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ORIGINAL ARTICLE

PLFA Profiling of Microbial Community Structure and Seasonal Shifts in Soils of a Douglas-fir Chronosequence

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Abstract The impact and frequency of forest harvesting could significantly affect soil microbial community (SMC) structure and functioning. The ability of soil microorganisms to perform biogeochemical processes is critical for sustaining forest productivity and has a direct impact on decomposition dynamics and carbon storage potential. The Wind River Canopy Crane Research Forest in SW, WA, provided a unique opportunity to study a forest chronosequence and the residual effects of harvesting on the SMC in comparison to old-growth forests. The objective of this study was to determine the effect of clear-cutting and stand age on temporal dynamics of SMC and physiological stress markers using phospholipid fatty acid (PLFA) profiling. Soil microbial PLFA profiles were determined seven times over 22 months (Nov. 02 to Sep. 04) in old-growth coniferous forest stands (300–500 years) and 8 (CC8)- or 25 (CC25)-year-old replanted clear-cuts. PLFA patterns of the SMC shifted because of clear-cutting, but seasonal temporal changes had greater shifts than differences among stand age. The microbial biomass (total PLFA) and bacterial, fungal, and selected other PLFAs were significantly reduced in CC8 but not in CC25 sites relative to the old-growth sites. An increase in stress indicators [PLFA ratios of saturated/monsaturated and (cy17:0+cy19:0)/(16:1ω7+18:1ω7)] in late summer was related to water stress. Although the canopy and litter input are quite different for a 25-year clear-cut compared to virgin old-growth forest, we conclude that the composition of the microbial communities, 25 years after clear-cutting, has recovered sufficiently to be much more similar to old-growth forests than a recent clear-cut at this Pacific Northwest forest site. The study shows the potential of PLFA analysis for profiling microbial communities and their stress status under field conditions, but wide temporal shifts emphasize the need for sampling over seasons to fully interpret ecosystem management impacts on microbial populations.

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Introduction

Microorganisms drive biogeochemical cycles that control functioning of ecosystems. This is particularly true of forests that, unlike agricultural systems, do not depend on external inputs but rather internal microbial-mediated processes such as litter decomposition and nutrient mineralization to sustain tree growth. Disturbance to the soil microbial community (SMC) structure may have significant impacts on forest productivity. At the same time, there is strong interest to optimize forest management to sequester C in soils to offset global climate change. During decomposition, SMC controls the partitioning of litter- and root-C between CO_2 via respiration and storage in semipermanent soil-C pools. Consequently, the impact and frequency of harvesting on SMC is important for ecosystems services and long-term forest sustainability.

The common practice of clear-cutting forests would be expected to have major impacts on forest landscapes and soils. Highly mechanized harvest activities cause soil compaction [16] and physical disruption of the litter layers. Clear-cut forests in the Pacific Northwest (PNW) result in homogenous replantings of Douglas-fir (*Pseudotsuga menziesii*)

that have a ground cover of grass and ferns. This change in the plant community affects both the amount and chemistry of C inputs through litter and root exudates and thus the availability of C to microorganisms [21]. Substantial C losses of >40% have been found in other regions depending upon method of harvest and the type of forest ecosystem [37–39]. Thus, the loss of canopy with clear-cutting would be expected to alter soil microclimate, physical, and chemical properties.

In contrast to clear-cuts, Douglas-fir and western hemlock (*Tsuga heterophylla*) old growth (OG) forests, are composed of a heterogeneous understory with multiple layers of different shrubs and trees [42]. Here, the microclimate, because of a mature canopy and thick litter layer, would be expected to have less extremes in temperature and moisture.

The loss of OG forests and the frequency of tree harvesting, therefore, should affect the complex and heterogeneous group of bacterial and fungal species, which often interact in consortia to carry out biogeochemical processes such as nutrient mineralization and transformations of C into organic matter. This, in turn, may affect the long-term productivity of forests. In addition, the Kyoto protocol called for maintenance of young forests because they are fast growing and capture more atmospheric carbon dioxide to reduce global climate change. However, this ignores the effect frequent harvests might have on soils and their microbial communities and associated activities.

The Wind River Experimental Forest (WREF) within the Gifford Pinchot National Forest provided a unique opportunity to study a landscape level, replicated, chronosequence ranging from an 8-year-old clear-cut to 300- to 500-year-old OG forests. Here, we investigated the SMC using phospholipid fatty acid (PLFA) profiling. PLFAs are essential components of every living cell and are useful biomarkers because of their wide structural diversity [47, 50]. They are considered to be representative of the viable microbial community because they are not found in storage products or in dead cells because, upon cell death, the phosphate group is quickly hydrolyzed [46, 51].

