

## Characterization of Humus Microbial Communities in Adjacent Forest Types That Differ in Nitrogen Availability

S.E. Leckie<sup>1</sup>, C.E. Prescott<sup>1</sup>, S.J. Grayston<sup>2</sup>, J.D. Neufeld<sup>3</sup> and W.W. Mohn<sup>3</sup>

(1) Department of Forest Sciences, University of British Columbia, 2424 Main Mall, Vancouver, BC, V6T 1Z4, Canada

(2) Macaulay Land Use Research Institute, Aberdeen, AB15 8QH, UK

(3) Department of Microbiology and Immunology, University of British Columbia, #300-6174 University Boulevard, Vancouver, BC, V6T 1Z3, Canada

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### Abstract

To address the link between soil microbial community composition and soil processes, we investigated the microbial communities in forest floors of two forest types that differ substantially in nitrogen availability. Cedar-hemlock (CH) and hemlock-amabilis fir (HA) forests are both common on northern Vancouver Island, B.C., occurring adjacently across the landscape. CH forest floors have low nitrogen availability and HA high nitrogen availability. Total microbial biomass was assessed using chloroform fumigation-extraction and community composition was assessed using several cultivation-independent approaches: denaturing gradient gel electrophoresis (DGGE) of the bacterial communities, ribosomal intergenic spacer analysis (RISA) of the bacterial and fungal communities, and phospholipid fatty acid (PLFA) profiles of the whole microbial community. We did not detect differences in the bacterial communities of each forest type using DGGE and RISA, but differences in the fungal communities were detected using RISA. PLFA analysis detected subtle differences in overall composition of the microbial community between the forest types, as well as in particular groups of organisms. Fungal PLFAs were more abundant in the nitrogen-poor CH forests. Bacteria were proportionally more abundant in HA forests than CH in the lower humus layer, and Gram-positive bacteria were proportionally more abundant in HA forests irrespective of layer. Bacterial and fungal communities were distinct in the F, upper humus, and lower humus layers of the forest floor and total biomass decreased in deeper layers. These

results indicate that there are distinct patterns in forest floor microbial community composition at the landscape scale, which may be important for understanding nutrient availability to forest vegetation.

### Introduction

Organic matter decay and nutrient cycling in forest soils are influenced by the interrelationships among climate, litter types, and the variety and abundance of soil organisms [7, 13, 49, 59]. Although there has been considerable research on the effects of climate and litter quality, it has been difficult to link the structure of soil communities to soil processes, because appropriate methods have only recently been developed for rapidly studying complex microbial communities. Several fingerprinting techniques have been developed to provide a rapid assessment of microbial communities, particularly for comparison or monitoring purposes. Soil microbial communities have been characterized using denaturing gradient gel electrophoresis (DGGE) [26, 36, 54] and ribosomal intergenic spacer analysis (RISA) [53], which are based on variability of ribosomal RNA genes and intergenic spacers, respectively. DNA from the community of organisms is amplified using the polymerase chain reaction (PCR) and the fragments are separated in a gel matrix based on length or sequence polymorphism to produce a visual pattern, or fingerprint, of the community. Phospholipid fatty acid (PLFA) profiling of soil microbial communities is a phenotypic fingerprinting method which has been used to study variability of forest soil communities [21, 47, 55]. Variation in the types of fatty acids present in the cell membrane of different organisms provides a profile of the community, and certain PLFAs are used as markers for particular groups of organisms [18, 22]. Total PLFA content has been shown to correlate with other measures of microbial biomass [3, 67].

Present address for S.J. Grayston: Department of Forest Sciences, University of British Columbia, 2424 Main Mall, Vancouver, BC, V6T 1Z4, Canada

Correspondence to: S.E. Leckie; E-mail: sleckie@interchange.ubc.ca

There are a number of studies which have used these recent techniques to address changes in soil communities in response to stress and disturbance; however, little is known about the variability of microbial communities in natural forest ecosystems [58]. By understanding how these communities are structured in functionally different ecosystems, we will be better able to understand the controls on soil processes and the consequent effects of shifts in community composition. Myers et al. [42] found that soil microbial communities in three forest types in Michigan had similar biomass, but distinct PLFA profiles, indicating differences in community composition. In Finland, Pennanen et al. [47] reported distinct PLFA profiles in forests of different fertility, with decreases in fungal PLFAs and increases in bacterial PLFAs as site fertility increased. Microbial communities have been shown to be influenced by tree species [27, 52, 55] and may mediate tree species effects on soils. Distinct microbial communities have also been found in different layers of the forest floor and mineral soil [21, 36]. Many of the studies undertaken so far have had insufficient replication to reliably indicate relationships with tree species or other site factors. There is a need for well-replicated studies, using standard comparable methods to understand the variability and distribution of organisms in forest soils.

In this study, we compared the humus microbial communities in two common forest types of northern Vancouver Island, B.C. Western redcedar (*Thuja plicata* Donn.)–western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) (CH type) forests have low nitrogen availability and poor tree performance on cutovers [50]. Adjacent forests of western hemlock–amabilis fir (*Abies amabilis* (Dougl.) Forbes) (HA type) have greater nitrogen availability and good regeneration on cutovers [50]. These two forest types provide an interesting contrast to explore forest soil communities and the potential links between the organisms and nutrient cycling processes.

Total microbial biomass and community composition in three forest floor layers in four CH and four adjacent HA forests were assessed using chloroform fumigation–extraction, DGGE analysis of the bacterial community, RISA profiles of the bacterial and fungal communities, and PLFA analysis of the whole microbial community. We hypothesized that microbial biomass and community composition differ between CH and HA forest floors and specifically addressed the following questions: (1) do the biomass and composition of forest floor microbial communities differ between CH and HA forests?, (2) how different is the biomass and community composition in different layers of the forest floor?, and (3) do composite forest floor samples adequately capture both the average community composition and the variability of a site?

