THE RELATIONSHIP BETWEEN PLANTS AND THEIR ROOT-ASSOCIATED MICROBIAL COMMUNITIES IN HYDROCARBON PHYTOREMEDIATION SYSTEMS

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ABSTRACT

Phytoremediation systems for petroleum hydrocarbons rely on a synergistic relationship between plants and their root-associated microbial communities. Plants exude organic compounds through their roots, which increase the density, diversity and activity of plant-associated microorganisms, which in turn degrade hydrocarbons. Understanding the mechanisms driving this relationship poses one of the more intriguing challenges in phytoremediation research. This study was designed to address that challenge. Plant-microbe interactions in a weathered-hydrocarbon contaminated soil were examined under controlled growth chamber, and field conditions. In both environments single-species grass treatments initially facilitated greater total petroleum hydrocarbon (TPH) degradation than *Medicago sativa* (alfalfa), mixed species, or control treatments. In growth chamber studies increased degradation was linked to increased aliphatic-hydrocarbon degrader populations within the rhizosphere. Under field conditions, specific recruitment of endophytic aliphatic-hydrocarbon degraders in response to high TPH levels may have facilitated increased degradation by the grass *Elymus angustus* (Altai wild rye, AWR). AWR stably maintained these communities during times of local drought, enabling them to act as subsequent source populations for rhizosphere communities. The broad phylogenetic diversity of AWR endophytes, compared to the *Pseudomonas*-dominated communities of other plants, contributed to the observed stability. The relative composition of exudates released by plants also impacted both degradation activity and potential. Alfalfa released higher concentrations of malonate, which hindered degradation by decreasing metabolic activity and concomitantly inhibiting catabolic plasmid transfer. In contrast, AWR exudates contained high levels of succinate, which was linked to increased catabolic gene expression and plasmid transfer. A reciprocal relationship between exudation patterns and endophytic community structure likely exists, and both parameters have a specific influence on rhizosphere degradation capacity. In this study, grasses were more successful in maintaining the specific balance of all parameters required for the transfer, preservation, and stimulation of hydrocarbon catabolic competency. .

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1.0 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. By 2002, the Canadian Council of Ministers of the Environment estimated that 60% of Canada's contaminated sites involved petroleum hydrocarbon (PHC) contamination (Canadian Council of Ministers of the Environment, 2002). 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 (Speer, 1999), 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. 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. PHCs are of concern due to their toxicity, mobility and persistence in the environment. Apart from ecological and health problems, there are significant financial repercussions associated with the release of these compounds into water and soil. Contaminated sites generally remain inactive until suitable clean-up has occurred, a process that may take substantial time and money. The cost of remediating hydrocarbon-contaminated sites in Canada is currently estimated at over forty billion dollars (CCME, 2008). While typical remediation options for both flare-pit and other hydrocarbon sites involves excavation and 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.

Phytoremediation systems for PHCs rely on a synergistic relationship between plants and their root-associated microbial communities. Degradation is facilitated through a rhizosphere effect; plants exude organic compounds through their roots,

increasing the density and activity of potential hydrocarbon degrading microorganisms in the zone surrounding the roots (Anderson et al., 1993; Siciliano and Germida, 1998). Although accumulating studies show that phytoremediation is a viable treatment option for PHCs (Arthur et al., 2005; Chaudhry et al., 2005; Pilon-Smits, 2005) this technology is still under-valued and under-utilized. The primary reason is the lack of consensus between study findings. Plant species vary greatly in their ability to increase the hydrocarbon degradation capacity of soil microbial communities (Liste and Prutz, 2006; Siciliano et al., 2003; Chiapusio et al., 2007; Parrish et al., 2004). Differences in degradation potential however, are not limited to different plant species. Separate phytoremediation studies often find contradictory results with regards to whether a specific plant species or species cultivar promotes hydrocarbon degradation (Binet et al. 2000; Rezek et al. 2008; Wiltse et al., 1998). These inconsistent results are linked to the paucity of knowledge surrounding the mechanisms whereby specific plants stimulate the growth and catabolic activity of rhizosphere microbial communities. How do plant-associated microbial communities differ, both structurally and functionally, between plant species? How do these differences impact degradation potential? Why do these plant-specific differences occur? How do differences in plant exudation patterns impact microbial degradation activity? Thus the mechanisms of this synergistic relationship remain ill-defined and pose one of the more intriguing challenges in phytoremediation research today. Until the determinant causes of increased degradation are elucidated, the full potential of phytoremediation will not be realized.

The studies undertaken in this thesis address these questions, with the primary objective of contributing to our understanding of the specific plant-microbe interactions that facilitate hydrocarbon degradation in the rhizosphere. This objective was pursued through a series of studies designed to address the following hypotheses:

1) Specific local plants promote increased net hydrocarbon degradation in weathered field soil
2) Individual plants promote hydrocarbon degradation by facilitating specific changes to the degradation potential and activity of dominant rhizosphere microbial communities

3) Endophytic microbial communities contribute to the overall degradation potential of individual plant species
4) Plant exudation patterns play a key role in determining plant-microbe inter-relationships
5) Specific components of root exudates facilitate catabolic gene expression by root associated bacteria

The studies presented in Chapters 3 through 9 are interlinked and overlapping, with each addressing more than one of the above hypotheses. Chapter 3 begins with a broad picture by examining the whole suite of plants being used at a phytoremediation site in south-eastern Saskatchewan. From there all studies focus down. By Chapter 9, very specific components of the exudates released by several of these plants are examined for their impact on the degradation potential and activity of soil microbial communities. Although each individual chapter has been written as a stand alone research paper in order to facilitate submission to peer-reviewed journals, the chapter introductions serve to tie in the primary objective for the reader. Due to the paper format, the thesis does contain a certain amount of redundant information. In order to reduce this, references were combined into a final list.

2.0 LITERATURE REVIEW

2.1 Phytoremediation: An overview

Phytoremediation is defined as the use of plants and their associated microbial communities to sequester, degrade, or stabilize xenobiotic contaminants. The past decade has produced an impressive body of research on the uses of plants to remediate a wide variety of both inorganic and organic compounds. Studies have shown that metal (Blaylock et al., 1997; Chen et al., 2006; McGrath et al., 2002), salts (Qadir et al., 2001; Qadir et al., 2003), pesticide (Karthikeyan et al., 2004; Newman and Reynolds, 2004; Olette et al., 2008), solvent (Taghavi et al., 2005; Newman and Reynolds, 2004), explosive (Cunningham et al., 1997; Siciliano and Greer, 2000), petroleum hydrocarbon (Phillips et al., 2006; Pilon-Smits, 2005), and radionuclide (Entry et al., 1996; McGrath et al., 2002) contamination in soil and water are all amenable to phytoremediation.

2.1.1 Advantages and limitations

Although generally considered slower than engineered remediation techniques, phytoremediation offers several advantages. Phytoremediation is usually more cost effective because it is an inherently naturally driven process. Once established, sites usually require little financial input with regards to maintenance. As contaminants remain on site, transportation costs associated with moving the contaminated material to a secondary treatment facility are eliminated. Practitioners estimate that, on average, phytoremediation is a minimum of ten times less expensive than other remediation technologies (Pilon-Smits, 2005). Elimination of transportation also reduces the possibility of spreading contaminants to other environments. Less site disturbance, contaminant containment via hydraulic gradients, erosion prevention, increased soil health and productivity, and a favourable public reaction to the “green aesthetics” of plant-based technologies all contribute to the growing popularity of phytoremediation.

Phytoremediation is not however, a panacea, and there are disadvantages and limitations. Bioaccumulation of metals within plant tissues may necessitate further treatment of harvested plants themselves. High contaminant concentrations may be toxic to plants, necessitating the pre-treatment of the site. The zone of effectiveness of phytoremediation is generally dictated by the depth of root growth, except in those cases where soluble contaminants are transported towards the root system by the evapotranspirative hydraulic gradient. Contaminants which are highly sorbed to soil particles may not be treatable due to limited bioaccessibility or bioavailability. Finally, the effectiveness of phytoremediation varies with environmental conditions, including soil physico-chemical properties and seasonal temperature fluctuations.

2.1.2 Modes of action

Plants facilitate contaminant removal via extraction, stabilization, volatilization, transformation, and degradation (Table 2.1). Degradation, the breakdown or mineralization of compounds, is the preferred outcome for organic contaminants such as petroleum hydrocarbons (Siciliano and Germida, 1998). Most hydrocarbon degradation is believed to occur through a rhizosphere effect; plants exude organic compounds through their roots, which increases the density and activity of specific microorganisms in the surrounding rhizosphere, which in turn metabolize or degrade hydrocarbons (Anderson et al., 1993). This relationship, and the underlying mechanisms that support hydrocarbon degradation, poses one of the more intriguing challenges in phytoremediation research today.

2.2 Phytoremediation of hydrocarbons

2.2.1 History of phytoremediation for petroleum hydrocarbons

The past two decades have produced an impressive body of research in the field of phytoremediation for petroleum hydrocarbon contamination. In 1990, Aprill and Simms (1990) reported that eight prairie grasses enhanced the removal of four different PAHs. Since then, numerous studies have noted the positive effect vegetation has on the degradation or dissipation of hydrocarbons. The degradation of both low molecular weight PAHs such as naphthalene and phenanthrene and high molecular weight PAHs

Table 2.1 Primary modes whereby plants remediate contaminants

Mode of action	Mechanism	Target contaminants
Phytoextraction	Uptake and concentration within plant tissues	Metals, radionuclides
Phytotransformation	Uptake and chemical modification within plant tissues	Chlorinated solvents, ammunition waste, herbicides, mono-aromatic hydrocarbons
Phytostabilization	Immobilization within root zone	Primarily metals
Phytovolatilization	Uptake and evapotranspiration	Volatile organics (TCE, toluene, MTBE), some metals (Hg, Se)
Phytodegradation	Enhanced degradation within root zone	Ammunition waste, pesticides, petroleum hydrocarbons

Information modified from Arthur et al., 2005; Pilon-Smits et al., 2005; Salt et al., 1998

such as pyrene, benzo(a)anthracene, and benzo(k)fluoranthene is reported to be enhanced in rhizosphere soil (Aprill and Simms, 1990; Binet et al. 2000; Reilly et al., 1996; Maila and Randima, 2005). Similarly, the degradation of complex mixtures of hydrocarbons including diesel fuel and weathered crude oil is also enhanced by plants (Banks et al., 2003; Günter et al. 1996; Kaimi et al., 2006; Liste and Prutz, 2006; Phillips et al., 2006). Phytoremediation studies have examined single plant species (Chen and Banks, 2004; Kaimi et al., 2004; Muratova et al., 2003; Parrish et al., 2004 and 2005) or single and mixed plant species (Kirk et al., 2005; Maila and Randima, 2005; Phillips et al., 2006; Siciliano et al., 2003). Although most studies find that specific plants are able to increase the degradation potential in hydrocarbon-contaminated soil there is little consensus as to the overall effectiveness of different treatments.

2.2.2 Effectiveness of phytoremediation for petroleum hydrocarbons

There is little doubt that plant species vary greatly in their ability to increase hydrocarbon degradation (Liste and Prutz, 2006; Siciliano et al., 2003; Chiapusio et al., 2007; Parrish et al., 2004), with reported degradation varying from as little as 5% to greater than 50%. Differences in degradation potential are not however, limited to different plant species. Contradictory results have been found in separate phytoremediation studies with regards to whether a specific plant species promotes hydrocarbon degradation. For example, the results of a recent study by Rezek et al. (2008) show little influence of *Lolium perenne* (perennial rye grass) on PAH degradation, while both Günther et al (1996) and Binet et al. (2000) reported a significant rhizosphere effect. When one considers however, that differences in degradation potential are observed even within clones of species cultivars (Schwab et al., 2006; Wiltse et al., 1998), it may be naïve to expect comparable results between plant species. There are numerous factors apart from the plant itself that will influence whether a specific plant will enhance hydrocarbon degradation. The nature of the hydrocarbon itself, contamination history and age, soil physico-chemical characteristics, soil and external environmental parameters, and the nature of the indigenous soil microbial communities will all influence degradation outcomes (Anderson et al., 1993).

What works in one soil and for one type of contamination often will not work under different conditions. Ultimately, for all situations, the efficiency of a given phytoremediation treatment is linked to the impact that the plant has on associated microbial communities.

2.3 Plant impacts on microbial communities

The relationship between plants and microbes spans the range of ecological interactions, from pathogenic to symbiotic. These associations occur on all plant surfaces, within all plant tissues, and in the rhizosphere zone impacted by plant root exudates. Up to 40% of the net photosynthetic carbon of a plant is exuded from root systems as organic compounds, including sugars, amino acids, and organic acids (Grayston et al., 1996). These exudates provide a steady and ready source of nutrients, which increases the overall magnitude of bacterial populations supported in the rhizosphere. These same exudates however, may limit diversity by selectively enhancing specific genotypes and phenotypes.

2.3.1 Rhizosphere microbial communities

Numerous studies have shown that plant species alter the general phylogenetic community structure of rhizosphere bacterial communities. While the specific nature of the phylogenetic changes varies according to plant species (Grayston et al., 1998; Johnson et al, 2003; Marschner et al., 2001), studies have shown that general shifts are often towards growing r-strategist bacteria such as *Pseudomonas* spp. (Espinosa-Urgel, 2004; Kosdroj and van Elsas, 2000; Grayston et al., 1998). While plant species are important in determining the genotypic structure of rhizosphere communities, this relationship itself is impacted by a myriad of environmental influences that change both with location and time. Factors such as electrical conductivity (Johnson et al., 2003), soil type (Johnson et al., 2003; Marschner et al., 2001), amendment inputs (Phillips et al., 2006; Siciliano et al., 2003; White et al., 2003), seasonal environmental changes (Smalla et al., 2001), and level of contaminant (Banks et al., 2003) all impact community structure. All factors that elicit genotypic changes invariably elicit phenotypic changes. In the case of hydrocarbon contamination, this generally results in increases to hydrocarbon degrading phenotypes.

As with community structure, the metabolic or functional diversity of soil bacteria is altered in the rhizosphere. Researchers typically find that both PAH and aliphatic hydrocarbon degrading phenotypes are increased in the rhizosphere of plants exposed to hydrocarbon contamination (Banks et al., 2003; Günther et al., 1006; Palmruth et al., 2007; Siciliano et al., 2003). Again, plant specific differences in types and magnitude of degrader phenotypes occur. Liste and Prutz (2006) examined thirteen separate horticultural and agricultural plant species and found that the prevalence of aromatic ring dioxygenase expressing bacteria (ARDB) was greatest in the rhizosphere of mustard (*Sinapis alba* L.), oat (*Avena sativa* L.) and cress (*Lepidium sativum* L.). Corgie et al. (2006) found higher numbers of both phenanthrene and pyrene degraders in rhizosphere zones of perennial rye grass roots. Legumes and grasses may stimulate degrader communities capable of utilizing different types of hydrocarbons. Several studies have found that PAH degraders are enhanced in legume over non-legume rhizospheres (Muratova et al., 2003; Parrish et al., 2005; Phillips et al., 2006), while alkane degrader populations are enhanced in grasses compared to legumes (Kirk et al., 2005; Phillips et al., 2006). Again however, the impacts of plants are moderated by other factors, including soil type. Chiapusio et al. (2007) found that the relative increases in PAH degraders in the rhizosphere of *Trifolium pretense* (red clover) compared to perennial rye grass was dependent on the clay content of the assessed soil.

2.3.2 Endophytic microbial communities

Endophytic bacteria are described non-pathogenic bacteria found within the interior tissues of healthy or symptomless plants (Schultz and Boyle, 2006). These bacteria are found in all plant species, span a wide range of bacterial phyla, and are known to have plant growth promoting and pathogen control activities (Hallmann et al., 1997; Hallmann and Berg, 2006; Ryan et al., 2008). Root colonization by endophytic bacteria is primarily rhizospheric via wounds, lateral root emergence zones and root hairs. Plant specific factors, including exudation patterns, root architecture, root surface structure, apoplastic nutrient composition, and systematic responses, influence bacterial colonization and distribution both pre- and post-colonization (Hallmann et al., 1997; Rosenblueth and Martinez-Ramiro, 2006). Endophytic community structure is also

influenced by soil related factors, with both soil type and soil inputs being determining factors of endophytic diversity in both wheat (Conn and Franco, 2004a) and canola (Germida et al., 1998). In the same soil however, plant species (Germida et al., 1998), plant cultivar (Porteous Moore et al., 2006; Siciliano and Germida, 1999), plant growth stage (Hallmann and Berg, 2006), and even root type within a single plant (Ofek et al., 2007) can all result in differences in endophytic distribution and diversity. Spatially heterogeneous colonization within roots is common and reflects both structural constraints, spatial variation in inorganic ions and nutrients, and, as may be the case with hydrocarbon degrading bacteria, organic contaminants.

Several recent studies have found that plants may harbour diverse endophytic communities which include bacteria capable of degrading hydrocarbons. As with other endophytes, the diversity of these degrader populations is influenced by plant-specific factors. Porteous Moore et al. (2006) isolated BTEX-degrading *Pseudomonas*, *Arthrobacte*r, *Enterobacter*, and *Bacillus* species from the root and stem tissues of different *Populus trichocarpa* × *deltoides* (poplar) cultivars growing at a car manufacturing plant. While all endophytic species were present in the different cultivars, their ratio and spatial distribution differed. Similarly, Siciliano et al. (2001) found that endophytic hydrocarbon degraders were ubiquitous in tall fescue (*Festuca arundinacea*) and rose clover (*Trifolium fragiferum*) at an aged-hydrocarbon contaminated site, with up to 4% of culturable endophytes possessing genes involved in hydrocarbon degradation. Fescue endophytes however, had an increased frequency of aromatic hydrocarbon degrading phenotypes while clover had an increased proportion of aliphatic hydrocarbon degrading phenotypes.

2.4 Hydrocarbon-degrading bacteria

Hydrocarbon-degrading bacteria have been found in habitats ranging from polar soils (Whyte et al., 2002) to marine environments (Yakimov et al., 2007). While these degraders are generally found at much lower concentrations in pristine environments (Margesin et al., 2003), the ubiquitous presence of hydrocarbons themselves, or of aromatic and aliphatic analogs, has resulted in the maintenance of degradation potential within most populations. Johnsen and Karlsen (2005) estimated the PAH degradation

capacity of 13 soils, from pristine to heavily contaminated, and found that the capacity to degrade pyrene and phenanthrene was ubiquitous except in the most pristine forest soils, which exhibited low pyrene mineralization. Although many sites were far from pollution sources, PAHs were present in all soils, likely due to atmospheric deposition of pyrogenic PAHs.

2.4.1 Diversity of hydrocarbon-degrading bacteria

The phylogenetic diversity of hydrocarbon degraders is vast, with degraders found in most, if not all, branches of the microbial family tree. Several recurrent groups are found in most phytoremediation studies. *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Arthrobacter*, *Flavobacterium*, *Rhodococcus*, *Ralstonia*, *Stenotrophomonas*, *Acinetobacter*, *Mycobacterium*, *Micrococcus, Alcaligenes*, and *Nocardioides* species are all hydrocarbon degraders regularly isolated from contaminated soils (Aislabie et al., 2008; Hamamura et al., 2006; Juck et al., 2000; Kästner et al., 1994; Liste and Prutz, 2006; Palmruth et al., 2007). There is some evidence that r-strategist groups become more prevalent in contaminated soils (Labbé et al., 2007; Margesin et al., 2003; Saul et al., 2005; Whyte et al., 2002). These studies found that β- and γ-Proteobacteria groups, including numerous *Pseudomonas* spp. and *Acinetobacter* spp., were increased in relative prevalence in contaminated soils compared to pristine soils, while groups such as Actinobacteria were maintained in both soil types. Other studies however, have found that while hydrocarbon contamination does shift microbial populations, community responses differ with soil type. In soils ranging from clay to loam, Hamamura et al. (2006) found that Actinobacteria, in particular *Rhodococcus* spp., increased in four of seven soil types in response to contamination. The dominance of a given degrader group may be a function of time. Kaplan and Kitts (2005) found that during early phases of hydrocarbon degradation in a land farming facility, when degradation rates were rapid, *Pseudomonas* and *Flavobacterium* dominated. After this initial period, slower growing *Alcaligenes*, *Microbacterium*, and *Bacteroides* became dominant.

2.4.2 Hydrocarbon metabolism

Crude oil and refined petroleum products contain complex mixtures of hydrocarbons, including aliphatic and aromatic hydrocarbons. Aliphatic hydrocarbons are linear, cyclic, or branched hydrocarbons which may be saturated (alkanes) or unsaturated (alkenes and alkynes). Aromatic hydrocarbons consist of one or more (polyaromatic) benzene rings. Soil bacterial communities degrade these hydrocarbons via numerous different catabolic pathways (Habe and Omori, 2003; van Beilan et al., 2007) which are subject to numerous regulatory mechanisms (Tropel and van der Meer, 2004). In order to understand how plants may impact the degradation ability of soil microbial communities, these pathways of metabolism must be understood.

2.4.2.1 Aerobic metabolism of aliphatic hydrocarbons

Aerobic metabolism of alkanes begins with the terminal or sub-terminal incorporation of oxygen into the hydrocarbon by a hydroxylase enzyme. The prevalence of naturally occurring alkanes, such as plant waxes, has led to the ubiquitous distribution of constitutive alkane hydroxylases. The best characterized system, common in *Pseudomonas* and *Rhodococcus* species, utilizes an integral membrane hydroxylase (encoded by *alkB*) to initiate metabolism of short to medium chain alkanes (C_5 to C_{16}) (van Beilan and Funhoff, 2007). Once oxidized to a primary alcohol, subsequent oxidation steps by alcohol and aldehyde dehydrogenases convert the compounds to fatty acids that may then be further metabolized via β-oxidation and the citric acid cycle (van Hamme et al., 2003). Recent studies have shown that some bacteria, including *Caulobacter* sp., *Acinetobacter* sp., and *Sphingomonas* sp., utilize an alternate or additional enzyme system, cytochrome P450 hydroxylase (van Beilan et al., 2006), to metabolize aliphatic hydrocarbons. Cytochrome P450 hydroxylases are inducible enzymes known to be up-regulated in the presence of alkanes (Sabirova et al., 2006). Although most alkane degraders contain several alkane hydroxylases which metabolize varying lengths of alkanes (van Beilan and Funhoff, 2007), *alkB* is commonly assessed to determine the degradation potential of microbial communities.

2.4.2.2 Aerobic metabolism of polyaromatic hydrocarbons

Aerobic degradation of polyaromatic hydrocarbons (PAH) is also initiated by the action of oxygenases, which introduce oxygen to the aromatic rings. PAH degradation has been best characterized in *Pseudomonas* species for phenanthrene and naphthalene (van Hamme et al., 2003). Phenanthrene is degraded via two different routes (Table 2.2, Figure 2.1). A series of enzymatic reactions converts phenanthrene to 1-hydroxy-2-naphthoic acid. This intermediate is then oxidized either to 1,2-dihydroxynaphthalene and enters the naphthalene degradation pathway or is cleaved to *trans*-2-carboxy-benzalpyruvate and enters the phthalate pathway (Ellis et al., 2006). Phenanthrene degraders which utilize the phthalate pathway, including *Alcaligenes* AFK2 and *Nocardioides* KP7, are generally less efficient degraders of naphthalene (Habe and Omori, 2003). Naphthalene and compounds entering the naphthalene pathway are converted via the upper pathway to salicylate (Figure 2.1), which is then converted to catechol and further metabolized by the lower pathway to citric acid cycle intermediates (van Hamme et al., 2003). Naphthalene dioxygenase is a versatile enzyme that acts on a number of aromatic compounds, including naphthalene, phenanthrene, anthracene, fluorene, and dibenzothiophene (Resnick et al., 1996), and as such is a primary target gene for studies assessing hydrocarbon degradation. Other key genes assessed during degradation studies are those that encode crucial first steps in degradation pathways, including phenanthrene 2,3 dioxygenase and catechol 2,3 dioxygenase.

2.4.3 Regulation of catabolic gene expression

There is a complex system of regulatory mechanisms that governs hydrocarbon degradation. Regulation may occur globally or locally. During global regulation, global control proteins such as the Crc regulatory proteins of *Pseudomonas* act on numerous catabolic operons (Collier et al., 1996). Physiological parameters such as the metabolic state of the bacteria and environmental parameters including temperature and pH (Cases and Lorenzo, 2001; Tropel and van der Meer**,** 2004) are also involved in global regulation of these operons. During local regulation, regulators which control the expression of specific catabolic operons either stimulate transcription of inducible pathways or repress transcription of constitutive pathways. These regulators themselves

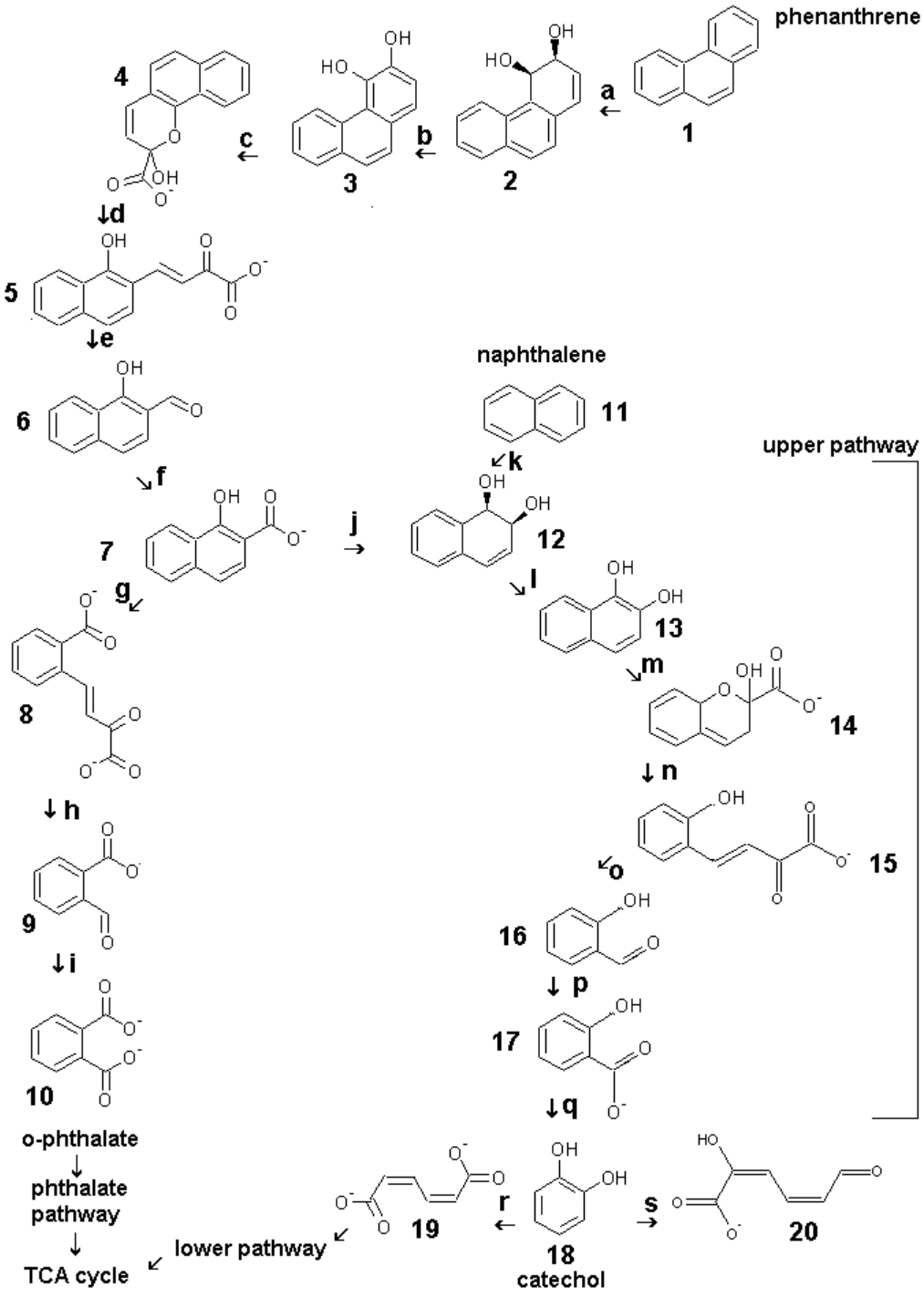

Figure 2.1 Key metabolic pathways for phenanthrene and naphthalene degradation. Numbers corresponding to substrates and letters corresponding to enzymes are listed in Table 2.2. Modified from Ellis et al. (2006).

Table 2.2 Substrates and enzymes in the phenanthrene and naphthalene biodegradation pathways featured in Figure 2.1

Phenanthrene pathway†				Naphthalene pathway†			
Substrate‡		Enzyme‡		Substrate‡		Enzyme‡	
1	phenanthrene	**a**	phenanthrene dioxygenase	**11**	naphthalene	**k**	naphthalene1,2-dioxygenase
2	*cis*-3,4-dihydroxy-3,4-dihydro-phenanthrene	**b**	*cis*-3,4-dihydroxy-3,4-dihydro-phenanthrene dehydrogenase	**12**	cis-1,2-dihydroxy-1,2-dihydro-naphthalene	**l**	1,2-dihydroxy-1,2-dihydro-naphthalene dehydrogenase
3	3,4-dihydroxy-phenanthrene	**c**	3,4-dihydroxy-phenanthrene dioxygenase	**13**	1,2-dihydroxy-naphthalene	**m**	1,2-dihydroxy-naphthalene dioxygenase
4	2-hydroxy-2*H*-benzo[*h*] chromene-2-carboxylate	**d**	2-hydroxy-2*H*-benzo[*h*] chromene-2-carboxylate isomerase	**14**	2-hydroxychromene-2-carboxylate	**n**	2-hydroxychromene-2-carboxylate isomerase
5	*trans*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate	**e**	*trans*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoate aldolase	**15**	trans-o-hydroxy-benzylidenepyruvate	**o**	trans-o-hydroxy-benzylidenepyruvate-hydratase aldolase
6	1-hydroxy-2-naphthaldehyde	**f**	1-hydroxy-2-naphthaldehyde dehydrogenase	**16**	salicylaldehyde	**p**	salicylaldehyde dehydrogenase
7	1-hydroxy-2-napthoate	**g**	1-hydroxy-2-napthoate 1,2-dioxygenase (to 8)	**17**	salicylate	**q**	salicylate hydroxylase
		j	1-hydroxy-2-naphthoate hydroxylase (to 13)	**18**	catechol	**r**	catechol 1,2-dioxygenase (to 19)
8	*trans*-2-carboxy-benzalpyruvate	**h**	*trans*-2-carboxy-benzalpyruvate aldolase	**19**	*cis,cis*-muconate		
9	2-carboxy-benzaldehyde	**i**	2-carboxy-benzaldehyde dehydrogenase	**18**	catechol	**s**	catechol 2,3-dioxygenase (to 20)
10	o-phthalate			**20**	2-Hydroxy-*cis,cis*-muconate semialdehyde		

†Modified from Ellis et al. (2006).

‡Numbers and letters correspond to substrates and enzymes outlined in the metabolic pathways illustrated in Figure 2.1

are subject to catabolite repression, substrate-induced activation, and cross-regulation from alternate metabolic pathways (Diaz and Prieto, 2000; Schingler, 200; Tropel and van der Meer, 2004). A very general overview of the regulatory mechanisms acting on catabolic operons involved in hydrocarbon degradation is provided in Figure 2.2. 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. Many intermediates of hydrocarbon degradation, including those listed in Table 2.2 and the subsequent down-stream pathway metabolites, may act as inducers or repressors. In addition, analogous compounds may mimic these effectors. Plant root exudates may have a significant and specific impact at this level on the hydrocarbon degradation potential of soil microbial communities.

2.4.4 Location of catabolic operons

Genetic information for the degradation of xenobiotic compounds is carried chromosomally, and on plasmids or other mobile genetic elements. While most bacterial species harbour plasmids, in environmental bacteria those occurring in *Pseudomonas* species have been best characterized (Table 2.3). Under non-selective conditions, the proportion of cells containing these degradative plasmids decreases, thereby reducing the overall metabolic load. When a compound such as naphthalene is encountered, rapid horizontal gene transfer (HGT) can transfer this degradative capacity to other members of the population. HGT can occur in several ways, including transposition and conjugation. Gene cassette homologies indicate that gene clusters and whole operons carried on a plasmid may act as modules, or discrete packages that may be transferred (van der Meer et al. 1992). For example, both the *xyl* gene cluster of plasmid pWWO and the *nah* gene cluster of plasmid NAH7 are located on highly mobile Class II transposons (Tsuda et al. 1999). Almost exact copies of the *xyl* operon have been found on plasmids belonging to different compatibility groups (Sentchilo et al. 2000). Conjugation, the transfer of entire plasmids bearing entire metabolic pathways between organisms, may be the most common mode of horizontal gene transfer that

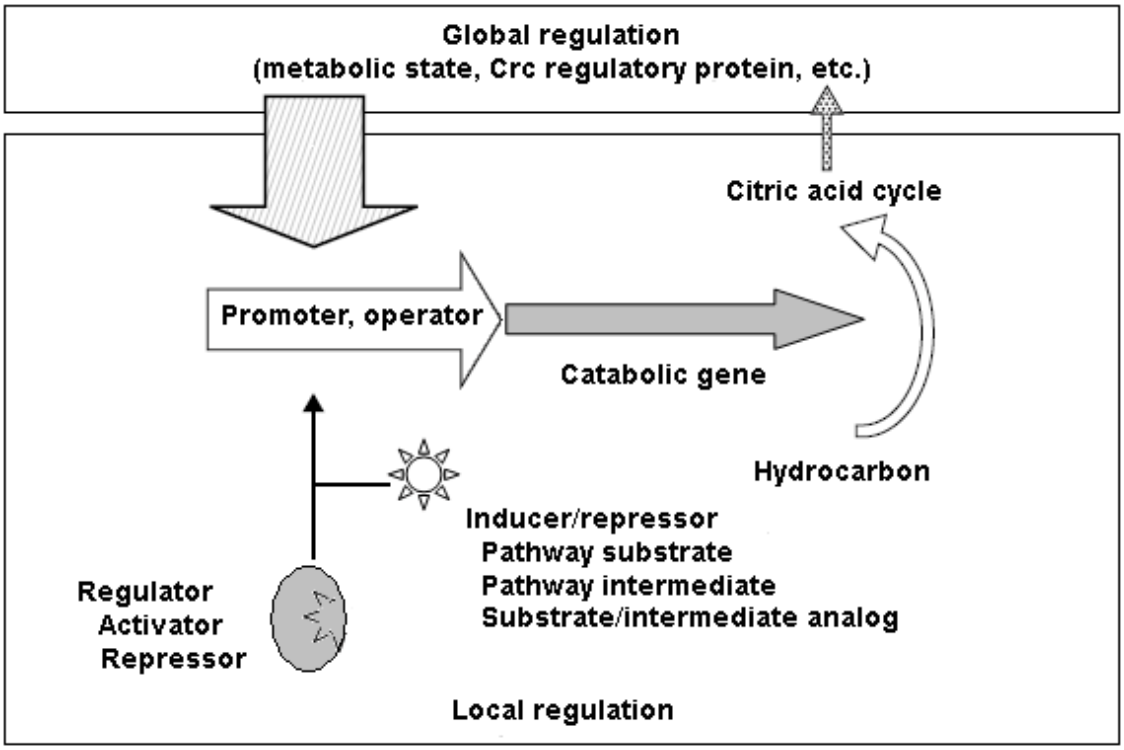

Figure 2.2 Schematic representation of local and global regulatory mechanisms acting on catabolic operons involved in hydrocarbon degradation. Modified from Diaz and Prieto (2000).

Table 2.3 Examples of catabolic plasmids conferring hydrocarbon degradation capacity to environmental bacteria

Plasmid	Bacterial species	Substrate	Gene clusters	Reference
pWWO	*P. putida*	xylene, toluene	*xyl*	Harayama et al., 1991
NAH7	*P. putida*	naphthalene	*nah*	Nojiri et al., 2004
pCg1	*P. putida*	naphthalene	*nah*	Park et al., 2003
pDK1	*P. putida*	xylene, toluene	*xyl*	Benjamin et al., 1991
pDTG1	*P. putida*	naphthalene	*nah*	Stuart-Keil et al., 1998
pDBF1	*Terrabacter* sp.	fluorene	*fln*	Nojiri et al., 2002
pDBT2	*P. alcaligenes*	dibenzothiophene	*nah*	Top et al., 2000
pNL1	*Sphingomonas* spp.	xylene, naphthalene	*xyl, pah, pch*	Romine et al., 1999
pKA1	*Pseudomonas* spp.	naphthalene, phenanthrene	*nah*	Sanseverino et al., 1993
pBS3	*P. fluorescens*	naphthalene	*nah*	Boronin et al., 1980
NA†	*Rhodococcus* sp.	naphthalene	*nar*	Kulakov et al., 1993
OCT	*P. putida*	alkanes	*alkB*	van Beilan et al, 2001

†Currently unspecified

occurs under environmental conditions (Nojiri et al., 2004). HGT is known to occur with high frequency in the rhizosphere, both within and between genera (Jussila et al., 2007; Kroer et al. 1998; van Elsas et al., 1988, van Elsas et al., 2003). High densities of bacterial populations combined with high nutrient availability generate conditions favourable for these events to occur.

2.5 Impact of plants on microbial degradation of hydrocarbons

There are several ways in which plants may increase the degradative potential of rhizosphere microbial communities, including general increases in microbial population densities, specific increases in degrader population densities, increased catabolic gene expression, increased horizontal transfer of catabolic genes, and enhanced bioavailability of hydrophobic hydrocarbons. A primary factor impacting these degradation outcomes is plant exudation.

2.5.1 Patterns of exudation

Plants release up to 40% of their total photosynthates through their roots in the form of low and high molecular weight secretions such as complex carbohydrates, plant mucilages, root lysates, gases, and low molecular weight exudates (Grayston et al., 1996). Low molecular weight organic acids comprise approximately 2% of the total carbon released by plant roots (Dakora and Phillips, 2002; Farrar and Jones, 2000) while other numerically and metabolically significant exudates include amino acids and some phenolic compounds (Grayston et al., 1996). Root exudation is neither consistent nor constant. Numerous factors, including plant species and cultivar (Cieslinski et al., 1998; LeSuffleur et al., 2007), soil composition (Cieslinski et al., 1998), environmental parameters (Henry et al., 2007), and the presence of xenobiotics and other stressors (Bais et al., 2004) influence exudation patterns. These differences in exudation patterns in turn impact the prevalence and activity of hydrocarbon degrading bacteria in the rhizosphere.

2.5.2 Impact of exudates on hydrocarbon degradation

Numerous studies have attempted to elucidate the specific impact of exudates on degradation potential. Root extracts and exudates have been shown to stimulate both

non-specific (da Silva et al., 2006; Miya and Firestone, 2001) and specific (Yoshitomi and Shann, 2001) increases in PAH degrader populations. Other studies however, have implicated exudates in the repression of hydrocarbon degradation potential. Corgie et al. (2004, 2006) observed distance-dependent repressive effects on PAH degradation by exudates released by *L. perenne*. Dibenzo[a,h]anthracene degradation was repressed in all planted soil while phenanthrene degradation was repressed at distances greater than 3mm from the roots. Similarly, Rentz et al. (2004) found that exudates from a variety of plants repressed the phenanthrene degrading activity of *P. putida* ATCC 17484. This repression was later found to be due at least in part to the repressive effects of exudates on *nahG*, a gene involved in naphthalene dioxygenase transcription (Kamath et al. 2004).

2.5.3 Exudation and degradation activity

There is an intimate and integral connection between the compounds that plant roots release and gene expression in the rhizosphere. Several recent studies have attempted to elucidate that interaction. Matilla et al. (2007) found ninety up-regulated genes in *P. putida* exposed to exudates from *Zea mays* (corn) roots. Preferentially expressed genes included those involved in the uptake of specific carbon and nitrogen sources such as those normally found at increased levels in the rhizosphere, as well as genes for the metabolism of aromatic compounds. Mark et al. (2005) examined the impact of exudates of two *Beta vulgaris* cultivars (beetroot) on gene expression in *Pseudomonas aeruginosa* PA01. Gene expression patterns promoted by the two exudates differed substantially, with only partial overlap. 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. These plant-specific impacts manifest as impacts on degradation potential. Specific components released by plants, including organic acids, amino acids, and phenolic compounds, both stimulate and repress expression of genes involved in hydrocarbon degradation. These compounds can act on any part of the metabolic pathways involved in hydrocarbon degradation, have different impacts in different bacterial species, and be moderated by the physiological state of the bacteria. In recent

years researchers have begun to elucidate the nature of these interactions, but their complexity ensures that it is no easy task.

2.5.3.1 Impact of organic acids on catabolic gene expression

Some organic acids exuded by plant roots, including the citric acid cycle intermediates succinate, citrate and fumarate, are believed to primarily exert catabolite repression impacts on aromatic compound metabolism (Diaz and Prieto, 2000). This repression however, is neither a single nor a simple mechanism and the stage at which it occurs differs with organic acid, aromatic compound, and bacterial species (Tropel and van der Meer**,** 2004). For example, Kuiper et al. (2002) found that succinate actually promoted a higher level of naphthalene dioxygenase expression in *P. putida* PCL1444 than did other organic acids. In studies on lower pathways involved in the degradation of aromatic compounds Brzostowicz et al. (2003) found that expression of the enzyme p-hydroxybenzoate hydroxylase, which catalyzes the metabolism of 4-hydroxybenzoate to protocatechuate (phthalate pathway, Figure 2.1), is highly expressed when *Acinetobacte*r strain ADP1 is grown in the presence of an inducer and citric acid cycle substrates and intermediates, including succinate, fumarate, and acetate. However, in *Pseudomonas* spp. succinate decreases expression of another key enzyme, protocatechuate 3,4 dioxygenase, that catalyzes the metabolism of protocatechuate to β-carboxy-cis,cis-muconate (Collier et al., 1996). Fumarate has been found to have disparate impacts on genes involved in aromatic compound degradation. For example, in chloro-aromatic metabolic pathways fumarate competitively inhibits a key transcriptional activator, ClcR, in a concentration dependent manner (McFall et al. 1997). In aromatic pathways however, a related transcriptional activator for a catechol degradation operon, CatR, is not repressed by fumarate (McFall et al., 1998).

The organic acid acetate has also been shown to be a strong inhibitor of aromatic catabolic gene expression in bacterial isolates. Kamath et al. (2004) found that *nahG* expression in *P. putida* ATCC 17484 was reduced approximately 40% relative to the control when the bacteria were exposed to naphthalene in the presence of 40 mg L^{-1} acetate. Similarly, acetate has been shown to be a strong repressor of *xyl* operon promoters on the *P. putida* TOL plasmid (Collier et al., 1996; Holtel et al., 1994).

Degradation by other bacterial groups is also impacted, as Ampe et al. (1996) found that utilization of phenol and its metabolic intermediates by *Alcaligenes eutrophus* was inhibited by acetate.

Catabolite repression also occurs during degradation of aliphatic hydrocarbons, with organic acids such as succinate repressing expression of the *alkB* operon (Dinamarca et al., 2003; Ruiz-Manzano et al., 2005; Yuste et al., 1998). The mechanisms of this repression differ between bacterial species, with repression occurring during the stationary phase in *Burkholderia* sp. (Marin et al., 2001) but during the exponential phase in *Pseudomonas* sp. (Ruiz-Manzano et al., 2005).

2.5.3.2 Impact of phenolic compounds on catabolic gene expression

Several studies have suggested that phenolic compounds released by plants may stimulate PAH degradation. Olson et al. (1999, 2001) found decreased PAH concentration in the upper root zone of *Morus* spp. (mulberry), a plant which is high in phenolic compounds (Hedge and Fletcher, 1996; Leigh et al., 2002). While one study has shown that mulberry root extracts stimulate phenanthrene mineralization by non-specifically increasing PAH degrader populations (da Silva et al., 2006), others have observed that mulberry roots and root extracts may actually repress PAH degradation (Mueller and Shann, 2006; Kamath et al., 2004). It has been shown however, that individual phenolic compounds such as salicylate (a known intermediate PAH degradation pathways, Figure 2.1) may initiate degradation of PAHs such as naphthalene (Singer et al., 2003) and phenanthrene (Chen and Aitken, 1999). In the Chen and Aitken study, pre-incubation of *P. saccharaphila* P15 with either phenanthrene or salicylate enhanced initial phenanthrene removal 10-fold compared to the control. As well, salicylate induction increased the initial rates of removal of the high molecular weight PAH's fluoranthene, pyrene, chrysene, benzo[a]pyrene, and benz[a]anthracene by up to 100 times. Further research has shown that salicylate induces PAH degradation in bacterial isolates in part by inducing expression of *nahG*, a gene involved in naphthalene dioxygenase transcription (Kamath et al., 2004). Complex soil communities may not, however respond to inputs in a manner comparable to

isolates, and further research has shown that salicylate does not induce PAH degradation when added to soil as a pure compound (Yi and Crowley, 2007).

2.5.3.3 Impact of amino acids on catabolic gene expression

There is little available information on the impact of amino acids on catabolic gene expression. At least one study however, reported that a complete amino acid mixture inhibited transcription of a phenol catabolic operon in *P. putid*a (Putrins et al., 2007). Another study found that the amino acid glutamate repressed the phenanthrene degrading activity of *P. putida* ATCC 17484 but that repression was not concentration dependent (Rentz et al., 2004).

2.5.4 Exudation and degradation potential

Another mechanism whereby plant root exudates may increase the degradation potential of microbial communities is by facilitating horizontal gene transfer (HGT) of genes involved in hydrocarbon degradation. Several studies have recorded increased horizontal gene transfer (HGT) in the rhizosphere compared to bulk soil (Kroer et al. 1998; Schwaner and Kroer, 2001; van Elsas et al., 1988, van Elsas et al., 2003). Increased densities of donor bacteria (Kroer et al.,1998; Schwaner and Kroer, 2001)and increased metabolic activities of rhizosphere (Lilley et al., 1994; Smets et al. 1993, van Elsas, 1988) bacteria have been proposed to account for these increased events. It is known that there are plant-specific differences in the frequency of HGT. Schwaner and Kroer (2001) found that transfer rates were highest in *Pisum sativum* (pea) rhizospheres, followed by *Hordeum vulgare* (barley) and *Triticum* spp. (wheat). Although the metabolic activity of the rhizosphere was comparable, there were approximately 10-fold differences in transfer rates. A later study by this group (Mølbak et al., 2007) again found that plasmid transfer rates were increased 10-fold in pea over barley rhizospheres. Increased transfer rates of the IncP1 plasmid pKJK5 between two *P. putida* species were correlated to increased donor densities in the pea rhizosphere, which itself was correlated to increased exudation. It is also possible however, that the proportion of exudate components played a role in determining the frequency of HGT.

Nielsen and van Elsas (2001) found that root exudates, including organic acids such as citrate and succinate and amino acids such as alanine and glycine stimulated

natural transformation in *Acinetobacter* sp. BD413. While all assessed compounds stimulated transformation in sterile soils, in a non-sterile soil transformation only occurred with the presence of additional phosphorous salts. In this case, acetate, lactate and alanine gave the highest transformation frequencies among the organic and amino acids tested. Of the carbohydrates tested, only glucose enhanced transformation. Pearce et al. (2000) also showed that plasmid transfer frequency between two *Enterobacter* sp. occurred in the presence of glucose. Transfer was highest at 10 $\mu g\ mL^{-1}$ glucose, but then decreased with increasing glucose concentration, concomitantly with decreased donor frequency.

Several studies have shown that HGT of catabolic plasmids likely occurs in contaminated soil environments. Herrick et al. (1997) examined sequence homologies of *nahAc* genes (naphthalene dioxygenase) from numerous bacterial strains isolated from a coal tar contaminated site, and found evidence of that horizontal gene transfer had contributed to microbial adaptation to contamination. A later correlative study found similar evidence of HGT of the naphthalene dioxygenase gene *phnAc* between *Burkholderia* at a coal tar contaminated site (Wilson et al., 2003). Although no studies have directly related increased catabolic fitness in the rhizosphere to increased HGT, Jussila et al. (2007) found evidence for the transfer of the pWWO plasmid (between *P. migulae* and *P. oryzihabitans* in the rhizosphere of the legume *Galega orientalis* (goat's rue). Given the high proportion of catabolic genes found on plasmids, it seems likely that increases in HGT by specific plant species, perhaps stimulated by different amounts or proportions of exudates, contributes to the increased degradative fitness observed in many phytoremediation studies.

2.5.5 Exudation and hydrocarbon bioavailability

Hydrocarbons are by nature hydrophobic, with octanol-water coefficients (log K_{ow}) for common PAH contaminants ranging from 3.0 for naphthalene to 6.04 for benzo[a]pyrene (Schnoor et al., 1995). Alkanes such as n-hexadecane are even more hydrophobic, with a log K_{ow} of approximately 9.1 (Bai et al., 1997). The biodegradation of hydrocarbons is directly related to their solubility, with mineralization rates being negatively correlated with increasing log K_{ow} (Stroud et al., 2007). Greater

hydrophobicity means lower bioavailability, which results in lower degradation rates. Further, as contaminants weather or age, their bioaccessibility is known to decrease as they become more strongly sorbed to soil minerals and organic compounds (Semple et al. 2003, 2007). There is evidence that specific plants may increase both the availability and accessibility of these compounds to microbial communities, through the release of surfactants and through intrinsic root physiology.

The solubility of n-hexadecane increases linearly with increasing concentrations of a microbial rhamnolipid surfactant (Bai et al., 1997). As different plant species produce different types and amounts of phospholipid surfactants (Read et al. 2003), their impact on hydrocarbon solubility likely differs. For example, Liste and Putz (2006) found that benzo[b]fluoranthene and benzo[a]pyrene concentrations in a weathered soil were increased by pea, cress, and pansy violets, but not by other plants. Similarly Parrish et al. (2005) found differences in the fraction of PAHs degraded in grass and legume treatments. Fescue was more effective at degrading the labile PAHs while clover degraded a higher percentage of strongly sorbed PAHs. These differences may also be related to root physiology. Legume roots have approximately twice the lipid content of grass roots and may adsorb significantly more PAHs (Gao and Zhu, 2004; Schwab et al., 1998). The removal of soluble PAHs from the soil water may in turn increase desorption of soil-bound PAHs, as solubility-maximums are maintained. Higher concentrations of PAHs may thus occur in the rhizosphere of legumes, with a concomitant selective impact on PAH degraders.

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3.0 CULTURE-BASED AND CULTURE-INDEPENDENT ASSESSMENT OF THE IMPACT OF MIXED AND SINGLE PLANT TREATMENTS ON RHIZOSPHERE MICROBIAL COMMUNITIES IN HYDROCARBON CONTAMINATED FLARE-PIT SOIL

3.1 Preface

Phytoremediation systems for organic compounds such as petroleum hydrocarbons rely on a synergistic relationship between plants and their root-associated microbial communities. Mixed plant treatments are commonly used for phytoremediation, with the underlying assumption that combinations of root types and exudate patterns will allow greater infiltration and stimulation of microbial communities, with a net positive impact on degradation. It is simply not known however, how plant-specific influences on rhizosphere microbial communities are impacted by inter-species competition in mixed plant treatments. In order to begin to elucidate the mechanisms of plant-bacterial interactions and work towards optimizing the potential of phytoremediation, we must first begin to unravel these plant-specific impacts. In this study, we examined six plants commonly used in phytoremediation, planted both singly and as two separate mixes, in order to address these issues.

3.2 Introduction

Earthen flare pits are located at well sites, battery and compressor stations throughout the oil and gas producing regions of North America. These pits are 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. Soil at these sites is impregnated with a complex mixture of aromatic, aliphatic, and asphaltene hydrocarbons and may be co-contaminated with high salt concentrations, metals, and other inorganic contaminants. These low-volume yet highly contaminated sites are widely scattered and number above 100,000 in western Canada alone (Speer, 1999). Typical treatments for these sites involve excavating the soil and removing it for treatment in biopiles, incinerators or slurry- and solid-phase reactors (Amatya et al., 2002). These treatments, though effective, are costly and involve extensive site

disturbance. Alternately, the soil may be remediated onsite by land farming or composting, with or without the injection of low or high temperature air to facilitate oxidation (Catalan et al., 2004). These in situ treatments are more cost effective, but, due to the recalcitrant nature of the contaminants present, may be limited in terms of effectiveness.

A novel potential remediation treatment for these sites is phytoremediation, the use of plants and their root associated bacteria to remove or degrade contaminants. A recent survey of western Canada (Robson et al., 2004) found that a wide variety of grasses and legumes were able to naturally re-vegetate flare pit soil. This finding suggests that, despite the complex nature of contamination at flare pits, phytoremediation may be used to stimulate the inherent degradative capacity of indigenous microbial communities. Although one study has been published on the survivability of plants in weathered flare pit soil (Rutherford et al., 2005) to the best of our knowledge no research has been published on the actual degradation potential of different plant treatments in this type of contamination.

The past decade has produced an extensive body of research on the phytoremediation of both organic and inorganic contaminants (for review see Anderson et al., 1993; Salt et al., 1998; Singer et al., 2003). Most hydrocarbon degradation is believed to occur through a rhizosphere effect; plants exude organic compounds through their roots, which increase the density, diversity, and activity of specific microorganisms in the surrounding rhizosphere, which in turn degrade hydrocarbons (Cunningham et al., 1996; Siciliano and Germida, 1998). To gain a better understanding of phytoremediation in hydrocarbon contaminated soils, including flare pit soils, the associated microbial community must be examined. Fungi isolated from flare pits have been shown to degrade aliphatic compounds (April et al., 2000) and it is likely that bacterial communities in these sites also play a key role in hydrocarbon degradation. Numerous halophytes have been shown to be capable of degrading aromatic compounds (Alva and Peyton, 2003; Garcia et al., 2005), suggesting that indigenous bacteria that have the potential to thrive in the high salt content of many flare pits may also degrade hydrocarbons. The effect that plants may have on these specialized communities is unknown.

This study examined the impact that plants had on the structural and functional diversity of flare-pit bacterial communities, in order to determine whether phytoremediation was a viable remediation option for weathered flare pit soil. In order to separate the effects of amendments required for plant growth from the effects of plants themselves, both non-amended and amended non-planted soils were assessed. Combinations of culture-based and culture-independent techniques were used in order to gain a thorough understanding of bacterial community shifts. Within this study an additional aspect of phytoremediation systems was also examined. Most in-situ phytoremediation systems are composed of mixed plant species, usually a combination of legumes and non-legumes such as grasses (Banks et al., 2003; Siciliano et al., 2003). Combinations of root types and exudate patterns are believed to allow greater infiltration of and stimulation of microbial communities, with a net positive stimulation of microbial catabolic potential. The underlying assumption is that the effects of mixed plant populations will be proportionally cumulative, with the positive benefits of each individual plant species summing to a greater whole. Few studies however, have examined the whole suite of plants generally used at phytoremediation sites. This study investigated a total of 6 plant species, planted both singly and as two separate mixes.

Our specific objectives were to determine what the impact of 1) amendments 2) mixed plant treatments and 3) mixed plant treatments versus the individual plant species that comprise the mix, were on the indigenous microbial communities and on the overall degradation potential of the soil.

