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ORIGINAL PAPER

# Effects of enrichment with phthalate on polycyclic aromatic hydrocarbon biodegradation in contaminated soil

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**Abstract** The effect of enrichment with phthalate on the biodegradation of polycyclic aromatic hydrocarbons (PAH) was tested with bioreactor-treated and untreated contaminated soil from a former manufactured gas plant (MGP) site. Soil samples that had been treated in a bioreactor and enriched with phthalate mineralized $^{14}$C-labeled phenanthrene and pyrene to a greater extent than unenriched samples over a 22.5-h incubation, but did not stimulate benzo[*a*]pyrene mineralization. In contrast to the positive effects on $^{14}$C-labeled phenanthrene and pyrene, no significant differences were found in the extent of biodegradation of native PAH when untreated contaminated soil was incubated with and without phthalate amendment. Denaturing-gradient gel electrophoresis (DGGE) profiles of bacterial 16S rRNA genes from unenriched and phthalate-enriched soil samples were substantially different, and clonal sequences matched to prominent DGGE bands revealed that $\beta$-Proteobacteria related to *Ralstonia* were most highly enriched by phthalate addition. Quantitative real-time PCR analyses confirmed that, of previously determined PAH-degraders in the bioreactor, only *Ralstonia*-type organisms increased in response to enrichment, accounting for 89% of the additional bacterial 16S rRNA genes resulting from phthalate enrichment. These findings indicate that phthalate amendment of this particular PAH-contaminated soil did not significantly enrich for organisms associated with high molecular weight PAH degradation or have any significant effect on overall degradation of native PAH in the soil.

**Keywords** Bioremediation · Biostimulation · Phthalate · Polycyclic aromatic hydrocarbons

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## Introduction

Biostimulation involves the addition of nutrients or amendments to an environment to grow or stimulate indigenous organisms, and it has been proposed as a strategy in the bioremediation of soil or sediment contaminated with polycyclic aromatic hydrocarbons (PAH). In particular, nitrogen and/or phosphate sources are commonly added to stimulate biodegradation (Atagana 2006; Bogan et al. 2001; Guerin 1999; Hwang and Cutright 2002; Negri et al. 2004; Sabate et al. 2006; Williams et al. 1999). Other substrates, such as easily degraded carbon sources (van Herwijnen et al. 2006), compost (Namkoong et al. 2002; Scelza et al. 2007), wood chips (Negri et al. 2004), manure (Cho et al. 1997), poultry litter (Williams et al. 1999), and sewage (Namkoong et al. 2002) have also been tested as biostimulants. Salicylate, an intermediate in the metabolism of some PAH, has been shown to induce PAH degradation in some bacteria (Chen and

Aitken 1999; Kamath et al. 2004; Tian et al. 2003) and has also been used as a biostimulant to increase the abundance of naphthalene-degrading bacteria in soils (Colbert et al. 1993; Ogunseitan et al. 1991; Ogunseitan and Olson 1993; Powell 2006; van Herwijnen et al. 2006). It has been hypothesized that the addition of relevant pathway intermediates might increase the numbers of bacteria capable of degrading a range of PAH (Chen and Aitken 1999).

Phthalate is an intermediate in some pathways for the aerobic bacterial metabolism of phenanthrene (Barnsley 1983; Kang et al. 2003; Keum et al. 2006; Seo et al. 2006; Stingley et al. 2004b), anthracene (van Herwijnen et al. 2003a), fluorene (Grifoll et al. 1994; van Herwijnen et al. 2003b), pyrene (Kim et al. 2007; Rehmann et al. 1998; Vila et al. 2001), and fluoranthene (Kweon et al. 2007; Lee et al. 2007). Although Gram-negative and Gram-positive organisms appear to utilize different pathways to degrade phthalate, both eventually proceed through protocatechuate (3,4-dihydroxybenzoate) to TCA cycle intermediates. The genetics and/or pathways of phthalate metabolism have been studied in detail for several bacterial genera, including *Burkholderia* (Chang and Zylstra 1998), *Arthrobacter* (Eaton 2001), *Pseudomonas* and *Acinetobacter* (Vamsee-Krishna et al. 2006), *Rhodococcus* (Choi et al. 2007; Choi et al. 2005), *Terrabacter* (Habe et al. 2003), and *Mycobacterium* (Stingley et al. 2004a), many of which are also capable of degrading PAH.

The addition of phthalate to PAH-contaminated soil might increase bioremediation through several mechanisms. Organisms that degrade PAH through pathways that include phthalate as an intermediate could potentially grow on phthalate as a carbon and energy source, increasing their numbers in the soil. Since the 5- and 6-ring PAH generally do not support bacterial growth (Juhasz et al. 1997; Kanaly and Harayama 2000), the addition of a potentially selective carbon source could enhance the cometabolism of these PAH (Dandie et al. 2004; Hwang and Cutright 2002). However, it is not known whether phthalate might induce PAH-degrading pathways in a manner similar to salicylate.

In this study, we added phthalate to PAH-contaminated soil treated in a lab-scale, aerobic bioreactor, identified the most abundant phthalate degraders and tracked their response to enrichment with phthalate. We also examined the effect of enrichment on degradation of the native PAH in untreated, contaminated soil from the same site.

## Methods and materials

### Chemicals

Phthalic acid (99%) was acquired from Acros Organics (Fairlawn, NJ). The radiolabeled compounds were obtained from Sigma (St. Louis, MO) and consisted of uniformly ring-labeled $^{14}$C-phthalic acid (>98%; 12.7 mCi/mmol), [9-$^{14}$C]phenanthrene (>98%; 8.3 mCi/mmol), [4,5,9,10-$^{14}$C]pyrene (95%; 61 mCi/mmol), and [7-$^{14}$C]benzo[*a*]pyrene (≥98%; 26.6 mCi/mmol). In subsequent discussion, phthalic acid dissolved in buffer is referred to as phthalate.

