Enhanced biodegradation of petroleum hydrocarbons in the mycorrhizosphere of sub-boreal forest soils

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Summary
Petroleum hydrocarbon (PHC) contamination is becoming more common in boreal forest soils. However, linkages between PHC biodegradation and microbial community dynamics in the mycorrhizosphere of boreal forest soils are poorly understood. Seedlings (lodgepole pine, paper birch, lingonberry) were established in reconstructed soil systems, consisting of an organic layer (mor humus, coarse woody debris, or previously oil-contaminated mor humus) overlying mineral (Ae, Bf) horizons. Light crude oil was applied to the soil surface after 4 months; systems were destructively sampled at 1 and 16 weeks following treatment. Soil concentrations of four PHC fractions were determined using acetone–hexane extraction followed by gas chromatography – flame ionization detection analysis. Genotypic profiles of root-associated bacterial communities were generated using length heterogeneity-PCR of 16S rDNA. Most plant–soil treatments showed significant loss in the smaller fraction PHCs indicating an inherent capacity for biodegradation. Concentrations of total PHCs declined significantly only in planted (pine-woody debris and birch-humus) systems (averaging 59% and 82% loss between 1 and 16 weeks respectively), reinforcing the importance of the mycorrhizosphere for enhancing microbial catabolism. Bacterial community structure was correlated more with mycorrhizosphere type and complexity than with PHC contamination. However, results suggest that communities in PHC-contaminated and pristine soils may become distinct over time.

Introduction
Boreal forest ecosystems are increasingly exposed to petroleum hydrocarbon (PHC) contamination due to expanding natural resource extraction and transportation activities in these regions (Kanaly and Harayama, 2000). The current understanding of PHC biodegradation dynamics in northern forest soils is extremely limited (Robertson et al., 2007). Long-term studies of oil-contaminated forest soils have reported reductions in PHC concentrations due to biodegradation (or biotransformation) by indigenous microbial communities (Braddock et al., 2003; Prince et al., 2003). Culture and microcosm experiments have demonstrated that many ubiquitous soil fungi and bacteria can biodegrade a variety of PHC compounds (Heinonsalo et al., 2000; Meharg and Cairney, 2000; Sarand et al., 2000) and numerous genetic and biochemical pathways have been elucidated (Watanabe, 2002; Díaz, 2004). It is generally accepted that the capacity to biodegrade PHCs is intrinsic to most soils (Meharg and Cairney, 2000; Chaillan et al., 2005). In forest soils, PHC biodegradation requires metabolic synergy among different functional guilds of organisms, including mycorrhizal fungi and the bacterial communities closely associated with the mycorrhizosphere (Alexander, 2000; Chaudhry et al., 2005).

The extraradical mycelia of ectomycorrhizal (ECM) and ericoid mycorrhizal (ERM) fungi generate extensive volumes of mycorrhizosphere soil, which supports microbial growth through exudation of energy-rich carbon (C) substrates and provides surfaces for bacterial colonization (Meharg and Cairney, 2000; Cairney, 2005). In lignin-rich humus layers and PHC-contaminated soils, mycorrhizosphere development and function play central roles in controlling bacterial community structure and activities related to biodegradation (Sarand et al., 1998; 2000; Heinonsalo et al., 2000). Enhanced PHC biodegradation in the mycorrhizosphere (i.e. mycorrhizosphere effect) is attributed to the greater metabolic activities of higher densities of microorganisms (Linderman, 1988; Ingham and Molina, 1991; Siciliano and Germida, 1998). Specific interactions between different combinations of host plants and mycorrhizal fungi may influence the rates and patterns of PHC biodegradation in the mycorrhizosphere due to differences in the quality of C in root...
exudates and nutrient availability (Rygiewicz and Anderson, 1994; Corgié et al., 2003; Mueller and Shann, 2007).

