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Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil

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ABSTRACT

Phytoremediation is a promising low cost technology for the cleanup of contaminated sites. However, specific plants may promote degradation under one set of conditions but not under another, and knowledge limitations surrounding the mechanisms of phytoremediation hamper attempts at optimization. We addressed this issue by examining how exudates released by *Elymus angustus* (wildrye) and *Medicago sativa* (alfalfa), grown under hydrocarbon-stressed or non-stressed conditions, impacted the degradation potential of microbial communities in a weathered hydrocarbon-contaminated soil. Degradation potential was assessed using mineralization assays with ¹⁴C-labeled hydrocarbons (hexadecane, naphthalene, phenanthrene) followed by DGGE of microbial communities and quantitative-PCR of genes associated with hydrocarbon degradation. All root exudates repressed hydrocarbon mineralization in soil microcosms, with exudates from hydrocarbon-stressed wildrye having the least repressive impact. Changes in degradation potential were not associated with changes in the dominant microbial community structure or with significant shifts in general microbial abundance. Degradation was, however, associated with functional changes in microbial communities. Mineralization of polyaromatic hydrocarbons (PAH) was highly correlated with copy numbers of catechol 2,3 dioxygenase and naphthalene dioxygenase, two genes involved in PAH degradation. Both gene copy numbers and mineralization parameters were significantly impacted by exudate composition, with specific compounds associated with either increased (acetate, alanine) or decreased (malonate) degradative capacity. The success of a given phytoremediation treatment is likely influenced by the relative amount of these and similar compounds within root exudates.

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1. Introduction

Phytoremediation, the use of plants and their associated microorganisms to remove or degrade contaminants, is a promising technology for the remediation of both inorganic and organic soil contaminants (Arthur et al., 2005; Chaudhry et al., 2005). Accumulating phytoremediation studies under both controlled environmental conditions and field conditions show that this technology is a feasible remediation option for petroleum hydrocarbons (Banks et al., 2003; Liste and Prutz, 2006; Phillips et al., 2006). The degradation of hydrocarbons is facilitated through a rhizosphere effect; plants exude organic compounds through their roots, influencing the abundance, diversity, or activity of potential hydrocarbon degrading microorganisms in the zone surrounding the roots (Anderson et al., 1993). The actual mechanisms of this

synergistic relationship remain ill-defined and pose one of the more intriguing challenges in phytoremediation research today.

Plant species vary greatly in their ability to increase the degradation capacity of soil microbial communities (Chiapusio et al., 2007; Liste and Prutz, 2006; Parrish et al., 2004; Siciliano et al., 2003). Differences in degradation potential are not however, limited to different plant species. Studies on the phytoremediation potential of specific plant species often provide contrasting results. For example, one recent study (Rezek et al., 2008) found little influence of *Lolium perenne* (perennial rye grass) on polyaromatic hydrocarbon (PAH) degradation, while another (Binet et al., 2000) reported a significant rhizosphere effect. Differences in degradation potential are also observed between specific cultivars of a plant. Genotypic clones of *Medicago sativa* cv Riley (alfalfa) have been found to promote different amounts of crude oil degradation (Schwab et al., 2006; Wiltse et al., 1998). Some genotypes increased degradation while others decreased degradation relative to an un-planted control. Such differences in degradation potential may be related to the composition of root exudates, as it has been shown that the exudates of related cultivars differentially impact bacterial gene

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expression. Mark et al. (2005) found that the exudates of two *Beta vulgaris* (beetroot) cultivars promoted substantially different patterns of gene expression in *Pseudomonas aeruginosa* PA01. For example, a gene encoding protocatechuate 3,4 dioxygenase, an aromatic ring-cleaving dioxygenase, was up-regulated by exudates from one cultivar but exhibited no change with exudates from the other cultivar. Such fundamental differences even within a plant species highlight the importance of specific exudation patterns on soil microbial community functioning.

Several recent studies have attempted to elucidate the impact of exudates on microbial communities. Artificial root exudates have been shown to increase bacterial densities, shift metabolic profiles (Baudoin et al., 2003), and stimulate hydrocarbon degrader populations (Joner et al., 2002). Similarly, root extracts and exudates have been shown to stimulate both general (Da Silva et al., 2006; Miya and Firestone, 2001) and group-specific (Yoshitomi and Shann, 2001) increases in PAH degrader populations. Other studies however, have implicated exudates in the repression of hydrocarbon degradation potential. Corgié et al. (2004, 2006) observed distance-dependent repressive effects on PAH degradation by exudates released by *L. perenne*. Rentz et al. (2004) found that exudates from a variety of plants repressed the phenanthrene degrading activity of a *P. putida* strain, in part due to the repressive effects of exudates on a gene involved in naphthalene dioxygenase transcription (Kamath et al., 2004). Individual compounds commonly found in root exudates caused either repression (glucose, glutamate, lactate and other organic acids) or stimulation (salicylic acid) of gene expression. Rhizosphere microbial communities are influenced by a myriad of chemical, physical, and biological factors and are unlikely to respond to root exudate inputs in a manner comparable to bacterial isolates. However, there is little doubt that the success of a given phytoremediation treatment is inextricably linked to the impact that the complex mixture of root exudates has on microbial metabolic activity. Until we develop a better understanding of this relationship and the determinant causes of increased degradation, the full potential of phytoremediation will not be realized.

This study investigated the impact of root exudates of *M. sativa* and *Elymus angustus* (Altai wildrye), two common plant species used in phytoremediation studies, on microbial communities indigenous to a weathered hydrocarbon contaminated soil. Both the plants and the soil used in the current study have been investigated in previous phytoremediation studies by our group, under both controlled (Phillips et al., 2006) and field (Phillips et al., 2009) conditions. The primary objective of this study was to determine how differences in exudation patterns, stimulated by the presence of hydrocarbons, impact the degradation potential of soil microbial communities.

