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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.

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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.*, 2004; Delille *et al.*, 2004). 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 (Díaz, 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; Delille *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 N_2 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; Díaz, 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

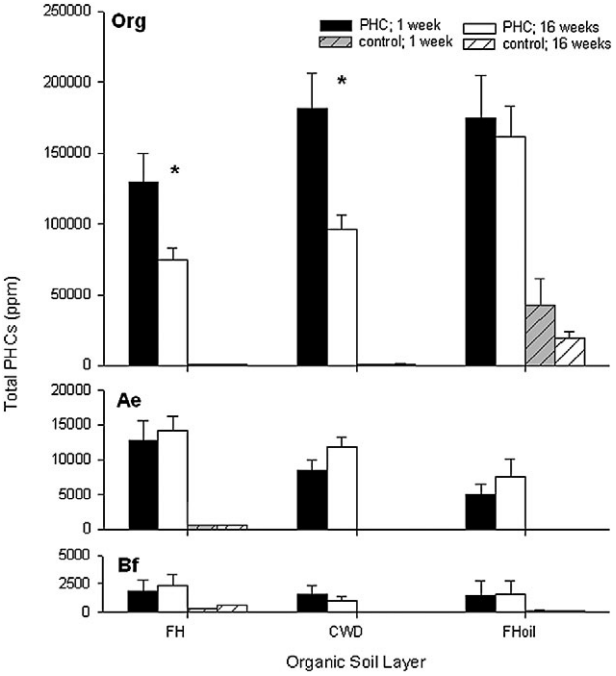

Fig. 1. Concentration of total PHCs (ppm = mg kg^{-1}) in three organic soil layers (FH, CWD and FHoil) for PHC-treated and untreated controls at 1 and 16 weeks (data pooled for plant treatment). Bars represent standard errors of the means. Significant (one-way ANOVA, $\alpha = 0.05$) losses of PHCs within organic soil treatment groups are indicated by *. Petroleum hydrocarbons were extracted from soils using a 4 h sequential shake method (Schwab *et al*., 1999; Siddique *et al*., 2006). Approximately 1 g of each soil layer was extracted with 10 ml of acetone:hexane (1:1, v/v) in glass vials that were shaken on a reciprocating platform shaker at 120 cycles per minute for 30 min (repeated three times). Solvent extracts were cleaned using a silica gel column procedure (CCME, 2001) and concentrated in 2 ml of AR grade toluene. Petroleum hydrocarbons were analysed on a Varian Model CP 3800 Gas Chromatograph equipped with a flame ionization detector and quantified using the CCME Canada-Wide Standard for Petroleum Hydrocarbons in Soil – Tier 1 Method (CCME, 2001).

up to 39% (mean of 15%) of PHCs extracted from PHC-treated FHoil soils. Petroleum hydrocarbon levels were negligible in all other untreated control soils. These residual PHCs likely consisted of a greater proportion of more recalcitrant compounds due to preferential 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 <nC16) through central metabolic pathways such as β-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 >nC16 (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

Table 1. Mean (± standard error) concentrations of F2 (nC10–nC16), F3a (nC16–nC23), F3b (nC23–nC34) and F4 (nC34–nC50) PHCs (ppt = mg g^{-1}) in organic layers of plant – soil systems compared at 1 and 16 weeks after PHC treatment ($n = 3$).

	PHC	FH layer – [PHC] (ppt)		CWD layer – [PHC] (ppt)		FHoil layer – [PHC] (ppt)	
Plant	Frxn	1 week	16 weeks	1 week	16 weeks	1 week	16 weeks
Pine	F2	32.4 ± 13.9	7.0 ± 1.0	**36.5 ± 4.7**	**3.6 ± 0.6**	18.6 ± 6.9	3.8 ± 1.7
	F3a	56.3 ± 2.3	43.6 ± 6.4	**82.7 ± 5.0**	**33.9 ± 3.2**	46.3 ± 23.2	38.5 ± 6.0
	F3b	52.9 ± 23.5	47.2 ± 7.2	**77.5 ± 5.7**	**39.8 ± 2.5**	44.4 ± 22.7	40.0 ± 7.0
	F4	13.8 ± 7.1	13.7 ± 1.8	**22.4 ± 1.4**	**11.7 ± 0.5**	13.6 ± 6.1	11.8 ± 5.4
Birch	F2	**36.6 ± 9.1**	**3.0 ± 0.2**	–	–	–	–
	F3a	**68.2 ± 5.9**	**17.8 ± 0.6**	–	–	–	–
	F3b	**57.3 ± 4.3**	**10.4 ± 2.3**	–	–	–	–
	F4	**22.6 ± 4.3**	**1.9 ± 1.0**	–	–	–	–
Lingonberry	F2	49.3 ± 20.6	12.6 ± 0.8	**34.7 ± 8.4**	**5.0 ± 1.5**	**38.1 ± 5.3**	**9.3 ± 3.7**
	F3a	77.7 ± 36.2	68.0 ± 8.4	48.6 ± 9.2	40.0 ± 16.2	57.0 ± 10.0	87.1 ± 18.3
	F3b	64.5 ± 32.2	63.2 ± 9.5	35.4 ± 6.5	35.2 ± 15.0	37.7 ± 7.7	63.1 ± 11.6
	F4	16.7 ± 8.4	16.6 ± 2.4	9.1 ± 1.9	9.5 ± 4.5	9.6 ± 2.2	17.4 ± 3.4
Pine + lingonberry	F2	39.2 ± 23.0	3.7 ± 0.9	11.6 ± 3.2	5.6 ± 1.6	–	–
	F3a	60.4 ± 30.8	38.5 ± 1.1	22.7 ± 3.3	32.4 ± 8.6	–	–
	F3b	34.4 ± 16.9	24.7 ± 0.9	12.4 ± 2.2	20.8 ± 3.6	–	–
	F4	18.4 ± 9.5	12. ± 0.6	3.2 ± 0.4	7.7 ± 1.7	–	–
No plant (control)	F2	**51.2 ± 15.4**	**7.6 ± 1.3**	**34.0 ± 10.6**	**4.9 ± 1.0**	35.4 ± 9.2	9.6 ± 3.4
	F3a	77.7 ± 16.8	43.0 ± 3.0	54.1 ± 16.6	28.7 ± 4.3	92.5 ± 13.6	81.0 ± 1.9
	F3b	51.5 ± 11.2	34.5 ± 6.0	30.4 ± 6.3	24.0 ± 4.9	101.9 ± 16.5	97.3 ± 1.6
	F4	15.4 ± 3.4	11.0 ± 1.3	8.1 ± 2.1	6.6 ± 1.4	28.2 ± 3.9	25.8 ± 0.2