## Methods

**Sites and Sampling.** Study sites were located within Western Forest Products Ltd. Tree Farm License 6 on northern Vancouver Island, BC, Canada (50°60'N, 127°35' W), in the very wet maritime subzone of the Coastal Western Hemlock (CWHvm) biogeoclimatic zone [29]. The climate is characterized by cool, moist summers, and mild, wet winters, with an average 1900 mm yearly precipitation, about 70% of which falls mainly as rain in the winter months (October to March). Mean daily temperatures range from 3.3°C in January to 14.1°C in August. The area is characterized by gentle topography with elevations no greater than 300 m above sea level. Mineral soils are well to poorly drained loamy Humo-Ferric Podzols which overlay unconsolidated morainal and fluvial outwash material [50]. Forest floors are up to 1 m thick and are predominantly humimors and lignomors, with well-developed humus horizons and large amounts of decomposing wood.

A pair of adjacent CH and HA forests was located at each of four study sites. CH forests are old-growth, dominated by western redcedar with western hemlock as a codominant species and in the understory. These forests are uneven-aged, with a relatively open canopy and an understory dominated by salal (*Gaultheria shallon* Pursh) [34]. HA forests are predominantly even-aged second-growth, established after a large-scale windstorm in 1906. Western hemlock and amabilis fir dominate the dense canopy, with a sparse understory of mosses and ferns [34].

Forest floors were sampled in early October 2001. Each of the eight forests was sampled within an area of approximately 400 m<sup>2</sup>, always more than 50 m from the transition between forest types or an edge. Within each forest, a small pit was dug at each of 10 points, at least 3 m from one another and variable distances from mature trees. The F layer [17], the uppermost humus (H<sub>U</sub>) layer, and the deepest humus (H<sub>L</sub>) layer directly above the mineral soil were sampled. Total forest floor depth was measured at each sampling point. The 10 samples of each of the three layers were combined for each of the four CH and HA forests, resulting in a total of 24 composite samples. At one site, within the HA forest, the 10 samples of the upper humus layer were also individually analyzed to address the degree of spatial variability within the site and to evaluate how accurately the composite sampling reflected the mean value.

Samples were kept cool (~4°C) during transport and processing. Each composite sample was sieved to <2 mm to remove large materials (wood pieces, roots, or stones) and to homogenize. Duplicate subsamples were dried at 70°C for 24 h to estimate moisture content. Moisture was also measured on uncomposited, unsieved samples. Fresh material was used within 1 week for microbial biomass

measurement (chloroform fumigations–extraction) and DNA extraction. Portions of each sample were frozen at  $-20^{\circ}\text{C}$  to be used later for PLFA and pH analyses. The pH was measured on 5 g (fresh weight) thawed samples mixed with 20 mL of  $\text{dH}_2\text{O}$ , using a Hanna Instruments 9025 pH meter.

**Microbial Biomass Carbon.** Microbial biomass carbon was measured using the chloroform fumigation–extraction (CFE) technique [60, 62]. Soluble organic carbon was measured in 0.5 M  $\text{K}_2\text{SO}_4$  extracts from fumigated and unfumigated samples, using the high-temperature combustion method, with a Shimadzu TOC-500 Carbon Analyzer. Microbial biomass carbon was estimated as the difference between fumigated and unfumigated samples and no correction factor was used. Measurements from unfumigated samples are reported as soluble organic carbon. Total microbial, bacterial, and fungal biomass were also estimated using PLFA analysis (see below).

**DNA Extraction and Purification.** DNA was extracted from equivalent dry weights of each sample (400–500 mg fresh weight). The Bio 101 Fast DNA Kit for Soil (La Jolla, California) was used for DNA extraction and purification, with some modifications from the manufacturer's directions based on empirical determination. Samples were vigorously shaken using a Mini Bead-Beater (BioSpec Products, Bartlesville, Oklahoma) for 2.5 min at 5000 beats per minute, then centrifuged for 10 min (14,000 rpm, Eppendorf Centrifuge 5415C). A 100- $\mu\text{L}$  aliquot of the supernatant (containing DNA) was drawn off and further purified with a protein precipitation and washed twice with ethanol–salt solution in spin filter columns. DNA was eluted in 100  $\mu\text{L}$  of water. DNA quality and quantity were confirmed on a 1% agarose gel and imaged using an AlphaImager 1200 (Alpha Innotech, CA). High molecular weight DNA indicated minimal shearing and, therefore, high-quality DNA. DNA was quantified by comparison with a range of known amounts of standard 1 kb DNA ladder (Invitrogen, Carlsbad, CA) using AlphaEase (version 3.3, Alpha Innotech, 1996) to measure the intensities of ethidium bromide and to construct a calibration curve. All samples were diluted to give equal DNA concentrations (10 ng  $\mu\text{L}^{-1}$ ) for subsequent PCR reactions.

**DGGE Analysis of Bacterial Communities.** Two primer pairs were used to amplify different regions of 16S rDNA (Table 1). The lengths of PCR products were approximately 585 bp for primer pair 357f-907r and 491 bp for 63f-518r. A GC-rich clamp was used on the forward primer of each pair, to ensure a halt of migration of the fragments in the denaturing gradient gel [40]. Polymerase chain reactions each consisted of 1  $\mu\text{L}$  purified DNA

template (10 ng), 5  $\mu\text{L}$  10 $\times$  PCR buffer (Qiagen, Valencia, CA) (final concentration of 1.5 mM  $\text{MgCl}_2$ ), 200  $\mu\text{M}$  each deoxynucleoside triphosphate, 500 nM each primer, 670  $\mu\text{g mL}^{-1}$  bovine serum albumin, and 1.25 units *Taq* DNA polymerase in a final volume of 50  $\mu\text{L}$ . Reactions were carried out in a PTC150 MiniCycler (MJ Research, Waltham, MA). Following a simplified hotstart (samples loaded into thermal cycler at  $95^{\circ}\text{C}$ ), an initial denaturation step was done at  $94^{\circ}\text{C}$  for 5 min. Subsequent cycles consisted of a 1-min denaturation step at  $94^{\circ}\text{C}$ , a 1-min primer-annealing step at  $55^{\circ}\text{C}$ , and a 1-min extension step at  $72^{\circ}\text{C}$ . At the end of 25 cycles, a final 7-min extension step was used to ensure that all PCR products were fully extended. Negative controls containing the same mixture, but without any DNA template, were included and never yielded detectable PCR products. PCR products were quantified on a 2% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

DGGE was performed using the Bio-Rad D-Code System (Bio-Rad, Hercules, CA), with modifications of the protocol of Muyzer et al. [40]. PCR products were loaded onto a 6% (37.5:1) polyacrylamide gel in 1 $\times$  TAE buffer (40 mM Tris base, 20 mM acetate, 1 mM  $\text{Na}_2\text{-EDTA}$ , pH 8). Gels had a denaturing gradient of 40–65%, where 100% denaturant contains 7.0 M urea and 40% deionized formamide. Standard markers, consisting of bands from cultured isolates as well as from trial DGGE fingerprints, were run on the outside and middle lanes of every gel to allow comparison of fingerprints within and among gels. Electrophoresis was carried out for 16 h at  $60^{\circ}\text{C}$  and 75 V. After electrophoresis, gels were stained using SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR) and immediately imaged using an AlphaImager 1200 (Alpha Innotech, CA).