3.3 Materials and Methods

3.3.1 Soil

Weathered-hydrocarbon contaminated soil was collected from a phytoremediation field site in south-eastern Saskatchewan. The soil at the site was excavated from an adjacent decommissioned flare-pit with a 30 year history of chronic releases. Soil analyses were done by EnviroTest Laboratories, Saskatoon, SK. The non-amended soil had a clay texture, pH of 8.0, EC 5.8 dS m^{-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. The amended soil had a clay texture, pH of 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 of 629, 989, and 4900 mg kg^{-1}, respectively. The soil was classified as moderately alkaline and saline/sodic. The average total hydrocarbon concentration at the site was 5300 mg kg^{-1} and consisted primarily of F3 and F4 fractions (C16 to C50). Collected soil was sieved through a 4.75 mm sieve to ensure homogeneity and used within one week. Moisture content was determined by oven-drying 10-g sub-samples of each soil at 100°C for 24 h.

3.3.2 Experimental design

Two groups of plants, a Remediation Technologies Development Forum (RTDF; http://www.rtdf.org) standard mix of *Lolium perenne* L. (perennial rye grass, PRG), *Medicago sativa* L. (alfalfa var. rambler. Alf), and *Festuca rubra* L. (creeping red fescue, CRF), and a mix of local species, *Agropyron elongatum* (Host) P. Beauv. (tall wheatgrass, TWG), *Elymus angustus* Trin. (Altai wild rye, AWR), *Puccinellia nuttalliana* (Schult.) A.S. Hitchc. (Nuttall's salt meadow grass, NSMG), and *M. sativa* were grown both singly and as mixes. Single species treatments (n = 6) were seeded in 0.5 kg of soil and mixed treatments (n = 6) were seeded in either 1.5 or 2.0 kg of soil (3- and 4-plant mixes, respectively). Gypsum ($CaSO_4.2H_2O$, 26 mg g^{-1} soil), straw (9 mg g^{-1} soil), 34-0-0 and 12-51-0 fertilizer (0.14 mg g^{-1} soil each), and the Real Thing™ farm compost (Agricore United, 200 mg g^{-1} soil) were incorporated prior to planting. Amendment rates were calculated to mimic rates used at the field site. Non-planted control treatments (n=9) consisted of contaminated soil with and without the above amendments. 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. Treatments were watered every second day to maintain approximately 20% gravimetric water content. After germination all pots were thinned to 10 plants per species. Natural attrition over the course of the experiment was not corrected and mixed plant treatments were allowed to establish natural species dominance patterns.

At each sampling point three replicates of each treatment were sacrificed. Control soils were processed at the time of seeding and all treatments were processed at 2.5 and 4.5 mo following germination. The study length represents an optimal growing

season for south-eastern Saskatchewan. Roots were shaken to dislodge loose soil and then attached rhizosphere soil was immediately used for culture-based analyses. Additional rhizosphere soil was archived at -20^{o}C for culture-independent and hydrocarbon analyses. Following analysis, washed roots and detached shoots were dried at room temperature for one week and weighed.

3.3.3 Cultural microbiological analyses

Soil from each replicate was serially diluted in monopotassium phosphate (MPP) buffer (0.65 g K_2HPO_4, 0.35 g KH_2PO_4, 0.10 g $MgSO_4$ in 1L water). Total culturable heterotrophic bacteria were enumerated by plating, in triplicate, 100 µL of each dilution series on 1/10 TSA plates containing 0.1g L^{-1} cycloheximide (Zuberer, 1994). Culturable hydrocarbon degraders in each treatment were determined using a modified MPN protocol (Wrenn and Venosa, 1996). Each treatment replicate was assessed for n-hexadecane, F2 diesel fuel, and polyaromatic hydrocarbon (PAH) degraders in separate 48-well microtiter plates. For n-hexadecane (Sigma-Aldrich) and diesel fuel (Imperial Oil Limited, Toronto, Canada) plates, 20 µL of filter-sterilized hydrocarbon was added to wells containing 720 µL Bushnell Haas (BH) mineral salts medium (per litre: 0.2 g $MgSO_4$-$7H_2O$, 0.02 g $CaCl_2$-$2H_2O$, 1.0 g KH_2PO_4, 1.0 g $(NH_4)_2HPO_4$, 1.0 g KNO_3, 0.05 g $FeCl_3$-$6H_2O$; pH 7.0). 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^{-8} to 10^{-2} serial dilutions (80 µL per well, one dilution per row) of soil 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 two weeks 200µL of filter-sterilized p-iodonitrotetrazolium violet (3g L^{-1}) was added to each well of the n-hexadecane and diesel fuel 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).

3.3.4 Molecular microbial analyses

Treatments which appeared to cause significant changes in hydrocarbon catabolic potential, as assessed during culture-based analyses, were selected for further molecular examination. Total community DNA was extracted from soil using a modified bead-beating protocol (Moré et al., 1994) which incorporated an initial soil washing step (Fortin et al., 2004) and a final PVPP/Sephacryl purification step (Berthelet et al., 1996; Fortin et al., 1998). 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 and re-suspended in 100 µL TE (pH 8.0). Two 0.5 g sub-samples of each treatment replicate were extracted and all replicates for a given treatment were pooled prior to purification. Fifty microliters of total community DNA from each treatment was sequentially purified using PVPP and Sephacryl™ S400 (Amersham) columns. DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Gibco-BRL) using a ChemiImager ™ 4400 imaging system (Alpha Innotech Corporation).

Community structure was examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments (Muyzer et al., 1993). DNA extracts from each treatment were amplified using the primer set U341-GC and U758 (Table 3.1). Each 50 µL final volume mixture contained 1 µL diluted DNA extract, 0.5 µM each primer, 200µM each dNTP, 2mM $MgCl_2$, 6.25 µg BSA (Amersham Biosciences), and 2.5 units rTaq polymerase in 5.5 µL 10X PCR buffer (Amersham Biosciences). Taq and buffer were added after an initial 5 min denaturing period at 96^{o}C. Touchdown PCR (Don et al., 1991), from 65 to 55^{o}C, was performed in order to minimize non-specific priming. Amplification proceeded for 10 cycles of 1 min denaturing at 94^{o}C, 1 min annealing at 65-55^{o}C and 1 min extension at 72^{o}C, followed by 20 cycles with an annealing temperature of 55 ^{o}C. Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1 V 3M sodium acetate and 2.5 V 100% ethanol at -20 ^{o}C overnight and re-suspended in 15 µL of TE buffer (pH 8.0). Final DNA concentration was quantified on ethidium bromide-

stained 1.4 % agarose gels by comparison with a 100 bp ladder (MBI Fermentas) using a ChemiImager™ 4400 imaging system (Alpha Innotech, Mississauga, Ontario, Canada).

DGGE was performed on a Bio-Rad DCode system (Bio-Rad, Mississauga, Ont.) as described by Lawrence et al. (2004). For each treatment, 800 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 16h at 80V and 60^{o}C. The resulting gel was stained with Vistra Green (Amersham Biosciences) in TAE buffer and scanned with a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA). DGGE bands of interest were excised from the gel using a scalpel and DNA was eluted in sterile deionized water by overnight incubation at 37^{o}C. DNA was re-amplified using the primer set U341 and U758 (Table 3.1), as described by Juck et al. (2000), with the addition of 6.25 μg BSA (Amersham Biosciences) to each 50 μL reaction mixture. Re-amplified DNA was purified with the QIAQuick PCR purification kit (Qiagen, Mississauga, Ont., Canada), prepared for sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) followed by Centre-Sep column purification (Princeton Separations, Adelphia, NJ), and sequenced using the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA). All procedures followed the manufacturers recommended protocols. Sequences were submitted for comparison to the GenBank database using the BLAST algorithm (Altschul et al., 1997) and checked for chimeras using the Chimera Check program of the Ribosomal Database Project (Cole et al., 2003).

A dendrogram was created by cluster analysis of the weighted (Muylaert et al., 2002) DGGE banding patterns, using Dendron 2.4 software (Solltech Inc., Oakdale, LA). This procedure was repeated with a replicate DGGE gel produced from separate amplification products in order to verify the stability of the dendrogram. In addition, sub-samples of RTDF soil were extracted using a gentler (no enzyme lysis) and a more rigorous (additional bead beating) variation of the outlined protocol. These DNA extracts were compared to final RTDF extracts by DGGE analysis in order to verify banding pattern reproducibility within a given treatment.

Table 3.1 Primers used for PCR amplification of bacterial genes and reference strains used for DIG-labelled probe generation

Target gene Primer	Primer Sequence (5' to 3')	Fragment size (bp)	Reference	Reference strains
PAH initial dioxygenase (*Burkolderia sp.* AF061751)				
phnAc-F	CAATTACGGTGATTTCG GACC	462	Laurie and Lloyd-Jones, 1999	*Burkholderia* sp.
phnAc-R	ACAAAATTCTCTGACGGCGC			
Alkane hydroxylase (Consensus)				
alk-H1-F	CIGIICACGAIITIGGICACAAGAAGG	549	Chénier et al., 2003	*P. putida* ATCC 29347
alk-H3-R	IGCITGITGATCIIIGTGICGCTGIAG			
Naphthalene dioxygenase (*P. putida* M23914)				
ndoB-F	CACTCATGATAGCCTGATTCCTGCCCCCGGCG	642	Whyte et al., 1996	*P. putida* ATCC 17484
ndoB-R	CCGTCCCACAACACACCC ATGCCGCTGCCG			
Naphthalene inducible dioxygenase (*Mycobacterium* sp. AF249301)				
nidA-F	ACCGCGCACTTCCAATGCCCGTACCACGG	323	Margesin et al., 2003	*Mycobacterium* sp. strain PYR1
nidA-R	AATTGTCGGCGGCTGTCTTCCAGTTCGC			
Catechol dioxygenase (*P. putida* imperfect consensus)				
C2,3O-F	AGGTGCTCGGTTTCTACCTGGCCGA	406	unpublished	*P. putida* ATCC 33015
C2,3O-R	ACGGTCATGAATCGTTCGTTGAG			
Universal 16S rDNA (Eubacteria)				
U758	CTACCAGGGTATCTAATCC	417	Rölleke et al., 1996, Lee et al., 1993	*P. putida* ATCC 33015
U341*	CCTACGGGAGGCAGCAG			

*Preceded by a GC clamp for DGGE: GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG; I, inosine

Treatments were analyzed for the presence of specific catabolic genes encoding enzymes involved in hydrocarbon degradation pathways. Total DNA extracts were assessed by PCR amplification using primers specific for the following genes: *alkB*, *ndoB*, *nidA*, *phnAc*, and C2,3O (Table 3.1). Primers for C2,3O were derived from conserved sequence regions of eight *P. putida* strains (GenBank M17159: *nahH*; M64747: *xylE*; X80765: *phlH*; X77856: *phhB*; M33263: *dmpB*; M65205: *xylE*; S77084: *C2,30*; U01825: *bphE*). For PCR amplification, approximately 50 ng of DNA was added to a 50 µL final volume mixture containing 0.5 µM each primer, 200µM each dNTP, 2mM $MgCl_2$, 6.25 µg BSA (Amersham Biosciences), and 2.5 units rTaq polymerase in 5.5 µL 10X PCR buffer (Amersham Biosciences). Taq and buffer were added after an initial 5 min denaturing period at 96^{o}C. Amplification then proceeded for 30 cycles of 1 min denaturing at 94^{o}C, 1 min annealing at 60 (*alkB*) or 65^{o}C (*ndoB, nidA, phnAc,* C2,3O), and 1 min extension at 72^{o}C, with a final extension of 3 min at 72^{o}C. PCR fragments were analyzed on ethidium bromide-stained 1.4% agarose gels.

Hybridization analysis was used to verify results obtained during PCR. PCR fragments were transferred from agarose gels to nylon membranes (Roche Molecular Biochemicals, Laval, Quebec) by vacuum transfer as previously described (Luz et al., 2004). DNA was UV cross-linked (120,000 µJ cm^{-2}) to membranes using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and the membranes were air-dried. Southern hybridization was performed using a DIG-labelling and detection kit (Roche Molecular Biochemicals, Laval, Quebec) following the manufacturer's protocols for pre-hybridization, hybridization, and detection. Probes were generated by incorporating DIG-dUTP into *alkB*, *ndoB*, *nidA*, *phnAc*, and C2,3O gene fragments by PCR amplification of reference strains with the appropriate primers (Table 3.1). Pre-hybridization, hybridization, and washing were performed under high stringency conditions at 65 ^{o}C. Hybridized probes were detected using chemiluminescence (CDP-star, Roche Molecular Biochemicals, Laval, Quebec) and revealed by exposure to x-ray film.

3.3.5 Hydrocarbon analysis

Soil was 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 of sodium sulphate and 4 mL 50:50 (v/v) hexane:acetone solvent, shaken for 10 min, and then centrifuged at 2000 rev min^{-1} for 10 min. Supernatants were transferred to a clean vial containing 0.5mL toluene, evaporated under nitrogen gas without heating to 0.5mL, transferred to a GC vial, and brought to a final volume of 1.8 mL with toluene. Duplicates of each plant/control replicate were processed, for a total of 6 replicates for a given treatment and time.

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), 15m x 0.25 mm i.d. and 0.25 µm stationary-phase film thickness, was used for analytical separation. Hydrogen carrier gas and helium makeup gas had flow rates of 25 mL min^{-1}. Air was supplied as an oxidant at a rate of 300 mL min^{-1}. Detector and injector temperatures were 320 and 300 ^{o}C, respectively. A 1 µL splitless injection volume was delivered with a 5-µL syringe. The initial column oven temperature was maintained at 40 ^{o}C for 1 min and then ramped at 20 ^{o}C min^{-1} to 300^{o}C. Total run time was 25 min. Alternating analytical controls of 250 mg kg^{-1} C34 standard (Supelco, Bellefonte, PA) and 250 mg kg^{-1} weathered diesel standard (Chromatographic Specialties Inc., Brockville, Ont., Canada) were run 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 a standard calibration curve derived from triplicate measurements of 100, 500, 1000, 2500, and 5000 mg kg^{-1} 30W motor oil (Federated Co-operatives Ltd. Saskatoon, SK, Canada) in toluene.

3.3.6 Statistical methods

MPN, CFU, and hydrocarbon data were examined for overall treatment effects by analysis of variance, 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. MPN and

CFU data were log transformed prior to analysis. Relationships between parameters were assessed by regression analysis and Pearson correlation. Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, Illinois).

3.4 Results

3.4.1 Growth chamber

Plant biomass varied substantially both between and within treatment types (data not shown). At 4.5 months root biomass ranged from 4.79 (CRF) to 20.57 (RTDF mix) mg dry weight g^{-1} soil, while shoot biomass ranged from 9.18 (alfalfa) to 19.48 (TWG) mg dry weight g^{-1} soil. CRF and NSMG consistently had the lowest shoot and root biomass, while mixed treatments and TWG had the largest overall biomass production. Species distribution in the RTDF mixture remained equivalent throughout the trial, with approximately 1/3 each of CRF, PRG, and alfalfa. In the local mix the ratio of alfalfa, NSMG, AWR, and TWG shifted from 1:1:4:4 at 2.5 months to 3:1:3:3 at 4.5 months, as alfalfa became a more dominant species.

3.4.2 Culturable microbial communities

Initial culturable heterotrophic populations for non-amended and amended control soils were 5.04 and 5.99 log10 CFUs g^{-1} dry soil, respectively. In non-amended control soil CFUs increased by an order of magnitude in the first 2.5 months and then decreased to levels comparable to time 0 by 4.5 months. In the amended soil however, no significant change in CFUs occurred during the study. All planted treatments except NSMG supported from 0.5 to 1.0 order of magnitude greater CFUs than the non-planted amended soil. Little significant difference was seen between rhizosphere CFUs.

At the beginning of the study the two non-planted soils had equivalent numbers of PAH and n-hexadecane degraders (Table 3.2). Over the course of the study however, degrader communities increased in the non-amended soil while all degrader communities decreased in the amended soil. By 4.5 months all degrader communities in the non-amended control soil were significantly higher than those in the amended control soil. When amended soil was planted however, there was an overall increase in hydrocarbon degrader populations (Table 3.2). In particular, PAH degrader populations

Table 3.2 Most probable number of hydrocarbon degraders (log10 degraders g^{-1} dry soil) in control and rhizosphere soil at time of seeding and at 4.5 months following germination

Treatment	n-hexadecane		Diesel fuel		PAH mixture	
	t0	4.5 mo	t0	4.5 mo	t0	4.5 mo
Control	5.99 (0.31) c	7.31 (0.33) a	5.74 (0.63) cd	7.12 (0.14) ab	4.19 (0.09) bc	4.72 (0.14) b
Amended	6.92 (0.63) abc	6.39 (0.45) bc	7.78 (0.35) a	5.32 (0.17) d	4.61 (0.49) bc	3.96 (0.15) c
Local mix	—	7.58 (0.12) ab	—	6.91 (0.43) ab	—	5.84 (0.21) a
RTDF mix	—	7.52 (0.21) ab	—	6.79 (0.10) abc	—	6.29 (0.20) a
Alfalfa	—	7.50 (0.21) ab	—	6.19 (0.17) bcd	—	6.40 (0.19) a
AWR	—	7.30 (0.27) ab	—	5.82 (0.51) cd	—	4.70 (0.28) bc
CRF	—	7.92 (0.40) a	—	6.99 (0.26) ab	—	4.55 (0.13) bc
NSMG	—	7.85 (0.33) a	—	7.07 (0.73) ab	—	4.61 (0.29) bc
PRG	—	7.27 (0.30) ab	—	6.74 (0.06) abc	—	4.35 (0.19) bc
TWG	—	7.34 (0.28) ab	—	7.29 (0.22) a	—	4.29 (0.12) bc

Control, non-planted non-amended soil; Amended, non-planted amended soil; RTDF mix, Remediation Technologies Development Forum mix; AWR, Altai wild rye; CRF, creeping red fescue; NSMG, Nuttall's salt meadow grass; PRG, perennial rye grass; TWG, tall wheatgrass; t0, time of seeding. Data are presented as log 10 transformed means (n = 3) with standard deviation in parentheses. Means in the same hydrocarbon column with the same letter are not significantly different ($p < 0.05$).

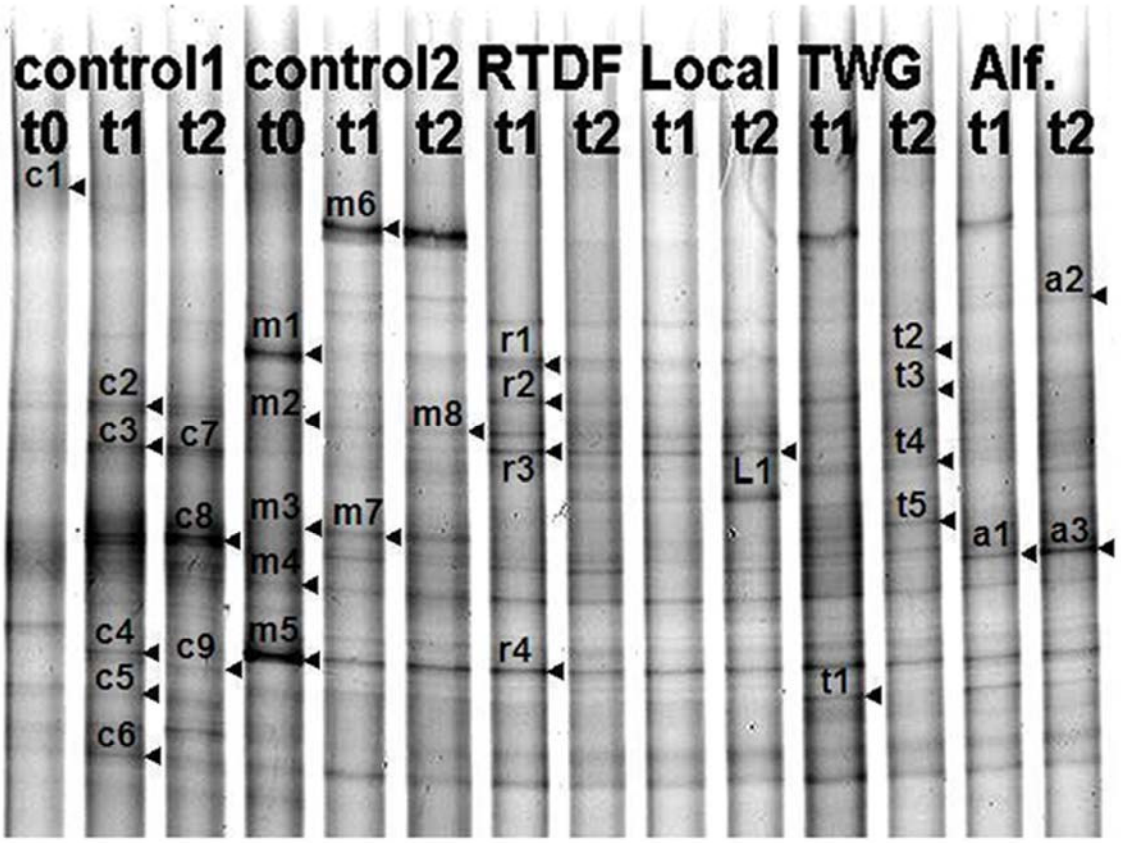

Figure 3.1 DGGE of PCR-amplified 16S rRNA fragments from non-amended (control) and amended (amended) control soils and mixed plant (RTDF, Remediation Technologies Development Forum; Local) and single plant (ALF, alfalfa; TWG, tall wheatgrass) rhizosphere soil. t0, time 0; t1, 2.5 months; t2, 4.5 months. Arrows indicate sequenced bands whose closest identities are provided in Table 3.3.

in alfalfa and both plant mixes were up to two orders of magnitude greater than in all other treatments by the end of the study. There was a significant relationship between CFUs and hydrocarbon degrader populations (data not shown). When assessed as individual replicates all degrader populations were positively correlated ($p < 0.01$) with heterotrophic populations.

3.4.3 Molecular analysis of microbial communities

Treatments which appeared to cause significant changes in hydrocarbon catabolic potential, as assessed during culture-based analyses, were selected for further molecular examination. Alfalfa and mixed-plant treatments, which contained significantly higher PAH degrader populations at 4.5 months than all other treatments, were compared to non-planted soils and to tall wheatgrass.

DGGE profiles (Figure 3.1) show that while numerous bands were common to all treatments, there were also changes in band presence and relative intensity due to treatment and temporal effects. Specific bands of interest were isolated and sequenced (indicated by arrows in Figure 3.1). Single-strand sequences 300 to 400bp in length were submitted for comparison to the GenBank databases and their closest database similarities are shown in Table 3.3.

The addition of amendments had a significant impact on the microbial community structure of the flare pit soil. Several of the dominant bands observed in the non-amended control are not present or are present at relatively reduced levels in amended treatments. These include two bacteria previously isolated from hydrocarbon contaminated soil, an α-Proteobacteria (C2: AF143762) and a Bacteroidetes (C7: AY758587), and an *Aequorivita* sp. previously isolated from high salt environments (C8: AY259510). However, other halo-tolerant bacteria were maintained in amended or planted treatments, including *Algoriphagus locisalis* (T4: AY835923), a Proteobacterium (C6: AY711644), a Flavobacter (M4: AY712425), and a Saprospiraceae (A2: AY509298).

Dendrogram analysis (Figure 3.2) of DGGE banding patterns confirms that the addition of amendments had a significant impact on the community structure. The treatments clustered into two main groups consisting of non-amended control soils and

Table 3.3 Phylogenetic affiliation of specific control and rhizosphere soil bacteria based on 16S rRNA sequences reamplified from DGGE bands

DGGE band	Closest identity	Accession number	% Similarity	Original source
C1	*Flavobacteriu*m sp. V4.B5.09	AJ244698	97	Marine sediment
C2, M2	Uncultured α- Proteobacteria	AF143762	96	Hydrocarbon contaminated soil
C3	*Sphingobacterium* sp. AC74	AJ717393	92	Non-saline alkaline soil
C4	Uncultured *Cytophaga* sp. SIMOCL-S30-76	AY149749	90	Salt marsh
C5	Uncultured γ-Proteobacteria	AJ871053	91	Oil degrading consortium
C6	Uncultured Proteobacterium clone SIMO-2278	AY711644	94	Salt marsh
C7	Uncultured Sphingomonadaceae bacterium	AY758587	97	Creosote contaminated soil
C8	*Aequorivita* sp. BSDS202	AY259510	96	Salt marsh
C9	*Sphingomonas* sp. SRS2	AJ251638	95	Agricultural soil
M1, R1,T2	Uncultured compost bacteria	AY489028	95	Compost
M3, M7,T5	*Algoriphagus locisalis* strain MSS-171	AY835923	98	Marine solar saltern
M4	Uncultured Flavobacteria bacterium clone SIMO-888	AY712425	95	Salt marsh
M5, R4	*Flexibacter aggregans*	AB078039	95	
M6	Uncultured soil bacterium clone N10	AY836593	95	Soil
M8	Flavobacteriaceae bacterium	AY319330	96	Agricultural soil
R2, T3	Rhizosphere soil bacterium clone RSC-11-67	AJ252691	97	Rhizosphere soil
R3, L1	Uncultured Bacteroidetes bacterium clone SIMO-1798	AY711164	96	Salt marsh
T1	Uncultured γ-Proteobacteria	AY515454	95	Intertidal mudflats
T4	*Sphingopyxis aleskensis* strain S23	AY509242	94	Rhizosphere soil
A2	Uncultured Saprospiraceae bacterium	AY509298	94	Marine sediment
A1, A3	Unidentified γ-Proteobacteria	AJ548928	94	Sulphurous biofilm

C, non-amended control; M, amended control; R, RTDF plant mix; T, tall wheat grass; L, local plant mix; A, alfalfa

planted and/or amended soils. Specific grouping was also observed within the amended cluster. Tall wheatgrass communities at both sampling periods were highly similar (70%) and showed the greatest difference from the other treatments. The mixed-plant treatments at the last sampling point were more similar to alfalfa (75 and 80% for local and RTDF mixes) than they were to the mixes at the first sampling point, indicating that the presence of alfalfa in the mix had a significant impact on the final microbial community structure. Many of the bands used for dendrogram analysis were relatively faint and much of the variation between treatments was the result of changes in these non-dominant microbial communities. Dendrogram analysis of a replicate DGGE gel created from separate amplification products indicated that the observed clustering is reproducible and stable.

Total community DNA was screened by PCR and hybridization analysis for the presence of five catabolic genotypes (*alkB, ndoB*, *nidA*, *phnAc*, and C2,3O). The *alkB*, *nidA*, and C2,3O genotypes were present in all treatments at all sampling times while the *ndoB* and *phnAc* genotypes were less widely distributed (Table 3.4). Neither amended nor non-amended control soils contained detectable levels of *ndoB* (*Pseudomonas putida*) by the end of the study. In contrast, ndoB was detected in all assessed planted treatments except the RTDF mix at the end of the study. PCR results for ndoB in RTDF treatments were positive but hybridization results were negative, indicating the presence of a distantly related genotype (Whyte et al., 2002).

3.4.4 Hydrocarbon concentration

Treatment specific reductions in extractable total petroleum hydrocarbon (TPH) concentrations were observed during this study (Table 3.5). The addition of amendments to the flare pit soil inhibited hydrocarbon degradation in non-planted treatments. When amended soil was planted however, hydrocarbon degradation increased. By the end of the trial TPH concentrations were reduced between 4 and 49% in planted soils. The initial soil was contaminated primarily with F3 (59%) and F4 (38%) fractions. Hydrocarbon degradation was distributed relatively evenly across these fractions for all treatments except creeping red fescue. In this treatment 66% of the F4 fraction and 35% of the F3 fraction were removed after 4.5 months, leaving an F3:F4 hydrocarbon ratio of

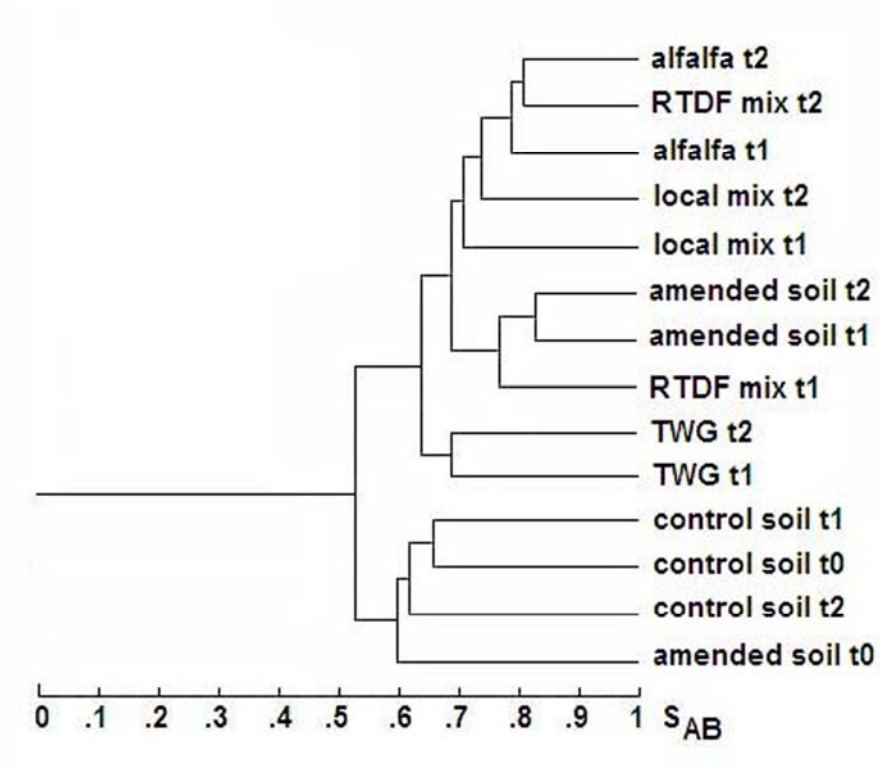

Figure 3.2 Dendrogram analysis of weighted DGGE banding patterns from control and rhizosphere soil**.** Control, non-planted non-amended soil; Amended, non-planted amended soil; RTDF mix, Remediation Technologies Development Forum mix; TWG, tall wheatgrass; t0, time 0; t1, 2.5 months; t2, 4.5 months.

Table 3.4 PCR and hybridization analyses of total community DNA from control and rhizosphere soils.

Treatment	*alkB*		C2,3O		*nidA*		*ndoB*		*phnA*c	
	PCR	Probe	PCR	Probe	PCR	Probe	PCR	Probe	PCR	Probe
Control t0	+	+	+	+	F	+	-	+	F	+
Control t1	+	+	+	+	F	+	-	+	F	+
Control t2	+	+	+	+	+	+	-	-	F	+
Amended t0	+	+	+	+	F	+	-	-	-	+
Amended t1	+	+	+	+	+	+	-	F	-	+
Amended t2	+	F	+	+	F	+	-	-	-	F
Alfalfa t1	+	F	+	+	F	+	F	+	-	+
Alfalfa t2	+	+	+	+	F	+	+	+	-	F
Local t1	+	+	+	+	F	+	-	-	-	-
Local t2	+	+	+	+	F	+	F	+	F	+
RTDF t1	+	+	+	+	F	+	+	-	-	+
RTDF t2	+	F	+	+	F	+	+	-	-	-
TWG t1	+	F	F	+	F	+	-	-	-	+
TWG t2	+	+	+	+	F	+	F	F	-	+

Control, non-planted non-amended soil; Amended, non-planted amended soil; Local, Local mix; RTDF, Remediation Technologies Development Forum mix; TWG, tall wheatgrass; t0, time 0; t1, 2.5 months; t2, 4.5 months; F, faint signal; -, no signal; +, positive signal

Table 3.5 Percent reduction in total petroleum hydrocarbon levels in control and rhizosphere soil at 4.5 months compared to time 0.

Treatment	F3 fraction	F4 fraction	TPH
Control	21 (10) ab	35 (4) b	28 (7) ab
Amended	0 (9) b	1 (1) d	0 (6) b
Local mix	10 (2) ab	7 (0) dc	9 (1) b
RTDF mix	15 (3) ab	12 (1) dc	14 (2) b
Alfalfa	10 (7) ab	8 (2) dc	10 (5) b
AWR	22 (3) ab	21 (4) bc	23 (5) b
CRF	35 (5) a	66 (3) a	49 (4) a
NSMG	30 (15) a	44 (28) abc	36 (21) ab
PRG	0 (21) b	14 (32) abcd	4 (26) ab
TWG	26 (9) ab	43 (22) abc	33 (14) ab

Control, non-planted non-amended soil; Amended, non-planted amended soil; RTDF mix, Remediation Technologies Development Forum mix; AWR, Altai wild rye; CRF, creeping red fescue; NSMG, Nuttal's salt meadow grass; PRG, perennial rye grass; TWG, tall wheatgrass. Data are presented means (n = 6) with standard deviation in parentheses. Means in the same column with the same letter are not significantly different ($p < 0.05$).

75:25. There was no correlation between TPH concentration and root or shoot biomass (data not shown). When data for individual replicates were analyzed, correlations emerged between the numbers of culturable degraders present and hydrocarbon concentrations over the course of the study. TPH concentration was negatively correlated with n-hexadecane degraders (Figure 3.3; $p < 0.001$).

3.5 Discussion

Flare pit soils represent a unique remediation challenge due to their complex mixture of organic and inorganic contaminants. The overall goal of this study was to determine whether phytoremediation was an effective treatment option to reduce hydrocarbon levels in this class of contaminated soils, by assessing the impact of plants on indigenous microbial communities and hence on overall degradation potential. As most phytoremediation studies require the addition of amendments such as fertilizer or compost at some point to facilitate plant growth, we also examined both amended and non-amended control soils. We found that the addition of amendments to flare pit soil caused significant changes to the functional and taxonomic structure of the microbial communities. These changes were detrimental with regards to degradation potential in at structural similarity between these two soils decreased to 50% by 4.5 months (Figure 3.2). At least two bacteria potentially capable of degrading hydrocarbons, an α-Proteobacteria (AF143762) and a Bacteroidetes (AY758587), which were dominant in non-amended soil, decreased in relative abundance in amended soil (Table 3.3, Figure 3.1). The cumulative result of the changes that occurred with the addition of amendments was that no detectable hydrocarbon degradation occurred in the amended control soil, whereas non-amended control soils exhibited a 28% decrease in total petroleum hydrocarbon (TPH) concentration (Table 3.5).

Increases in heterotrophic and degrader populations in the non-amended control soil indicate that a priming effect associated with soil preparation may have stimulated the 28% TPH reduction seen in this soil. A comparable priming effect might be expected to increase degradation in the amended soils. However, the amended control shows no sign of such an effect occurring, as heterotrophic numbers do not increase beyond the initial 5.99 log10 CFUs g^{-1} dry soil, degrader populations decrease (Table

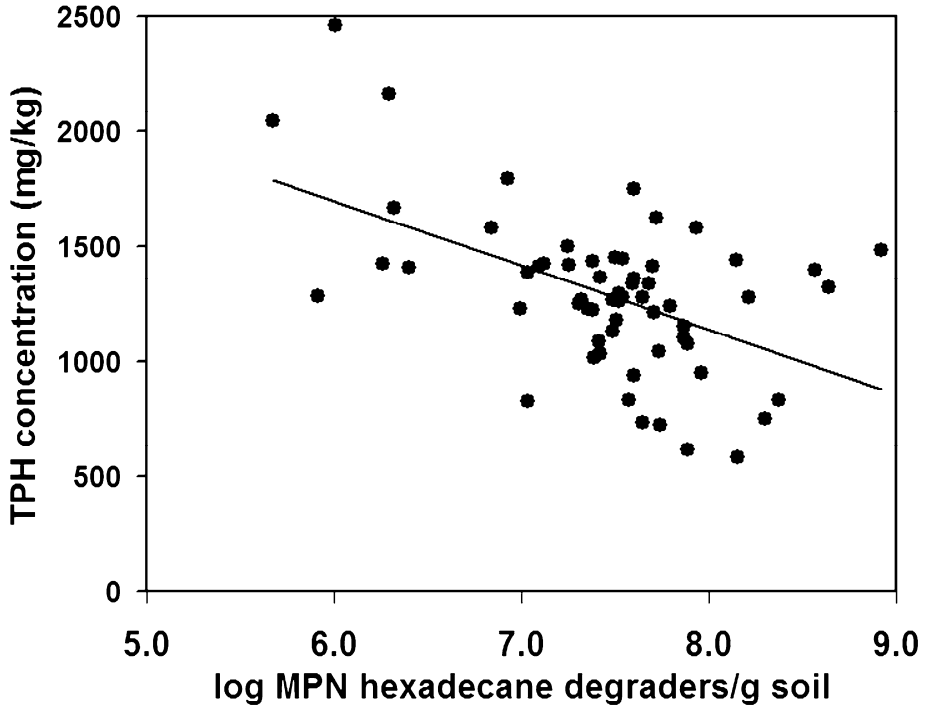

Figure 3.3 Relationship ($r = -0.520$, $p < 0.001$) between n-hexadecane degraders and hydrocarbon levels based on individual treatment replicates.

3.2), and no TPH reduction is observed (Table 3.5). The impact of the amendments themselves on the degradation potential of indigenous microbial populations appears to have negated the potentially beneficial impact of a priming effect. TPH reductions in planted treatments are therefore most likely due to plant specific impacts, and not, as seen in other studies, enhanced or promoted by the addition of amendments (Siciliano et al., 2003; White et al., 2003).

Hydrocarbon degradation did increase when amended soil was planted, although there were large differences in overall degradation between the treatments (Table 3.5). Two grasses, creeping red fescue and perennial rye grass, facilitated the highest and the lowest overall reductions, 49% and 4% respectively, in TPH concentration. Mixed plant treatments and alfalfa treatments facilitated the next lowest overall reduction of approximately 10%. Although no one factor may be singled out to account for these different treatment responses, several overall trends emerged that may help elucidate their occurrence.

TPH concentrations over the course of this study were correlated ($p < 0.001$) to the absolute number of culturable n-hexadecane degraders found in the treatments (Figure 3.3). The role that aliphatic hydrocarbon degraders play in TPH degradation has not been extensively examined, as most studies focus on the role that degraders play in the degradation of more recalcitrant PAHs. Some studies have found that alkane degrader populations (Kirk et al., 2005) or *alkB* genotypes (Siciliano et al., 2003) are present at higher levels in rhizosphere soil than bulk soil but no direct link to TPH reduction has been shown. Previous research (Margesin et al., 2003, Whyte et al., 2002) has revealed that specific *alkB* genotypes, particularly those belonging to r-strategist groups such as Pseudomonads, are more prevalent in contaminated than pristine soil. As many plant exudates are known to attract and stimulate microbial communities such as Pseudomonads (Espinosa-Urgel, 2004), the introduction of plants to a contaminated soil should further stimulate the growth and activity of indigenous r-strategist alkane degraders, resulting in substantial initial increases in both the presence of *alkB* genotypes and overall degradation. In this study we found that alkane degrader populations were maintained at 1-1.5 orders of magnitude greater in rhizosphere compared to amended control soil (Table 3.2). Creeping red fescue rhizosphere soil had

the highest number of these degraders, while perennial rye grass had the lowest. Although n-hexadecane degraders were positively correlated with heterotrophic populations (data not shown), CFUs in rhizosphere treatments exceeded those in the control by only half an order of magnitude, suggesting that degrader populations were also influenced by selective factors. When the presence of alkane monoxygenase genes was assessed by PCR and hybridization analysis all treatments, including the control soils, contained detectable levels of *alkB*. Since consensus *alkB* primers targeting both Gram-positive and -negative bacteria were used, it is impossible to determine if specific *alkB* genotypes increased in prevalence in any given treatment.

Trends were also seen in the prevalence of specific genotypes capable of stimulating aromatic hydrocarbon degradation. PAH degrader populations were up to 2 orders of magnitude greater in both the mixed plant and the mono-alfalfa treatments by the end of the study than in all other treatments (Table 3.2). Contrary to expectations however, these treatments did not have the highest degradation rates. Overall TPH degradation in these treatments was lower than in all treatments except perennial rye grass and the non-planted amended soil (Table 3.5). Other studies have also implicated legumes in an overall decrease in total hydrocarbon degradation. During field trials in California with tall fescue and mixes of native grasses and legumes, Banks et al. (2003) found that mixes containing legumes exhibited only 12 to 18% reduction in TPH concentrations, while tall fescue alone facilitated a 65% reduction in TPH concentration. Conversely, studies have linked specific increases in PAH degraders in legume rhizospheres to PAH degradation (Muratova et al., 2003; Parrish et al., 2005). Although not specifically measured, degradation of PAHs may have occurred in alfalfa-containing treatments in our study, while the degradation observed in treatments not containing alfalfa may have occurred primarily in the aliphatic fraction. The enhancement of specific organisms may lead to the preferential degradation of either aliphatics or aromatics, with degradation of the remaining class of compound being inhibited as a result of catabolite repression (Rentz et al., 2004, Kamath et al., 2004, Yuste et al., 1998), competitive exclusion (Espinosa-Urgel, 2004), or niche competition (Kästner and Mahro, 1996). In our study increased PAH degrader activity in the alfalfa rhizosphere

may have led to a decrease in the activity or presence of microbes capable of degrading aliphatic hydrocarbons, with a resultant concomitant inhibition of TPH reduction.

If such selective mechanisms were occurring one would expect to see measurable changes in the taxonomic or functional diversity of alfalfa rhizosphere communities. DGGE results indicate that alfalfa does appear to have a dominant effect on microbial community patterns. For example, the most prevalent species in the local plant mix was tall wheatgrass, which represented approximately 40% of the mix at both sampling points. Alfalfa was present in these mixes at approximately 10% (2.5 months) and 30% (4.5 months). Local mix DGGE banding patterns however, show 70% and 75% similarity (2.5 months, 4.5 months) to alfalfa profiles, while only showing 65% similarity to tall wheatgrass profiles (Figure 3.2). A similar trend was observed by Kirk et al. (2005) in a study that examined microbial diversity in perennial ryegrass (PRG), alfalfa, and mixed plant treatments in petroleum contaminated soil. The DGGE profile for the mixed plant community was 82% similar to the alfalfa profile, but only 55% similar to the PRG profile. In our study, some of the observed difference was accounted for by several predominant bands, as an unidentified γ-Proteobacteria (Figure 3.1, band A1/A2) and several other un-sequenced bands increased in relative prevalence in alfalfa-containing soil. Most of the observed difference however, was due to the fluctuating presence of minor bands. This suggests that plant-specific impacts in this type of contamination may be more significant in terms of overall community composition, rather than on one or two dominant species. Focusing primarily on the impact that plants have on dominant species may skew our understanding of the actual dynamics that may be important for increased degradation.

3.6 Conclusions

This study has shown that phytoremediation can be an effective treatment option for flare pit soils contaminated with weathered hydrocarbons. Plant specific factors, both selective and non-selective, had a substantial impact on the degradation potential of indigenous hydrocarbon degrading microorganisms. Final degrader populations were higher, there was an increase in catabolic genotypes in specific planted treatments, and overall hydrocarbon degradation was enhanced in planted compared to un-planted

amended soil. This enhanced potential was independent of the addition of amendments, as non-planted amended soil experienced an overall decrease in degradation potential. The common in situ practice of using mixed plant species however, may slow or inhibit degradation in these soils, as there appears to be a dominant and selective influence by alfalfa on degrader populations that does not immediately favour increased overall degradation. More research is required to determine if the suppressive effect that alfalfa had on degradation is limited to an initial adaptation period.

4.0 FIELD-SCALE ASSESSMENT OF WEATHERED HYDROCARBON DEGRADATION BY MIXED AND SINGLE PLANT TREATMENTS

4.1 Preface

In Chapter 3 we saw that the common practice of using mixed plant species may limit the effectiveness of phytoremediation in flare pit soils contaminated with weathered hydrocarbons. Specific plants such as alfalfa exerted a dominant impact on rhizosphere microbial populations, which suppressed overall degradation. Plant impacts on rhizosphere microbial communities under controlled environment conditions however, may not correlate with those that occur under field conditions, particularly in cold climate regions. Thus, corroborating field studies are required. In this two-year field study changes to both rhizosphere and endophytic bacterial communities were monitored with respect to long term hydrocarbon degradation potential.

4.2 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 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; Chaudhry 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^{o}C in the winter to 35^{o}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 (TPH), 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.

4.3 Materials and Methods

4.3.1 Phytoremediation site and sampling

A two year phytoremediation study was established at a hydrocarbon-contaminated site located in south-eastern Saskatchewan, Canada. Soil at the site, derived from an adjacent decommissioned flare-pit with a 30 year history of chronic releases, was classified as moderately alkaline and saline/sodic. The soil had a pH of 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 of 629, 989, and 4900 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 (1m × 1m) 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 wheatgrass), *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 ($CaSO_4.2H_2O$, 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. 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 0.5m 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-wk 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 seeds. 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 ^{o}C until processing and analysis (within 36 h of sampling for soil samples and 72 h for endophytic samples). For hydrocarbon analysis, two 0- to 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^{o}C until analysis.

4.3.2 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 5 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 1mL 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^{o}C for 24 h while awaiting results from sterility assessments. All other samples were immediately assessed using culturable techniques.

4.3.3 Microbial community analysis

Endophytic extracts were produced by macerating 2.5-g surface-sterile root from each treatment replicate in 22.5mL 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 10-fold 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^{o}C for 24 h.

4.3.4 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 0.1g L^{-1} cycloheximide. Plates were incubated at room temperature for 7 d.

4.3.5 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, 1g 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} to 10^{-1} serial dilutions (80 µL per well, 5 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 two weeks, 200µL of filter-sterilized p-iodonitrotetrazolium violet (3g 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).

4.3.6 Hydrocarbon degrading activity of microbial communities

The hydrocarbon degrading activities of control soil, rhizosphere soil and endophytic microbial populations were assessed using C-14 hydrocarbon mineralization assays. Microcosms were set up and sampled as outlined in Chenier 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 0.5mL 1M 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 10mL 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 one wk resting interval).

4.3.7 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 30m × 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 ^{o}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 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).

4.3.8 Statistical methods

Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, Illinois). 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).

4.4 Results

4.4.1 Decrease in total petroleum hydrocarbon concentration

Treatment specific reductions in extractable total petroleum hydrocarbons (TPH) concentrations were observed over the course of the study. Altai wild rye (AWR) treatments stimulated the greatest overall decrease (>50%) in TPH during the first growing season (Table 4.1). 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 4.1, Figure 4.1). Although all treatments reached comparable TPH levels by the end of the second season, the single grass treatments AWR and TWG exhibited the highest overall

Table 4.1 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†	Fraction 3 (C16-34)	Fraction 4 (C34-50)	Total hydrocarbons (C10-C50)
	2005		
Control	+5.4 (28.4)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 wheatgrass	-35.1 (34.2)ab	-33.9 (16.6)ab	-35.4 (28.1)ab
	2006		
Control	-32.6 (10.8)a‡	-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 wheatgrass	-51.8 (25.7)a	-45.3 (15.1)a	-50.1 (22.1)a

†Control, non-planted control soil; AWR, Altai wild rye; TWG, tall wheat grass; Mixed plants, mixture of alfalfa, AWR, and TWG. Data are presented as means (n = 4) with SD in parentheses.

‡Means in a single sub-column followed by a different letter are significantly different at $p \leq 0.05$

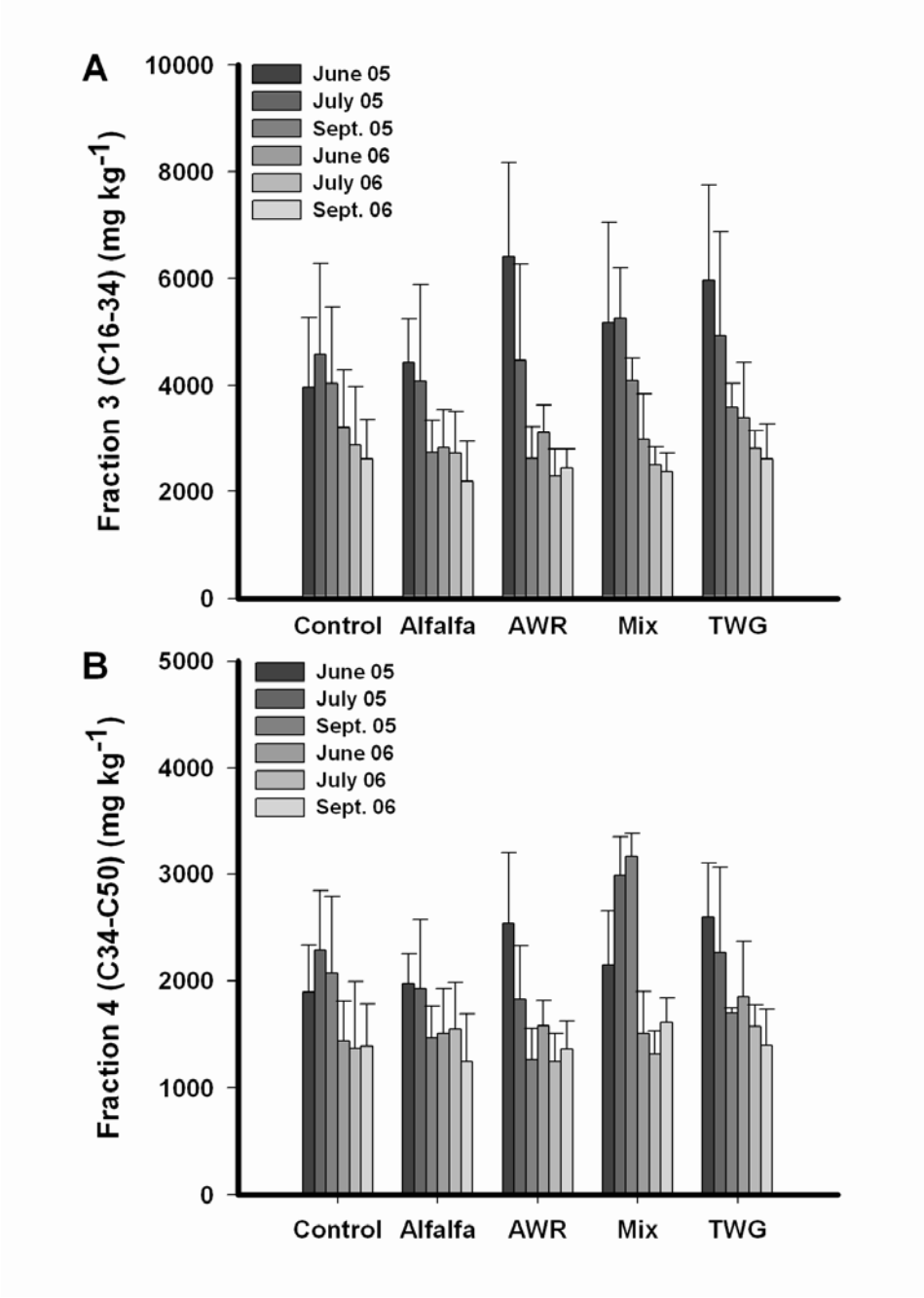

Figure 4.1 Fraction 3 (A) and fraction 4 (B) hydrocarbons in control and planted plots over a two season phytoremediation field trial. Treatments include non-planted control soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as means (n = 4) with error bars representing 1 SD.

TPH degradation (56 and 50% respectively). High spatial variability in initial total petroleum hydrocarbon (TPH) levels was observed at the beginning of the study, even though the plots were distributed over a relatively small total area (5.5m × 7m) 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 5,000 and 7,000 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 4.1) were often not statistically significant.

4.4.2 Heterotrophic microbial communities

Endophytic CFUs were highly variable both within and between treatments during the first season, and then stabilized over time (Figure 4.2A). 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 4.2). 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. Heterotrophic communities were relatively stable over the course of the study, averaging between 10^7 and 10^8 CFUs g^{-1} soil (Figure 4.2B). 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 4.2), 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 in the unplanted control (Figure 4.2B).

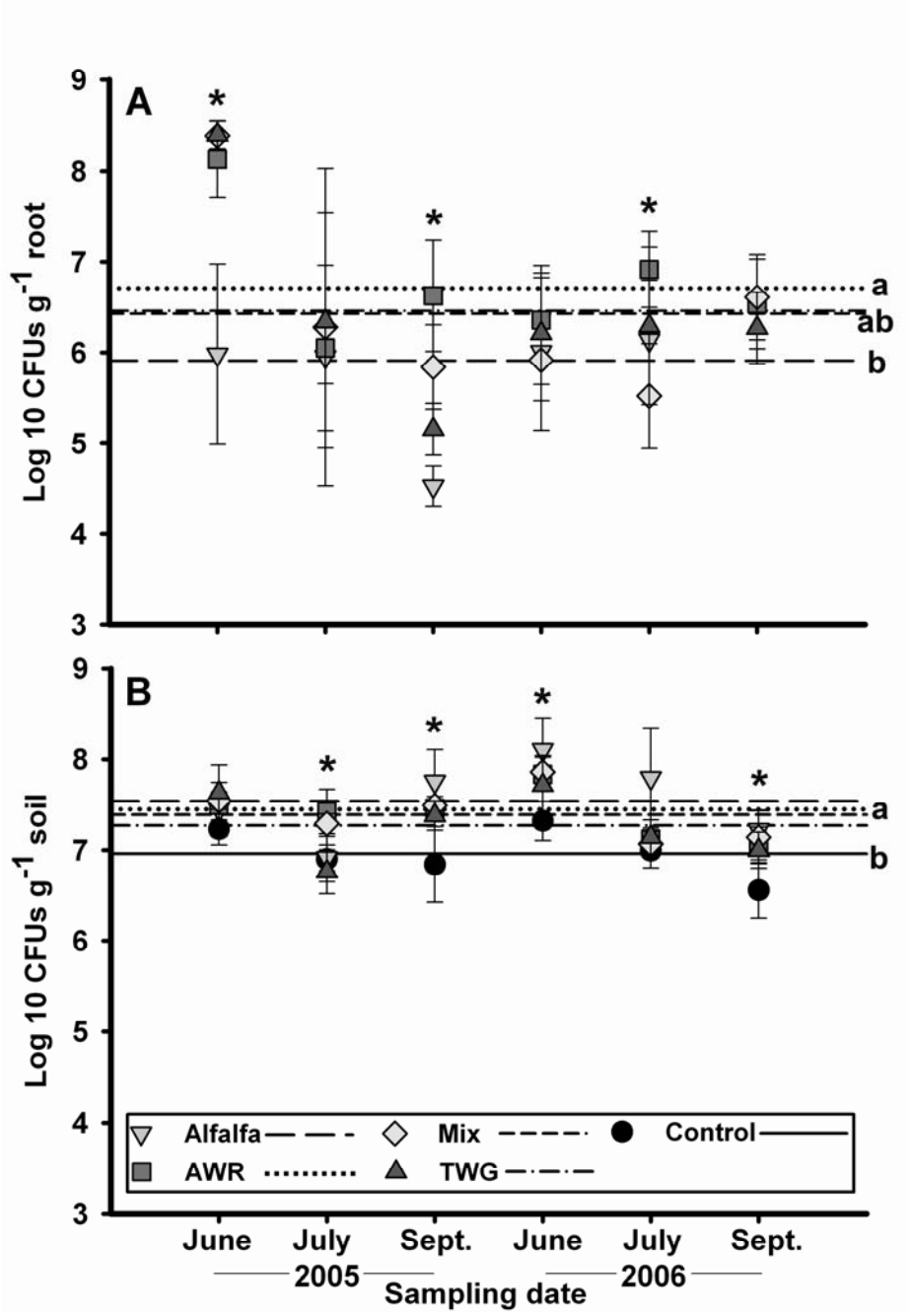

Figure 4.2 Heterotrophic microbial populations within (A) plant roots and (B) rhizosphere or control soil during a two season phytoremediation field trial. Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means (n = 4) with error bars representing ± 1 SD. Error bars may be obscured by data points. * indicates 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 4.2 Correlation coefficients for soil and endophytic microbial communities, sampled at 6-wk intervals over two growing seasons**.**

Microbial communities	Year	Soil communities†		Endophytic communities		Moisture	TPH
		Hexadecane degraders	PAH degraders	Heterotrophic	Hexadecane degraders		
Soil communities†							
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‡	2005			0.443**	0.517***	0.205*	0.325*
	2006			0.016	-0.069	0.103	-0.135

*Significant at $p \leq 0.05$
**Significant at $p \leq 0.01$
***Significant at $p \leq 0.001$
† Control and/or rhizosphere soil
‡Endophytic PAH degraders were not correlated with soil microbial communities

4.4.3 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 (Figure 4.3A), 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 (Figure 4.3B), 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 increased TPH concentration, while in the second season they were strongly correlated with heterotrophic communities (Table 4.2). 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 4.2). An additional correlation was observed between endophytic hexadecane degraders and TPH concentration during the first growing season (Table 4.2), 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 4.2).

