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Pyrosequence analyses of bacterial communities during simulated *in situ* bioremediation of polycyclic aromatic hydrocarbon-contaminated soil

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Abstract

Barcoded amplicon pyrosequencing was used to generate libraries of partial 16S rRNA genes from two columns designed to simulate *in situ* bioremediation of polycyclic aromatic hydrocarbons (PAHs) in weathered, contaminated soil. Both columns received a continuous flow of artificial groundwater but one of the columns additionally tested the impact of biostimulation with oxygen and inorganic nutrients on indigenous soil bacterial communities. The penetration of oxygen to previously anoxic regions of the columns resulted in the most significant community changes. PAH-degrading bacteria previously determined by stable-isotope probing (SIP) of the untreated soil generally responded negatively to the treatment conditions, with only members of the *Acidovorax* and a group of uncharacterized PAH-degrading Gammaproteobacteria maintaining a significant presence in the columns. Additional groups of sequences associated with the Betaproteobacterial family Rhodocyclaceae (including those associated with PAH degradation in other soils), and the *Thiobacillus*, *Thermomonas*, and *Bradyrhizobium* genera were also present in high abundance in the biostimulated column. Similar community responses were previously observed during biostimulated *ex situ* treatment of the same soil in aerobic, slurry-phase bioreactors. While the low relative abundance of many SIP-determined groups in the column libraries may be a reflection of the slow removal of PAHs in that system, the similar response of known PAH-degraders in a higher-rate bioreactor system suggests that alternative PAH-degrading bacteria, unidentified by SIP of the untreated soil, may also be enriched in engineered systems.

Keywords

pyrosequencing; polycyclic aromatic hydrocarbons; bioremediation

Introduction

Physical and chemical methods are often used for the cleanup of polycyclic aromatic hydrocarbon (PAH)-contaminated sites (Gan et al. 2009; U. S. Environmental Protection Agency 2007; U.S. Environmental Protection Agency 2004), but engineered systems that rely on bioremediation are a potentially more cost-effective method for the treatment of soils (Gan et al. 2009). However, a wide variety of variables can impact the success of a biological treatment process. For example, in a system such as *ex situ* treatment of PAH-

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contaminated soil in bioreactors, multiple factors that affect PAH removal have been examined, including enhancement of PAH availability with solvents or surfactants (Dean-Ross 2005; Di Gennaro et al. 2008; Rehmann et al. 2008; Wang et al. 2010), oxygen concentration (Venkata et al. 2008), bioaugmentation (Dean-Ross 2005), loading rate (Dean-Ross 2005; Singleton et al. 2011), chemical oxidation (Gryzenia et al. 2009), temperature (Antizar-Ladislao et al. 2006), and amendment with carbon sources (Antizar-Ladislao et al. 2006). Ultimately, the success of any bioremediation method will be predicated on the presence and activity of microorganisms expressing a desired phenotype within the system. It is important to understand the effect of controllable variables on the abundance and activity of those specific organisms in order to maximize a system's PAH-removal capabilities.

Few studies have identified specific members of a microbial consortium that are actively degrading PAHs in contaminated soils within the confines of an engineered system. Indeed, identifying which members of a community may be involved in the process is a difficult task for any number of reasons. A wide variety of organisms are capable of PAH degradation, and it is unlikely that all such organisms have been identified. Communities in soil are particularly difficult to study given the high diversity of microbes in that environment, and PAH-degraders may represent only a small fraction of those communities. Finally, subtle changes in environmental conditions may differentially affect PAH-degraders.

We have previously attempted to overcome some of these issues by identifying organisms involved in the degradation of PAHs ranging from two to four rings in contaminated soil with DNA-based stable-isotope probing (SIP), a molecular technique that does not rely on the cultivation of organisms (Jones et al. 2011a; Jones et al. 2011b; Singleton et al. 2005; Singleton et al. 2006). We have also examined the effect of various environmental conditions on the abundances of these known PAH-degrading groups, generally by quantitative real-time PCR (qPCR) (Richardson et al. 2011; Zhu et al. 2010). While effective, this method is complicated by the need for specific qPCR primers for each group of target organisms and the time and expense of analyzing multiple samples.

An alternative approach, and the one utilized in this study, is to analyze the total bacterial community and then examine it for known or identified PAH-degrading organisms. Until recently this approach was infeasible for most applications, but the emergent technology of barcoded amplicon pyrosequencing now allows for the recovery of large numbers of microbial sequences from multiple environmental samples simultaneously (Hamady et al. 2008; Liu et al. 2007). We previously used this technique to examine the communities in aerobic, lab-scale, slurry-phase bioreactors treating PAH-contaminated soil from a former manufactured-gas plant (MGP) site (Singleton et al. 2011). In that study we were able to quantify the relative abundances of bacterial 16S rRNA gene sequences that were similar to those recovered from SIP experiments on the untreated soil material (Jones et al. 2011a; Jones et al. 2011b).

The same PAH-contaminated soil treated *ex situ* in the earlier bioreactor study was also treated under simulated *in situ* conditions in lab-scale, flow-through columns that received either simulated groundwater (a control column) or simulated groundwater amended with inorganic nutrients and oxygen (a biostimulated column) (Richardson et al. 2012). While biodegradation accounted for only 18% of total PAH removal in the control column over 534 days, 57% of the total PAHs were removed by biodegradation in the biostimulated column (Richardson et al. 2012). Much of the success of the biostimulated column was attributed to the penetration of oxygen throughout the column profile.

