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Field-scale assessment of weathered hydrocarbon degradation by mixed and single plant treatments

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ABSTRACT

Phytoremediation is gaining recognition as a viable treatment option for hydrocarbon contaminated soil. However, there is a recognized need for field research to corroborate the findings of controlled environment studies, particularly in cold climate regions. In this study, a two-year field trial was conducted at a weathered hydrocarbon flare-pit site in southeastern Saskatchewan, Canada. Three plants commonly used in phytoremediation mixes (tall wheat grass, Altai wild rye (AWR) and alfalfa), a mix of all plants, and non-planted controls were established and sampled regularly over two growing seasons. Total petroleum hydrocarbon (TPH) concentration and microbial parameters, including endophytic and rhizosphere degrader populations, were measured at 6-week intervals during the growing seasons. Significant differences occurred in degradation trends in the first growing season, with AWR promoting greater than 50% TPH degradation while no cumulative degradation occurred in mixed plant or control treatments. Some of the discrepancy in degradation potential was related to microbial population dynamics. Results show that AWR selectively recruits endophytic hexadecane degraders in response to high TPH concentration (r, 0.795; $p < 0.01$) and then maintains these communities during times of environmental stress. During local drought, when most plants experienced significant decreases in rhizosphere and endophytic degrader communities, AWR supported up to 100 times more endophytic hexadecane degraders than the other plants. It is probable that the increased degradation seen in AWR treatments is related to its ability to act as a refuge and hence subsequent source for hydrocarbon degrader communities. As both plants and microbial communities mature, these discrepancies in degradation potential are decreased and cumulative TPH degradation increases in all treatments. Although this study shows that the use of mixed plant treatments may initially hinder the achievement of remediation goals, extenuating factors, including the increased desorption of previously unextractable hydrocarbons, may cause an underestimation of the actual amount of degradation occurring in all treatments.

1. Introduction

Fossil fuels are an integral component of our modern industrial society. The extraction, transport, and use of these fuels, however, pose inevitable environmental risks. Spills, leaks, and discharges of petroleum hydrocarbons occur simply due to the nature of resource extraction, and to both human and mechanical error. In western Canada alone there are over 300,000 small-volume hydrocarbon contaminated sites consisting of current and former oil and gas wells (Canadian Council of Ministers of the Environment, 2008). Of these, an estimated 100,000 contain earthen flare pits, which were used to store and then burn liquid waste hydrocarbons (condensate and crude oils), chemicals, salt water, bitumen, and other waste products associated with petroleum extraction (Speer, 1999). Soil at these sites is impregnated with a complex mixture of recalcitrant hydrocarbons and may be co-contaminated with high salt concentrations and inorganic compounds. The cost of remediating these and other hydrocarbon contaminated sites in Canada is estimated at over forty billion dollars (CCME, 2008). While typical remediation options for both flare-pit and other hydrocarbon sites involves excavation and

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off-site treatment in biopiles, incinerators or slurry- and solid-phase reactors (Amatya et al., 2002), more cost-effective and less destructive alternate treatments are desired. Phytoremediation is one such treatment being investigated.

The usefulness of phytoremediation to enhance the degradation of organic contaminants has been extensively researched in recent years (for review see Arthur et al., 2005; Chaudry et al., 2005; Pilon-Smits, 2005). Whereas mounting evidence from studies under controlled environmental conditions show that phytoremediation is a feasible remediation option for petroleum hydrocarbons (Kirk et al., 2005; Liste and Prutz, 2006; Phillips et al., 2006) there is a recognized need for corroborating field studies, particularly in those climate regimes found in areas such as western Canada (Pilon-Smits, 2005; Frick et al., 1999). These regions typically see temperatures ranging from −35 °C in the winter to 35 °C in the summer and often experience long periods of summer drought. The impact that this will have on the success of phytoremediation is unknown. For phytoremediation to be considered a viable treatment option for Canadian sites, results derived under controlled environmental conditions must also be shown to occur under natural conditions.

In hydrocarbon contaminated soils, plants enhance degradation by both specifically and non-specifically stimulating the density, diversity and activity of hydrocarbon-degrading microorganisms within plant roots (Newman and Reynolds, 2005; Ryan et al., 2008) and in the surrounding rhizosphere (Anderson et al., 1993; Siciliano and Germida, 1998). Because combined root types and exudate patterns are believed to allow greater infiltration and stimulation of microbial communities, phytoremediation systems are often composed of mixes of monocots and dicots, such as grasses and legumes. However, several studies on the efficacy of phytoremediation for weathered hydrocarbon contaminated soil have found that mixtures of grasses and legumes result in a decrease in degradation potential compared to single grass treatments. During a 15-month field trial on weathered contaminated soil in California, Banks et al. (2003) found that mixed plant treatments exhibited less than 20% reduction in total petroleum hydrocarbons (TPHs), compared to more than 50% in fescue treatments. Similarly, a recent study by our group (Phillips et al., 2006) found that single grass treatments facilitated reductions of up to 50% of TPH in weathered flare-pit soil within 4.5 months, compared to less than 15% by mixed plant treatments. The underlying mechanisms that drive this phenomenon are currently unknown and merit further research.

This study was designed to address the knowledge gaps discussed above, by assessing the long term impacts of both mixed and single plant treatments on endophytic and rhizosphere microbial communities under field conditions in western Canada. Specific objectives were to (1) determine if the reduced degradation potential observed in mixed plant treatments under controlled conditions also occurs under field conditions and (2) investigate the probable role of endophytic and rhizosphere microbial communities in degradation responses.