2. Materials and methods

2.1. Plant growth and exudate collection

E. angustus Trin. (Altai wildrye) and *M. sativa* L. (alfalfa var. Rambler) were grown under sterile conditions in modified Leonard jars. Seeds were surface disinfected by washing for 1 min with 95% ethanol, followed by a 5 min (alfalfa) or 10 min (wildrye) wash with 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. Seeds were then incubated on 1/10th strength tryptic soy agar (TSA) plates for a minimum of 2 days and only those seeds which showed no bacterial or fungal growth were used.

Each modified Leonard jar consisted of a quartz-sand (400 g) filled 330 ml straight-bodied glass funnel inverted into a 950 ml wide-mouth bottle containing 600 ml 0.5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). A fiberglass wick (Specialty Gaskets, Mississauga Ont.) connected the liquid

reservoir to the solid growth substrate. All components were sterilized by autoclaving both pre- and post-assembly. The sand in half of all systems was subsequently spiked with 200 mg kg⁻¹ of both pyrene and phenanthrene (98% pure; Sigma Aldrich, Mississauga, Ont.) in acetone (40 mg ml⁻¹), and the acetone was allowed to evaporate off for 24 h. PAH-spiked and non-spiked systems were then planted with surface sterilized seeds (PAH-spiked 15 seeds each, non-spiked 10 seeds each), or left un-planted for controls, and covered by a sterile Sunbag with a 0.02 µm gas exchange filter (Sigma Aldrich, Mississauga, Ont.). Six treatments were established with four replicates each; Altai wildrye, alfalfa, and a non-planted control, in PAH-contaminated and non-contaminated sand.

Plants were grown in a growth chamber with a 16 h/25 °C day (1500 µmol m⁻²) and 8 h/15 °C night cycle for 6 weeks. Exudates and control eluates were collected by flushing the sand with 300 ml sterile deionized water. Exudate and eluate sterility was assessed by plating 100 µl aliquots on 1/10th strength TSA plates. An additional 1 ml aliquot, boiled to release DNA, was assessed by PCR using the eubacterial primers outlined in the following sections. All exudates and eluates were then filtered through a 0.45 µm filter and frozen until use. Replicates were not pooled and were maintained as separate units throughout the study. Plant roots and shoots were separated, dried at 60 °C for 1 week, and weighed.

Any bacteria found in the growth systems post-harvest were isolated and assessed by FAME analysis (Hewlett Packard 5890 Series II GC), according to previously outlined procedures (de Freitas et al., 1997). Isolates were identified by library comparison using MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80, Microbial ID, Inc., Newark, DE, USA).

2.2. TOC analysis

A 5 ml sub-sample of each exudate or control eluate, acidified to a pH of less than 2.0 using HCl, was assessed for total organic carbon (TOC) using a Shimadzu TOC-5050A TOC Analyzer. TOC concentration was determined by comparison with a potassium hydrogen phthalate standard curve.

2.3. Identification and quantification of root exudate components

Organic acids, amino acids, and phenolic compounds of the plant root exudates were isolated by solid phase extraction (SPE) of bulk exudates using cation and anion exchange membranes (organic acids, amino acids) or resins (phenolics) and quantified by gas chromatography–mass spectrometry (GC–MS) analysis of derivatized sub-samples. Organic acid and amino acid recovery methods were modified from previously developed protocols (Gillespie, 2003). SPE and GC–MS were used to determine if residual PAHs were present in exudates from PAH spiked Leonard jars. Unless otherwise stated, all standards and derivatizing reagents were obtained from Sigma–Aldrich (Mississauga, Ont.) and all solvents were high purity GC grade (OmniSolv).

Ion exchange membranes were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Cation and anion exchange membranes were prepared by washing 4 times with 1.0 M NaOH or 1.0 M HCl, respectively, followed by a regeneration step of washing 4 times with 1.0 M HCl or 1.0 M NaOH, respectively. Exudates and control eluates were thawed, transferred to a sterile Erlenmeyer flask containing an anion and a cation membrane, and shaken in the dark on a rotary shaker for 12 h. All membranes were then removed from solution, rinsed with deionized water, placed in separate sterile 6 ml vials containing either 5 ml of 0.5 M HCl (anion membranes) or 5 ml of 2.0 M NH₄OH (cation membranes), and shaken in the dark for 8 h.

For organic acid analysis, a 1 ml aliquot of anion exchange eluate was transferred to a 4 ml vial containing 20 µl of glutaric acid

(100 $\mu\text{g ml}^{-1}$, internal standard). The eluate was shaken for 10 s with 2 ml diethyl ether (DEE), the phases were allowed to separate, and the non-aqueous phase was transferred to a 1.8 ml autosampler vial containing 40 μl triethylamine (EM Science) and evaporated under N_2 at room temperature. The DEE extraction process was repeated for a total of 3 extractions. Organic acids were derivatized to *tert*-butyldimethylsilyl (*t*-bdms) ester derivatives by dissolving the final DEE residue in 75 μl isooctane and 25 μl *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), capping the vial, and heating at 70 °C for 45 min.

For amino acid analysis, a 1 ml aliquot of cation exchange eluate was transferred to a 1.8 ml autosampler vial containing 20 μl of norleucine (100 $\mu\text{g ml}^{-1}$, internal standard) and evaporated to dryness under N_2 at room temperature. Amino acids were derivatized to *t*-bdms ester derivatives by dissolving the dried residue in 100 μl *N*-*N*-dimethylformamide, and 100 μl MTBSTFA (Sigma–Aldrich, Mississauga, Ont.), capping the vial, and heating at 70 °C for 30 min.