DGGE fingerprint patterns were compared using Gel Compar II (Applied Maths, Belgium). Standard markers were used to normalize fingerprints within and among gels. Patterns were analyzed using Pearson's product moment correlation, which gives pairwise percent similarity for the entire fingerprint image of all patterns. This method was used instead of band-matching approaches to avoid the high subjectivity of identifying and matching individual bands in these complex fingerprints.

**RISA Profiles of Bacterial and Fungal Communities.** The same DNA extracts (see above) were used for RISA. Primers 1406f and 23Sr were used to amplify the spacer region between the 16S and 23S ribosomal subunit genes of the bacterial community (Table 1). Primers ITS1-Ff and ITS4r were used to amplify the ribosomal internal transcribed spacer region of the fungal community (Table 1). The forward primers of each pair were 5' end labeled with the phosphoramidite dye 5-FAM. Reaction mixtures were as above except that they

**Table 1. Primers used for DGGE and RISA profiling of soil bacterial and fungal community DNA**

Primer <sup>a</sup>	Target site	Sequence (5' to 3')	Specificity	Reference
357f-GC <sup>c</sup>	16S: 341–357 <sup>b</sup>	CCT ACG GGA GGC AGC AG	Bacterial	[40]
907r	16S: 907–926 <sup>b</sup>	CCG TCA ATT CMT TTG ACT TT	Universal	[41]
63f-GC	16S: 43–63 <sup>b</sup>	CAG GCC TAA CAC ATG CAA GTC	Bacterial	[39]
518r	16S: 518–534 <sup>b</sup>	ATT ACC GCG GCT GCT GG	Universal	[40]
1406f	16S: 1392–1406 <sup>b</sup>	TGY ACA CAC CGC CCG T	Universal	[9]
23Sr	23S: 115–130 <sup>b</sup>	GGG TTB CCC CAT TCR G	Bacterial	[9]
ITS1-Ff	3' end of 18S	CTT GGT CAT TTA GAG GAA GTA A	Fungal	[25]
ITS4r	5' end of 28S	TCC TCC GCT TAT TGA TAT GC	Universal	[64]

<sup>a</sup>f (forward) and r (reverse) indicate the orientation of the primers with respect to the 16 rDNA sequence.

<sup>b</sup>*Escherichia coli* numbering of the 16S or 23S rRNA gene of Brosius et al. [10].

<sup>c</sup>GC indicates a 40 bp GC-rich sequence attached to the 5' end of the primer.

The sequence is 5'-CGCCGCGCGCCCCGCGCCCGTCCGCGCCCCGCCCCG-3'.

contained 2 µL purified DNA template (20 ng) and 2 mM MgCl in the fungal reactions. Cycling was as described above except annealing temperatures of 56°C and 58°C were used for fungi and bacteria, respectively, and the extension step was 1.5 min in each cycle to account for longer products. Controls and quantification were as described above, but PCR products were not purified. A RoboCycler gradient 96 (Stratagene, La Jolla, CA) thermal cycler was used for RISA PCR reactions.

DNA fragments were resolved using an automated approach [20, 53] on 5% Long Ranger gels (6.0 M Urea, TTE) using an ABI Prism 377. Samples were loaded with GS2500 Rox size standards and run under denaturing conditions for 15 h at 4000 V. GeneScan software was used to convert fluorescence data to electropherograms, and these data were analyzed using Gel Compar II. Very high resolution and extremely precise normalization among lanes could be achieved with this technique. Therefore, band-matching and Ochiai's similarity among samples were used to analyze the RISA fingerprints, based on presence and absence of each identified band, to generate a similarity matrix for the whole data set.

**PLFA Profiles of Microbial Communities.** Lipids were extracted from approximately 650 mg (fresh weight) thawed material using a Bligh and Dyer [8] extraction, as modified by White et al. [63] and Frostegård et al. [24]. The polar lipid fraction was subject to a mild alkaline methanolysis to yield fatty acid methyl esters (FAMES). FAMES were separated and quantified by gas chromatography (Hewlett Packard E5895 Series II) using splitless injection, helium as a carrier gas, and a polar column, and were identified relative to standards. Fatty acid nomenclature follows Frostegård et al. [23]. Fatty acids 16:1ω7c, 16:1ω7t, cy17:0, and 18:1ω7 were used to represent Gram-negative bacteria [65] and fatty acids i15:0, a15:0, i16:0, a17:0, br17:0, and 10Me17:0 were chosen to represent Gram-positive bacteria [45]. Bacterial biomass was represented by 15:0 and cy19:0 in addition to the above fatty acids (except 10Me17:0) [18, 23, 37]. The fatty acid 18: 2ω6,9 was used to represent fungal biomass

[18, 22]. Actinomycetes were represented by 10Me18:0 [38]. The ratio of fungal-to-bacterial PLFAs was calculated without 16:1ω7c in the measure of bacterial biomass, to make the ratios comparable to those presented by Frostegård and Bååth [22].