PLFAs can be analyzed with multivariate procedures to determine statistically significant changes in SMC structure and relative abundances from soils under different management regimes. Unlike nucleic acid profiling, where there is high species specificity, PLFAs largely profile functional groups. However, PLFA analyses are quantitative and allow relatively high throughput, which is an advantage over nucleic acid profiling for field-based microbial ecology studies. This makes PLFA biomarkers well suited for field studies of ecosystem management [3] and to track microbial communities over seasons.

The PNW forests are important for wood production and recreation and could be a large sink for atmospheric carbon dioxide [43]. There have been no microbial community composition studies on young or older clear-cuts relative to virgin OG forests of the PNW. Therefore, the objectives of this study were to determine how clear-cut forests and OG forests affect SMC structure along a chronosequence on a seasonal basis.

Materials and Methods

Stand Description and Selection

The study site is located within the Gifford Pinchot National Forest in the southern Cascade Range of Washington State. A detailed description of the ecological setting is provided by [42]. The forests are predominately Douglas-fir (*P. menziesii*) stands of varying ages. The area is characterized by a cool, moist climate with average annual precipitation and temperature of 222 cm and 8.7°C, respectively. Only 5% of this precipitation falls during June, July, and August; the average monthly air temperature in July is 17.7°C [42].

The soils are medial, mesic, Entic Vitrands (Stabler series, http://ortho.ftw.nrcs.usda.gov/cgi-bin/osd/osdname.cgi). They are deep (2–3 m), well-drained loams and silt loams, generally stone-free and derived from air-deposited, mixed volcanic tephra between 3500 and 12,000 years old.

The three forest stand ages in our study were (1) OG ranging from 300 to 500 years old, (2) an 8-year-old clear-cut stand (CC8) that was cut in 1994, and (3) a 25-year-old clear-cut stand (CC25) that was cut in 1977. Both clear-cuts were immediately replanted with Douglas-fir (*P. menziesii*). The CC8 and CC25 had two landscape level replications with which each had an adjacent paired OG stand giving OG four replications. The OG sites contain Douglas-fir and western hemlock (*T. heterophylla*) as the dominant tree species. Dominant under story shrub species are vine maple (*Acer circinatum*), salal (*Gaultheria shallon*), and dwarf Oregon grape (*Berberis nervosa*) and constitute about 57% of the total under story composition [42]. The CC8 site is an open reestablishing Douglas-fir forest with bracken ferns (*Pteridium aquilinum*) dominating the ground cover, whereas the CC25 site has a closed-canopy Douglas-fir overstory with weak understory development (very few shrubs, if any). The O horizon (i.e., organic or litter layer) of OG sites is between 2 and 4 cm deep, whereas the O horizon for the clear-cut stands is between 0 and 0.5 cm deep. Soil properties of the chronosequences are shown in Table 1.

All of the stands except for CC8 are located within the WREF—a 4208-ha area where active forest research has been conducted since 1908. The CC8 stands are adjacent to the WREF on United States Department of Agriculture Forest Service land.

Table 1 Total C, N, bulk density, and pH for litter and soil samples collected in June 2002

Forest stand	Layer	Depth (cm)	Bulk density (Mg m^{-3})	Carbon		Nitrogen		*C/N*	pH
				% C	Mg C ha^{-1}	% N	Mg N ha^{-1}		
OG	Litter	4.2	0.04	33.96	6.29	0.93	0.17	36.7	4.3
	Soil	0–10	0.89	3.72	32.9	0.15	1.33	24.8	5.0
		10–30	0.95	2.16	41.0	0.13	2.48	17.3	5.2
CC8	Litter	1.0	0.09	28.89	2.63	0.81	0.07	35.6	4.6
	Soil	0–10	0.91	3.42	31.2	0.14	1.28	24.4	5.3
		10–30	0.99	1.92	38.2	0.10	1.99	19.2	5.4
CC25	Litter	2.0	0.07	21.33	2.93	0.85	0.12	25.2	4.6
	Soil	0–10	0.87	4.30	37.4	0.14	1.22	30.7	5.1
		10–30	0.97	2.37	45.9	0.11	2.13	21.5	5.1

Sample Collection

In November 2002, open-ended polyvinyl chloride (PVC) microcosms (10-cm dia.×12- to 16-cm long columns) were installed in three sub-replicate plots in each forest stand. Two of the three sub-replicate plots were used in this study, and the third was used in a subsequent ^{13}C litter decomposition study (see [28] for details). To install microcosms, a custom-made steel soil corer with beveled edges (10-cm dia. by 16-cm; Scotty's Welding, Wenatchee, WA) that held a PVC column was used to extract intact soil cores that received root or litter bag treatments. After these amendments, the PVC column was placed in the hole, and the soil was pushed back out of the corer into the column so that minimal disturbance occurred and soil layers remained intact.