Phenolic compounds were extracted using Discovery DPA-6S SPE columns (Supelco, Bellefonte, PA). Columns were conditioned with 5 ml ethyl acetate, and rinsed with 5 ml methanol followed by 3 $\times$ 5-ml deionized water. Samples (50 ml) were acidified to pH 3.0, run through at a rate of 3 ml min^{-1} , and then the column was rinsed with 10 ml deionized water. Columns were allowed to dry for 1 h under vacuum and phenolic compounds were eluted with 10 ml ethyl acetate. The eluted phenolics were transferred to a 1.8 ml autosampler vial containing 50 μl of naringenin (100 $\mu\text{g ml}^{-1}$, internal standard) and dried under N_2 at room temperature. Phenolics were derivatized by dissolving the ethyl acetate residue in 75 μl acetonitrile and 25 μl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, Bellefonte, PA), capping the vial, and heating at 70 °C for 40 min.

Residual PAHs were extracted from 10 ml subsamples of eluate from each PAH spiked treatment using Supelclean ENVI-18S SPE tubes (Supelco, Bellefonte, PA). Columns were conditioned with 9 ml acetonitrile followed by 12 ml deionized water:isopropanol (90:10; pH 2.5 with HCl) at a flow rate of 3 ml min^{-1} . Samples amended with 10% isopropanol were run through columns at a flow rate of 10 ml min^{-1} , columns were washed with 30 ml deionized water:isopropanol (90:10; pH 2.5 with HCl), dried under vacuum for 30 min, and then PAHs were eluted with 2 $\times$ 4-ml aliquots of hexane:acetone:isopropanol (90:5:5) at a flow rate of 1 ml min^{-1} . Toluene (0.5 ml) was added to each final eluate, which was then dried under N_2 to a final volume of 0.5 ml, diluted to 1.8 ml with toluene, and assessed by GC–MS. Eluates from Leonard jars containing no PAHs served as controls. Serial dilutions of the target compounds were used to establish a 0.004–2.000 $\mu\text{g ml}^{-1}$ calibration curve.

Samples were analysed on a Varian CP-3800 GC equipped with a Saturn 2200 MS detector and an 8400 autosampler. A Varian FactorFourTM column (VF-Xms) with dimensions of 30 m $\times$ 0.25 mm i.d. and a 0.25 μm stationary-phase film thickness was used for analytical separation. The carrier gas was helium with a constant flow rate of 1 ml min^{-1} . The injection port temperature was 250 °C. A 1 μl injection volume was delivered with a 5 μl syringe. The MS was operated in the EI ionization mode and was set to scan from *m/z* 45 to 650 with a scan rate of 0.420 scans s^{-1} . Spectral data were acquired using a linked Saturn GC/MS Workstation version 5.5 (Varian Inc., Walnut Creek, CA). For organic and amino acid analysis, the initial column oven temperature was maintained at 60 °C for 1 min, and then ramped at 5 °C min^{-1} to 300 °C where it was held for 1 min or 6 min, respectively. For phenolic analysis the initial column temperature was maintained at 80 °C for 1 min, then ramped at a rate of 20 °C min^{-1} to 250 °C and held for 1 min, then ramped at a rate of 6 °C min^{-1} to 300 °C and held for 2 min, and then ramped at a rate of 20 °C min^{-1} to 320 °C and held for 4 min.

Mixed standards for each group of compounds (see [supplementary Table S1](#)) were derivatized as outlined above and used to identify and quantify individual compounds within the exudates. For PAH analysis, the initial column oven temperature was maintained at 100 °C for 1 min and then ramped at 10 °C min^{-1} to 320 °C, where it was held for 17 min. A targeted *m/z* scan between the range of *m/z* 60 and 650 and a scan rate of 0.50 scans s^{-1} was performed from 3 min onward.

2.4. Hydrocarbon mineralization potential

[¹⁴C]hydrocarbon mineralization assays were used to determine what impact each exudate or control eluate had on the hydrocarbon degrading activity of soil microbial communities. The soil, collected from a weathered-hydrocarbon contaminated site in south-eastern Saskatchewan, Canada, had a clay texture, pH of 8.0, electrical conductivity 5.8 dS m^{-1} , sodium absorption ratio 20.3, cation exchange capacity 18.46 cmol kg^{-1} , and $\text{NO}_3\text{-N}$, P and K concentrations of 1.6, 1.0, and 332 mg kg^{-1} , respectively. Total hydrocarbon concentration (C16–C50) was 3700 mg kg^{-1} ; PAH concentration was less than 0.50 mg kg^{-1} . Soil was sieved through a 4.75 mm sieve to ensure homogeneity, stored at 4 °C for one month until use, and acclimatized to room temperature for 2 weeks prior to microcosm setup. Moisture content was determined by oven-drying 10 g sub-samples of each soil at 100 °C for 24 h. Serum vial microcosms were set up and [¹⁴C]₂ was sampled (at days 1, 3, 5, 8, 12, 15, 21 and 28) as outlined in [Phillips et al. \(2006\)](#). Exudates/eluates were added to microcosms containing 6 g soil at a rate of 10 $\mu\text{g TOC g}^{-1}$ soil, and final moisture was adjusted to 30% using sterile deionized water. Serum vials were amended with 50,000 dpm (100 mg kg^{-1}) of [¹⁻¹⁴C]n-hexadecane, [¹⁻¹⁴C]naphthalene, or [⁹⁻¹⁴C]phenanthrene (specific activities 12, 6.2, and 8.2 mCi mmol^{-1} , respectively; Sigma–Aldrich, Mississauga, Ont.). The final mineralization set-up consisted of six exudate/eluate treatments (Altai wildrye exudates, alfalfa exudates, and non-planted control eluates from PAH-spiked and non-spiked systems, as outlined in Section 2.1) of four replicates each, assessed in separate mineralization microcosms with the three different hydrocarbons (72 microcosms total). Abiotic controls ($n=3$) for each hydrocarbon treatment were established using autoclaved soil (2 $\times$ 1-h with a 1 week resting interval). Non-radioactive duplicate microcosms (72 microcosms total) were established for all mineralization microcosms for use in subsequent molecular analyses.