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 (Figure 4.4). PAH degraders in soil niches were positively correlated with soil heterotrophic communities throughout the study duration (Table 4.2). 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 4.2).

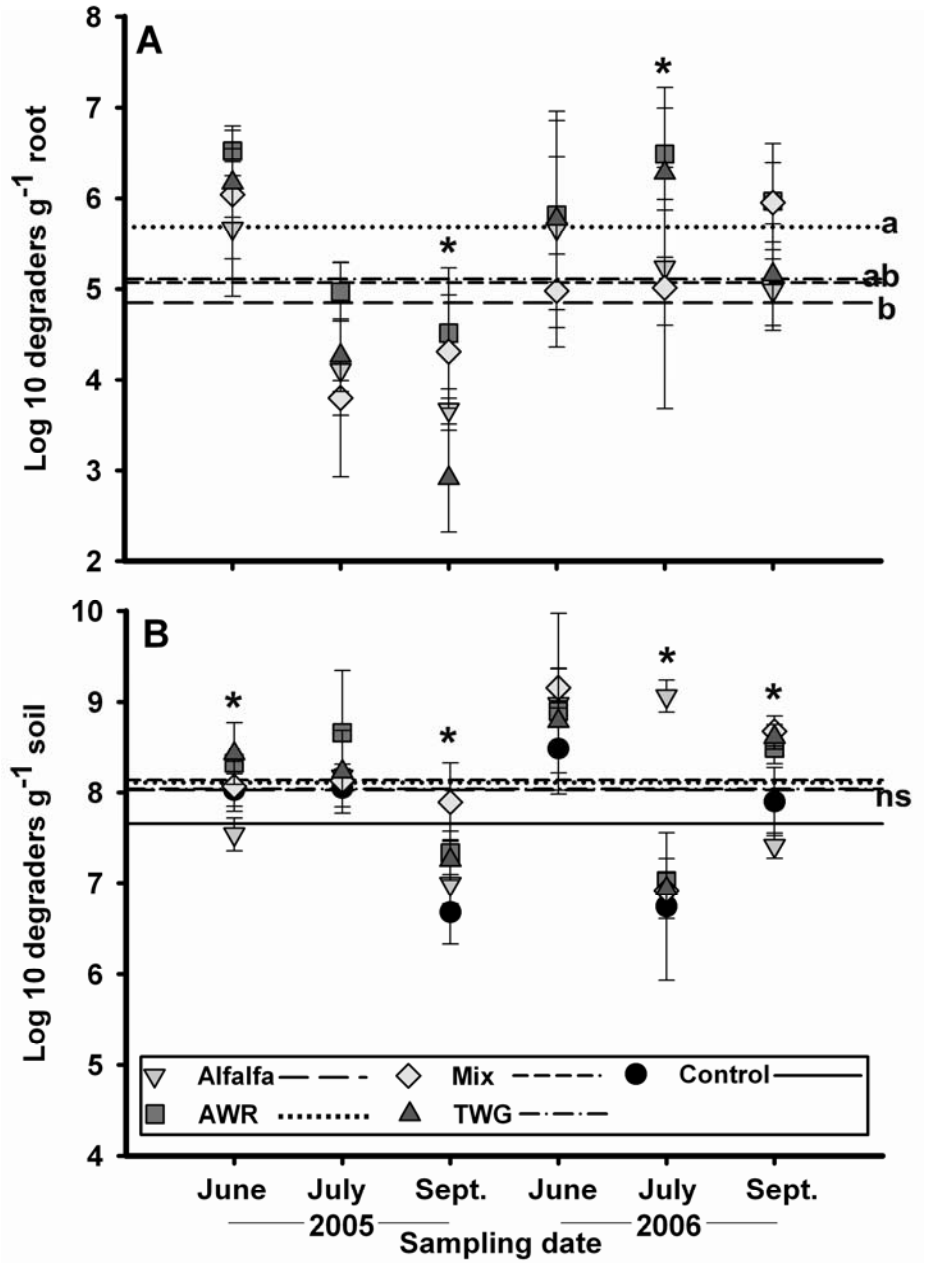

Figure 4.3 Most probable number of microbial n-hexadecane degraders within (A) plant roots and (B) rhizosphere or control soil during a two season phytoremediation field trial**.** Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means (n = 4) with error bars representing ± 1 SD. Error bars may be obscured by data points. * indicates 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 ($p > 0.05$).

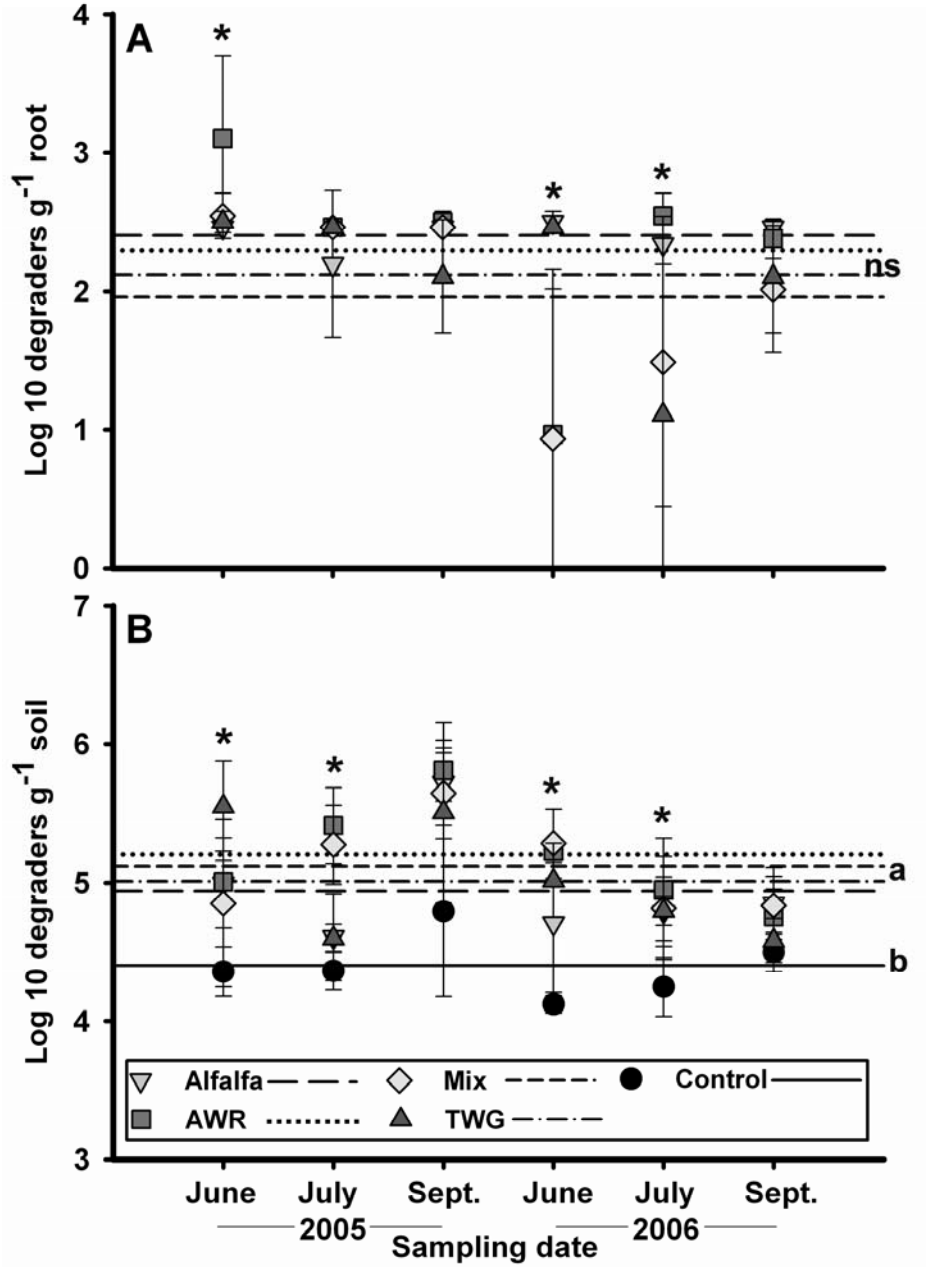

Figure 4.4 Most probable number of microbial PAH degraders within (A) plant roots and (B) rhizosphere or control soil during a two season phytoremediation field trial**.** Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Data are presented as log transformed means (n = 4) with error bars representing ± 1 SD. Error bars may be obscured by data points. * indicates 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 ($p > 0.05$).

4.4.4 Hydrocarbon degrading activity of microbial communities

Endophytic hexadecane mineralization was highly variable within treatments, between treatments, and over time (Figure 4.5A). Endophytic hexadecane mineralization, although occurring only sporadically, was positively correlated with MPN-enumerated hexadecane degraders during the first growing season, but negatively correlated with both soil heterotrophic and hexadecane degrader communities during the second growing season (Table 4.3). 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 (Figure 4.5B). A strong negative correlation was observed between hexadecane degrader populations as assessed using the MPN technique and hexadecane mineralization during both growing seasons (Table 4.3).

All rhizosphere microbial communities mineralized approximately 40% of the added phenanthrene in three weeks, compared to an average of less than 30% in control soils (Figure 4.6). 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 4.3) and negatively correlated with hexadecane degraders during the 2006 season (Table 4.3). 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).

4.5 Discussion

This study compared the potential of three single-plant treatments and a combination of these plants to facilitate the degradation of weathered hydrocarbons at a field site in south eastern Saskatchewan, Canada. We found treatment specific decreases in total petroleum hydrocarbons (TPH) during the first growing season, with degradation

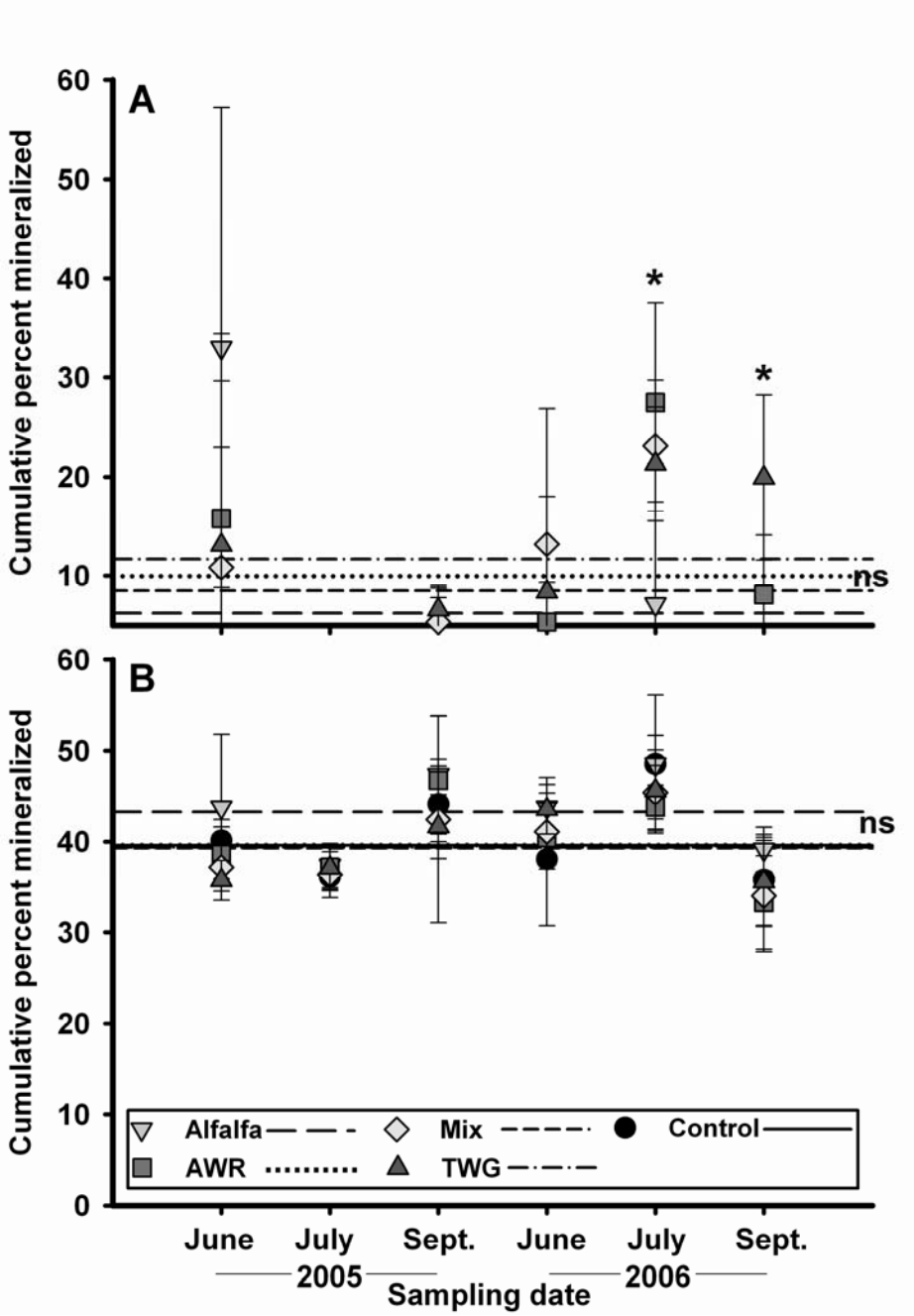

Figure 4.5 Cumulative percent n-hexadecane mineralized by (A) endophytic microbial communities and (B) rhizosphere or control microbial communities during a two season phytoremediation field trial**.** Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Error bars representing ± 1 SD and may be obscured by data points. * indicates 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 ($p > 0.05$).

Table 4.3 Correlation coefficients for hydrocarbon mineralization and soil and endophytic microbial communities, sampled at approximately 6-wk intervals over two growing seasons.

Hydrocarbon mineralization	Year	Soil microbial communities†			Endophytic microbial communities		
		Heterotrophic	Degrader		Heterotrophic	Degrader	
			Hexadecane	PAH		Hexadecane	PAH
Soil †							
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

*Significant at $p \leq 0.05$
**Significant at $p \leq 0.01$
***Significant at $p \leq 0.001$
† Control and/or rhizosphere soil

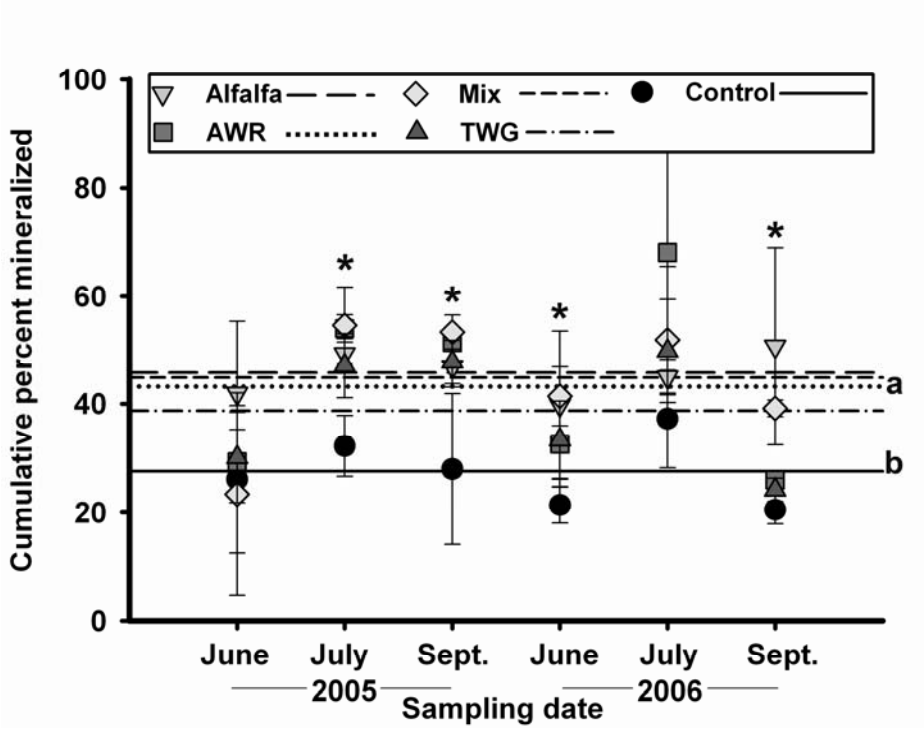

Figure 4.6 Cumulative percent phenanthrene mineralized by rhizosphere or control microbial communities during a two season phytoremediation field trial**.** Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Error bars representing ± 1 SD and may be obscured by data points. * indicates 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 single plant treatments of up to 54% (Table 4.1) 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 used 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 show 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.

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 4.2). 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., under review) 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 (Figure 4.2A) and degrader populations (Figure 4.3A) in September of 2005 and July of 2006, times of local drought (Figure 4.7). During 2005 a strong correlation was observed between endophytic communities and rhizosphere aliphatic degrader communities (Table 4.2), 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 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 (Figures 4.3 and 4.4). Previous research has shown that grasses and legumes have different impacts on both microbial

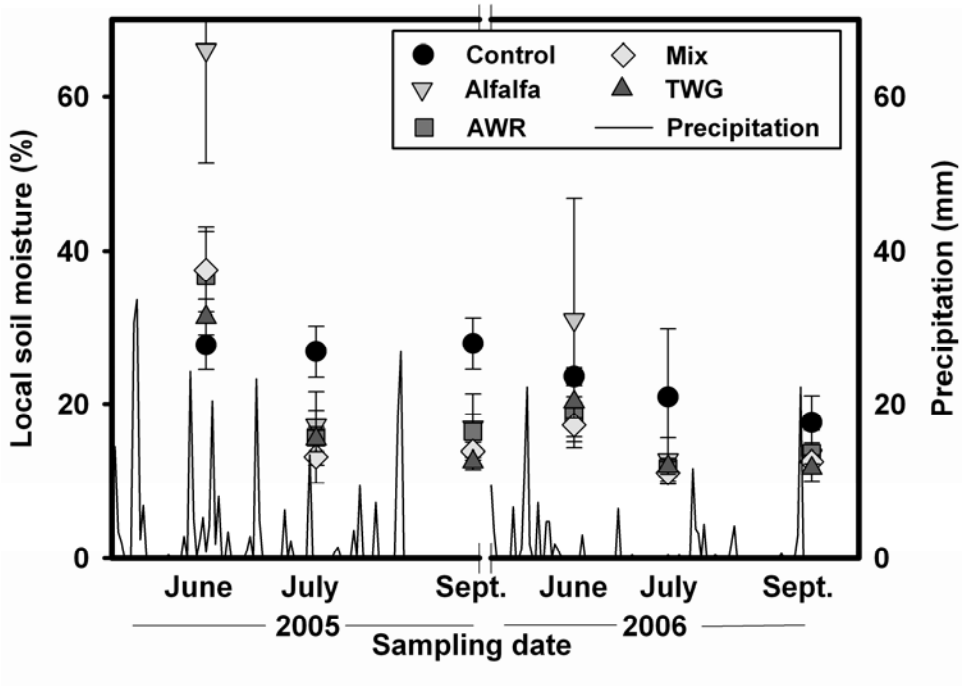

Figure 4.7 Average soil moisture in treatment plots at each sampling point during a two season phytoremediation field trial, compared to local precipitation over the growing season (Environment Canada)**.** Treatments include non-planted soil (control), alfalfa, Altai wild rye (AWR), tall wheat grass (TWG), and a mixture of alfalfa, AWR, and TWG (mix). Error bars representing ± 1 SD and may be obscured by data points.

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} (Figure 4.1). Whereas both the chemical extractability and bioaccessibility of contaminants is 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 Putz (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; Nuran 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 (Figure 4.1). A similar phenomenon was recorded during a mixed-plant phytoremediation field study of aged contaminated soil in Finland, (Palmruth 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. These results 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 4.3). 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^{o}C by two soils with 10,700 and 2,738 hexadecane degraders g^{-1} 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 4.2). While decreased hexadecane mineralization has been linked to increased soil moisture and decreased oxygen availability (Borresen and Rike (2007) no direct correlation between moisture and hexadecane mineralization occurred in either season. Thus 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-Manzano 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.

4.6 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 wheatgrass 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.

5.0 HYDROCARBON DEGRADATION POTENTIAL AND ACTIVITY OF ENDOPHYTIC BACTERIA ASSOCIATED WITH PRAIRIE PLANTS

5.1 Preface

In the previous chapter we saw that both rhizosphere and endophytic bacteria play a role in determining the degradation potential of a given plant. During initial plant establishment the recruitment and maintenance of specific endophytic degrader phenotypes may enhance degradation potential, contributing to the discrepancies in total hydrocarbon degradation between plant treatments. To date, little is known about the diversity, distribution, and activity of potentially useful endophytic communities capable of contributing to the overall degradation potential of plants. To address this knowledge gap this study assessed the endophytic communities of five different plants from a long-term phytoremediation site within which the previous study was established.

5.2 Introduction

Phytoremediation, the use of plants to remove or degrade contaminants, has been extensively researched in recent years (for review see Arthur et al., 2005; Chaudhry et al., 2005). Phytoremediation systems for organic compounds such as petroleum hydrocarbons rely on a synergistic relationship between plants and their associated rhizosphere microbial communities. Hydrocarbon degradation is believed to occur through a rhizosphere effect; plants exude organic compounds through their roots, which increase the density, diversity and activity of specific microorganisms in the surrounding rhizosphere, which in turn degrade hydrocarbons (Anderson et al., 1993; Siciliano and Germida, 1998). As a consequence, most phytoremediation research has focused on specific plant and contaminant influences on rhizosphere community structure, with respect to degradation of petroleum compounds. Current research however, also indicates a probable role for endophytic bacteria in phytoremediation systems (Newman and Reynolds, 2005; Ryan et al., 2008).

Endophytic bacteria are described non-pathogenic bacteria found within the interior tissues of healthy or symptomless plants (Schultz and Boyle, 2006). These bacteria are found in most, if not all, plant species, span a wide range of bacterial phyla, and are known to have plant growth promoting and pathogen control activities (Hallmann et al., 1997; Hallmann and Berg, 2006; Ryan et al., 2008). Recent research suggests that these beneficial impacts may, in the case of plants growing at contaminated sites, extend to the degradation of xenobiotic compounds. Germaine et al. (2006) inoculated pea plants with a *Pseudomonas putida* strain capable of degrading 2,4-dichlorophenoxyacetic acid. The strain actively colonized endophytic and rhizoplane niches, resulting in increased 2,4-D removal from soil and decreased 2,4-D accumulation by plants. Barac et al. (2004) demonstrated that a genetically modified endophytic strain of *Burkholderia cepacia* was able to increase the tolerance of yellow lupine to toluene, with a concomitant decrease in toluene evapotranspiration. Apart from direct in planta degradation, endophytic inoculants have also been shown to transfer their degradative plasmids to other endophytes, thus increasing the overall degradation potential of endogenous endophytic communities (Taghavi et al., 2005; Wang et al., 2007).

Although endogenous bacterial endophytes capable of degrading hydrocarbons are likely to be widespread, to date only a few studies have assessed the degradation potential inherent to endophytic communities of common plants used in phytoremediation studies. Porteous Moore et al. (2006) found that *Pseudomonas*, *Arthrobacte*r, *Enterobacter*, and *Bacillus* strains isolated from the root and stem tissues of different poplar cultivars growing at a car manufacturing plant were able to degrade BTEX compounds. The distribution of these bacteria was spatially limited both within and between individual poplar cultivars. Siciliano et al. (2001) assessed the bacterial populations associated with tall fescue and red clover growing at an aged-hydrocarbon contaminated site. The researchers found from 0.1 to 4% of culturable heterotrophic endophytic bacteria possessed genes involved in hydrocarbon degradation, but that there were significant plant specific differences in the prevalence of degrader genotypes. In some cases, as with *alkB* genotypes in tall fescue endophytic communities, there was

increased degrader prevalence in endophytic communities relative to rhizosphere communities.

Optimization of the degradation potential of endophytic communities could allow researchers to optimize phytoremediation treatments for specific sites. The plant interior represents a stable environment, where specialized degrader communities may be relatively unaffected by changing external environmental conditions. In some cases, as seen in Siciliano et al. (2001), plant conditions may favour the establishment of specific degrader communities. Such endophytic communities may act as a source of degrader communities for the rhizosphere, allowing swift re-establishment of such communities following periods of environmental stress such as drought (Lori Phillips, unpublished data). Further, endophytic inoculants derived from a specific plant are already adapted to conditions within that plant, so such inoculants would face less competitive pressure during population establishment and maintenance.

The potential benefits of endophytic research with regards to phytoremediation are clear. Currently, more research is required to assess the diversity, distribution, and activity of potentially useful endophytic communities capable of contributing to the overall degradation potential of plants used in phytoremediation studies. Thus, this study assessed the hydrocarbon degradation potential of endophytic communities of five different plant species at a long-term phytoremediation field site. The primary objectives were 1) to determine and compare the degradation potential and activity of endophytic communities of different plant species, 2) to compare the degradation potential and activity of endophytic and rhizosphere communities, and 3) to evaluate and compare microbial community structure in the two niches.

5.3 Materials and Methods

5.3.1 Phytoremediation site and sampling

Three year old plants were harvested from a hydrocarbon-contaminated phytoremediation site located in south-eastern Saskatchewan, Canada. Soil at the site, derived from an adjacent decommissioned flare-pit with a 30 year history of chronic releases, was classified as moderately alkaline and saline/sodic. Plots from which plants

were harvested had total hydrocarbon concentrations between 3000 to 3500 mg kg^{-1}, which consisted primarily of F3 and F4 fractions (C16 to C50). The soil had a pH of 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 of 629, 989, and 4900 mg kg^{-1}, respectively (EnviroTest Laboratories, Saskatoon, SK) .

Plots (3m × 3m) were arranged in a randomized complete block design, with 4 treatments replicated four times. The treatments consisted of an unfertilized unplanted control, a fertilized unplanted control, and two fertilized planted treatments. The planted treatments were a Remediation Technologies Development Forum (RTDF; http://www.rtdf.org) standard mix consisting of *Lolium perenne* L. (perennial rye grass), *Medicago sativa* L. (alfalfa var. rambler), and *Festuca rubra* L. (creeping red fescue), and a mix of local species consisting of Agropyron *elongatum* (Host) P. Beauv. (tall wheatgrass), *Elymus angustus* Trin. (Altai wild rye), *Puccinellia nuttalliana* (Schult.) A.S. Hitchc. (Nuttall's salt meadow grass), and *M. sativa.* Perennial rye grass, alfalfa, tall wheatgrass, Altai wild rye, and Nuttal's salt meadow grass plants from well isolated clumps of plants within the original treatment mixes were collected from the site in the fall of 2004. At this point, all plants were flowering and had begun to set seed. Plants with attached roots and root-associated soil were placed on ice and returned to the laboratory for processing and analysis. Control soil samples were taken from adjacent non-planted plots.

5.3.2 Sample processing

Bulk, rhizosphere and endophytic communities were evaluated for all plants. Roots were initially lightly shaken to remove any loose soil, which was discarded. Next, bulk soil was collected by vigorously shaking the roots onto a sterile surface. Roots with attached soil were then suspended in excess sterile deionized water in a sterile Erlenmeyer flask, shaken on a rotary shaker for 0.5 h, and the resulting soil slurry was decanted into a sterile Falcon tube, centrifuged (5 min/7600rpm/4 ^{o}C in Beckman centrifuge) and the supernatant decanted. 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 5 rinses with sterile water. To assess

surface sterility, 100 µL aliquots of the final rinse water were spread on $1/10^{th}$ TSA plates. An additional 1mL 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^oC for 24h while awaiting results from sterility assessments. All other samples were immediately assessed using culturable techniques. Sub-samples were archived at -20^oC for subsequent molecular analysis.

5.3.3 Cultural microbial community analysis

Endophytic extracts were produced by macerating 1g of surface-sterile root from each treatment replicate in 9mL 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. Rhizosphere soil and root extract were serially diluted in MPP and these ten-fold 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^oC for 24 h.

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 0.1g L^{-1} cycloheximide. Plates were incubated at room temperature for 7 d. 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, F2 diesel fuel, and polyaromatic hydrocarbon (PAH) degraders in separate 48-well microtiter plates. For n-hexadecane (Sigma-Aldrich) and diesel fuel (Imperial Oil Limited, Toronto, Canada) 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, 1g 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^{-8} to 10^{-2} serial dilutions (80 µL per well, 5 wells per row, one dilution per row) of soil extracts in MPP buffer. A final control row was inoculated with 80 µL MPP buffer. After two weeks, 200µL of filter-sterilized p-iodonitrotetrazolium violet (3g Ll^{-1}) was added to each well of the n-hexadecane and

diesel fuel 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).

5.3.4 Molecular microbial community structure analysis

Total community DNA was extracted from all samples using a bead-beating protocol previously outlined in 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 100 µL TE (pH 8.0), and purified using PVPP columns. Four grams of bulk and rhizosphere soil and 5-g of surface disinfected root from each treatment replicate were extracted. DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Gibco-BRL) using a ChemiImager ™ 4400 imaging system (Alpha Innotech Corporation, Ontario, Canada).

Community structure and taxonomic diversity were examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments. Total DNA extracts from each treatment were amplified using the primer set U341-GC and U758 (Table 5.1) following the PCR protocol outlined in Phillips et al. (2006). Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1 V 3M sodium acetate and 2.5 V 100% ethanol at -20 ^{o}C overnight, re-suspended in 15 µL of TE buffer (pH 8.0), and quantified on ethidium bromide-stained 1.4 % agarose gels by comparison with a 100 bp ladder (MBI Fermentas).

DGGE was performed on a Bio-Rad DCode system (Bio-Rad, Mississauga, Ont.) essentially as described by Lawrence et al. (2004). For each treatment, 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 16h at 80V and 60^{o}C. The resulting gels was stained with Vistra Green (Amersham Biosciences) in TAE buffer and scanned with a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA).

Table 5.1 Primers used for PCR amplification of bacterial genes and reference strains used for DIG-labelled probe generation

Target gene/ primer	Primer Sequence (5' to 3')	Fragment size (bp)	Reference	Reference strains
PAH initial dioxygenase (*Burkolderia sp.* AF061751)				
phnAc-F	CAATTACGGTGATTTCG GACC	462	Laurie and Lloyd -Jones, 1999	*Burkholderia* sp.
phnAc-R	ACAAAATTCTCTGACGGCGC			
Alkane hydroxylase (*Rhodococcus/Pseudomonas* consensus)				
alk-H1-F	CIGIICACGAIITIGGICACAAGAAGG	549	Chénier et al., 2003	*P. putida* ATCC 29347
alk-H3-R	IGCITGITGATCIIIGTGICGCTGIAG			
Naphthalene dioxygenase (*P. putida* M23914)				
ndoB-F	CACTCATGATAGCCTGATTCCTGCCCCCGGCG	642	Whyte et al., 1996	*P. putida* ATCC 17484
ndoB-R	CCGTCCCACAACACACCC ATGCCGCTGCCG			
Naphthalene inducible dioxygenase (*Mycobacterium* sp. AF249301)				
nidA-F	ACCGCGCACTTCCAATGCCCGTACCACGG	323	Margesin et al., 2003	*Mycobacterium* sp. strain PYR1
nidA-R	AATTGTCGGCGGCTGTCTTCCAGTTCGC			
Catechol dioxygenase (*P. putida* imperfect consensus)				
C2,3O-F	AGGTGCTCGGTTTCTACCTGGCCGA	406	Luz et al., 2004	*P. putida* ATCC 33015
C2,3O-R	ACGGTCATGAATCGTTCGTTGAG			
Universal 16S rRNA (Eubacteria)				
U758	CTACCAGGGTATCTAATCC	417	Rölleke et al., 1996	*P. putida* ATCC 33015
U341†	CCTACGGGAGGCAGCAG		Lee et al., 1993	

†Preceded by a GC clamp for DGGE, GCGGGCGGGGCGGGGGCACGGGGGGCGCGG CGGGCGGGGCGGGGG; I, inosine

DGGE bands of interest were excised from gels using a sterile scalpel and DNA was eluted in sterile deionized water by overnight incubation at 37°C. DNA was re-amplified using primer set U341 and U758 essentially as described by Juck et al. (2000), with the addition of 6.25 µg BSA (Amersham Biosciences) to each 50 µL reaction mixture. Amplification proceeded for 25 cycles of 1 min denaturation at 94 °C, 1 min annealing at 64 °C, 1 min extension at 72 °C, and a final extension of 3 min at 72 °C. Re-amplified DNA was purified with the QIAQuick PCR purification kit (Qiagen, Mississauga, Ont., Canada), prepared for sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) followed by Centre-Sep column purification (Princeton Separations, Adelphia, NJ), and sequenced using theABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA). All procedures followed the manufacturers recommended protocols. Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul et al., 1997).

5.3.5 Molecular microbial catabolic gene analysis

Treatments were analyzed for the presence of specific catabolic genes encoding enzymes involved in hydrocarbon degradation pathways. Total DNA extracts were assessed by PCR amplification using primers specific for the following genes: *alkB*, *ndoB*, *nidA*, *phnAc*, and C2,3O (*xylE*, imperfect consensus). Primer and gene information is listed in Table 5.1. For PCR amplification, approximately 50 ng of DNA was added to a 50 µL final volume reaction mixture containing 0.5 µM of each primer, 200µM of each dNTP, 2mM $MgCl_2$, and 6.25 µg BSA (Amersham Biosciences). Following an initial 5 min denaturing period at 96°C the temperature was brought down to 80°C and 2.5 units rTaq polymerase (Amersham Biosciences) in 5.5 µL Taq polymerase buffer (Amersham Biosciences) were added. Amplification then proceeded for 30 cycles of 1 min denaturing at 94°C, 1 min annealing at 60 (*alkB*) or 65°C (*ndoB, nidA, phnAc,* C2,3O), and 1 min extension at 72°C, with a final extension of 3 min at 72°C. PCR fragments were analyzed on ethidium bromide-stained 1.4% agarose gels.

Hybridization analysis was used to verify results obtained during PCR. PCR fragments were transferred from agarose gels to nylon membranes (Roche Molecular

Biochemicals, Laval, Quebec) by vacuum transfer as previously described (Luz et al., 2004). DNA was UV cross-linked (120,000 μJ cm^{-2}) to membranes using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and the membranes were air-dried and stored at 4^{o}C for up to one week until use. Southern hybridization was performed using a DIG-labelling and detection kit (Roche Molecular Biochemicals, Laval, Quebec) following the manufacturer's protocols for pre-hybridization, hybridization, and detection. Probes were generated by incorporating DIG-dUTP into *alkB*, *ndoB*, *nidA*, *phnAc*, and C2,3O gene fragments by PCR amplification of reference strains with the appropriate primers (Table 5.1). Pre-hybridization, hybridization, and washing were performed under high stringency conditions at 65 ^{o}C. Hybridized probes were detected using chemiluminescence (CDP-star, Roche Molecular Biochemicals, Laval, Quebec) and revealed by exposure to x-ray film.

5.3.6 Hydrocarbon mineralization assay

The hydrocarbon degrading activity of rhizosphere and endophytic microbial populations was assessed using C-14 hydrocarbon mineralization assays. Microcosms were set up and sampled as outlined in Chenier et al. (2003). Serum vials containing either 2 g rhizosphere or control soil or 2 g sterilized soil (gamma irradiated, 2 × 3.0 Mrad with a one week resting interval) plus 0.5 mL root extract were amended with 50,000 dpm (100 mg kg^{-1}) of [1-^{14}C]n-hexadecane, [1-^{14}C]naphthalene, or [9-^{14}C]phenanthrene (specific activities, 12, 6.2, and 8.2 mCi $mmol^{-1}$ respectively; Sigma-Aldrich, Mississauga, Ontario, Canada). A 1.8 mL glass vial with 0.5 mL 1M 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.

5.3.7 Statistical methods

Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, Illinois). Mineralization and culturable bacteria data (log transformed prior to analysis) 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 regression analysis and Kendall Tau correlation. Dendrograms were created by cluster analysis of the non-weighted DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths). Bands identified as representing plant plastid DNA were excluded from the analysis. Mineralization rate constants (*k*) were determined by fitting the data to a first order rate equation (Ostrofsky et al. 2002) using SigmaPlot Enzyme Kinetics 1.3 (Systat Software, Inc.). The coefficient of determination (R^2) was calculated to assess how well the data were described by the first order rate equation.

5.3.8 Accession numbers

All sequences obtained with this study have been deposited in GenBank under accession numbers EU635955-EU635978.

5.4 Results

5.4.1 Heterotrophic microbial communities

Standard plate counts were used to determine the overall heterotrophic populations found in each treatment niche. These populations were relatively consistent regardless of plant species (Table 5.2) and averaged $1.00x10^7$ CFUs g^{-1} dry soil and $1.00x10^8$ CFUs g^{-1} wet root. Control soil CFUs were an order of magnitude lower than those of rhizosphere populations. There was no direct correlation between the magnitude of heterotrophic communities in the rhizosphere and endophytic niches. Although high endophytic heterotrophic populations have been associated with plant pathogens (Hallmann and Berg, 2006), all plants appeared healthy and asymptomatic.

5.4.2 Hydrocarbon degrading potential of microbial communities

A MPN method was used to enumerate microbial populations with the potential to degrade n-hexadecane, diesel fuel, and PAHs. Large hydrocarbon degrader communities were found in the rhizosphere and roots of all plants (Table 5.2). Aliphatic

Table 5.2 Total culturable heterotrophic and hydrocarbon-degrader microbial communities found in rhizosphere and control soil and within plant roots.

Rhizosphere	Total heterotrophs (CFUs) g^{-1} soil***	Hydrocarbon degraders g^{-1} soil***		
		n-hexadecane	Diesel fuel	PAH†
Control soil	6.05 (0.06)c‡	7.85 (0.07)b‡	7.47 (0.11)b	4.23 (0.18)c
Alfalfa	7.19 (0.03)ab	9.35 (0.16)a	9.00 (0.27)a	5.37 (0.24)a
Altai wild rye	7.02 (0.35)ab	8.00 (0.60)ab	7.47 (0.44)b	4.45 (0.11)bc
Nuttal's salt meadow grass	6.86 (0.11)bc	8.59 (0.34)ab	8.23 (0.45)ab	5.04 (0.24)a
Perennial rye grass	7.81 (0.60)a	8.56 (0.61)ab	8.45 (0.26)a	5.02 (0.07)a
Tall wheat grass	7.20 (0.17)ab	8.94 (0.44)ab	8.72 (0.11)a	4.92 (0.17)ab
Endophytic	Total heterotrophs (CFUs) g^{-1} root*	Hydrocarbon degraders g^{-1} root*		
		n-hexadecane	Diesel fuel	PAH
Alfalfa	8.59 (0.15)a	3.51 (0.09)a	3.86 (0.31)b	nd§
Altai wild rye	8.57 (0.02)a	3.91 (0.37)a	4.67 (1.56)ab	1.21 (2.09)
Nuttal's salt meadow grass	7.93(0.04)b	3.62 (0.16)a	5.31 (0.16)a	nd
Perennial rye grass	7.60(1.12)ab	3.46 (0.00)a	4.52 (0.17)b	nd
Tall wheat grass	7.91(0.92)ab	4.01 (0.69)a	4.06 (0.78)ab	1.15 (2.00)

Data are presented means (n = 3) of log transformed values with standard deviation in parentheses.

† Polyaromatic hydrocarbon mixture (phenanthrene, anthracene, fluorene, dibenzothiophene)

‡ Means in a single sub-column followed by a different letter are significantly different at *$p \leq 0.05$ or ***$p \leq 0.001$

§ Not detected

(n-hexadecane) degrader populations were found at consistent levels irrespective of plant species. Rhizosphere soil averaged $1.00x10^8$ n-hexadecane degraders g^{-1} dry soil while plant roots averaged $1.00x10^4$ n-hexadecane degraders g^{-1} wet root. There were significant differences however, in populations capable of degrading the more complex diesel fuel and PAH mixtures (Table 5.2). Alfalfa rhizosphere soil contained the largest absolute number of both these degrader communities ($1.00x10^9$ and $2.34x10^5$ diesel and PAH degraders g^{-1} soil, respectively) while those in AWR were approximately an order of magnitude lower. Large rhizosphere degrader populations however, did not result in large endophytic degrader populations. Instead, alfalfa supported lower diesel fuel degrader populations within its roots than all other plants and no detectable populations of PAH degraders. Endophytic PAH degraders were only detected in AWR and TWG roots, and then only in one replicate (AWR replicate 3 and TWG replicate 2). These two replicates also contained the highest number of diesel fuel degraders (data not shown) and contributed to the high variation observed for endophytic degrader populations in these plants. Although there was no direct correlation in degrader populations between the two niches, there was strong positive correlation between all rhizosphere degrader populations ($r > 0.644$, $p < 0.01$). In contrast, endophytic degrader communities showed no inter-group correlation. Finally, there was no correlation between hydrocarbon degrader communities in either niche and the corresponding heterotrophic populations.

PCR and hybridization analysis revealed that although catabolic genes associated with hydrocarbon degradation were widespread in all niches, there were plant specific differences in the presence of individual catabolic genotypes (Table 5.3). For example, PRG endophytic communities did not have detectable levels of genes for two key enzymes involved in PAH degradation, catechol 2,3 dioxygenase and naphthalene dioxygenase (*ndoB*), within its roots, and PRG-associated bulk and rhizosphere soil had no detectable levels of *ndoB*.

5.4.3 Hydrocarbon degrading activity of microbial communities

Mineralization assays with C-14 labeled hydrocarbons were used to assess hydrocarbon degradation activity in the rhizosphere and endophytic niches (Figure 5.1). Mineralization in abiotic controls, if any, was subtracted from the data prior to analysis.

Table 5.3 PCR and hybridization analysis of field samples for catabolic genes involved in hydrocarbon degradation

Treatment	Alfalfa			AWR				NSMG			PRG			TWG				Control soil
	B	R	E	B	R	E	E3	B	R	E	B	R	E	B	R	E	E2	
alkB	+	+	-	+	+	-	-	+	+	-	+	+	+	+	+	+	-	+
C2,3O	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
nidA	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
phnAc	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ndoB	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-	+

-, no signal; +, positive signal. AWR, Altai wild rye; NSMG, Nuttal's salt meadow grass; PRG, perennial rye grass; TWG, tall wheat grass; B, bulk; R, rhizosphere; E, endophytic; E3, AWR endophytic subsample 3; E2, TWG endophytic subsample 2. *alkB*, alkane monoxygenase; C2,3O, catechol 2,3 dioxygenase; *nidA*, naphthalene inducible dioxygenase; *phnAc*, phenanthrene dioxygenase; *ndoB*, naphthalene dioxygenase

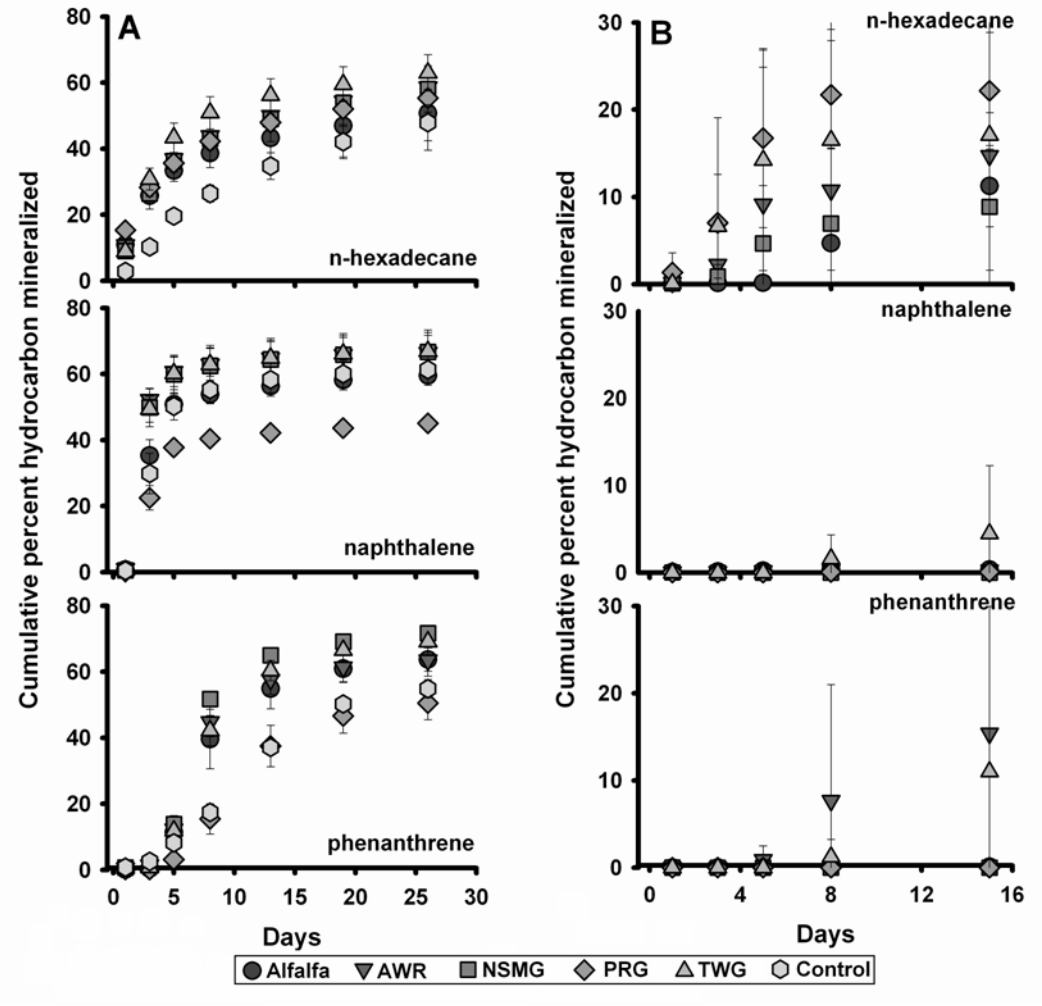

Figure 5.1 Time course data for the mineralization of C14-labelled hydrocarbons by control and rhizosphere soil**.** Error bars represent ± 1 SD and may be obscured by data points. (A) Rhizosphere and control soil microbial communities. (B) Endophytic microbial communities.

Distinct and highly reproducible hydrocarbon utilization patterns were seen in soil microbial communities (Figure 5.1A). These hydrocarbon degradation trends were well described by first-order rate expressions, with R^2 values ranging from 0.834 to 0.986 (Table 5.4). The more complex the hydrocarbon, the more likely it was that mineralization rate and extent would be significantly increased in rhizosphere soil compared to control soils. Lag periods prior to mineralization were observed in the control soil for all hydrocarbons and in rhizosphere soil for naphthalene (approx. 1day) and phenanthrene (approx. 4 days) (Figure 5.1A). Although the final cumulative mineralization of n-hexadecane did not significantly differ between treatments, the kinetic data revealed higher degradation rates in the rhizosphere of alfalfa and PRG than in control soil (Table 5.4). Similarly, although all rhizosphere soils except those of PRG exhibited final cumulative naphthalene mineralization comparable to the control soils (Figure 5.1A and Table 5.4), mineralization rates in rhizosphere soils were significantly greater (Table 5.4). Within the rhizosphere soils, the grasses AWR, NSMG, and TWG had significantly higher naphthalene mineralization rates than alfalfa or PRG. For the third and most complex assessed hydrocarbon, phenanthrene, cumulative mineralization and mineralization rates were significantly higher in all rhizosphere soils except PRG (Table 5.4). There was no correlation between rhizosphere hydrocarbon degradation activity and total MPN degrader populations

There was a strong relationship between the degradation of naphthalene and phenanthrene in rhizosphere soils (cumulative mineralization: $r = 0.818$, $p < 0.001$; mineralization rate constants: $r = 0.816$, $p < 0.001$), but no relationship with n-hexadecane degradation. There were also significant relationships with rhizosphere heterotrophic populations. Total rhizosphere CFUs were negatively correlated with both naphthalene ($r = -0.716$; $p < 0.01$) and phenanthrene ($r = -0.854$; $p < 0.001$) rate constants, but did not impact n-hexadecane rate constants.

In contrast to the high reproducibility of replicate mineralization patterns observed in the rhizosphere soil, there was little reproducibility observed in endophytic mineralization patterns (Figure 5.1B). Instead, each endophytic replicate, derived from 1 g of surface sterilized root, exhibited individual hydrocarbon degradation activity. For

Table 5.4 Average hydrocarbon mineralization potential of control and rhizosphere soil

Treatment	Rhizosphere									Endophytic[§]		
	n-hexadecane			naphthalene			Phenanthrene			n-hexadecane		
	Max[†]	$k(d^{-1})^{*}$	R^2	Max[***]	$k(d^{-1})^{***}$	R^2	Max[***]	$k(d^{-1})^{***}$	R^2	Max	$k(d^{-1})^{*}$	R^2
Alfalfa	50.7 (11.2)a	0.234 (0.139)a	0.838	59.6 (3.0)a	0.273 (0.021)b	0.899	63.7 (3.5)a	0.055 (0.015)a	0.884	11.2 (4.7)a	$3.57E^{-4}$ ($2.42E^{-4}$)b	0.751
AWR	58.6 (1.0)a	0.208 (0.008)ab	0.986	66.5 (5.2)a	0.348 (0.014)a	0.840	63.5 (4.9)a	0.068 (0.005)a	0.875	14.7 (15.6)a	0.052 (0.090)ab	0.702
NSMG	58.1 (7.0)a	0.195 (0.018)ab	0.904	66.7 (6.7)a	0.331 (0.005)a	0.834	71.7 (1.1)a	0.069 (0.007)a	0.881	8.9 (10.8)a	0.027 (0.044)ab	0.901
PRG	55.4 (2.6)a	0.239 (0.016)a	0.945	45.1 (1.0)b	0.245 (0.020)bc	0.910	50.5 (5.0)b	0.011 (0.010)b	0.895	22.2 (6.7)a	0.169 (0.152)ab	0.825
TWG	63.0 (5.5)a	0.224 (0.016)ab	0.949	66.9 (5.7)a	0.326 (0.007)a	0.850	69.2 (1.1)a	0.055 (0.009)a	0.900	17.1 (15.5)a	0.198 (0.042)a	0.834
Control	47.9 (5.5)a	0.081 (0.016) b	0.963	61.3 (4.1)a	0.237 (0.016)c	0.902	54.9 (2.8)b	0.015 (0.004)b	0.954			

AWR, Altai wild rye; NSMG, Nuttal's salt meadow grass; PRG, perennial rye grass; TWG, tall wheat grass. Data are presented as means (n = 3) with standard deviations in parentheses. R^2 values indicate the goodness of fit of the first order rate equation used to determine the mineralization constant, *k*.

† Maximum cumulative percent mineralization of C14-labelled hydrocarbon after 28 days.

‡ Means in a single sub-column followed by a different letter are significantly different at $*p \leq 0.05$ or $***p \leq 0.001$

§ Presented kinetic data for endophytic replicates were calculated for each replicate prior to averaging

comparative purposes, first order rate expressions were calculated for each replicate and then kinetic data were averaged (Table 5.4). Although the endophytic communities of most root sub-samples were able to mineralize n-hexadecane, there were distinct differences in mineralization pattern and extent. Cumulative n-hexadecane degradation by endophytic bacterial communities ranged from 1% in one NSMG replicate to greater than 30% degradation in AWR replicate 3 and TWG replicate 2 (data not shown). PRG endophytic replicates exhibited the greatest average amount and highest consistency of cumulative n-hexadecane mineralization. PRG endophytes, along with TWG endophytes, also exhibited the greatest rate constants and shortest lag period (Table 5.4, Figure 5.1B). Alfalfa endophytes, in contrast, generally exhibited the lowest n-hexadecane degradation activity of all plants. With two exceptions, none of the endophytic populations were able to effectively use naphthalene or phenanthrene. AWR replicate 3 and TWG replicate 2, both of which had positive PAH MPN assays, were the sole endophytic samples able to mineralize these two hydrocarbons (Figure 5.1b). There was no correlation between endophytic hydrocarbon degradation activity and total MPN degrader populations, or with rhizosphere hydrocarbon degradation activity.

5.4.4 Microbial community structure

DGGE analysis of 16S rRNA gene fragments was used to assess changes in the microbial community structure associated with bulk, rhizosphere, and endophytic niches. Due to the heterogeneous nature of endophytic community colonization seen in the culture based analyses, trial DNA extractions and DGGE analyses were used to determine the amount of root tissue required to generate reproducible community patterns. Although stable banding patterns were generated from one to three grams of root material (data not shown), as an added precaution all subsequent endophytic DGGE analyses were performed on DNA extracted from 5 g of root (Seghers et al., 2004). Additionally, the original endophytic replicates that showed aromatic hydrocarbon degradation potential in the culture based analyses (AWR3 and TWG2) were analyzed to ascertain if increases in specific bacteria occurred.

DGGE analysis showed a decreasing order of complexity from bulk to endophytic microbial communities, with approximately 25 distinct bands found in bulk

samples and approximately 10 bands found in endophytic samples (Figure 5.2). Accordingly, when assessed by dendrogram analysis, endophytic communities formed a separate cluster distinct from bulk and rhizoplane communities (Figure 5.3). Within this cluster, banding patterns further revealed that the bacterial communities within the roots of a given plant species were distinct and exhibited no more than 50% similarity with the endophytic communities of other plant species. Banding patterns from AWR and TWG roots clustered with those of the AWR3 and TWG2 replicates at approximately 50% similarity. This low similarity was expected as DNA from these sub-samples was extracted from the original sub-samples used for culture-based analyses, and thus less than 1g of root was available. Additionally, the presence of several distinct bands (bands 6 and 9, Figure 5.2) within these replicates may be seen. Rhizosphere communities of different plant species were also distinct and showed no clustering trends (Figure 5.3). Finally, microbial communities from bulk soils exhibited the highest similarity (≥ 50%), although this was distinct (<30% similarity) from microbial communities found in control soil. It is worth noting the high similarity (>75%) between communities in the bulk and rhizosphere niches of AWR and TWG. It is possible that un-noticed fine roots were co-extracted in the bulk soil of these treatments. However, this finding, combined with the observed difference between bulk and control soil microbial communities, suggests the possibility that the rhizosphere effect extends further into the bulk soil than anticipated.

