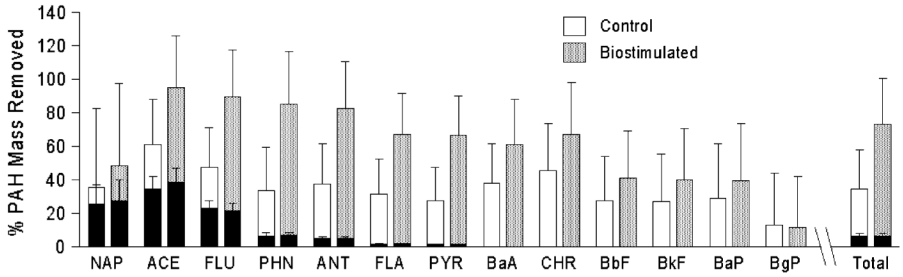

Fig. 2.

Percentage of PAHs removed from the biostimulated and control *columns*. Removal of a PAH by dissolution alone is represented by the *black bars*. Error associated with the percent removal values was the result of sample variability of the column soil and propagation of error in calculating total mass and percent removal of each PAH in the *columns*

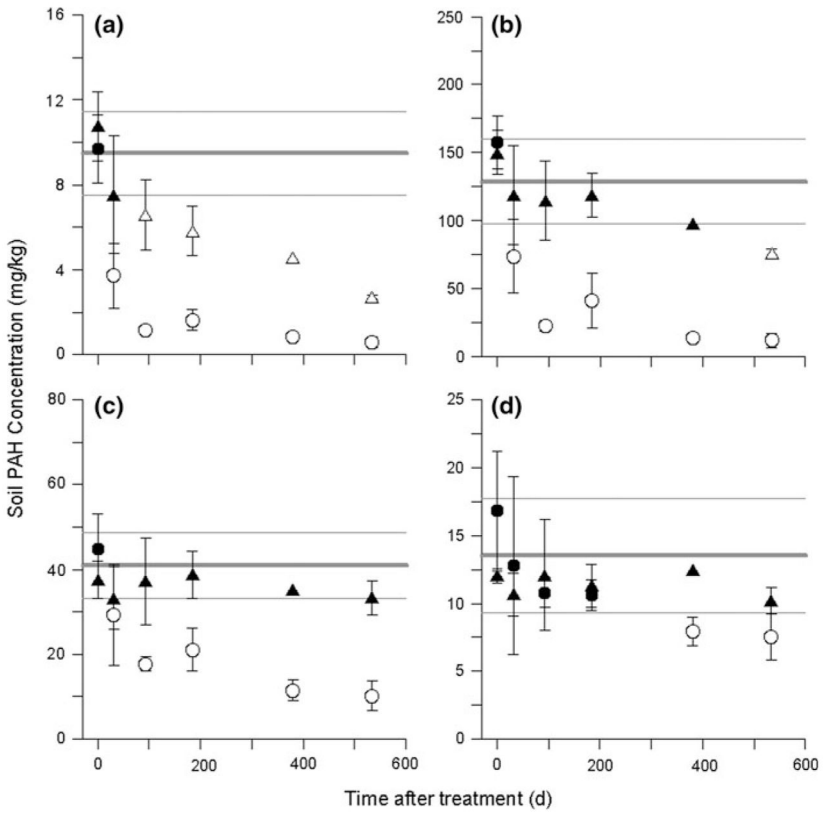

Fig. 3.

Time courses of fluorene (**a**), phenanthrene (**b**), pyrene (**c**), and benzo[*a*]pyrene (**d**) concentrations for soil samples collected from Port A of the biostimulated (*filled circle*, *open circle*) and control (*filled triangle*, *open triangle*) columns. Each *point* represents the mean value of duplicate or triplicate samples and the *error bars* are the range or standard deviation, respectively. An *open symbol* represents an analyte concentration that is significantly different ($p < 0.05$) from the initial column soil concentration. *Horizontal lines* represent the mean and standard deviation of the PAH in the original column soil (Table 1)