2. Materials and methods

2.1. Phytoremediation site and sampling

A two-year phytoremediation study was established at a hydrocarbon contaminated site located in southeastern Saskatchewan, Canada. Soil at the site, derived from an adjacent decommissioned flare-pit with a thirty-year history of chronic releases, was classified as moderately alkaline and saline/sodic. The soil had a clay texture, pH of 8.0, EC 5.8 d Sm^{-1}, SAR 20.3, CEC 18.46 cmol kg^{-1}, bulk density 1.13 g cm^{-3}, and NO_3-N, P and K concentrations of 1.6, 1.0, and 332 mg kg^{-1}, respectively (EnviroTest Laboratories, Saskatoon, SK). Initial hydrocarbon concentrations at the site averaged 7000 mg kg^{-1} and consisted primarily of F3 (70%) and F4 (30%) hydrocarbon fractions. Plots (1 m × 1 m) were arranged in a randomized complete block design, with five treatments replicated four times. The treatments consisted of an unplanted control, *Medicago sativa* L. (alfalfa var. rambler), *Agropyron elongatum* (Host) P. Beauv. (tall wheat grass), *Elymus angustus* Trin. (Altai wild rye) and a mixed plant treatment containing all three plants. Plants were selected based on their performance in growth chamber studies (Phillips et al., 2006). All plots were amended with gypsum ($CaSO_4{\cdot}2H_2O$, 5 kg m^{-2}), straw (1.62 kg m^{-2} soil), 34-0-0 (26.4 mg m^{-2}) and 12-51-0 fertilizer (9.6 mg m^{-2}), and the Real Thing™ farm compost (Agricore United, 22 kg m^{-2}), which were rototilled to a 0.3 m depth prior to planting. Post-amendment, soil pH was 7.4, EC 5.2 dS m^{-1}, SAR 20.9, CEC 25.5 cmol kg^{-1}, bulk density 0.94 g cm^{-3}, and NO_3-N, P and K concentrations were 629, 989, and 4900 mg kg^{-1}, respectively. A final layer of compost (39.6 kg m^{-2}) was added to each plot to serve as a seed bed. Each plot was separated from other plots by a 0.5-m non-amended buffer strip.

Plots were fall-seeded in October 2004 and then sampled during the growing seasons of 2005 and 2006. Sampling was done at approximately 6-week intervals and occurred in June, July, and September. The three sampling periods were designed to accommodate both site access and plant growth stages. By the June sampling periods shoots were actively growing, by the July sampling periods plants were flowering, and by the September sampling period plants were setting seed. For microbial analysis, both control soil and representative plants and their attached roots/soil were excavated to a 25 cm depth, the roots and soil were placed in a sterile bag, and the entire plant was placed on ice for transport back to the laboratory, where they were stored at 4 °C until processing and analysis (within 36 h of sampling for soil samples and 72 h for endophytic samples). For hydrocarbon analysis, two 0–25-cm samples were taken with a soil auger from each plot, composited, packed into glass soil jars with PTFF lined lids (Life Sciences, Peterborough, Ont.), placed on ice for transport back to the laboratory, and then frozen at −20 °C until analysis.

2.2. Plant sampling and processing

Rhizosphere and endophytic communities were evaluated for all plants. Roots were shaken to remove any loose soil, which was discarded. Next, rhizosphere soil was collected by vigorously shaking the roots onto a sterile surface, and by rolling roots on a sieve to remove any remaining soil. For endophytic community assessment, rinsed roots were surface disinfected by sequential washes with 95% ethanol and 5.25% sodium hypochlorite, followed by a minimum of five rinses with sterile water. To assess surface sterility, 100 μL aliquots of the final rinse water were spread on 1/10th TSA plates. An additional 1 mL aliquot of the final wash water, boiled to release DNA, was assessed by PCR using the eubacterial primers outlined in the following sections. Roots were stored at 4 °C for 24 h while awaiting results from sterility assessments. All other samples were immediately assessed using culturable techniques.

2.3. Microbial community analysis

Endophytic extracts were produced by macerating 2.5 g surface-sterile root from each treatment replicate in 22.5 mL monopotassium phosphate (MPP) buffer (0.65 g K_2HPO_4, 0.35 g KH_2PO_4, 0.10 g $MgSO_4$ L^{-1} water) using a sterile mortar and pestle. Control and rhizosphere soil and root extracts were serially diluted in MPP and these tenfold dilutions were used for culturable microbial enumeration and most probable number (MPN) assays

for hydrocarbon degraders. To determine moisture content 10 g sub-samples of each soil were oven-dried at 105 °C for 24 h.

2.3.1. Heterotrophic microbial communities

Total culturable heterotrophic bacteria were enumerated by plating in triplicate 100 μL of each dilution (10^{-2}–10^{-7}) from each treatment on 1/10 TSA plates containing 0.1 g L^{-1} cycloheximide. Plates were incubated at room temperature for 7 days.

2.3.2. Hydrocarbon degrading potential of microbial communities

Hydrocarbon degrading bacteria were enumerated using a modified MPN protocol (Wrenn and Venosa, 1996) as described in Phillips et al. (2006). Each treatment replicate was assessed for n-hexadecane and polyaromatic hydrocarbon (PAH) degraders in separate 48-well microtiter plates. For n-hexadecane (Sigma–Aldrich) plates, 20 μL of filter-sterilized hydrocarbon was added to wells containing 720 μL Bushnell Haas (BH) mineral salts medium. For PAH plates, 40 μL of a PAH mixture dissolved in pentane (per litre: 10 g phenanthrene, 1 g anthracene, 1 g fluorene, 1 g dibenzothiophene; Sigma–Aldrich) was added to each well and the pentane was allowed to evaporate off prior to BH addition. Each plate was inoculated with 10^{-7}–10^{-1} serial dilutions (80 μL per well, five wells per row, one dilution per row) of soil or root extracts in MPP buffer. A final control row was inoculated with 80 μL MPP buffer. All plates were incubated in the dark at room temperature. After 2 weeks, 200 μL of filter-sterilized p-iodonitrotetrazolium violet (3 g L^{-1}) was added to each well of the n-hexadecane plates, plates were incubated overnight, and positive wells were counted. PAH plates were incubated for an additional week and positive wells were scored by the presence of yellow to brown colour due to the partial oxidation of aromatic compounds (Wrenn and Venosa, 1996).