Surface-spilled PHCs initially spread laterally within the lignin-rich forest floor (FH), where heavier fractions may strongly sorb (i.e. adsorb or absorb) to native soil organic matter. With time, lighter PHC fractions may move down the soil profile, along the paths of roots and fissures, resulting in uneven distribution within the soil profile (Trofimov and Rozanova, 2003; Suleimanov et al., 2005). Petroleum hydrocarbon contamination may lead to an initial loss of bacterial diversity, followed by proliferation of metabolically competent populations capable of inhabiting the new environmental conditions imposed by the chemical contaminants (Diaz, 2004). The major impacts of PHCs on microbial communities are often associated with disturbances to water, nutrient and oxygen regimes, related to the hydrophobicity and fluidity of oily products, rather than with toxicity of PHC constituents such as polycyclic aromatic hydrocarbons (PAHs), which usually constitute <2% of crude oil mixtures (McGill et al., 1981; Tarradellas and Bitton, 1997). Loss of populations that are susceptible to chemical toxicity or to changes in the soil physical–chemical habitat may lead to differences in soil community diversity that can be observed years after initial spill events (Lindstrom et al., 1999; Braddock et al., 2003; Deilie et al., 2004). Whether these differences alter soil microbial processes depends on the composition and degree of functional redundancy within the original soil community (Setälä et al., 2000).

In this study, we examined relationships between PHC biodegradation and bacterial community structure in bioassay experiments using reconstructed sub-boreal forest soils. Our first objective was to compare biodegradation patterns of four PHC fractions in plant–soil systems that varied with respect to type of organic soil layer (i.e. forest floor (FH), coarse woody debris (CWD), or forest floor previously contaminated with PHCs (FHoil)) and plant species (lodgepole pine, Pinus contorta Dougl. Ex Loud. var. latifolia Engelm.; paper birch, Betula papyrifera Marsh.; or lingonberry, Vaccinium vitis-idaea L.). Our second objective was to compare bacterial community structure in PHC-treated and untreated (control) systems of the different plant–soil combinations. Forest soils were layered [i.e. Ae (~1 cm) and Bf (~15 cm)] mineral soil layers beneath organic (FH, CWD or FHoil) soil layers (~2 cm)] in pots that were then planted with surface-sterilized pine or birch seeds or rooted cuttings of lingonberry. Pine seeds and lingonberry cuttings were also co-planted to generate double-plant systems; unplanted soil systems were used as controls. We hypothesized that the type of organic layers or previous exposure to PHCs would influence the initial composition of bacterial communities in the developing mycorrhizosphere. Individual mycorrhizosphere properties associated with the different plant treatments, such as density and depth of fine root tips, type of mycorrhizal association (ecto- or ericoid), and single or double-plant systems, were expected to further shape bacterial community structure. We anticipated that treatment of soil systems with ecologically relevant levels (i.e. approximately 22 tonnes ha⁻¹) of crude oil would enhance bacterial communities capable of PHC biodegradation, leading to differences in community structure and a reduction of soil PHCs between 1 and 16 weeks.

Results and discussion

Petroleum hydrocarbon biodegradation

After 4 months of seedling establishment under greenhouse conditions, half of all plant–soil systems were treated with BC light crude oil (volatile fraction removed by N₂ bubbling) by pipetting 3 ml (219 mg cm⁻²) of the oil onto the organic soil surface (see Appendix S1 in Supporting information). After 1 and 16 weeks, PHCs were extracted from soil layers using acetone–hexane extraction (Schwab et al., 1999; Siddique et al., 2006) followed by gas chromatography – flame ionization detection (GC-FID) (CCME, 2001). Our results showed that the process of PHC biodegradation in reconstructed sub-boreal forest soil systems over 16 weeks is closely related to the nature of the organic soil layer. We found significant reduction of total PHC levels following addition of crude oil to plant–soil systems with pristine FH (47% reduction; P < 0.001) and CWD (43% reduction; P = 0.02) organic soil layers (Fig. 1). These lignin-rich and metabolically active soil layers form substantial surface components of northern forest landscapes (Lundström et al., 2000; Prescott et al., 2000). Their tendency to retain PHCs in the soil organic matter was evident in our study, as PHC concentrations were far greater (>90%) than those extracted from the underlying mineral soils (Ae and Bf soil layers averaged ~7.3% and 1% respectively) in each system. The high level of variation within treatment groups is likely due to the uneven distribution of oil within the surface soil layers. Soil analyses showed a significantly lower pH and higher C:N ratio in CWD compared with FH layers at both 1 and 16 weeks (see Table S1 in Appendix S2 in Supporting information); however, these differences were minor and did not appear to play a role in the biodegradation capacity of these soil communities.