We examined the bacterial communities of the column systems by barcoded amplicon pyrosequencing. Of particular interest were whether biological treatment of the same soil in the columns and the bioreactors resulted in similar bacterial communities, how *in situ* treatment affected previously determined groups of PAH-degrading bacteria, and how the presence of oxygen and amended nutrients affected the column communities.

Methods and Materials

Summary of treatment systems

The design and operation of each of the column treatment systems has been described previously (Richardson et al. 2012; Richardson et al. 2011), however, a brief summary is provided below. Each of two stainless steel columns (Figure 1) was packed with a 50:50 mixture of soil excavated from a former MGP site in Salisbury, NC, USA (Richardson et al. 2011) and sterile sand. A simulated groundwater based on historical data from the area was pumped into each of the columns for eight months at 20°C during an equilibration period. At the end of this period, the inlet groundwater for the biostimulated column was amended with NH_4NO_3 and K_2HPO_4 to provide N and P concentrations of 1.0 and 0.3 mg/L, respectively, and was continuously sparged with pure oxygen; the control column continued to receive unamended groundwater saturated with air for the duration of the study. The dissolved oxygen concentration was monitored through ports spaced throughout the length of the columns. Soil samples taken 0, 31, 93, 184, 380, and 534 days after the end of the equilibration period from the surface soil and ports spaced 30, 55, and 80 cm below the top of the columns (designated ports A, B, and C, respectively; Figure 1) were used for PAH quantification and analyses of the bacterial communities.

Sample selection

Soil samples for pyrosequence analysis from the control and biostimulated columns were selected with a particular interest in samples collected before and after transitions from an anoxic to oxic state at the various sample ports. Bacterial 16S rRNA genes from DNA extracted from soil samples from the day 0 time point (at the end of the equilibration phase but prior to the implementation of biostimulation) and at days 93, 380, and 534 were partially sequenced. Samples from port C of the control column for all time points and the surface soil of both columns at day 534 were not analyzed.

DNA extraction was performed on triplicate 0.5–0.7 g (wet wt.) aliquots of soil collected as previously described (Richardson et al. 2011). The mass of DNA in each extract was measured using a NanoDrop ND-3300 Fluorospectrometer (ThermoScientific; 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.

Pyrosequencing and analyses

Barcoded amplicon pyrosequencing was performed using a 454 Life Sciences GS FLX Titanium platform as previously described (Singleton et al. 2011). Libraries were processed using mothur v.1.22.2 (Schloss et al. 2009) and analyses followed the “Schloss standard operating procedure (SOP)” (http://www.mothur.org/wiki/Schloss_SOP). The analysis included quality control of the sequences, grouping of sequences into operational taxonomic units (OTUs), diversity analyses, and classification of the OTUs. Sequences were organized into OTUs using a distance criterion of 0.03. Community comparisons within mothur were processed using libraries randomly reduced to the smallest library (560 sequences); the calculation of relative abundance and quantification of selected bacterial groups was performed considering all available sequences. The quantification of selected bacterial

groups (e.g., “Anthracene Group 1” and “Pyrene Group 2”; AG1 and PG2) and abundant OTUs was performed using local BLAST searches against representative sequences as identified by mothur to identify OTUs with significant similarity (≥ 97% to the query sequence) as previously described (Singleton et al. 2011). Alignments for phylogenetic trees were generated with ClustalX 2.1 (Thompson et al. 1997) and trimmed using GBlocks to allow direct comparison of GenBank sequences to the shorter pyrosequences (Talavera and Castresana 2007). Bootstrapped neighbor-joining trees (1000 replicates) were generated using ClustalX.

Results

Summary of pyrosequencing

A total of 305,780 sequences were obtained from the 454 sequencing run. Of 19,681 unique sequences analyzed, the UCHIME implementation in mothur (Edgar et al. 2011) detected 5,021 potential chimeras. The chimeric sequences, those that were shorter than 225 bases, sequenced from the reverse primer, or were otherwise unsuitable were removed from further analysis. A total of 76,131 sequences were retained for final analyses. The smallest library (the biostimulated column, port B, day 0) contained 560 sequences, while the largest library (the control column, surface, day 93) contained 8,423 sequences (Table 1). The mean number of sequences and standard deviation for all libraries was 2,719 ±1,628 and the median was 2,600 sequences.

Diversity analyses of column libraries

The soil samples represented a range of conditions based on depth in the columns, biostimulation with oxygen and inorganic nutrients, and oxygen penetration (Table 1). Bacterial diversity was generally the highest at the surface of both columns, which had been exposed the longest to oxygen (Table 1). Lower diversity was observed for most of the anoxic and lower port samples, comparable to that of the untreated soils. Interestingly, even the samples exposed to oxygen early in treatment (e.g., port A of the biostimulated column) exhibited lower diversity than the surface samples at days 93 and 380.

Principal coordinate analyses (PCoA) of the libraries indicated clear differences in the 16S rRNA gene libraries derived from oxic and anoxic regions of the soil columns (Figure 2). The PCoA analyses additionally indicated similarity between libraries created from the untreated soil and samples from the initial sampling event (day 0) for the lower, subsurface regions of the columns, as well as similarity between soil regions exposed to oxygen the longest, generally the surface samples and the uppermost region (port A) of the biostimulated column. The library created from port C of the biostimulated column at 534 days also clustered with this latter group; its grouping with samples taken closer to the surface was attributed to oxygen penetration through the bottom of the column by the 534-d sampling event (note that port C of the control column was not examined by pyrosequencing). Variability between samples due to heterogeneity of the soil and resultant bacterial communities may have influenced the clustering of some samples. For example, the 0-d samples from the control and biostimulated columns were dissimilar in this analysis; this can likely be attributed to the large number of sequences associated with the *Thiobacillus* genus in the biostimulated column (see below).