2.4. Hydrocarbon degrading activity of microbial communities

The hydrocarbon degrading activity of control soil, rhizosphere soil and endophytic microbial populations was assessed using C-14 hydrocarbon mineralization assays. Microcosms were set up and sampled as outlined in Chénier et al. (2003). Serum vials containing either 2 g rhizosphere or control soil or 2.5 g macerated root and associated buffer were amended with 50,000 dpm (100 mg kg^{-1}) of [1-^{14}C]n-hexadecane or [9-^{14}C]phenanthrene (specific activities 12 and 8.2 mCi $mmol^{-1}$, respectively; Sigma–Aldrich, Mississauga, Ontario, Canada). A 1.8-mL glass vial with 0.5 mL 1 M KOH was inserted into each microcosm prior to crimp sealing to function as a $^{14}CO_2$ trap. The KOH was periodically aspirated, added to 10 mL scintillation cocktail (ACSII, Amersham), and counted by liquid scintillation spectrometry (Beckman LS 3801). Abiotic controls for each hydrocarbon treatment were established using gamma-irradiated soil (2 × 3.0 Mrad with a 1-week resting interval).

2.5. Hydrocarbon analysis

Treatments were analyzed for F2 to F4 hydrocarbon fractions using a modified shaking extraction method (Schwab et al., 1999) followed by GC-FID analysis. A 2-g sub-sample of soil was mixed with 1 g sodium sulphate and 10 mL 50:50 (v/v) hexane:acetone solvent (OmniSolv, EMD Chemicals, Germany), shaken for 1 h, and then centrifuged at 2000 rpm for 10 min. Supernatants were transferred to a clean vial containing 0.5 mL toluene, evaporated under nitrogen gas without heating to 0.5 mL, transferred to a GC vial, and brought to a final volume of 1.8 mL with toluene. Duplicate sub-samples of each plant/control replicate were extracted and analyzed at each sampling point, then averaged to give a final value for that treatment replicate. Samples were analyzed on a Varian CP-3800 GC equipped with a FID detector and an 8400 autosampler. A Varian 5-CB fused silica column (100% dimethylpolysiloxane) with dimensions of 30 m × 0.25 mm i.d. and a 0.25-μm stationary-phase film thickness was used for analytical separation. The carrier gas was hydrogen with a flow rate of 50 mL min^{-1} and the makeup gas was helium with a flow rate of 30 mL min^{-1}. Air was supplied as an oxidant at a rate of 330 mL min^{-1}. Detector and injection port temperatures were 320 and 300 °C, respectively. A 0.5-μL splitless injection volume was delivered with a 10-μL syringe. The initial column oven temperature was maintained at 40 °C for 1 min and then ramped at a rate of 20 °C min^{-1} to 300 °C where it was held for 14.30 min. Total run time was 28.30 min. Toluene blanks and analytical controls of 100 mg kg^{-1} C34 standard (Supelco, Bellefonte, PA) were run for every 10 samples. Data were analyzed using a linked Varian Star Chromatography Workstation v. 6.2 (Varian Inc., Walnut Creek, CA). Integrated GC areas were converted to hydrocarbon concentrations using the average response factor for a calibration curve series of C10, C16 and C34 hydrocarbon standards (Supelco, Bellefonte, PA), according to Remediation Technologies Development Forum standard protocol (http://www.rtdf.org).

2.6. Statistical methods

Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, IL). Hydrocarbon and microbial 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 using Pearson's correlation (parametric data) or Spearman's rank correlation (non-parametric data).

3. Results

3.1. Decrease in total petroleum hydrocarbon concentration

Treatment specific reductions in extractable TPH concentrations were observed over the course of the study. AWR treatments stimulated the greatest overall decrease (>50%) in TPH during the first growing season (Table 1). In contrast, mixed plant and non-planted control treatments exhibited a net increase of 6% in TPH levels at the end of the first growing season, at least partially due to increases in the extractable F4 fraction hydrocarbons (Table 1, Fig. 1). Although all treatments reached comparable TPH levels by the end of the second season, the single grass treatments AWR and tall wheat grass (TWG) exhibited the highest overall TPH degradation (56 and 50%, respectively). High spatial variability in initial TPH levels was observed at the beginning of the study, even though the plots were distributed over a relatively small total area (5.5 m × 7 m) consisting of contaminated material that had been homogenized prior to site establishment. Although the initial starting concentrations in the majority of the plots was between 5000 and 7000 mg kg^{-1}, several hotspots up to 11,000 mg kg^{-1} were found in AWR, TWG, and mixed plant treatment plots (data not shown). Due to this high variability, large absolute differences in degradation, such as that observed between AWR and control treatments at the end of the study (Table 1) were often not statistically significant.

3.2. Heterotrophic microbial communities

Endophytic CFUs were highly variable both within and between treatments during the first season, and then stabilized over time

Table 1
Percent change in total petroleum hydrocarbon concentration after one (2005) and two (2006) growing seasons by planted and non-planted treatments, compared to time 0.

Treatment[a]	Fraction 3 (C16–34)	Fraction 4 (C34–50)	Total hydrocarbons (C10–C50)
2005			
Control	+5.4 (28.4)b[b]	+9.2 (22.7)bc	+6.4 (25.2)b
Mixed plants	−14.1 (27.7)ab	+53.8 (37.2)c	+6.0 (30.6)b
Alfalfa	−35.8 (23.1)ab	−23.1 (27.1)ab	−31.9 (24.3)ab
Altai wild rye	−56.1 (17.5)a	−46.2 (22.2)a	−53.4 (18.6)a
Tall wheat grass	−35.1 (34.2)ab	−33.9 (16.6)ab	−35.4 (28.1)ab
2006			
Control	−32.6 (10.8)a[b]	−27.4 (7.8)a	−31.0 (8.6)a
Mixed plants	−49.2 (18.7)a	−20.7 (25.4)a	−40.7 (21.0)a
Alfalfa	−47.7 (24.2)a	−34.2 (29.3)a	−43.5 (25.9)a
Altai wild rye	−60.5 (8.2)a	−44.0 (12.6)a	−55.9 (9.4)a
Tall wheat grass	−51.8 (25.7)a	−45.3 (15.1)a	−50.1 (22.1)a

[a] Control: non-planted control soil; mixed plants: mixture of alfalfa, Altai wild rye and tall wheat grass. Data are presented as means ($n = 4$) with S.D. in parentheses.
[b] Means in a single sub-column followed by a different letter are significantly different at $p \leq 0.05$.