Many of the bands found in the final DGGE gel were sequenced and their closest identities are presented in Table 5.5. Clear shifts were seen in the relative prevalence of specific bacteria in bulk soil, rhizosphere, and endophytic niches of specific plants (Figure 5.2). For example, a bacterium identified as an *Erwinia* sp. (Band 18, Figure 5.2) decreased in relative prevalence from within alfalfa roots to the alfalfa bulk soil. Other bacteria, such as an unknown alpha proteobacterium (Band 23, Figure 5.2) distinctly present in the bulk soil associated with most plants and the control soil,

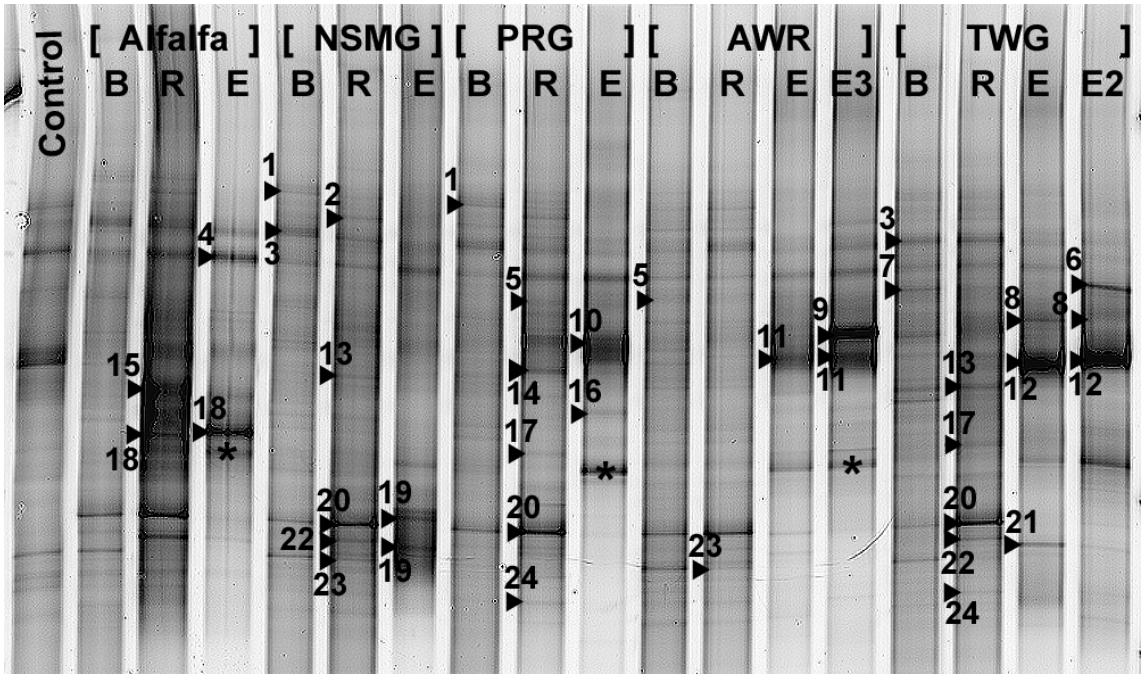

Figure 5.2 Representative DGGE of PCR-amplified 16S rRNA gene fragments from bulk (B), rhizosphere (R), and endophytic (E) microbial communities. Control, non-planted control soil; NSMG, Nuttal's salt meadow grass; PRG, perennial rye grass; AWR, Altai wild rye; TWG, tall wheat grass; E3, AWR endophytic sub-sample, E2, TWG endophytic sub-sample. Arrows indicate sequenced bands whose closest identities are provided in Table 5.5; * indicates plant plastid DNA

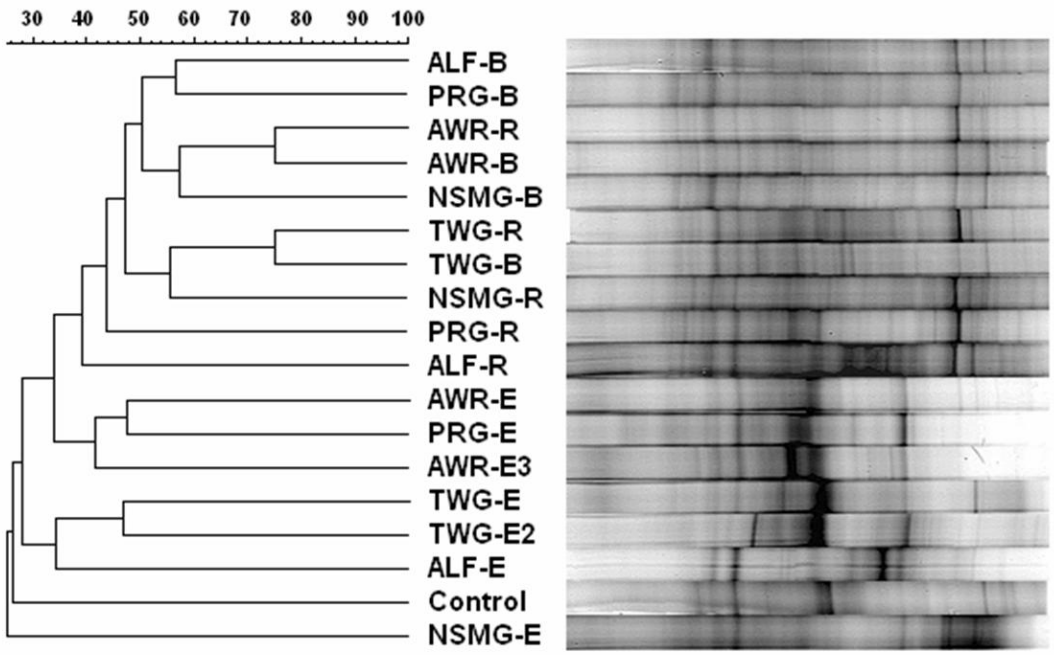

Figure 5.3 Dendrogram analysis of non-weighted DGGE banding patterns from bulk (B), rhizosphere (R), and endophytic (E) microbial communities**.** Bands identified as plastid DNA were excluded. Control, non-planted control soil; NSMG, Nuttal's salt meadow grass; PRG, perennial rye grass; AWR, Altai wild rye; TWG, tall wheat grass; AWR-E3, AWR endophytic sub-sample; TWG-E2, TWG endophytic sub-sample. Distance matrix indicates percent similarity between banding patterns.

Table 5.5 Phylogenetic affiliation of specific bulk, rhizosphere, and endophytic bacteria based on 16S rRNA gene sequences reamplified from DGGE bands.

DGGE band	Phylogenetic group	Closest relative	Percent similarity	Accession number	Notes on original source
1	Alphaproteobacteria	Uncultured alpha proteobacterium clone AKYG624	97	AY922043	Farm soil
2	Bacteroidetes	Uncultured *Bacteroidetes* bacterium clone GASP-MA1S3_A07	94	EF662493	Cropland
3	Alphaproteobacteria	*Sphingomonas* sp. KAR7	99	EF451637	High Arctic permafrost soil
4	Alphaproteobacteria	*Agrobacterium tumefaciens* strain CCBAU 85035	99	EU256460	
5	Alphaproteobacteria	Uncultured *Rhizobium* sp. clone GASP-WC2S2_B12	98	EF074979	Pasture soil
6	Gammaproteobacteria	*Pseudomonas rhodesiae* strain PCL1761	99	DQ313384	
7	Bacteroidetes	*Algoriphagus locisalis* strain MSS-171	99	AY835923	Marine solar saltern
8	Gammaproteobacteria	*Pseudomonas syringae* isolate Lz4W	99	AJ576247	Antarctic soil
9	Alphaproteobacteria	*Brevundimonas bullata* strain HPC 1014	99	AY948226	Nitro- and chloro-aromatic treatment plant
10	Gammaproteobacteria	*Pseudomonas fulgida* strain DSM 14938T	100	AJ492830	Grass phyllosphere
11	Gammaproteobacteria	*Pseudomonas fluorescens* strain ATCC 17397	99	AF094727	
12	Gammaproteobacteria	*Pseudomonas poae* strain DSM 14936T	100	AJ492829	Grass phyllosphere
13	Alphaproteobacteria	*Ochrobactrum anthropi* strain HAMBI2402	97	AF501340	Oil-contaminated rhizosphere of *Galega orientalis*
14	Alphaproteobacteria	*Ochrobactrum* sp. RI54	98	DQ530151	Soybean rhizosphere

Table 5.5 cont.

DGGE band	Phylogenetic group	Closest relative	Percent similarity	Accession number	Notes on original source
15	Alphaproteobacteria	*Rhizobium* sp. Alm-1	98	EF364372	Shrub-legume nodules in gypsum contaminated soils
16	Betaproteobacteria	Uncultured *Duganella* sp. clone GASP-KC1S1_F04	97	EU299108	Restored grassland
17	Alphaproteobacteria	*Paracoccus alcaliphilus* strain mku-E2	98	DQ402044	Elephant yam tubers
18	Gammaproteobacteria	*Erwinia* sp. 14Hsp45d	100	DQ457577	Wild legume nodules
19	Gammaproteobacteria	*Serratia grimesii* strain DSM 30063	100	AJ233430	
20	Actinobacteria	*Arthrobacter* sp. Pi4 strain Pi4	100	AM905949	Contaminated aquifer
21	Gammaproteobacteria	*Pantoea agglomerans* strain A57	100	AF130927	Plant endophytic populations
22	Actinobacteria	*Arthrobacter* sp. 12HD5	96	AB242640	Tomato phyllosphere
23	Alphaproteobacteria	Uncultured alpha proteobacterium clone KCM-B-15	98	AJ581585	Heavy metal contaminated soils
24	Actinobacteria	*Arthrobacter aurescens* TC1	98	CP000474	

decreased in relative prevalence in rhizosphere communities and became undetectable within plant roots. Finally, there were specific bacteria, such as an *Arthrobacter* sp. previously identified in a contaminated aquifer (Band 20, Figure 5.2) that were dominant in all rhizosphere soils, yet decreased in relative prevalence in bulk soil, and became undetectable in most plant roots. The only identified bacteria that were maintained at relatively equal prevalence in all niches of all plants and in the control soil were identified as a *Sphingomonas* sp. and an *Agrobacterium* species (Bands 3 and 4, respectively, Figure 5.2).

5.5 Discussion

In this study we assessed the degradation potential of endophytic and rhizosphere communities from five plants currently growing in a phytoremediation field trial in south-eastern Saskatchewan, Canada. In a soil with the same physico-chemical characteristics and the same contamination and treatment history, we found a strong plant influence on endophytic community diversity and activity. Both community structure, as assessed by DGGE and sequencing analyses (Figure 5.2, Table 5.5), and community function, as assessed by mineralization (Figure 5.1, Table 5.4), MPN (Table 5.2), and catabolic gene analyses (Table 5.3) of hydrocarbon degradation potential show substantial inter-species variation. In general, we found that all assessed plants, Altai wild rye (AWR), Nuttal's salt meadow grass (NSMG), tall wheat grass (TWG), perennial rye grass (PRG), and alfalfa, developed unique endophytic communities which exhibited high hydrocarbon degradation potential.

5.5.1 Endophytic community structure

All plant species developed unique endophytic communities containing at least one dominant endophytic bacterium, which was either not found in other plant species or found at relatively reduced levels (Figure 5.2). The alfalfa endophytic community was dominated by an *Erwinia* species (Band 18), previously identified in wild legume nodules. NSMG was dominated by a *Serratia grimesii* strain (Band 19), PRG by a *Pseudomonas fulgida* strain (Band 10), AWR by a *P. fluorescens* strain (Band 11), and TWG by a *P. poae* strain (Band 12). All of these bacteria had higher relative prevalence within plant roots than in the plant rhizosphere.

Endophytic community structure may be influenced by both soil related and plant specific factors. Endophytic diversity in both wheat (Conn and Franco, 2004a) and canola (Germida et al., 1998) has been shown to be determined by soil type and soil inputs (Seghers et al., 2004). Plants in the current study however, were harvested from within 14 meters of each other, at a site with the same soil contamination and treatment history. While endophytic bacteria likely originated in the soil (Hallmann and Berg, 2006), DGGE analysis of community structure revealed that mature plants developed unique endophytic communities with little similarity to those colonizing the bulk soil (Figure 5.2). Previous research has shown that in the same soil plant species (Germida et al., 1998), plant cultivar (Porteous Moore et al., 2006; Siciliano and Germida, 1999), plant growth stage (Hallmann and Berg, 2006), and even root type within a single plant (Ofek et al., 2007) can all result in differences in endophytic distribution and diversity. As DGGE banding patterns showed no more than 50% similarity between endophytic communities (Figure 5.3), it may be surmised that plant specific factors, including nutrient composition and systemic plant responses (Rosenblueth and Martinez-Ramiro, 2006), were the dominant determining factors.

5.5.2 Hydrocarbon degradation potential of endophytic communities

Although all endophytic microbial communities were found to support equivalent numbers of microorganisms capable of degrading n-hexadecane (Table 5.2), there were significant differences in their in situ degradation activity (Table 5.4), both inter and intra-specifically. A relationship was observed between the detection of *alkB*, a well-studied alkane hydroxylase common in *Pseudomonas* species (van Beilan and Funhoff, 2007), n-hexadecane mineralization activity, and the dominance of *Pseudomonas* species within endophytic communities. The endophytic communities of the two grasses, PRG and TWG, which exhibited the highest rates (0.169 and 0.198 d^{-1} respectively) and shortest lag periods ($\leq$ 2 days, Figure 5.1) for n-hexadecane mineralization, also had detectable levels of *alkB*. DGGE and sequencing analysis revealed that *Pseudomonas* spp. were dominant bacterial endophytes of these grasses (Figure 5.2: bands 6, 8, 10, 12). In contrast, the endophytic communities of alfalfa and NSMG which exhibited the lowest rates (0.0004 and 0.027 d^{-1} respectively) and longest

lag periods (≥3 days, Figure 5.1) for n-hexadecane mineralization, did not have detectable levels of *alkB* and were not dominated by *Pseudomonas* species. Recent studies have shown that some bacteria, including *Caulobacter* sp., *Acinetobacter* sp., and *Sphingomonas* sp., use an alternate or additional enzyme system, cytochrome P450 hydroxylase (van Beilan et al., 2006), to metabolize medium to long length aliphatic hydrocarbons. The *alkB* primers used in the study were specific for the integral membrane hydroxylase from *Pseudomonas* spp. and *Rhodococcus* spp., and if endophytic n-hexadecane mineralization in alfalfa and NSMG used the cytochrome P450 hydroxylase, this would not be detected in PCR/hybridization assay. AWR endophytic communities, which had relatively higher rates of n-hexadecane mineralization than alfalfa and NSMG, also had no detectable levels of *alkB*, but DGGE and sequencing analysis revealed the presence of a *Brevundimonas* sp. belonging to the family Caulobacteraceae. While increased lag periods may reflect a growth period required for sufficient degrader populations to be reached such that measurable mineralization occurs, it may also represent the dominance of degrader populations for which alkane catabolism is not constitutive.

In contrast to the relatively ubiquitous degradation of aliphatic hydrocarbons by endophytic microbial communities, few endophytic communities were able to effectively degrade aromatic hydrocarbons. Although molecular assays indicated that numerous genes involved in aromatic degradation were found in endophytic communities (Table 5.3), only two endophytic subsamples, AWR3 and TWG2, showed any aromatic degradation ability, both in MPN and mineralization assays. AWR3 and TWG2 mineralized 46% and 32% of the available phenanthrene in 2 weeks, and TWG2 mineralized 14% of the available naphthalene in 2 weeks. DGGE and sequence analysis of these specific sub-samples revealed an increased prevalence of specific bacteria which may be responsible for these degradation responses. TWG2 contained relatively higher levels of a *P. rhodesiae* strain (Band 6, Figure 5.2), while AWR 2 contained relatively higher levels of a *Brevundimonas* sp. (Band 9, Figure 5.2). Some strains of *P. rhodesiae* are known to degrade aromatic PAHs (Kahng et al., 2002), and *Brevundimonas* and *Caulobacter* species have also been reported to have PAH degradation capacity (Viñas et al., 2005a). Although it is not currently possible to posit a

definitive connection between the presence of these bacteria and the degradation capacity seen in these root samples, there is a highly suggestive causal relationship.

5.5.3 Endophytic degrader community establishment

The heterogeneous distribution of endophytic hydrocarbon degrading communities seen in this study may be affected by a combination of factors, including colonization pathway, root physiological characteristics, and plant uptake of hydrocarbons. Root colonization by endophytic bacteria is primarily rhizospheric via wounds, lateral root emergence zones and root hairs. Plant specific factors, including exudation patterns, root architecture, root surface structure, apoplastic nutrient composition, and systematic responses, influence bacterial colonization and distribution both pre- and post-colonization (Hallmann et al., 1997; Rosenblueth and Martinez-Ramiro, 2006). Endophytes may become localized at the site of entry or spread to different regions of the plant. Spatially heterogeneous colonization within roots is common and reflects both structural constraints, spatial variation in inorganic ions and nutrients, and, as may be the case with hydrocarbon degrading bacteria, organic contaminants. The development and maintenance of endophytic hydrocarbon degrading communities is dependent on the presence of selection pressures (van der Lelie et al., 2005). If hydrocarbons are not taken up by roots, there is little pressure on the endophytic community to maintain degrader phenotypes.

Plant uptake of hydrocarbons is, in general, governed by the hydrophobicity of the hydrocarbon. Moderately hydrophobic hydrocarbons, with a log K_{ow} between 0.5 and 3.0, are readily taken up by plant roots while highly hydrophobic hydrocarbons, with a log K_{ow} greater than 3.0, bind strongly to lipids found on the root surface (Schnoor et al 1995; Siciliano and Germida, 1998). Significant positive correlations have been reported for PAH adsorption and root epidermis lipid content (Gao and Zhu, 2004). Alfalfa roots, with approximately twice the lipid content of grass roots (10 g kg^{-1} vs. 4.5 g kg^{-1}), have been found to adsorb nearly twice the amount of naphthalene (log K_{ow} 3.36; Schwab et al., 1998). Preferential partitioning to the epidermis may limit hydrocarbon translocation to other root tissues. Consequently, translocation of hydrocarbons within alfalfa roots may be lower than that of grasses, resulting in

decreased selective pressure on endophytic communities, and contributing to the decreased hydrocarbon degradation activity seen in alfalfa endophytic communities (Figure 5.1, Table 5.4). In contrast, a recent study reported on the uptake and translocation of both anthracene and phenanthrene (log k_{ow} 4.45 and 4.46 respectively) in maize and wheat roots (Wild et al., 2005). Uptake occurred primarily in the root hair and branching zones, after which compounds were transported vertically and apoplastically within the cortex to the base of the root. The presence of strong localized hot-spots of aromatic hydrocarbons in the root cortex of specific lateral roots could result in the localized presence of aromatic hydrocarbon degraders, such as that seen in AWR and TWG roots in the current study.

Alkanes such as n-hexadecane have a log K_{ow} of approximately 9.1, a reported solubility of up to 0.0263 mg L^{-1} (Bai et al., 1997), and in an isolated state are unlikely to be effectively taken up by plants. However, biosurfactants released by bacteria and plants can significantly increase that solubility. Bai et al. (1997) found that n-hexadecane solubility increased linearly with increasing concentrations of a rhamnolipid surfactant derived from *P. aeruginosa*. At a rhamnolipid concentration of 500mg L^{-1} solubility of n-hexadecane was 19 mg L^{-1}. Solubility was further increased up to 25-fold when both rhamnolipids and divalent cations such as sodium and magnesium were present (Bai et al., 1998). Given these factors, it is likely that plant uptake of alkanes at the saline-sodic phytoremediation site examined in the current study was relatively high. High uptake of n-hexadecane by plant roots, correlated again with root lipid content, would result in high selective pressure on endophytic bacterial communities, and degrader communities would become well established.

5.5.4 Relationship between rhizosphere and endophytic hydrocarbon degrading communities

This study, like other studies over the past decade (for review see Arthur et al., 2005; Chaudhry et al., 2005) shows that plant species influence hydrocarbon degradation in the rhizosphere. Although it is difficult to make generalized conclusions due to the large numbers of parameters evaluated, several trends were seen in rhizosphere hydrocarbon degradation. In general, rhizospheric soils of the grasses TWG,

AWR, and NSMG exhibited higher naphthalene and phenanthrene and lower n-hexadecane mineralization activity (Table 5.4). PAH degradation rates were impacted by the size of the total heterotrophic populations. Naphthalene and phenanthrene mineralization rates were negatively correlated with rhizospheric heterotrophic populations ($r = -0.716$, $p < 0.01$; $r = -0.854$, $p < 0.001$, respectively). Increased heterotrophic populations, particularly in PRG rhizosphere soil, may have inhibited PAH degradation due to competitive exclusion (Espinosa-Urgel, 2004) or niche competition (Kästner and Mahro, 1996). However, large populations of either heterotrophic or hydrocarbon degraders in the rhizosphere did not impact degradation potential within the plant roots, reaffirming that mature plants develop specific and unique degrader communities distinct from those colonizing the rhizosphere.

Alkane degrader communities represented a much smaller proportion of the total endophytic communities than did comparable degrader communities in the rhizosphere (1:10,000 vs. 10:1 degraders:heterotrophs, respectively, Table 5.2). Siciliano et al. (2001) found that alkane degrading genotypes were increased or comparable in endophytic communities of fescue and clover compared to rhizosphere communities. A direct comparison between studies may not be valid however, due to differences in plant species and age, soil and contaminant types, and assessment methodology. The generally longer lag rates in endophytic n-hexadecane mineralization likely reflects this disparity in degrader population size, particularly considering the small amount of root material (0.05g) ultimately spiked into mineralization microcosms. Specific differences in endophytic lag rates, as previously discussed, likely reflect plant specific differences in endophytic community structure.

Plant specific differences in endophytic degrader communities may, however, have an impact on rhizosphere degradation of hydrocarbons. For example, cumulative mineralization and mineralization rates of aromatic hydrocarbons in PRG rhizosphere soil were significantly lower than most other plants (Figure 5.1, Table 5.4). In contrast, rhizosphere n-hexadecane mineralization rates were equivalent or higher (Figure 5.1, Table 5.4). PRG endophytic communities, while also not showing any aromatic hydrocarbon degradation, exhibited large and consistent cumulative n-hexadecane mineralization with little lag time (Figure 5.1). It is possible that the dominant

Pseudomonas sp. found in PRG roots (band 10, Figure 5.2) and also found in relatively high levels in the PRG rhizosphere, substantially contributed to this increased alkane degradation. While it is not possible in the current study to determine movement of this species from the endophytic community to the rhizosphere (or vice versa), the potential remains for the endophytic community to act as a source for hydrocarbon degrading populations in the rhizosphere.

5.6 Conclusions

This study has shown that diverse plant species growing in weathered-hydrocarbon contaminated soil maintain distinct, heterogeneously distributed endophytic microbial populations, which may impact the ability of plants to promote the degradation of specific types of hydrocarbons. Both endophytic degrader populations and endophytic degrader activity showed substantial inter-species variation, which was independent of that shown by the respective rhizosphere populations. Plant specific factors, likely including root morphology and physiology, had a greater impact on the degradation potential of bacterial endophytes than on rhizosphere bacteria. Further research is required to determine whether the increase observed in specific endophytes was directly related to degradation. To that end, we have isolated a number of the endophytic bacteria identified in this study and are currently assessing their degradation potential.

6.0 ISOLATION AND CHARACTERIZATION OF BACTERIAL ENDOPHYTES INDIGENOUS TO PRAIRIE GRASSES

6.1 Preface

In Chapter 5 we found that diverse plant species growing in weathered-hydrocarbon contaminated soil maintained distinct, heterogeneously distributed endophytic microbial populations. These endophytic communities exhibited substantial hydrocarbon degradation capacity and may have impacted the ability of plants to promote the degradation of specific types of hydrocarbons. In addition, these endophytic degraders represent an important source of potential inoculants, which may enable researchers to enhance the degradation capacity of specific phytoremediation treatments. In the current study, we isolated and identified both specific and randomly chosen bacterial endophytes and assessed their ability to degrade aliphatic and aromatic hydrocarbons.

6.2 Introduction

Beneficial plant-bacterial interactions have been extensively studied with regards to agricultural applications. One of the best understood of all such interactions occurs between members of the plant family Fabaceae and nitrogen fixing bacteria of the family Rhizobiaceae. Non-pathogenic endophytic bacteria spanning a wide range of bacterial phyla have also been found to be associated with most plant species (Hallmann et al., 1997; Hallmann and Berg, 2006). These bacteria may promote plant growth, either directly by enhancing nutrient uptake or indirectly by competing with phyto-pathogenic bacteria. Current research suggests that in contaminated-soil conditions, these bacteria may also help to reduce toxicity and increase contaminant degradation (Newman and Reynolds, 2005; Ryan et al., 2008).

In an elegant study Barac et al. (2004) demonstrated that inoculating *Lupinus luteus* (yellow lupine) with a genetically modified endophytic strain of *Burkholderia*

cepacia increased plant tolerance to toluene and concomitantly decreased evapotranspirative release of toluene. Similarly, Huang et al. (2004, 2005) demonstrated that inoculating *Festuca arundinacea* phytoremediation systems with plant growth promoting *Enterobacter* sp. reduced toxic impacts to both germination and growth, and increased degradation of PAH and total petroleum hydrocarbons by up to 45% compared to non-inoculated plants. Other studies have found that endophytic bacteria indigenous to phytoremediator plants have intrinsic degradative capabilities (Germaine et al., 2006; Moore et al. 2006; Siciliano et al., 2001). These indigenous degraders are of particular interest as they are already acclimatized to the specialized niches found within their host plant and thus face less competition during population establishment and maintenance. Conn and Franco (2004b) compared the colonization patterns of a mixed microbial soil inoculant to that of single endophytic inoculants that had previously been isolated from wheat roots. The researchers found that the single endophytic inoculants had higher survival rates and did not adversely impact indigenous microbial endophytic populations while the mixed inoculant reduced both endophytic diversity and colonization.

Previous research by our group found that common grasses used in petroleum phytoremediation harbour a diverse group of potential hydrocarbon degrading endophytes (Phillips et al., under review; Chapter 5), which may enhance and/or specifically limit the hydrocarbon degradation activity of rhizosphere communities in weathered hydrocarbon contaminated soil. The successful isolation and archiving of these bacteria would represent a significant resource for future phytoremediation studies in Canadian prairie ecosystems. In this study we isolated and identified both specific and randomly chosen endophytes from three grass species. The primary objectives were to assess plant-specific endophytic diversity and hydrocarbon degradation ability.

6.3 Materials and Methods

6.3.1 Isolation of endophytic bacteria

Endophytic bacteria were obtained from samples of a previous study (Chapter 5), which assessed the hydrocarbon degradation potential of endophytic communities of different plant species at a long-term weathered hydrocarbon phytoremediation field

site. Briefly, 3 yr old *Lolium perenne* L. (perennial rye grass), *Agropyron elongatum* (Host) P. Beauv. (tall wheatgrass), and *Elymus angustus* Trin. (Altai wild rye) were harvested in the fall of 2004. At this point, all plants were flowering and had begun to set seed. Plants with attached roots and root-associated soil were placed on ice and returned to the laboratory for processing and analysis. Roots were rinsed to remove adhering soil and were then surface disinfected by sequential washes with 95% ethanol and 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. To assess surface sterility, 100µL aliquots of the final rinse water were spread on $1/10^{th}$ TSA plates. An additional 1mL aliquot of the final wash water, boiled to release DNA, was assessed by PCR using the universal 16S rRNA primers U758 (5'-CTA CCA GGG TAT CTA ATC C; Rölleke et al., 1996) and U341 5'-CCT ACG GGA GGC AGC AG; Lee et al., 1993) according to Phillips et al. (2006). Roots were stored at 4^oC for 24 hours while awaiting results from sterility assessments.

Endophytic extracts were produced by macerating 1g of surface-sterile root in 9ml 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. Isolates were obtained by serially diluting and plating endophytic extracts on 1/10 TSA plates containing 0.1g L^{-1} cycloheximide. Colonized plates were stored at 4^oC until use.

6.3.2 Isolation of *Brevundimonas* spp.

6.3.2.1 Probe design

A probe was designed to target the V7 variable region (*E. coli* numbering 589-630) of *Brevundimonas bullata* strain HPC 1014 (AY948226). The probe was designed using the Picky v2.0 oligomer design program (Chou et al., 2004) and probe quality was assessed using OligoAnalyzer v1.0.2 (Integrated DNA Technologies, Coralville, IA, USA). Initial specificity of the final 29 bp probe (5'-GGT ATG TGG AAC TCC GAG TGT AGA GGT GA-3') was assessed by comparison to the GenBank databases using the BLAST algorithm (Altschul et al., 1997). A final DIG-labelled probe was obtained from Proligo (Boulder, CO, USA).

6.3.2.2 Hybridization analysis

Altai wild rye endophytic extracts were serially diluted in MPP and plated on *Caulobacter*-specific agar plates (CBA: 2 g peptone, 1g yeast extract, 0.2g MgSO4, 10g agar L^{-1} water). Colony lifts were performed on plates containing approximately 40 colonies each. Plated *Brevundimonas dimunata* (U of S Soil Microbiology Culture Collection) isolates were used as a positive control and *Pseudomonas putida* ATCC 17484 was used as a negative control. DNA was transferred from plates to nylon membranes following the manufacturer's colony lift protocol (Roche Molecular Biochemicals, Laval, Quebec). DNA was cross-linked to the membranes by baking at 80^{o}C for 1hr. Hybridization analysis was performed using a DIG detection kit (Roche Molecular Biochemicals, Laval, Quebec) following the manufacturer's protocols for pre-hybridization, hybridization, and detection. Pre-hybridization, hybridization, and washing were performed at 68 ^{o}C. Hybridized probes were detected using a colorimetric assay (NBT/BCIP, Roche Molecular Biochemicals, Laval, Quebec).

6.3.3 Isolate identification

Initial identification of all isolates was done using FAME analysis (Hewlett Packard 5890 Series II GC), according to procedures outlined in 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).

Subsequent identification was via sequencing analysis. Isolates were inoculated into 120 µL LB broth (10g Bacto-tryptone, 5g yeast extract, 10g NaCl L^{-1} water) in a 96 well microtitre plate, grown overnight at 33^{o}C, then transferred to PCR multiwell plates containing 5% (V/V) glycerol. Near full-length 16S rRNA gene fragments were sequenced directly from isolates using the bacterial primers PB36 (5'-AGRGTTTGATCMTGGCTCAG-3'; Saul et al. 2005) and PB38 (5'-GKTACCTTGTTACGACTT-3'; Saul et al. 2005). Sequencing of both strands was performed at the Plant Biotechnology Institute, Saskatoon, SK, Canada using the AB 3730xl capillary electrophoresis DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul et al., 1997).

6.3.4 Hydrocarbon utilization assays

6.3.4.1 MPN assays

Randomly selected isolates were assessed for hydrocarbon degrading potential using a modified MPN protocol. Triplicates of each isolated endophyte were inoculated into separate wells of a 48-well microtiter plates containing hydrocarbons as the sole carbon source. For hexadecane plates, 20 µL of filter-sterilized hydrocarbon was added to wells containing 720 µL Bushnell Haas (BH) mineral salts medium. For PAH plates, 80 µL naphthalene, fluorene, or phenanthrene (Sigma-Aldrich; 5g L^{-1}pentane), was added each well and the pentane was allowed to evaporate off prior to BH addition. Control wells contained hydrocarbon and BH but no bacteria. After two weeks, 200µL of filter-sterilized p-iodonitrotetrazolium violet (3g L^{-1}) was added to each well of the n-hexadecane, 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).

6.3.4.2 Mineralization assays

The hydrocarbon degrading activity of isolated *Brevundimonas* spp. and several other isolates was assessed using ^{14}C-hydrocarbon mineralization assays. Assays were performed in the absence of additional carbon sources (BH media), in the presence of additional carbon sources (sterile soil), and in the presence of additional carbon sources and bacteria (non-sterile soil). In each case, serum vials were amended with 50,000 dpm of [1-^{14}C]n-hexadecane, [1-^{14}C]naphthalene, or [9-^{14}C]phenanthrene (specific activities, 12, 6.2, and 8.2 mCi $mmol^{-1}$ respectively; Sigma-Aldrich, Mississauga, Ontario, Canada). For all assays, bacteria were pre-cultured for 48 hours in CB media, then cultures were centrifuged (5 min at 5000ppm) and extra media was decanted. The bacterial pellet was re-suspended and washed with phosphate buffered saline 4 times, and the final washed pellet was re-suspended in BH media. Total bacterial density was determined by optical density using a Klett-Summerson Photoelectric Colorimeter, using previously determined standard curves.

BH serum vials were spiked with 10^7 bacterial cells mL^{-1} BH and supplemental non-radioactive naphthalene or phenanthrene (20 and 2 mg L^{-1}, respectively). A 20 mL glass test tube with 1M 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). Four replicate serum vials for each bacteria and each hydrocarbon were incubated in the dark on a rotary shaker (100 rpm). Abiotic controls were established for each hydrocarbon treatment.

Soil microcosms were established using soil collected from the above described phytoremediation site. Soil was sieved through a 4.75 mm sieve to ensure homogeneity, stored at 4°C until use, and acclimatized to room temperature for 2 wk prior to microcosm setup. For sterile soil experiments, soil was sterilized by autoclaving (2× with a one wk resting period). Soil microcosms were spiked with 10^7 bacterial cells g^{-1} soil, to a final moisture of 30%, and supplemental non-radioactive hydrocarbon to a final concentration of 100 mg kg^{-1}. A 1.8ml glass vial with 0.5mL 1M KOH was inserted into each microcosm prior to crimp sealing to function as a $^{14}CO_2$ trap and was sampled as described. Abiotic controls for each hydrocarbon treatment were established using autoclaved soil, and bacterial biomass controls were established using killed bacterial cultures.

6.3.5 Statistical methods

Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, Illinois). Mineralization 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.

6.4 Results

6.4.1 Isolation of *Brevundimonas* sp.

While the initial BLAST assessment of the probe indicated a high specificity for *Brevundimonas* and other Caulobacteraceae, only 3 of 20 hybridization-positive isolates

were identified as probable *Brevundimonas* spp. using FAME identification (SIM > 0.575). The remaining bacteria included 10 *Alcaligene*s spp., 3 *Arthrobacter* spp., and 4 *Stenotrophomonas* spp. One of the putative *Brevundimonas* spp. was successfully sequenced and showed 99% similarity (1267 of 1276 bp) to *Brevundimonas alba* CB88 (AJ227785).

6.4.2 Identification of bacterial endophytes

Randomly selected endophytic isolates were identified by FAME and by sequencing of 16S rRNA genes. Identification agreement between the two methods was generally to the genus level (Table 6.1 to 6.3). Perennial rye grass and tall wheat grass endophytic communities were dominated by Pseudomonas species (Table 6.1 and 6.2) while Altai wild rye communities were dominated by other bacterial species (Table 6.3).

6.4.3 Hydrocarbon degrading activity

6.4.3.1 MPN assays

All endophytic isolates listed in Tables 6.1 to 6.3 were assessed by MPN for their ability to degrade hydrocarbons. With the exception of PRG-E4, identified as a *Comamonas acidovorans* (Table 6.1), all isolates were able to use n-hexadecane as a sole carbon source. Only a few isolates however, were able to use the assessed PAHs. Two *Flavobacterium* spp. (TWG-E15. PRG-E20), a *Pseudomonas* sp. (TWG-E18), and a *Kocuria* sp. (TWG-E19) were able to degrade fluorene. Two *Pseudomonas* spp. (PRG-E7 and AWR-E2) were able to degrade phenanthrene. An *Arthrobacter* sp. (TWG-E16) and a *Flavobacterium* sp. (TWG-E15) were able to degrade naphthalene.

6.4.3.2 Mineralization assays

Brevundimonas sp., *Stenotrophomonas maltophilia* (TWG-E13), *Sphingobacterium* sp. (AWR-E7), and a *Burkholderia* sp. (AWR-E6) were assessed by mineralization assays using C-14 labelled hydrocarbons. When these specific isolates were assessed individually and as a consortia in either sterile liquid or sterile solid media (Bushnell Haas and soil, respectively), no significant mineralization of hydrocarbon occurred. When assessed individually in a non-sterile soil, mineralization was not

Table 6.1 Endophytic bacteria isolated from perennial rye grass

Sample ID	FAME Identity	SIM†	16S rRNA Identity	% Similarity	Accession number	Agreement level
PRG-E1	*Janthinobacterium lividum*	0.893	NA			
PRG-E2	*P. syringae*	0.559	*P. putida* ATCC 17494	99	AF094740	Genus
PRG-E3	*Paenibacillus macerans*	0.067	*P. synxantha*	99	AF267911	Genus
PRG-E4	*Comamonas acidovorans*	0.712	NA			
PRG-E5	*P. chlororaphis*	0.860	*P. syringae*	99	AM184090	Genus
PRG-E6	*P. chlororaphis*	0.672	NA			
PRG-E7	*P. syringae*	0.443	NA			
PRG-E8	*Corynebacterium bovis*	0.320	*Arthrobacter aurescens* TC1	100	CP000474	Order
PRG-E9	*P. syringae*	0.596	*Pseudomonas* sp. 4_C7/16_5	99	EF540490	Genus
PRG-E10	*P. chlororaphis*	0.879	*P. frederiksbergensis*	99	AY785733	Genus
PRG-E11	*P. fluorescens*	0.698	*P. trivialis* BIHB 763	99	DQ885947	Genus
PRG-E12	*P. chlororaphis*	0.794	*Pseudomonas* sp. S5-28	99	DQ525593	Genus
PRG-E13	*P. syringae*	0.691	*P. fluorescens* K30-2	99	EU102273	Genus
PRG-E14	*P. syringae*	0.835	*P. trivialis* BIHB 749	99	DQ885949	Genus
PRG-E16	*Clavibacter michiganense*	0.678	NA			
PRG-E17	*F. johnsoniae*	0.564	*Flavobacterium* sp. WB 3.3.42	99	AM177619	Genus
PRG-E18	*P. syringae*	0.510	NA			
PRG-E19	*P. fluorescens*	0.518	*P. trivialis* BIHB 749	99	DQ885949	Genus
PRG-E20	*F. johnsoniae*	0.569	*Flavobacterium* sp. WB 3.3.42	99	AM177619	Genus
PRG-E21	*P. chlororaphis*	0.471	NA			
PRG-E22	*P. chlororaphis*	0.842	NA			
PRG-E23	*P. syringae*	0.511	NA			

†Similarity index

Table 6.2 Endophytic bacteria isolated from tall wheat grass

Sample ID	FAME Identity	SIM†	16S rRNA Identity	% Similarity	Accession number	Agreement level
TWG-E1	*P. putida*	0.759	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E2	*P. putida*	0.726	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E3	*P. putida*	0.228	*Pseudomonas* sp. K94.08	AY456703	100	Genus
TWG-E4	*P. syringae*	0.121	*Pseudomonas* sp. K94.08	AY456703	100	Genus
TWG-E5	*P. putida*	0.488	*P. fluorescens* strain F	DQ146946	100	Genus
TWG-E6	*P. fluorescens*	0.558	*P. fluorescens* strain F	DQ146946	100	Species
TWG-E7	*P. putida*	0.810	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E8	*Bacillus pumilus*	0.371	NA			
TWG-E9	*P. putida*	0.802	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E10	*P. putida*	0.439	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E11	*P. putida*	0.708	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E12	*P. chlororaphis*	0.610	*Pseudomonas* sp. EP25		100	Genus
TWG-E13	*Stenotrophomonas maltophilia*	0.310	*S. maltophilia* LMG 20578	AY040357	100	Species
TWG-E14	*Chromobacterium violaceum*	0.415	NA			
TWG-E15	*F. johnsoniae*	0.457	NA			
TWG-E16	*Arthrobacter ramosus*	0.616	*Arthrobacter* sp. 4_C16_21	EF540487	100	Genus
TWG-E17	*P. fluorescens*	0.377	*P. marginalis* ATCC 10844T	AB021401	100	Genus
TWG-E18	No match		*Pseudomonas* sp. K94.08	AY456703	100	
TWG-E19	*Kocuria kristinae*	0.828	NA			
TWG-E20	*P. fluorescens*	0.207	*P. veronii* INA06	AB056120	99	Genus
TWG-E21	*P. fluorescens*	0.185	*P. marginalis* ATCC 10844T	AB021401	100	Genus
TWG-E22	*S. maltophilia*	0.383	*Stenotrophomonas* sp. DM1-41	DQ109991	100	Species
TWG-E23	*P. putida*	0.714	*P. putida* ATCC 17494	AF094740	99	Species
TWG-E24	*P. putida*	0.805	*P. putida* ATCC 17494		100	Species
TWG-E25	*P. putida*	0.802	*P. putida* AGL 13	EU118779	99	Species

†Similarity index

Table 6.3 Endophytic bacteria isolated from Altai wild rye

Sample ID	FAME Identity	SIM†	16S rRNA Identity	SIM	Accession number	Agreement level
AWR-E1	*Alcaligenes xylosoxydans*	0.642	*A. xylosoxidans* strain F	AJ491845	99	Species
AWR-E2	*P. syringae*	0.708	*Pseudomonas* sp. 1/4_O_3	EF540467	100	Genus
AWR-E3	*A. piechaudii*	0.605	*Burkholderia* sp. 56	AY177370	100	Order
AWR-E4	NA	0.407	*Sphingomonas* sp. A1-13	AY512602	98	
AWR-E5	*S. maltophilia*	0.455	*Burkholderia* sp. 56	AY177370	100	
AWR-E6	*S. maltophilia*	0.479	*Burkholderia* sp. 56	AY177370	100	
AWR-E7	*Sphingobacterium multivorum*	0.751	*Sp. faecium* DSM 11690T	AJ438176	99	Genus
AWR-E8	*Sp. multivorum*	0.589	*A. xylosoxidans* strain F	AJ491845	100	
AWR-E9	*P. putida*	0.864	*Pseudomonas* sp. 1/4_O_3	EF540467	99	Genus
AWR-E10	NA	0.374	*A. xylosoxidans* strain F	AJ491845	100	
AWR-E11	*Sp. multivorum*	0.693	NA			
AWR-E12	*Sp. multivorum*	0.419	*S. maltophilia* ISSDS-429	EF620448	99	
AWR-E13	*Ar. oxydans*	0.559	NA			
AWR-E14	*Ar. ilicis*	0.790	NA			
AWR-E15	*A. xylosoxydans*	0.799	NA			
AWR-E16	*P. chlororaphis*	0.854	NA			
AWR-E17	*S. maltophilia*	0.550	NA			
AWR-E18	*A. piechaudii*	0.725	NA			
AWR-E19	*Ar. globiformis*	0.730	NA			
AWR-E20	*B. pumilus*	0.921	NA			
AWR-E21	*Paenibacillus polymyxa*	0.950	NA			
AWR-E22	*Ar. globiformis*	0.804	NA			
AWR-E23	*B. megaterium*	0.583	NA			
AWR-E24	*K. varians*	0.664	NA			

†Similarity index

significantly different either between endophytes or between endophyte-amended and non-amended soil (data not shown). When assessed as a consortium on non-sterile soil however, mineralization of both naphthalene and phenanthrene was higher than in the control soils (Table 6.4)

6.5 Discussion

Over 70 endophytic bacteria were isolated from mature perennial rye grass (PRG), tall wheat grass (TWG), and Altai wild rye (AWR) plants harvested from a long-term phytoremediation field site (Tables 6.1-6.3). Of these endophytes, all but one exhibited the ability to use the aliphatic hydrocarbon hexadecane. Large populations of alkane degrading bacteria have been found in habitats ranging from polar soils (Whyte et al., 2002) to marine environments (Yakimov et al., 2007). While these degraders are generally found at much lower concentrations in pristine environments (Margesin et al., 2003), plants themselves are high in naturally occurring alkanes such as those of waxes. These plant-derived alkanes, combined with any plant uptake of hydrocarbons at the phytoremediation site, would have favoured the selection and maintenance of endophytic alkane degraders. Plant uptake of hydrocarbons may have also favoured the establishment of PAH degrader endophytes. Although only seven of the assessed isolates (three *Flavobacterium* spp., three *Pseudomonas* spp., an *Arthrobacter* sp. and a *Kocuria* sp.) were able to use the different PAHs in MPN assays, this still represented 10% of the population. In comparison Siciliano et al. (2001) found that less than 1% of the total endophytic heterotrophs isolated from hydrocarbon-exposed *Festuca arundinacea* contained genes involved in PAH degradation. In our previous studies on the endophytic communities of plants at these sites, we found that hexadecane degraders outnumbered PAH degraders at a consistent ratio of 1000:1 as assessed by MPN (Chapters 4 and 5). The relatively high numbers of endophytic PAH degraders found in the current study may be linked to the increased mass of root from which the communities were isolated. The larger sampling size may have fortuitously included greater proportions of root hair and branching zones, areas where both hydrocarbon uptake and endophytic establishment are higher (Wild et al., 2005).

Table 6.4. Cumulative percent hydrocarbon mineralized by control and endophyte amended soil after 2 and 4 weeks.

	Hexadecane		Naphthalene		Phenanthrene	
Treatment	2 weeks	4 weeks	2 weeks*	4 weeks*	2 weeks*	4 weeks*
Control mixture†	35.6 (2.4)	47.1 (1.7)	43.4 (0.2)	48.9 (0.9)	23.2 (0.8)	43.9 (2.7)
Endophytic mixture‡	38.1 (2.1)	48.7 (2.7)	47.2 (0.4)	52.4 (1.3)	31.6 (4.1)	52.9 (3.5)

*Difference significant at $p \leq 0.05$

†Soil amended with killed endophytic bacterial culture

‡ Soil amended with endophytic mixture of *Brevundimonas* sp., *Burkholderia* sp., *Stenotrophomonas* sp., and *Sphingobacterium* sp.

Data are presented as means (n = 3) with SD in parentheses

The hydrocarbon degrading ability of several isolates, *Brevundimonas* sp., *Stenotrophomonas maltophilia*, *Sphingobacterium* sp., and *Burkholderia* sp., was further tested by mineralization assays. The *Brevundimonas* was specifically selected due to its association with enhanced PAH degradation in AWR endophytic communities in a previous study (Chapter 5). The other isolates were selected to represent the broad bacterial phylogeny of known endophytic bacteria with potential hydrocarbon degrading ability. Neither the isolates nor a consortium of the isolates were able to degrade the added hydrocarbons under sterile conditions. As the isolation techniques used in the study were not hydrocarbon-selective, and many culturing steps occurred between MPN and mineralization assays, it may be that the bacteria lost their hydrocarbon degradation capability. However, while the isolates also did not significantly enhance mineralization on non-sterile soil, a consortium of the isolates significantly enhanced both naphthalene and phenanthrene mineralization (Table 6.4). Several recent studies have shown that bacterial consortia exhibit greater PAH degrading capacity than the individual isolates comprising the consortia (Guo et al., 2005; Viñas et al., 2005b). There are numerous mechanisms whereby this might occur, including co-metabolism and elimination of toxic metabolites (Bouchez et al., 1995). Our results suggest that re-inoculating plants with common endophytes might increase the degradative fitness of the rhizosphere community. If specifically selected endophytic inoculants also re-colonize plant roots, the potential immediate increase in degradation would reduce toxic impacts on plants. Further, as previous research has shown that endophytic inoculants transfer their degradative plasmids to other endophytes (Taghavi et al., 2005; Wang et al., 2007), the long-term degradative fitness of the endophytic community could also be enhanced.

Although endophytes from one plant species have been successfully inoculated into another while maintaining degradation potential (Taghavi et al., 2005), our results suggest that for long-term studies inoculants should be tailored to the specific plant species. Mature AWR developed a distinct endophytic community from that of PRG and TWG. The endophytic communities of the latter plants (Tables 6.1 and 6.2) were dominated by *Pseudomonas* species, while those of AWR contained a phylogenetically diverse group of bacteria (Table 6.3). Comparable differences in inherent diversity were observed in a previous study with these plants (Chapter 5). In a two year field site study

which included AWR and TWG (Chapter 4), we confirmed that plant-specific factors become the dominant determinant of endophytic community structure as plants mature, and that differences in this community structure have a significant impact on the overall effectiveness of a given phytoremediation treatment.

6.6 Conclusions

This study has shown that common prairie grasses maintain diverse populations of hydrocarbon-degrading endophytic bacteria that are readily cultured. These potential endophytic inoculants represent an enormous resource for phytoremediation, and could effectively shorten the typically long time-frames required to reach target contaminant levels. Further research is required to select appropriate plant-bacterial pairs and to assess their impact in situ.

7.0 DIVERSITY AND ACTIVITY OF HYDROCARBON DEGRADING BACTERIA IN SOIL AMENDED WITH PLANT ROOT EXUDATES

7.1 Preface

In the previous chapters we have seen that both rhizosphere and endophytic communities are integral to determining the success of a given phytoremediation technique. Although it is generally accepted that plant release of organic compounds via exudation increases the diversity and activity of rhizosphere hydrocarbon degraders, the mechanisms whereby this occurs remains ill-defined. If the success of a given phytoremediation treatment is inextricably linked to the impact that root exudates have on indigenous microbial communities, then a better understanding of that relationship could facilitate site-specific treatment optimization . In the current study this relationship was addressed by examining the impact of plant root exudates on the degradation potential and activity of soil microbial communities indigenous to the phytoremediation sites used in the previous studies.

7.2 Introduction

Phytoremediation, the use of plants and their associated microorganisms to remove or degrade contaminants, is an emerging technology for the remediation of both inorganic and organic contaminants (for review see 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; Phillips et al., submitted for publication). The degradation of hydrocarbons is facilitated through a rhizosphere effect; plants exude organic compounds through their roots, increasing the density, diversity, and activity of potential hydrocarbon degrading microorganisms in the zone surrounding the roots (Anderson et al., 1993; Siciliano and Germida, 1998). The actual mechanisms of this synergistic relationship remain ill-

defined and pose one of the more intriguing challenges in phytoremediation research today. Until the determinant causes of increased degradation are elucidated, the full potential of phytoremediation will not be realized.

Plant species vary greatly in their ability to increase the degradation capacity of soil microbial communities (Liste and Prutz, 2006; Siciliano et al., 2003; Chiapusio et al., 2007; Parrish et al., 2004). Differences in degradation potential are not however, limited to different plant species. Separate phytoremediation studies often find contradictory results with regards to whether a specific plant species promotes hydrocarbon degradation. For example, the results of a recent study by Rezek et al. (2008) show little influence of *Lolium perenne* (perennial rye grass) on PAH degradation, while Binet et al. (2000) reported a significant rhizosphere effect. Moreover, differences in degradation potential are observed even within specific plant cultivars. Wiltse et al. (1998) found that different genotypic clones of *Medicago sativa* cv *Riley* (alfalfa) promoted different amount of crude oil degradation. Some genotypes increased degradation while others decreased degradation relative to an un-planted control. These disparate hydrocarbon-degradation traits of the Riley cultivar clones were later shown to be stably heritable, with increased-degradative phenotypes manifesting even under varied environmental conditions (Schwab et al., 2006). The exudates of related cultivars are known to differentially impact bacterial gene expression. Mark et al. (2005) examined the impact of exudates of two *Beta vulgaris* cultivars (beetroot) on gene expression in *Pseudomonas aeruginosa* PA01. Gene expression patterns promoted by the two exudates differed substantially, with only partial overlap. 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 specific impact of exudates on microbial phylogenetic and metabolic structure. 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 non-specific (da Silva et al., 2006; Miya and Firestone, 2001) and specific (Yoshitomi and Shann, 2001) increases in PAH degrader populations. Other studies however, have implicated exudates in the repression of hydrocarbon degradation potential. Corgie et al. (2004, 2006) observed distance-dependent repressive effects on PAH degradation by exudates released by *L. perenne*. Dibenzo[a,h]anthracene degradation was repressed in all planted soil while phenanthrene degradation was repressed at distances greater than 3mm from the roots. Rentz et al. (2004) found that exudates from a variety of plants repressed the phenanthrene degrading activity of *P. putida* ATCC 17484, in part due to the repressive effects of exudates on *nahG*, 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 pressures and are unlikely to respond to root exudate inputs in a manner comparable to bacterial isolates. However, there is no doubt that the success of a given phytoremediation treatment is inextricably linked to the impact that the complex mixture of root exudates has gene expression in indigenous microbial communities.

This study investigated the impact of root exudates of *M. sativa* and *Elymus angustus*, two common plant species used in phytoremediation studies, on microbial communities indigenous to a weathered hydrocarbon contaminated soil. The primary objectives were (1) to assess differences in exudation patterns between and within plant species in response to hydrocarbon contamination, (2) to examine the impact of these exudates on total microbial community structure, (3) to determine if exudates selectively enrich aromatic and aliphatic hydrocarbon-degrading microorganisms and (4) to evaluate causative relationships between root exudate components and degradation responses.

7.3 Materials and methods

7.3.1 Plant growth and exudate collection

Elymus angustus Trin. (Altai wild rye; AWR) and *Medicago sativa* L. (alfalfa var. rambler) were grown aseptically in modified Leonard jars. Prior to planting seeds were surface disinfected by washing for 1minute with 95% ethanol, followed by a 5 (alfalfa) or 10 (AWR) min wash with 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. Seeds were then incubated on $1/10^{th}$ TSA plates for a minimum of 2 d, and only those seeds which showed no bacterial or fungal growth were used.

Each modified Leonard jar was comprised of a 330 mL narrow-neck bottle, with the bottom cut off, that was inverted into a 950 mL amber wide-mouth bottle (Life Science, Peterborough Ont.) which served as the liquid media reservoir. A fiberglass wick (Specialty Gaskets, Mississauga Ont.) extended from the top of the inverted bottle to the bottom of the media reservoir and was held in place by a washed glass wool plug. Quartz sand was repeatedly washed until the rinse water ran clear, dried at 100^{o}C, autoclaved twice for 1h with a week resting period, and then 200 g of fine sand was layered on top of 200 g of coarse sand around the wick in the inverted bottle. All other dry components were autoclaved for 1 hour and Hoagland's media was autoclaved for 20 min prior to system assembly. Each media reservoir was filled with 600 mL 0.5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), sand was pre-wetted with 100 mL 0.5 strength Hoagland's, and each assembled system was then wrapped in tin-foil and autoclaved again for 30 min. All further manipulations of the assembled systems were performed using the best aseptic techniques in a sterile laminar flow hood. The sand in half of all sterile systems was subsequently spiked with 2 mL of a pyrene/phenanthrene (98% pure; Sigma Aldrich, Mississauga, Ont.) mixture in acetone (40mg mL^{-1}) using a sterile glass syringe, to incremental depths up to 6-cm at 6 injection sites, and acetone was allowed to evaporate off for 24 h, for a final concentration of 200 mg kg^{-1} of each hydrocarbon. All systems were then planted with surface sterilized seeds (PAH-spiked 15 seeds each, non-spiked 10 seeds each). Once each Leonard jar was planted, all jars were covered by a sterile Sunbag with a 0.02 μm gas exchange

filter (Sigma Aldrich, Mississauga, Ont.), which was secured in place around the neck of the media reservoir. Six treatments were established with four replicates each; AWR, alfalfa, and a non-planted control, in PAH-contaminated and non-contaminated soil.

Plants were grown in a growth chamber with a 16h/25 ^{o}C day (1500 µmol m^{-2}) and 8h/15^{o}C night cycle for 6 weeks. As the enclosing Sunbag limited the effects of evapotranspiration, no additional Hoagland's solution was required during this period, which facilitated the maintenance of an aseptic system. Exudates and control eluates were collected by transferring the solid media bottle to a sterile Erlenmeyer flask and rinsing the sand with 300 mL sterile deionized water. Exudate sterility was initially assessed by plating 100 µL aliquots on 1/10th TSA plates. An additional 1mL aliquot, boiled to release DNA was assessed by PCR using the eubacterial primers outlined in the following sections. All exudates were then filtered through a 0.45µm filter and frozen until use. Plant roots and shoots were separated, dried, and weighed.

7.3.2 TOC analysis

A 5 mL subsample 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.