2.5. Microbial community assessment

Total community DNA was extracted from all non-radioactive mineralization microcosms (two separate extractions of 0.50 $\pm$ 0.01 g soil per microcosm) as previously outlined ([Phillips et al., 2006](#)). Briefly, this method used a combination of bead-beating, proteinase K, and sodium dodecyl sulphate to lyse cells. Proteins and cellular debris were precipitated using 7.5 M ammonium acetate, and DNA was subsequently precipitated using isopropanol, re-suspended in TE (pH 8.0), and purified using polyvinylpyrrolidone (PVPP) columns. DNA yield was quantified pre- and post-PVPP purification on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen) and by spectrophotometer evaluation.

Microbial community structure in each treatment replicate was examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments. PCR amplification was performed using universal eubacterial 16S rRNA gene primers ([Lee et al., 1993](#); [Röllerle et al., 1996](#); [supplementary Table S2](#)), and following the PCR protocol outlined in [Phillips et al. \(2006\)](#). Pooled PCR replicates were concentrated and precipitated with 0.1 volume 3 M sodium acetate and 2.5

volumes 100% ethanol at -20°C overnight, re-suspended in $15\ \mu\text{l}$ of TE buffer (pH 8.0), and quantified on ethidium bromide-stained 1.4% agarose gels by comparison with a 100 bp ladder (Invitrogen). DGGE was performed on a Bio-Rad DCode system (Bio-Rad, Mississauga, Ont.) essentially as described by Lawrence et al. (2004). For each gel, 600 ng of amplified 16S rRNA gene product was loaded per lane onto an 8% acrylamide gel with a 40–60% urea-formamide denaturing gradient. Electrophoresis was performed for 16 h at 80 V and 60°C . The resulting gels were stained with SYBR Green I (Sigma–Aldrich, Mississauga, Ont.) in TAE buffer and visualized using a digital gel documentation system (GelDocMega; BioSystematica, Devon, United Kingdom). All treatment replicates were assessed for intra-treatment banding pattern similarity on separate DGGE's prior to combining replicates for final inter-treatment comparative gels.

Quantitative PCR (Q-PCR) was performed on microbial community DNA extracted from all mineralization microcosms. The 16S rRNA gene (Lee et al., 1993; Rölleke et al., 1996) and three genes involved in hydrocarbon degradation, catechol 2,3 dioxygenase (Luz et al., 2004), naphthalene dioxygenase (Baldwin et al., 2003), and alkane monooxygenase (Powell et al., 2006) were assessed. Each separate assay was performed at least twice to verify qPCR reproducibility. PCR assays were performed on an ABI 7500 RT PCR system (Applied Biosystems, Foster City, Calif.) using QuantiTect SYBR green PCR kits (Qiagen, Mississauga, Ont). Amplification reactions were set up according to the manufacturer's protocol. Primer concentrations, amplification conditions, and positive-control strains are listed in supplementary Table S2. Absolute quantification was performed by comparison with standard curves generated from positive-control strains (supplementary Table S2). Ten fold dilutions of DNA standards ranging from 10^2 to 10^6 gene copy numbers were prepared from DNA extracts of these strains (supplementary Table S2). Gene copy numbers were calculated from the concentrations of positive-control strains, quantified spectrophotometrically, assuming a molecular mass of 660 Da per dsDNA bp, 6.0 Mbp per genome and one copy per genome (Park and Crowley, 2006). Standard curves were linear over 4–5 orders of magnitude with R^2 values >0.990 and $E > 0.83$. Correct product size of standards and samples were periodically verified on agarose gels. Q-PCR plates were set-up such that each gene for all replicate microcosms was assessed in a single reaction (Smith et al., 2006), and each plate included a full range of the relevant DNA standards.

2.6. Statistical analyses

Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, Illinois). Data were examined for overall treatment effects using ANOVA, followed by a Tukey test (variances equal) or a Games Howell test (variances unequal) to determine whether significant differences occurred between treatments. Homogeneity of variance was assessed using the Levene statistic. Relationships between parameters were assessed by stepwise multiple regression analysis and Spearman's rank correlation. Relationships in microbial community structure between treatments were assessed by cluster analysis of non-weighted DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths).

3. Results

3.1. Plant growth

Alfalfa and wildrye grew well in both non-amended and PAH amended sand. At the end of the study all plants appeared healthy, with no visible chlorosis or other signs of stress. All wildrye and

control systems were free of contamination at the end of the study. Most alfalfa systems however, harboured bacteria at approximately $10^4\ \text{CFU ml}^{-1}$. *Pantoea agglomerans* ($\text{SIM} \geq 0.391$) was found in all PAH systems while *Microbacterium esteraromaticum* ($\text{SIM} \geq 0.623$) was found in two non-PAH systems. Both of these bacteria have previously been recovered from beneath the seed coat of surface-sterilized alfalfa seeds (Moline and Kulik, 1997).