On December 16, 2002, April 25, June 20, August 31, and November 23, 2003, and June 14 and September 1, 2004, one pair of intact microcosms (one containing each of the amendments) was randomly chosen from each sub-replicate plot and transported to the laboratory within 8 h. Samples were stored at 4°C and processed within 3 days. The 0- to 5-cm mineral soil (below litter or O horizon) was sieved to pass 4.75 mm to remove wood pieces, roots, or stones and homogenized and analyzed for PLFA and organic C content. Subsamples were dried at 65°C for 24 h to calculate moisture content.

Soil Temperature and Moisture Measurements

In April 2003, soil moisture data from the CC8 site were collected using a handheld time-domain reflectometer (TDR; Hydrosense probe, Decagon Devices, Pullman, WA). Beginning on June 11, 2003, soil moisture data at one of the CC8 stands were collected every 15 s and averaged hourly using water content reflectometers (model cs615; Campbell Scientific Inc., Logan, UT) mounted vertically through the soil profile with 30-cm rods (3.2-mm diameter and 3.2-mm spacing) and a data logger (model 23×, Campbell Scientific, Inc., Logan, UT). Beginning in December 2002 (before sampling), soil temperature at both CC8 stands was measured every 30 min by temperature sensors (HOBO, Onset Computer Corp, Bourne, MA) that were buried between 10 and 15 cm in the soil.

Soil moisture and temperature measurements in 2003 and 2004 for one of the OG sites were provided by the Wind River Canopy Crane Research Facility data archive (http://depts.washington.edu/wrccrf/database.html). Soil moisture data were collected every 30 min at a depth of 15 cm using two TDRs with 30-cm rods (0.48-cm diameter, and 4.5-cm spacing between outer rods, model cs610, Campbell Scientific, Inc., Logan, UT). Soil temperature probes (model cs107B, Campbell Scientific, Inc.) were placed at 15 cm below surface between the TDR waveguides. Soil moisture and temperature data were collected every 5 s, and 30-min averages were stored by a data logger (model cr10×, Campbell Scientific, Inc.).

A soil moisture release curve was generated by measuring soil water potential (mega Pascal) with a WP4 Dewpoint PotentiaMeter (Decagon Devices, Pullman, WA) over a range of soil moisture values (grams of water per gram of dry soil) and plotting the results. This plot was used to convert soil moisture readings from the field (volumetric) into water potential. The water potential of samples from TDR data was back-calculated from volumetric water content to gravimetric water content using soil bulk density data (described below) and a density of water of 1 g cm^{-3}.

Soil temperature for OG and CC8 for the December 2002 and April 2003 sample dates was estimated using temperature data measured for 2003. A linear regression between CC8 and OG resulted in a strong correlation (r= 0.95) between the two sites for data collected in 2003.

Soil temperature at the CC25 sites were measured from June 2002 through April 2004 by HOBO temperature sensors placed at 15 cm in the mineral soil.

Total C and N, Bulk Density, and pH

Soil and litter samples were collected in June 2002 to obtain total C and N concentration, bulk density, and pH values. Soil samples were collected from 0 to 10 cm and 10 to 30 cm depths after removing the litter layer. Total C and N were determined by dry combustion using a LECO CNS-2000 Macro Analyzer (LECO Inc, St. Joseph, MI).

To express soil and litter total C and N values on an area basis, bulk density measurements for mineral soil were conducted using a Troxler nuclear density gauge (Troxler Electronic Laboratories Inc., Research Triangle Park, NC), and litter samples were done by the volumetric ring (VR) method. For the VR method, steel rings (5-cm diameter) were pushed into the litter layer and the depth inside the ring recorded. The litter was placed in sealed plastic bags, weighed and dried for 48 h. The weight of the litter occupying a given volume was calculated and reported as grams per centimeter. Soil and litter pH were measured using a soil/water ratio of 1:2 (*w/v*) and a litter/water ratio of 1:10 (*w/v*). Samples were mixed using a glass stir rod and allowed to stand for 30-min before pH reading.