7.3.3 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 GC-MS analysis of derivatized sub-samples. Organic acid and amino acid recovery methods were modified from previously developed protocols (Gillespie, 2003). Percent recovery was determined by extracting 100 µg mL^{-1} solutions of all target compounds using the protocols outlined below**.** 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).

7.3.3.1 Ion exchange membrane preparation and use

Ion exchange membranes were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Cation exchange and anion 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, 200 mL each were transferred to a sterile Erlenmeyer flask containing a single anion and single cation exchange membrane, and samples were 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 6mL vials containing either 5 mL of 0.5 M HCl (anion membranes) or 5 mL of 2.0 M NH_4OH (cation membranes), and shaken in the dark for 8 h. Recovered eluates were stored at 4^{o}C until use.

7.3.3.2 Organic acid quantification

A 1mL aliquot of anion exchange eluate was transferred to a 4 mL vial containing 20 µL of glutaric acid (100µg mL^{-1}, used as an internal standard). The eluate was shaken for 10s 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 was repeated an additional 2 times, with the non-aqueous phase being added to the original DEE residue in the autosampler vial and evaporated under N_2 at room temperature. Organic acids were derivatized to *tert*-butyldimethylsilyl (*t*-bdms) 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 ^{o}C for 45 min. The vial was cooled to room temperature and the *t*-bdms ester derivatives of the organic acids were assessed by GC-MS. Serial dilutions of the following 100 µg mL^{-1} mixed organic acid standard were derivatized as above: acetic, aconitic, azeleic, butyric, caffeic, citric, citraconic, fumaric, hydroxymalonic, itaconic, maleic, malonic malic, methylglutaric, methylmalonic, phthalic, tartaric, suberic, and succinic acids.

7.3.3.3 Amino acid quantification

A 1 mL aliquot of cation exchange eluate was transferred to a 1.8 mL autosampler vial containing 20 µL of norleucine (100 µg mL^{-1}, used as an internal standard) and evaporated to dryness under N_2 at room temperature. Amino acids were derivatized to *t*-bdms 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 ^{o}C for 30 min. GC vials were cooled to room temperature and the amino acid *t*-bdms ester derivatives were assessed by GC-MS. Serial dilutions of the following 100 µg mL^{-1} mixed amino acid standard were derivatized as above: alanine, asparagine, aspartic acid, cysteine, cystine, histidine, hydroxyproline, isoleucine, glutamine, glutamic acid, glycine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

7.3.3.4 Phenolic quantification

Phenolic compounds were extracted from exudates 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 × 5-mL deionized water. Samples (100 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 final eluate was divided in half. One half was transferred into a 10 mL vial and one half successively transferred to a 1.8 mL autosampler vial containing 50 µL of naringenin (100 µg mL^{-1}, used as an internal standard). Both vials were dried under N_2 at room temperature. The 10 mL vials were frozen at -20^{o}C for future use and the phenolics in the 1.8 mL vial 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 for 40 min at 70^{o}C. Vials were allowed to cool and were then analyzed by GC-MS. Serial dilutions of the following 100 µg mL^{-1} standard solution of phenolics were derivatized as above: caffeic acid, catechin, catechol, cinnamic acid, ferulic acid, flavone, gentisic acid, hesperetin,

hydroxyquinone, isovanillic acid, p-coumaric p-salicylic, naringenin, protocatechuic acid, quercetin, salicylic, sinapic, syringic ubelliferone, and vanillic acid.

7.3.3.5 PAH quantification

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 (Busetti et al. 2006) 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 × 4-mL aliquots of hexane:acetone:isopropanol (90:5:5) at a flow rate of 1mL min^{-1}. Toluene (0.5mL) was added to each final eluate, which was then dried under N_2 to a final volume of 0.5mL, 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 to 2.000 µg mL^{-1} calibration curve.

7.3.3.6 GC-MS analysis

Samples were analyzed on a Varian CP-3800 GC equipped with a Saturn 2200 MS detector and an 8400 autosampler. A Varian FactorFour™ column (VF-Xms) with dimensions of 30 m x 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^{o}C. A 1 µL splitless injection volume was delivered with a 5 µL syringe. For organic acid analysis, the initial column oven temperature was maintained at 60^{o}C for 1 min, and then ramped at 5^{o}C min^{-1} to 300^{o}C where it was held for 1 min, for a total run time of 50 min. The MS was operated in the EI ionization mode and was set to scan from *m/z* 45-650 with a scan rate of 0.420 scans s^{-1}. The MS remained off for the initial 10 min of the run to allow solvent and derivatization reagent to elute. Spectral data were acquired using a linked Saturn GC/MS Workstation v. 5.5 (Varian Inc., Walnut Creek, CA). For amino acid analysis the initial column temperature was held at 60^{o}C for 1 min, then ramped at 5^{o}C min^{-1} to 300^{o}C and held for 6 min, for a total run time of 55 min. An open scan was performed

from 7.25 min onwards. For phenolic analysis the initial column temperature was maintained at 80^{o}C for 1min, then ramped at a rate of 20 ^{o}C min^{-1} to 250^{o}C and held for 1min, then ramped at a rate of 6 ^{o}C min^{-1} to 300^{o}C and held for 2 min, and then ramped at a rate of 20 ^{o}C min^{-1} to 320^{o}C and held for 4 min, for a total run time of 25.83 min. An open scan was performed from 3.5 min onwards.

For PAH analysis, the initial column oven temperature was maintained at 100^{o}C for 1 min and then ramped at 10^{o}C min^{-1} to 320^{o}C, where it was held for 17 min, for a total run time of 40 min. A targeted *m/z* scan between the range of *m/z* 60 to 650 and a scan rate of 0.50 scans s^{-1} was performed from 3 min onward.

7.3.4 Isolation and identification of contaminating bacteria

Any contaminating bacteria found in the growth systems post-harvest were isolated and assessed by FAME analysis (Hewlett Packard 5890 Series II GC), according to procedures outlined in 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).

7.3.5 Hydrocarbon mineralization potential

C-14 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, EC 5.8 dS m^{-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. The total hydrocarbon concentration was 3700 mg kg^{-1}, and consisted of F3 and F4 fractions (C16 to C50). Soil was sieved through a 4.75 mm sieve to ensure homogeneity, stored at 4^{o}C 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^{o}C for 24 h. Microcosms were set up and sampled as outlined in Chenier et al. (2003).

Exudates/eluates were added to serum vials containing 6g soil at a rate of 10.0 µg TOC/g soil, and final moisture was adjusted to 30% water content using sterile deionized water. Serum vials were amended with 50,000 dpm (100 mg kg^{-1}) of [1-^{14}C]n-

hexadecane, [1-^{14}C]naphthalene, or [9-^{14}C]phenanthrene (specific activities, 12, 6.2, and 8.2 mCi mmol^{-1} respectively; Sigma-Aldrich, Mississauga, Ont.). A 1.8 mL glass vial with 0.5 mL 1M 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 autoclaved soil (2 × 1-h with a one week resting interval). Non-radioactive replicate microcosms were established for all exudate replicates with all assessed hydrocarbons for use in subsequent molecular analyses.

7.3.6 Microbial community assessment

7.3.6.1 Microbial community DNA extraction

Total community DNA was extracted from all soil samples using a bead-beating protocol previously outlined in 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 100 µL TE (pH 8.0), and purified using PVPP columns. Exudate amended soil (0.50 ± 0.01g) from each non-radioactive replicate mineralization microcosm was extracted. Following PVPP purification of 50 µL total DNA extract all volumes were standardized. Final DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen) and by spectrophotometer evaluation.

7.3.6.2 DGGE analysis of community structure

Community structure was examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments. Total DNA extracts from each treatment replicate were amplified using the universal eubacterial 16S rRNA gene primers listed in Table 7.1, using the PCR protocol outlined in Phillips et al. (2006). Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1 V 3 M sodium acetate and 2.5 V 100% ethanol at -20 ^{o}C overnight, re-suspended in 15 µL of TE buffer (pH 8.0), and quantified on ethidium bromide-

Table 7.1 Primers and amplification conditions used for PCR and Q-PCR amplification

Target gene Primer sequence†	T_a‡ (^oC)	Primer (μM)	Expected product size (bp)	Reference	Control strain
Naphthalene dioxygenase (*nahAc*)					
F: 5'-CAA AAR CAC CTG ATT YAT GG	47	0.3	377	Baldwin et al., 2003	*P. putida*
R: 5'-AYR CGR GSG ACT TCT TTC AA					ATCC 17484
Alkane monoxygenase (*alkB*)					
F: 5'-AAC TAC ATC GAG CAC TAC GG	50	0.5	100	Powell et al., 2006	*P. putida*
R: 5'-TGA AGA TGT GGT TGC TGT TCC					ATCC 29347
Catechol 2,3 dioxygenase (C2,3O)					
F: 5'- AGG TGC TCG GTT TCT ACC TGG CCGA	65	0.3	406	Luz et al.,	*P. putida*
R: 5'- ACG GTC ATG AAT CGT TCG TTG AG				2004	ATCC 33015
Universal eubacterial 16S rRNA (16S)					
F: 5'-CTA CCA GGG TAT CTA ATC C	55	0.15	450	Rölleke et al., 1996	*P. putida*
R: 5'-CCT ACG GGA GGC AGC AG§				Lee et al., 1993	ATCC 17484

†Forward (F) and reverse (R) primers are indicated.

‡T_a, annealing temperature used during real-time PCR.

§Preceded by a GC clamp for DGGE: GCGGGCGGGGCGGGGGCACGGGGGGCGCGG CGGGCGGGGCGGGGG

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 treatment, 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 16h at 80V and 60^{o}C. The resulting gel was 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).

7.3.6.3 Quantitative PCR assessment

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, catechol 2,3 dioxygenase (C2,3O), naphthalene dioxygenase (*nahAc*), and alkane monoxygenase (*alkB*), were assessed. 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 manufacturers protocol incorporating the primer concentration listed in Table 7.1. After the initial 15 min denaturing period, PCR amplification proceeded for 38 cycles of 30s denaturing at 94^{o}C, 40s annealing at the appropriate temperature (Table 7.1), 1 min extension at 72 ^{o}C, and a data collection step of 45 s at 80 ^{o}C. A final melt curve analysis from 5 ^{o}C below amplification temperature to 95 ^{o}C concluded the cycling program. Absolute quantification was performed by comparison with standard curves. Ten fold DNA standards ranging from 10^2 to 10^6 gene copy numbers were prepared from serial dilutions of DNA extracts from positive control strains (Table 7.1). 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. 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).

7.3.6.4 Microbial community diversity

The general microbial community composition found in the study soil was assessed by cloning the 16S rRNA products obtained above (section 7.3.6.1) into the pCR2.1 TOPO vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA), according to the manufacturers protocol. Ninety six randomly chosen clones were inoculated into 120 µL LB broth (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl L^{-1} water) in a 96 well microtitre plate, grown overnight at 33^{o}C, then transferred to PCR multiwell plates containing 5% (V/V) glycerol. 16S rRNA gene fragments (approx. 450 bp) were sequenced directly from the clones using the TOPO TA kit M13 forward (5´-GTAAAACGACGGCCAG) and reverse (5´-CAGGAAACAGCTATGAC) primers. Sequencing of both strands was performed at the Plant Biotechnology Institute, Saskatoon, SK, Canada using the AB 3730xl capillary electrophoresis DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul et al., 1997). A phylogenetic tree was constructed using the ClustalW2 multiple sequence alignment software of the European Bioinformatics Institute (Lopez and Lloyd, 1997).

7.3.7 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 weighted (Muylaert et al. 2002) DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths).

7.4 Results

7.4.1 Modified Leonard jar performance

Alfalfa and AWR grew well in both non-amended and PAH (-pp) amended sand. At the end of the study all plants appeared healthy, with no visible chlorosis or other signs of stress. As there were differences in the number of seeds which germinated in each system, the root:shoot ratio was used to determine if PAHs had a significant negative impact on plant growth. The alfalfa and alfalfa-pp treatments had average root:shoot ratios of 0.30 and 0.38, respectively, and were not significantly different, indicating that growth was not significantly impacted by the presence of the PAHs. AWR and AWR-pp had average root:shoot ratios of 0.52 and 0.28 respectively ($p < 0.1$), indicating some stress due to the presence of PAHs.

All AWR and control systems were free of contamination at the end of the study, as assessed by TSA plate counts and PCR analysis. Most alfalfa systems however, harboured bacteria at a concentration of approximately 10^4 CFU mL^{-1}. Two non-PAH systems were contaminated with *Microbacterium esteraromaticum* (SIM ≥ 0.623) and all PAH systems were contaminated with *Pantoea agglomerans* (SIM ≥ 0.391). Both of these bacteria have previously been recovered from beneath the seed coat of surface-sterilized alfalfa seeds (Moline and Kulik, 1997).

7.4.2 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 AWR replicates contained 2.9, 1.9, 8.0, and 1.1 ppb phenanthrene, respectively.

7.4.3 Plant exudate qualification and quantification

Organic acids formed the major component of identified compounds in the root exudates, with concentrations of individual organic acids ranging from less than 1 $\mu g\ g^{-1}$ root to greater than 300 $\mu g\ g^{-1}$ root (Figure 7.1). Although the very high variability meant that differences between plant exudates were often not significant, several trends were observed. AWR-pp generally produced higher concentrations of organic acids than AWR, while higher concentrations of organic acids were generally found in alfalfa

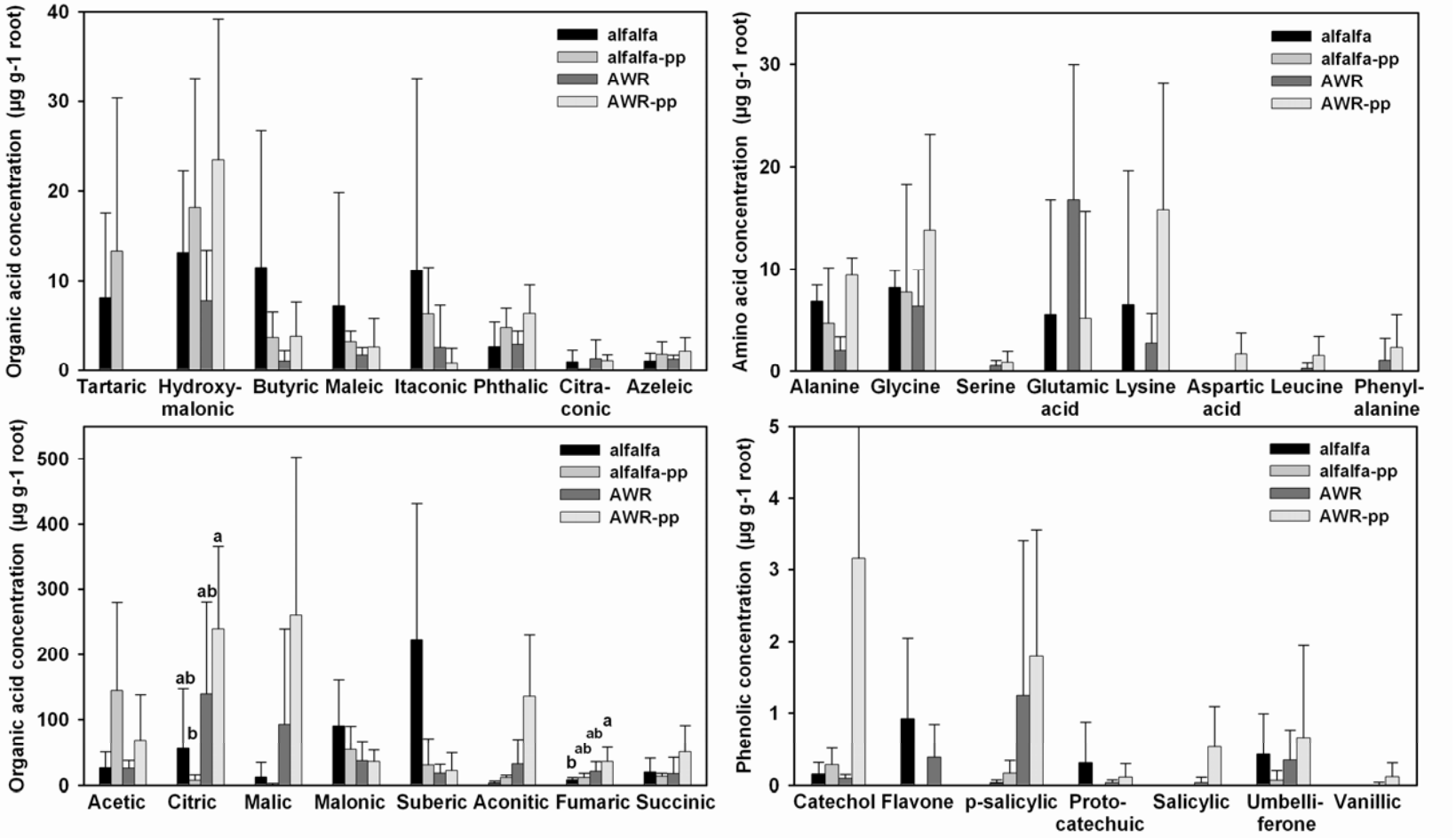

Figure 7.1 Organic acid, amino acid, and phenolic content of alfalfa and Altai wild rye (AWR) root exudates**.** The tag "-pp" indicates that the exudates were collected under phenanthrene/pyrene stimulated conditions. Data are presented as means (n = 4) with error bars representing ± 1 SD. Differences are significant at $p \leq 0.05$ where indicated.

compared to alfalfa-pp. Higher levels of those organic acids which are intermediates of the citric acid cycle were detected in AWR compared to alfalfa treatments. These substrates, which include citric, cis-aconitic, succinic, fumaric, and malic acids, were all inter-correlated with correlation coefficients ranging from 0.493 ($p \leq 0.05$) to 0.788 ($p \leq 0.001$). Amino acid and phenolic compounds were detected at relatively low concentrations with high variability in all treatments (Figure 7.1). As with organic acids, higher levels of both groups were detected in AWR-pp versus AWR exudates, while higher levels of both groups were generally detected in alfalfa versus alfalfa-pp exudates.

The fatty acids palmitic and stearic were also identified, though not quantified, in exudates from all plants.

7.4.4 General soil microbial community diversity

In order to obtain a general picture of the bacterial species distribution in the soil, 16S rRNA gene fragments were sequenced and identified via Topoclone analysis. Figure 7.2 shows a phylogenetically grouped summary of bacteria identified in the soil, at a frequency of one to 9 replicates. The closest match for each sequence group is given along with the associated NCBI accession number. Approximately 80% of the matches on the 450 bp sequence fragments were above 97% (data not shown). The largest proportion of identified bacteria (52%) belonged to γ-Proteobacteria, and of these approximately 35% have high sequence homology with Pseudomonadales (uncultured clones PYR10dll, Pitesti, and 91-7, Figure 7.2). Most bacteria were previously identified from environmental samples, with 34% from hydrocarbon impacted sediments and soils, 16% from general agricultural and forest soils, 33% from marine/salt impacted environments, including a number from oil impacted coastal sediments, and 15% from other environments including mine tailings and metal contaminated aquifers.

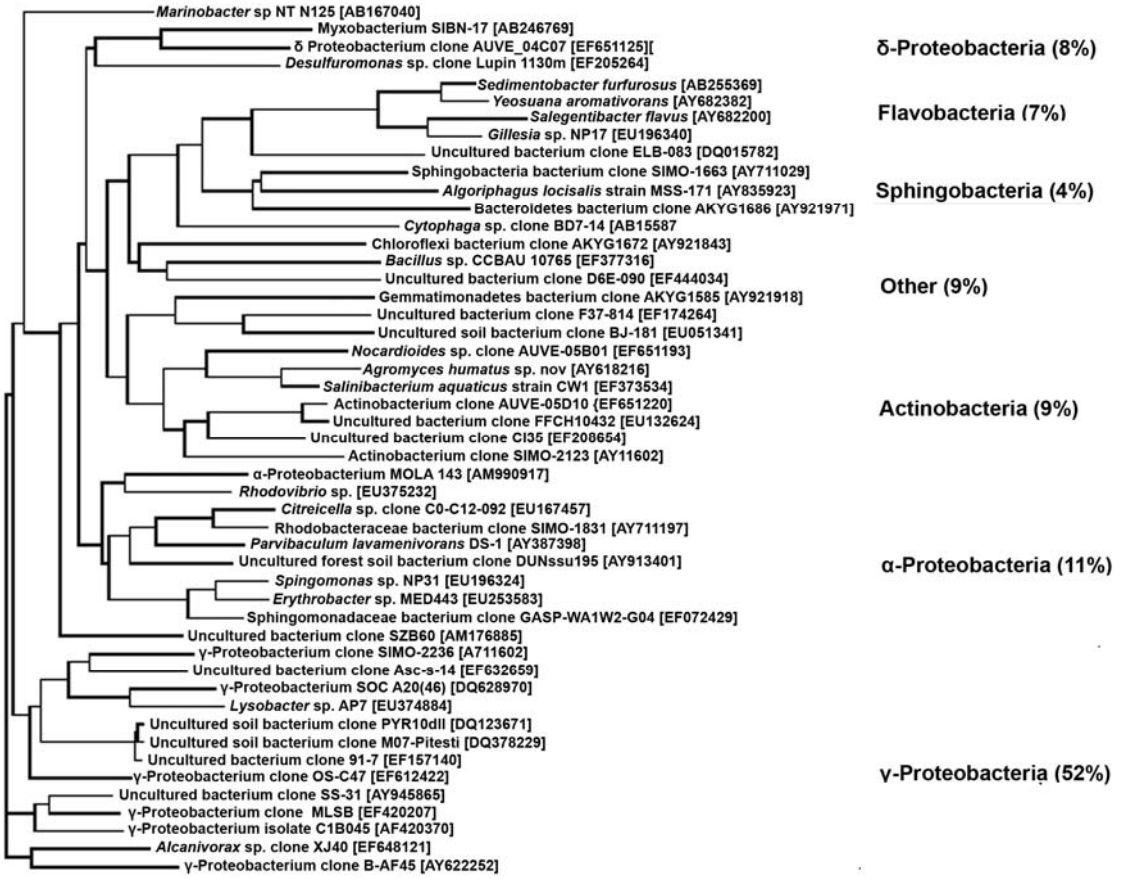

Figure 7.2 Phylogenetic tree representing 98 randomly chosen bacterial isolates (Topoclone isolates) found in the study soil.

7.4.5 Impact of exudates on hydrocarbon mineralization potential

Mineralization assays with C-14 labeled hydrocarbons were used to assess the impact of exudates on hydrocarbon degradation activity in a weathered-hydrocarbon contaminated soil. $^{14}CO_2$ evolution in abiotic controls, if any, was subtracted from the data prior to analysis. Three very distinct trends were observed in the mineralization assays. First, the addition of plant root exudates initially repressed the mineralization of all hydrocarbons compared to that observed in control eluate-amended microcosms (Figure 7.3). Second, exudates or control eluates derived from PAH-amended growth systems in general stimulated greater hydrocarbon mineralization than those from non-PAH growth systems. The more complex the assessed hydrocarbon, the more likely it was that a non-specific priming effect would occur. Third, exudates derived from PAH-stimulated AWR plants (AWR-pp) were generally more effective at stimulating hydrocarbon mineralization than other plant root exudates.

In phenanthrene microcosms, although differences in cumulative mineralization in the first week were minimal, significant differences between treatments evolved over time. The lag times (defined as the time to 5% measurable mineralization) of alfalfa and AWR exudate amended treatments were significantly higher, maximum mineralization rates were lower, and cumulative mineralization was significantly less than that of control-eluate amended treatments (Table 7.2). AWR-pp exudates however, while still promoting significantly lower cumulative mineralization than control eluates, exhibited fewer repressive effects. Although only trace amounts of phenanthrene were detected in one control-pp replicate, a definite priming effect was observed for all mineralization parameters (Figure 7.3, Table 7.2). In naphthalene microcosms there were large initial differences in mineralization potential between control and planted treatments, and between PAH and non-PAH treatments (Figure 7.3, Table 7.2). Contrary to phenanthrene mineralization patterns, these differences became less pronounced over time. After 4 weeks of sampling there was no significant difference in cumulative mineralization between control and alfalfa-pp and AWR-pp treatments, or between individual PAH and non-PAH treatments.

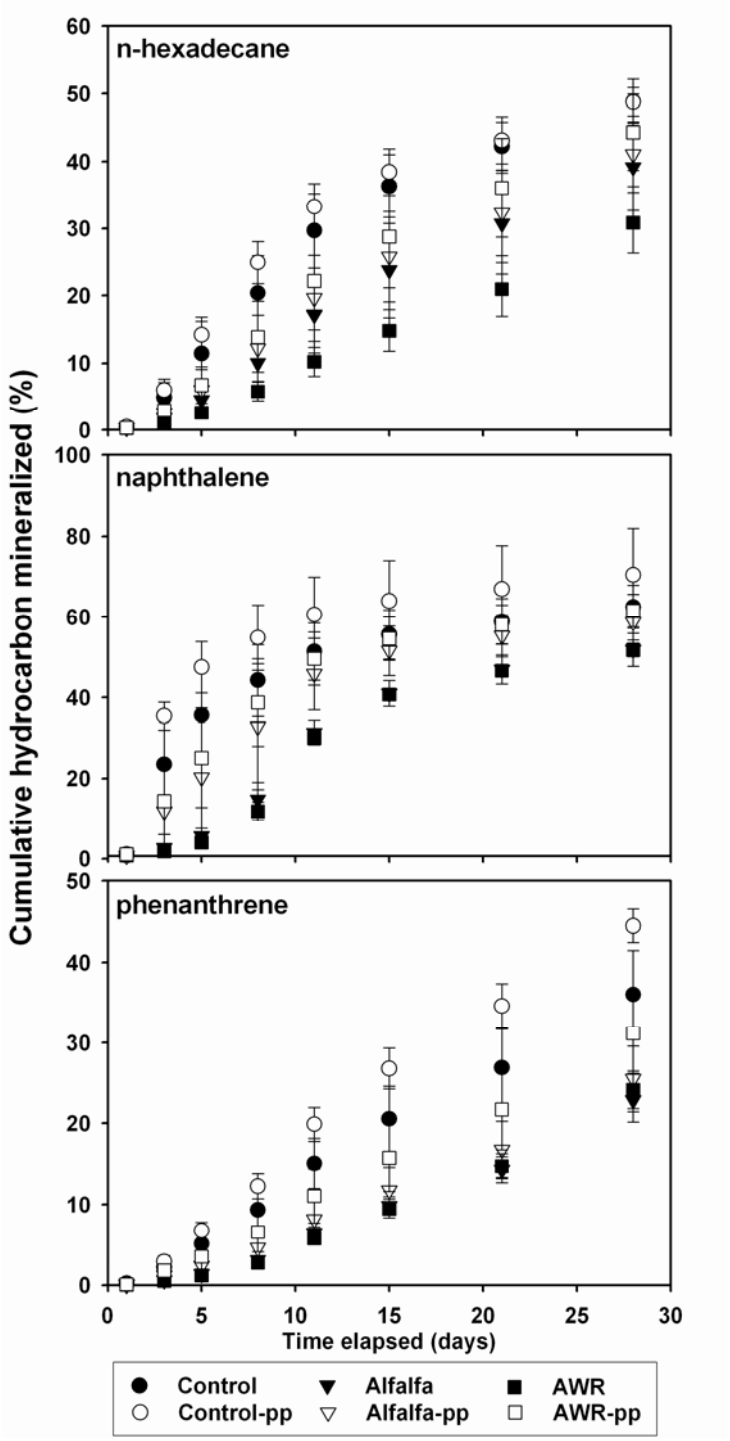

Figure 7.3 Cumulative percent hydrocarbon mineralized by soil microbial communities amended with plant exudates or control eluates. Treatments included alfalfa and Altai wild rye (AWR) exudates and non-planted control eluates. The tag "-pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions. Data are presented as means (n = 4) with error bars representing ± 1 SD, which may be obscured by data points.

Table 7.2 Hydrocarbon mineralization parameters and total gene copy number of soil mineralization microcosms amended with plant bulk exudates or control eluates.

Mineralization Microcosms	Lag (days)	Maximum rate (%$^{14}CO_2$ day^{-1})	Cumulative % mineralized	Gene copy number g^{-1} dry soil (log 10 scale)†		
Phenanthrene	Lag ***§	Rate***	Cumulative***	16S rRNA	C2,3O***	*nahAc****
Control	5.3 (1.0) cd	1.8 (0.1) b	36.0 (5.4) ab	7.57 (0.11)a	7.78 (0.14)a	6.59 (0.12)a
Control-pp‡	4.1 (0.5) d	2.3 (0.2) a	44.5 (2.0) a	7.52 (0.19)a	7.84 (0.17)a	6.63 (0.22)a
Alfalfa	9.9 (0.5) a	1.2 (0.2) b	22.9 (2.7) c	7.58 (0.09)a	7.03 (0.26)c	5.63 (0.26)bc
Alfalfa-pp	8.5 (2.0) ab	1.2 (0.1) b	25.5 (4.1) c	7.54 (0.09)a	7.29 (0.45)abc	5.84 (0.50)bc
AWR	10.3 (0.6) a	1.3 (0.2) b	24.2 (2.3) c	7.65 (0.04)a	7.09 (0.09)bc	5.59 (0.16)c
AWR-pp	6.9 (1.7) bc	1.5 (0.3) ab	31.2 (4.9) bc	7.69 (0.07)a	7.64 (0.32)ab	6.29 (0.03)ab
Naphthalene	Lag ***	Rate***	Cumulative***	16S rRNA	C2,3O***	*nahAc****
Control	1.6 (0.3) b	11.6 (4.9) ab	62.5 (5.4) ab	7.37 (0.20)a	7.75 (0.32)a	6.57 (0.39)ab
Control-pp	1.5 (0.0) b	17.2 (1.8) a	70.5 (11.5) a	7.42 (0.10)a	7.88 (0.11)a	6.74 (0.16)a
Alfalfa	4.9 (0.9) a	5.6 (1.2) b	51.8 (4.1) b	7.27 (0.23)a	6.92 (0.33)b	5.41 (0.41)cd
Alfalfa-pp	2.8 (1.6) ab	7.9 (3.5) b	58.8 (4.5) ab	7.37 (0.07)a	7.26 (0.35)ab	5.91 (0.42)bcd
AWR	5.4 (0.6) a	6.1 (0.4) b	51.8 (1.7) b	7.22 (0.10)a	6.80 (0.04)b	5.30 (0.06)d
AWR-pp	2.0 (0.4) b	7.5 (3.3) b	61.6 (4.1) ab	7.38 (0.15)a	7.27 (0.46)ab	6.11 (0.38)abc
Hexadecane	Lag ***	Rate***	Cumulative***	16S rRNA	*alkB*	
Control	3.3 (1.2) bc	3.1 (0.4) ab	48.8 (2.1) a	7.60 (0.03)a	5.95 (0.05)a	
Control-pp	2.9 (0.3) c	3.7 (0.3) a	48.8 (3.4) a	7.65 (0.20)a	6.06 (0.46)a	
Alfalfa	5.6 (1.3) ab	2.0 (0.5) bcd	39.2 (6.5) ab	7.69 (0.17)a	5.93 (0.24)a	
Alfalfa-pp	4.9 (1.4) bc	2.2 (0.6) bcd	41.1 (4.8) ab	7.47 (0.13)a	5.63 (0.33)a	
AWR	7.4 (1.0) a	1.2 (0.2) d	30.8 (4.5) b	7.63 (0.13)a	5.87 (0.20)a	
AWR-pp	4.4 (1.1) bc	2.5 (0.7) bc	44.3 (5.6) a	7.57 (0.18)a	6.07 (0.20)a	

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

† 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

‡The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

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

There was no general priming effect associated with eluates from PAH systems in n-hexadecane mineralization assays, but there were significant differences between treatments. As with the PAH mineralization assays, plant exudates had a repressive effect on cumulative hydrocarbon mineralization and on associated mineralization parameters (Figure 7.3, Table 7.2). This repression was most pronounced in AWR microcosms, which exhibited mineralization trends significantly lower than AWR-pp or control microcosms (Table 7.2).

Strong correlations were found between all mineralization parameters, with both lag time and mineralization rate being strongly associated with cumulative mineralization for all hydrocarbons (Table 7.3). Stepwise multiple regression revealed that specific root exudate components, including the organic acids malonate, acetate and fumarate, and the amino acid alanine, significantly influenced mineralization parameters (Table 7.4). In particular, malonate exerted a significant repressive effect on mineralization, increasing lag times for all hydrocarbons and decreasing rates and extent of hexadecane mineralization.

7.4.6 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, catechol 2,3 dioxygenase, naphthalene dioxygenase, and alkane monoxygenase, 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 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 microbial community structure (Figure 7.4). DGGE banding patterns and the relative abundance of individual bands were highly similar in all treatments. The amount of 16S rRNA gene was also consistent between all treatments (Table 7.2), indicating

Table 7.3 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 number†			Mineralization‡	
Phenanthrene	16S	C2,30	*nahAc*	Rate	Cumulative
Lag‡	0.002	-0.810***	-0.866***	-0.807***	-0.951***
Rate	0.073	0.619***	0.711***		0.897***
Cumulative	-0.018	0.759***	0.809***		
Naphthalene	16S	C2,30	*nahAc*	Rate	Cumulative
Lag	-0.525**	-0.916***	-0.952***	-0.636***	-0.873***
Rate	0.349	0.671***	0.653***		0.793***
Cumulative	0.457*	0.806***	0.839***		
Hexadecane	16S	*alkB*		Rate	Cumulative
Lag	-0.118	-0.317		-0.952***	-0.937***
Rate	0.135	0.356			0.915***
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 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

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

Table 7.4 Linear multiple regression for hydrocarbon degradation parameters (mineralization and gene copy number) as a function of root exudate components

Hydrocarbon treatment		
Phenanthrene	Constant ± standardized β-coefficients	R^2
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(azeleic) +0.683 (aconitate) –0.459(succinate)	0.755
C2,30***	7.269 –0.734(malonate) +0.343(alanine) +0.329(acetate)	0.750
*nahAc****	5.758 –0.713(malonate) +0.474(alanine) +0.268(acetate)	0.785
Naphthalene	Constant ± standardized β-coefficients	R^2
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
C2,30**	7.225 –0.615(malonate) +0.370(acetate)	0.514
*nahAc***	6.077 –0.659(malonate)	0.434
Hexadecane	Constant ± standardized β-coefficients	R^2
Lag***	4.777 +0.586(malonate) –0.364(alanine) +0.438(fumarate)	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	
alkB	NS	

Regression equation significant at: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$
NS: No significant associations found
†Lag, days to 5% mineralization; rate, maximum rate of mineralization (%$^{14}CO_2$ day^{-1}); cumulative, cumulative % mineralized
‡ Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

that none of the eluates/exudates had a significant impact on overall bacterial population densities.

Although population densities did not significantly differ between treatments, there were significant differences in copy numbers of both catabolic genes involved in PAH degradation. Both C2,3O and *nahAc* gene copy numbers were increased in those microcosms which exhibited increased PAH mineralization (Table 7.2). The abundance of these genes was strongly positively correlated with both mineralization rate and cumulative mineralization and strongly negatively correlated with lag times (Table 7.3). In naphthalene mineralization microcosms, there were also significant correlations between these catabolic genes and 16S rRNA genes (Table 7.5) and between 16S rRNA gene abundance and mineralization parameters (Table 7.3). No comparable correlation was observed in microbial communities from phenanthrene mineralization microcosms. In both PAH mineralization microcosms however, the abundance of both catabolic genes was highly positively correlated (Table 7.5). As seen with mineralization parameters, specific exudate components had a significant impact on gene copy numbers (Table 7.4). Malonate had a repressive effect on C2,3O and *nahAc* copy numbers in both PAH treatments, which was offset by alanine and acetate.

The addition of eluates did not directly impact the total copy number of *alkB* genes, which were comparable between all treatments at the end of the study and averaged approximately 10^6 gene copies per gram of soil (Table 7.2). Although no significant difference was observed in *alkB* abundance, in general total *alkB* copy numbers were positively correlated with 16S rRNA gene numbers (Table 7.5).

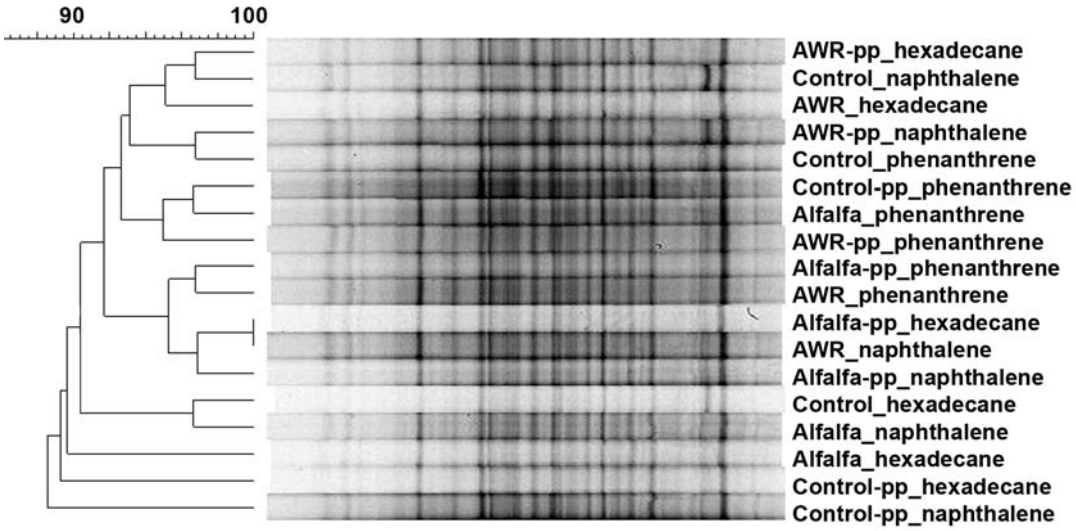

Figure 7.4 Dendrogram analysis of DGGE banding patterns from all exudate and eluate amended mineralization (14C- labelled naphthalene, phenanthrene, and n-hexadecane) microcosms. Treatments included alfalfa and Altai wild rye (AWR) exudates and non-planted control eluates. The tag "-pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 7.5 Spearman's rank correlation coefficients (n = 24) for microbial gene copy numbers in eluate and exudate amended soil microcosms**.**

Hydrocarbon microcosm	Gene copy number†	
Phenanthrene	C2,30	*nahAc*
16S rRNA	0.278	0.136
C2,30		0.939***
Naphthalene	C2,30	*nahAc*
16S rRNA	0.712***	0.663***
C2,30		0.960***
Hexadecane	*alkB*	
16S rRNA	0.779**	

Significant at: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

7.5 DISCUSSION

7.5.1 Plant exudation responses

The general plant growth in the enclosed systems provides a first indicator of the differences in plant response to hydrocarbon stress. The ratio of root to shoot production is known to increase under conditions of xenobiotic induced stress, as plants distribute a greater proportion of their photosynthetic resources to roots (Kaimi et al., 2007). Alfalfa plants in the current study had statistically comparable root:shoot ratios (alfalfa, 0.30 and alfalfa-pp, 0.38) indicating that significant stress due to the presence of PAHs did not occur. Factors which may have reduced stress in the alfalfa-pp systems include the presence of the potential hydrocarbon-degrading *Pantoea agglomerans* (Vasileva-Tonkova and Gesheva, 2007) and a higher root lipid content compared to AWR roots. Root adsorption of PAHs is positively correlated to epidermis lipid content (Gao and Zhu, 2004) and alfalfa roots, with approximately twice the lipid content of grass roots, have been shown to adsorb proportionally more PAHs than grass roots (Schwab et al., 1998). Preferential partitioning to the epidermis may limit hydrocarbon translocation to other root tissues, and decrease overall plant stress responses. AWR treatments, where the root:shoot ratio in PAH growth systems was doubled compared to that of non-PAH systems, did exhibit stress responses to the presence of PAHs. When plants are stressed by hydrocarbons, changes in gene expression (Peña-Castro et al., 2006) lead to changes in root exudation patterns (Bais et al. 2004; Farrar and Jones, 2000). These differences in exudation patterns contribute to alterations in rhizosphere microbial community structure and functional diversity (Grayston et al., 1998). In the current study, very distinct differences were seen in exudation patterns, both between plant species and between PAH and non-PAH stimulated plants.

AWR-pp released approximately three times the amount of organic acids and phenolics, and double the amount of amino acids per gram of root than other plants (Figure 7.1). These increases occurred along with a general increase in total organic

carbon (TOC) released by the roots, which at 90mg TOC g^{-1} root was more than double that of other plants (data not shown). In contrast, in alfalfa treatments generally greater quantities of exudates were detected in non-PAH systems. Due to the low levels contaminating seed endophytes found in most alfalfa systems, it is not possible to attribute the entirety of the observed exudation patterns to alfalfa alone. However, at least one other study that compared root exudation in axenic and non-axenic systems found that microorganisms primarily used the sugar components and that organic acid composition was not changed (Kraffczyk et al., 1984). Identified organic acids comprised approximately 2% of the TOC released by all plants, which is comparable to that found in other studies (Farrar and Jones, 2000). Although the high degree of variation found in all exudate components precluded the finding of statistically significant differences in most cases, it was generally found that AWR and AWR-pp released more of those organic acids which are citric acid cycle intermediates than did alfalfa treatments (Figure 7.1). Amino acids were identified at low levels in most exudates, with the primary amino acids detected being glutamic acid, lysine, alanine and glycine. It is important to note that amino acids released by roots may also be taken back up by roots (Farrar and Jones, 2000; LeSuffleur et al. 2007), thereby distorting actual exudation patterns. LeSuffleur et al. (2007) observed differences in influx and efflux rates of amino acids by different legumes and grasses and noted that influx can actually equal or exceed efflux, as was the case for ryegrass and serine. The final identified root exudate components, phenolic compounds, represented less than 0.02% of the TOC.

The overall composition of root exudates found in this study was comparable to that found in numerous other studies (Cieslinski et al., 1998; Fletcher and Hedge, 1995; Henry et al; 2007; Kraffczyk et al., 1983; LeSuffleur et al.; 2007 Rentz et al. 2004). While the temptation to compare exudation rates between studies is strong, numerous variables impact the relative ratios observed. Plant type (Cieslinski et al., 1998; LeSuffleur et al., 2007), soil composition (Cieslinski et al., 1998), environmental parameters (Henry et al; 2007), and the presence of xenobiotics and other stressors (Bais et al., 2004**)** have all been found to all impact exudation patterns. Cieslinski et al. (1998) found that two cultivars of durum wheat (*Triticum turgidum* var. *durum*) produced different types and quantities of organic acids when grown in the same soil. The

researchers also found significant differences in organic acid exudation when the same cultivar was grown in different soil. The above referenced differences in exudation patterns likely influence the often conflicting findings of phytoremediation studies, and the exudation patterns observed in this study contributed to differences in degradation potential between plant species.

7.5.2 Impact of exudates on microbial community structure

The addition of exudates or control eluates had no significant impact on the general microbial community structure in the study soil. At the end of the study, all treatments in all hydrocarbon assays contained comparable microbial population densities, as enumerated by 16S rRNA gene quantification (Table 7.2). As different bacteria may contain from 1 to 15 copies of the 16S rRNA operon (Acinas et al., 2004), it is possible that differences in community populations were masked by the proliferation of different types of bacteria. However, DGGE analysis revealed little to no difference in the type or relative abundance of bacterial species found in the different treatments (Figure 7.4), supporting the conclusion that microbial population densities did not differ between treatments.

It is well known that plants exert a stimulatory effect on rhizosphere microbial populations, and generally increase microbial population densities and alter metabolic potential (for review see Anderson et al., 1993; Siciliano and Germida, 1998). Previous phytoremediation studies on this soil (Phillips et al., 2006; Chapter 5) have shown that plants do exert a selective influence both on rhizosphere community structure and population density. There are several related factors which may have contributed to the stability observed in the current study. The microbial community present in the soil includes many species adapted to both hydrocarbon and salt contaminated environments. The observed distribution (Figure 7.2) is comparable to that previously found in the study soil, when assessed at different times over a several year period (Phillips et al., 2006; Phillips et al. 2008, submitted for publication). The indigenous microbial communities are well adapted to the weathered-hydrocarbon saline-sodic soil, and a greater nutrient input may be required before changes to the overall community structure occur. In a standard agricultural soil, genotypic shifts were only observed when

amendment rates were increased from 50 to 100 μg C g^{-1} soil (Griffiths et al., 1999). In the current study, experimental limitations associated with maintaining exudates in an axenic state resulted in soil being amended with a single aliquot of exudates at a rate of 10 μg C g^{-1}dry soil. It is probable that larger amendment rates would have resulted in changes to microbial community composition.

While the conditions of this study do not mimic those that would be seen in planted soil systems, they do allow significant insight into the mechanisms whereby specific plant root exudates impact hydrocarbon degradation potential.

7.5.3 Impact of exudates on degradation trends

7.5.3.1 PAH mineralization

There was a general priming effect associated with eluates or exudates from PAH-amended growth systems on C^{14}-PAH mineralization (Figure 7.3). As the observed stimulation of PAH mineralization was more significant in phenanthrene microcosms than in naphthalene microcosms, it is likely that a stimulation comparable to co-metabolism occurred. SPE-GC-MS analysis of these eluates/exudates revealed only trace amounts of phenanthrene (≤ 8ppb) in a limited number of control, alfalfa, and AWR replicates. Although most growth systems were successfully maintained in an aseptic condition such that no bacterial degradation of either phenanthrene or pyrene would have occurred, it is possible that photolytic (Matsuzawa et al. 2001; Niu et al. 2007) and/or plant-mediated (Eapen et al., 2007) breakdown of the PAHs occurred. As we did not specifically quantify PAHs in the solid growth media, or PAH breakdown products in the liquid media or eluates, it is not possible to determine to what extent such a breakdown may have occurred. Given the observed results however, it is reasonable to assume that some breakdown did occur, and that these breakdown products contributed to the observed increase in mineralization. Kanaly and Bartha (1999) observed that the addition of complex mixtures of hydrocarbons stimulated greater degradation of high molecular weight PAHs via co-metabolism than did single PAHs, and it is likely that a comparable effect is occurring in our systems. This was not however, the only factor involved in increased degradation with specific treatments, as both plant and hydrocarbon specific differences were also observed (Figure 7.3).

Plant root exudates, including those derived under PAH stimulated conditions, initially repressed mineralization of both PAHs (Figure 7.3). Several other recent studies have reported a comparable inhibitory effect for root exudates. One set of studies observed a distance-dependent repressive effect on PAH degradation by exudates released by *Lolium perenne*. Dibenzo[a,h]anthracene degradation was repressed in all planted soil (Corgie et al., 2006), while phenanthrene degradation was repressed at distances greater than 3mm from the roots (Corgie et al., 2004; 2006). Rentz et al. (2004) found that both real exudates and single-compound potential root exudate substrates repressed the phenanthrene degrading activity of *P. putida* ATCC 17484. Root extracts from a wide range of plants, including the grass *Avena sativa*, had a greater repressive effect than known inhibitors such as succinate. A concomitant study by the research group showed that inhibition was at least partly due to the repressive effects of exudates on *nahG*, a gene involved in naphthalene dioxygenase transcription (Kamath et al. 2004). Catabolite inhibition commonly occurs during PAH degradation (Rentz et al., 2004) where exudate components such as succinate and glucose are known to inhibit catabolic gene expression (Stulke and Hillen, 1999). The complex and variable nature of exudate components undoubtedly have both complementary and opposing impacts on degradation trends. In the current study, we found that the concentration of specific exudate compounds significantly influenced hydrocarbon degradation (Table 7.4). When examining these results it is important to re-iterate that exudates were added to mineralization microcosms as a proportion of TOC, rather than as a proportion based on root weight (Figure 7.1).

Increased malonate concentration resulted in increased lag periods and decreased catechol 2,3 dioxygenase and naphthalene dioxygenase gene copy numbers for both PAHs (Table 7.4). While alanine and several organic acids mitigated these repressive effects malonate concentration was clearly a dominant determinant of PAH degradation in this soil. Malonate is a cellular respiration inhibitor that acts by competitively inhibiting the active site of succinate dehydrogenase in the TCA cycle. Increased levels of malonate in the exudates of alfalfa plants compared to AWR plants are likely associated with the decreased levels of TCA intermediates found in alfalfa (Figure 7.1), as well as the reduced hydrocarbon degradation potential (Figure 7.3). Malonate is

commonly found in legume exudates (Li and Copeland, 2000; Pearse et al., 2006), where it is believed to play a defensive role against herbivory and microbial pathogens. In the current study, malonate impacted degradation potential by either repressing microbial activity and/or by affecting the proliferation of degradative genotypes.

Previous studies have linked increased hydrocarbon degradation 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, all exudate/eluate treatments in all hydrocarbon assays contained comparable microbial population densities at the end of the study, as enumerated by 16S rRNA gene quantification (previous section and Table 7.2). In contrast, PAH catabolic gene copy numbers were significantly increased in those treatments exhibiting increased PAH mineralization (Table 7.2), indicating that specific changes to catabolic potential were occurring. While non-specific bacterial proliferation may not have been the major factor governing PAH degradation potential, minor population fluctuations did have some impact. In naphthalene microcosms, cumulative naphthalene degradation was weakly correlated with total 16S rRNA gene copy numbers as well as catabolic gene copy numbers, indicating that some of the observed degradation was associated with non-specific increases in microbial populations (Table 7.3). However, as the overall magnitude of population densities between treatments did not significantly differ (Table 7.2), there were likely other, more specific, factors involved.

The mechanisms involved in the specific increases in degradation potential become clearer when factors involved in phenanthrene mineralization are examined. As with naphthalene, the degradation of phenanthrene was highly correlated with catabolic gene copy number. In contrast however, neither mineralization nor catabolic gene copy number was associated with overall population densities (Table 7.3 and 7.5). The naphthalene dioxygenase and catechol 2,3 dioxygenase primers used in this study target plasmid-borne genes (*nahAc* and *nahH*/*xylE* respectively), including those found on conjugative plasmids such as NAH7 (Nojiri et al. 2004). The very high correlation observed between detected catabolic gene copy numbers ($r > 0.939$, $p < 0.001$; Table 7.5), independent of 16S rRNA gene copy number, confirmed that the targeted genes are found on the same plasmids. As some catabolic plasmids carry two C2,3O genes (Li et

al., 2004; Sentchilo et al., 2000), increased C2,3O relative to *nahAc* gene copy numbers may be expected (Table 7.2). Increased catabolic gene copy number, independent of comparable changes in bacterial population densities (Table 7.2) suggests that increased hydrocarbon degradation in this soil may be associated with increased catabolic plasmid transfer. Horizontal gene transfer (HGT) is known to occur with high frequency in the rhizosphere, both within and between genera (Jussila et al., 2007; Kroer et al. 1998; van Elsas et al., 1988, van Elsas et al., 2003). Over half of the bacterial species initially identified in this study soil belonged to the γ-Proteobacteria (Figure 7.2), a group that includes many genera known for high transformation rates (van Elsas et al., 2003). Enhanced HGT in the rhizosphere has been linked to increased exudation (Mølbak et al. 2007) and to specific exudate components, including organic acids, amino acids (Nielsen and van Elsas, 2001) and sugars (Pearce et al., 2000). The actual mechanism that facilitates these increased transfer rates is under debate.

While some studies suggest that conjugative transfer of plasmids is associated with increased cellular metabolic activity, (Lilley et al., 1994; Smets et al. 1993, van Elsas, 1988), others have found no direct correlation between these parameters in the rhizosphere (Kroer et al.,1998; Schwaner and Kroer, 2001). In the current study, we observed a strong negative relationship between exudate concentrations of the cellular respiration inhibitor malonate (Table 7.4) and catabolic gene copy number in root-exudate amended microcosms, suggesting that decreased plasmid transfer rates were associated with decreased metabolic activity. HGT events are also impacted by exudate concentration and type. Pearce et al. (2000) showed that plasmid transfer frequency between two *Enterobacter* sp. was highest at 10 μg mL^{-1} glucose, but then decreased with increasing glucose concentration, concomitantly with decreased donor frequency. Similarly, Mølbak et al. (2007) suggested that increased transfer rates in pea versus barley rhizosphere were due to increased exudation by pea, which resulted in increased donor densities. In *Acinetobacter*, natural transformation is stimulated by numerous compounds found in root exudates, including organic acids such as citrate and succinate and amino acids such as alanine and glycine (Nielsen and van Elsas, 2001). The generally improved phenanthrene degradation parameters associated with AWR-pp

exudates may be related to the higher levels of these compounds found in these exudates (Figure 7.1).

7.5.3.2 Alkane mineralization

As with the PAH assays, the addition of exudates initially repressed hexadecane mineralization. Once again, there were differences in the inhibitive effects exerted by the different exudates, with AWR exudates resulting in the greatest repression (Figure 7.3 and Table 7.2). Mineralization was not however, associated with *alkB* copy numbers (Table 7.3). The *alkB* primers used in this study were designed to target integral membrane hydroxylase genes from various Actinobacteria and α- and β-Proteobacteria (Powell et al., 2006), and a GenBank search of the primers shows that a comparable phylogenetic range of bacteria are matched (data not shown). The bacterial species identified in the soil in the current study span the range of targeted species (Figure 7.2), and Q-PCR results indicate that high copy numbers of *alkB* are present in the study soil (Table 7.2) and that they are highly correlated with total bacterial population densities (Table 7.5). However, there was no significant difference between *alkB* gene copy numbers between treatments (Table 7.2). Assessment of this gene may reflect the alkane degradation capabilities of some soils (Powell et al., 2006), but is not an appropriate indicator of degradation capacity in this soil.

As with PAHs, the organic acid malonate was associated with repression of hexadecane mineralization, increasing lag times and decreasing both rate and cumulative mineralization (Table 7.4). Fumarate and alanine were also implicated in hexadecane degradation, with alanine associated with increased and, contrary to PAH microcosms, fumarate associated with decreased hexadecane mineralization. This repressive effect was not, however, associated with *alkB* or 16S rRNA gene copy numbers (Table 7.4), indicating that other alkane degrader phenotypes were involved. Recent studies have shown that some bacteria use an alternate or additional enzyme system, cytochrome P450 hydroxylase, to metabolize medium to long chain aliphatic hydrocarbons. These bacteria include *Alcanivorax* spp. (Sabirova et al., 2006), *Acinetobacter* spp., and *Sphingomonas* spp. (van Beilan et al., 2006), all of which were present in the current study soil (Figure 7.2). Cytochrome P450 hydroxylases are known

to be up-regulated in the presence of alkanes (Sabirova et al., 2006), and it is equally probable that some form of catabolite inhibition will occur. As comparable amounts of malonate were added to the soil with both alfalfa and AWR exudates, yet alfalfa showed significantly less repression, there are undoubtedly additional factors involved. On a per gram of soil basis, the AWR exudates contained approximately 4 times more fumarate and 2 times less alanine than other exudates (data not shown), and this balance undoubtedly impacted the degradation potential.

7.6 Conclusion

In this study we found that plant root exudates initially inhibited hydrocarbon degradation in a weathered hydrocarbon-contaminated soil. Several root exudate components identified in this study were associated with changes in degradation potential. The organic acid malonate, associated with decreased PAH degradation potential, may have decreased rates of catabolic plasmid transfer. This inhibition effect was mitigated by the amino acid alanine and the organic acid acetate, which appeared to have specific stimulatory effects on plasmid transfer. Decreased degradation of the aliphatic hydrocarbon hexadecane was also associated with increased concentrations of malonate and decreased concentrations of alanine. Concentrations of these root exudate components differed between and within plant species under different external stimuli, and had an immediate and specific impact on hydrocarbon degradation potential. These findings do not presume to be predictive of real-world situations, but they do provide substantial insights into why specific plant species may promote degradation under one set of circumstances, yet inhibit it under another. The differences in degradation potential observed in this study reflect the balance of having sufficient and appropriate nutrient sources such that transfer of catabolic competency can occur, without incurring a concomitant reduction in degradation potential due to catabolite or general metabolic inhibition.