3.2. PAH quantification

Trace amounts of phenanthrene were detected in the eluates of several PAH-amended microcosms. The eluate/exudates of one alfalfa replicate, one control replicate, and two wildrye replicates contained 2.9, 1.9, 8.0, and $1.1\ \mu\text{g l}^{-1}$ phenanthrene, respectively.

3.3. Plant exudate qualification and quantification

PAH-stressed wildrye released twice as much total organic carbon (TOC) as other plants, at approximately $90\ \text{mg TOC g}^{-1}$ root. The quantity of identified exudates varied greatly both between and within treatments (Fig. 1). Individual organic acids ranged from less than $1\ \mu\text{g g}^{-1}$ root to more than $300\ \mu\text{g g}^{-1}$ root. Although exudation patterns were highly variable, several trends were observed. Higher concentrations of organic acids were found in PAH-stimulated wildrye treatments than in non-stimulated systems, while the opposite was found in the alfalfa treatments. Amino acid and phenolic compounds were detected at relatively low concentrations with high variability in all treatments (Fig. 1). As with organic acids, higher levels of both groups were detected in wildrye-PAH versus wildrye exudates, with the opposite occurring in the alfalfa treatments.

3.4. Impact of exudates on hydrocarbon mineralization potential

Distinct trends were observed in the mineralization assays. Plant root exudates had a repressive effect on the mineralization of all hydrocarbons compared to control eluates (Table 1; supplementary Fig. S1). Lag times (defined as the time to 5% measurable mineralization) of exudate amended treatments were usually higher, maximum mineralization rates were lower, and cumulative mineralization less than that of control-eluate amended treatments. In general however, exudates derived from PAH-stimulated wildrye plants exhibited fewer repressive effects than other exudates on the mineralization of all assessed hydrocarbons. Mineralization parameters in these wildrye-PAH amended microcosms were usually not significantly different from those of control microcosms (Table 1). A priming effect was observed in control-PAH compared to control microcosms, though these differences in mineralization parameters were usually not statistically significant (Table 1).

A strong relationship was found between lag time, rate, and cumulative mineralization for each individual hydrocarbon ($p \leq 0.001$ for all correlations, data not shown). Stepwise multiple regressions revealed that specific root exudate components, including the organic acids malonate, acetate and fumarate, and the amino acid alanine, were significantly associated with these mineralization parameters (Table 2). In particular, malonate was associated with decreased mineralization, both with increased lag times for all hydrocarbons and decreased rate and extent of hexadecane mineralization.

3.5. Impact of exudates and hydrocarbons on microbial communities

Quantitative PCR (Q-PCR) was performed on microbial community DNA extracted from all treatment replicates. The 16S rRNA gene and three genes involved in hydrocarbon degradation,

Fig. 1. Organic acid, amino acid, and phenolic content of alfalfa and Altai wildrye (wildrye) root exudates. The tag -PAH indicates that the exudates were collected under phenanthrene/pyrene stimulated conditions. Data are presented as means ($n=4$) with error bars representing ± 1 standard deviation. Differences are significant at $p \leq 0.05$ where indicated.

catechol 2,3 dioxygenase, naphthalene dioxygenase, and alkane monooxygenase, were quantified by comparison to standards. Standard curves for the four genes had average linear correlation coefficients of 0.999, 0.987, 0.995, and 0.993, respectively. Each primer set yielded DNA bands of the appropriate size for the assessed gene sequence, with no observable primer-dimer formation (data not shown). The absence of non-specific amplification and primer-dimer formation was confirmed by melt curve analyses (data not shown).

The addition of control eluates or plant root exudates did not result in significant changes in general microbial community structure (Fig. 2). DGGE banding patterns and the relative abundance of

individual bands were highly similar in all treatments. The abundance of 16S rRNA gene was also consistent between all treatments (Table 3), indicating that none of the eluates/exudates had a significant impact on overall bacterial population abundance.

Although bacterial population densities did not statistically differ between treatments, there were significant differences in copy numbers of both catabolic genes involved in PAH degradation (Table 3). The abundance of these catabolic genes was highly positively inter-correlated ($r > 0.939$, $p < 0.001$). Both catechol 2,3 dioxygenase (C2,3O) and naphthalene dioxygenase (*nahAc*) gene copy numbers were increased in those microcosms which exhibited increased PAH mineralization, and their abundance was

Table 1

Hydrocarbon mineralization parameters of soil microcosms amended with plant bulk exudates or control (non-planted) eluates and one of three hydrocarbons.

Mineralization microcosm	Lag*** (days) [†]	Maximum rate*** (% ¹⁴ C day ⁻¹)	Cumulative*** % mineralized
Phenanthrene			
Control	5.3 (1.0)cd	1.8 (0.1)b	36.0 (5.4)ab
Control-PAH [‡]	4.1 (0.5)d	2.3 (0.2)a	44.5 (2.0)a
Alfalfa	9.9 (0.5)a	1.2 (0.2)b	22.9 (2.7)c
Alfalfa-PAH	8.5 (2.0)ab	1.2 (0.1)b	25.5 (4.1)c
Wildrye	10.3 (0.6)a	1.3 (0.2)b	24.2 (2.3)c
Wildrye-PAH	6.9 (1.7)bc	1.5 (0.3)ab	31.2 (4.9)bc
Naphthalene			
Control	1.6 (0.3)b	11.6 (4.9)ab	62.5 (5.4)ab
Control-PAH	1.5 (0.0)b	17.2 (1.8)a	70.5 (11.5)a
Alfalfa	4.9 (0.9)a	5.6 (1.2)b	51.8 (4.1)b
Alfalfa-PAH	2.8 (1.6)ab	7.9 (3.5)b	58.8 (4.5)ab
Wildrye	5.4 (0.6)a	6.1 (0.4)b	51.8 (1.7)b
Wildrye-PAH	2.0 (0.4)b	7.5 (3.3)b	61.6 (4.1)ab
Hexadecane			
Control	3.3 (1.2)bc	3.1 (0.4)ab	48.8 (2.1)a
Control-PAH	2.9 (0.3)c	3.7 (0.3)a	48.8 (3.4)a
Alfalfa	5.6 (1.3)ab	2.0 (0.5)bcd	39.2 (6.5)ab
Alfalfa-PAH	4.9 (1.4)bc	2.2 (0.6)bcd	41.1 (4.8)ab
Wildrye	7.4 (1.0)a	1.2 (0.2)d	30.8 (4.5)b
Wildrye-PAH	4.4 (1.1)bc	2.5 (0.7)bc	44.3 (5.6)a