### Soil samples

PAH-contaminated soil was obtained from the site of a former manufactured gas plant (MGP) in Charlotte, NC. This soil has been used for several years in a project to study the biological removal of PAH in a lab-scale, aerobic bioreactor (Singleton et al. 2005). The reactor was operated semi-continuously as previously described except that the solids content of the reactor was reduced to 8–10% (wt:wt) and untreated soil was added to the bioreactor on a monthly basis. Soil for this study was either sampled from the bioreactor at the time of the experiment or from a storage container of untreated soil maintained at 4°C.

### Chemical analysis

To determine phthalate concentrations in amended samples, soil slurries were centrifuged to remove solid particles and the supernatant filtered through a syringe filter with a Magna 0.45 μm pore-size nylon filter (GE Osmonics, Trevose, PA). The filtrate was stored in amber, crimp-top gas chromatography vials at −20°C until analysis by high-pressure liquid chromatography (HPLC). The HPLC system consisted of a Waters (Milford, MA) 600E system controller, a Waters 717 Plus autosampler, and a Kratos (Chestnut Ridge, NY) Spectroflow 757 UV absorbance detector set at 230 nm. Samples were run

through a Supelcosil™ LC-PAH column with 3 μm particle size (Supelco, Bellefonte, PA), using an isocratic mobile phase of 30% acetonitrile: 70% reagent water containing 0.1% trifluoracetic acid. Concentrations of phthalate were determined by comparisons to standard curves generated from external standards.

The concentrations of PAH in the soil for each incubation were determined by a two-day solvent extraction followed by HPLC analysis. Following each incubation, the slurry from each incubation flask was transferred to 35-ml glass centrifuge vials, centrifuged for 15 min at 3,500 rpm, and the supernatant discarded (previous experience indicated that aqueous-phase PAH concentrations are negligible). Sodium sulfate (5 g) was added to each vial to absorb the remaining water and 5-mm glass beads were included to improve mixing. To each vial was added 0.2 ml of 100 mg/l anthracene-$d_{10}$ (Cambridge Isotope Laboratories, Andover, MA) in acetonitrile to serve as an internal standard for evaluating recovery efficiency. Finally, dichloromethane and acetone (10 ml each) were added. All vials were placed on a wrist-action shaker for 24 h and then centrifuged as described above. The supernatant from each vial was filtered through a 0.2 μm pore-size nylon filter (Millipore; Burlington, MA) and collected in a 50 ml volumetric flask. The vials were replenished with 10 ml each of dichloromethane and acetone and placed on the shaker for an additional 24 h. The second-day extracts were centrifuged, filtered, and combined with the initial filtered extracts. The combined extracts were brought to volume with acetonitrile, transferred to amber serum vials, and stored in the dark at 4°C prior to analysis. All extracts were diluted 10-fold with acetonitrile and analyzed using the HPLC system described above but with a Perkin Elmer (Beaconsfield, UK) LS40 fluorescence detector. The total run time for the samples was 26 min including a 6-min delay between samples to enable pressure recovery. A gradient mobile phase of filtered acetonitrile and reagent water was used with initial conditions of 60% acetonitrile and 40% water at a flowrate of 1 ml/min. The proportion of acetonitrile was increased linearly to 100% during the first 10 min of each sample run, followed by a flowrate increase to 2 ml/min at 12.5 min. PAH concentrations in each sample were calculated on a dry mass basis using moisture content values of the centrifuged slurry prior to extraction.

## Mineralization experiments

Incubations were prepared containing contaminated soil (with or without phthalate enrichment), a $^{14}C$-labeled compound, and a $CO_2$ trap to determine the rate and extent of mineralization of selected compounds by soil microorganisms. To first create a phthalate-enriched microbial community, soil slurry was sampled from the bioreactor and aliquots of 25 ml of slurry (containing approximately 2 g dry soil) were added to 25 ml of reactor buffer (RB; 10 mM phosphate buffer, pH 7.0, and 2.3 mM $NH_4NO_3$) containing phthalic acid to a final concentration of 100 mg/l in each of a series of replicate shake flasks. Killed controls were prepared similarly except the flasks were amended with 85% phosphoric acid to reduce the pH to $< 2$. Additional flasks each containing 25 ml of soil slurry and an equal volume of RB without phthalate were prepared to serve as unenriched controls. The flasks were incubated in the dark at 25°C with shaking at 150 rpm for 43 h. The removal of phthalate to below the detection limit in the phthalate-amended flasks, and the lack of phthalate removal in the killed controls, were confirmed by HPLC.

After the 43-h incubation period, soil slurry from each condition (unenriched, phthalate-enriched, and killed controls) was used to evaluate the mineralization of phthalate, phenanthrene, pyrene, or benzo[*a*]pyrene (BaP); triplicate vials were prepared for each condition and each compound. Each incubation consisted of a 40-ml amber EPA vial containing 2 ml of soil slurry, a glass test tube containing a piece of filter paper saturated with 60 μl of 2 N KOH to trap $^{14}CO_2$, and a target of 20,000 disintegrations per min (dpm) of one of the radiolabeled compounds. Each vial was sealed with an aluminum foil-lined septum and screw-top lid. The $^{14}C$-labeled compound was added to the vial in a solvent that was allowed to evaporate prior to slurry addition. All vials were incubated at 25°C and shaken at 150 rpm. The filter paper was removed and replaced at intervals of 0.5, 1, 2, 4, and 22.5 h, and the amount of trapped $^{14}CO_2$ from each time interval was measured with a Packard (Meriden, CT) Tri-Carb Liquid Scintillation Analyzer, Model 1900 TR. After the final time point (22.5 h), all samples were acidified to pH $< 2$ with phosphoric acid and shaken for an additional 48 h for a final counting of trapped $^{14}CO_2$. Increases in recovered $^{14}CO_2$ after

acidification were negligible in all cases. To estimate concentrations of residual radiolabeled compound in the soil slurry, an equal volume of ethyl acetate was added to each slurry, shaken for 3 h, and the radioactivity in a portion of the ethyl acetate counted. Unaccounted-for radioactivity was presumed to have been incorporated into biomass, lost due to volatility, or strongly sorbed to the soil matrix.