(Fig. 2A). In general AWR maintained higher endophytic CFUs than other plant treatments over the duration of the study. Endophytic heterotrophic communities in general were positively related to local soil moisture conditions and TPH concentration during the first growing season, but not during the second growing season (Table 2). However, there were plant-specific influences on the strength of these correlations. The correlation between moisture and endophytic communities was influenced by AWR, TWG, and mixed plant treatments ($r \geq 0.774$; $p < 0.01$) but not by alfalfa. The correlation between TPH concentration and endophytic communities was influenced by AWR and TWG treatments ($r \geq 0.614$; $p \leq 0.05$) but not by alfalfa or mixed plant treatments.

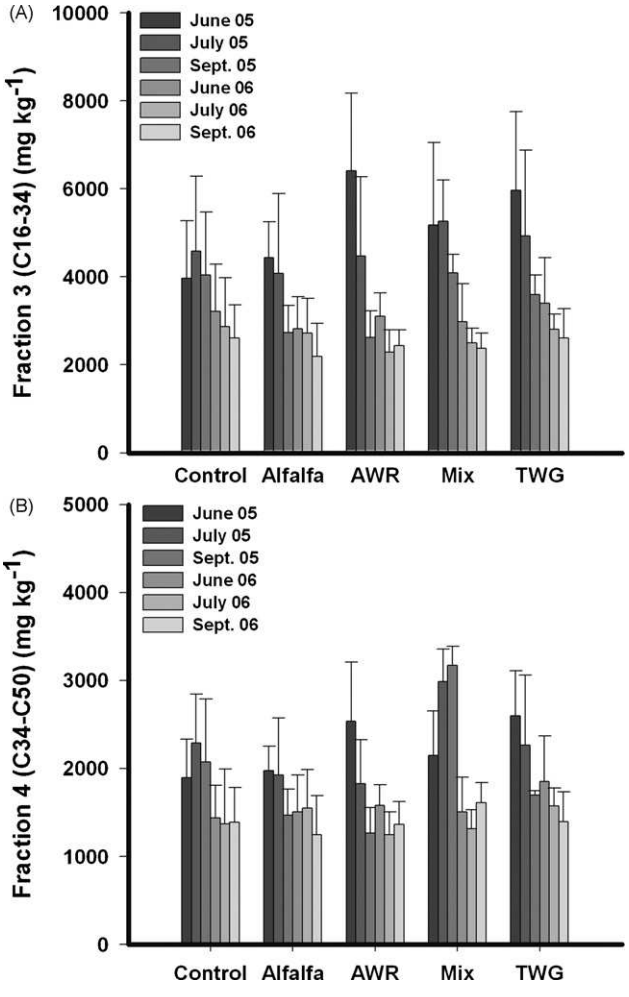

Fig. 1. Fraction 3 (A) and fraction 4 (B) hydrocarbons in control and planted plots over a two-season phytoremediation field trial. Treatments include non-planted control soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as means ($n = 4$) with error bars representing 1 S.D.

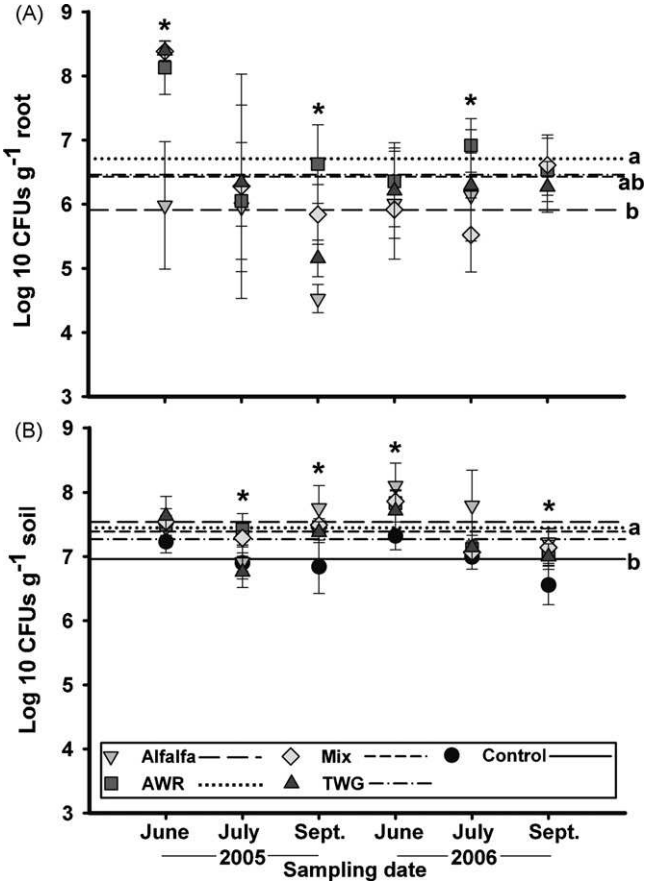

Fig. 2. Heterotrophic microbial populations within (A) plant roots and (B) rhizosphere or control soil during a two-season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means ($n = 4$) with error bars representing ±1 S.D. Error bars may be obscured by data points. *Significant difference at individual sampling points ($p \leq 0.05$). Horizontal lines indicate average populations for each treatment over the duration of the study; lowercase letters indicate a significant difference between treatments ($p \leq 0.05$).

Table 2
Correlation coefficients for soil and endophytic microbial communities, sampled at 6-week intervals over two growing seasons.

Microbial communities	Year	Soil communities[a]		Endophytic communities		Moisture	TPH
		Hexadecane degraders	PAH degraders	Heterotrophic	Hexadecane degraders		
Soil communities[a]							
Heterotrophic	2005	0.074	0.624***	0.054	0.206	0.153	0.022
	2006	0.573***	0.340**	−0.142	−0.117	0.335**	0.037
Hexadecane degraders	2005		−0.097	0.372**	0.397***	−0.036	0.368**
	2006		0.232	−0.018	−0.077	0.191	0.165
PAH degraders	2005			−0.138	−0.214	−0.222	−0.188
	2006			0.125	0.188	−0.306**	0.002
Endophytic communities							
Heterotrophic	2005				0.825***	0.278**	0.483***
	2006				0.501***	−0.046	0.034
Hexadecane degraders	2005					0.558***	0.442**
	2006					−0.241	−0.070
PAH degraders[b]	2005			0.443**	0.517***	0.205*	0.325*
	2006			0.016	−0.069	0.103	−0.135

[a] Control and/or rhizosphere soil.
[b] Endophytic PAH degraders were not correlated with soil microbial communities.
* Significant at $p \leq 0.05$.
** Significant at $p \leq 0.01$.
*** Significant at $p \leq 0.001$.