PLFA Analysis

PLFAs were extracted in three steps using a modified [5] procedure. In brief, lipids were extracted from soils by a one-phase chloroform, methanol and water extractant, then fractionated into neutral lipids, glycolipids and phospholipids on a silicic acid column. The phospholipids were then subjected to alkaline methanolysis and analysis on a gas chromatograph (Agilent Ultra 2 column; carrier gas, helium; temperature ramping 120 to 260°C at a rate of 5° C per minute) with a flame ionization detector [15].

Individual fatty acids were identified relative to several standards: 37 fatty acid methyl ester (FAME) mixtures (FAME 37 47885-4; Supelco, Inc., Bellefonte, PA), 24 bacterial FAME mixtures (P-BAME 24 47080-U; Supelco, Inc.), and MIDI standards (Microbial ID, Inc., Newark, DE). Quantification of FAMES was accomplished by using varying concentrations of tridecanoic FAME (Supelco, Inc.) and allowed peak areas to be converted to a molar basis. Fatty acids with less than 0.5% of the total relative abundance were not included in the data set.

Standard nomenclature is used to describe FAMEs. They are designated by the total number of carbon atoms/number of double bonds, followed by the position of the double bond from the methyl (aliphatic) end (*w*) of the molecule. The prefixes "a" and "i" refer to anteiso- and iso-branched fatty acids. The prefix "10Me" indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule, "OH" indicates a hydroxyl group, and "cy" indicates cyclopropane fatty acids. A sum of the fatty acids indicative of Gram-positive bacteria (GM+), Gram-negative bacteria (GM−), and actinomycetes (actinos) plus the five additional fatty acids listed are used as a measure of total bacterial biomass (Table 2) [15]. The 18:2ω6,9 is used as a measure of fungal biomass. A ratio of the fungal/bacteria PLFAs is used as a biomass index as an indication of the changes in the ratio of fungal to bacterial biomass [2].

Total PLFA ($PLFA_{tot}$) concentration (nanomole PLFA per gram C) was used as an index of the total viable microbial biomass. The sum of PLFAs characteristic of general bacteria, GM+ bacteria, GM− bacteria, actino-

Table 2 PLFA markers used for taxonomic microbial groups and as stress indicators

Taxonomic group	PLFA group	Specific PLFA markers	Reference
PLFA biomarkers			
Bacteria	Multiple groups	Sum of i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, 16:1ω7, 18:1ω7, and 17:1ω9	[15]
Gram-positive bacteria	Branched PLFAs	Sum of i15:0, a15:0, i16:0, i17:0, and a17:0	[36]
Gram-negative bacteria	Cyclopropyl and mono PLFAs	Sum of cy17:0, 16:1ω7, 18:1ω7, and 17:1ω9	[48]
Actinomycetes	10Me-PLFAs	Sum of 10Me16:0, 10Me17:0, and 10Me18:0	[26]
Fungi	Polyunsaturated PLFAs	18:2ω6,9	[12, 15]
F/B ratio	Multiple groups	Fungi/bacteria	[12, 15]
Protozoan	Polyunsaturated PLFAs	Sum of 20:2ω6 and 20:4ω6	[41]
Microbial stress indicators			
S/M ratio		SAT/MONO PLFAs	[6]
SAT	Saturated PLFAs	14:0, 15:0, 16:0, 17:0, 18:0	
MONO	Monosaturated PLFAs	i16:1, 16:1ω11, 16:1ω7, 16:1ω5, 17:1ω9, a17:1, 17:1ω8, 18:1ω9, 18:1ω7, 18:1ω5, 11Me18:1ω7	
cy/pre ratio		(cy17:0+cy19:0)†/(16:1ω7+18:1ω7)	[23]

mycetes, fungi, and the fungal to bacteria ratio (*F/B*) were used as broad taxonomic microbial groupings (Table 2). Lastly, the ratio of saturated (SAT) and monounsaturated (MONO) PLFAs were used in conjunction with the ratios of the sum of cyclopropyl PLFAs to the sum of their monoenoic precursors (cy17:0+cy19:0)/(16:1ω7+18:1ω7; abbreviated as cy/pre) as indicators of physiological or nutritional stress in bacterial communities [6, 23] (Table 2). Fatty acids were calculated on a per gram C soil basis. Total bacteria and fungal PLFAs were calculated using both absolute (nanomole per gram C) and relative abundances (mole percent of $PLFA_{tot}$). All other groups were calculated using mole percent of $PLFA_{tot}$.