### Effect of phthalate enrichment on degradation of native PAH

Two separate incubations were performed to analyze the effect of phthalate amendment on the microbial community and PAH degradation in untreated, contaminated soil. In the first incubation, a 10% (w/v) slurry of untreated soil was created in fresh RB containing 100 mg/l phthalate. Approximately 60 ml of slurry was transferred to sterile, 250-ml glass flasks with aluminum foil-lined screw caps. Duplicate samples were incubated in the dark at room temperature (~23°C), with shaking at 150 rpm for 15 d. Aliquots taken during the incubation were used to determine phthalate concentrations (as described above) as well as for DNA extraction for use in molecular analyses.

In the second set of incubations, untreated soil was manually mixed, and 2 g (wet weight) transferred to sterile, 125-ml glass flasks with aluminum foil-lined screw caps. To each flask was added fresh RB to a final concentration of 10% (w/v). Separate 2-g soil samples were oven-dried for the determination of soil moisture content. Phthalate-enriched samples contained 100 mg/l of phthalic acid in RB. Killed controls were created by adding phosphoric acid to pH < 2. Five replicate flasks were created for each of four conditions: soil in RB without incubation (day 0), soil in RB for 14-d incubations, soil in RB with phthalate for 14-d incubations, and acidified soil in RB for 14-d incubations. Flasks were incubated in the dark at room temperature (~23°C) with shaking at 150 rpm. Concentrations of PAH were determined as described previously.

### Data analysis

One flask in each incubation condition for the experiment testing the degradation of native PAH in untreated soil contained much higher concentrations of total PAH than the other four replicates. We attribute this to the likely presence of a "hot spot" in the initial soil sample that was distributed to one of the flasks for each incubation condition. To determine if the concentration of an individual PAH in these incubations might be an outlier, the median, upper and lower quartiles ($Q_3$ and $Q_1$, respectively), and interquartile range (IQR) of the five concentration values for every PAH in each condition were calculated. Values greater than $Q_3 + 3(IQR)$ or less than $Q_1 - 3(IQR)$ were considered extreme outliers and not considered in the calculations for the mean and standard deviation of PAH concentrations. Differences in the extent of degradation between initial, unenriched, and phthalate-enriched conditions used all concentration values regardless of outlier status and were tested for significance using a one-tailed Wilcoxon Rank Sum test with the ProStat software program (v.4.02; Poly Software International, Inc., Pearl River, NY).

### Molecular analyses

For molecular analyses, bioreactor-treated and untreated soil samples were treated similarly. DNA was extracted from soil samples using a MoBio UltraClean Soil DNA Kit (Carlsbad, CA) according to the manufacturer's recommendations, including an optional 10-min incubation of the sample at 70°C prior to physical lysis. Real-time quantitative PCR (qPCR) was performed to measure the number of 16S rRNA genes of members of the *Ralstonia*, *Acidovorax*, and *Sphingomonas* genera, as well as the uncultivated Pyrene Groups 1–3, in extracted DNA samples using methods and primer sets previously described (Powell 2006; Singleton et al. 2007; Singleton et al. 2006; Smits et al. 2004). Primers used to target *Mycobacterium* species were MYCO583F (Leys et al. 2005) and MycR (Cheung and Kinkle 2001) with an annealing temperature of 55°C and an expected product of ~259 base pairs. Genomic DNA from *Mycobacterium vanbaalenii* was used to generate standard curves for the *Mycobacterium*-specific primer set. Aliquots of 1 µl of DNA (of 50 µl total) from each of the duplicate incubations in each condition were run twice for all primer sets to obtain an average number of 16S rRNA genes for each bacterial group.

Denaturing gradient gel electrophoresis (DGGE) was used to compare bioreactor slurry before and after

phthalic acid enrichment. DGGE-PCR was performed with primers 63F-GC (Marchesi et al. 1998) and 517R (Muyzer et al. 1993) targeting the V1–V3 region of the gene, using the PCR method of Yu and Morrison (Yu and Morrison 2004) except that the denaturing, annealing, and extension times for the PCR were 1 min, 1 min, and 3 min, respectively. DGGE was performed with a DCode™ Universal Mutation Detection system (Bio-Rad, Hercules, CA) using a 6% polyacrylamide gel with a denaturant range of either 20–70% or 35–55%. The gels were run at 60 V for 16 h before post-staining with ethidium bromide.

A clone library of DGGE-PCR products was constructed using a TOPO TA Cloning® Kit for Sequencing (Invitrogen; Carlsbad, CA). Plasmids of selected clones were purified with a QIAprep® Spin Miniprep Kit (Qiagen; Valencia, CA) and used as template for DGGE-PCR. Products were run alongside DGGE-PCR products from DNA obtained from phthalate-enriched soil samples on a DGGE gel, and clones matching bands of interest were sequenced at the UNC-CH Genomics Analysis Facility. Sequence inserts were trimmed using Sequencher 4.7 (Gene Codes; Ann Arbor, MI) and close relatives determined by BLASTN searches of GenBank (Altschul et al. 1990). Phylogenetic trees were constructed by aligning selected sequences with the pileup program of the Genetics Computer Group (Wisconsin Package version 1.1; Accelrys Inc., San Diego, CA) and the ClustalX program (Thompson et al. 1994). Sequences were deposited in GenBank with accession numbers EU109674–EU109679.