Data are presented as means ($n=4$) with ± 1 standard deviation in parentheses.

[†] Means in a single sub-column followed by a different letter are significantly different at *** $p \leq 0.001$.

[‡] The tag -PAH indicates that the original plant exudates/control eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 2

Linear multiple regression for hydrocarbon degradation parameters (mineralization and gene copy number) as a function of the concentration of specific root exudate components.

Hydrocarbon treatment	Constant ± standardized β -coefficients	R^2
Phenanthrene		
Lag [†]	9.298 + 0.521(malonate) – 0.495(alanine)	0.500
Rate [*]	1.238 + 0.458 (fumarate)	0.153
Cumulative	NS	
16S rRNA ^{†***}	7.591 + 0.402(glycine) – 0.551(azelaic) + 0.683(aconitate) – 0.459(succinate)	0.755
C2,3O ^{***}	7.269 – 0.734(malonate) + 0.343(alanine) + 0.329(acetate)	0.750
<i>nahAc</i> ^{***}	5.758 – 0.713(malonate) + 0.474(alanine) + 0.268(acetate)	0.785
Naphthalene		
Lag ^{**}	3.340 + 0.819(malonate) – 0.553(hydroxymalonate)	0.573
Rate	NS	
Cumulative [*]	58.781 + 0.433(acetate)	0.187
16S rRNA ^{**}	7.518 – 0.768(malonate) – 0.404(glycine) + 0.390(maleate)	0.708
C2,3O ^{**}	7.225 – 0.615(malonate) + 0.370(acetate)	0.514
<i>nahAc</i> ^{**}	6.077 – 0.659(malonate)	0.434
Hexadecane		
Lag ^{***}	4.777 + 0.586(malonate) + 0.438(fumarate) – 0.364(alanine)	0.730
Rate ^{**}	2.037 – 0.606(malonate) + 0.381(alanine)	0.497
Cumulative ^{**}	47.109 – 0.601(malonate) – 0.429(fumarate)	0.606
16S rRNA	NS	
<i>alkB</i>	NS	

Regression equation significant at: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

[†] Lag, days to 5% mineralization; rate, maximum rate of mineralization (%¹⁴C day⁻¹); cumulative, cumulative % mineralized.

[‡] Gene copy numbers g⁻¹ dry soil: 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxxygenase; *nahAc*, naphthalene dioxxygenase.

strongly correlated with the mineralization parameters (Table 4). In naphthalene mineralization microcosms, there were significant correlations between these catabolic genes and 16S rRNA genes ($r > 0.663$, $p < 0.001$) and between 16S rRNA gene abundance and mineralization parameters (Table 4). However, no comparable correlation was observed in microbial communities from phenanthrene mineralization microcosms. As seen with mineralization parameters, specific exudate components were significantly associated with gene copy numbers (Table 2). In particular, malonate was associated with reduced C2,3O and *nahAc* copy numbers in both PAH treatments, while alanine and acetate were associated with increased copy numbers of these genes.

No relationship was found between exudate composition and the total copy number of alkane hydroxylase (*alkB*) genes, which were comparable between all treatments at the end of the study and averaged approximately 10⁶ gene copies per gram of soil (Table 3). Although no significant difference was observed in *alkB* abundance, in general total *alkB* copy numbers were positively correlated with 16S rRNA gene numbers ($r = 0.779$, $p < 0.001$).

Fig. 2. Dendrogram analysis of final DGGE banding patterns from all exudate and eluate amended mineralization (¹⁴C- labeled naphthalene, phenanthrene, and n-hexadecane) microcosms. Treatments included alfalfa and Altai wildrye (wildrye) exudates and non-planted control eluates. The tag -PAH indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 3

Total gene copy numbers of soil mineralization microcosms amended with plant bulk exudates or control (non-planted) eluates.