8.0 IMPACT OF CLASS-SPECIFIC EXUDATE FRACTIONS ON THE HYDROCARBON DEGRADATION POTENTIAL OF INDIGENOUS SOIL MICROBIAL COMMUNITIES

8.1 Preface

A primary goal of this research has been to elucidate the mechanisms whereby specific plants enhance the degradation potential of indigenous rhizosphere bacteria. In the previous chapter (Chapter 7), we found plant exudates as a whole inhibited overall degradation in the short term. However, specific exudate components were implicated in both decreased and increased degradation potential via two different mechanisms, catabolite repression and increased degradative plasmid transfer. The current study was designed to further pursue this insight. The complex mixture of exudates used in the previous study was separated into class specific fractions and their impact on microbial degradation potential was assessed.

8.2 Introduction

Phytoremediation systems for organic contaminants such as petroleum hydrocarbons rely on a synergistic relationship between plants and their root associated microbial communities. Root exudates are a primary factor governing this interaction, and are known to influence both microbial community structure (Anderson et al., 1993; Siciliano and Germida, 1998) and function (Grayston et al., 1998). Root exudation is not, however, either consistent or constant. Numerous factors, including plant species and cultivar (Cieslinski et al., 1998; LeSuffleur et al., 2007), soil composition (Cieslinski et al., 1998), environmental parameters (Henry et al., 2007), and the presence of xenobiotics and other stressors (Bais et al., 2004**)** influence exudation patterns. These differences in exudation patterns contribute to the often contradictory results observed in phytoremediation studies, where a specific plant such as *Lolium perenne* facilitates remediation under one set of conditions (Binet et al., 2000) but not under another (Rezek

et al., 2008). The complexity both of exudation and of soil microbial communities, combined with fluctuating soil environmental conditions and fundamental differences in contamination type and history makes research into plant-bacterial interactions extremely challenging. However, in order to further the potential of phytoremediation as an accepted treatment option, the specific role of exudates in these interactions must be determined.

Several recent studies have attempted to address this question, with mixed conclusions. Research using artificial root exudates often observe a stimulation of hydrocarbon degradation potential, both specific (Yoshitomi and Shann, 2001) and non-specific (da Silva et al., 2003; Miya and Firestone, 2001). Studies assessing the impact of actual root exudation however, often find a repressive impact (Corgie et al., 2004; Corgie et al., 2006; Rentz et al., 2004). These contradictory observations may be related to the limited composition of artificial root exudates, which may not contain key repressive compounds normally found in root exudates. A recent study by our group that assessed the impact of complete root exudates on the degradation potential of a soil microbial community indigenous to a saline-sodic weathered-hydrocarbon contaminated soil found that specific compounds exerted a significant repressive effect (Chapter 7). For example, decreased copy numbers of plasmid-borne genes involved in PAH degradation were significantly and specifically associated with increased concentrations of the organic acid malonate, the concentration of which varied both inter- and intra-specifically. Other root exudate components, including the amino acid alanine, had a mitigating impact and were associated with increased degradation potential.

The specific classes of compounds found in exudates, including organic acids (Collier et al., 1996; Dinamarca et al., 2003; Kuiper et al., 2002), amino acids (Putrins et al., 2007; Sze and Shingler, 1999), and phenolics (Chen and Aitken, 1999; da Silva et al., 2006) have been implicated in either increased or decreased degradation responses in model organisms such as *Pseudomonas* spp. The goal of the current study was to characterize the impact of these specific exudate fractions on the degradation potential of soil microbial communities. Organic acids, amino acids, and phenolic compounds were extracted from whole root exudates and amended to weathered-hydrocarbon soil microcosms and the responses of the indigenous bacterial communities were monitored.

The primary objectives were to 1) to determine if specific class fractions of exudates had a dominant impact on degradation response, 2) to determine if individual components of these fractions had a dominant impact on degradation response, and 3) to characterize the probable mechanisms whereby exudate components impacted soil microbial community responses.

8.3 Materials and methods

8.3.1 Plant growth and exudate collection

Elymus angustus Trin. (Altai wild rye; AWR) and *Medicago sativa* L. (alfalfa var. rambler) were grown aseptically in modified Leonard jars. Prior to planting, seeds were surface disinfected by washing for 1min with 95% ethanol, followed by a 5 (alfalfa) or 10 (AWR) min wash with 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. Seeds were then incubated on $1/10^{th}$ TSA plates for a minimum of 2 d, and only those seeds which showed no bacterial or fungal growth were used.

Each modified Leonard jar was comprised of a 330 mL narrow-neck bottle, with the bottom cut off, that was inverted into a 950 mL amber wide-mouth bottle (Life Science, Peterborough Ont.) which served as the liquid media reservoir. A fiberglass wick (Specialty Gaskets, Mississauga Ont.) extended from the top of the inverted bottle to the bottom of the media reservoir and was held in place by a washed glass wool plug. Quartz sand was repeatedly washed until the rinse water ran clear, dried at $100^{o}C$, autoclaved twice for 1h with a week resting period, and then 200 g of fine sand was layered on top of 200 g of coarse sand around the wick in the inverted bottle. All other dry components were autoclaved for 1 hour and Hoagland's media was autoclaved for 20 min prior to system assembly. Each media reservoir was filled with 600 mL 0.5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), sand was pre-wetted with 100 mL 0.5 strength Hoagland's, and each assembled system was then wrapped in tin-foil and autoclaved again for 30 min. All further manipulations of the assembled systems were performed using the best aseptic techniques in a sterile laminar flow hood. The sand in half of all sterile systems was subsequently spiked with 2 mL of a pyrene/phenanthrene (98% pure; Sigma Aldrich, Mississauga, Ont.) mixture in acetone

(40 mg mL^{-1}) using a sterile glass syringe, to incremental depths up to 6-cm at 6 injection sites, and acetone was allowed to evaporate off for 24 h, for a final concentration of 200 mg kg^{-1} of each hydrocarbon. All systems were then planted with surface sterilized seeds (PAH-spiked 15 seeds each, non-spiked 10 seeds each). Once each Leonard jar was planted, all jars were covered by a sterile Sunbag with a 0.02 μm gas exchange filter (Sigma Aldrich, Mississauga, Ont.), which was secured in place around the neck of the media reservoir. Six treatments were established with four replicates each; AWR, alfalfa, and a non-planted control, in PAH-contaminated and non-contaminated soil.

Plants were grown in a growth chamber with a 16h/25 ^{o}C day (1500 μmol m^{-2}) and 8h/15^{o}C night cycle for 6 weeks. As the enclosing Sunbag limited the effects of evapotranspiration, no additional Hoagland's solution was required during this period, which facilitated the maintenance of an aseptic system. Exudates and control eluates were collected by transferring the solid media bottle to a sterile Erlenmeyer flask and rinsing the sand with 300 mL sterile deionized water. Exudate sterility was initially assessed by plating 100 μL aliquots on 1/10th TSA plates. An additional 1mL aliquot, boiled to release DNA was assessed by PCR using the eubacterial primers outlined in the following sections. All exudates were then filtered through a 0.45μm filter and frozen until use. Plant roots and shoots were separated, dried, and weighed.

8.3.2 Isolation, 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 GC-MS analysis of derivatized sub-samples. Organic acid and amino acid recovery methods were modified from previously developed protocols (Gillespie, 2003). Unless otherwise stated, all standards and derivatizing reagents were obtained from Sigma-Aldrich (Mississauga, Ont.) and all solvents were high purity GC grade (OmniSolv).

8.3.2.1 Ion exchange membrane preparation and use

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, 200 mL each transferred to a sterile Erlenmeyer flask containing a single anion and single cation exchange membrane, and samples were shaken in the dark on a rotary shaker for 12 h. 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 NH_4OH (cation membranes), and shaken in the dark for 8 h. Recovered eluates were stored at 4^{o}C until use.

8.3.2.2 Organic acid isolation and quantification

A 1.5 mL aliquot of anion exchange eluate was transferred to a 6 mL glass vial. 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 new 10 mL glass vial, and evaporated under N_2 at room temperature. The DEE extraction was repeated an additional 2 times, with the non-aqueous phase being added to the original DEE residue in the autosampler vial and evaporated under N_2 at room temperature. Extraction controls of 1.5 mL sterile de-ionized (DI) water were extracted and dried as above. All dried extracts were re-suspended in 6.5 mL sterile DI water, shaken in the dark for 24 h, then filter-sterilized through a 0.45 µm filter and frozen at -20 ^{o}C until use. A 150 µL sub-sample of each re-suspended extract was added to a 1.8 mL GC vial containing 25 µL of glutaric acid (100 µg mL^{-1}, used as an internal standard), and dried under N_2 at room temperature. Organic acids were derivatized to *tert*-butyldimethylsilyl (*t*-bdms) 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 ^{o}C for 45 min. The vial was cooled to room temperature and the *t*-bdms ester derivatives of the organic acids were assessed by GC-MS. Serial dilutions of the following 100 µg mL^{-1} mixed organic acid standard were derivatized as above: acetic, aconitic, azeleic, butyric, caffeic, citric, citraconic, fumaric, hydroxymalonic, itaconic, maleic, malonic malic, methylglutaric, methylmalonic, phthalic, tartaric, suberic, and succinic acids.

8.3.2.3 Amino acid isolation and quantification

A 3.0 mL aliquot of cation exchange eluate was transferred to a 10 mL glass vial and evaporated to dryness under N_2 at room temperature. All dried extracts were re-suspended in 6.5 mL sterile DI water, shaken in the dark for 24 h, then filter-sterilized through a 0.45µm filter and frozen at -20 ^{o}C until use. An additional 1.0 mL aliquot was transferred to a 1.8 mL autosampler vial containing 20 µL of norleucine (100 µg mL^{-1}, used as an internal standard) and evaporated to dryness under N_2 at room temperature. Amino acids were derivatized to *t*-bdms 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 ^{o}C for 30 min. GC vials were cooled to room temperature and the amino acid *t*-bdms ester derivatives were assessed by GC-MS. Serial dilutions of the following 100 µg mL^{-1} mixed amino acid standard were derivatized as above: alanine, asparagine, aspartic acid, cysteine, cystine, histidine, hydroxyproline, isoleucine, glutamine, glutamic acid, glycine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

8.3.2.4 Phenolic isolation and quantification

Phenolic compounds were extracted from exudates 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 x 5-mL deionized water. Samples (100 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 final eluate was divided into two equal volumes. One half was transferred into a 10 mL vial and one half successively transferred to a 1.8 mL autosampler vial containing 50 µL of naringenin (100µg mL^{-1}, used as an internal standard). Both vials were dried under N_2 at room temperature. Eluates in the 10 mL vials were re-suspended in 6.5 mL sterile DI water, shaken in the dark for 24 h, then filter-sterilized through a 0.45 µm filter and frozen at -20 ^{o}C until use. Eluates in the 1.8 mL vial were derivatized by dissolving the ethyl acetate residue in 75µL acetonitrile and 25µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, Bellefonte, PA), capping the

vial, and heating for 40 min at 70°C. Vials were allowed to cool and were then analyzed by GC-MS. Serial dilutions of the following 100 µg mL^{-1}standard solution of phenolics were derivatized as above: caffeic acid, catechin, catechol, cinnamic acid, ferulic acid, flavone, gentisic acid, hesperetin, hydroxyquinone, isovanillic acid, p-coumaric, p-salicylic, naringenin, protocatechuic acid, quercetin, salicylic, sinapic, syringic, ubelliferone, and vanillic acid.

8.3.2.5 GC-MS analysis

Samples were analyzed on a Varian CP-3800 GC equipped with a Saturn 2200 MS detector and an 8400 autosampler. A Varian FactorFour™ column (VF-Xms) with dimensions of 30 m x 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 splitless injection volume was delivered with a 5 µL syringe. For organic 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 minute, for a total run time of 50 min. The MS was operated in the EI ionization mode and was set to scan from *m/z* 45-650 with a scan rate of 0.420 scans s^{-1}. The MS remained off for the initial 10 min of the run to allow solvent and derivatization reagent to elute. Spectral data were acquired using a linked Saturn GC/MS Workstation v. 5.5 (Varian Inc., Walnut Creek, CA). For amino acid analysis the initial column temperature was held at 60°C for 1 min, then ramped at 5°C min^{-1} to 300°C and held for 6 min, for a total run time of 55 min. An open scan was performed from 7.25 min onwards. For phenolic analysis the initial column temperature was maintained at 80°C for 1min, then ramped at a rate of 20 °C min^{-1} to 250°C and held for 1min, 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, for a total run time of 25.83 min. An open scan was performed from 3.5 min onwards.

8.3.3 Hydrocarbon mineralization potential

C-14 hydrocarbon mineralization assays using were used to determine what impact each exudate fraction 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, EC 5.8 dS m^{-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. The total hydrocarbon concentration was 3700 mg kg^{-1}, and consisted of F3 and F4 fractions (C16 to C50). Soil was sieved through a 4.75 mm sieve to ensure homogeneity and stored at 4°C until use. Soil moisture content was determined by oven-drying 10 g sub-samples at 100°C for 24 hr. Microcosms were set up and sampled as outlined in Chenier et al. (2003). Microcosm soil was acclimatized to room temperature for a minimum of 2 wk and to 18% moisture content (with sterile de-ionized water) for a minimum of 48 h prior to microcosm setup. Isolated exudate fractions (500 µL) were added to serum vials containing 4 g soil, for a final moisture content of 30%. Sterile water-amended microcosms were used as a control to ascertain if exudate fraction extraction procedures impacted mineralization potential. Abiotic controls were established using autoclaved soil (2 × 1-h with a 1-wk resting interval). Serum vials were amended with 50,000 dpm (100 mg kg^{-1}) of [1-^{14}C]n-hexadecane, [1-^{14}C]naphthalene, or [9-^{14}C]phenanthrene (specific activities, 12, 6.2, and 8.2 mCi $mmol^{-1}$ respectively; Sigma-Aldrich, Mississauga, Ont.). A 1.8 mL glass vial with 0.5 mL 1M 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). After 2 wk incubation, an additional 500 µL of each fraction was added to microcosms using a sterile syringe. Non-radioactive replicate microcosms were established for all exudate fractions with all assessed hydrocarbons for use in subsequent molecular analyses.

8.3.4 Microbial community assessment

8.3.4.1 Microbial community DNA extraction

Total community DNA was extracted from all samples using a bead-beating protocol previously outlined in 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 100 µL TE (pH 8.0),

and purified using PVPP columns. Soil sub-samples (0.50 ± 0.01 g) from each non-radioactive replicate mineralization microcosm for all treatments and hydrocarbons were extracted. Following PVPP purification of 50 µL total DNA extract all volumes were standardized. Final DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen) and by spectrophotometer evaluation.

8.3.4.2 DGGE analysis of community structure

Community structure was examined by DGGE analysis of PCR-amplified 16S rRNA gene fragments. Total DNA extracts from each treatment replicate were amplified using the universal eubacterial 16S rRNA gene primers listed in Table 8.1, using the PCR protocol outlined in Phillips et al. (2006). Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1 V 3 M sodium acetate and 2.5 V 100% ethanol at -20 oC overnight, re-suspended in 15 µL 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 treatment, 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 16h at 80V and 60oC. The resulting gel was 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). Intra-treatment banding pattern reproducibility was assessed before comparing inter-treatment community structure.

8.3.4.3 Quantitative PCR assessment

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, catechol 2,3 dioxygenase (C2,3O), naphthalene dioxygenase (*nahAc*), and alkane monoxygenase (*alkB*), were assessed. PCR assays were performed on an ABI 7500 RT PCR system (Applied Biosystems, Foster City, Calif.) using

Table 8.1 Primers and amplification conditions used for PCR and Q-PCR amplification

Target gene Primer sequence†	T_a‡ (°C)	Primer (μM)	Expected product size (bp)	Reference	Control strain
Naphthalene dioxygenase (*nahAc*)					
F: 5'-CAA AAR CAC CTG ATT YAT GG	47	0.3	377	Baldwin et al., 2003	*P. putida*
R: 5'-AYR CGR GSG ACT TCT TTC AA					ATCC 17484
Alkane monoxygenase (*alkB*)					
F: 5'-AAC TAC ATC GAG CAC TAC GG	50	0.5	100	Powell et al., 2006	*P. putida*
R: 5'-TGA AGA TGT GGT TGC TGT TCC					ATCC 29347
Catechol 2,3 dioxygenase (C2,3O)					
F: 5'- AGG TGC TCG GTT TCT ACC TGG CCGA	65	0.3	406	Luz et al.,	*P. putida*
R: 5'- ACG GTC ATG AAT CGT TCG TTG AG				2004	ATCC 33015
Universal eubacterial 16S rRNA (16S)					
F: 5'-CTA CCA GGG TAT CTA ATC C	55	0.15	450	Rölleke et al., 1996	*P. putida*
R: 5'-CCT ACG GGA GGC AGC AG§				Lee et al., 1993	ATCC 17484

†Forward (F) and reverse (R) primers are indicated.

‡T_a, annealing temperature used during real-time PCR.

§Preceded by a GC clamp for DGGE: GCGGGCGGGGCGGGGGCACGGGGGGCGCGG CGGGCGGGGCGGGGG

QuantiTect SYBR green PCR kits (Qiagen, Mississauga, Ont). Amplification reactions were set up according to the manufacturers protocol incorporating the primer concentration listed in Table 8.1. After the initial 15 min denaturing period, PCR amplification proceeded for 38 cycles of 30s denaturing at 94^{o}C, 40s annealing at the appropriate temperature (Table 8.1), 1min extension at 72 ^{o}C, and a data collection step of 45s at 80 ^{o}C. A final melt curve analysis from 5 ^{o}C below amplification temperature to 95 ^{o}C concluded the cycling program. Absolute quantification was performed by comparison with standard curves. Ten fold DNA standards ranging from 10^2 to 10^6 gene copy numbers were prepared from serial dilutions of DNA extracts from positive control strains (Table 8.1). Gene copy numbers were calculated from the concentrations of positive-control strains, quantified spectrophotometrically, assuming a molecular mass of 660Da 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 >0.99. 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).

8.3.5 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 analysis. Relationships in microbial community structure between treatments were assessed by cluster analysis of weighted (Muylaert et al., 2002) DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths).

8.4 Results

8.4.1 Concentration of exudate fractions

Organic acids, amino acids, and phenolic acids were successfully recovered from bulk root exudates. The actual amount of any individual exudate component added to any one microcosm varied greatly (Table 8.2). Phenolics represented the smallest component identified in the root exudates and were added to microcosms at trace amounts only. Amino acids were the next largest identified root exudate components and were added at concentrations ranging from 0 to 200 ng individual amino acid g^{-1} dry soil. Organic acids were the largest identified components and were added at concentrations ranging from 0 to 300 ng individual organic acid g^{-1} dry soil, for cumulative TOC amounts ranging from 100 to 1200 ng g^{-1} dry soil. Organic acids were also identified in the extracted and re-suspended eluates from control microcosms, although at lower concentrations and diversity than those found in the plant root exudates. Although low levels of acetic acid were detected in water controls extracted by the same process (data not shown), this cannot account for the organic acids found in the control eluates. Instead, it is likely that some bacterial contamination of these eluates occurred when they were opened for initial organic acid analysis.

8.4.2 Impact of phenolic compounds on degradation potential

The addition of plant-derived phenolic compounds had no significant impact on PAH mineralization potential (Figure 8.1A). Some difference was observed during n-hexadecane mineralization, with control eluate-amended microcosms exhibiting significantly more mineralization than AWR-phenolic amended microcosms (55.9 and 48.5% respectively, $p \leq 0.05$). Other mineralization parameters for all hydrocarbons were comparable between water-amended and phenolic-amended microcosms. Lag and mineralization rates for phenanthrene, naphthalene, and n-hexadecane averaged 10.0, 1.7, and 2.0 days (respectively) and 1.7, 7.3, and 5.4% $^{14}CO_2$ day^{-1}(respectively) for all treatments.

Table 8.2 Concentration of individual identified components in organic acid, amino acid, and phenolic fractions added to mineralization microcosms

	Alfalfa	Alfalfa-pp[†]	AWR[‡]	AWR-pp	Control	Control-pp
Organic acid	ng organic acid g-1 soil					
Succinic	114 (54)	133 (74)	264 (56)	124 (43)	42 (21)	82 (41)
Acetic	82 (34)	104 (57)	98 (47)	98 (70)	57 (4)	58 (21)
Fumaric	37 (345)	82 (90)	53 (52)	59 (72)	21 (4)	42 (55)
Maleic	29 (23)	61 (40)	30 (23)	39 (27)	28 (17)	33 (15)
Oxalic	89 (59)	104 (74)	102 (77)	38 (45)	76 (88)	65 (78)
Phthalic	19 (6)	20 (5)	15 (6)	20 (0)	14 (10)	14 (11)
Itaconic	121 (154)	40 (80)	46 (86)	34 (69)	nd[§]	Nd
Azelaic	6 (5)	7 (3)	5 (1)	3 (2)	nd	Nd
Aconitic	2 (4)	4 (2)	128 (185)	35 (37)	nd	Nd
Malonic	49 (31)	80 (43)	46 (24)	75 (15)	nd	Nd
Malic	nd	nd	41 (48)	2 (5)	nd	Nd
Amino acid	ng amino acid g-1 soil					
Alanine	32 (6)	18 (23)	21 (10)	37 (13)	nd	Nd
Glycine	39 (4)	27 (31)	65 (17)	49 (35)	nd	Nd
Lysine	29 (58)	nd	23 (20)	70 (114)	nd	Nd
Glutamic acid	25 (49)	nd	264 (340)	23 (46)	nd	Nd
Leucine	nd	nd	2 (4)	6 (7)	nd	Nd
Serine	nd	nd	6 (4)	3 (4)	nd	Nd
Phenylalanine	nd	nd	8 (17)	9 (11)	nd	Nd
Aspartic acid	nd	nd	nd	7 (8)	nd	Nd
Isoleucine	nd	nd	nd	4 (5)	nd	Nd
Phenolic	ng phenolic g-1 soil					
Catechol	0.1 (0.1)	0.4 (0.3)	0.2 (0.1)	1.9 (2.2)	nd	Nd
Salicylic	nd	nd	0.1 (0.1)	0.3 (0.3)	nd	Nd
Protocatechuic	0.4 (0.7)	nd	0.1 (0.1)	0.1 (0.1)	nd	Nd
Flavone	0.9 (1.0)	nd	0.7 (0.8)	nd	nd	Nd
Umbelliferone	< 0.1 (0.0)	< 0.1 (0.0)	< 0.1 (0.0)	0.1 (0.1)	nd	Nd
p-salicylic	< 0.1 (0.0)	0.2 (0.2)	2.0 (3.3)	1.3 (1.2)	nd	Nd

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

†The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

‡ AWR, Altai wild rye

§ nd, not detected

No significant change in microbial community structure was observed (Figure 8.2). Banding patterns both within and between hydrocarbon treatments were greater than 94% similar (data not shown). Total microbial densities were also equivalent, with an average of 3.16×10^7 16S rRNA gene copy numbers g^{-1} dry soil in all microcosms. Copy numbers of the catabolic genes *alkB*, C2,3O, and *nahAc*, were also comparable between hydrocarbon microcosms and treatments, and averaged 3.31×10^5, 1.00×10^6, and 2.82×10^4 copies g^{-1} soil (respectively). Increased copy numbers of all catabolic genes were associated with increased microbial population density, but, with the exception of phenanthrene, not associated with mineralization parameters (Table 8.3). In phenanthrene microcosms increases in general population densities and in catabolic gene copy numbers were negatively associated with mineralization parameters (Table 8.3). Only two phenolic compounds, catechol and p-salicylic acid (p-hydroxybenzoic acid) were found in all exudates, at concentrations up to 7 ng g^{-1} dry soil (Table 8.2). When regression analysis was performed on the impact of these two compounds on all degradation parameters, significant correlations were found for phenanthrene mineralization parameters only (Table 8.4). Increasing concentrations of p-salicylic acid were associated with decreased rate and extent of phenanthrene mineralization, but with increased catabolic gene copy numbers. No other direct associations between added phenolics and hydrocarbon degradation potential were found.

8.4.3 Impact of amino acids on degradation potential

There was no significant treatment effect of the added amino acid eluates on mineralization parameters for either PAHs or for n-hexadecane (Figure 8.1B). Lag rates for phenanthrene, naphthalene, and n-hexadecane averaged 8.8, 3.9, and 7.5 days (respectively) and mineralization rates averaged 2.1, 4.6, and 2.1 % $^{14}CO_2$ day^{-1} (respectively) for all treatments. Microbial communities within a given hydrocarbon treatment were highly stable, with ≥95% similarity between banding patterns in hexadecane microcosms and 100% similarity between banding patterns in phenanthrene and in naphthalene microcosms (Figure 8.3). The type of hydrocarbon did have some impact on community structure, decreasing similarity in banding patterns between

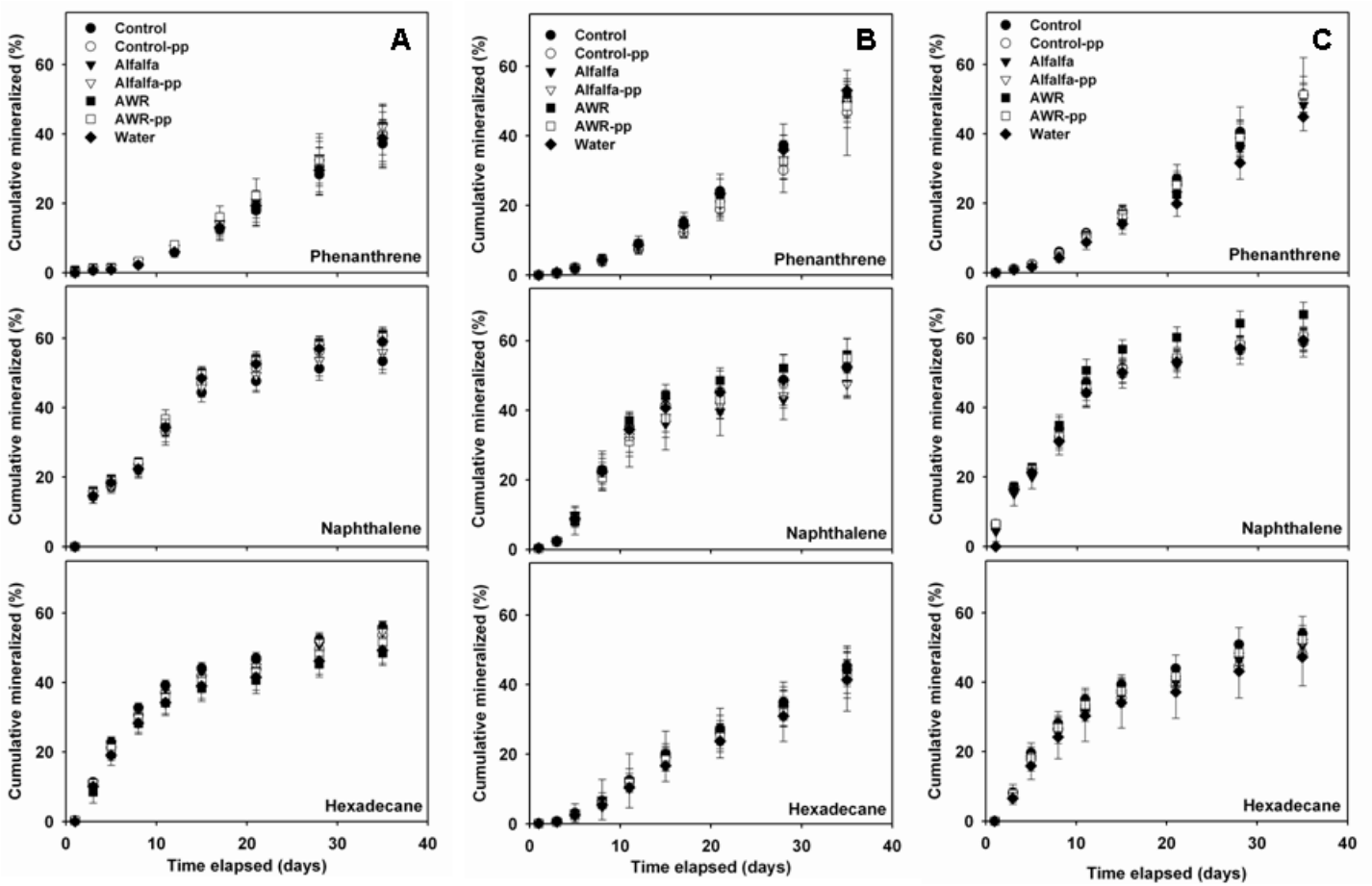

Figure 8.1 Cumulative percent mineralization for 14C-phenanthrene, 14C-naphthalene and 14C-hexadecane in soil microcosms treated with extracted root exudate A) phenolic fractions, B) amino acid fractions, and C) organic acid fractions. Treatments include fractions extracted from alfalfa and Altai wild rye (AWR) exudates and non-planted control eluates, and water only experimental controls. The tag –pp indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

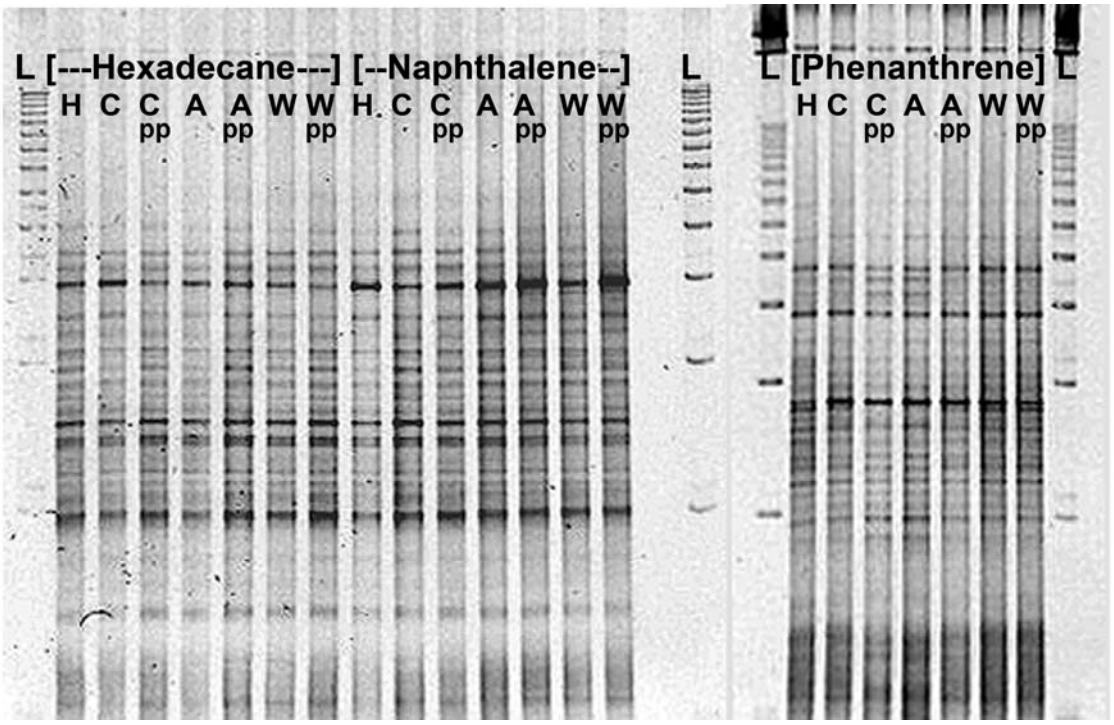

Figure 8.2 Representative DGGE of PCR-amplified 16S rRNA gene fragments from phenolic-amended soil microcosms spiked with n-hexadecane, naphthalene, or phenanthrene. Treatments included phenolics extracted from alfalfa (A) and Altai wild rye (W) exudates and non-planted control (C) eluates, and water-only (H) experimental controls. The tag –pp indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 8.3 Spearman's rank correlation coefficients (n=27) for hydrocarbon mineralization parameters and microbial gene abundance in phenolic fraction amended soil microcosms.

Hydrocarbon microcosm	Gene copy number†		Mineralization‡		
Phenanthrene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.583**	0.684***	0.553**	-0.394*	-0.456*
C,23O		0.941***	0.573**	-0.539**	-0.548**
nahAc			0.559**	-0.483*	-0.521**
Naphthalene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.552**	0.680***	-0.036	-0.004	-0.073
C,23O		0.902***	-0.079	-0.131	0.066
nahAc			0.087	-0.061	0.055
Hexadecane	alkB		Lag	Rate	Cumulative
16S rRNA	0.495**		-0.250	-0.340	0.020
alkB			-0.144	-0.225	-0.138

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

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

Table 8.4 Linear multiple regression for hydrocarbon degradation parameters (mineralization and gene copy number) as a function of phenolic concentration.

Hydrocarbon treatment		
Phenanthrene	Constant ± standardized β-coefficients	R^2
Rate‡	1.748 -0.478 p-salicylic acid*	0.190
Cumulative	41.201 -0.443 p-salicylic acid*	0.197
C2,3O	5.920 +0.439 p-salicylic acid*	0.192
nahAc	4.345 +0.389 p-salicylic acid†	0.106

Regression equation significant at *$p \leq 0.05$ and †$p < 0.10$

‡Degradation parameters: Rate, maximum rate of mineralization ($\%^{14}CO_2$ day^{-1}); cumulative, cumulative % mineralized; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

hydrocarbon treatments. Several additional bands were observed in phenanthrene treatments, which decreased the banding pattern similarity with hexadecane and naphthalene treatments to 90%.

Heterotrophic population densities in phenanthrene treatments were also impacted by the amino acids. Approximately four times more ($p \leq 0.05$) 16S rRNA gene copies were found in alfalfa phenanthrene microcosms than in control or water amended microcosms (3.02 versus 0.79 x 10^7 copies g^{-1} soil, respectively). However, copy numbers of the catabolic genes C2,3O and *nahAc* were comparable between treatments and averaged approximately 2.63 x 10^7 and 5.25 x 10^5 copies g-1 dry soil, respectively. These genes, while highly correlated with each other, were not correlated to general increases in 16S rRNA copy numbers (Table 8.5). In naphthalene microcosms 16S rRNA, C2,3O and *nahAc* genes averaged 7.21 x 10^7 , 7.31 x 10^7, and 6.36 x 10^6 copies g^{-1} soil (respectively) with no treatment specific differences, and all genes were positively correlated (Table 8.5). In hexadecane microcosms, AWR and control-pp treatments had the lowest 16S rRNA and *alkB* copy numbers (approximately 3.70 x 10^7 and 2.82 x10^5 copies g^{-1} dry soil, respectively), significantly lower than all other treatments except AWR-pp. These decreased gene copy numbers were associated with general decreases in total heterotrophic populations (Table 8.5).

While there was no specific association between gene copy numbers and mineralization parameters (Table 8.5), regression analysis revealed some impact by amino acid concentrations. The amount of individual amino acid amended to each microcosm varied significantly, with generally higher quantities and diversity in AWR microcosms than alfalfa microcosms (Table 8.2). Only two amino acids, alanine and glycine, were found in all plant exudate replicates, and were amended at concentrations ranging from 11 to 86 ng g^{-1} soil. Increased alanine concentrations had a weak stimulatory effect on hexadecane mineralization rates, and a stronger impact on increased copy numbers of C2,3O and *nahAc* relative to 16S rRNA in naphthalene microcosms (Table 8.6). Although not associated with the observed mineralization, both 16S rRNA and *alkB* copy numbers in hexadecane mineralization microcosms decreased with increasing glycine concentration (Table 8.6).

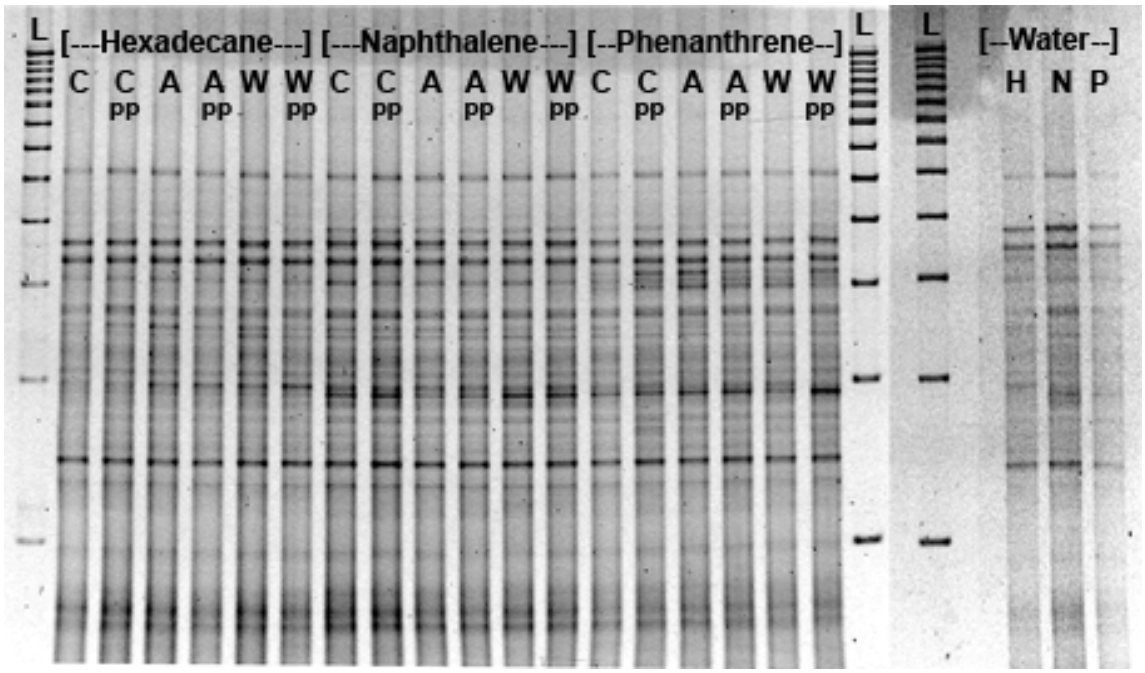

Figure 8.3 Representative DGGE of PCR-amplified 16S rRNA gene fragments from amino acid-amended soil microcosms spiked with n-hexadecane (H), naphthalene (N), or phenanthrene (P)**.** Treatments included phenolics extracted from alfalfa (A) and Altai wild rye (W) exudates and non-planted control (C) eluates, and water-only experimental controls. The tag –pp indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 8.5 Spearman's rank correlation coefficients (n=27) for hydrocarbon mineralization parameters and microbial gene abundance in amino acid fraction amended soil microcosms.

Hydrocarbon microcosm	Gene copy number†		Mineralization‡		
Phenanthrene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.333	0.293	0.088	0.094	0.170
C,23O		0.892***	0.108	0.101	0.015
nahAc			0.072	0.141	0.036
Naphthalene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.408*	0.324*	-0.119	0.124	0.216
C,23O		0.919***	0.112	-0.179	0.001
nahAc			0.211	-0.280	-0.008
Hexadecane	alkB		Lag	Rate	Cumulative
16S rRNA	0.777***		0.270	-0.267	0.051
alkB			0.100	-0.092	0.072

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

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

8.4.4 Impact of organic acids on degradation potential

The addition of extracted organic acids had no significant impact on cumulative phenanthrene or hexadecane mineralization (Figure 8.1C). Lag periods for all phenanthrene and hexadecane treatments averaged 8.0 and 2.4 days (respectively) and maximum mineralization rates averaged 2.1 and 5.1 % $^{14}CO_2$ day^{-1}(respectively). Naphthalene mineralization however, was impacted by the organic acids. Organic acids decreased lag periods for naphthalene mineralization relative to the water control, from 2.5 to an average of 1 day ($p < 0.01$). Mineralization was also increased by organic acids from AWR treatments, which exhibited approximately 67% cumulative mineralization, significantly different than water, alfalfa, control and control-pp ($p < 0.05$).

As with both amino acid and phenolic fractions, organic acids had little impact on overall microbial community structure (Figure 8.4). DGGE banding pattern similarity was greater than 95% within hydrocarbon treatments, and greater than 90% between hydrocarbon treatments. Absolute gene copy numbers within phenanthrene and naphthalene treatments were also not significantly different between treatments. 16S rRNA, C2,3O and *nahAc* genes averaged 4.8 $x10^7$, 6.31 x 10^6, and 2.63 x 10^5 copies g^{-1} soil for phenanthrene microcosms, and 5.75 x 10^7, 4.90 x 10^6, and 2.45 x 10^5 copies g^{-1} soil for naphthalene microcosms (respectively). In n-hexadecane microcosms, AWR treatments had significantly lower ($p < 0.001$) copy numbers of both 16S rRNA and *alkB* genes (6.31 x 10^7 and 2.0 x 10^6 copies g^{-1} soil, respectively) than water or control-pp treatments. Increased copy numbers of all catabolic genes were associated with increased microbial population density, but not associated with mineralization parameters (Table 8.7).

Although treatment specific differences in mineralization potential did not occur, specific organic acids had opposing stimulatory and repressive impacts on hydrocarbon degradation (Table 8.8). Phthalic acid had a stimulatory effect on phenanthrene mineralization, increasing both rate and extent of mineralization, while malonate had a repressive effect on both parameters. Similarly, increased concentrations of succinate were associated with increased naphthalene degradation, while increased fumarate concentrations repressed naphthalene degradation. Both malonate and fumarate

Table 8.6 Linear multiple regression for hydrocarbon degradation parameters (mineralization and gene copy number) as a function of amino acid concentration.

Hydrocarbon treatment		
Naphthalene	Constant ± standardized β-coefficients	R^2
C2,3O:16S rRNA‡	0.995 +0.546 alanine*	0.299
nahAc:16S rRNA	0.866 +443 alanine*	0.197
Hexadecane	Constant ± standardized β-coefficients	R^2
Rate	1.885 +0.335 alanine†	0.112
alkB	5.895 -0.514 glycine*	0.264
16S rRNA	7.906 -0.454 glycine*	0.202

Regression equation significant at *$p \leq 0.05$ and †$p \leq 0.10$

‡ Indicates ratio between catabolic gene and 16S rRNA gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

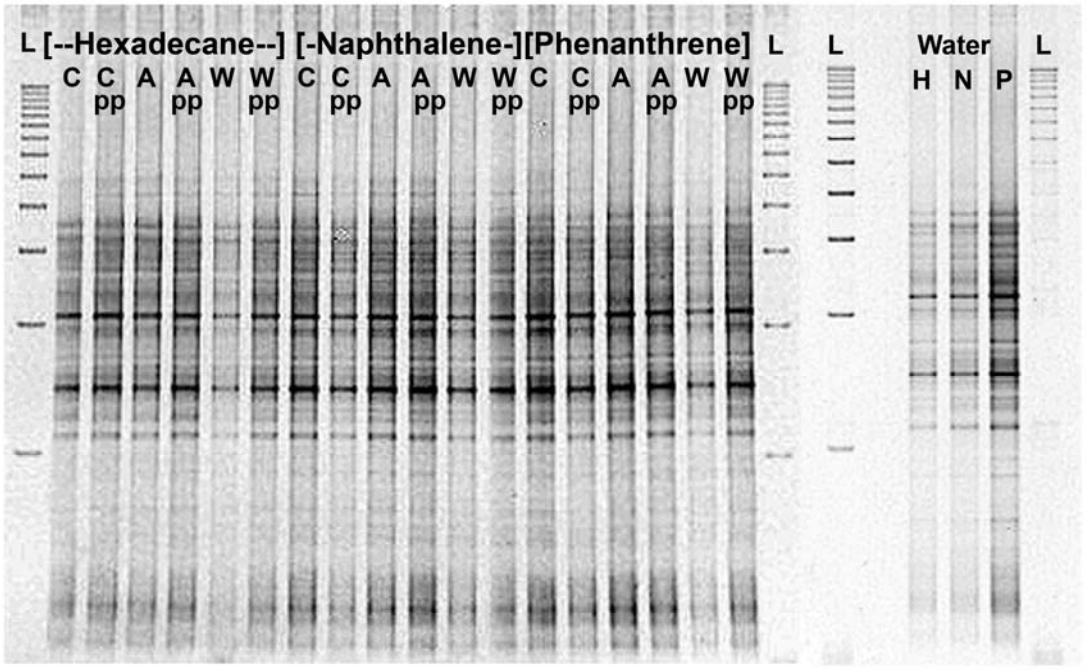

Figure 8.4 Representative DGGE of PCR-amplified 16S rRNA gene fragments from organic acid-amended soil microcosms spiked with n-hexadecane (H), naphthalene (N), or phenanthrene (P). Treatments included phenolics extracted from alfalfa (A) and Altai wild rye (W) exudates and non-planted control (C) eluates, and water-only experimental controls. The tag –pp indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

Table 8.7 Spearman's rank correlation coefficients (n=27) for hydrocarbon mineralization parameters and microbial gene abundance in organic acid fraction amended soil microcosms.

Hydrocarbon microcosm	Gene copy number†		Mineralization‡		
Phenanthrene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.510**	0.419*	-0.015	-0.043	0.089
C,23O		0.941***	0.045	0.247	0.338
nahAc			-0.016	0.277	0.371
Naphthalene	C2,30	*nahAc*	Lag	Rate	Cumulative
16S rRNA	0.516***	0.632***	0.093	0.159	0.129
C,23O		0.841***	-0.072	0.021	0.029
nahAc			-0.059	0.062	-0.029
Hexadecane	alkB		Lag	Rate	Cumulative
16S rRNA	0.792***		0.342	0.000	-0.317
alkB			0.218	0.214	-0.153

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

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

Table 8.8 Linear multiple regression for hydrocarbon degradation parameters (mineralization and gene copy number) as a function of organic acid concentration.

Hydrocarbon treatment		
Phenanthrene	Constant ± standardized β-coefficients	R^2
Lag†	7.668 +0.409 fumarate*	0.167
Rate	1.760 +0.554 phthalic -0.538 malonate**	0.390
Cumulative	45.359 +0.535 phthalic -0.406 malonate*	0.267
Naphthalene	Constant ± standardized β-coefficients	R^2
Rate	4.508 + 0.850 succinate -0.658 fumarate***	0.663
Cumulative	58.716 +0.725 succinate -0.502 fumarate**	0.457
Hexadecane	Constant ± standardized β-coefficients	R^2
Rate	4.682 -0.365 malonate +1.286 maleate -0.990 fumarate***	0.587
16S rRNA‡	7.999 -0.384 succinate*	0.147
alkB	6.701 -0.568 acetate**	0.322
alkB:16SrRNA	0.838 -0.650 acetate***	0.423

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 (%$^{14}CO_2$ day^{-1}); cumulative, cumulative % mineralized

‡ 16S rRNA, eubacterial 16S rRNA; *alkB*, alkane monoxygenase

repressed hexadecane mineralization, while maleate was associated with increased mineralization. Direct effects were also observed on gene copy numbers. Increased acetate concentration was associated with decreased *alkB* copy numbers, independent of 16S rRNA copy numbers.

8.5 Discussion

The primary objective of this study was to isolate and compare the impact of specific exudate fractions on microbial hydrocarbon degradation potential in a long-term hydrocarbon contaminated soil. Amino acid, organic acids, and phenolic compounds extracted from the exudates of alfalfa and Altai wild rye, grown axenically under PAH stressed or non-stressed conditions, were evaluated for their impact on microbial community structure, catabolic potential, and in-situ hydrocarbon mineralization. There was high variability in the actual amount of exudate components added to the microcosms, both within and between exudate treatments (Table 8.2), such that impacts on hydrocarbon degradation potential were not treatment specific (Figure 8.1).

Contrary to our previous study (Chapter 7), there was little correlation between the assessed catabolic genes and hydrocarbon mineralization. (Tables 8.3, 8.5 and 8.7). Soil bacterial communities are diverse and degrade hydrocarbons via numerous different catabolic pathways (Habe and Omori, 2003; van Beilan et al., 2007). In the current study, we assessed the relative quantities of two genes involved in PAH degradation and one gene involved in alkane degradation, chosen because they represent ubiquitous routes for the degradation of both polyaromatic and aliphatic hydrocarbons in soil communities. However, this still only provides us with a simple snapshot in time of the indisputably complex interactions occurring in the soil microbial community. Amending the soil with a single class-fraction, instead of the whole spectrum of exudates, stimulated specific bacterial responses that were not directly correlated with increased degradation in the short term. The concentration-dependent impact of specific compounds within those fractions on degradation potential however, does provide further insight into the mechanisms whereby plant root exudates impact the overall degradation potential of indigenous soil microbial communities.

8.5.1 Phenolic compounds and degradation potential

Several studies have suggested that phenolic compounds released by plants may stimulate PAH degradation. Olson et al. (1999, 2001) found decreased PAH concentration in the upper root zone of *Morus* spp. (mulberry), a plant which is high in phenolic compounds (Hedge and Fletcher, 1996; Leigh et al., 2002). While one study has shown that mulberry root extracts stimulate phenanthrene mineralization by non-specifically increasing PAH degrader populations (da Silva et al., 2006), others have observed that mulberry roots and root extracts may actually repress PAH degradation (Mueller and Shann, 2006; Kamath et al., 2004). It has been shown however, that individual phenolic compounds such as salicylic acid can induce PAH degradation by bacterial isolates (Chen and Aitken, 1999), in part by inducing expression of *nahG*, a gene involved in naphthalene dioxygenase transcription (Kamath et al., 2004). Complex soil communities may not, however respond to inputs in a manner comparable to isolates, and further research has shown that salicylic acid does not induce PAH degradation when added to soil as a pure compound (Yi and Crowley, 2007). While the efficacy of utilizing phenolic releasing plants to remediate PAHs may still be under debate, many common phytoremediation plants release only low levels of phenolic compounds (Rentz et al., 2004). In the current study, only trace amounts of phenolics were extracted from alfalfa and Altai wild rye root exudates which, when added to soil mineralization microcosms (Table 2), had no resultant treatment-specific impact on degradation potential (Figure 8.1A). There were however, specific trends within the phenolic treatments that warrant discussion.

Increased gene copy numbers of the catabolic genes *alkB*, *nahAc* and C2,3O in phenolic treatments were associated with general increases in microbial population in all hydrocarbon treatments (Table 8.3). These non-specific increases in degradation potential did not however, correlate to increased mineralization potential. Instead, in phenanthrene microcosms increased heterotrophic and catabolic microbial populations were significantly associated with decreased phenanthrene mineralization (Table 8.3). There are several possible explanations for these counterintuitive results. Phenolic compounds were extracted from exudates using SPE, with ethyl acetate as the final eluting solvent. In this soil acetate has been shown to increase copy numbers of both

nahAc and C2,3O, yet decrease the mineralization of both phenanthrene and naphthalene (Chapter 9). Ethyl acetate may have had a comparable impact. However, all phenolic extraction columns were extracted with the same amount of ethyl acetate, such that the addition of residual solvent would have been comparable between microcosms. Additionally, water controls did not stimulate significantly greater phenanthrene mineralization (Figure 8.1A). Thus, while residual solvent may contribute to the negative correlation between degradation potential and actual hydrocarbon mineralization, other factors must be involved.

Regression analysis revealed that rate and extent of phenanthrene mineralization decreased but that C2,3O and *nahAc* copy numbers increased in association with increased p-salicylic acid concentration (Table 8.4). Soil bacteria are known to degrade phenanthrene through several routes. In general, a series of enzymatic reactions degrade phenanthrene to 1-hydroxy-2-naphthoic acid, which then either enters the naphthalene degradation pathway (catechol) or the phthalate pathway (protocatechuate) before converging on the β-ketoadipate pathway (Ellis et al., 2006). One of the downstream intermediates of the phthalate pathway is p-salicylic acid, which is a concentration-dependent inducer of genes involved in protocatechuate metabolism (Bertani et al., 2001). Although downstream intermediates of the naphthalene pathway, including salicylic acid, induce PAH dioxygenase activity and PAH mineralization (Chen and Aitken (1999), we found no information as to the impact of p-salicylic acid on upstream processes. Given the results of this study, we hypothesize that increased levels of p-salicylic had a catabolite inhibition effect on upstream catabolic gene expression, thus reducing phenanthrene mineralization, but had a concomitant stimulatory effect on the growth of hydrocarbon-degrading bacteria via increased influx of compounds to the TCA cycle. As expression of numerous catabolic genes is known to be repressed during exponential growth phases (Cases and de Lorenzo, 2005; Ruiz-Manzano et al., 2005), a simple stimulation of growth may also have accounted for the observed patterns. We acknowledge however, that the amended levels of p-salicylic acid may have been too low to actually impact degradation and that the observed correlations may have been simply fortuitous. Further study is warranted before firm conclusions may be drawn.

8.5.2 Amino acids and degradation potential

Amino acids are a numerically and metabolically significant component of plant root exudates (Grayston et al., 1996). As with all root exudate components, the amount and type of amino acids released by a given plant species is highly variable, and both efflux and influx impact availability to rhizosphere microbial communities (Lesuffleur et al., 2007). While amino acids have been implicated in aromatic gene repression (Putrins et al., 2007; Sze and Shingler, 1999), little information is available about their impact on the hydrocarbon degradation potential of soil bacteria. One study found that the amino acid glutamate repressed the phenanthrene degrading activity of *P. putida* ATCC 17484 but that repression was not concentration dependent (Rentz et al., 2004). A previous study by our group however, found the concentration of glycine and alanine had both repressive and stimulatory (respectively) impacts on soil microbial communities and on hydrocarbon degradation (Chapter 7). The impacts of amino acids did not occur in isolation however, as organic acids within the root exudates made synergistic and antagonistic contributions to all parameters. In the current study we found that specific compound concentrations within the isolated amino acid fraction also impacted degradation parameters, even in the absence of co-substrates.

Regression analysis showed that increased concentrations of alanine were associated with increased rates of hexadecane mineralization (Table 8.6). This finding supports results of our previous study, which found that alanine was associated with the stimulation of hexadecane mineralization (Chapter 7). As in that previous study, alanine did not impact *alkB* copy numbers and *alkB* copy numbers were not associated with increased hexadecane mineralization. Although *alkB* copy number did decrease with increasing glycine concentration (Table 8.6), this did not adversely impact hexadecane mineralization. We previously hypothesized that hexadecane mineralization in the current study soil may occur via an alternate aliphatic degradation enzyme, cytochrome P450 hydroxylase (van Beilan et al., 2007), and results of this study do not contradict that hypothesis. Cytochrome P450 hydroxylases are up-regulated in the presence of alkanes (Sabirova et al., 2006) and it is possible that root exudate components, including amino acids, also have stimulatory effects. Alanine was also associated with relative increases in C2,3O and *nahAc* copy numbers (Table 8.6), catabolic genes which are

typically carried on conjugative plasmids (Nojiri et al., 2002). The very high correlation between copy numbers of these genes in this study, usually irrespective of increased 16S rRNA copy numbers (Table 8.3, 5, 7), indicates that increased plasmid transfer may be the primary mode by which the degradative fitness of this soil microbial community is increased. While there is little information available on the impact of amino acids on conjugation frequency, alanine has been shown to stimulate natural transformation in *Acinetobacter* spp. (Nielsen and van Elsas, 2001) and may exert a comparable stimulatory impact on other horizontal gene transfer events.