## Results and discussion

### Effect of phthalic acid enrichment on mineralization of selected PAH

The extent to which enrichment with phthalate selected for bacteria capable of mineralizing phthalate, phenanthrene, pyrene, or benzo[*a*]pyrene was evaluated in aliquots of bioreactor-treated soil enriched with 100 mg/l phthalate, in unenriched slurry, and in killed controls (Fig. 1). As expected, the initial rate and extent of phthalate mineralization was greatly increased in enriched samples (Fig. 1a). Although the amounts of phenanthrene and pyrene mineralized by phthalate-enriched slurry were ultimately greater than the corresponding unenriched samples after 22.5 h of incubation, the initial rates of mineralization were not different (Fig. 1b, 1c). This observation might be explained by the need for the added radiolabeled PAH to dissolve into the aqueous phase, such that the rate of mineralization over the first few hours was limited by the rate of dissolution. It is also possible that the most abundant phthalate-degrading organisms did not degrade these PAH or that they metabolized them very slowly, in which case the enhancement of phenanthrene and pyrene mineralization at 22.5 h might indicate that other, less substantially enriched organisms were capable of transforming phenanthrene and pyrene. Unlike the other compounds, benzo[*a*]pyrene mineralization was unaffected by phthalate amendment and was mineralized similarly by both unenriched and enriched soil samples. Furthermore, only slightly more radiolabel was recovered in the $^{14}CO_2$ traps from experimental incubations than in the traps from the killed controls (Fig. 1d).

The average total recovery of $^{14}C$ at the end of the experiment for the killed controls containing radiolabeled phthalate, phenanthrene, pyrene, and BaP was 117%, 93%, 90%, and 50%, respectively, indicating that strong sorption to soil appears to have been significant only for BaP. The extent to which this influenced BaP mineralization is unknown, although BaP mineralization was very low even over the first few hours of incubation.

### Identification and quantification of phthalic acid-degrading bacteria

Duplicate samples of slurry from the bioreactor were incubated in the presence of phthalate, and the bacterial community before and after enrichment was compared by DGGE and real-time qPCR. The microbial community of the slurry was significantly different after enrichment, as illustrated by a number of new DGGE bands (Fig. 2). Partial 16S rRNA gene clone libraries constructed from the DGGE-PCR were matched to prominent bands in the enriched samples by DGGE, and selected inserts sequenced. Five of six sequenced clones, representing some of the dominant DGGE bands, were most closely related or identical to *Ralstonia* sequences, including many previously determined (Singleton et al. 2005; Powell

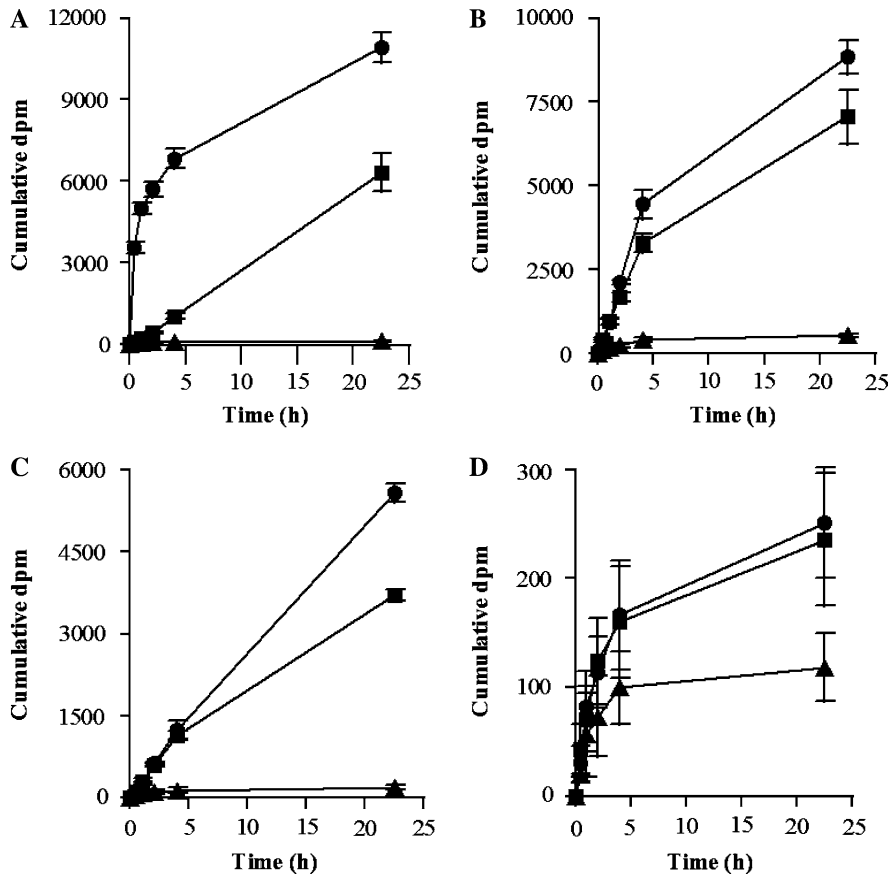


**Fig. 1** Mineralization of $^{14}$C-labeled (**a**) phthalic acid, (**b**) phenanthrene, (**c**) pyrene, and (**d**) benzo[*a*]pyrene by unenriched slurry (■), phthalate-enriched slurry (●), and acidified killed controls (▲). Each point represents the cumulative average dpm of triplicate incubations and the error bars are the standard deviations

2006) to be degraders of naphthalene and salicylate in the bioreactor (Fig. 3). The sixth clone was an α-Proteobacterium related to nearly full-length sequences previously detected in unenriched bioreactor slurry that have not yet been associated the metabolism of any PAH (Singleton et al. 2006).