Heterotrophic communities were relatively stable over the course of the study, averaging between 10^7 and 10^8 CFUs g^{-1} soil (Fig. 2B). Although differing from control soil, no significant differences were observed between the rhizosphere CFUs of any planted treatment either at any given sampling time, or over the course of the study. These general heterotrophic populations were positively correlated with local soil moisture conditions during the 2006 season (Table 2), with all treatments except alfalfa contributing to the correlation. During both seasons, rhizosphere heterotrophic communities of all planted treatments were maintained at significantly higher levels than those in the control soil (Fig. 2B).

3.3. Hydrocarbon degrading potential of microbial communities

Degrader communities showed less seasonal stability than heterotrophic communities. There were both sampling date and seasonal differences in hexadecane degrader populations and AWR consistently maintained the largest populations of these endophytic degraders (Fig. 3A), at approximately an order of magnitude greater than other plants. Although average rhizosphere hexadecane degrader populations did not significantly differ between planted treatments over the duration of the study, sampling date specific and seasonal differences were again observed (Fig. 3B), and in 2005 AWR maintained significantly higher hexadecane degraders than alfalfa ($p < 0.01$, data not shown). Three measurable factors were associated with the magnitude of hexadecane degraders; total heterotrophic communities, local soil moisture and TPH concentration. In the first season, increased populations of rhizosphere degraders were associated with higher TPH concentration, while in the second season they were strongly correlated with heterotrophic communities (Table 2). Endophytic degraders of all treatments were strongly correlated with endophytic heterotrophic communities throughout the study, and strongly influenced by soil moisture during the first growing season (Table 2). An additional correlation was observed between endophytic hexadecane degraders and TPH concentration during the first growing season (Table 2), which was largely driven by AWR treatments ($r = 0.795$; $p < 0.01$). Finally, during the first growing season a strong correlation also was observed between the hexadecane degrader communities in each niche (Table 2).

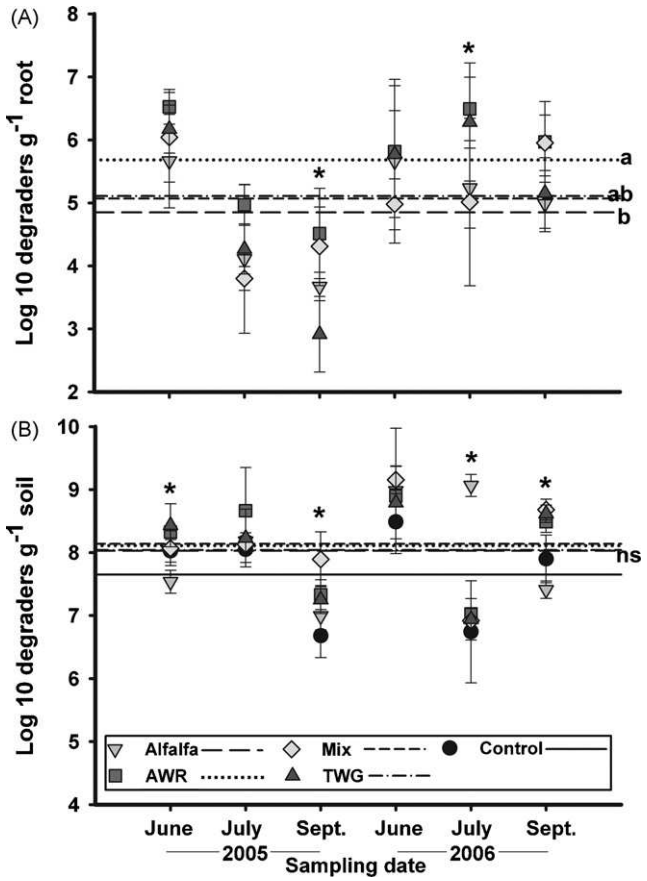

Fig. 3. Most probable number of microbial n-hexadecane degraders within (A) plant roots and (B) rhizosphere or control soil during a two-season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means ($n = 4$) with error bars representing ±1 S.D. Error bars may be obscured by data points. *Significant difference at individual sampling points ($p \leq 0.05$). Horizontal lines indicate average populations for each treatment over the duration of the study; lowercase letters indicate a significant difference in average populations ($p \leq 0.05$); ns: no significant difference in average populations ($p > 0.05$).

Rhizosphere and endophytic PAH degrader populations showed sampling date specific differences but were not significantly different between planted treatments either seasonally or over the duration of the study (Fig. 4). PAH degraders in soil niches were positively correlated with soil heterotrophic communities throughout the study duration (Table 2). AWR rhizosphere PAH degraders also showed a strong negative correlation with TPH concentration during the first growing season ($r = -0.767$; $p < 0.01$; data not shown). Endophytic PAH degraders in general were correlated with endophytic heterotrophic communities, endophytic hexadecane degrader communities, soil moisture, and TPH concentration during the first growing season (Table 2).

3.4. Hydrocarbon degrading activity of microbial communities

Endophytic hexadecane mineralization was highly variable within treatments, between treatments, and over time (Fig. 5A). Endophytic hexadecane mineralization, although occurring only sporadically, was positively correlated with MPN-enumerated endophytic hexadecane degraders during the first growing season, but negatively correlated with soil heterotrophic and hexadecane degrader communities during the second growing season (Table 3). All soil microbial communities actively mineralized from 40 to 50% of the added hexadecane, with no significant differences observed between any treatments during any given sampling point or time frame (Fig. 5B). A strong negative correlation was observed between soil hexadecane degrader populations as assessed using the MPN technique and hexadecane mineralization during both growing seasons (Table 3).