Impact of column incubation on known PAH-degraders

PAH-degrading bacteria in the untreated, contaminated soil used to pack the columns were previously identified through DNA-SIP with six PAHs ranging from two to four rings: naphthalene (NAP; 2 rings), phenanthrene (PHN; 3 rings), anthracene (ANT; 3 rings), benz[*a*]anthracene (BAA; 4 rings), fluoranthene (FLA; 4 rings), and pyrene (PYR; 4 rings)

(Jones et al. 2011a; Jones et al. 2011b). The relative abundances of sequences in column pyrosequence libraries to these SIP-determined PAH- degraders were calculated based on high similarity to representative sequences of OTUs, considering all available sequences (Table 2).

Only two bacterial groups previously associated with PAH degradation in the untreated soil maintained a significant presence during column operation or increased in abundance (Table 2). The most significant group of 16S rRNA gene sequences associated with PAH degradation in the columns were highly similar to uncharacterized Gammaproteobacterial sequences previously designated “Pyrene Group 2” (PG2; Table 2) which have been associated with the degradation of phenanthrene, pyrene, fluoranthene, and benz[*a*]anthracene (Jones et al. 2011b; Singleton et al. 2007; Singleton et al. 2006). Although sequences from this group were poorly represented in libraries constructed from the untreated soil (average relative abundance of 0.8%; n = 2), they were well-represented in libraries from both columns. Sequences related to PAH-degrading *Acidovorax* (associated with the degradation of NAP and PHN) averaged 2.8% of the libraries derived from untreated soil and were particularly abundant in both columns at the day 0, post-equilibration sampling event (maximum relative abundances of 13% - 15%). *Acidovorax* sequences remained significant for all examined ports at day 93 (1.4% – 7.7%), but decreased in relative abundance in all ports at day 380, and at the final time point (534 days) were a significant percentage of libraries only for ports A and B of the control column (2.3% – 3.7%).

Data on other sequences in the column libraries previously associated with PAH-degradation in the untreated soils are additionally provided in Table 2. A group of naphthalene-degraders associated with the genus *Achromobacter* displayed low abundance in the untreated soil (0.1 – 0.2% of those libraries), but comprised up to 1.4% of the column libraries from the day 0 (post-equilibration) samples, particularly in the lower, anoxic ports. However, by the next examined sampling point (day 93), sequences associated with this group had decreased to less than 0.5% of the libraries and did not increase in any subsequent samples. Sequences similar to anthracene-degrading *Variovorax* species were poorly represented in the untreated soil and represented a maximum of 1.4% of any library at the day 0 sample point. *Varioxorax*-associated sequences rose to a maximum of 1.0% and 5.2% of surface soils in the control and biostimulated columns, respectively. They were particularly well represented (4.0% – 5.2%) at the surface of the biostimulated column at the day 93 and 380 time points (the surface communities were not examined at day 534), but similar to *Achromobacter* sequences were not abundant in lower regions of both columns after the equilibration period. That sequences from these groups were at low abundance below the column surface (particularly the biostimulated column) after the equilibration phase suggests that they were not from organisms that were among the most active or dominant PAH-degraders in these systems.

Some of the known PAH-degrading groups displayed a pattern of high initial abundance in the untreated soil followed by a substantial decrease during column operation. Two groups in particular, composed of sequences linked to the *Sphingobium* (associated with growth on FLA) and *Pseudoxanthomonas* (associated with growth on NAP and ANT) genera, were very highly represented in libraries constructed from untreated soil, with an average relative abundance of 19% and 31%, respectively. *Pseudoxanthomonas*-related sequences remained in high abundance in anoxic regions of the soil columns after the equilibration phase, but steadily decreased in abundance during column operation, particularly in the biostimulated column (Table 2).

Sequences associated with *Sphingobium* were not abundant in the columns in samples taken after the equilibration period (Table 2). Sequences associated with "Anthracene Group 1" (AG1), a group of uncharacterized anthracene-degrading bacteria within the order Sphingomonadales, comprised an average of 4.4% of the untreated soil libraries but were not detectable in most pyrosequence libraries at any subsequent time points. Similarly, additional groups within the *Sphingobium* and *Pseudomonas* genera, which were both associated with naphthalene degradation and whose 16S rRNA genes comprised an average of 4.9% and 3.9% of the untreated soil communities, respectively, displayed decreasing abundances in samples retrieved during column operation. As with other bacterial groups that did not maintain a significant presence in the columns, it is unlikely that these organisms were dominant PAH-degraders in the columns.

Other bacterial groups identified by SIP as PAH-degraders had low abundance in every sample, including the untreated soil. These included groups of sequences associated with the genera *Pigmentiphaga* (associated with growth on NAP, PHN, and ANT), *Pseudomonas* (NAP), *Sphingomonas* (FLA), and *Rhizobium* (ANT).