The abundance of *Ralstonia*-type sequences in enriched bioreactor slurry was also confirmed by qPCR of DNA extracted before and after enrichment. *Ralstonia* was the only PAH-degrading bacterial group tested that showed a significant increase after incubation with phthalate, rising from an average of $1.3 \times 10^5$ to $4.5 \times 10^8$ 16S rRNA genes per g dry weight of soil over the incubation period. Other known PAH-degrading bacterial groups, many of which have been found in previous studies of the bioreactor, including *Acidovorax*, *Sphingomonas*, *Mycobacterium*, "Pyrene Group 1", "Pyrene Group 2", and "Pyrene Group 3" (Singleton et al. 2005; Singleton et al. 2006; Singleton et al. 2007), either showed no increase in 16S rRNA gene copy number in response to phthalate enrichment or a slight decrease (data not shown). Using qPCR standard curves generated from the *Ralstonia* template, 89% of the new total bacterial 16S rRNA genes resulting from enrichment with phthalate could be attributed to genes from the *Ralstonia* group. This is consistent with the intensity of bands in the DGGE profiles and sequences recovered in the clone library, even though the number of clones recovered from DGGE analyses was small.

*Ralstonia* spp. have previously been associated with growth on naphthalene (Singleton et al. 2005) and salicylate (Powell 2006; Singleton et al. 2005) in the soil from our lab-scale bioreactor. However, it is somewhat surprising that *Ralstonia* would dominate a phthalate-enriched community. In a previous study of PAH-degrading isolates, Widada et al. (2002) found that two isolates most closely related to *Ralstonia* by 16S rRNA gene sequences were incapable of growth on phthalic acid (Widada et al. 2002). *Ralstonia* sp. strain U2 (previously described as a *Pseudomonas*

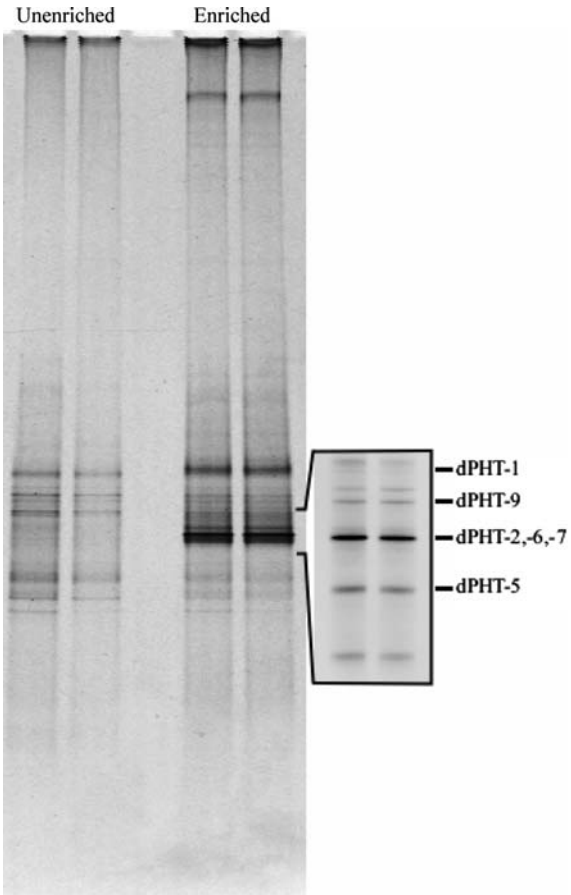


**Fig. 2** DGGE gels (20–70% denaturant, large gel; 35–55% denaturant, small gel) showing 16S rRNA genes from bioreactor-treated slurry prior to and after 44 h of enrichment with phthalate. The smaller gel was cropped to show the banding pattern of the region of the larger gel with the most significant changes relative to the unenriched soil. Designations of clones containing partial 16S rRNA genes matching the migration pattern of selected bands are indicated next to the gel

sp.) have been shown to degrade naphthalene through a salicylate-gentisate pathway (Fuenmayor et al. 1998; Zhou et al. 2001). *Ralstonia* spp. have not been associated with PAH that are degraded through a pathway that includes phthalate as an intermediate, and *Ralstonia* genes were not found in abundance in DNA associated with phenanthrene or pyrene consumption in previous studies of the microbial community in this bioreactor (Singleton et al. 2005; Singleton et al. 2006). Much of what is known of phthalate degradation comes from research with the $\beta$-Proteobacterium *Burkholderia cepacia* (Batie et al. 1987; Chang and Zylstra 1998, 1999a, b) and from the results of the present study it appears that other members of this phylogenetic group, such as *Ralstonia* spp., share this metabolic capability. An alternative explanation for the dominance of *Ralstonia* spp. after phthalate addition might be that phthalate inhibited other organisms, allowing *Ralstonia* spp. to flourish while growing on substrates other than phthalate. However, it would still be expected that one or more other organisms would have grown substantially on the relatively large amount of readily available phthalate added to the slurry, yet no evidence was found for this.

As evidenced in this experiment, *Ralstonia* spp. grow fairly rapidly. Added phthalate (100 mg/l) was degraded in less than 2 days, even though the *Ralstonia* population was a fairly insignificant percentage of the total microbial community at the start of the enrichment (0.12% of all 16S rRNA genes). Members of the *Ralstonia* genus likely grow more rapidly than organisms such as *Mycobacterium* spp. or any of the undescribed groups of Proteobacterial pyrene degraders identified by stable-isotope probing (Singleton et al. 2006). Thus, the fact that there are fast-growing members of *Ralstonia* capable of growth on phthalate likely precluded any of the slower-growing PAH degraders from significantly increasing in number. The enrichment of primarily *Ralstonia* spp. by phthalate is also consistent with a minimal effect on the short-term mineralization of phenanthrene, pyrene, and BaP. To our knowledge, *Ralstonia* spp. has not been associated with the degradation of PAH containing three or more rings.