Unexpectedly, prior in situ treatment of the FH with PHCs did not appear to have enhanced microbial biodegradation capacity for subsequent PHC treatment in these soils (Braddock et al., 2003; Diaz, 2004). The lack of significant PHC reduction over the study period found in the previously contaminated (FHoil) soil layer may have been partly due to the presence of residual PHCs in control FHoil soils (Fig. 1), which may have accounted for
compounds biodegrade more slowly and also tend to decomposer organisms (Alexander, 2000). Contaminants in soil generally reduce their availability to oxidation) lead to distinct chemical profiles in soil; a chemically complex and heterogeneous environment with biodegradation of smaller substrates and contributed to a more chemically complex and heterogeneous environment with respect to biodegradation (Chaillan et al., 2004). Aging of contaminants in soil generally reduces their availability to decomposer organisms (Alexander, 2000).

Processes that reduce PHC concentrations over time (i.e. biodegradation, evaporation, water washing, or photooxidation) lead to distinct chemical profiles in soil; a decrease in smaller compound concentrations relative to larger compounds is typical of biodegradation profiles (Braddock et al., 2003). Larger, more complex PHC compounds biodegrade more slowly and also tend to sorb more strongly to the solid phase in soils (Alexander, 1999; 2000). In general, we found a trend of reduced F2 concentrations for all treatment groups between 1 and 16 weeks (Table 1). Significant decreases in F2 concentrations were found in most planted and unplanted organic layers, including the pine-CWD ($P = 0.002$), birch-FH ($P = 0.02$), lingonberry-CWD ($P = 0.03$) lingonberry-FHoil ($P = 0.001$), no plant-FH ($P = 0.02$) and no plant-CWD ($P = 0.02$) treatments. This finding was not surprising as many soil bacteria are known to completely mineralize smaller aliphatic compounds (e.g. alkanes $<\text{nC}16$) through central metabolic pathways such as $\beta$-oxidation and Kreb’s cycle (Alexander, 1999). Our results suggest that biodegradation capacity for F2 PHCs exists within all the soil systems tested. In addition, unplanted (control) soils continued to support biodegradative microbial communities for at least 8 months after soil collection.

In contrast, significant decreases of larger PHCs (i.e. F3a, F3b and F4 fractions) occurred only in planted systems (Table 1). Pine-CWD and birch-FH systems showed significant decreases in F3a ($P = 0.001$ for both treatments), F3b ($P = 0.004$ and 0.001) and F4 ($P = 0.002$ and 0.009) concentrations over the study period. Overall, concentrations of F3a, F3b and F4 PHCs decreased by 59%, 49% and 48% respectively, between 1 and 16 weeks in pine-CWD layer soils; the corresponding reductions in birch-FH layer soils were 74%, 82% and 92% respectively. Enhanced PAH biodegradation in rhizosphere compared with non-rhizosphere soils has been reported by Joner and colleagues (2006). This increased biodegradative potential in planted compared with unplanted soils is likely due to the mycorrhizosphere effect, where plant–fungal exudates support proliferation of functional groups of organisms with enhanced capacity for cometabolic biodegradation of larger PHCs (Linderman, 1988; Ingham and Molina, 1991; Rygiewicz and Anderson, 1994; Alexander, 2000). The presence of a mycorrhizosphere appears necessary to enhance biodegradation of $>\text{nC}16$ (i.e. F3a and greater) PHCs over the course of this 16 week study.

The finding that significant reductions of the larger PHC (i.e. nC16+) fractions occurred in only pine and birch systems provides indirect evidence for a stimulatory role of ECMs in the biodegradation process. The extensive extraradical mycelia of ECM fungi provide colonization surfaces and C substrates that enhance bacterial metabolism (Sarand et al., 1998; 2000; Heinonsalo et al., 2000) and secrete oxidative enzymes that open aromatic ring structures (Burke and Cairney, 2002). In our study, establishment of diverse ECM communities on the fine roots of pine and birch likely limited competition for reduced C by free-living soil fungi in the rhizosphere (Lindahl et al., 2007). The three-dimensional space created by incorporating depth in our layered soil systems was also
expected to reduce competition for plant-derived C and reduced C substrates in PHC mixtures between saprotrophic ECM fungi and associated bacterial communities, resulting in a generally stimulatory effect on biodegradation (Koivula et al., 2004; Cairney, 2005; Morgan et al., 2005). One explanation for the lack of significant PHC biodegradation in lingonberry (i.e. ericoid host) systems may be that the fine root systems were shallow and lacked extensive mycelia; secreted enzymes and exudates may have been diluted with distance from the ERM roots. For PAH degradation, the mycorrhizosphere effect may not extend more than a few millimetres from roots colonized by arbuscular mycorrhizae (Corgié et al., 2003; Joner and Leyval, 2003).