Other significant bacterial groups in the biostimulated column

Three of the most abundant groups of sequences in the column systems that were not previously associated with PAH metabolism by DNA-SIP of the untreated soil were uncharacterized Betaproteobacteria within the family Rhodocyclaceae (Table 3). While none of these Rhodocyclaceae OTUs were explicitly identified as PAH-degraders in this soil, OTU-59 was very closely related (>99% 16S rRNA gene similarity) to sequences recovered during DNA-SIP with [U-^{13}C]pyrene of an MGP soil from Charlotte, NC designated "Pyrene Group 1" (PG1; Figure 3) (Singleton et al. 2006); those PG1 organisms were also shown to grow on phenanthrene (Singleton et al., 2007). The other two groups of sequences (OTU-5 and OTU-70) also possessed high similarity to PG1 sequences and to environmental sequences recovered from hydrocarbon-contaminated environments but not to any characterized isolates (Figure 3). OTU-5 sequences were particularly abundant in the control column (up to 38% of sequences in one library) and lower portions of the biostimulated column after the start of biostimulated conditions (Table 3). Sequences most closely related to PG1 (OTU-59) were primarily associated with the oxygen front in the biostimulated column. However, these sequences were not particularly abundant in libraries derived from the control column, which suggests a positive response of organisms associated with this group to the amended nutrients and oxygen of the biostimulated column. Sequences in OTU-70 appeared to be primarily associated with samples from the control column; therefore organisms associated with this OTU may have been better adapted to either the anoxic and/or oligotrophic conditions generally found in that column. Libraries constructed from the untreated soil in this experiment did not contain any sequences from two of these three unclassified Rhodocyclaceae OTUs, and the one OTU that was detected (OTU-5) comprised an average of only 0.1% of the untreated soil libraries.

Several groups of sequences in the columns were associated with the *Thiobacillus* genus, including one (OTU-1) which comprised 38% of all sequences from port A of the biostimulated column at day 0 (Table 3). It is unclear why these sequences were abundant in the biostimulated column but not the control column, as both columns had been operated under the control conditions prior to the day 0 sampling event. Their presence may represent a localized and ephemeral enrichment of that group of organisms, perhaps related to inevitable micro-heterogeneity of the soil used to fill the columns. OTU-1 sequences declined substantially in the Port A sample at day 93 in the biostimulated column, but were most abundant at the downstream ports B and C of the biostimulated column at day 93 (6–7% of the libraries); this observation suggests transport of the organisms represented by OTU-1 to the lower parts of the column. The abundance of these sequences dropped to 1–

2% throughout the column for the remainder of the experiment, suggesting that these organisms were transient. Another group of *Thiobacillus*-associated sequences (OTU-100) were primarily associated with the anoxic sections of the control column after the equilibration phase and maintained relative abundances of 4–8% in those libraries. Two additional *Thiobacillus*-associated groups (OTU-3 and OTU-7) were primarily associated with the surfaces of both column systems but were found at lower abundances at the lower ports as well.

Bacteria represented by other OTUs appeared to respond favorably to conditions in the biostimulated column (Table 3). Sequences associated with the *Bradyrhizobium* genus (OTU-20) comprised 2–7% of sequences at the surface of the column and up to 7% of sequences in lower ports during column operation. Subsequent to the day 0 sampling event, sequences associated with the genus *Thermomonas* (OTU-461) were highly enriched (3–36%) below the surface of the biostimulated column, particularly at port A (20–36%; Table 3). The fact that these sequences were not detected anywhere in that column prior to day 93 and were rarely encountered in the control column libraries suggests that biostimulation was required for their growth.

Discussion

Prior work on the columns analyzed in this study indicated oxygen penetration was positively correlated with PAH biodegradation (Richardson et al. 2012), and it now appears to be a prime factor in determining the bacterial community composition as well. Principal coordinate analyses of the 16S rRNA gene clone libraries derived from various regions of the columns showed a clear differentiation between oxic and anoxic samples, with an exception being the oxic sample from port A of the control column at day 534 (Figure 2). This may be partially explained by the fact that oxygen had just recently penetrated to that point on the control column and the bacterial community may not have had time to significantly change prior to the sampling event. The lack of additional nutrients in the simulated groundwater may also explain in part the slow community response in the control column to advancement of the oxygen front. The most diverse libraries were generated from the surface of both column samples (Table 1). The higher diversity of surface samples may be partially attributable to mixing of the surface soil during sampling as well as the continual exposure to higher concentrations of amended nutrients and oxygen (albeit at lower overall concentrations in the control column).

Prior work examining the result of biostimulated treatment of this MGP soil in lab-scale, aerobic slurry-phase bioreactors indicated that only a few of the many PAH-degrading bacterial groups identified by SIP of the untreated soil appeared to respond favorably to bioremediation under those conditions (Singleton et al. 2011). The organisms that did respond favorably (demonstrating high and persistent relative abundance of 16S rRNA genes) were associated with the *Acidovorax* and *Sphingomonas* genera, as well as members of the uncultivated PG2. Except for the lack of a positive response from members of the *Sphingomonas* genus, the resultant bacterial communities in soils treated in the columns in this experiment (particularly the biostimulated column) shared many similarities to the communities of the bioreactors.

Acidovorax sequences were most commonly associated with phenanthrene degradation in SIP of untreated soil, and as phenanthrene was the most abundant quantified PAH in this soil (Richardson et al. 2012), their presence in the columns and bioreactors was not unexpected. *Acidovorax* 16S rRNA genes were also the most commonly encountered sequences from SIP with phenanthrene of bioreactor-treated, PAH-contaminated soil from a different MGP site in Charlotte, NC (Singleton et al. 2005) and were found in clone libraries

constructed from ^{13}C-enriched DNA derived from phenanthrene in another soil (Martin et al. 2012). The data suggest, however, that after an initial bloom *Acidovorax* organisms in the biostimulated column decreased in abundance after passage of the oxygen front and consumption of phenanthrene and other readily available carbon sources.