## Effects of phthalate enrichment on degradation of native PAH

We also evaluated the extent to which enrichment with phthalate might stimulate the degradation of PAH in the untreated contaminated soil from an MGP site. In the first experiment, slurried, untreated soil was amended with phthalate and added to duplicate flasks. Phthalate was removed to below the detection limit in the samples after 31 h (data not shown). During this time the number of *Ralstonia* 16S rRNA genes in the sample increased from $6.3 \times 10^4$ to $4.0 \times 10^8$ genes per g soil. The average number of *Ralstonia* 16S rRNA genes decreased by 70% during the remainder of the 15-d incubation, to a final

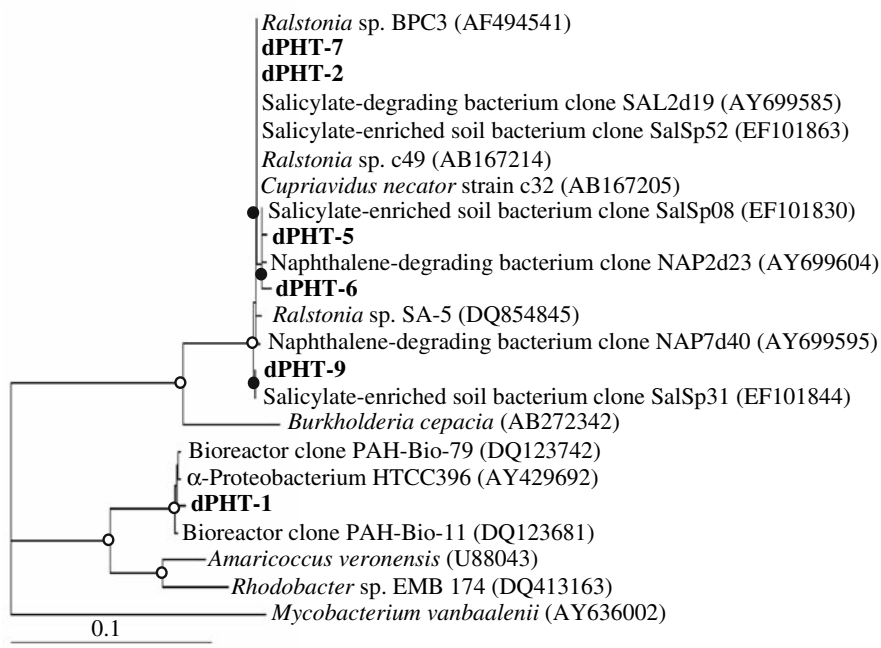


**Fig. 3** Phylogenetic tree showing sequenced 16S rRNA gene fragments representing prominent DGGE bands in phthalate-enriched bioreactor slurry (in bold) and their close relatives. GenBank accession numbers for reference sequences are shown in parentheses. Bootstrap values of greater than 50% and 95% are indicated on nodes with a closed (•) or open circle (○), respectively

abundance of $1.2 \times 10^8$ genes per g soil. Thus, the response of *Ralstonia* to phthalate amendment in untreated soil appeared similar to that of bioreactor-treated samples.

In a second series of incubations, significant reductions in PAH concentration were observed for a number of compounds in both phthalate-enriched and unenriched samples, particularly those of 2–4 rings (Fig. 4). Among the PAH which displayed marked disappearance, the overall trend was that the highest levels of removal were evidenced in soil without phthalate enrichment. The concentrations of PAH in phthalate-enriched soil were generally similar to those in the unenriched samples with the exception

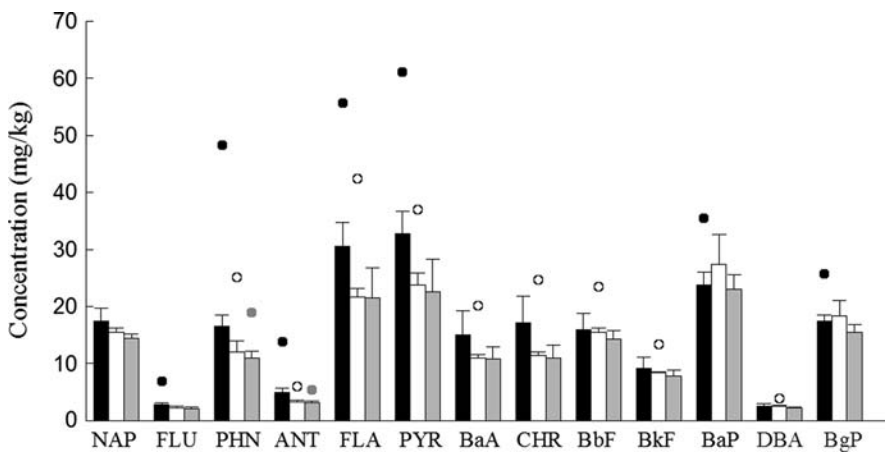


**Fig. 4** Concentrations of various PAH in contaminated soil prior to incubation (black bars), after 14 days with phthalate enrichment (white bars), and after 14 days without phthalate enrichment (gray bars). Bars are the average values of the replicates ($n = 5$, or when an outlier is present $n = 4$) and error bars are the standard deviation. Samples designated as outliers by statistical analysis are plotted as individual points above the corresponding bars. The concentration of acenaphthene was below the detection limit for all samples. NAP, naphthalene; FLU, fluorene; PHN, phenanthrene; ANT, anthracene; FLA, fluoranthene; PYR, pyrene; BaA, benz[*a*]anthracene; CHR, chrysene; BbF, benzo[*b*]fluoranthene; BkF, benzo[*k*]fluoranthene; BaP, benzo[*a*]pyrene; DBA, dibenz[*a,h*]anthracene; BgP, benzo[*g,h,i*]perylene

of naphthalene and dibenz[*a*,*h*]anthracene, which showed significantly more removal in unenriched samples ($P < 0.05$). There was no significant reduction of any PAH in the killed controls compared to the untreated soil (data not shown).