Significant (one-way ANOVA, $\alpha = 0.05$) differences are shown in bold.
Total PHCs were divided into fractions based on equivalent normal straight-chain hydrocarbon (nC) boiling point ranges in soil: F2 (nC10–nC16), F3a (nC16–nC23), F3b (nC23–nC34) and F4 (nC34–nC50) (CCME, 2001). Petroleum hydrocarbon fractions were determined on the GC-FID chromatograms using peak retention times of the external standards decane (nC10), hexadecane (nC16), nonadecane (nC19), eicosane (nC20), tricosane (nC23), dotriacontane (nC32) and tetratriacontane (nC34); external standards were run concurrently with samples at concentrations of 10, 25, 50, 125 and 250 mg l^{-1}.

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 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

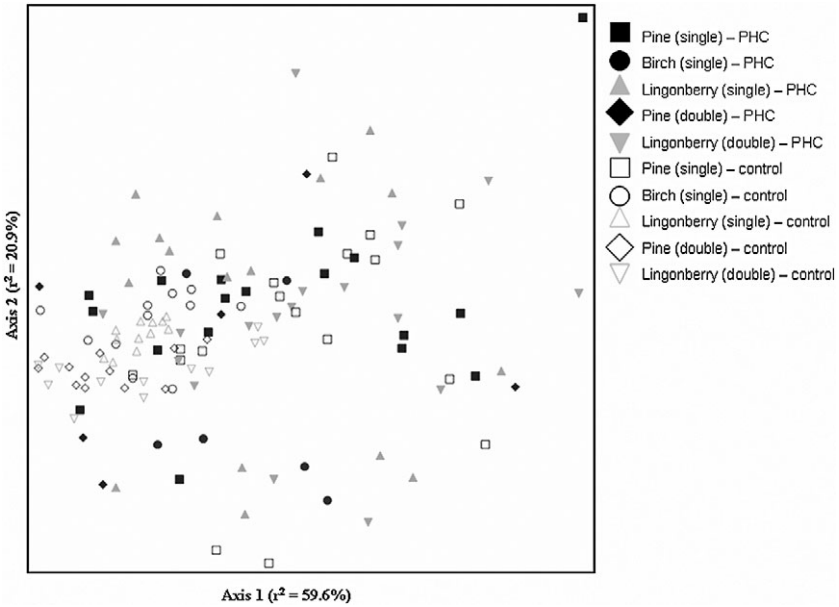

Fig. 2. Non-metric multidimensional scaling (NMS) ordination of bacterial community structure associated with single- (pine, birch or lingonberry) and double- (pine + lingonberry) plants in PHC-treated and untreated (control) soil systems (stress = 16.42; instability = 0.06). Solid symbols represent PHC-treated systems; open symbols represent untreated control systems. DNA was extracted from mycorrhizal roots using a modified CTAB protocol (Fujimura *et al.*, 2008) and amplified via PCR using 10 μM forward (D4 fluorescent dye-labelled 27F) and reverse (unlabelled 355R) primers (Mills *et al.*, 2003). DNA samples (2 μl) were analysed using the amplicon fragment length polymorphism program of the CEQ™ 8000 sequencer (Beckman-Coulter). Community structure was assessed graphically with NMS, which was calculated on the basis of a Sørensen (Bray–Curtis) distance measure with 50 runs with real and randomized data (compared using Monte Carlo simulations) and a maximum of 500 iterations to assess stability (McCune and Mefford, 1999; McCune and Grace, 2002). Pairwise comparisons between treatment groups were tested with Multi-Response Permutation Procedures (MRPP) using the Sørensen distance measure (McCune and Grace, 2002) (see Appendix S1 in *Supporting information*).

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 *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 *et al.*, 1999; Braddock *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 *et al.*, 2000; Delille *et al.*, 2004), seems to remain intact in PHC-contaminated soils for some time in the absence of further disturbance.

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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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