Trends in the relative abundance of PG2 organisms determined through pyrosequencing generally followed trends in PG2 total abundance over time at each sample location in each column as measured by quantitative PCR (qPCR) of the same DNA samples in our previous work (Richardson et al. 2012). Increases in abundance of PG2 gene sequences had been correlated to the loss of PAHs, but the increases at some locations in the columns, whether as relative abundance (Table 2) or by qPCR (Richardson et al. 2012), preceded the passing of the oxygen front. Changes in PG2 abundance over time and location in the columns was previously attributed to both growth at the expense of PAHs and transport through the columns (Richardson et al. 2012). As sequences from this group have been associated with growth on a number of PAHs, these sequences may represent one of the dominant PAH-degrading bacterial groups in the columns under the conditions tested.

From these data and from prior work concerning *ex situ* treatment of this same soil in aerobic, slurry-phase bioreactors (Singleton et al. 2011), the majority of SIP-identified bacterial groups determined from analyses of the untreated soil were not particularly abundant under the examined treatment regimens. In the column systems, in which PAH-removal was relatively slow, the low abundance of SIP-identified organisms may not have been a barrier to their significant contribution to the biodegradation of PAHs. However, in the more efficient bioreactors, which removed a higher percentage of PAHs in a matter of days rather than months or years, the low abundance of those same sequences might indicate that they were unlikely to be major contributors to PAH removal within those systems. It is not certain whether the few identified groups that were abundant and persistent (*Acidovorax* and “Pyrene Group 2” in both the columns and bioreactors, as well as *Sphingomonas* organisms in the bioreactors) were responsible for the bulk of PAH removal or whether less abundant groups of SIP-identified PAH-degraders, or entirely different PAH-degrading bacteria unidentified by SIP of the untreated soil, were the most significant contributors. It is certainly possible that SIP analyses or other methods of determining indigenous PAH-degrading bacteria based on untreated, contaminated soil may be relatively poor predictors of the active populations of PAH-degraders in engineered, biological treatment systems.

“Pyrene Group 1” (PG1) sequences were recently identified in the same soil as used in this study after persulfate oxidation and correlated to the recovery of phenanthrene mineralization (Richardson et al. 2011), and similar sequences have also been recovered from heavy DNA extracted from a soil in France enriched with ^{13}C-phenanthrene (Martin et al. 2012). As a number of other environmental sequences closely related to the PG1-related OTUs have been recovered from petroleum and PAH-impacted environments (Figure 3), some members of this uncharacterized group may have contributed to PAH removal in the columns. The low abundances of PG1-related OTUs in the untreated soil prior to biological treatment could explain why these potential PAH-degrading organisms were not previously detected by SIP. However, as little is known of the metabolic potential of this group outside of the sequences directly linked to phenanthrene and pyrene degradation, the extent to which the PG1-related organisms were involved in PAH removal in the column system remains uncertain.

Additionally, while there is no direct evidence in these data to suggest that organisms from abundant groups in the column systems were PAH degraders in this system, some members of the identified genera do have links to PAH removal. For example, *Thiobacillus* organisms have been associated with PAH degradation, particularly of phenanthrene (Bodour et al.

2003; Martin et al. 2012). Although sequences associated with the *Bradyrhizobium* genus were found in abundance in the columns members of this genus are not typically associated with hydrocarbon utilization; there is, however, one report of an isolate removing phenanthrene (Radwan et al. 2007). Sequences associated with this genus were also recently identified by our group during an SIP experiment using ^{13}C- labeled anthracene and bioreactor-treated soil (Dunlevy 2012). Kaplan and Kitts (Kaplan and Kitts 2004) demonstrated that members of the *Thermomonas* genus increased and were likely associated with the degradation of petroleum hydrocarbons in weathered soil subsequent to a period of high microbial activity, but they were not linked to PAH degradation in that study. Alternatively, some *Thermomonas* strains are capable of nitrate and nitrite reduction (Kim et al. 2006) and *Thermomonas* strains have been isolated from a denitrification reactor (Mergaert et al. 2003). The high abundance of sequences associated with this genus in the biostimulated column may be a reflection of the amended ammonium nitrate during column operation. Further experiments are required to determine the extent to which PAH-degrading populations derived from soils treated *in situ* or *ex situ* differ from those identified in untreated soils, and whether any of the abundant groups observed in the column systems can be definitively linked to PAH degradation.

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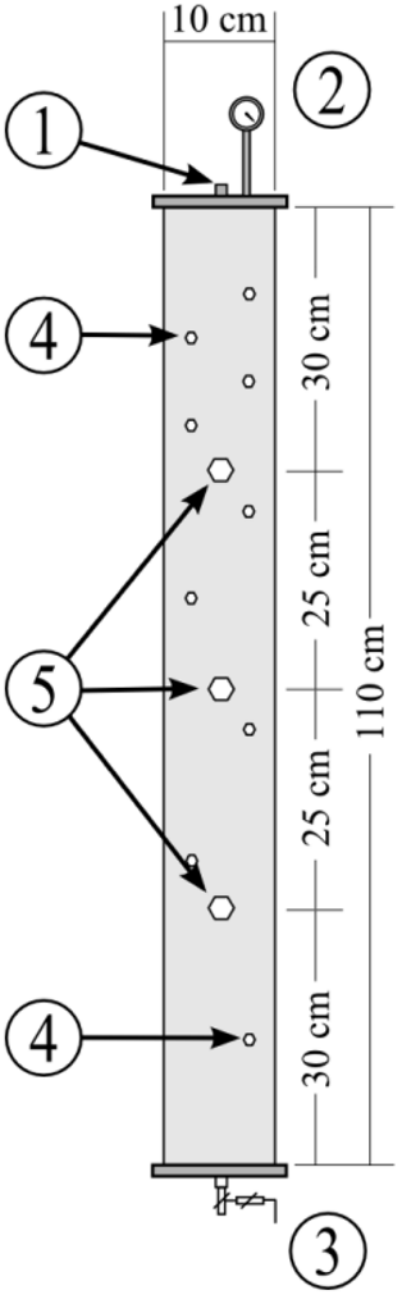