Mineralization microcosms	Gene copy numbers g ⁻¹ dry soil (log scale)		
	16S rRNA	C2,3O ^{***†}	<i>nahAc</i> ^{***}
Phenanthrene			
Control	7.57 (0.11)a	7.78 (0.14)a	6.59 (0.12)a
Control-PAH [‡]	7.52 (0.19)a	7.84 (0.17)a	6.63 (0.22)a
Alfalfa	7.58 (0.09)a	7.03 (0.26)c	5.63 (0.26)bc
Alfalfa-PAH	7.54 (0.09)a	7.29 (0.45)abc	5.84 (0.50)bc
Wildrye	7.65 (0.04)a	7.09 (0.09)bc	5.59 (0.16)c
Wildrye-PAH	7.69 (0.07)a	7.64 (0.32)ab	6.29 (0.03)ab
Naphthalene			
Control	7.37 (0.20)a	7.75 (0.32)a	6.57 (0.39)ab
Control-PAH	7.42 (0.10)a	7.88 (0.11)a	6.74 (0.16)a
Alfalfa	7.27 (0.23)a	6.92 (0.33)b	5.41 (0.41)cd
Alfalfa-PAH	7.37 (0.07)a	7.26 (0.35)ab	5.91 (0.42)bcd
Wildrye	7.22 (0.10)a	6.80 (0.04)b	5.30 (0.06)d
Wildrye-PAH	7.38 (0.15)a	7.27 (0.46)ab	6.11 (0.38)abc
Hexadecane			
Control	7.60 (0.03)a	5.95 (0.05)a	
Control-PAH	7.65 (0.20)a	6.06 (0.46)a	
Alfalfa	7.69 (0.17)a	5.93 (0.24)a	
Alfalfa-PAH	7.47 (0.13)a	5.63 (0.33)a	
Wildrye	7.63 (0.13)a	5.87 (0.20)a	
Wildrye-PAH	7.57 (0.18)a	6.07 (0.20)a	

16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxxygenase; *nahAc*, naphthalene dioxxygenase; Data are presented as means ($n = 4$) with ± 1 standard deviation in parentheses.

[†] Means in a single sub-column followed by a different letter are significantly different at *** $p \leq 0.001$.

[‡] The tag -PAH indicates that the original plant exudates/control eluates were collected under phenanthrene/pyrene stimulated conditions.

4. Discussion

Phytoremediation researchers often find that specific plants may promote degradation under one set of conditions but not under another. To date, we know relatively little about how

Table 4

Spearman's rank correlation coefficients ($n=24$) for hydrocarbon mineralization parameters and microbial gene abundance in eluate and exudate amended soil microcosms.

Hydrocarbon microcosm	Gene copy numbers [†]		
	16SrRNA	C2,30	<i>nahAc</i>
Phenanthrene			
Lag [‡]	0.002	−0.810***	−0.866***
Rate	0.073	0.619***	0.711***
Cumulative	−0.018	0.759***	0.809***
Naphthalene			
Lag	−0.525**	−0.916***	−0.952***
Rate	0.349	0.671***	0.653***
Cumulative	0.457*	0.806***	0.839***
Hydrocarbon microcosm	Gene copy numbers [†]		
	16SrRNA	<i>alkB</i>	
Hexadecane			
Lag	−0.118	−0.317	
Rate	0.135	0.356	
Cumulative	0.123	0.335	

Significant at: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

[†] Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16S rRNA; C2,30, catechol 2,3 dioxxygenase; *nahAc*, naphthalene dioxxygenase.

[‡] Mineralization parameters: Lag, days to 5% mineralization; rate, maximum rate of mineralization ($\%^{14}CO_2 \text{ day}^{-1}$); cumulative, cumulative % mineralized.

different plant species impact the structure and function of soil microbial communities, and how these impacts affect the degradation potential of the system as a whole. We addressed this issue by examining how differences in exudation patterns impact the degradation potential of microbial communities indigenous to a weathered hydrocarbon contaminated soil. Root exudates collected from Altai wildrye and alfalfa initially repressed mineralization of all assessed hydrocarbons, resulting in increased lag periods and decreased overall mineralization rates compared to eluates derived from control treatments (Table 1). In general, exudates collected from wildrye grown under hydrocarbon stress were the least repressive, while those collected from non-stressed wildrye had the greatest repressive effect. Other studies have reported a comparable inhibitory effect for root exudates. *L. perenne* exudates have been found to induce a distance-dependent repressive effect on PAH degradation (Corgié et al., 2004, 2006). Rentz et al. (2004) found that both real exudates from a wide range of plants and single-compound potential root exudate substrates repressed the phenanthrene degrading activity of a *P. putida* strain. Although in our study cumulative mineralization of both hexadecane and naphthalene in most treatments reached parity after 28 days (Table 1), the initial and variable repressive effects offer insights into the underlying mechanisms of plant-microbe interactions in these systems.

Previous studies have linked increased hydrocarbon degradation in exudate amended systems to non-specific increases in bacterial populations (Da Silva et al., 2006; Kamath et al., 2004; Rentz et al., 2004; Tuomi et al., 2004). In this study however, DGGE analysis revealed no detectable difference in the type or relative abundance of the dominant bacterial species found in the different treatments (Fig. 2), and 16S rRNA gene quantification showed no statistically significant shifts in microbial population abundance (Table 3). While shifts in numerically minor yet active microbial species (Corgié et al., 2006) may have occurred, they were not detected. Previous research on the soil used in this study (Phillips et al., 2006, 2008) has shown that over time plants do exert a selective influence on both rhizosphere community structure and population density. However, the indigenous microbial community includes many species adapted to hydrocarbon contaminated environments (Phillips et al., 2006 and supplementary Fig. S2) and a

greater nutrient input may be required before changes to the overall community structure occur (Griffiths et al., 1999). The added levels of exudates were sufficient however, to elicit changes to the metabolic capacity of the soil microbial communities. Our results show that these changes in degradation potential were associated with specific differences in exudation patterns.