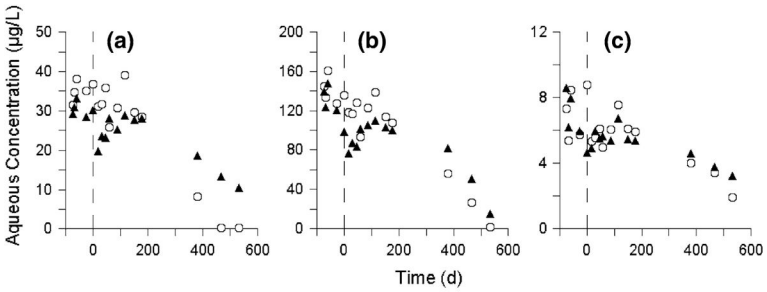

Fig. 4.

Fluorene (**a**), phenanthrene (**b**), and pyrene (**c**) concentrations in effluent samples collected from the biostimulated (*open circle*) and control (*filled triangle*) columns over time. Time 0 (depicted with the vertical *dashed line*) refers to the time from samples collected during the equilibration phase. Concentrations of BaA, CHR, BbF, BkF, BaP, DBA, and BgP were below detection. Abbreviations are as in Table 1

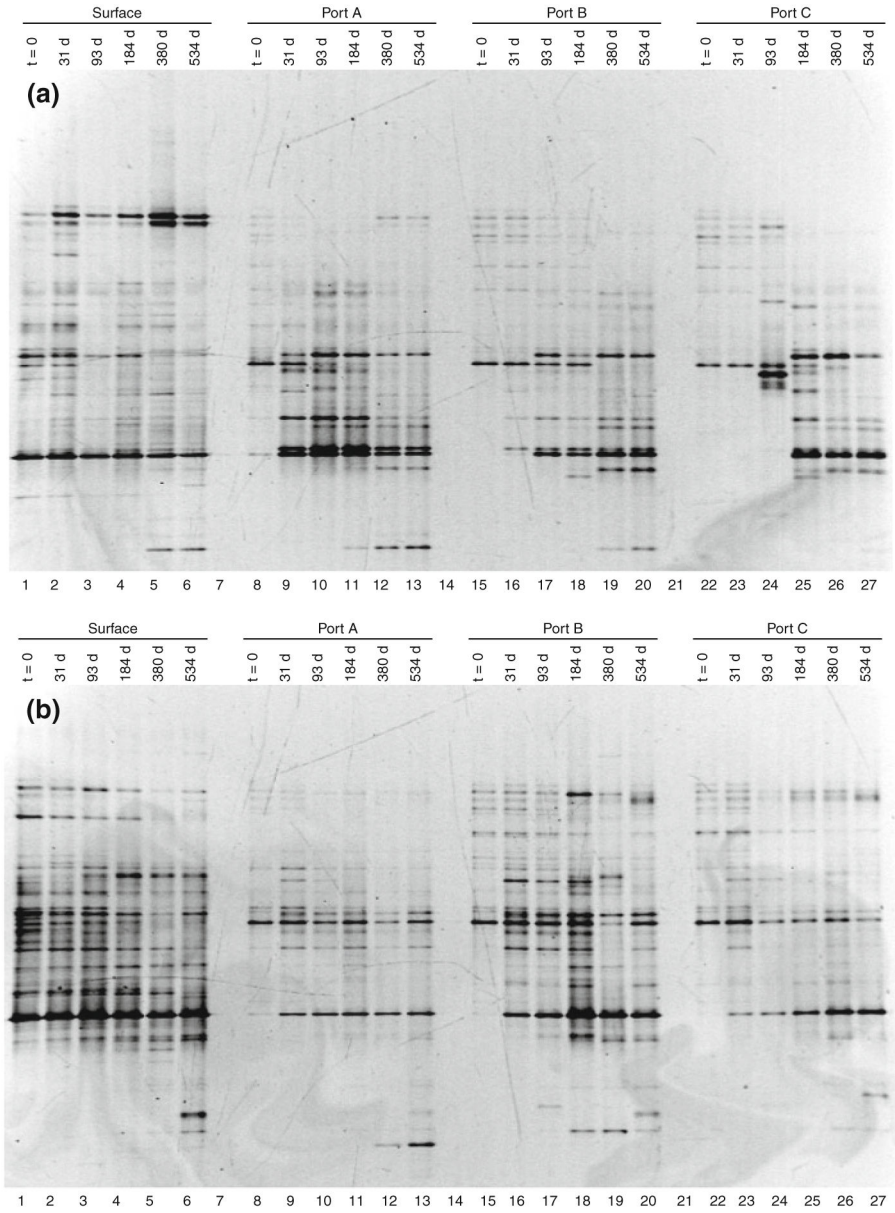

Fig. 5.

Negative image of DGGE gels for the biostimulated (**a**) and control (**b**) columns delineating 16S rRNA gene profiles before biostimulation commenced ($t = 0$) and at the indicated sampling times after treatment at the surface soil (*lanes 1–6*), Port A (*lanes 8–13*), Port B (*lanes 15–20*) and Port C (*lanes 22–27*). *Lanes 7*, *14*, and *21* were left blank for clarity

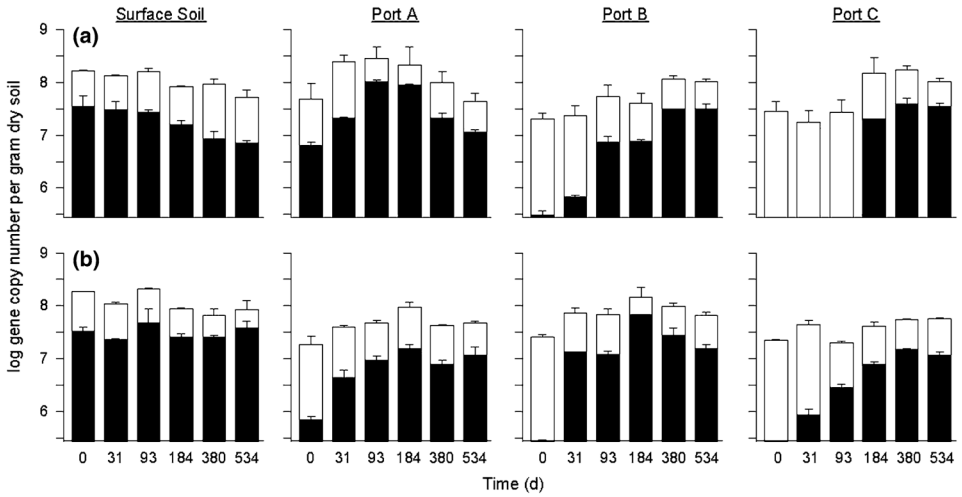

Fig. 6.