Figure 1.
Simplified schematic of the stainless-steel column treatment systems utilized in this study. Tubes, fittings, pumps, and power supplies are not shown. 1 – groundwater influent port, 2 – inlet pressure gauge, 3 – effluent control valve, 4 – dissolved oxygen monitoring ports, 5 – soil sampling ports A, B, and C (top to bottom, respectively). Adapted from Richardson et al. (Richardson et al. 2012).

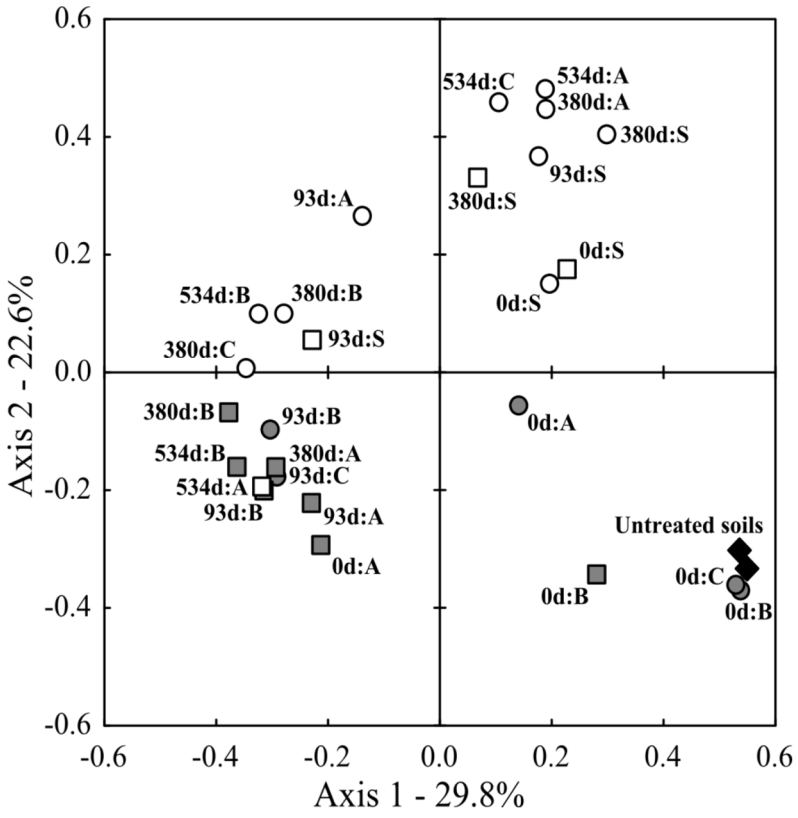

Figure 2.

Principal coordinate analyses (PCoA) of pyrosequence libraries. All libraries were randomly reduced to the size of the smallest library (560 sequences) for comparison. Labels indicate time of sampling (in days) and location in column: S – surface, A – port A, B – port B, C – port C. Symbol shapes and shading are indicative of source and oxygen presence, respectively: diamonds (◇) – untreated soil, circles (○) – biostimulated column, squares (□) – control column. Shaded symbols indicate anoxic conditions and open symbols indicate oxic conditions.

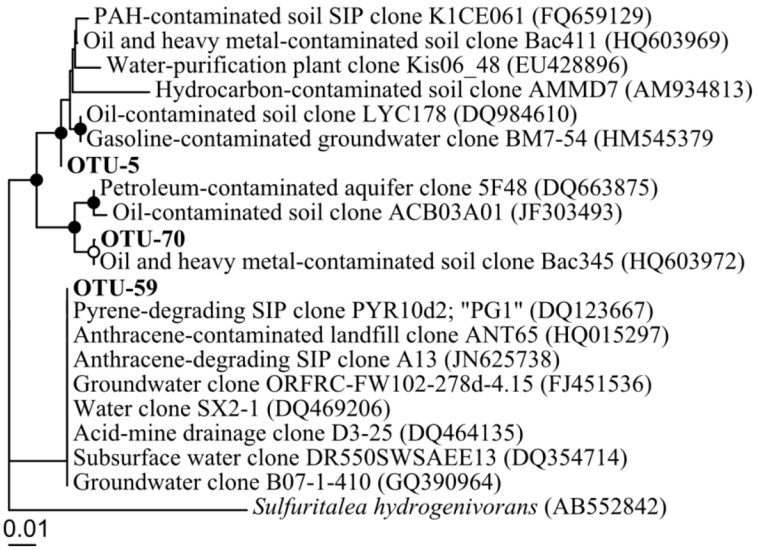

Figure 3.

Neighbor-joining tree showing relationship of uncharacterized members of the family Rhodocyclaceae to GenBank-deposited sequences. Representative sequences from OTUs in this study are in bold. Closed (●) and open (○) circles on nodes indicate ≥50% and ≥95% bootstrap support, respectively. GenBank accession numbers are in parentheses. The tree used *Sulfuritalea hydrogenivorans*, the closest described relative to the sequences, as an outgroup.