Statistical Analysis

All statistical analyses were conducted using SAS (version 8) and PC-ORD (version 4) statistical software [33]. Results from the pair of microcosms (sub-replicates) were averaged at each field replication because they were statistically the same ($p<0.05$; four values for each). The stand by age treatment effects were analyzed as a completely randomized design.

The mean of each PLFA for each site ($N=4$ for OG and $N=2$ for CC25 and CC8) was used after transformation for nonmetric multidimensional scaling (NMS) plots and as mole percent for analysis of variance (ANOVA) analyses. Data used in the NMS plots were transformed using sample unit totals to represent relative abundance of each PLFA (mole percent of total PLFA). A monotonic transformation (square root) of all PLFAs was also conducted to create a more normally distributed data set and to reduce the coefficient of variation among PLFAs. The square root transformation is similar in effect to the log transform but is less drastic and is commonly used in ecological studies [33]. A second matrix containing summed values for the taxonomic groups and stress indicators listed in Table 2 was used to construct a joint plot overlaid on the NMS. This joint plot is used to visualize the relationship between a set of variables (in this case, taxonomic groups and stress indicators) and NMS scores. The angle and length of a line indicate the direction and strength of the relationship [32].

The data were examined using NMS with the Sorensen distance measure [27, 31]. Forty runs with real data were conducted, and Monte Carlo simulations were conducted using 50 randomized runs and a stability criterion of 0.0001.

The NMS scores from each axis were then loaded into SAS (version 8) using a repeated measures program to determine if stand age, sample date, or their interaction was significantly different ($P\leq0.05$) along a given axis. To determine how the structure and composition of soil microbial communities differed between stand age and sample dates, repeated measures ANOVAs were conducted on group (summed) PLFA markers and stress indicators. This is considered a more reliable result than analysis of individual PLFAs because the groupings provide a more robust analysis to evaluate and interpret microbial community changes [51]. For all repeated measures programs, the compound symmetry variance–covariance matrix was used because this matrix was the only matrix that does not require equal spacing between recorded times and meet convergence criteria required by the program. Tukey's honestly significantly different tests were used to adjust *P* values for pair-wise comparisons to control for the experiment-wise error rate with significant differences defined at $P\leq0.05$.

Results

Soil Temperature and Moisture

To coincide with the seven sample dates, soil moisture and temperature measurements were averaged over the previous 3 days before each sampling date (Table 3). Averaged

Table 3 Soil temperature and moisture values averaged over 3 days prior to each of the sample dates for this study

	Soil temperature (°C)			Soil moisture (%)		
	OG	CC8	CC25	OG	CC8	CC25
Dec-02	7.3	4.5	4.3	nd	nd	nd
Apr-03	11.6	10.2	6.6	29.4	30.2	nd
Jun-03	13.2	16.2	12.4	28.2	21.7	nd
Aug-03	16.2	16.0	14.3	17.1	12.7	nd
Nov-03	4.1	2.7	2.7	34.3	30.9	nd
Jun-04	11.4	13.3	nd	33.4	30.6	nd
Sep-04	16.6	16.7	nd	30.3	24.2	nd

nd Not determined

across stand age, the coldest sample month was November 2003 (3.2°C), and the warmest month was September 2004 (16.7°C). Soils in August 2003 were between 10.1 and 17.7% drier than soils collected during all other dates, and between 5 and 12.7°C were warmer than samples collected in December 2002 and in April and November 2003. Between August 22 and August 27, 2004, approximately 118 mm of rain was recorded at a weather station in an open field nearby OG and is reflected by the relatively high soil moisture values recorded on September 1, 2004 (30.3 and 24.2% for OG and CC8, respectively) compared to those recorded the previous year on August 31, 2003 (17.1 and 12.7% for OG and CC8, respectively). The latter moisture contents correspond to soil water potentials of −0.9 and −1.3 MPa, respectively (data not shown).

Effects of Stand Age and Sample Date on Total PLFA, and Bacterial and Fungal PLFA Abundances

There was a significant effect of stand age (Fig. 1) and sample date (Fig. 2) on total PLFA ($PLFA_{tot}$) concentrations, which is an indicator of the active living biomass. Averaged across sample dates, there was a significant difference in $PLFA_{tot}$ between OG (2144 nmol g^{-1} C) and CC8 (1405 nmol g^{-1} C) but not between OG and CC25 (1808 nmol g^{-1} C) or between CC25 and CC8 (Fig. 1). Averaged across stand age, the $PLFA_{tot}$ concentrations were significantly lower in August 2003 (1116 nmol g^{-1} C) compared to other sampling dates (Fig. 2). The greatest concentration occurred in June 2003 (2611 nmol g^{-1} C), and this value was significantly greater than all other dates except for December 2002 and June 2004 samples.