**Statistical Analyses.** To test for differences in microbial biomass carbon, pH, moisture, extractable carbon, and PLFA biomarkers, we used analysis of variance (General Linear Model procedure of SAS) of a split-plot experiment in a randomized complete block design, with forest type as the whole-plot factor, and forest floor layer as the subplot factor. Total depth of the forest floor was analyzed as a completely randomized block design. Data for fungal PLFAs and Gram-negative bacterial PLFAs were log-transformed, extractable carbon data were square-root transformed, and microbial carbon data were inverse square-root transformed to meet the assumptions of homogeneity of variance and normality. Data for the actinomycete marker did not meet the assumption of homogeneity of variances, even after transformation, and were therefore not analyzed statistically. A *t*-test was used to test if the composite sample differed from the mean value of the uncomposited samples for estimates of microbial biomass, forest floor measurements, and marker PLFA groups. An *F*-test was used to determine if the variance of the ten uncomposited samples differed from the variance of the four sites (with composite samples). An alpha value of 0.05 was considered significant for all analyses. Analysis of variance was followed by pairwise *t*-test comparisons of the least-square means, with the alpha level (0.05) adjusted for the number of comparisons using Bonferroni's adjustment to control the overall type I error rate. Where significant interactions occurred, comparisons were made only within each forest type and within each layer.

Principal component analysis (PCA) was used to analyze the DGGE, RISA, and PLFA profiles of the microbial community. For DGGE and RISA, the similarity matrix calculated with Gel Compar II was analyzed by PCA. Proportional PLFA data (% mol) were log-trans-

**Table 2.** Forest floor properties and microbial biomass of F, upper humus (H<sub>U</sub>), and lower humus (H<sub>L</sub>) layers in cedar–hemlock (CH) and hemlock–amabilis fir (HA) forests<sup>a</sup>

Forest floor layer	Moisture (%)		pH		Soluble organic C (mg C g <sup>-1</sup> dry humus)		CFE biomass C (mg C g <sup>-1</sup> dry humus)		Total PLFA <sup>b</sup> (nmol g <sup>-1</sup> dry humus)	
	CH	HA	CH	HA	CH	HA	CH	HA	CH	HA
F	76.1 a (1.3)	76.9 x (1.0)	<u>4.37</u> a (0.09)	<u>3.92</u> x (0.03)	1.86 a (0.14)	0.94 x (0.05)	4.27 a (1.76)	3.75 x (0.79)	1730 a (120)	1540 x (50)
H <sub>U</sub>	78.4 ab (1.3)	77.8 x (1.0)	3.92 b (0.08)	3.64 x (0.04)	0.85 b (0.06)	0.58 x (0.02)	2.88 b (0.38)	2.77 y (0.50)	1350 b (70)	1240 y (30)
H <sub>L</sub>	<u>80.3</u> b (1.1)	<u>77.4</u> x (1.0)	3.79 b (0.09)	3.71 x (0.09)	0.50 b (0.10)	0.48 x (0.06)	1.84 c (0.15)	1.49 z (0.34)	890 c (50)	970 z (70)

<sup>a</sup>The means of four sites are presented, with standard errors of the mean in parentheses below. Within each forest type, values followed by the same letter are not significantly different, and underlined values indicate significant differences between the two forest types for a particular layer.

<sup>b</sup>Significant site × forest interaction ( $F = 4.1$ ,  $p = 0.031$ , at one site CH > HA).

formed prior to analysis to remove the effect of skewed distributions, and correlation matrices were used. Data from the uncomposited samples were also used in the principal component analyses of PLFA data. The uncomposited samples were used as supplementary samples and thus did not influence the analysis. The first three components for each fingerprint type were analyzed with analysis of variance as described above to detect significant effects of the site, forest type, and layer on the sample scores on each component. All statistical analyses were performed using SAS (version 8.2, SAS Institute Inc., 1999, Cary, NC), except PCA of the PLFA data, which was performed using CANOCO (Version 4, Microcomputer Power, Ithaca, NY).

## Results

**Forest Floor Characteristics.** There was no difference in mean depth of the forest floor in CH (95% CI: 28.8–36.6 cm) and HA (95% CI: 26.1–32.8 cm) forests. Forest floor moisture was greater in CH than HA forests in the deepest humus layer and the pH of the F layer was greater in CH than HA forests (Table 2). Soluble organic carbon was greater in F than humus layers ( $F = 20.1$ ,  $p < 0.001$ ), but although differences in soluble organic carbon between CH and HA forests appeared large, they were not significant (Table 2).

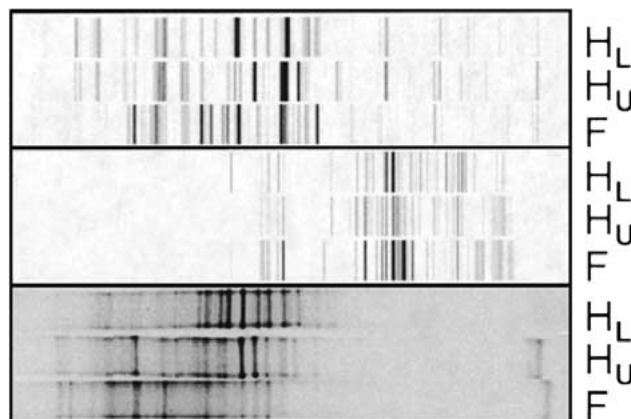
**Microbial Biomass.** Microbial biomass estimates using CFE and PLFA showed significant reductions in biomass in successively deeper forest floor layers (Table 2). There was no significant difference in total microbial biomass between the two forest types.

### Microbial Community Composition

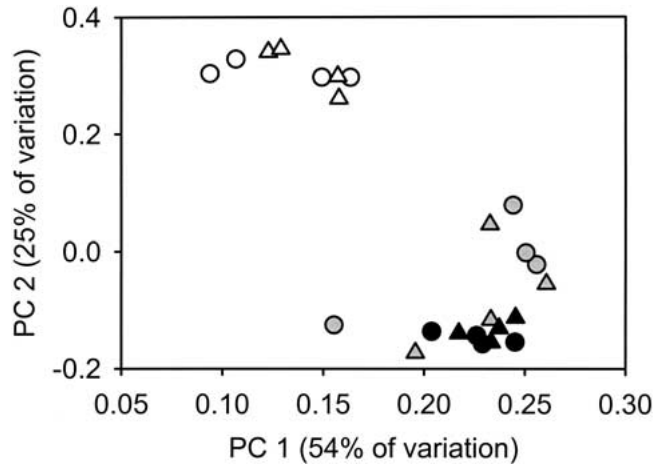
**DGGE Profiles of the Bacterial Community.** The two different primer pairs used to amplify regions of the 16S rDNA gave similar results, so results are only presented for primer pair 357f-907r. Fingerprints of each forest

floor layer were distinct (Fig. 1) and showed no more variability than replicate analyses of the same sample. Layers were significantly different on principal component (PC) 1 ( $F = 34.2$ ,  $p < 0.001$ ), with F-layer samples clustering separately from lower or upper humus samples (Fig. 2). The three forest floor layers were significantly different on PC2 ( $F = 155.7$ ,  $p < 0.001$ ).