8.5.3 Organic acids and degradation potential

Studies with bacterial isolates have found that organic acids may have either a stimulatory or a repressive effect on PAH hydrocarbon degradation potential. Kuiper et al. (2002) found that succinate promoted a higher level of naphthalene degradation gene expression in *P. putida* PCL1444 than did fumarate or other organic acids. Similarly, acetate was found to be a strong inhibitor of the *P. putida* TOL plasmid *xyl* operon, while citrate and succinate had little negative impact (Collier et al., 1996). Catabolite repression also occurs during degradation of aliphatic hydrocarbons, with organic acids such as succinate repressing expression of *alkB* promoters on the *P. putida* OCT plasmid (Dinamarca et al., 2003). In the current study, we found that organic acids also had comparable impacts on the degradation potential of complex soil microbial communities. Although not linked to a treatment specific effect (Figure 8.1C), the assessed degradation parameters were strongly impacted by the concentration of specific organic acids amended to the microcosms. Regression analysis revealed that fumarate, malonate, and acetate were associated with decreased degradation potential, while succinate, phthalate, and maleate were associated with increased degradation potential (Table 8.8).

Previous research on the current study soil (Chapter 7) revealed that malonate was associated with decreased degradation, potentially acting by repressing microbial metabolic activity and decreasing catabolic plasmid transfer. In the current study, malonate was also associated with decreased mineralization of both phenanthrene and hexadecane (Table 8.8). The repressive effect of malonate on phenanthrene

mineralization was mitigated by increased concentrations of phthalic acid, an aromatic dicarboxylic acid. As previously discussed, the phthalate pathway is one route by which soil bacteria degrade phenanthrene (Habe and Omori, 2003). That phthalic acid was not associated with increased naphthalene degradation suggests that a particular subset of bacteria was involved in phenanthrene degradation. Phenanthrene degraders which use the phthalate pathway, including *Alcaligenes* AFK2 and *Nocardioides* KP7, are generally less efficient degraders of naphthalene (Habe and Omori, 2003). Both these genera of bacteria have been previously identified in the study soil (Chapters 5 and 7), and the difference in DGGE assessed community structure between phenanthrene and naphthalene microcosms (Figure 8.4) may represent the increased prevalence of such phthalate-utilizing bacteria. As phthalic acid was not associated either with increased heterotrophic populations or with increased catabolic gene copy number (Table 8.8), its impact is likely to be directly on gene expression (Doddamani et al., 2000), possibly in a manner comparable to salicylic acid (Chen and Aitken, 1999; Kamath et al., 2004), a key downstream intermediate of the naphthalene or catechol degradation pathway. It is worth noting that a later downstream intermediate of the phthalate pathway, p-salicylic acid, was associated with increased catabolic gene copy numbers (Table 8.4) but decreased phenanthrene mineralization, possibly due to specific catabolite repression.

Succinate and fumarate also had opposing impacts on naphthalene mineralization. Naphthalene mineralization was positively associated with increased succinate concentration, but negatively associated with increased fumarate concentration (Table 8). Catabolite repression of genes involved in aromatic degradation is a common phenomenon with TCA cycle intermediates such as succinate and fumarate (Diaz and Prieto, 2000). These repressive effects are not, however, equivalent. Several studies have reported that succinate has a lower inhibitory effect than other organic acids (Kamath et al., 2004), no inhibitory effect (Collier et al., 1996), or an actual stimulatory effect on catabolic gene expression (Kuiper et al., 2002). As succinate may also stimulate horizontal gene transfer events (Nielsen and van Elsas, 2001), a combination of increased gene expression, increased gene transfer, and generally increased degrader populations (Table 8.7) may have contributed to the relationship between increased

succinate concentration and increased naphthalene degradation. More research is required to further elucidate the impact of succinate in the current study soil.

8.6 Conclusion

Specific compounds within class specific fractions of root exudates were found to be associated with changes to the degradation potential of soil microbial communities indigenous to weathered hydrocarbon contaminated soil. In this study, we found that, in general, malonate and fumarate were associated with decreased degradation potential while phthalic acid, succinate, and alanine were associated with increased degradation potential. Several specific yet inter-related mechanisms, including catabolite repression, catabolite stimulation, and changes to horizontal gene transfer rates were hypothesized to account for replicate- specific differences in degradation potential. The results of the current study corroborate results of a previous study by our group which used whole root exudates (Chapter 7), where organic and amino acids exerted an influence on degradation potential even in the presence of dominant root exudate components such as sugars. While sugars such as glucose are known to exert global control of catabolic pathways, organic acids, amino acids, and phenolic compounds exert local yet dominant impacts on catabolic pathways and degradation potential. This research shows that the ratio of specific compounds released by plant roots, rather than total amount of exudation, will be key to determining whether degradation of hydrocarbons is increased or inhibited. While the impacts observed in this study soil may not be universal to other soil or contamination types, correlative impacts undoubtedly occur. Further research into these interactions may provide a means for standardizing phytoremediation treatments, and thus alleviate the current state of ambiguity surrounding the efficacy of phytoremediation for hydrocarbon contamination.

9.0 IMPACT OF SINGLE ORGANIC ACIDS ON THE HYDROCARBON DEGRADATION POTENTIAL OF INDIGENOUS SOIL MICROBIAL COMMUNITIES

9.1 Preface

In the previous two studies (Chapters 7 and 8) we found that organic acids had a dominant influence on the hydrocarbon degradation potential of soil microbial communities. Some organic acids, such as malonate, which may have repressed microbial metabolic activity and decreased the transfer of plasmid-borne catabolic genes, were consistently associated with repressed degradation potential. Others, such as succinate, were consistently associated with increased degradation potential. A final group, including fumarate and acetate, were associated either with repressed or increased degradation potential. These general relationhships occurred when organic acids were added to soil as part of a complex mixture of root exudates (Chapter 7) or when added as an exudate-extracted mixture of organic acids (Chapter 8). The current study was designed to further elucidate the mechanisms whereby specific organic acids either repress or stimulate hydrocarbon degradation potential. Increasing concentrations of single organic acids were added to weathered hydrocarbon contaminated soil and the impact on indigenous microbial communities was assessed. Four organic acids with the potential to increase degradation were assessed; acetate, citrate, fumarate and succinate.

9.2 Introduction

Phytoremediation of hydrocarbons occurs primarily via rhizodegradation, where plants provide a suitable physical and nutritional environment for hydrocarbon-degrading microbial communities. Plants release up to 40% of their total photosynthates through their roots (Grayston et al., 1996), and these exudates are known to be an important factor governing plant-rhizosphere microbial community interaction (for review see Bertin et al., 2003; Patterson, 2003; Walker et al., 2003). Exudates may

increase the degradative fitness of the indigenous microbial community by increasing general population densities, increasing specific degrader population densities, increasing catabolic gene expression, and increasing horizontal transfer of catabolic genes. Both whole root exudates (Corgie et al., 2003; Rentz et al., 2004) and separate classes of root exudates (Chen and Aitken, 1999; Collier et al., 1996; da Silva et al., 2006; Kuiper et al., 2002; Putrins et al., 2007; Sze and Shingler, 1999) have been implicated in one or all of these mechanisms.

Individual compounds within an exudate class may also have opposing impacts on degradation potential. Specific organic acids, which as a group comprise up to 2% of total root exudates (Dakora and Phillips, 2002; Farrar and Jones, 2000), may increase the degradation potential of a soil by increasing horizontal gene transfer events (Nielsen and van Elsas, 2001) and by stimulating catabolic gene expression (Kuiper et al., 2002), but may also decrease the degradation potential via catabolite repression (Rentz et al., 2004; Kamath et al., 2004). Catabolite repression is classically understood as the preferred utilization of one carbon substrate over another by bacteria. In the presence of the preferred substrate, global or local regulatory mechanisms repress expression of genes required to use the non-preferred substrate. In global regulation, global control proteins such as the Crc regulatory proteins of *Pseudomonas* act on numerous catabolic operons (Collier et al., 1996). During local regulation specific pathway intermediates or downstream substrates act on key operons (Schingler, 2003).

Downstream intermediates of hydrocarbon degradation pathways have been shown to both repress and activate upstream gene expression, depending on the particular regulatory system involved (Tropel and van der Meer, 2004). Some common root exudate components, including the citric acid cycle intermediates succinate, citrate and fumarate, are believed to have a primarily repressive impact on aromatic compound metabolism (Diaz and Prieto, 2000). Previous research by our group however, found that specific organic acids, including fumarate and succinate, had a stimulatory effect on both hydrocarbon degradation potential and activity (Chapters 7 and 8). Although some of the increased degradation was hypothesized to be linked to increased catabolic gene transfer, if **c**atabolite repression was dominant then degradation should have been suppressed.

In order to further clarify the impacts of these organic acids on the hydrocarbon degradation potential of a complex microbial community, increasing concentrations of acetate and specific citric acid cycle intermediates were added to weathered hydrocarbon contaminated soil and the impact on indigenous microbial communities was assessed.

9.3 Materials and methods

9.3.1 Hydrocarbon mineralization potential

Soil ^{14}C- hydrocarbon mineralization microcosms were set up and sampled as previously described (Chapter 8**)**. Briefly, 50,000 dpm (100 mg kg^{-1}) of [1-^{14}C]n-hexadecane, [1-^{14}C]naphthalene, or [9-^{14}C]phenanthrene (specific activities, 12, 6.2, and 8.2 mCi $mmol^{-1}$ respectively; Sigma-Aldrich, Mississauga, Ont.) was added to a weathered hydrocarbon-contaminated (3700 mg kg^{-1}) saline-sodic soil. Microcosm soil was acclimatized to room temperature and to 20% moisture for 2 weeks prior to microcosm setup. Increasing concentrations (0, 25, 50, 75, 100 and 200 mg kg^{-1}) of filter sterilized acetate, citrate, fumarate, or succinate (Sigma-Aldrich, Mississauga, Ont.) solutions were added in 500 µl aliquots to serum vials containing 4 g soil, for a final moisture content of 30%. The pH of the 200 mg kg^{-1} organic acid solutions ranged from 6.7 (acetate) to 6.9 (fumarate) and all other concentrations of solutions had an average pH of 7.0. Three replicates were established for each treatment. Abiotic controls were established using autoclaved soil (2 × 1hr with a one week resting interval). Phenanthrene microcosms were sampled after 2 and 4 wks. Non-radioactive replicate microcosms were established for all treatment replicates with all assessed hydrocarbons for use in subsequent molecular analyses.

9.3.2 Microbial hydrocarbon degradation potential

9.3.2.1 Microbial community DNA extraction

Total community DNA was extracted from all samples using a bead-beating protocol previously outlined in 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 100 µl TE (pH 8.0), and purified using PVPP columns. Soil sub-samples (0.50 ± 0.01g) from each non-radioactive replicate mineralization microcosm for all treatments and hydrocarbons were extracted. Following PVPP purification of 50 µl total DNA extract all volumes were standardized. Final DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen) and by spectrophotometer evaluation.

9.3.2.2 Quantitative PCR assessment

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, catechol 2,3 dioxygenase (C2,3O), naphthalene dioxygenase (*nahAc*), and alkane monoxygenase (*alkB*), were assessed. 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 manufacturers protocol incorporating the primer concentration listed in Table 9.1. After the initial 15 min denaturing period, PCR amplification proceeded for 38 cycles of 30 s denaturing at 94^{o}C, 40 s annealing at the appropriate temperature (Table A), 1 min extension at 72 ^{o}C, and a data collection step of 45 s at 80 ^{o}C. A final melt curve analysis from 5 ^{o}C below amplification temperature to 95 ^{o}C concluded the cycling program. Absolute quantification was performed by comparison with standard curves. Ten fold DNA standards ranging from 10^2 to 10^6 gene copy numbers were prepared from serial dilutions of DNA extracts from positive control strains (Table 9.1). Gene copy numbers were calculated from the concentrations of positive-control strains, quantified spectrophotometrically, assuming a molecular mass of 660Da 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 > 0.99. 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).

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Table 9.1 Primers and amplification conditions used for Q-PCR amplification

Target gene Primer sequence†	T_a‡ (^oC)	Primer (μM)	Expected product size (bp)	Reference	Control strain
Naphthalene dioxygenase (*nahAc*)					
F: 5'-CAA AAR CAC CTG ATT YAT GG	47	0.3	377	Baldwin et al., 2003	*P. putida*
R: 5'-AYR CGR GSG ACT TCT TTC AA					ATCC 17484
Alkane monoxygenase (*alkB*)					
F: 5'-AAC TAC ATC GAG CAC TAC GG	50	0.5	100	Powell et al., 2006	*P. putida*
R: 5'-TGA AGA TGT GGT TGC TGT TCC					ATCC 29347
Catechol 2,3 dioxygenase (C2,3O)					
F: 5'- AGG TGC TCG GTT TCT ACC TGG CCGA	65	0.3	406	Luz et al.,	*P. putida*
R: 5'- ACG GTC ATG AAT CGT TCG TTG AG				2004	ATCC 33015
Universal eubacterial 16S rRNA (16S)					
F: 5'-CTA CCA GGG TAT CTA ATC C	55	0.15	450	Rölleke et al., 1996	*P. putida*
R: 5'-CCT ACG GGA GGC AGC AG				Lee et al., 1993	ATCC 17484

†Forward (F) and reverse (R) primers are indicated.

‡T_a, annealing temperature used during real-time PCR

9.3.3 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 organic acid concentration and degradation parameters were assessed by Kendall tau rank correlation analysis. Relationships between microbial gene quantification and mineralization were assessed by Spearman's rank correlation analysis

9.4 Results

9.4.1 Phenanthrene degradation potential

The assessed organic acids had very different impacts on phenanthrene mineralization and on overall degradation potential. After 2 weeks, mineralization in all acetate, citrate and fumarate replicates was approximately 10%, relative to approximately 15% in succinate and water microcosms (data not shown). At this stage, the repressive impact of the organic acids was not dose dependent. By 4 weeks succinate still exerted neither repressive nor stimulatory effects on mineralization (Figure 9.1A), while both citrate and fumarate decreased mineralization in a dose dependent response (Figure 9.1A), with increased concentrations of organic acid generally resulting in decreased mineralization. With acetate this trend was reversed, such that the lowest concentration had the greatest repressive effect (Figure 9.1A).

With the exception of fumarate, the assessed gene copy numbers were not specifically associated with cumulative mineralization. Decreased mineralization at two weeks in fumarate microcosms was associated with non-specific decreases in the catabolic genes C2,3O and *nahAc* (Table 9.2). By 4 weeks however, this relationship was no longer significant. In contrast to the non-specific decreases in catabolic genes that occurred in fumarate microcosms, acetate stimulated specific increases in C2,3O and *nahAc* gene copy numbers (Figure 9.1, Table 9.2). 16S rRNA gene copy numbers decreased (Figure 9.1B) but catabolic gene copy numbers increased (Figure 9.1C and

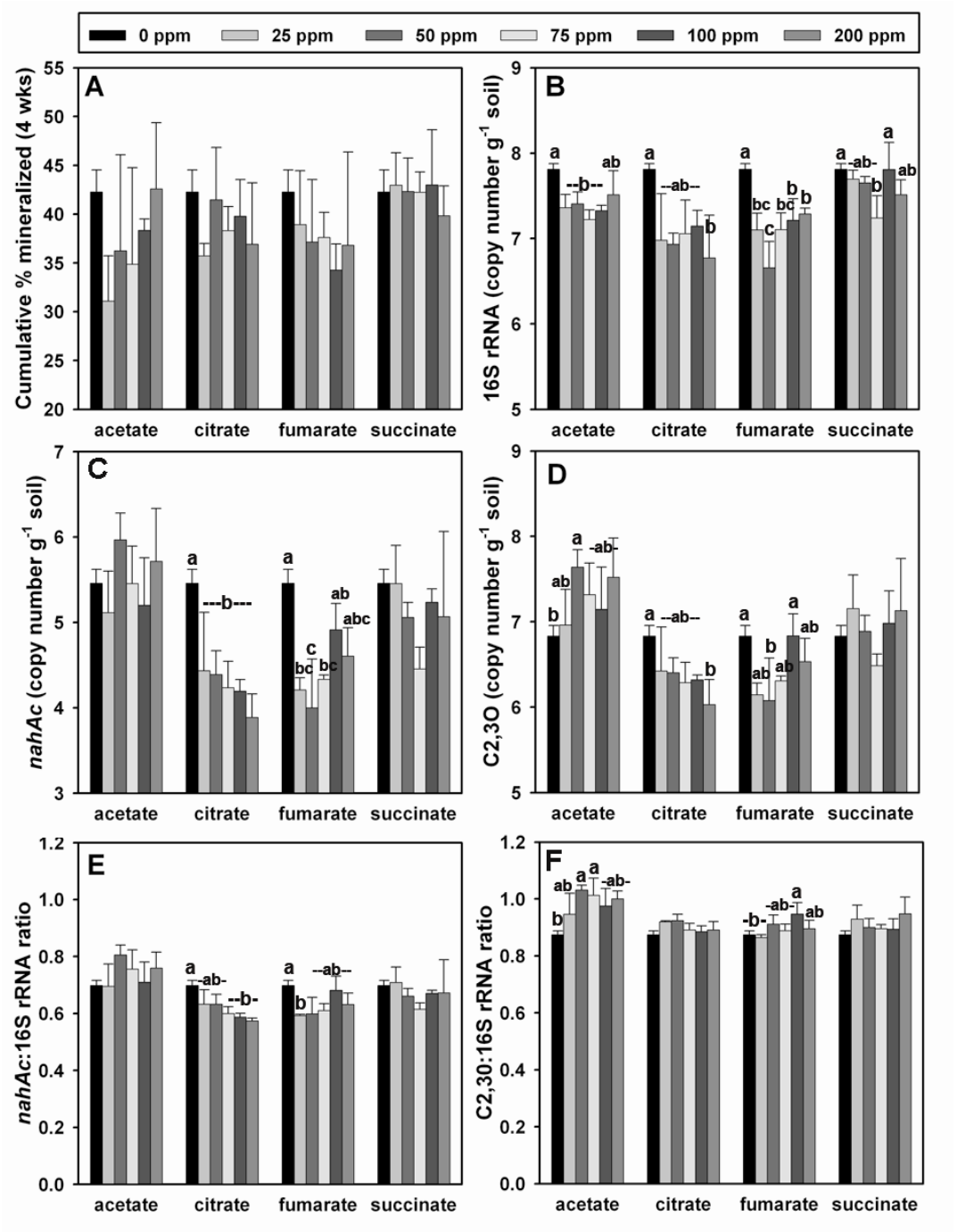

Figure 9.1 Hydrocarbon degradation parameters for increasing concentrations of single organic acids added to 14C-phenanthrene spiked mineralization microcosms. A) Cumulative percent mineralization at 4 weeks; B) 16S rRNA gene copies g^{-1} dry soil (log scale); C) *nahAc* gene copies g^{-1} dry soil (log scale); D) C2,3O gene copies g^{-1} dry soil (log scale); E) Ratio of *nahAc* to 16S rRNA copy numbers; F) Ratio of C2,3O to 16S rRNA copy numbers. Data are presented as means (n = 3) and error bars indicate 1 SD. Significant differences ($p \leq 0.05$) within a given organic acid treatment are indicated by different letters.

Table 9.2 Correlation coefficients (n = 18) for microbial gene abundance and hydrocarbon mineralization parameters in phenanthrene soil microcosms.

Organic acid	Gene copy number†			Mineralization‡	
Acetate	16S rRNA	C2,3O	*nahAc*	2 weeks	4 weeks
Concentration§	-0.285	0.369*	0.188	-0.077	0.035
16S rRNA		0.018	0.267	0.445	0.434
C,23O			0.926***	-0.128	0.001
nahAc				-0.020	0.146
Succinate	16S rRNA	C2,3O	*nahAc*	2 weeks	4 weeks
Concentration	-0.386*	0.007	-0.355*	-0.216	-0.257
16S rRNA		0.459*	0.810***	0.127	0.141
C,23O			0.676**	0.255	0.098
nahAc				0.234	0.018
Fumarate	16S rRNA	C2,3O	*nahAc*	2 weeks	4 weeks
Concentration	-0.090	0.118	0.021	-0.285	-0.244*
16S rRNA		0.734***	0.756***	0.635**	0.191
C,23O			0.953***	0.470*	0.049
nahAc				0.459*	0.063
Citrate	16S rRNA	C2,3O	*nahAc*	2 weeks	4 weeks
Concentration	-0.383*	-0.522**	-0.578***	-0.104	-0.132
16S rRNA		0.841***	0.897***	0.253	0.249
C,23O			0.971***	0.146	0.273
nahAc				0.170	0.346

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

‡Mineralization parameters: cumulative % mineralized.

§Concentration of added organic acid assessed using Kendall tau rank correlation. Spearmans rank correlation used for all other parameters.

D), resulting in increased catabolic genes relative to general microbial densities (Figure 9.1E and F). While this may have impacted the mineralization trend seen with acetate, there were no statistically significant correlations (Table 9.2). Similar to fumarate, changes in degradation potential in succinate microcosms were associated with non-specific changes in general microbial densities (Table 9.2). This did not however, significantly impact final phenanthrene degradation. The final organic acid, citrate, effected a concentration-dependent non-specific decrease in both microbial densities and associated catabolic genes (Table 9.2, Figure 9.1).

9.4.2 Naphthalene degradation potential

All organic acids except succinate had a repressive effect on naphthalene mineralization. Increasing concentrations of acetate were highly correlated with decreased naphthalene mineralization (Figure 9.2A, Table 9.3), while increased concentrations of succinate generally increased mineralization (Figure 9.2A). Decreased mineralization in acetate microcosms was strongly associated with specific increases in copy numbers of both C2,3O and *nahAc* genes (Table 9.3). Although the general increase in gene copy numbers found with increased acetate concentration was significant only at $p < 0.10$ (Figure 9.2C and D), correlation analysis indicated that the relative ratio of catabolic genes to 16S rRNA genes increased with increasing acetate ($r > 0.313$, $p < 0.05$; data not shown). Citrate and fumarate had significant repressive impacts on 16S rRNA gene copy numbers (Figure 9.2B, Table 9.3) and general, though not significant, repressive impacts on catabolic gene copy numbers (Figure 9.2C and D). Increased concentrations of succinate had very different overall impacts on degradation potential and resulted in general increases in mineralization (Figure 9.2A) concomitant with significant increases in the relative ratio of catabolic genes found in the soil (Figure 9.2E and F). The increased relative catabolic gene ratio in succinate microcosms was due both to decreases in 16S rRNA gene copy numbers and increases in catabolic gene copy numbers.

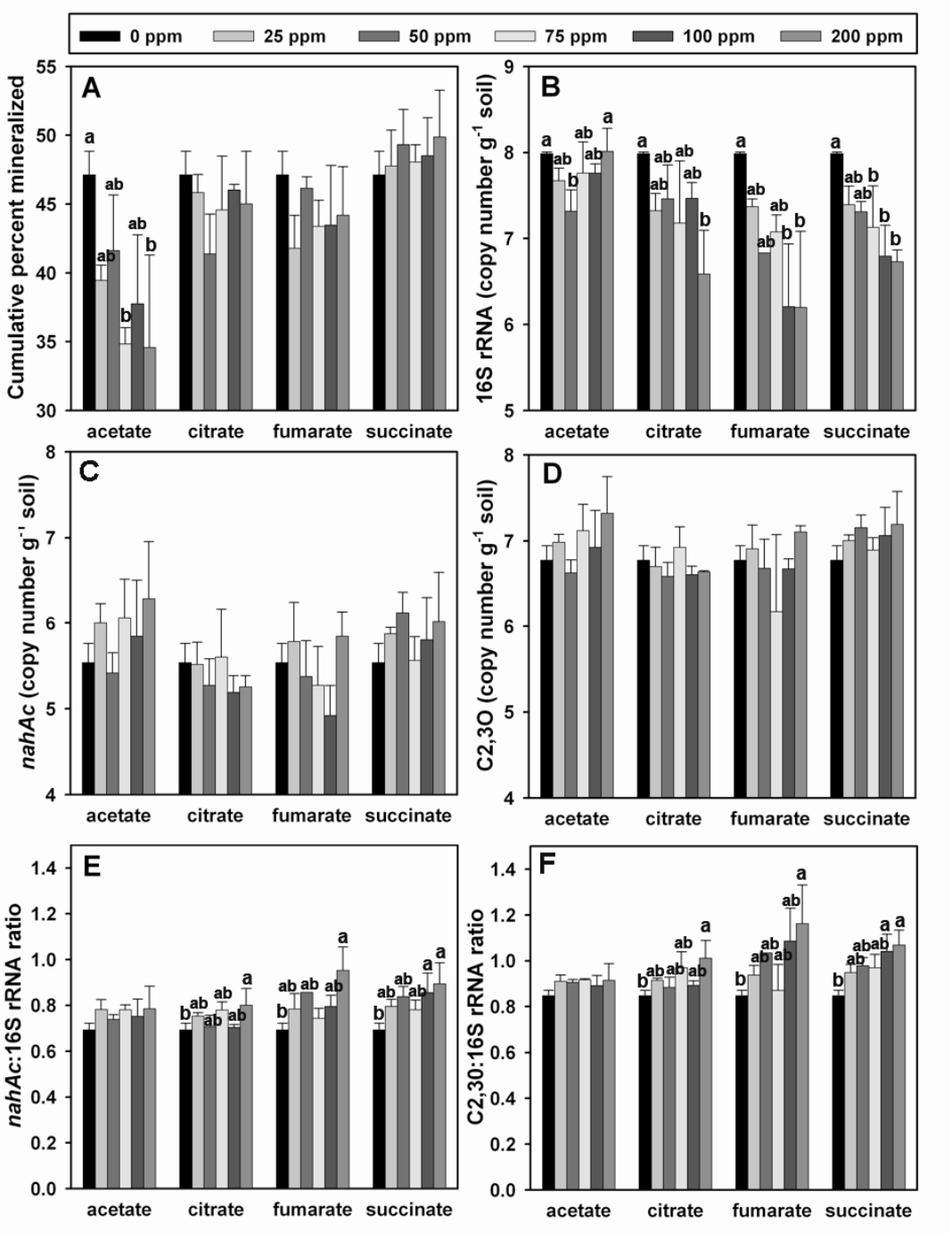

Figure 9.2 Hydrocarbon degradation parameters for increasing concentrations of single organic acids added to 14C-naphthalene spiked mineralization microcosms. A) Cumulative percent mineralization at 4 weeks; B) 16S rRNA gene copies g^{-1} dry soil (log scale); C) *nahAc* gene copies g^{-1} dry soil (log scale); D) C2,3O gene copies g^{-1} dry soil (log scale); E) Ratio of *nahAc* to 16S rRNA copy numbers; F) Ratio of C2,3O to 16S rRNA copy numbers. Data are presented as means (n = 3) and error bars indicate 1 standard deviation. Significant differences ($p \leq 0.05$) within a given organic acid treatment are indicated by different letters.

Table 9.3 Correlation coefficients (n = 18) for microbial gene abundance and hydrocarbon mineralization parameters in naphthalene soil microcosms.

Organic acid	Gene copy number†			Mineralization‡
Acetate	16S rRNA	C2,30	*nahAc*	2 weeks
Concentration§	0.077	0.313	0.293	-0.508**
16S rRNA		0.387	0.311	0.030
C,23O			0.981***	-0.730***
nahAc				-0.748***
Succinate	16S rRNA	C2,30	*nahAc*	2 weeks
Concentration	-0.717***	0.293	0.146	0.230
16S rRNA		-0.381	-0.162	-0.263
C,23O			0.922***	0.177
nahAc				0.185
Fumarate	16S rRNA	C2,30	*nahAc*	2 weeks
Concentration	-0.704***	0.090	-0.104	-0.104
16S rRNA		-0.068	0.247	0.035
C,23O			0.860***	0.069
nahAc				-0.005
Citrate	16S rRNA	C2,30	*nahAc*	2 weeks
Concentration	-0.532**	-0.188	-0.313	-0.188
16S rRNA		0.377	0.522*	0.071
C,23O			0.814***	-0.125
nahAc				0.079

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase

‡Mineralization parameters: cumulative % mineralized.

§Concentration of added organic acid assessed using Kendall tau rank correlation. Spearmans rank correlation used for all other parameters.

9.4.3 Hexadecane degradation potential

The impact of organic acids on the mineralization of the aliphatic hydrocarbon hexadecane was similar to that observed on PAHs. Acetate, citrate and fumarate decreased mineralization, while succinate stimulated mineralization at an extent comparable to the control (Figure 9.3A). The decreased mineralization in both acetate and citrate microcosms was significantly correlated to increased organic acid concentration (Table 9.4), while that in fumarate microcosms was more generally repressed (Figure 9.3A). The addition of organic acids resulted in relatively small decreases in overall microbial abundance (Figure 9.3B), but significant decreases in catabolic gene abundance (Figure 9.3C), resulting in a general decrease in the relative ratio of *alkB* to 16S rRNA (Figure 9.3D) For succinate, fumarate and citrate, decreases in *alkB* were correlated to increased organic acid concentration, while *alkB* decreases in acetate microcosm were not a function of concentration (Table 9.4, Figure 9.3C). The organic acids clearly had a repressive effect on both hexadecane mineralization and on microbial populations, but these two components were not in themselves highly correlated (Table 9.4).

9.5 Discussion

The primary goal of this study was to determine how specific exudate components impacted degradation potential. Several possible mechanisms may increase degradation fitness in rhizosphere microbial communities; increased general microbial population densities, increased specific degrader population densities, increased catabolic gene expression, and increased horizontal transfer of catabolic genes. In previous studies we concluded that root exudates increased the degradation fitness of the current study soil by the latter mechanism, but that this increased fitness was moderated by decreased microbial activity and by catabolite repression. Results from the current study further validate this hypothesis, as single organic acids had a comparable effect to that observed when they were part of a complex solution of exudates (Chapter 7 and 8). In general, acetate decreased hydrocarbon mineralization but increased the degradation potential, citrate and fumarate moderately and variably repressed both mineralization

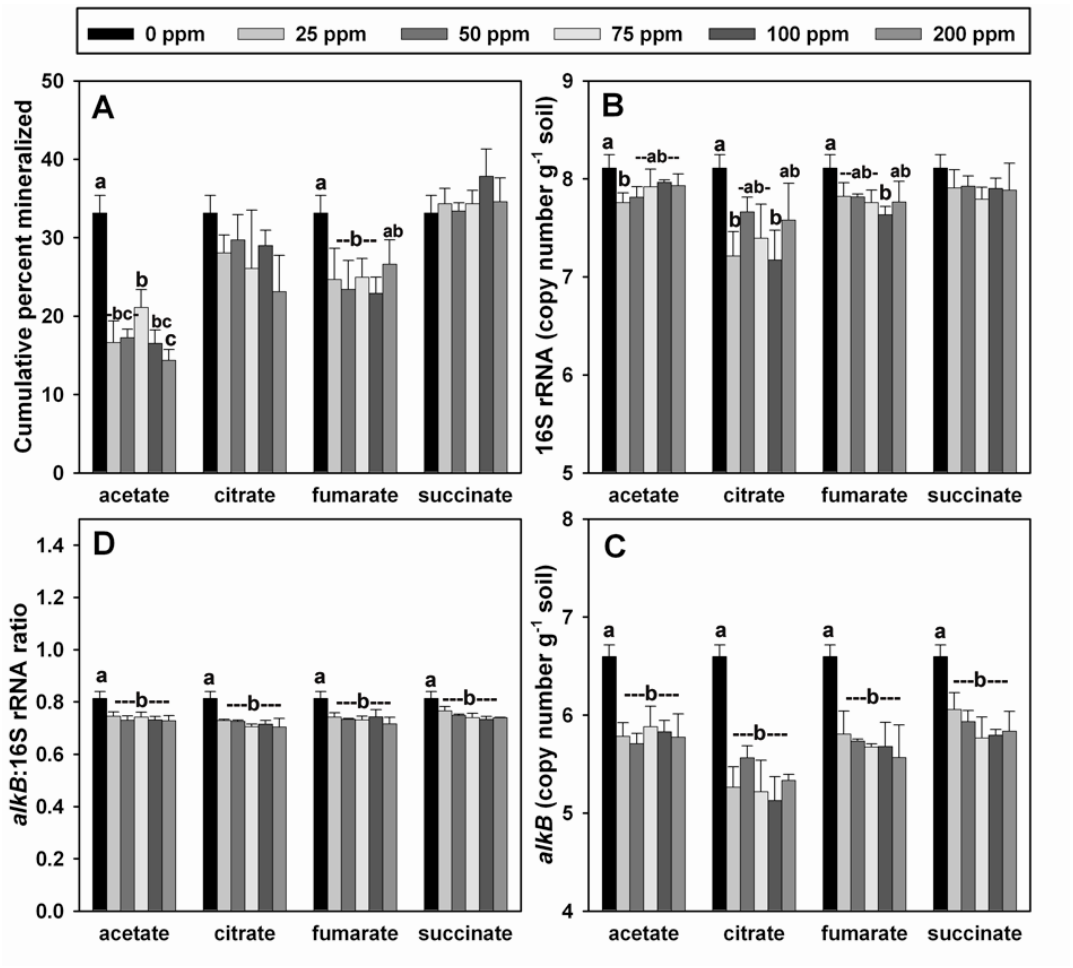

Figure 9.3 Hydrocarbon degradation parameters for increasing concentrations of single organic acids added to 14C-hexadecane spiked mineralization microcosms. A) Cumulative percent mineralization at 4 weeks; B) 16S rRNA gene copies g^{-1} dry soil (log scale); C) Ratio of *alkB* to 16S rRNA copy numbers; D) *alkB* gene copies g^{-1} dry soil (log scale). Data are presented as means (n = 3) and error bars indicate 1 standard deviation. Significant differences ($p \leq 0.05$) within a given organic acid treatment are indicated by different letters.

Table 9.4 Correlation coefficients (n = 18) for microbial gene abundance and hydrocarbon mineralization parameters in n-hexadecane soil microcosms.

Organic acid	Gene copy number†		Mineralization‡
Acetate	16S rRNA	*alkB*	2 weeks
Concentration§	-0.021	-0.257	-0.480**
16S rRNA		0.621***	0.255
alkB			0.346*
Succinate	16S rRNA	*alkB*	2 weeks
Concentration§	-0.271	-0.564**	0.244
16S rRNA		0.569***	-0.294
alkB			-0.255
Fumarate	16S rRNA	*alkB*	2 weeks
Concentration§	-0.438*	-0.489**	-0.230
16S rRNA		0.590***	0.451**
alkB			0.302
Citrate	16S rRNA	*alkB*	2 weeks
Concentration§	-0.265	-0.433*	-0.383*
16S rRNA		0.809***	0.302
alkB			0.420*

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

†Gene copy numbers g^{-1} dry soil: 16S rRNA, eubacterial 16SrRNA; *alkB*, alkane monoxygenase

‡Mineralization parameters: cumulative % mineralized.

§Concentration of added organic acid assessed using Kendall tau rank correlation. Spearmans rank correlation used for all other parameters.

and degradation potential, and succinate either did not impact or increased both mineralization and degradation potential.

9.5.1 Impact of single organic acids on PAH degradation potential

Previous studies have shown that acetate is a strong inhibitor of aromatic catabolic gene expression in bacterial isolates. Kamath et al. (2004) found that *nahG* expression in *P. putida* ATCC 17484 was reduced approximately 40% relative to the control when the bacteria were exposed to naphthalene in the presence of 40 mg L^{-1} acetate. Similarly, acetate has been shown to be a strong repressor of *xyl* operon promoters on the *P. putida* TOL plasmid (Collier et al., 1996; Holtel et al., 1994). Degradation by other bacterial groups is also impacted, as Ampe et al. (1996) found that utilization of phenol and its metabolic intermediates by *Alcaligenes eutrophus* was inhibited by acetate. Although no comparable studies have been done on complex soil microbial communities, the results of this study indicate that a catabolite repression effect also occurs. In naphthalene microcosms increased acetate concentrations were correlated with decreased mineralization (Table 9.3, Figure 9.2A). This decreased degradation was not however, associated with a decrease in the actual degradation potential of the soil community. Instead, both absolute (Figures 9.2C and 9.2D) and relative (Figures 9.2E and 9.2F) PAH catabolic gene copy numbers increased with increasing acetate concentration, indicating that a specific increase in catabolic potential occurred. While repressing catabolic gene expression, acetate concomitantly stimulated catabolic gene transfer. A comparable impact occurred in PAH mineralization microcosms amended with both total and fraction-specific exudates (Chapters 7 and 8). In both cases, increased copy numbers of C2,3O and *nahAc* were associated with increased acetate concentration. Nielsen and van Elsas (2001) found that natural transformation of *Acinetobacter* sp. BD413 was stimulated by acetate, and in the current study soil acetate appears to increase the degradative potential of soil microbial communities by stimulating increased catabolic plasmid transfer.

A comparable increase in degradation potential occurred in acetate-amended phenanthrene microcosms (Figures 9.1C-F), but mineralization patterns were significantly different. With this PAH the lowest concentrations of acetate (25 mg kg^{-1})

repressed mineralization by 25% relative to the control, but then mineralization increased with each increase in concentration (Figure 9.2A). Although no direct correlation was observed between the increased mineralization and increased catabolic gene copy numbers (Table 9.2), a relationship likely exists. The longer experimental time-frame used for phenanthrene microcosms compared to naphthalene microcosms (4 vs. 2 weeks) may have been sufficient for the increased degradative fitness of the microbial community to outweigh the repressive effects of any remaining acetate. However, given the complex system of regulatory mechanisms that govern aromatic degradation (Tropel and van der Meer**,** 2004), it is also equally possible that acetate exerts an as yet unknown stimulatory effect on phenanthrene degradation pathways.

As previously discussed, citric acid cycle intermediates are known to impact aromatic compound metabolism via catabolite repression (Diaz and Prieto, 2000). Catabolite repression is neither a single nor a simple mechanism and the stage at which it occurs differs with organic acid, aromatic compound, and bacterial species (Tropel and van der Meer**,** 2004). For example, in chloro-aromatic metabolic pathways fumarate competitively inhibits a key transcriptional activator, ClcR, in a concentration dependent manner (McFall et al. 1997). In aromatic pathways however, a related transcriptional activator, CatR, is not repressed by fumarate (McFall et al., 1998). In this study, although both citrate and fumarate generally reduced the mineralization of both PAHs relative to the controls (Figures 9.1A and 9.2A), their impact on the soil microbial community differed. Citrate reduced phenanthrene degradation at least in part by non-specifically decreasing both heterotrophic and degrader microbial communities in a concentration-dependent manner (Table 9.2). In contrast, increased concentrations of fumarate were directly correlated with decreased phenanthrene mineralization (Table 9.2) but not with decreased microbial community abundance. This suggests that fumarate acts as a catabolite inhibitor of gene expression in phenanthrene degradation pathways in the current study soil, while citrate does not. In naphthalene microcosms however, both organic acids exerted comparable impacts. Heterotrophic communities decreased in a concentration-dependent manner (Table 9.3) but the relative abundance of catabolic genes increased (Figure 9.2E and F), indicating the potential occurrence of catabolic gene transfer. The disparate responses of the soil microbial communities to

the two PAHs indicate that an interactive effect of organic acid and hydrocarbon is occurring, one that increases degradation potential, if not immediate degradation, in the case of naphthalene. While the design of the current study does not allow us to parse out the nature of this interaction, it may be related to the greater solubility of naphthalene compared to phenanthrene (Schwab et al, 1995). Increased uptake of naphthalene by bacteria in and of itself may trigger transfer of catabolic genes within the microbial community. Hohnstock et al. (2002) found that naphthalene was required to stimulate horizontal gene transfer between environmental isolates from a hydrocarbon contaminated site. In our study, this transfer is enhanced in an un-elucidated manner by the organic acids. These responses also highlight the complexity of the impacts of exudates on microbial degradation pathways.

If the impact of fumarate as a catabolite repressor of aromatic degradation was dominant, naphthalene mineralization would have been repressed in a manner comparable to phenanthrene. While the naphthalene response was likely moderated by the increased degradative fitness of the microbial community, it is also likely that different catabolic pathways (Ellis et al., 2006) and/or different microbial communities were active in degrading the two PAHs, and that the impact of fumarate in these different systems was not comparable. Although community structure was not specifically evaluated in the current study, previous research on isolated exudate components (Chapter 8) indicated that hydrocarbon type had some influence on community structure, with DGGE banding patterns between hydrocarbon treatments differing up to 10%. Although the overall pattern did not change, several additional bands occurred, indicating the increased relative prevalence of specific bacterial species in response to specific hydrocarbons.

Unlike the other three assessed organic acids, succinate had no repressive effect on PAH mineralization and slightly increased mineralization relative to the water control in naphthalene microcosms (Figure 9.2). Increased mineralization in succinate-amended microcosms was not associated with general increases in microbial population densities, as succinate was negatively correlated with 16S rRNA gene copy numbers in both PAH treatments (Tables 9.2 and 9.3). There was a definite interactive effect between type of PAH, succinate concentration, and PAH catabolic gene prevalence. In

phenanthrene microcosms, increased concentrations of succinate decreased both total heterotrophic communities and catabolic gene copy numbers concomitantly (Table 9.2). That mineralization was not similarly repressed indicates that succinate may increase degradation potential in this soil by increasing catabolic gene expression. Succinate has been found to have disparate impacts on genes involved in aromatic compound degradation. Kuiper et al. (2002) found that succinate promoted a higher level of naphthalene dioxygenase expression in a *P. putida* strain than did other organic acids. In studies on lower pathways involved in the degradation of aromatic compounds Brzostowicz et al. (2003) found that expression of the enzyme p-hydroxybenzoate hydroxylase, which catalyzes the metabolism of 4-hydroxybenzoate to protocatechuate, is highly expressed when *Acinetobacte*r strain ADP1 is grown in the presence of an inducer and citric acid cycle substrates and intermediates, including succinate, fumarate, and acetate. However, in *Pseudomonas* spp. succinate also decreases expression of another key enzyme, protocatechuate 3,4 dioxygenase, that catalyzes the metabolism of protocatechuate to β-carboxy-cis,cis-muconate (Collier et al., 1996). As both groups of bacteria are present in the current study soil (see Chapter 7), succinate may specifically stimulate gene expression in one group of bacteria, while depressing it in others.

Succinate exerted a comparable impact with other organic acids on the degradation potential of naphthalene microcosms, where the relative copy number of catabolic gene to 16S rRNA gene increased (Figure 9.2 E and F). As with acetate, succinate has been found to stimulate natural transformation of *Acinetobacter* spp. (Nielsen and van Elsas, 2001), although at a much lower rate and only in non-sterile soils in the presence of phosphorous salts. Potentially increased horizontal gene transfer in the presence of succinate and other organic acids in the current study soil may be linked to the increased release of bound phosphorous, which occurs in soils amended with organic acids (Jones et al., 2003).

9.5.2 Impact of single organic acids on hexadecane degradation potential

The general impact of organic acids on hexadecane mineralization followed similar patterns to that PAH mineralization, with acetate greatly decreasing mineralization and succinate slightly increasing mineralization (Figure 9.3A, Table 9.4).

In contrast to PAH microcosms however, the impact on the overall microbial community was comparable, with all organic acids resulting in decreased heterotrophic and degrader communities. For all citric acid cycle intermediates, decreases in the *alkB* gene copy number were significantly correlated with increases in organic acid concentration (Table 9.4). While some of this decrease may be accounted for by non-selective decreases in total heterotrophic populations (Figure 9.3B, Table 9.4), a decrease in the relative ratio of *alkB* to 16S rRNA gene copies indicates that a selective decrease in bacteria harbouring the *alkB* enzyme system also occurred (Figure 9.3D).

Although at least one other study (Siciliano et al., 2003) found that *alkB* genotypes were more prevalent in rhizosphere than non-rhizosphere soil, our current findings are in line with previous research on the current study soil. In studies with whole root exudates (Chapter 7), and root exudate fractions (Chapter 8), we found little difference between control and exudate treatments in the magnitude of assessed *alkB* genes. The higher levels of simulated exudation used in this study likely stimulated shifts in the indigenous microbial community, which was previously well-adapted to the nutrient-deficient soil, with decreased *alkB* genotypes resulting from competitive exclusion (Espinosa-Urgel, 2004) or niche competition (Kästner and Mahro, 1996). That correlative decreases in mineralization did not occur in succinate microcosms suggests either that succinate has a stimulatory impact on gene expression or that alternate un-characterized enzyme systems were being used.

Previous research has shown that organic acids, including succinate, inhibit alkane degradation by catabolite repression of the *alkB* operon (Yuste et al., 1998). The mechanisms of this repression differ between bacterial species, with repression occurring during the stationary phase in *Burkholderia* sp. (Marin et al., 2001) but during the exponential phase in *Pseudomonas* sp. (Ruiz-Manzano et al., 2005). Although comparable information on the impact of organic acids in complex soil microbial community is not available, it is possible that a similar repression is occurring in our soil. If succinate does repress *alkB* expression, then the increased mineralization observed in succinate amended microcosms may have been due to the stimulation of alternate bacteria and/or enzyme systems. We previously hypothesized (Chapter 7) that differences in hexadecane mineralization that occurred with whole root exudates may

have been due to differential stimulation of cytochrome P450 hydroxylase systems (van Beilan et al. 2007), which have been found in numerous soil bacteria (Sabirova et al., 2006; van Beilan et al., 2006). Although specific research on the impacts of organic acids on that enzyme system would be required to validate that hypothesis, the results of the current study do support it.

9.5.3 Impact of single organic acids on microbial community magnitude

This study found a significant relationship between increased organic acid concentration and a decreased magnitude of heterotrophic microbial communities (Tables 9.2 to 9.4). Previous research on this soil found that the addition of whole or partial plant root exudates did not significantly impact on heterotrophic populations (Chapters 7 and 8). However, although not resulting in significant differences between treatments, in hexadecane microcosms amended with organic acid fractions (Table 8.8) and in phenanthrene microcosms amended with total exudates (Table 7.4), decreased 16S rRNA copy numbers were associated with succinate. While the magnitude of these changes was not comparable to the current study, these results show that the addition of readily available carbon in the form of root exudates has a specific negative impact on the indigenous heterotrophic microbial communities. We previously hypothesized that a greater nutrient input was required to change the overall structure of the indigenous microbial community, which was well adapted to the weathered-hydrocarbon saline-sodic soil (see Chapter 7), and the current study supports this hypothesis. The amount of root exudates added to the soil in those studies was lower than in the current study (≤ 10 vs. ≥ 25 $\mu g\ C\ g^{-1}$ soil, respectively). However, as the genotypic community structure was not assessed, more specific conclusions on genotypic shifts are not possible.

9.6 Conclusions

The results of this study support the conclusions of the two previous chapters, which found that the relative ratio of specific components within the root exudates was more important than the overall amount of root exudate in determining the degradation responses of soil microbial communities. Each of the four assessed organic acids had very specific and often concentration dependent impacts on degradation potential. There

were definite interactive impacts between organic acids and the PAH naphthalene. In the presence of naphthalene, all organic acids facilitated a concentration dependent increase in catabolic gene copy numbers. Acetate, citrate, and fumarate also however, concomitantly depressed degradation activity. Acetate had a comparable impact in the presence of phenanthrene, while both citrate and fumarate repressed both degradation activity and potential. However, fumarate appears to act as a concentration-dependent catabolite inhibitor of gene expression in phenanthrene degradation pathways in the current study soil, while citrate represses degradation more generally by decreasing total microbial populations. Succinate, in contrast, may increase degradation potential in this soil by increasing catabolic gene expression. Finally, all citric acid cycle intermediates had a selective negative impact on bacteria harbouring the *alkB* enzyme system.

10.0 SUMMARY AND CONCLUSIONS

10.1 Of endophytes and exudates

Researchers in the field of phytoremediation typically rely on one of a few dozen workhorse plants, including alfalfa, perennial rye grass, tall wheat grass, poplar, and others. Plant selection is often determined by past performance in other studies, with some initial screening to assess suitability for the environment under study. Attempts to correlate results from such studies have been inconclusive. Specific plants, such as perennial rye grass, which promote hydrocarbon degradation under one set of conditions (Binet et al., 2000; Günther et al., 1996), do not under another (Rezek et al., 2008). These inconsistent results are caused in part by the paucity of knowledge surrounding the mechanisms of phytoremediation. To date, we know relatively little about how different plant species used for phytoremediation impact the structure and function of soil microbial communities, and how these impacts affect the hydrocarbon degradation potential of the system as a whole. Thus, the primary goal of this study was to address these knowledge gaps by seeking the answer to a number of interlinked questions. How and why do the structure and function of plant-associated microbial communities differ between plant species, and how do these differences impact degradation potential? Given the inherently complex nature of the soil environment, simple answers were neither expected nor received. Instead, this thesis work revealed that there are layers of interlinked reasons why one plant may be more effective than another at promoting degradation.

Plants assessed in this study were grown under comparable environmental conditions, either in the growth chamber or in the field, yet facilitated different amounts of total petroleum hydrocarbon (TPH) degradation. Under controlled environmental conditions a contributing factor to this difference in degradation potential was the type of rhizosphere degrader community stimulated by the different plants. The highest degradation occurred in those plant treatments, primarily single species grasses, which

supported the largest populations of aliphatic hydrocarbon degraders. The lowest degradation occurred in those treatments, primarily alfalfa-containing, which supported the largest populations of PAH degraders. In the absence of external environmental stresses, the presence of alfalfa within a treatment was found to exert a dominant influence on rhizosphere microbial communities. While many phytoremediation studies have focused on the ability of plants to enhance PAH degraders, many hydrocarbon contaminated sites contain large proportions of aliphatic hydrocarbons. An enhanced PAH degrader population in the rhizosphere, which is the active zone in which enhanced TPH degradation would occur, could limit the presence and the activity of aliphatic degraders, via competitive exclusion, niche competition, or catabolite repression. Numerous factors would contribute to the selective influence of plant species on degrader composition within the rhizosphere. The higher lipid content of alfalfa roots compared to grass roots has been shown to concentrate PAHs, thus providing a selective factor for the establishment of PAH degrader communities. Endophytic communities maintained by the plants may also contribute to rhizosphere community structure and function.

In field studies with a sub-set of the plants assessed in growth chambers, environmental factors influenced both rhizosphere and endophytic microbial communities. In general, both heterotrophic and degrader communities experienced declines in population following extended periods of no precipitation. Some plants however, were able to mitigate these adverse impacts. The grass Altai wild rye (AWR) supported substantially higher endophytic degrader populations over the course of the study than other plants, due at least in part to its ability to maintain these communities during periods of environmental stress. These endophytic degrader communities were selectively recruited by AWR during the first growing season in response to high TPH concentration. The higher initial TPH degradation facilitated by AWR during this season (greater than 50%) may have been related to its ability to attract and provide a refuge for these degraders, which could then act as a subsequent source population for rhizosphere communities once environmental conditions improved.

The maintenance of a greater magnitude of endophytic degrader populations by AWR was undoubtedly related to the diverse nature of that community. Phenotypic and

genotypic assessments of mature endophytic communities revealed that AWR maintained very distinct communities from other grasses and alfalfa. While the endophytic communities of most plants were dominated by r-strategist *Pseudomonas* species, AWR harboured a phylogenetically diverse population that included *Pseudomonas*, *Sphingomonas*, *Sphingobacterium*, *Burkholderia*, *Brevundimonas*, *Alcaligenes*, and *Stenotrophomonas* species. As all of these bacteria have been previously demonstrated to degrade hydrocarbons, their diverse nature may have conferred functional redundancy. If conditions within the root changed such that conditions became unfavourable for one population, as perhaps in times of local soil drought, then other populations with different requirements and different life strategies could readily become dominant. Simply put, a diverse community is less likely to suffer a catastrophic event. Differences in root morphology likely contributed to the differences in degrader populations maintained by the plants under study, which were growing at the same site under the same conditions. Differences in exudation patterns however, were also shown to play a significant role.

Both the absolute amount and ratio of exudates components released by AWR and alfalfa differed. Until hydrocarbon stress, AWR released approximately three times the amount of organic acids and double the amount of amino acids per gram of root than alfalfa. While not specifically assessed, the quantity and type of exudates released by AWR undoubtedly contributed to the magnitude and diversity of its endophytic community. These differences in exudation patterns did have a direct impact on both the degradation potential and the degradation activity of soil microbial communities. AWR exudates contained greater proportional amounts of compounds, such as the organic acid succinate, which may have increased degradation potential by stimulating catabolic plasmid transfer and catabolic gene expression within the indigenous microbial community. Conversely, alfalfa exudates contained greater proportional amounts of compounds, such as the organic acid malonate, which had the opposite effect on degradation potential and activity. The ratio of these types of compounds released by the plants was key to determining whether the degradation potential of the microbial communities was increased or decreased. As alfalfa was previously shown to have a dominant impact on rhizosphere community structure, the lower degradation observed

with mixed plant treatments in both growth chamber and field studies may have been linked to the repressive effect of alfalfa exudates on overall degradation activity. It is also probable however, that allelopathic interactions between plants would have affected the overall composition of root exudates, contributing to the decreased degradation potential.

A reciprocal relationship between exudation patterns and endophytic community structure likely exists, and both parameters have a specific influence on rhizosphere degradation capacity. This thesis work has shown that all parameters contribute to the degradation success of specific plant treatments. In this study, grasses were more successful in maintaining the specific balance required for the transfer, preservation, and stimulation of hydrocarbon catabolic competency. While the impact of these factors will differ with both plant species and specific environmental conditions, the findings of this study point to potential ways to optimize phytoremediation research. Future phytoremediation research may be best served by finding simple ways to monitor probable exudation patterns prior to establishing field sites, finding ways to manipulate exudation patterns, and developing specific endophytic inoculants.

10.2 Future research

10.2.1 Hydrocarbon-degrading microbial inoculants

Research suggests that it may be possible to use microbial inoculants to reduce contaminant toxicity to plants and increase contaminant degradation. Huang et al. (2004, 2005) demonstrated that inoculating *Festuca arundinacea* phytoremediation systems with plant growth promoting *Enterobacter* spp. reduced toxic impacts to both germination and growth, and increased degradation of PAH and total petroleum hydrocarbons by up to 45% compared to non-inoculated plants. There are problems associated with using non-indigenous microbial inoculants however, including reduced survivability and adverse impacts on indigenous microbial populations. Conn and Franco (2004b) inoculated wheat with a mixed microbial soil consortia and with a single endophyte that had previously been isolated from wheat roots. The endophytic-derived

inoculant had a higher survival rate and did not adversely impact indigenous microbial endophytic populations, while the mixed inoculant reduced both endophytic diversity and colonization. Thus endophytic inoculants may also be a better choice for the purposes of phytoremediation.

Studies have shown that endophytic inoculants are capable of enhancing the degradation of xenobiotic compounds. Inoculation of pea plants with an endophytic 2,4-dichlorophenoxyacetic acid-degrading *P. putida* strain resulted in increased 2,4-D removal from soil and decreased 2,4-D accumulation by plants (Germaine et al., 2006). Similarly, a genetically modified endophytic strain of *Burkholderia cepacia* was shown to increase the tolerance of *Lupinus luteus* (yellow lupine) to toluene, while decreasing toluene evapotranspiration (Barac et al.; 2004). A concomitant study inoculated poplar trees with the same *B. cepacia* strain and found that, apart from increasing tolerance and in-planta degradation, the inoculants transferred their degradative plasmids to other endophytes, thus increasing the overall degradation potential of endogenous endophytic communities (Taghavi et al., 2005). The inoculant itself did not however, survive in detectable numbers within the plant tissue. In the course of this thesis work, we determined that plants developed specific and stable mature endophytic communities capable of degrading hydrocarbons. The utilization of these plant-specific endophytic degraders should result in better survivability and subsequently contribute to the overall degradation potential of surrounding rhizosphere communities.