In general, phthalate amendment did not appear to stimulate the biodegradation of any compound to a greater extent than was found by the addition of inorganic nutrients alone. Interestingly, despite the massive increase in numbers of *Ralstonia* in enriched incubations, the degradation of naphthalene (a known growth substrate for *Ralstonia* spp. in the bioreactor) was no greater in phthalate-enriched samples than in unenriched incubations; this finding suggests that naphthalene might be of limited bioavailability in the soil.

## Conclusions

Based on results from this study, phthalate does not appear to be a promising biostimulant for PAH degradation in contaminated soil. Phthalate substantially increased the abundance of organisms that have been associated with the degradation of naphthalene, but not with higher molecular weight PAH. Phthalate addition led to a slight increase in the extent of mineralization of phenanthrene and a greater increase in the extent of pyrene mineralization, but had no effect on benzo[*a*]pyrene mineralization. Additionally, phthalate enrichment did not have a positive impact on overall PAH degradation in contaminated soil, and may actually have been slightly inhibitory for some compounds.

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## References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410

Atagana HI (2006) Biodegradation of polycyclic aromatic hydrocarbons in contaminated soil by biostimulation and bioaugmentation in the presence of copper(II) ions. World J Microbiol Biotechnol 22:1145–1153

Barnsley EA (1983) Phthalate pathway of phenanthrene metabolism: formation of 2′-carboxybenzalpyruvate. J Bacteriol 154:113–117

Batie C, LaHaie E, Ballou D (1987) Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from *Pseudomonas cepacia*. J Biol Chem 262:1510–1518

Bogan BW, Lahner LM, Trbovic V, Szajkovics AM, Paterek JR (2001) Effects of alkylphosphates and nitrous oxide on microbial degradation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:2139–2144

Chang HK, Zylstra GJ (1998) Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J Bacteriol 180:6529–6537

Chang HK, Zylstra GJ (1999a) Role of quinolinate phosphoribosyl transferase in degradation of phthalate by *Burkholderia cepacia* DBO1. J Bacteriol 181:3069–3075

Chang HK, Zylstra GJ (1999b) Characterization of the phthalate permease OphD from *Burkholderia cepacia* ATCC 17616. J Bacteriol 181:6197–6199

Chen S-W, Aitken MD (1999) Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. Environ Sci Technol 33:435–439

Cheung P-Y, Kinkle BK (2001) *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. Appl Environ Microbiol 67:2222–2229

Cho BH, Chino H, Tsuji H, Kunito T, Nagaoka K, Otsuka S, Yamashita K, Matsumoto S, Oyaizu H (1997) Laboratory-scale bioremediation of oil-contaminated soil of Kuwait with soil amendment materials. Chemosphere 35:1599–1611

Choi KY, Kim D, Sul WJ, Chae JC, Zylstra GJ, Kim YM, Kim E (2005) Molecular and biochemical analysis of phthalate and terephthalate degradation by *Rhodococcus* sp. strain DK17. FEMS Microbiol Lett 252:207–213

Choi KY, Kim D, Chae JC, Zylstra GJ, Kim E (2007) Requirement of duplicated operons for maximal metabolism of phthalate by *Rhodococcus* sp. strain DK17. Biochem Biophys Res Commun 357:766–771

Colbert S, Schroth M, Weinhold A, Hendson M (1993) Enhancement of population densities of *Pseudomonas putida* PpG7 in agricultural ecosystems by selective feeding with the carbon source salicylate. Appl Environ Microbiol 59:2064–2070

Dandie CE, Thomas SM, Bentham RH, McClure NC (2004) Physiological characterization of *Mycobacterium* sp. strain 1B isolated from a bacterial culture able to degrade high molecular weight polycyclic aromatic hydrocarbons. J Appl Microbiol 97:246–255

Eaton RW (2001) Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B. J Bacteriol 183: 3689–3703

Fuenmayor SL, Wild M, Boyes AL, Williams PA (1998) A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J Bacteriol 180:2522–2530

Grifoll M, Selifonov SA, Chapman PJ (1994) Evidence for a novel pathway in the degradation of fluorene by

*Pseudomonas* sp. strain F274. Appl Environ Microbiol 60:2438–2449

Guerin TF (1999) Bioremediation of phenols and polycyclic aromatic hydrocarbons in creosote contaminated soil using ex-situ landtreatment. J Hazard Mater 65:305–315

Habe H, Miyakoshi M, Chung J, Kasuga K, Yoshida T , Nojiri H, Omori T (2003) Phthalate catabolic gene cluster is linked to the angular dioxygenase gene in *Terrabacter* sp. strain DBF63. Appl Microbiol Biotechnol 61:44–54

Hwang S, Cutright TJ (2002) Biodegradability of aged pyrene and phenanthrene in a natural soil. Chemosphere 47: 891–899

Juhasz AL, Britz ML, Stanley GA (1997) Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a*,*h*]anthracene by *Burkholderia cepacia*. J Appl Microbiol 83:189–198

Kamath R, Schnoor JL, Alvarez PJ (2004) Effect of root-derived substrates on the expression of nah-lux genes in *Pseudomonas fluorescens* HK44: implications for PAH biodegradation in the rhizosphere. Environ Sci Technol 38:1740–1745

Kanaly RA, Harayama S ( 2000) Biodegradation of high molecular weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–67

Kang H, Hwang SY, Kim YM, Kim E, Kim Y-S, Kim S-K, Kim SW, Cerniglia CE, Shuttleworth KL, Zylstra GJ (2003) Degradation of phenanthrene and naphthalene by a *Burkholderia* species strain. Can J Microbiol 49:139–144

Keum YS, Seo JS, Hu Y, Li QX (2006) Degradation pathways of phenanthrene by *Sinorhizobium* sp. C4. Appl Microbiol Biotechnol 71:935–941

Kim SJ, Kweon O, Jones RC, Freeman JP, Edmondson RD, Cerniglia CE (2007) Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. J Bacteriol 189:464–472