**RISA Profiles of the Bacterial Community.** Bacterial community fingerprints using RISA showed similar clustering to that seen with DGGE, but there was much greater variability among samples. There were no significant effects of forest type or layer on PC1 scores, which accounted for only a third of the variation in the data. Layers were significantly different on PC2 ( $F = 5.7$ ,  $p = 0.018$ ) and on PC3 ( $F = 7.3$ ,  $p = 0.008$ ). F-layer samples had higher scores on PC2 than lower humus samples (Fig. 3), and the upper humus samples were significantly different from the other two layers on PC3.



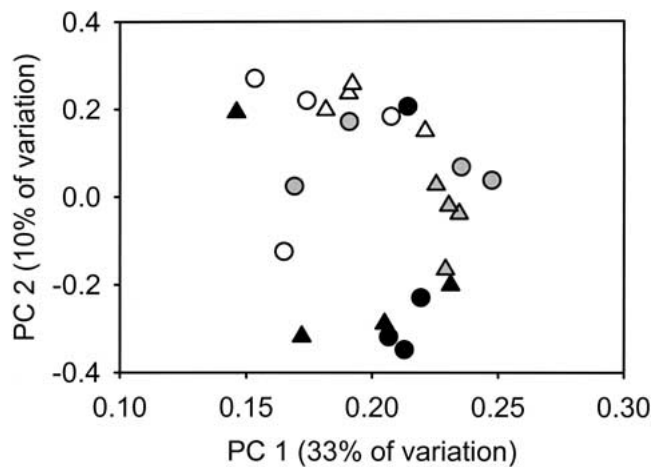
**Figure 1.** Representative images of DGGE (bottom panel), bacterial RISA (middle panel), and fungal RISA (top panel) fingerprints from F, upper humus (H<sub>U</sub>), and lower humus (H<sub>L</sub>) forest floor layers of cedar hemlock (CH) forests. RISA images were generated by Gel Compar II from electropherograms.



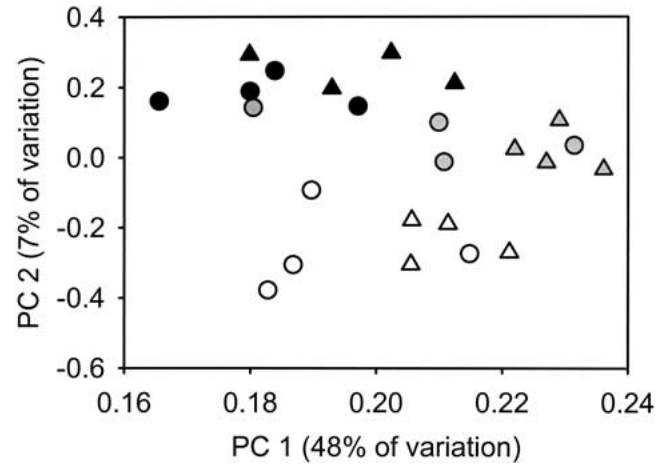
**Figure 2.** Principal component plot of bacterial community DGGE fingerprints from four sites in F (white), upper humus ( $H_U$ ) (gray), and lower humus ( $H_L$ ) (black) layers of cedar-hemlock (CH) (circle) and hemlock-amabilis fir (HA) (triangle) forest floors, using primers 357f-907r.

The similarity among clustered samples for RISA (35–55% similarity) was much lower than for DGGE (80–95% similarity); however, reproducibility of the PCR and the electrophoresis was extremely high for RISA fingerprints of bacteria and fungi, with two runs of the same sample being nearly identical.

*RISA Profiles of the Fungal Community.* The fungal community RISA fingerprints were differentiated by both forest type and forest floor layer, but there was relatively low similarity overall among the clustered samples (Fig. 4). Forest type ( $F = 9.6$ ,  $p = 0.053$ ) and layer ( $F = 15.1$ ,  $p = 0.001$ ) effects were significant for PC1. HA forests had higher PC1 scores than CH forests and upper humus



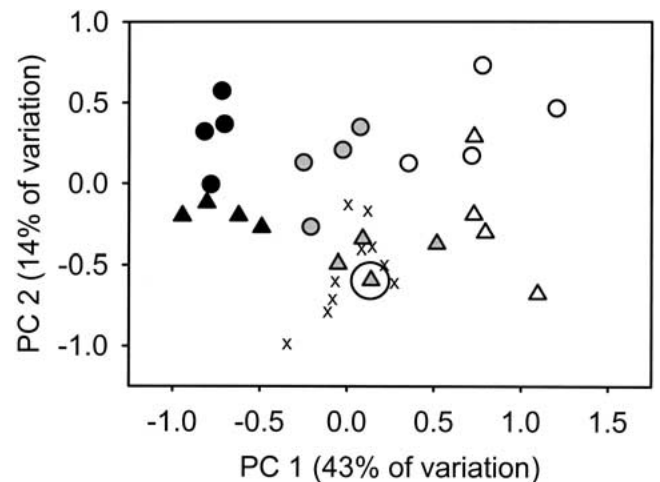
**Figure 3.** Principal component plot of bacterial community RISA fingerprints from four sites in F (white), upper humus ( $H_U$ ) (gray), and lower humus ( $H_L$ ) (black) layers of cedar-hemlock (CH) (circle) and hemlock-amabilis fir (HA) (triangle) forest floors.



**Figure 4.** Principal component plot of fungal community RISA fingerprints from four sites in F (white), upper humus ( $H_U$ ) (gray), and lower humus ( $H_L$ ) (black) layers of cedar-hemlock (CH) (circle) and hemlock-amabilis fir (HA) (triangle) forest floors.

samples had greater scores than the other two layers (Fig. 4). The three forest floor layers had significantly different PC2 scores ( $F = 126.0$ ,  $p < 0.001$ ). Similarity among clustered samples was 45–60%, in the same range as for bacterial RISA profiles.