**Bacterial communities**

Overall, surface application of crude oil appeared to have little impact on the composition of bacterial communities in the mycorrhizosphere. Pairwise comparisons of PHC treatment effects within plant groups using Multi-Response Permutation Procedures revealed few significant differences between PHC-treated and control communities, as shown by the overlap in distribution of open (PHC-treated) and closed (untreated control) symbols depicted in the non-metric multidimensional scaling ordination (Fig. 2). Significant differences ($P = 0.04$) were found only between the bacterial communities of PHC-treated and untreated pine in the double-plant systems (depicted by diamond symbols). The heterogeneity in the soil matrix may provide a protective effect against the solvent shock associated with initial PHC contamination (Huertas et al., 2000).

Bacterial community structure varied between lingonberry and pine ($P = 0.01$) and lingonberry and birch ($P = 0.02$) in the single-plant systems and between pine and lingonberry in the double-plant systems ($P < 0.001$). Furthermore, community structure varied significantly ($P < 0.001$) in single- compared with double-plant systems for both pine and lingonberry. Figure 2 shows tight clustering of communities from the double-plant systems (i.e. diamonds for pine and inverted triangles for lingonberry) within the more dispersed distribution of single-plant system communities (i.e. squares for pine, circles for birch and triangles for lingonberry). This differing effect of plant when planted together versus when planted singly was intriguing. The soil conditions being the same, we presume that a double-plant system augments the effect of plant when planted together versus when planted singly was intriguing. The soil conditions being the same, we presume that a double-plant system augments the level of competitive interactions (perhaps by providing more specialized niches owing to different root systems) compared with a single-plant system, which is reflected in the bacterial diversity. Although significant PHC biodegradation was not observed in either of the double-plant (pine and lingonberry - FH or - CWD) systems tested, this may...
represent an example of increased environmental complexity where within-group variation was too great to detect PHC reduction over 16 weeks.

Significant differences in bacterial community structure were found between the FH and FHoil organic soil layers \((P < 0.001)\) and the CWD and FHoil organic soil layers \((P = 0.009)\), but not between FH and CWD groups. It appears that prior treatment with crude oil (i.e. PHCs applied to the FH at a rate of 2 l m\(^{-2}\) and weathered \textit{in situ} for 4 months prior to soil collection) gave rise to a distinct bacterial community in the mycorrhizosphere of FHoil systems, representing proliferation of populations capable of inhabiting the contaminated soil environment (Díaz, 2004). It is possible that rapid biodegradation of some smaller PHC compounds occurred over the first week in the FHoil soils due to presence of a more acclimatized microbial community (Alexander, 1999). Whether these communities represent populations specialized for PHC biodegradation over time was not apparent from this study as few significant decreases in PHCs were detected after 16 weeks. Other studies have found that differences in soil community diversity between PHC-contaminated and uncontaminated forest sites are present for many years after initial spill events (Lindstrom \textit{et al.}, 1999; Braddock \textit{et al.}, 2003). In the current study, comparisons between PHC-treated and control groups after the 16 week harvest also showed significant \((P = 0.036)\) differences in bacterial community structure. From a forest soil management perspective, a key challenge is to discover if modifications of the environment resulting from PHC contamination lead to changes in microbial communities that may inhibit future ecosystem functions. In general, the capacity for biodegradation of various organic substrates, which is controlled by the functional redundancy of the community originally present (Setälä \textit{et al.}, 2000; Delille \textit{et al.}, 2004), seems to remain intact in PHC-contaminated soils for some time in the absence of further disturbance.

\section*{Acknowledgements}

We thank Paul Sanborn for access to the field site, Steve Storch and John Orlowsky for greenhouse assistance, Quanji Wu for PHC analysis, Anna Scarpino and Allen Esler for soil analysis, Dana O’Bryan and Mark Thompson, for DNA analysis, and Ralph Alm (Husky Oil, Prince George, BC), Dawn Stubley (BC Ministry Tree Seed Centre, Surrey, BC) and Barb Rayment (Birch Creek Nursery, Prince George, BC) for providing bioassay supplies. Funding for this project was provided by the Natural Sciences and Engineering Research Council of Canada. Two anonymous reviewers provided constructive comments that strengthened the paper.
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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Experimental procedures.

**Appendix S2.** Soil properties.

**Table S1.** Mean (± standard error) total soil C concentration, total soil N concentration and soil pH at 1 and 16 weeks. Total soil C and N concentrations are reported on an air-dry mass basis.

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