10.2.2 Enhancement of root exudation

Both hydrocarbon degradation activity and potential are influenced by the proportional composition of exudates released by plants under hydrocarbon stress. In order to fine tune phytoremediation treatments, it may be possible to modify the exudation patterns of these plants, such that gene transfer or activation within the rhizosphere community is enhanced. A significant amount of work has been done on manipulating organic acid efflux from plants to manage metal toxicity (for review see Rengel, 2002). Increased exudation of organic acids such as citrate and malate increases chelation of some metals, such as Al^{3+}, in the rhizosphere and effectively limits plant uptake of the toxic compound. While many plants selected for increased exudation have

been bred via mutation studies, there has been limited success in directly genetically modifying plants for this trait. For example, Delhaize et al. (2003) assessed transgenic *Nicotiana tabacum* (tobacco) with increased mitochondrial citrate synthase activity. The transgenic plants had up to 5 times more enzyme activity, but this did not translate into increased citrate production. As these techniques mature however, it may be possible to modify plant exudate production to serve a number of needs, including organic contaminant degradation (Macek et al., 2008). Until then, traditional breeding techniques have been shown to be effective in identifying plants that facilitate hydrocarbon degradation. Wiltse et al. (1998) found that different genotypic clones of *Medicago sativa* cv *Riley* (alfalfa) promoted different amount of crude oil degradation. Schwab et al. (2006) demonstrated that it was possible to stably breed these cultivar clones, with increased-degradative phenotypes manifesting even under varied environmental conditions. Whether through genetic manipulation or breeding, the optimization of plant-specific patterns of exudation to increase overall degradation potential could greatly benefit future research into phytoremediation

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APPENDIX A

Carlyle field site set-up

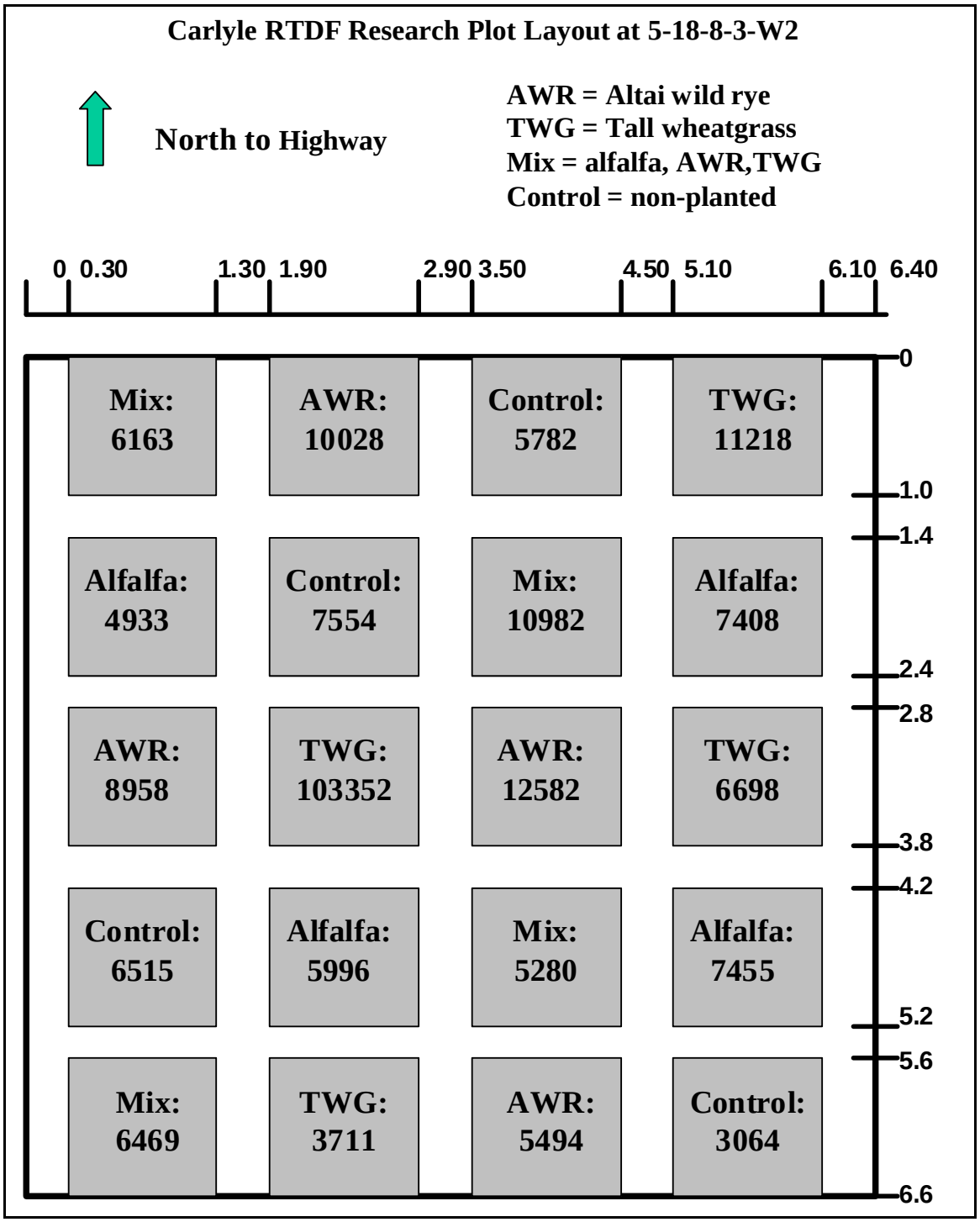

Figure A.1 Carlyle field site set-up.

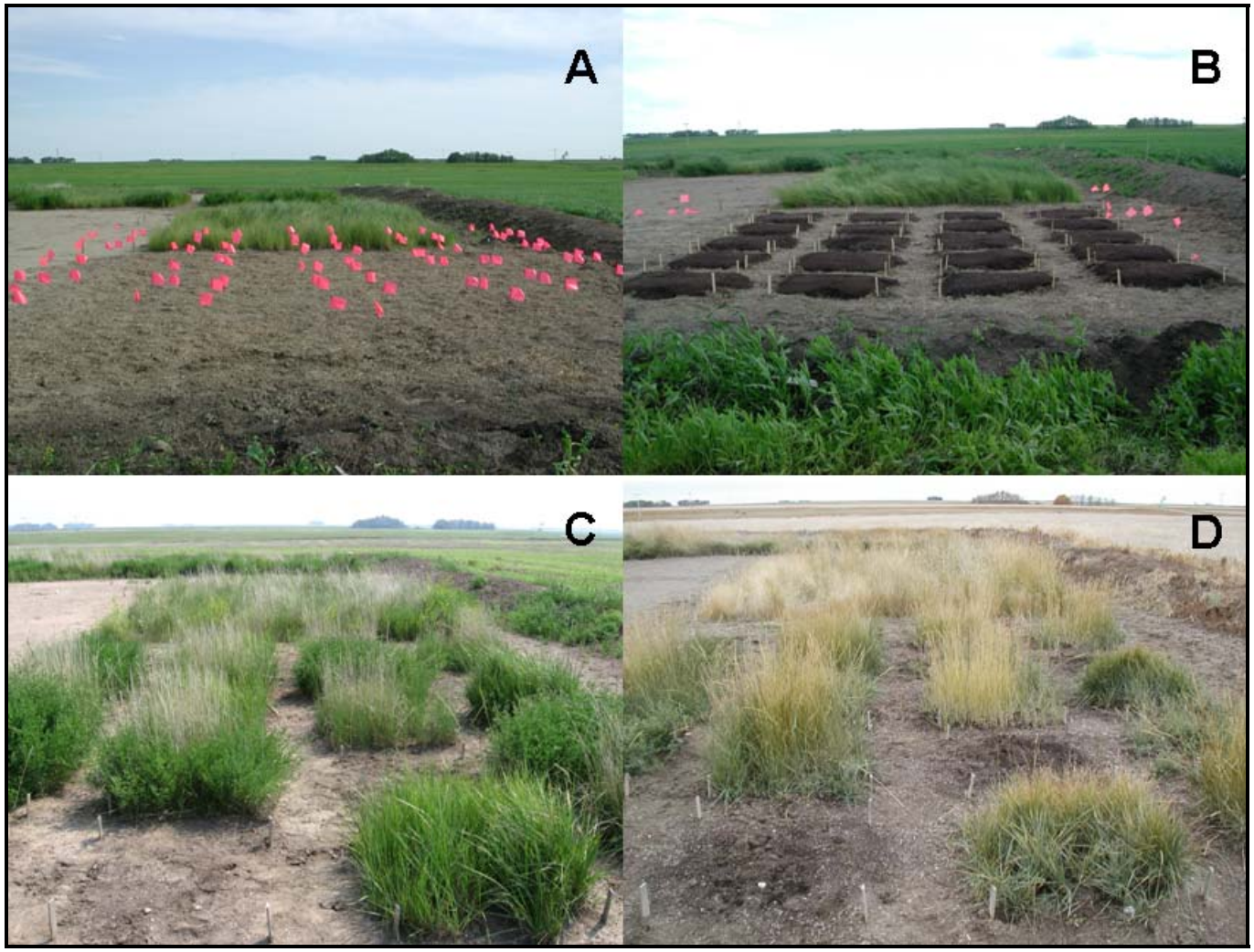

Figure A.2 Carlyle field site (view southwards) during (A, B) site preparation, (C) summer 2006 (D) fall 2006.

APPENDIX B

Chromatograms used in exudate qualification and standard curve data used for exudate quantification.

Table B.1. Retention time and standard curve for assessed amino acids.

	Chromatogram		Standard curve	
Amino acid	RT†	Label‡	Equation	R^2
alanine	20.016	a	y = 3775.9x	0.999
glycine	20.836	b	y = 3406.6x	0.993
valine	22.646	c	y = 3084.1x	0.999
leucine	23.480	d	y = 3056.1x	0.997
isoleucine	24.197	e	y = 2965.7x	0.998
norleucine	24.610		y = 3017.7x	0.998
proline	25.262	f	y = 4474.4x	0.997
methionine	29.286		y = 2925.0x	0.999
serine	29.368	g	y = 4779.2x	0.998
threonine	29.704	h	y = 2515.3x	0.997
phenylalanine	31.588	i	y = 1985.0x	0.998
aspartic acid	32.386	j	y = 2512.5x	0.992
cysteine	33.313	k	y = 1206.6x	0.994
glutamic acid	34.458	l	y = 2920.4x	0.996
asparagine	35.033	m	y = 1702.2x	0.991
lysine	36.171	n	y = 1880.0x	0.997
histidine	39.618	o	y = 2180.3x	0.999
tyrosine	40.169	p	y = 4517.3x	0.998
tryptophan	43.901	q	y = 1937.5x	0.994
cystine	47.089	r	y = 1825.1x	0.985

† Retention time of compounds during GC-MS analysis

‡ Label in Figure B.1

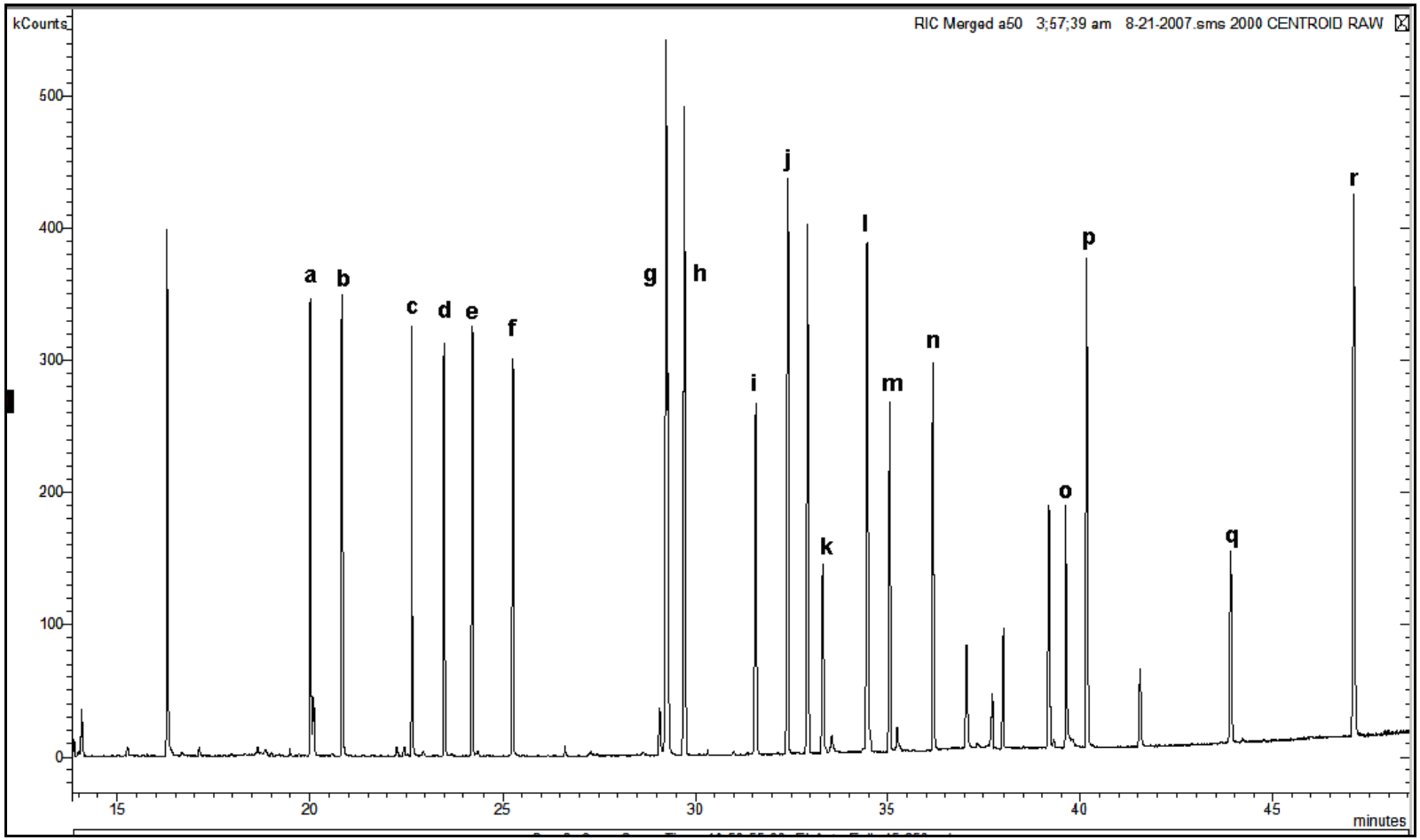

Figure B.1. Sample chromatogram of a 50 mg L^{-1} amino acid standard. Letters indicate peak identity as listed in Table B.1.

Table B.2. Retention time and standard curve for assessed organic acids.

	Chromatogram		Standard curve	
Organic acid	RT†	Label‡	Equation	R^2
acetic	19.733	a	y = 45073x	1.000
butyric	21.683	b	y = 45946x	1.000
malonic	22.962	c	y = 37119x	1.000
hydroxy-malonic	23.136	d	y = 44388x	1.000
maleic	25.249	e	y = 33621x	1.000
succinic	25.547	f	y = 41969x	1.000
citraconic	25.884	g	y = 31310x	0.999
itaconic	26.116	h	y = 34487x	0.999
fumaric	26.345	i	y = 24067x	0.994
malic	31.886	j	y = 328076x	0.999
azeleic	35.200	k	y = 62322x	0.997
tartaric	36.606	l	y = 52777x	0.986
aconitic	36.755	m	y = 79524x	0.996
hydroxy-mandelic	37.167	n	y = 26365x	0.990
citric	39.771	o	y = 402880x	0.999
phthalic	32.860		y = 35337x	0.995

† Retention time of compounds during GC-MS analysis

‡ Label in Figure B.2

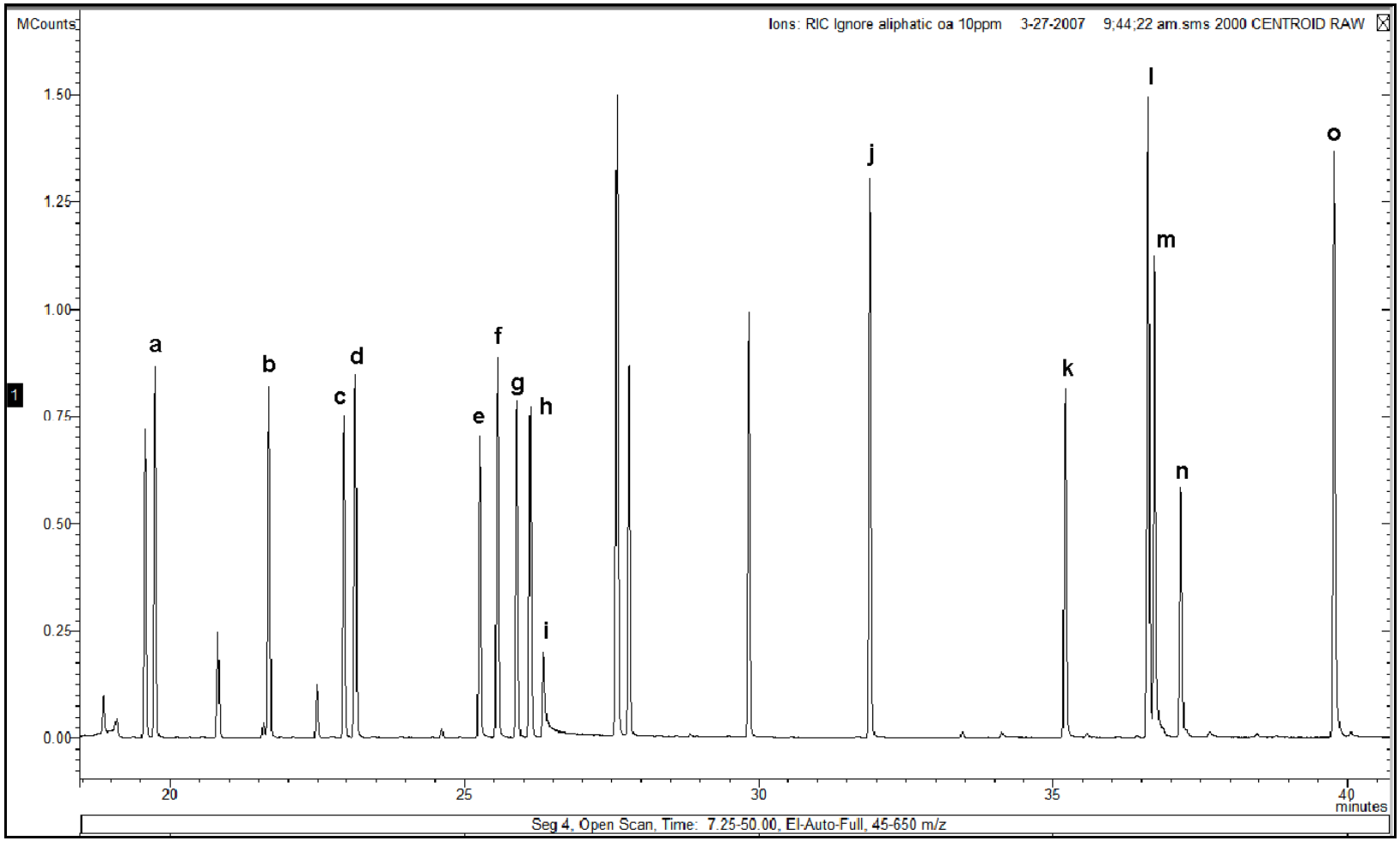

Figure B.2. Sample chromatogram of a 10 mg L^{-1} aliphatic organic acid standard. Letters indicate peak identity as listed in Table B.2

Table B.3. Retention time and standard curve for assessed phenolics.

	Chromatogram		Standard curve	
Phenolic	RT†	Label‡	Equation	R2
catechol	5.483	a	y = 37105x	0.998
salicylic	6.895	b	y = 30107x	0.989
p-hydroxybenzoic	7.495	c	y = 50451x	0.999
vanillic	8.256	d	y = 64625x	0.999
gentisic	8.414	e	y = 30799x	0.994
protocatechuic	8.442	f	y = 42590x	0.999
3,5 dihydroxybenzoic	8.532		y = 32178x	0.998
umbelliferone	8.987	g	y = 5596.3x	0.999
flavone	11.951	h	y = 2475.5x	0.992

† Retention time of compounds during GC-MS analysis

‡ Label in Figure B.3

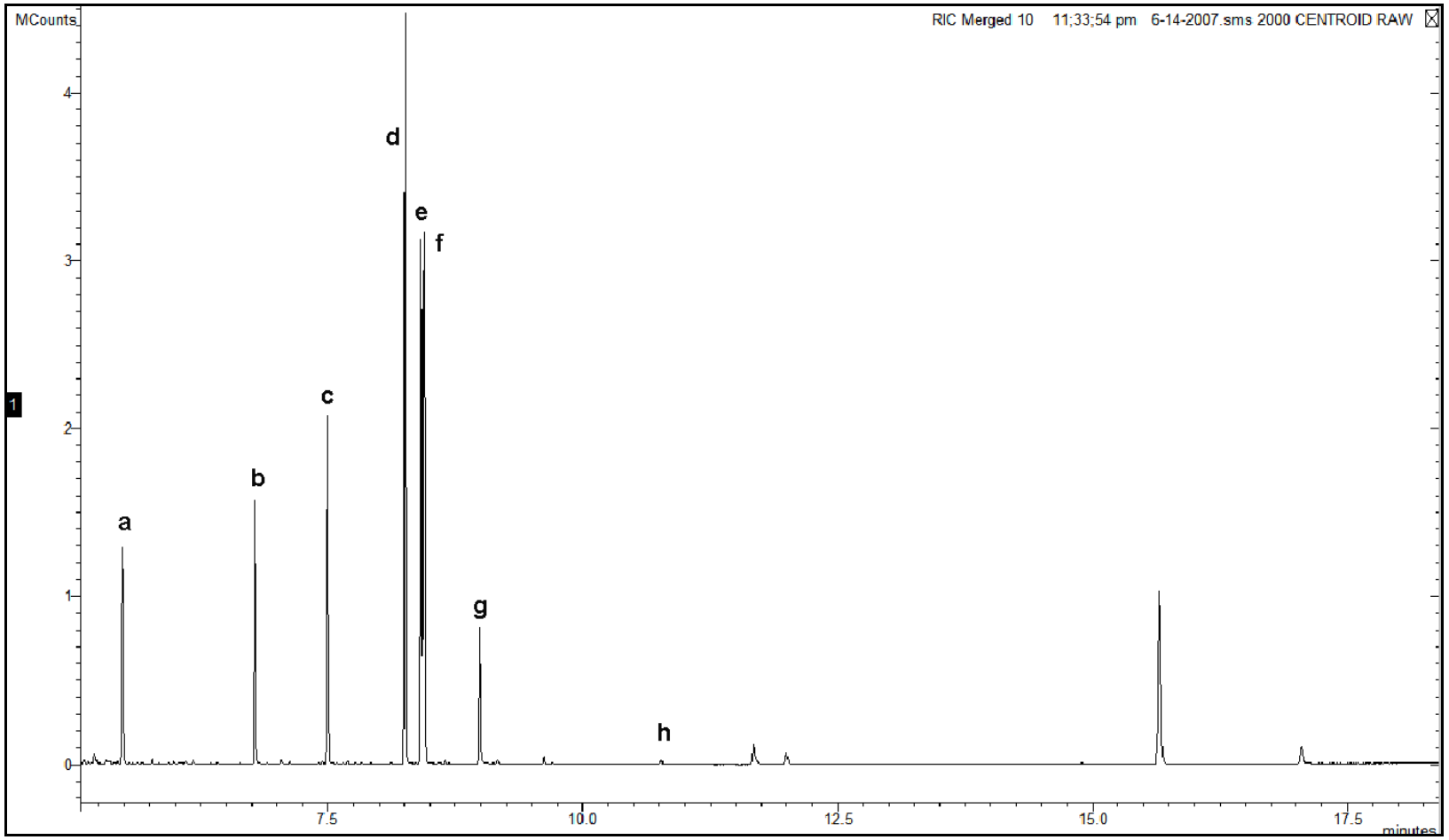

Figure B.3. Sample chromatogram of a 10 mg L^{-1} phenolic standard. Letters indicate peak identity as listed in Table B.3

APPENDIX C

Hydrocarbon mineralization parameters and gene copy numbers for all phenolic, amino acid, and organic acid fraction-specific exudate studies.

Table C.1: Hydrocarbon mineralization parameters and total gene copy number of soil mineralization microcosms amended with phenolic fractions of plant root exudates or control eluates

Mineralization Microcosms	Lag (days)	Maximum rate (%$^{14}CO_2$ day^{-1})	Cumulative % mineralized	Gene copy number g^{-1} dry soil (log scale)†		
Phenanthrene	Lag*	Rate	Cumulative	16S rRNA	C2,3O	*nahAc*
Control	10.9 (0.6)ab	1.5 (0.3)	37.2 (7.0)	7.64 (0.12)	6.04 (0.17)	4.59 (0.20)
Control-pp‡	11.2 (1.1)a	1.7 (0.5)	39.9 (8.7)	7.53 (0.11)	6.04 (0.15)	4.57 (0.24)
Alfalfa	9.8 (0.7)ab	1.8 (0.3)	42.4 (5.6)	7.09 (0.75)	5.63 (0.42)	3.96 (0.72)
Alfalfa-pp	10.0 (0.8)ab	1.8 (0.2)	42.0 (4.4)	7.29 (0.19)	5.93 (0.22)	4.30 (0.30)
AWR§	10.6 (1.2)ab	1.6 (0.3)	38.0 (6.0)	7.34 (0.34)	6.01 (0.17)	4.34 (0.26)
AWR-pp	9.5 (0.4)b	1.6 (0.4)	39.3 (8.8)	7.34 (0.27)	5.93 (0.34)	4.28 (0.45)
Water	11.0 (0.6)ab	1.7 (0.2)	38.8 (4.7)	7.54 (0.25)	6.25 (0.05)	4.80 (0.09)
Naphthalene	Lag	Rate	Cumulative*	16S rRNA	C2,3O	*nahAc*
Control	1.7 (0.0)	7.2 (0.5)	53.3 (2.5)b	7.46 (0.19)	6.20 (0.24)	4.84 (0.27)
Control-pp	1.7 (0.0)	7.3 (0.6)	58.9 (4.3)ab	7.48 (0.08)	6.16 (0.13)	4.79 (0.16)
Alfalfa	1.7 (0.0)	7.6 (0.1)	60.1 (2.3)ab	7.42 (0.44)	6.04 (0.24)	4.59 (0.34)
Alfalfa-pp	1.7 (0.1)	7.0 (0.8)	55.9 (4.9)ab	7.61 (0.06)	6.22 (0.13)	4.84 (0.16)
AWR	1.6 (0.0)	8.1 (0.4)	61.2 (0.9)a	7.49 (0.22)	6.11 (0.12)	4.68 (0.14)
AWR-pp	1.7 (0.0)	7.7 (0.2)	60.4 (1.0)a	7.60 (0.14)	5.99 (0.73)	4.96 (0.24)
Water	1.7 (0.1)	7.3 (1.1)	59.1 (3.7)ab	7.49 (0.09)	5.90 (0.11)	4.57 (0.13)
Hexadecane	Lag	Rate	Cumulative*	16S rRNA	*alkB*	
Control	1.9 (0.1)	5.7 (0.4)	55.9 (1.6)a	7.55 (0.14)	5.68 (0.30)	
Control-pp	1.9 (0.1)	5.3 (0.3)	53.7 (2.1)ab	7.49 (0.20)	5.50 (0.42)	
Alfalfa	1.9 (0.1)	5.7 (0.4)	54.6 (1.5)ab	7.14 (0.37)	5.21 (0.26)	
Alfalfa-pp	1.9 (0.1)	5.4 (0.2)	54.7 (3.0)ab	7.55 (0.15)	5.33 (0.21)	
AWR	2.3 (0.5)	5.5 (0.4)	48.5 (3.6)b	7.61 (0.08)	5.61 (0.34)	
AWR-pp	1.9 (0.0)	5.4 (0.5)	51.4 (6.0)ab	7.59 (0.26)	5.44 (0.31)	
Water	2.0 (0.1)	5.0 (0.4)	49.3 (1.4)ab	7.65 (0.04)	6.03 (0.59)	

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

*Means in a single sub-column followed by a different letter are significantly different at $p \leq 0.05$

† 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

‡The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

§AWR, Altai wild rye

Table C.2: Hydrocarbon mineralization parameters and total gene copy number of soil mineralization microcosms amended with amino acid fractions of plant root exudates or control eluates

Mineralization Microcosms	Lag (days)	Maximum rate (%$^{14}CO_2$ day^{-1})	Cumulative % mineralized	Gene copy number g^{-1} dry soil (log scale)†		
Phenanthrene	Lag	Rate	Cumulative	16S rRNA*	C2,3O	*nahAc*
Control	8.3 (0.3)	2.2 (0.5)	49.8 (5.8)	6.97 (0.33)b	7.49 (0.20)	5.72 (0.31)
Control-pp‡	9.3 (0.6)	1.8 (0.5)	46.7 (12.2)	7.01 (0.18)ab	7.47 (0.32)	5.87 (0.42)
Alfalfa	8.6 (0.8)	2.0 (0.1)	50.3 (4.5)	7.48 (0.15)a	7.60 (0.19)	5.94 (0.28)
Alfalfa-pp	9.3 (1.0)	1.9 (0.3)	50.3 (5.3)	7.25 (0.30)ab	7.54 (0.13)	5.92 (0.18)
AWR§	8.4 (0.3)	2.1 (0.3)	51.9 (3.7)	7.06 (0.28)ab	7.49 (0.23)	5.87 (0.39)
AWR-pp	8.6 (1.1)	1.9 (0.3)	48.6 (6.2)	7.19 (0.16)ab	7.11 (0.30)	5.18 (0.10)
Water	8.8 (1.8)	3.0 (0.3)	53.1 (3.3)	6.89 (0.11)b	7.24 (0.39)	5.52 (0.61)
Naphthalene	Lag	Rate	Cumulative	16S rRNA	C2,3O	*nahAc*
Control	3.8 (0.3)	4.8 (0.4)	52.2 (4.9)	7.47 (0.10)	7.34 (0.11)	6.40 (0.09)
Control-pp	4.0 (0.7)	4.7 (0.5)	52.5 (1.8)	7.14 (0.22)	7.31 (0.14)	6.35 (0.21)
Alfalfa	3.9 (0.5)	4.2 (1.2)	47.8 (4.4)	6.81 (0.60)	7.17 (0.38)	6.17 (0.59)
Alfalfa-pp	4.1 (0.9)	4.6 (0.4)	47.5 (3.7)	7.23 (0.38)	7.39 (0.17)	6.49 (0.32)
AWR	3.9 (0.3)	5.2 (0.5)	55.9 (4.8)	7.21 (0.23)	7.32 (0.27)	6.38 (0.38)
AWR-pp	3.8 (0.3)	4.2 (0.4)	55.0 (1.7)	7.15 (0.19)	7.30 (0.18)	6.35 (0.38)
Water	3.8 (0.3)	4.7 (0.3)	52.4 (8.1)	7.45 (0.23)	7.33 (0.38)	6.35 (0.61)
Hexadecane	Lag	Rate	Cumulative	16S rRNA***	*alkB****	
Control	6.9 (1.3)	2.0 (0.3)	45.3 (1.6)	7.91 (0.09)ab	5.80 (0.12)a	
Control-pp	7.5 (0.6)	2.1 (0.2)	44.2 (3.0)	7.56 (0.08)c	5.38 (0.05)b	
Alfalfa	7.6 (2.1)	2.1 (0.5)	43.6 (7.5)	7.88 (0.09)ab	5.88 (0.16)a	
Alfalfa-pp	8.0 (0.0)	2.1 (0.3)	44.8 (4.6)	7.94 (0.06)a	5.79 (0.07)a	
AWR	7.4 (0.8)	2.3 (0.3)	44.4 (4.8)	7.58 (0.18)c	5.51 (0.18)b	
AWR-pp	7.5 (1.0)	2.2 (0.5)	41.9 (4.4)	7.68 (0.10)bc	5.78 (0.05)a	
Water	7.7 (0.6)	1.7 (0.2)	41.4 (9.0)	7.87 (0.11)ab	5.87 (0.19)a	

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

Means in a single sub-column followed by a different letter are significantly different at *$p \leq 0.05$ and ***$p \leq 0.001$

† 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase

‡The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

§AWR, Altai wild rye

Table C.3: Hydrocarbon mineralization parameters and total gene copy number of soil mineralization microcosms amended with organic acid fractions of plant root exudates or control eluates

Mineralization Microcosms	Lag (days)	Maximum rate (%$^{14}CO_2$ day^{-1})	Cumulative % mineralized	Gene copy number g^{-1} dry soil (log scale)†		
Phenanthrene	Lag	Rate	Cumulative	16S rRNA	C2,3O	*nahAc*
Control	7.3 (0.5)	2.5 (0.7)	51.4 (10.6)	7.57 (0.17)	6.81 (0.17)	5.53 (0.18)
Control-pp‡	7.6 (0.5)	1.9 (0.2)	51.1 (5.4)	7.73 (0.03)	6.76 (0.08)	5.39 (0.09)
Alfalfa	8.4 (0.5)	1.9 (0.2)	48.3 (3.6)	7.67 (0.08)	6.77 (0.13)	5.35 (0.18)
Alfalfa-pp	8.0 (0.7)	2.0 (0.3)	50.2 (4.0)	7.76 (0.11)	6.85 (0.12)	5.44 (0.17)
AWR§	8.1 (0.8)	2.1 (0.1)	51.1 (3.5)	7.78 (0.15)	6.97 (0.14)	5.56 (0.14)
AWR-pp	8.1 (0.6)	2.2 (0.3)	51.4 (5.2)	7.50 (0.35)	6.62 (0.30)	5.20 (0.34)
Water	8.8 (1.4)	1.8 (0.1)	44.9 (4.0)	7.81 (0.06)	6.83 (0.13)	5.46 (0.16)
Naphthalene	Lag**	Rate	Cumulative*	16S rRNA	C2,3O	*nahAc*
Control	0.9 (0.1)b	4.5 (0.3)	59.4 (3.4)b	7.55 (0.34)	6.62 (0.19)	5.37 (0.25)
Control-pp	0.9 (0.1)b	4.5 (0.7)	58.8 (2.4)b	7.82 (0.08)	6.75 (0.01)	5.49 (0.03)
Alfalfa	1.2 (0.6)b	4.8 (0.1)	58.6 (4.0)b	7.69 (0.19)	6.62 (0.13)	5.30 (0.17)
Alfalfa-pp	0.8 (0.1)b	4.6 (0.0)	60.2 (1.0)ab	7.63 (0.06)	6.75 (0.05)	5.36 (0.09)
AWR	0.9 (0.1)b	5.3 (0.3)	66.8 (3.5)a	7.72 (0.38)	6.60 (0.21)	5.24 (0.28)
AWR-pp	0.8 (0.0)b	4.5 (0.3)	60.6 (2.4)ab	7.91 (0.05)	6.71 (0.10)	5.39 (0.07)
Water	2.5 (0.0)a	4.7 (0.6)	59.5 (2.9)b	7.99 (0.02)	6.78 (0.17)	5.54 (0.22)
Hexadecane	Lag	Rate	Cumulative	16S rRNA***	*alkB****	
Control	2.3 (0.4)	5.8 (0.3)	54.0 (5.0)	7.95 (0.06)ab	6.57 (0.10)ab	
Control-pp	2.3 (0.2)	5.2 (0.4)	48.0 (1.2)	8.12 (0.17)a	6.76 (0.17)a	
Alfalfa	2.4 (0.1)	4.7 (0.1)	50.6 (2.4)	7.94 (0.04)ab	6.50 (0.09)ab	
Alfalfa-pp	2.4 (0.3)	5.2 (1.5)	51.6 (3.9)	7.90 (0.11)ab	6.42 (0.10)ab	
AWR	2.2 (0.1)	5.0 (0.3)	52.6 (3.8)	7.83 (0.08)b	6.29 (0.21)b	
AWR-pp	2.4 (0.1)	5.1 ()0.7	52.3 (2.6)	7.79 (0.04)b	6.28 (0.16)b	
Water	2.6 (0.4)	4.6 (1.1)	47.3 (8.2)	8.11 (0.13)a	6.60 (0.12)ab	

Data are presented as means (n = 4) with ± 1 SD in parentheses. Means in a single sub-column followed by a different letter are significantly different at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

† 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase.

‡The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions.

§AWR, Altai wild rye.

Table C.4: Ratio of catabolic gene to 16S rRNA gene copy numbers, in phenanthrene, naphthalene, and hexadecane microcosms amended with phenolic, amino acid, or organic acid fractions derived from plant root exudates.

Mineralization Microcosms	Phenanthrene		Naphthalene		Hexadecane
Phenolics	*nahAc*:16SrRNA†	C2,3O:16SrRNA	*nahAc*:16SrRNA	C2,3O:16SrRNA	*alkB*:16SrRNA
Control	0.600 (0.017)	0.790 (0.013)	0.648 (0.028)	0.831 (0.022)	0.753 (0.027)
Control-pp‡	0.607 (0.030)	0.802 (0.018)	0.640 (0.021)	0.824 (0.017)	0.734 (0.035)
Alfalfa	0.578 (0.015)	0.782 (0.015)	0.618 (0.012)	0.814 (0.020)	0.730 (0.018)
Alfalfa-pp	0.589 (0.028)	0.813 (0.011)	0.636 (0.017)	0.818 (0.012)	0.706 (0.018)
AWR	0.590 (0.012)	0.819 (0.017)	0.625 (0.010)	0.816 (0.012)	0.738 (0.049)
AWR-pp	0.583 (0.051)	0.808 (0.038)	0.652 (0.026)	0.789 (0.097)	0.718 (0.057)
Water	0.637 (0.011)	0.829 (0.025)	0.611 (0.025)	0.788 (0.023)	0.789 (0.081)
Amino acids	*nahAc*:16SrRNA*	C2,3O:16SrRNA	*nahAc*:16SrRNA	C2,3O:16SrRNA	*alkB*:16SrRNA***
Control	0.822 (0.050)ab	1.077 (0.046)	0.857 (0.018)	0.983 (0.024)	0.733(0.013)abc
Control-pp	0.837 (0.047)a	1.065 (0.030)	0.890 (0.056)	1.025(0.050)	0.712(0.004)c
Alfalfa	0.794 (0.052)ab	1.016 (0.044)	0.905 (0.012)	1.056(0.041)	0.746(0.015)ab
Alfalfa-pp	0.817 (0.046)ab	1.042 (0.053)	0.899 (0.057)	1.024 (0.048)	0.730(0.003)abc
AWR	0.831 (0.039)a	1.062 (0.032)	0.885 (0.027)	1.016 (0.016)	0.726(0.007)bc
AWR-pp	0.721 (0.012)b	0.989 (0.046)	0.888 (0.046)	1.022 (0.026)	0.752(0.012)a
Water	0.801 (0.084)ab	1.050 (0.045)	0.852 (0.060)	0.984(0.030)	0.746(0.015)ab
Organic acids	*nahAc*:16SrRNA	C2,3O:16SrRNA	*nahAc*:16SrRNA**	C2,3O:16SrRNA**	*alkB*:16SrRNA
Control	0.731(0.022)	0.900(0.023)	0.711(0.007)a	0.877 (0.015)ab	0.827 (0.015)
Control-pp	0.696(0.013)	0.874(0.013)	0.702(0.004) ab	0.863 (0.009)ab	0.833 (0.013)
Alfalfa	0.698(0.019)	0.883(0.016)	0.689 (0.012)ab	0.862 (0.011)ab	0.819 (0.011)
Alfalfa-pp	0.701(0.022)	0.882(0.015)	0.703(0.015) ab	0.884 (0.005)a	0.812 (0.004)
AWR	0.714(0.015)	0.896(0.014)	0.679(0.006) b	0.855 (0.018)ab	0.803 (0.018)
AWR-pp	0.694(0.018)	0.883(0.013)	0.681(0.007) b	0.848 (0.009)b	0.807 (0.018)
Water	0.699(0.017)	0.874(0.014)	0.693(0.029) ab	0.848 (0.023)b	0.813 (0.026)

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

Means in a single sub-column followed by a different letter are significantly different at *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

† 16S rRNA, eubacterial 16S rRNA; C2,3O, catechol 2,3 dioxygenase; *nahAc*, naphthalene dioxygenase; *alkB*, alkane monoxygenase.

‡The tag "pp" indicates that the original exudates/eluates were collected under phenanthrene/pyrene stimulated conditions

§AWR, Altai wild rye.

Table C.5. Spearman's rank correlation coefficients (n=27) for hydrocarbon mineralization parameters in organic acid, amino acid, and phenolic amended microcosms**.**

Treatment	Organic acid		Amino acid		Phenolic	
Phenanthrene	Lag	Cumulative	Lag	Cumulative	Lag	Cumulative
Rate†	-0.308	0.826***	-0.346	0.744***	-0.572**	0.925**
Lag		-0.602*		-0.304		-0.697***
Naphthalene	Lag	Cumulative	Lag	Cumulative	Lag	Cumulative
Rate	0.148	0.656**	0.202	0.487*	-0.936***	0.686**
Lag		-0.315		-0.168		-0.675***
Hexadecane	Lag	Cumulative	Lag	Cumulative	Lag	Cumulative
Rate	-0.414*	0.641**	-0.398*	0.556**	-0.556**	0.241
Lag		-0.234		-0.262		-0.517**

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

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

APPENDIX D

Quantitative PCR dissociation curves

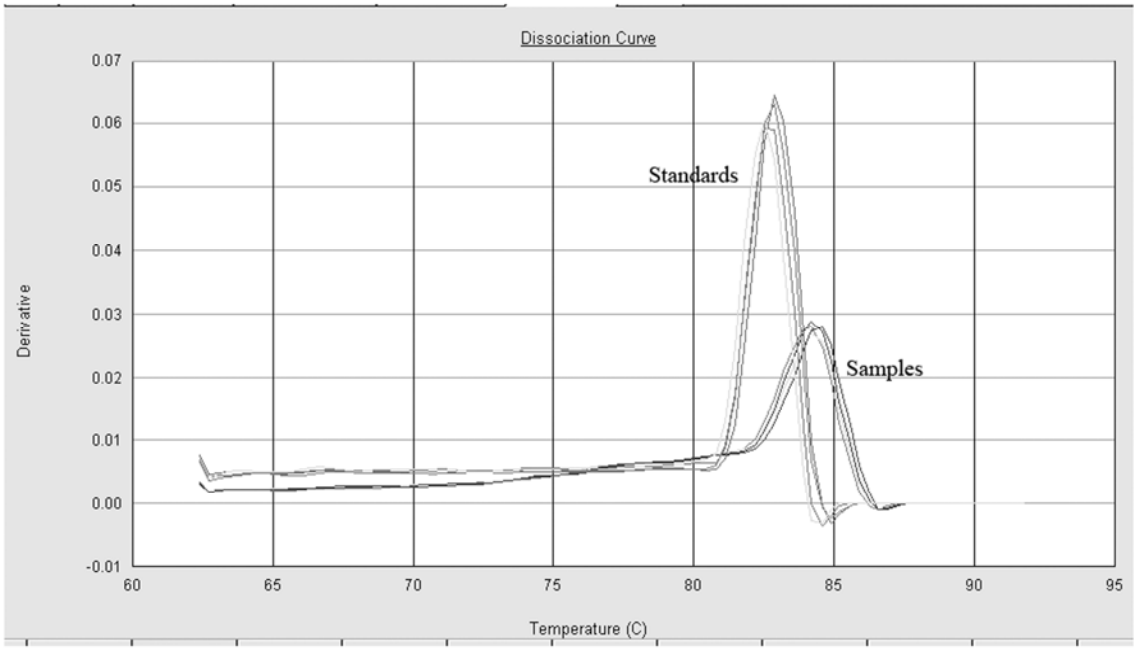

Figure D.1 Sample dissociation curve for the quantitative PCR of 16S rRNA genes

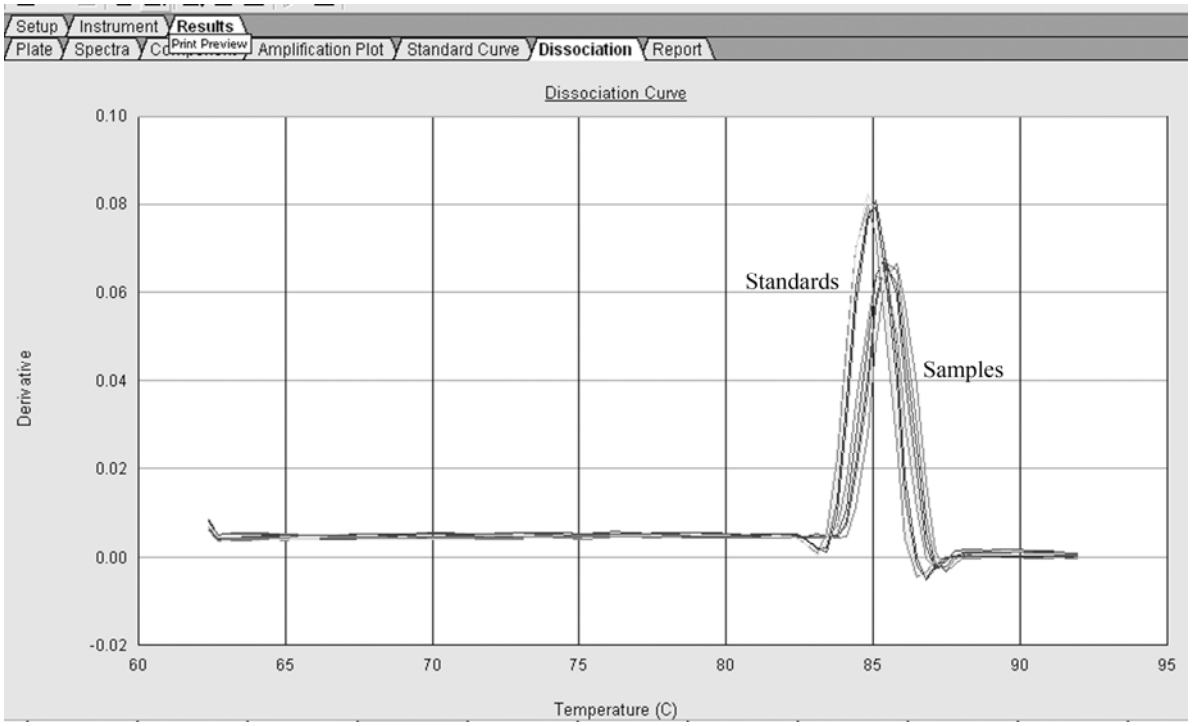

Figure D.2. Sample dissociation curve for the quantitative PCR of catechol 2,3 dioxygenase genes

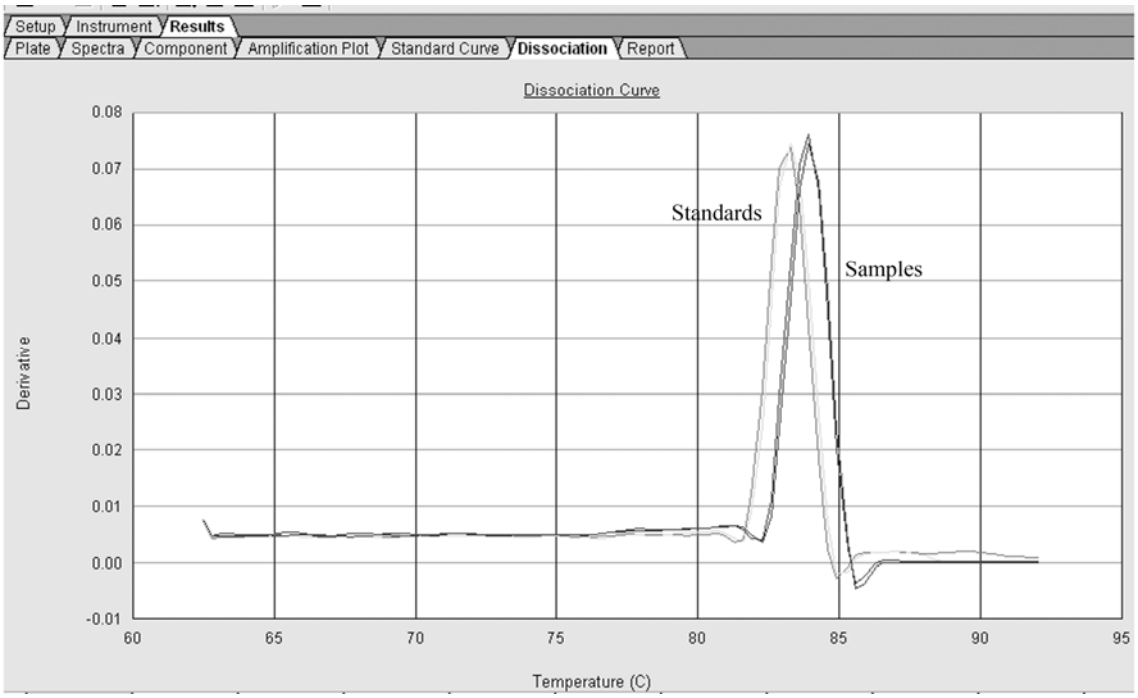

Figure D.3 Sample dissociation curve for the quantitative PCR of naphthalene dioxygenase (*nahAc*) genes

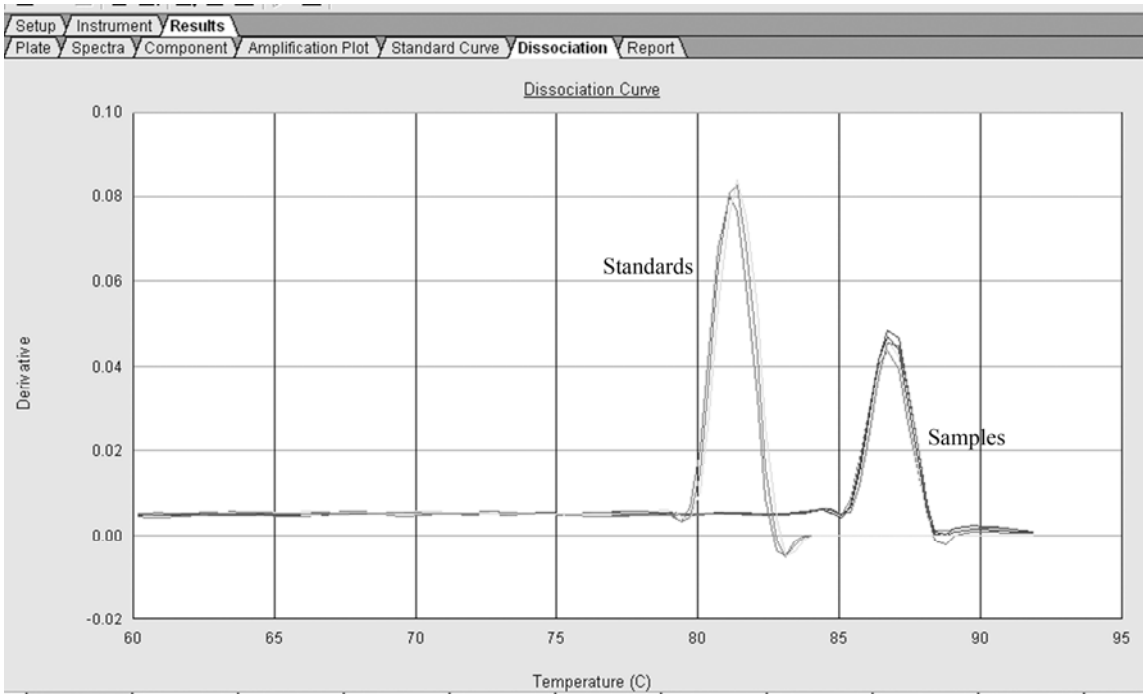

Figure D.4 Sample dissociation curve for the quantitative PCR of alkane monoxygenase (*alkB*) genes

APPENDIX E

Representative standards and analytical controls used for hydrocarbon analyses

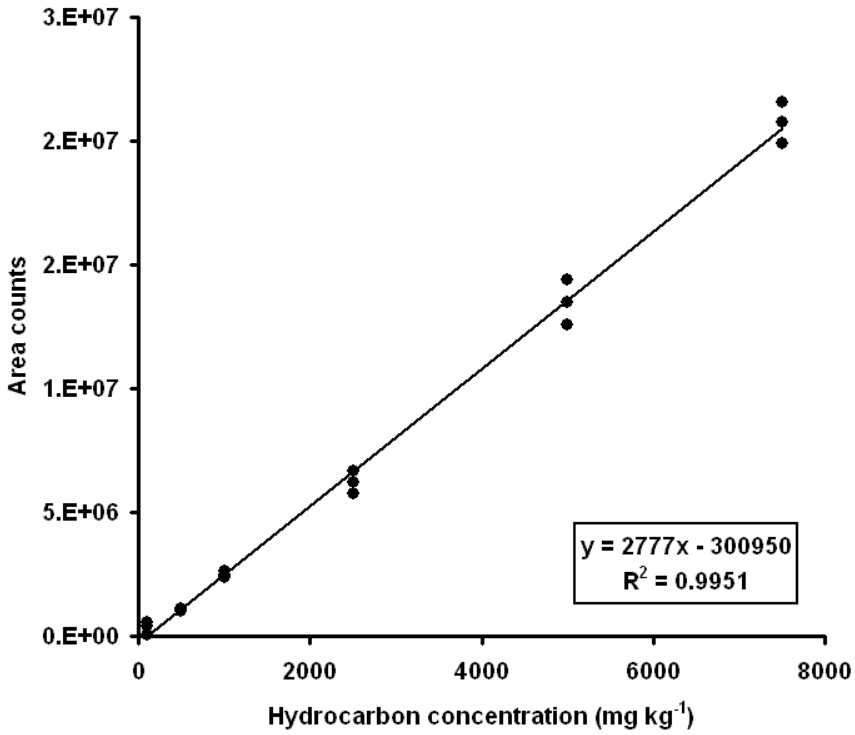

Figure E.1 Sample motor oil standard curve used for analysis of hydrocarbon concentration from GC-FID area counts

Table E.1. Representative table summarizing the reproducibility of analytical standards used during the GC-FID assessment of hydrocarbon concentration

	Average area counts of analytical controls		
Concentration (mg kg^{-1})	30W motor oil†		
100	3.29E+05 (2.74E+05)		
500	1.07E+06 (4.43E+04)		
1000	2.48E+06 (1.30E+05)		
2500	6.20E+06 (4.60E+05)		
5000	1.35E+07 (9.15E+05)		
7500	2.07E+07 (8.35E+05)		
	C10, C16, C34 standard mixture‡		
Concentration (mg kg^{-1})	C10	C16	C34
10	5.19E+04 (5.59E+02)	5.15E+04 (1.09E+02)	7.03E+04 (1.01E+03)
25	1.34E+05 (2.04E+03)	NA	1.80E+05 (8.52E+03)
50	2.74E+05 (5.26E+03)	1.52E+05 (1.17E+03)	3.41E+05 (1.39E+04)
100	6.80E+05 (2.43E+04)	6.13E+05 (3.01E+04)	6.68E+05 (1.03E+04)
	C16, C34 single standards§		
Concentration (mg kg^{-1})	C16	C34	
100	1.79E+05 (1.12 E+04)	1.77E+05 (1.32E+04)	

†.2004 GC-FID analytical controls for a single sample run. Data are presented as means (n = 3) with ± 1 SD in parentheses.
‡ 2005 GC-FID analytical controls for a single sample run. Data are presented as means (n = 3) with ± 1 SD in parentheses
§ 2006 GC FID analytical controls for a single sample run. Data are presented as means (n = 10) with ± 1 SD in parentheses