*PLFA Profiles.* In the principal component analysis, the two forest types were discriminated along the second principal component ( $F = 119.26$ ,  $p = 0.002$ ) while forest floor layers were discriminated by the first principal component ( $F = 156.5$ ,  $p < 0.001$ ) (Fig. 5). There was



**Figure 5.** Principal component plot of PLFA composition data from four sites in F (white), upper humus ( $H_U$ ) (gray), and lower humus ( $H_L$ ) (black) layers of cedar-hemlock (CH) (circle) and hemlock-amabilis fir (HA) (triangle) forest floors, including 10 HA  $H_U$  uncomposited samples that were analyzed separately (x). Circled triangle indicates the composite HA  $H_U$  sample corresponding to uncomposited samples.

**Table 3.** Analysis of variance results for proportion (% mol) of marker PLFAs and ratio of fungal-to-bacterial PLFAs in F, upper humus (H<sub>U</sub>), and lower humus (H<sub>L</sub>) layers of cedar-hemlock (CH) and hemlock-amabilis fir (HA) forest floors<sup>a</sup>

	% Bacterial	% Fungal	% Gram-negative bacterial	% Gram-positive bacterial	Fungal: bacterial
<b>Site effect</b>					
<i>F</i> ( <i>p</i> ) df=3	2.2 (0.147)	2.9 (0.080)	2.4 (0.124)	2.7 (0.092)	2.8 (0.083)
<b>Forest type effect</b>					
<i>F</i> ( <i>p</i> ) df=1	5.1 (0.109)	2.9 (0.188)	4.5 (0.124)	<b>30.6 (0.012)</b>	2.0 (0.248)
<b>Site × Forest effect</b>					
<i>F</i> ( <i>p</i> ) df=3	1.4 (0.289)	2.9 (0.076)	0.3 (0.842)	1.7 (0.228)	<b>3.5 (0.050)</b> (at one site, CH > HA)
<b>Layer effect</b>					
<i>F</i> ( <i>p</i> ) df=2	<b>9.5 (0.003)</b>	<b>72.4 (&lt;0.001)</b>	<b>6.0 (0.016)</b>	<b>9.0 (0.004)</b>	<b>74.2 (&lt;0.001)</b>
<b>Forest × Layer effect</b>					
<i>F</i> ( <i>p</i> ) df=2	<b>10.0 (0.003)</b>	2.6 (0.115)	<b>6.0 (0.015)</b>	1.4 (0.291)	3.5 (0.063)

<sup>a</sup>*F* values are presented, with *p* values in parentheses, and significant effects are indicated in bold.

separation of both the layers and forest types by PCA into distinct clusters with minimal overlap of the six sample types.

There were clear differences among forest floor layers, with all microbial groups, except actinomycetes, declining in abundance with depth. The proportion of bacterial PLFAs in HA forests increased with depth and was greater than in CH forests in the lower humus layer (Tables 3, 4). Gram-positive bacterial PLFAs were proportionally more abundant in HA forests than CH (Table 3). For Gram-negative bacteria, the PLFAs declined proportionally with depth in CH forests (Table 4). The differences in Gram-negative bacteria between forest types in the F layer, both proportionally and as total abundance, seemed large but were not significant (Table 4). Fungal PLFAs were significantly more abundant in CH forests than HA ( $F = 28.8$ ,  $p = 0.013$ ). The ratio of fungal-to-bacterial PLFAs decreased with depth (Table 5), and although there was a tendency for the ratio to be greater in CH forests, there was a significant difference at only one site (Table 3).

**Within-Plot Variability.** For estimates of microbial biomass, PLFA markers, and other forest floor measurements, there were no differences between the value of

the composite sample and the mean value for the 10 uncomposited samples, except for pH. The composite pH value was greater than the mean of 10 within-plot samples. For all data except actinomycetal PLFAs, the variance was greater within one site than among four sites (composite sampling).

For DGGE fingerprints, there was high similarity among all samples from the same layer, regardless of forest type or site. Fingerprints of uncomposited samples were similar to the other humus samples, regardless of sampling location. The variability among the 10 uncomposited samples was similar to that among the composited samples clustered by forest floor layer. For fungal community fingerprints, the uncomposited samples were similar to each other and clustered with the composited humus samples from the HA forest type. Variability among these 10 samples was similar to that among the other clusters of forest floor layer and type, indicating that there was considerable variability among the 10 samples. RISA fingerprints of the bacterial community were more variable, but uncomposited samples generally clustered with other upper humus samples.

The 10 uncomposited samples fell within the group of HA upper humus samples in the PCA of the PLFA data (Fig. 5). They showed more variability than the four

**Table 4.** Proportion (% mol) of marker PLFAs in F, upper humus (H<sub>U</sub>), and lower humus (H<sub>L</sub>) layers of cedar-hemlock (CH) and hemlock-amabilis fir (HA) forest floors<sup>a</sup>

Forest floor layer	% Bacterial		% Fungal		% Gram-positive bacterial		% Gram-negative bacterial		% Actinomycetal <sup>b</sup>	
	CH	HA	CH	HA	CH	HA	CH	HA	CH	HA
F	43.0 a (0.6)	41.1 x (0.6)	9.9 a (0.4)	10.0 x (0.4)	<u>13.0 a</u> (0.8)	<u>15.4 x</u> (0.3)	25.0 a (0.9)	20.7 x (0.1)	0.7 (0.2)	0.5 (0.0)
H <sub>U</sub>	42.1 a (0.8)	43.8 x (0.6)	7.2 b (0.8)	5.7 y (0.5)	<u>14.4 ab</u> (0.2)	<u>16.5 xy</u> (0.4)	20.2 ab (0.4)	19.0 x (0.3)	0.9 (0.1)	1.1 (0.1)
H <sub>L</sub>	<u>42.8 a</u> (1.2)	<u>48.1 y</u> (1.2)	5.1 c (0.7)	3.0 z (0.6)	<u>14.3 b</u> (0.8)	<u>17.9 y</u> (0.5)	18.7 b (2.0)	21.5 x (1.3)	1.5 (0.2)	1.6 (0.1)

<sup>a</sup>The means of four sites are presented, with standard errors of the mean below in parentheses. Within each forest type, values followed by the same letter are not significantly different and underlined values indicate significant differences between the two forest types.