Exudate patterns are known to be influenced by a myriad of factors, including plant species (Ciéslinski et al., 1998; Lesuffleur et al., 2007), soil composition (Ciéslinski et al., 1998), environmental parameters (Henry et al., 2007), and the presence of xenobiotics (Bais et al., 2004). Similarly, we found differences in the overall exudation patterns both between plant species and between PAH stressed and non-stressed plants of the same species (Fig. 1). We also found however, that the composition of exudates released by replicates of a given treatment was highly variable (Fig. 1). Other studies have noted that individual plants of the same species (Lesuffleur et al., 2007) and the same variety (Ciéslinski et al., 1998; Micallef et al., 2009) may have substantially different exudation patterns when grown under the same conditions. This intra-species exudate variation has been shown to have a significant impact on the composition of rhizosphere microbial communities (Micallef et al., 2009) and on bacterial gene expression (Mark et al., 2005). With respect to hydrocarbon degradation, such exudate variation may have a significant and specific impact on the degradative fitness of the microbial community. In the current study, increased concentrations of the organic acid malonate in exudates were associated with decreased degradation potential (Table 2). In contrast, increased concentrations of the organic acid acetate and the amino acid alanine were in general associated with increased degradation potential.

Exudates may specifically influence the degradative fitness of an existing microbial community via a number of mechanisms, including shifts in catabolic gene expression, general metabolic status, and catabolic gene transfer (Da Silva et al., 2006; van Elsas et al., 2003). Both catabolite repression and induction are particularly important phenomena for environmental bacteria, which must quickly adapt to rapidly changing carbon sources without loss of competitive fitness. Although gene expression was not specifically assessed in the current study, if catabolic repression was the dominant factor we would expect to see minimal changes in catabolic gene copy number, yet still see decreased hydrocarbon mineralization. This occurred in hexadecane microcosms, where alkane hydroxylase gene copy numbers were equivalent in all microcosms (Table 3) and not correlated with mineralization parameters (Table 4). Previous research has shown that rich carbon sources inhibit alkane degradation by catabolite repression of the *alkB* operon in a *Pseudomonas* species (Yuste et al., 1998). Similarly, numerous organic acids, including fumarate, have been shown to repress *PalkB* promoter expression in *Burkholderia cepacia* (Marín et al., 2001). In our study, the decreased alkane mineralization associated with the organic acids fumarate and malonate (Table 2) may be indicative of catabolite repression.

Similar repression may have occurred in the exudate-amended PAH microcosms, as exudates from a wide variety of plants have been shown to repress *nahG*, a gene involved in naphthalene dioxxygenase transcription (Kamath et al., 2004). However, we also observed significant changes in the copy number of both catechol 2,3 dioxxygenase and naphthalene dioxxygenase genes (Table 3), and the abundance of these genes was highly correlated with PAH mineralization parameters (Table 4). Bacterial gene copy number within a soil may be altered by several mechanisms, including shifts in general microbial populations, DNA replication preceding bacterial division, and plasmid transfer. In naphthalene microcosms, increases in general microbial populations may have accounted for some of the change in degradation potential, as 16S rRNA and catabolic gene copy numbers were highly correlated ($r > 0.600$,

$p < 0.001$). One factor which may have impacted microbial abundance in these microcosms is the organic acid malonate, which was negatively associated with 16S rRNA gene copy numbers (Table 2). Malonate is a cellular respiration inhibitor that acts by competitively inhibiting the active site of succinate dehydrogenase in the citric acid cycle. Thus malonate may have impacted the metabolic status of the soil microbial communities such that replication was inhibited. If depressed microbial populations were the dominant factor impacting catabolic gene copy number however, we would have expected to see significant population differences between treatments. As previously discussed, this did not occur, nor was there any correlation between population densities and catabolic genes in phenanthrene microcosms.

Many bacterial genes involved in PAH degradation are carried on conjugative plasmids (Nojiri et al., 2004). The catabolic genes assessed in the current study are commonly found on the same plasmid, with C2,3O often present in multiple copies (Li et al., 2006; Sentchilo et al., 2000). While gene location was not directly assessed, the very high correlation between *nahAc* and C2,3O copy numbers ($r > 0.939$, $p < 0.001$), independent of 16S rRNA gene copy number, suggests that they were located on the same plasmid. If so, then plasmid transfer may have been a significant factor influencing the changes in degradation potential. Horizontal gene transfer (HGT) is known to occur with high frequency in the rhizosphere (Jussila et al., 2007; van Elsas et al., 1988, 2003), stimulated in part by increased exudation (Mølbak et al., 2007). In our study, *nahAc* and C2,3O copy numbers were positively associated with the organic acid acetate and the amino acid alanine (Table 2), two compounds which have been shown to directly increase HGT events (Nielsen and van Elsas, 2001). We also observed a negative association between these genes and the organic acid malonate. As previously discussed, malonate may repress the metabolic status of microbial communities. Several studies suggest that conjugative transfer of plasmids is associated with increased cellular metabolic activity (Lilley et al., 1994; Smets et al., 1993; van Elsas et al., 1988), therefore increased concentrations of malonate within the exudates could play a role in decreasing catabolic gene transfer. As plasmid copy number was not directly assessed in our study, no definitive link may be made with the decreased degradative capacity of some exudate-amended microcosms. However, this mechanism certainly warrants further investigation in phytoremediation studies.

Our findings provide insight into why specific plant species may promote degradation under one set of circumstances, yet inhibit it under another. Comparable differences in exudation patterns may have contributed to the heritable differences in crude oil degradation facilitated by genotypic clones of alfalfa in another study (Schwab et al., 2006; van Elsas et al., 2003). Our results suggest that the patterns of exudation are the driving factors behind the degradation traits. Recent research with *Arabidopsis thaliana* accessions (Micallef et al., 2009) suggests that some exudation traits are also stably heritable. If so, then plant-specific selection and breeding for specific patterns of exudation could greatly further the effectiveness of phytoremediation treatments, and remove some of the current uncertainty associated with plant selection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apsoil.2011.10.009.

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