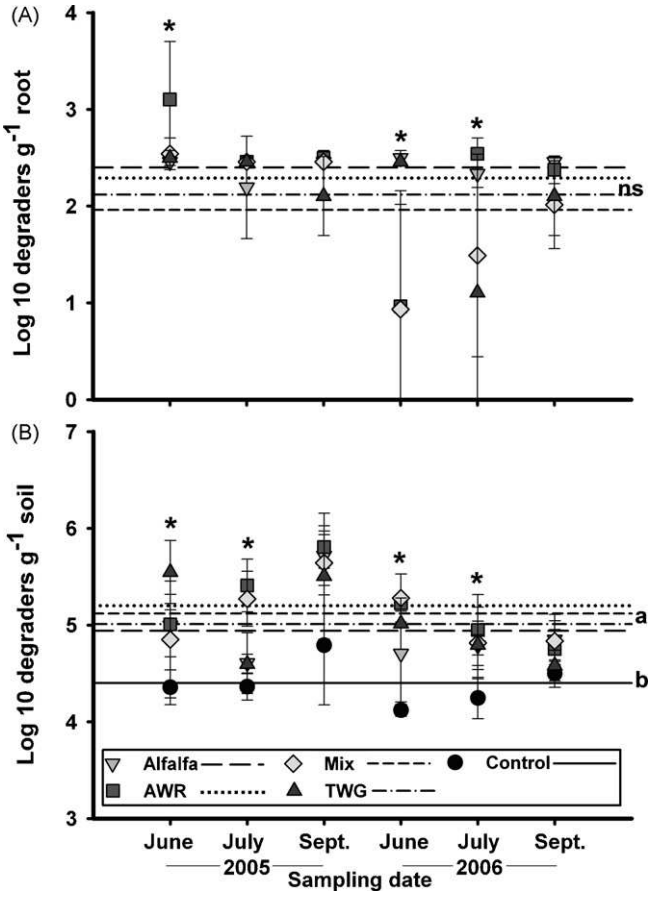

Fig. 4. Most probable number of microbial PAH degraders within (A) plant roots and (B) rhizosphere or control soil during a two-season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means ($n = 4$) with error bars representing ± 1 S.D. Error bars may be obscured by data points. *Significant difference at individual sampling points ($p \leq 0.05$). Horizontal lines indicate average populations for each treatment over the duration of the study; lowercase letters indicate a significant difference in average populations ($p \leq 0.05$); ns: no significant difference in average populations ($p > 0.05$).

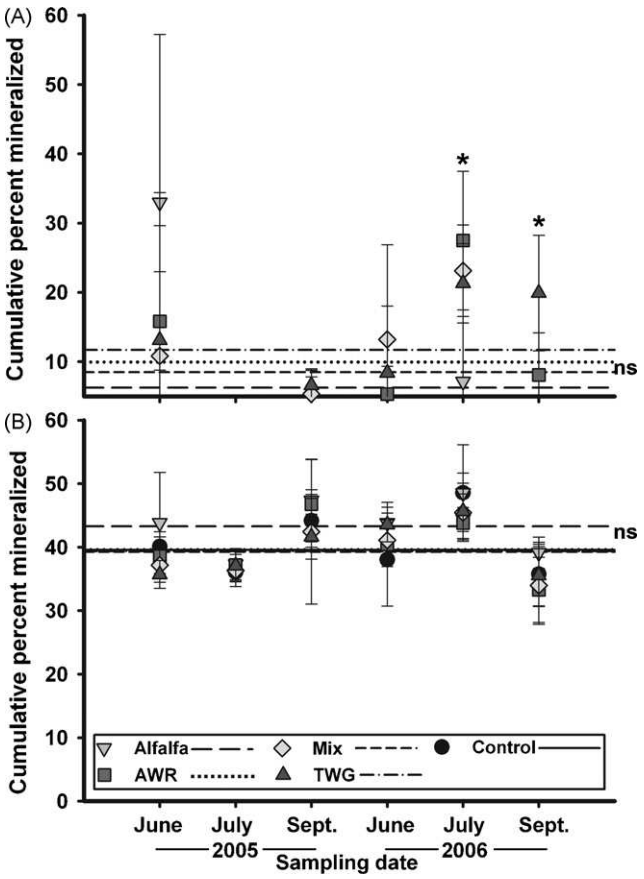

Fig. 5. Cumulative percent n-hexadecane mineralized by (A) endophytic microbial communities and (B) rhizosphere or control microbial communities during a two-season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as means ($n = 4$). Error bars represent ± 1 S.D. and may be obscured by data points. *Significant difference at individual sampling points ($p \leq 0.05$). Horizontal lines indicate average populations for each treatment over the duration of the study; ns: no significant difference in average populations ($p > 0.05$).

All rhizosphere microbial communities mineralized approximately 40% of the added phenanthrene in 3 weeks, compared to an average of less than 30% in control soils (Fig. 6). In contrast to trends seen with hexadecane mineralization, phenanthrene mineralization by rhizosphere communities was positively correlated with MPN-enumerated PAH degrader populations during both growing seasons (Table 3) and negatively correlated with hexadecane degraders during the 2006 season (Table 3). Rhizosphere phenanthrene mineralization was also negatively correlated with both endophytic heterotrophic and hexadecane degrader communities during the first growing season. There was a general correlation between the mineralization of both hydrocarbons over the duration of the study ($r = 0.410$; $p < 0.001$). Only a few sub-samples of endophytic communities were able to actively mineralize phenanthrene during the first growing season, and in the 2006 season no such mineralization was observed (data not shown).

4. Discussion

This study compared the potential of three single plant treatments and a combination of these plants to facilitate the

Table 3
Correlation coefficients for hydrocarbon mineralization and soil and endophytic microbial communities, sampled at approximately 6-week intervals over two growing seasons.