<sup>b</sup>These data were not statistically analyzed because they did not meet the assumptions of analysis of variance.

**Table 5. Summary of the ratio of fungal-to-bacterial PLFAs for forest floors and forest and non-forest mineral soils**

<i>Ecosystem<sup>a</sup></i>	<i>Depth sampled (cm)</i>	<i>Fungal: bacterial PLFAs</i>	<i>Reference</i>
<b>Forest Floors</b>			
Pine forest (poor site)	n/a	0.59	[47]
Pine forest	3	0.56	[3]
Spruce and pine forest	6	0.52	[3]
Scots pine forest	n/a	0.49	[47]
Spruce forest	4	0.43	[56]
Spruce forest	n/a	0.42	[47]
Spruce forest	5	0.42	[22]
Spruce forest	4	0.39	[56]
Spruce forest (fertile site)	n/a	0.38	[47]
Spruce forest (fertile site)	3.8	0.33	[21]
Pine forest (poor site)	5.1	0.33	[21]
<b>Hemlock–fir forest</b>	<b>2.6 (F)</b>	<b>0.31</b>	<b>This study</b>
Pine–spruce forest	4.7	0.30	[21]
<b>Cedar–hemlock forest</b>	<b>2.3 (F)</b>	<b>0.29</b>	<b>This study</b>
Spruce forest (poor site)	7	0.25	[52]
Birch forest (poor site)	3	0.23	[52]
<b>Cedar–hemlock forest</b>	<b>2.3–6.3 (H)</b>	<b>0.21</b>	<b>This study</b>
Pine forest (poor site)	5	0.20	[52]
<b>Hemlock–fir forest</b>	<b>2.6–6.6 (H)</b>	<b>0.16</b>	<b>This study</b>
Birch forest (fertile site)	2	0.15	[52]
<b>Cedar–hemlock forest</b>	<b>28–32 (H)</b>	<b>0.14</b>	<b>This study</b>
Spruce forest (fertile site)	5	0.13	[52]
Pine forest (fertile site)	4	0.12	[52]
<b>Hemlock–fir forest</b>	<b>25–29 (H)</b>	<b>0.07</b>	<b>This study</b>
<b>Mineral Soils</b>			
Spruce forest (fertile site)	6.5 (A horizon)	0.16	[21]
Birch forest (poor site)	3	0.14	[52]
Pine forest (poor site)	3.1 (A horizon)	0.13	[21]
Pine forest (poor site)	3	0.12	[52]
Spruce forest (poor site)	3	0.12	[52]
Grassland	5	0.12	[22]
Birch forest (fertile site)	3	0.11	[52]
Pine–spruce forest	5 (A horizon)	0.11	[21]
Spruce forest (fertile site)	6.6–16.5 (B horizon)	0.11	[21]
Pine forest (poor site)	3.1–13.1 (B horizon)	0.09	[21]
Pine–spruce forest	5–15 (B horizon)	0.09	[21]
Spruce forest (fertile site)	3	0.08	[52]
Spruce forest	(B horizon)	0.08	[22]
Arable field	5	0.08	[22]
Grassland	5	0.06	[22]
Pine forest (fertile site)	3	0.05	[52]
Beech forest	sampled at 40 cm	0.05	[22]
Beech forest	5	0.04	[22]
Beech forest	5	0.04	[22]
Grassland	5	0.03	[22]

<sup>a</sup>In studies from the literature, entire forest floors (F + H) were sampled, with the depth of the layer indicated, and mineral soil samples were taken from the surface of mineral soil to the depth indicated or from the layer and depth indicated. Values in bold are from this study with entire F layers (depth indicated) sampled, and humus layers sampled at the average depth indicated. n/a indicates data not presented in the reference.

composite samples for the four sites, but overlapped minimally with the other composite samples.

## Discussion

Microbial biomass was similar in CH and HA forest floors at the time of sampling, which may suggest a similarity in the availability of resources and environmental factors affecting microorganisms [4]. Chang et al. [12] found no significant differences among biomass

measurements in CH forest floors obtained in May, July, August, and October 1992. However, it is not known whether microbial biomass in HA forests varies seasonally or whether differences between the two forests might be larger during winter or spring months. The differences in nitrogen mineralization and availability between these two forests [50] indicate that process rates differ substantially. Two other studies have found equal soil microbial biomass, but different community composition in forests that differed in nutrient cycling process rates and



nutrient availability [42, 47]. Most other studies that reported differences in both biomass and composition among sites have compared very different or altered systems. Our result illustrates that although microbial biomass may indicate the decomposability of a substrate [4], it does not adequately explain differences in process rates among forests.

The two forest types did not have distinct bacterial communities, but did have different fungal communities, as assessed using molecular fingerprinting. There were also significant differences in the abundance and proportion of different groups of microorganisms in the two forest types, as measured by marker PLFAs. The greater fungal biomass in CH than HA forest floors is consistent with greater C:N ratios in CH forests [34, 50]. It has been assumed that fungal biomass is greater in nutrient-poor forests because fungi are more able to use recalcitrant materials and are better adapted to acidic conditions than bacteria [59]. However, while both CH and HA forests are relatively acidic, the F layer in CH forests had higher pH than HA forests, which is probably related to the presence of cedar litter which has high concentrations of base cations and creates forest floors of higher pH [51]. It remains unclear whether microbial community composition and activity can be predicted by climate and litter chemistry. It is possible that dominance by fungi resulting from other site factors or biological interactions could also affect soil fertility.

It seems unlikely that greater fungal biomass in CH forests is directly caused by the different tree species in CH and HA forests. Western redcedar, found in CH forests but not HA, has been associated with lower forest floor fungal biomass, and lower ratios of fungal-to-bacterial biomass than hemlock, spruce, and Douglas fir [28]. Forest floor and mineral soil under western redcedar has also been found to have lower fungal spore counts, and higher bacterial counts and populations of ammonia-oxidizers compared to hemlock [61]. Thus, microbial communities found in CH forests are not consistent with those seen in other western redcedar forests and may be more related to site factors than effects of tree species [51].