Table 1

Summary of column samples and conditions, library sequence data, and diversity estimation.

Sample	Oxic[a]	No. of sequences	No. of OTUs[b]	Inv. Simpson[b,c]
Control column				
Surface				
0 d	+	3779	131	26.5
93 d	+	8423	101	15.8
380 d	+	5014	125	32.3
Port A				
0 d	-	3245	83	7.5
93 d	-	2398	101	13.1
380 d	-	3782	105	10.3
534 d	+	2762	120	11.7
Port B				
0 d	-	3335	94	12.9
93 d	-	3368	113	11.3
380 d	-	2901	92	8.8
534 d	-	1819	118	10.4
Biostimulated column				
Surface				
0 d	+	1396	125	32.1
93 d	+	3479	130	43.3
380 d	+	3184	122	30.5
Port A				
0 d	−	1597	77	5.9
93 d	+	2437	103	11.3
380 d	+	5118	95	7.3
534 d	+	1688	116	11.9
Port B				
0 d	−	560	118	11.8
93 d	+	1091	90	7.1
380 d	+	2089	118	18.3
534 d	+	2317	119	13.8
Port C				
0 d	−	574	106	13.4
93 d	−	1635	93	5.6
380 d	+	2974	111	9.5
534 d	+	2890	128	32.5
Untreated soil				
Sample 1	n.d.[d]	1012	83	9.1
Sample 2	n.d.[d]	1264	93	6.8

[a] Refers to whether measurable dissolved oxygen (> 0.1 mg/L) was present. Based on previously published data (Richardson et al. 2012).

[b] Values based on 560 random sequences per library.

[c] Inv. Simpson - the inverse of the Simpson diversity indicator.

[d] n.d. – not determined; samples were stored in a sealed container under aerobic conditions.

Table 2

Percent relative abundance[a] of sequences in column libraries possessing sequence similarity to SIP-determined PAH-degraders.

Sample[b]	*Achromobacter*	*Acidovorax*	AG1	*Pigmentiphaga*	*Pseudomonas* (72)	*Pseudomonas* (78)	*Pseudoxanthomonas*	PG2	*Rhizobium*	*Sphingobium* (61)	*Sphingobuim* (92)	*Sphingomonas*	*Variovorax*
Control column													
Surface													
0 d	0.2	**13.2**	-	-	-	0.2	0.5	**2.1**	-	0.6	0.2	-	**1.0**
93 d	0.1	**4.3**	-	0.0	-	0.0	0.1	**5.0**	-	0.2	0.0	-	0.1
380 d	0.0	0.5	-	-	-	0.0	0.0	**7.1**	-	0.4	0.2	0.0	-
Port A													
0 d	0.6	**11.0**	-	-	0.2	0.3	**11.0**	0.7	0.0	0.9	**1.3**	0.1	0.4
93 d	0.1	**5.5**	-	-	-	0.0	**5.7**	**3.7**	-	0.3	0.5	-	0.1
380 d	0.1	**1.9**	-	-	0.1	0.0	**2.1**	**3.7**	-	0.2	0.3	-	0.1
534 d	-	**2.3**	-	-	0.0	0.0	**3.3**	**4.6**	-	0.0	0.0	0.1	0.2
Port B													
0 d	0.4	**15.4**	0.0	0.0	0.4	**1.5**	**16.2**	0.1	0.0	**2.2**	**4.5**	0.0	0.6
93 d	0.1	**4.0**	-	0.1	-	0.1	**3.1**	**1.9**	-	0.6	**1.4**	-	0.2
380 d	0.0	0.4	-	-	-	0.0	0.5	**6.7**	-	0.0	0.1	-	-
534 d	0.1	**3.7**	0.1	-	-	0.0	**2.3**	**3.9**	-	0.3	0.6	-	0.1
Biostimulated column													
Surface													
0 d	0.6	**7.7**	-	-	-	0.1	**1.4**	**5.1**	0.1	**1.2**	**1.5**	0.1	0.1
93 d	0.3	**3.3**	-	-	-	1.0	0.2	**4.3**	-	0.8	0.4	-	**4.0**
380 d	0.1	0.6	-	-	-	**6.5**	0.1	**1.9**	-	0.3	0.1	-	**5.2**
Port A													
0 d	0.2	**4.0**	0.1	-	-	0.3	**8.1**	0.8	-	0.5	0.2	0.1	0.5
93 d	-	**1.4**	-	-	-	0.0	0.2	**4.0**	-	-	0.0	0.0	0.6
380 d	-	0.0	-	-	-	0.1	0.0	**3.4**	-	0.0	0.0	-	0.0
534 d	0.1	0.1	-	-	-	0.0	0.2	**3.9**	-	0.1	0.0	-	0.1
Port B													
0 d	0.7	**12.9**	-	-	0.4	**2.0**	**29.8**	0.2	-	**2.0**	**4.5**	-	**1.4**
93 d	0.2	**6.0**	-	-	0.1	0.1	**4.0**	**2.1**	-	0.1	0.5	-	-