Abundances of 16S rRNA genes for total bacteria (*white bars*) and group PG2 (*black bars*) in the surface soil and at Ports A–C for the biostimulated (**a**) and control (**b**) columns over time. *Values* represent the log-transformed mean and range of duplicate qPCR runs of pooled DNA extracts. The minimum value on the *y*-axis is the average detection limit for all primer sets (3×10^5 genes/g). Absent values were below detection

Table 1

Initial PAH concentrations in the column soil

PAH	No. of rings	Concentration (mg/kg)[a]
Naphthalene (NAP)	2	9.5 ± 4.2
Acenaphthene (ACE)	3	11.9 ± 2.7
Fluorene (FLU)	3	9.5 ± 2.0
Phenanthrene (PHN)	3	129 ± 31
Anthracene (ANT)	3	10.5 ± 2.2
Fluoranthene (FLA)	4	25.2 ± 5.0
Pyrene (PYR)	4	40.9 ± 7.8
Benz[*a*]anthracene (BaA)	4	13.8 ± 3.0
Chrysene (CHR)	4	14.0 ± 3.6
Benzo[*b*]fluoranthene (BbF)	5	6.9 ± 1.7
Benzo[*k*]fluoranthene (BkF)	5	4.2 ± 1.1
Benzo[*a*]pyrene (BaP)	5	13.5 ± 4.2
Benzo[*g*,*h*,*i*]perylene (BgP)	6	5.3 ± 1.6
Total		295 ± 65