It has generally been thought that heterotrophic soil bacteria and fungi are carbon-limited [1, 15], although there is evidence that soil microbes can be limited by nitrogen [15, 31, 33]. Gram-negative bacteria generally respond faster to nutrient enrichment [30] and are thought to be very competitive in nutrient-rich environments. Indeed, Gram-negative bacteria were most abundant in the F layer of CH forests, where soluble organic carbon was also most abundant. Thus, while CH forests are poor in nutrients required for plant growth, there may be a greater availability of carbon for soil microorganisms than in HA forests. Moreover, the same nutrients may not limit both plant productivity and decomposition processes [32, 48].

Decreases in microbial biomass with depth have been reported for other forest soils and have been attributed to a decrease in root density and available carbon as well as changes in microenvironment, trophic interactions, and organic matter composition [6, 16, 18, 21, 35]. The forests in this study are unusual in their large accumulations of humus, and the deepest humus layers sampled (at an average depth of 30 cm and a maximum of 60 cm) still had significant amounts of plant fine roots [5]. Soluble organic carbon declined with depth in CH and HA forest floors, suggesting that there was less carbon available for microbes in deeper layers. Patterns of moisture and pH with depth were inconsistent between CH and HA forests, suggesting these factors were not responsible for the overall decrease in biomass with depth.

A trend of declining ratio of fungal-to-bacterial PLFAs from surface layers of the forest floor through deeper humus layers and mineral soil is evident across a range of ecosystems (Table 5). The ratio of fungal-to-bacterial PLFAs in the lower humus layers of this study are comparable to mineral soils in other studies, however, the depth of forest floor in these forests is much greater than usually found in other coniferous forests. Therefore, there seems to be a decline in fungal-to-bacterial ratios with depth within the forest floor, as well as in mineral soils compared to forest floor.

Based on PLFA markers, Gram-positive bacteria (including actinomycetes) made up an increasing component of the bacterial community with depth, while Gram-negative bacteria were proportionally less abundant in the lower humus layer than F layer in CH forests. Fritze et al. [21] found somewhat contradictory results, with greater proportions of Gram-negative bacterial PLFAs in the deep elluvial mineral soil layer than the humus above. Their study involved sampling layers down to about 45 cm, although the humus layer was <6 cm deep. Fritze et al. [21] also found greater proportions of actinomycetes with depth and a reduction in fungal PLFAs with depth, in agreement with the present study. Patterns of microbial community structure related to depth are likely affected by the same factors mentioned above related to biomass. Reductions in fungal PLFAs with depth may also be due to decreases in plant tissue, as 18:2 $\omega$ 6,9 is also found in plants [67].

The distinction among fungal communities in different forest floor layers could be related to differences in mycorrhizal associations and rooting distributions among the plant species in the two forest types. The dominant plants in CH forests are arbuscular, ecto-, and ericoid mycorrhizal plant species, while HA forests are dominated by ectomycorrhizal tree species. However, fungal community differences detected with RISA may include both mycorrhizal and saprotrophic fungi, and we could not distinguish which groups were responsible for the patterns detected.

The marked similarity between the two forest types in the DGGE fingerprints of the bacterial communities may indicate that these different forest floor environments select similar bacterial communities or alternatively, that although litter inputs and process rates differ, these two forest types are not actually very different habitats for bacteria. Considering differences detected using PLFA analysis, however, DGGE analysis of the whole bacterial community may be less effective for discerning differences. Indeed, many other studies using DGGE have found no difference in DGGE profiles among different soils or treatments [14, 19, 57]. Those that have detected differences have compared very different soil types [26, 43], environmental conditions [46], or soil layers [36]. The number of detectable bands in DGGE fingerprints is far lower than the estimated species richness of forest soil samples, and thus low resolution of the fragments may be responsible for the difficulty in detecting small changes in the community. Thus, DGGE may be more useful for comparing very different systems, monitoring after a large perturbation, or comparing a small portion of the community using taxon-specific primers, than for studying the extent of variability within and between soils of forest ecosystems.

It may be expected that RISA should detect more variability in a sample than DGGE and therefore offer greater resolution when comparing samples. Indeed, similarity values were lower for RISA than for DGGE, despite similar overall clustering of the bacterial profiles with each analysis. It is possible that community patterns are consistent at different levels of taxonomic resolution thought to be distinguished with DGGE and RISA. However, because of the very different electrophoresis systems used for DGGE and RISA in this study, we cannot determine the reason for low similarity values for RISA fingerprints compared to DGGE. Given that RISA fingerprints of both fungal and bacterial communities showed similar variability and were both run using the DNA sequencing system, it seems likely that differences in variability between DGGE and RISA were at least partly due to the electrophoresis systems used.

The molecular fingerprinting methods generally showed clustering of the uncomposited samples with the composite samples of the same type. The greater variability in the PLFA profiles among the 10 uncomposited samples compared to the composite samples from the four sites suggests considerable variability at small spatial scales. This agrees with other studies that have shown the scale of variability of forest soil microbial communities to be around 3–4 m [47, 55]—similar to the distances between sampling points in each plot in this study. Other studies have shown high variability at very small spatial scales [11, 44]. This potentially high variability at small spatial scales, however, did not mask clear patterns in the communities based on forest floor layer and forest type

when composite sampling was used. The composite sampling was therefore likely adequate to characterize the microbial communities in each forest.

Differences in community composition detected between CH and HA forests support the idea that different forest types have distinct soil microbial communities, as suggested by Myers et al. [42]. It must be remembered, however, that community composition differences detected in CH and HA forests do not reflect the activity levels of the whole microbial biomass or different components. The detection of differences in the forest floor microbial community between CH and HA forests needs to be put into context by further study of similar and very different ecosystem types. By understanding the extent to which communities differ in ecosystems with similar and very different process rates, we will be better able to interpret the potential functional implications of the community differences documented in this and other studies. Investigation of the factors contributing to differences in the fungal and bacterial communities in these forests will help to determine whether the patterns observed between the two forest types are determined by, or are to some degree a cause of, the differences in nutrient availability. This will require the coupling of manipulative and observational studies of forest soil microbial communities. Future research should thus be directed to understanding the factors controlling the composition and activities of these communities, as well as to experimentally elucidating the link between the microbial community structure and soil processes.

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