Sample[b]	*Achromobacter*	*Acidovorax*	AG1	*Pigmentiphaga*	*Pseudomonas* (72)	*Pseudomonas* (78)	*Pseudoxanthomonas*	PG2	*Rhizobium*	*Sphingobium* (61)	*Sphingobuim* (92)	*Sphingomonas*	*Variovorax*
380 d	-	**2.3**	-	-	0.0	0.0	0.1	**6.9**	-	0.1	0.0	-	0.3
534 d	-	0.2	-	-	-	0.0	0.1	**5.1**	-	-	0.0	-	0.1
Port C													
0 d	**1.4**	**13.1**	-	-	0.9	**1.4**	**25.8**	0.0	-	**2.1**	**3.1**	-	-
93 d	0.2	**7.7**	-	-	0.4	0.1	**8.0**	0.2	-	0.1	**1.2**	-	-
380 d	-	0.4	-	-	-	0.0	0.0	**3.8**	-	-	0.1	-	-
534 d	-	0.3	-	-	-	0.0	0.0	**8.4**	-	-	0.1	-	-
Untreated soil													
Sample 1	0.2	**1.9**	**4.3**	0.3	**2.7**	**1.8**	**26.4**	0.6	0.7	**22.2**	**5.6**	0.6	-
Sample 2	0.1	**3.6**	**4.5**	-	**5.0**	**1.3**	**36.3**	0.9	0.5	**15.3**	**4.2**	0.3	0.4

[a] Values ≥ 1% are in bold. Dashes indicate that no sequences associated with that group were found in the library.

[b] Numbers in parentheses represent clone designations from prior SIP experiments (Jones et al. 2011a; Jones et al. 2011b). AG1 – “Anthracene Group 1”; PG2 – “Pyrene Group 2”

Table 3

Percent relative abundance of well-represented sequences in column libraries not directly associated with PAH-degradation[a].

	Uncl. Rhodocyclaceae[b]			*Thiobacillus*				*Brady-rhizobium*	*Thermo-monas.*
Sample	**OTU-5**	**OTU-59**	**OTU-70**	**OTU-1**	**OTU-3**	**OTU-7**	**OTU-100**	**OTU-20**	**OTU-461**
Control column									
Surface									
0 d	**3.8**	**6.3**	0.1	-	0.3	**3.5**	0.0	**5.6**	-
93 d	**19.3**	**5.1**	0.6	0.2	**4.3**	**9.3**	0.3	**2.5**	-
380 d	**5.6**	**3.9**	0.2	-	**4.5**	**4.3**	0.7	**7.4**	-
Port A									
0 d	**34.0**	0.1	**2.5**	-	0.5	0.2	0.6	0.4	-
93 d	**20.3**	0.8	**15.1**	0.1	**1.5**	0.8	**7.4**	0.8	0.0
380 d	**23.8**	0.7	**22.0**	0.0	**1.5**	**1.7**	**5.6**	**1.4**	-
534 d	**26.5**	0.9	**11.4**	0.1	**2.1**	**1.7**	**3.9**	0.4	-
Port B									
0 d	**6.6**	0.1	0.6	-	-	0.2	0.1	0.2	-
93 d	**23.7**	**1.2**	**11.2**	0.7	**1.2**	0.9	**7.9**	0.3	-
380 d	**28.5**	**3.5**	0.8	0.1	0.6	0.1	**5.3**	0.6	0.0
534 d	**30.4**	0.6	**6.2**	0.1	0.3	0.2	**3.6**	**1.3**	-
Biostimulated column									
Surface									
0 d	**4.8**	**4.6**	-	0.1	**1.4**	**3.9**	0.1	**2.3**	-
93 d	**3.5**	**4.7**	-	0.3	**3.9**	**6.8**	-	**5.5**	0.5
380 d	0.2	**2.3**	-	0.1	**1.5**	0.5	-	**7.2**	**4.4**
Port A									
0 d	**9.0**	0.4	0.1	**38.0**	**9.6**	**2.2**	0.1	0.5	-
93 d	**19.8**	**2.0**	0.2	**2.1**	**2.3**	0.6	-	0.9	**20.4**
380 d	**2.4**	**6.0**	-	**1.7**	**1.2**	0.5	-	**6.8**	**36.2**
534 d	**2.5**	**7.1**	0.1	0.5	**2.0**	0.9	0.0	**7.5**	**24.6**
Port B									
0 d	0.5	-	-	0.9	**1.3**	0.2	-	-	-

	Uncl. Rhodocyclaceae[b]			*Thiobacillus*				*Brady-rhizobium*	*Thermo-monas.*
Sample	**OTU-5**	**OTU-59**	**OTU-70**	**OTU-1**	**OTU-3**	**OTU-7**	**OTU-100**	**OTU-20**	**OTU-461**
93 d	**33.7**	0.7	0.5	**6.9**	**2.9**	0.4	-	**1.7**	**7.7**
380 d	**18.8**	**5.2**	-	**1.4**	0.4	0.4	-	**4.2**	**3.3**
534 d	**19.9**	**4.3**	0.3	**1.4**	0.6	0.3	-	**6.6**	**5.8**
Port C									
0 d	-	-	-	-	0.3	-	-	0.2	-
93 d	**37.8**	0.5	0.7	**6.3**	**1.5**	0.1	-	0.1	**4.5**
380 d	**29.3**	0.8	0.1	**1.8**	0.2	0.1	-	**2.0**	**5.9**
534 d	**5.6**	**3.6**	-	**2.1**	0.8	0.4	-	**6.7**	**9.2**
Untreated soil									
Sample 1	0.2	-	-	-	-	-	-	0.3	-
Sample 2	0.1	-	-	-	-	-	-	0.2	-

[a] Notation as in Table 2.

[b] Uncl. – unclassified.