Hydrocarbon mineralization	Year	Soil microbial communities[a]			Endophytic microbial communities		
		Heterotrophic	Degrader		Heterotrophic	Degrader	
			Hexadecane	PAH		Hexadecane	PAH
Soil[a]							
Hexadecane	2005	0.263	−0.528***	0.243	−0.335*	−0.257	−0.061
	2006	0.228	−0.368**	0.177	−0.055	−0.046	0.365
Phenanthrene	2005	0.148	0.041	0.415***	−0.643***	−0.611***	−0.305*
	2006	0.130	−0.420***	0.244*	−0.082	−0.092	0.070
Endophytic							
Hexadecane	2005	0.334*	0.008	−0.018	0.184	0.395**	0.243
	2006	−0.363*	−0.431**	−0.013	0.114	0.153	−0.044

[a] Control and/or rhizosphere soil.
* Significant at $p \leq 0.05$.
** Significant at $p \leq 0.01$.
*** Significant at $p \leq 0.001$.

degradation of weathered hydrocarbons at a field site in south-eastern Saskatchewan, Canada. We found treatment specific decreases in TPH during the first growing season, with degradation in single plant treatments of up to 54% (Table 1) compared to no degradation in the mixed plant or control treatments. These field degradation trends match those observed in a related growth chamber study that utilized similar plants and soil from the current field site. In that study by our group (Phillips et al., 2006), single-species grass treatments also promoted greater TPH degradation than mixed plant and control treatments. At the field site however, this difference in total degradation decreased during the second growing season, after which there were no significant treatment effects on cumulative degradation. These results suggest the possibility of extrapolating results from the large number of controlled environment phytoremediation studies to real world situations, including contaminated sites in cold regions.

Both rhizosphere and endophytic heterotrophic and hydrocarbon degrader microbial communities were evaluated at 6-week intervals over the two growing seasons. Correlations were observed between many of these parameters that were significant across the duration of the study. Examination of seasonal data however, often revealed that strong correlations in either 2005 or 2006 were sufficient to influence the trends seen over the entire study. Examination of individual treatments within each season further revealed that for some parameters, specific planted treatments contributed strongly to the observed correlations. Our data shows that very specific relationships occurred during each individual season, corresponding to initial plant establishment in 2005 and mature plant growth in 2006, and that planted treatments did not respond equally.

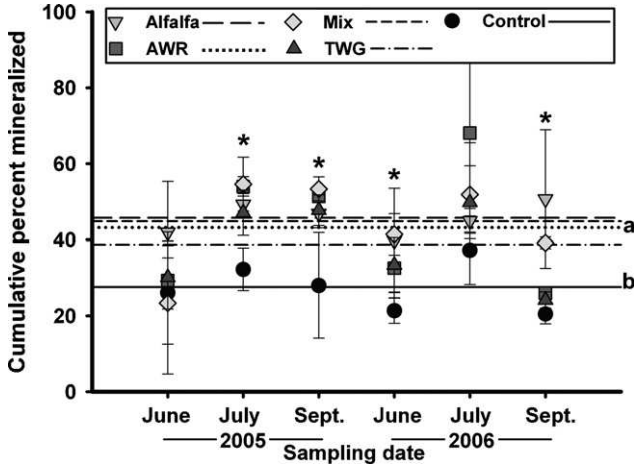

Fig. 6. Cumulative percent phenanthrene mineralized by rhizosphere or control microbial communities during a two-season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Error bars representing ±1 S.D. and may be obscured by data points. *Significant difference at individual sampling points ($p \leq 0.05$). Horizontal lines indicate average populations for each treatment over the duration of the study; lowercase letters indicate a significant difference in average populations ($p \leq 0.05$).

In the first growing season, external pressures were significant in determining endophytic community structure. When external moisture was high, plants in general supported higher endophytic communities and when external hexadecane degraders were abundant, higher levels of endophytic degraders also were found (Table 2). As plant treatments matured however, these relationships disappeared and by the 2006 season no such correlations occurred, indicating that plant-specific factors had become the dominant determinant. Three-year-old plants harvested from the same phytoremediation site used in this study (Phillips et al., 2008) also developed unique plant-specific endophytic communities that differed in community structure and in hydrocarbon degradation potential from each other and from rhizosphere microbial communities. In the current study, some plants appeared to be more effective at shielding their endophytic populations from initial external environmental stresses. Altai wild rye, a grass with a high salinitiy/sodicity tolerance, maintained higher endophytic heterotrophic (Fig. 2A) and degrader populations (Fig. 3A) in September of 2005 and July of 2006, times of local drought (Fig. 7). During 2005 a strong correlation was observed between endophytic communities and rhizosphere aliphatic degrader communities (Table 2), suggesting that if AWR roots serve as a refuge for bacteria during times of stress, they may also act as a subsequent source for rhizosphere populations once environmental condition become favourable.

Altai wild rye endophytic aliphatic hydrocarbon degrader communities also showed a significant positive correlation to TPH concentration in the first growing season ($r = 0.795$; $p < 0.01$). Increased hexadecane degrader populations in AWR roots could result from non-specific factors. Research has shown that hydrocarbon uptake in grass roots occurs primarily in the root hair and branching zones (Wild et al., 2005), the same regions associated with high endophytic colonization (Hallmann and Berg, 2006). Increased hydrocarbon uptake in grass roots due to increased soil volume colonization (Gregory, 2006), lower hydrocarbon-adsorbing root lipid content (Gao and Zhu, 2004), or other factors, could therefore result in a concomitant non-specific

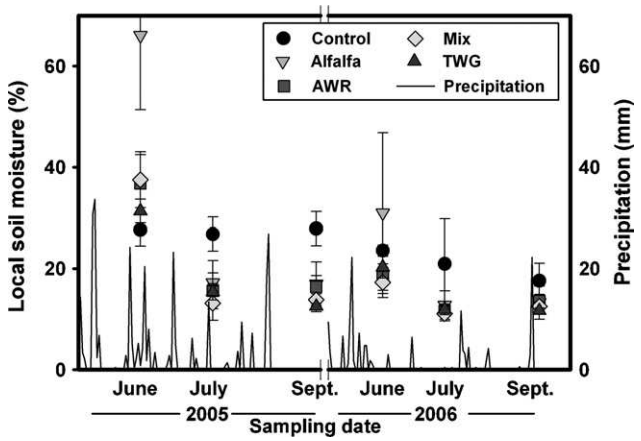

Fig. 7. Average soil moisture in treatment plots at each sampling point during a two-season phytoremediation field trial, compared to local precipitation over the growing season (Environment Canada). Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Error bars representing ±1 S.D. and may be obscured by data points.

increase in endophytic hydrocarbon degraders. If this were the only factor governing endophytic degrader populations however, one would expect to see comparable correlative increases in such degraders in both the TWG and mixed plant treatments. That this is not the case suggests that AWR specifically recruits aliphatic hydrocarbon degraders when external hydrocarbon concentrations are high. These results support previous research (Siciliano et al., 2001) which suggested that some plant endophytic communities were differentially established in response to soil contaminant levels.