[a]Values are presented as mean ± standard deviation (n = 33)

Table 2

Percentage of PAH mass removed by dissolution, sampling, and biodegradation in the biostimulated column after 534 days

PAH[a]	Percent of PAH mass removed			
	Total	Dissolution	Sampling	Biodegradation[b]
NAP	48.5 ± 49.0	27.5 ± 12.1	12.9 ± 5.7	8.1 ± 50.8
ACE	95.1 ± 31.1	38.3 ± 8.6	4.3 ± 1.1	52.5 ± 32.3
FLU	89.3 ± 28.1	21.5 ± 4.5	5.8 ± 1.3	62.0 ± 28.4
PHN	84.9 ± 31.8	6.6 ± 1.6	7.1 ± 1.8	71.2 ± 31.9
ANT	82.4 ± 27.9	4.9 ± 1.0	7.4 ± 1.7	70.1 ± 28.0
FLA	67.1 ± 24.5	1.6 ± 0.3	10.5 ± 2.2	55.0 ± 24.6
PYR	66.6 ± 23.5	1.2 ± 0.2	10.3 ± 2.1	55.1 ± 23.6
BaA	60.9 ± 27.1	*c*	12.5 ± 2.9	48.5 ± 27.3
CHR	67.0 ± 31.2	*c*	14.6 ± 3.8	52.3 ± 31.4
BbF	40.8 ± 27.9	*c*	16.0 ± 4.1	24.7 ± 28.2
BkF	40.1 ± 30.2	*c*	15.7 ± 4.4	24.4 ± 30.5
BaP	39.2 ± 34.1	*c*	16.1 ± 5.1	23.0 ± 34.5
BgP	11.0 ± 31.0	*c*	22.6 ± 6.9	NA
Total	73.0 ± 27.7	6.5 ± 1.4	9.5 ± 2.2	56.9 ± 27.8
3-Ring[d]	85.7 ± 31.4	9.7 ± 2.3	6.8 ± 1.7	69.1 ± 31.5
4-Ring[d]	66.0 ± 24.5	*c*	11.3 ± 2.4	54.6 ± 24.7
5-Ring[d]	40.7 ± 32.0	*c*	15.8 ± 4.7	24.8 ± 32.3

NA not applicable (calculated value was negative)

[a] See Table 1 for definitions of abbreviations

[b] Calculated by subtracting removal by dissolution and removal by sampling from the total removal and accounting for error propagation

[c] Value was below detection

[d] Represents the sum of the mass removed for PAHs with the same number of rings (Table 1); NAP and BgP are not included in these values

Table 3

Spearman rank coefficients (r_S) representing significant correlations between individual PAH concentrations (mg/ kg) and DO concentration (mg/L) in the biostimulated column

PAH[a]	Spearman rank coefficients (r_s) with DO concentration[b]		
	Port A	Port B	Port C
ACE			**−0.85**
FLU	*−0.71*		**−0.78**
PHN	*−0.71*		**−0.78**
ANT	*−0.71*	**−0.76**	**−0.78**
FLA	*−0.71*	**−0.88**	**−0.78**
PYR	*−0.71*	**−0.76**	**−0.78**
BaA	**−0.83**	**−0.76**	**−0.82**
CHR	**−0.83**	**−0.76**	**−0.85**
BbF	*−0.71*		
BaP	**−0.83**		
BgP	**−0.77**		

[a] Spearman rank coefficients for NAP, BkF, and DBA were not significant at all sample locations and are, therefore, omitted from the table; see Table 1 for definitions of abbreviations

[b] Only r_S values representing correlations with $p < 0.15$ are reported ($p < 0.05$ in bold and underlined; $0.05 < p < 0.1$ in bold; $0.1 < p < 0.15$ in italics); r_S values with $p > 0.15$ are omitted for clarity; no significant correlations were identified in the surface soil