Kweon O, Kim SJ, Jones RC, Freeman JP, Adjei MD, Edmondson RD, Cerniglia CE (2007) A polyomic approach to elucidate the fluoranthene degradative pathway in *Mycobacterium vanbaalenii* PYR-1. J Bacteriol 189: 4635–4647

Lee SE, Seo JS, Keum YS, Lee KJ, Li QX (2007) Fluoranthene metabolism and associated proteins in *Mycobacterium* sp. JS14. Proteomics 7:2059–2069

Leys NM, Ryngaert A, Bastiaens L, Wattiau P, Top EM, Verstraete W, Springael D (2005) Occurrence and community composition of fast-growing *Mycobacterium* in soils contaminated with polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 51:375–388

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795–799

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Namkoong W, Hwang EY, Park JS, Choi JY (2002) Bioremediation of diesel-contaminated soil with composting. Environ Pollut 119:23–31

Negri M, Manfredini A, Saponaro S, Sorlini C, Bonomo L, Valle A, Zanardini E (2004) Solid phase treatment of an aged soil contaminated by polycyclic aromatic hydrocarbons. J Environ Sci Health A Tox Hazard Subst Environ Eng 39:1–17

Ogunseitan OA, Olson BH (1993) Effect of 2-hydroxybenzoate on the rate of naphthalene mineralization in soil. Appl Microbiol Biotechnol 38:799–807

Ogunseitan OA, Delgado IL, Tsai YL, Olson BH (1991) Effect of 2-hydroxybenzoate on the maintenance of naphthalene-degrading pseudomonads in seeded and unseeded soil. Appl Environ Microbiol 57:2873–2879

Powell SN (2006) Stable isotope probing of salicylate-degrading bacteria in polycyclic aromatic hydrocarbon-contaminated soil. Dissertation. University of North Carolina

Rehmann K, Noll HP, Steinberg CE, Kettrup AA (1998) Pyrene degradation by *Mycobacterium* sp. strain KR2. Chemosphere 36:2977–2992

Sabate J, Vinas M, Solanas AM (2006) Bioavailability assessment and environmental fate of polycyclic aromatic hydrocarbons in biostimulated creosote-contaminated soil. Chemosphere 63:1648–1659

Scelza R, Antonietta Rao M, Gianfreda L (2007) Effects of compost and of bacterial cells on the decontamination and the chemical and biological properties of an agricultural soil artificially contaminated with phenanthrene. Soil Biol Biochem 39:1303–1317

Seo JS, Keum YS, Hu Y, Lee SE, Li QX (2006) Phenanthrene degradation in *Arthrobacter* sp. P1-1: initial 1,2-, 3,4- and 9,10-dioxygenation, and meta- and ortho-cleavages of naphthalene-1,2-diol after its formation from naphthalene-1,2-dicarboxylic acid and hydroxyl naphthoic acids. Chemosphere 65:2388–2394

Singleton DR, Powell SN, Sangaiah R, Gold A, Ball LM, Aitken MD (2005) Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. Appl Environ Microbiol 71:1202–1209

Singleton DR, Hunt M, Powell SN, Frontera-Suau R, Aitken MD (2007) Stable-isotope probing with multiple growth substrates to determine substrate specificity of uncultivated bacteria. J Microbiolog Meth 69: 180–187

Singleton DR, Sangaiah R, Gold A, Ball LM, Aitken MD (2006) Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. Environ Microbiol 10:1736–1745

Smits THM, Devenoges C, Szynalski K, Maillard J, Holliger C (2004) Development of a real-time PCR method for quantification of the three genera *Dehalobacter*, *Dehalococcoides*, and *Desulfitobacterium* in microbial communities. J Microbiolog Meth 57:359–378

Stingley RL, Brezna B, Khan AA, Cerniglia CE (2004a) Novel organization of genes in a phthalate degradation operon of *Mycobacterium vanbaalenii* PYR-1. Microbiology 150: 3749–3761

Stingley RL, Khan AA, Cerniglia CE (2004b) Molecular characterization of a phenanthrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1. Biochem Biophys Res Commun 322:133–146

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tian L, Ma P, Zhong J-J (2003) Impact of the presence of salicylate or glucose on enzyme activity and phenanthrene degradation by *Pseudomonas mendocina*. Proc Biochem 38:1125–1132

Vamsee-Krishna C, Mohan Y, Phale P (2006) Biodegradation of phthalate isomers by *Pseudomonas aeruginosa* PP4, *Pseudomonas* sp. PPD and Acinetobacter lwoffii ISP4. Appl Microbiol Biotechnol 72:1263–1269

van Herwijnen R, Springael D, Slot P, Govers HA, Parsons JR (2003a) Degradation of anthracene by *Mycobacterium* sp. strain LB501T proceeds via a novel pathway, through *o*-phthalic acid. Appl Environ Microbiol 69:186–190

van Herwijnen R, Wattiau P, Bastiaens L, Daal L, Jonker L, Springael D, Govers HA, Parsons JR (2003b) Elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthene, anthracene and dibenzothiophene by *Sphingomonas* sp. LB126. Res Microbiol 154:199–206

van Herwijnen R, Joffe B, Ryngaert A, Hausner M, Springael D, Govers HA, Wuertz S, Parsons JR (2006) Effect of bioaugmentation and supplementary carbon sources on degradation of polycyclic aromatic hydrocarbons by a soil-derived culture. FEMS Microbiol Ecol 55:122–135

Vila J, Lopez Z, Sabate J, Minguillon C, Solanas AM, Grifoll M (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67: 5497–5505

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T (2002) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol 58:202–209

Williams CM, Grimes JL, Mikkelsen RL (1999) The use of poultry litter as co-substrate and source of inorganic nutrients and microorganisms for the ex situ biodegradation of petroleum compounds. Poult Sci 78:956–964

Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 70:4800–4806

Zhou NY, Fuenmayor SL, Williams PA (2001) *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. J Bacteriol 183: 700–708