While enhanced maintenance of degrader populations by AWR may contribute to the differences seen in degradation during the first growing season, there are undoubtedly other factors. General trends indicate that grass treatments maintained higher rhizosphere and endophytic degrader populations than alfalfa in 2005, which likely contributed to the overall degradation patterns (Figs. 3 and 4). Previous research has shown that grasses and legumes have different impacts on both microbial community composition and hydrocarbon degradation (Kirk et al., 2005; Phillips et al., 2006) which are related both to exudate production and to root morphology and physiology (Gao and Zhu, 2004; Gregory, 2006; Schwab et al., 1998). Evidence also suggests that grasses and legumes differ in the types of PAHs that are degraded. Parrish et al. (2005) found that fescue was more effective at degrading labile PAHs while clover degraded a higher percentage of strongly sorbed PAHs. If the above positive impacts were cumulative in mixed plant treatments, one would expect to see higher degradation. However, several previous studies on phytoremediation in weathered soils also have shown reduced TPH degradation when grasses and legumes are grown together (Banks et al., 2003; Phillips et al., 2006).

One contributing factor to the decreased degradation observed in mixed plant treatments during the first growing season was an increase in the detection of higher molecular weight hydrocarbons. The extractable C34 to C50 hydrocarbons in this treatment were significantly increased, from 2000 to 3000 mg kg^{-1} (Fig. 1). While both the chemical extractability and bioaccessibility of contaminants are known to decrease with aging or weathering (Semple et al., 2003, 2007), there is evidence that specific plants may increase the chemical extractability of strongly sorbed compounds in aged or weathered contaminated soils. Joner et al. (2002) found that the concentration of 5- and 6-ring PAHs was increased in soil microcosms amended with artificial plant root exudates. Liste and Prutz (2006) found that pea, cress, and pansy increased the extractability of PAHs by up to 60% and of TPHs by up to 16%. These increases in hydrocarbon concentration have been attributed to both mobilization and movement of contaminants to the rhizosphere and to desorption of previously unextractable compounds (Liste and Alexander, 2000). Increased desorption may occur due to the action of microbial surfactants (Christofi and Ivshina, 2002), plant derived surfactants (Read et al., 2003), and plant exudation patterns that impact physico-chemical soil properties (Joner et al., 2002). Allelopathic interactions between the grasses and alfalfa used in the mixed plant treatment may have altered both microbial and plant inputs, resulting in increased desorption of hydrocarbons (Chung and Miller, 1995; Nunan et al., 2005; Schenk, 2006).

In this study we also observed several cases where the TPH concentration (both C16–C34 and C34–C50) increased following the overwintering period (Fig. 1). A similar phenomenon was recorded during a mixed-plant phytoremediation field study of aged contaminated soil in Finland (Palmroth et al., 2006) where increased TPH levels were found in the first sampling following repeated winter seasons. Rezek et al. (2008) also found seasonal accumulation of PAHs following a simulated winter period. Early spring root growth or increased residue decomposition may have facilitated increased hydrocarbon desorption by the previously discussed mechanisms. Alternately, the results also suggest an influence of larger environmental impacts such as freeze–thaw on biotic- and abiotic-mediated desorption of hydrocarbons. If both biotic and abiotic factors act to increase the extractability of hydrocarbons during phytoremediation, it is likely that the actual amount of degradation is being underestimated, both in mixed and single plant treatments.

A final observation was the negative correlation between MPN-assessed hexadecane degraders and hexadecane mineralization in soil, but not endophytic, microbial communities, which occurred throughout the study (Table 3). At least one other study appears to have had comparable findings, although the relationship was not discussed. A study examining the degradation potential of Antarctic soils found that mineralization of hexadecane at 15 °C by two soils with 10,700 and 2738 hexadecane degraders per gram of soil was 15 and 30%, respectively (Aislabie et al., 2008). While it is possible in the current study that the two separate assays were simply measuring the degradation potential of separate populations, the strong negative correlation suggests a different relationship. In 2006, hexadecane degraders were positively associated with increased heterotrophic communities, which in turn increased with increasing moisture levels (Table 2). While decreased hexadecane mineralization has been linked to increased soil moisture and decreased oxygen availability (Børresen and Rike, 2007) no direct correlation between moisture and hexadecane mineralization occurred in either season. A more likely link lies in fundamental differences in the two assays.

Hexadecane is the primary carbon source in MPN assays, whereas mineralization assays occur in soil in the presence of significant additional carbon sources. The soil carbon sources, including root exudates such as succinate, may have stimulated the growth of bacteria capable of degrading hydrocarbons (as enumerated by the MPN assay) yet concurrently inhibited the expression of *alkB* genes involved in hexadecane catabolism by catabolite inhibition (Ruiz-Manxano et al., 2005; Yuste and Rojo, 2001). Large populations of these potential degraders could also have competitively excluded other potential alkane degrading bacteria (Espinosa-Urgel, 2004; Kästner and Mahro, 1996), resulting in the observed negative correlation between hexadecane degrader potential and activity. Regardless of the cause, these results illustrate the difficulty in parsing out degradation trends in complex soil environments, and highlight the need for caution

when using single assays to make general conclusions on degradation potential.

4.1. Conclusions

This study has shown that phytoremediation can be an effective treatment for petroleum hydrocarbon contaminated sites in western Canada. The use of mixed plant treatments however, may initially hinder the achievement of remediation goals, as no cumulative degradation was recorded in these treatments in the first growing season. Although all treatments reached comparable TPH levels by the end of the second season, the single grass treatments Altai wild rye and tall wheat grass exhibited the highest overall TPH degradation. Increased hydrocarbon degradation by Altai wild rye is likely related to this plant's ability to selectively increase and maintain endophytic hydrocarbon degraders. All treatments exhibited transient increases in hydrocarbon levels, either during the growing season as with mixed plant treatments, or after the overwintering period, as with the single plant treatments. These increases in measurable hydrocarbons are probably due to increased desorption of previously unextractable compounds by biotic and abiotic pressures. This phenomenon may bias interpretations on the effectiveness of phytoremediation and requires further study.

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