Total absolute bacterial and fungal PLFA concentrations followed similar patterns as $PLFA_{tot}$ throughout the sample period (Figs. 1 and 2). Averaged across sample dates, there was a significant decrease (P=0.01) in total bacterial PLFA for CC8 (459±46 nmol g^{-1} C) samples compared to OG (688±36 nmol g^{-1} C; Fig. 1). Samples from CC25 (569± 62 nmol g^{-1} C) were not significantly different than OG or CC8. Total bacterial PLFA also significantly differed among sample dates when averaged across stand age (Fig. 2). Mean amount of bacterial PLFAs in June 2003 (860 nmol g^{-1} C) was greater than the mean amounts in April (451 nmol g^{-1} C) and August (436 nmol g^{-1} C) of 2003 and in September 2004 (480 nmol g^{-1} C).

The fungal PLFA concentration was significantly greater in OG and CC25 samples compared to CC8 but did not differ between clear cuts (Fig. 1). Fungal concentrations were lowest in August samples but not significantly lower for all dates (Fig. 2). There was a significant stand age by date interaction on total fungal PLFA concentration (P=0.0015). For OG samples, fungal concentrations (nmol g^{-1} C) were significantly higher in December 2002 (391±27.4 nmol g^{-1} C) compared to all other sample dates (range from 102 nmol g^{-1} C in April 2003 to 215 nmol g^{-1} C in June 2003) except from those collected in June 2004 (274±67.3 nmol g^{-1} C). Samples collected in December 2002 from OG sites were also significantly greater for the fungal marker than those collected from CC25 (129±37.5 nmol g^{-1} C) or CC8 (95± 7.5 nmol g^{-1} C) sites. The fungal/bacteria ratio, however, was not significantly different because of stand ages or sample dates (Table 4). In addition, stand age or sample date did not significantly affect any other absolute concentrations for the other microbial groups (e.g., Gm+, Gm−, actinomycetes; data not shown).

Effects of Stand Age and Sample Date on SMC Structure

Shifts in the SMC structure among stand age (Fig. 3a) and sample date (Fig. 3b) were observed as indicated by the two-dimensional ordination plots using NMS of PLFA profiles (as square root of mole percent). Within these plots, the closer the points, the more similar they are in SMC composition. Using repeated measures ANOVA of NMS scores, OG and CC25 were significantly separated from CC8 samples along axis three (P<0.05). NMS scores also were significantly separated on the basis of sample date along axes two and three (P<0.001), although a much stronger effect of sample date was observed along axis three (Fig. 3b). Along axis three, August samples were significantly different (P≤0.0001) from those collected for all other sample dates. In addition, samples from June and November 2003 were significantly different from those collected in December 2002 and June and September 2004. Along axis two, June 2003 samples were also significantly different from samples collected in August 2003. An examination of taxonomic markers used to construct the joint plot (Fig. 3c) suggests that OG and CC25 plots tended

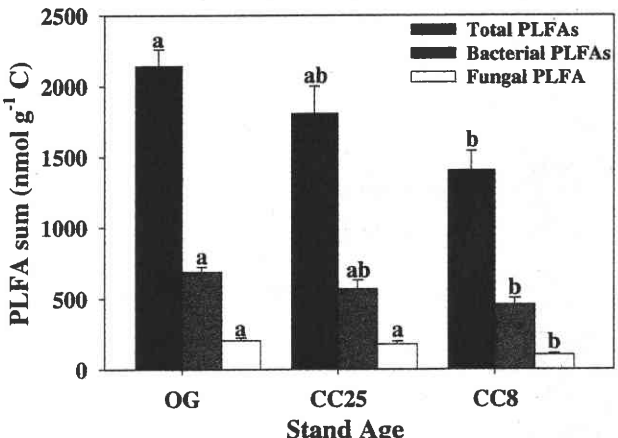

Figure 1 Total PLFA and sum of bacterial and fungal PLFA concentrations for OG, CC25, and CC8 stands averaged over the seven sample dates. *Error bars* represent standard error (n=8). Letters denote significant differences (P≤0.05) between stand ages

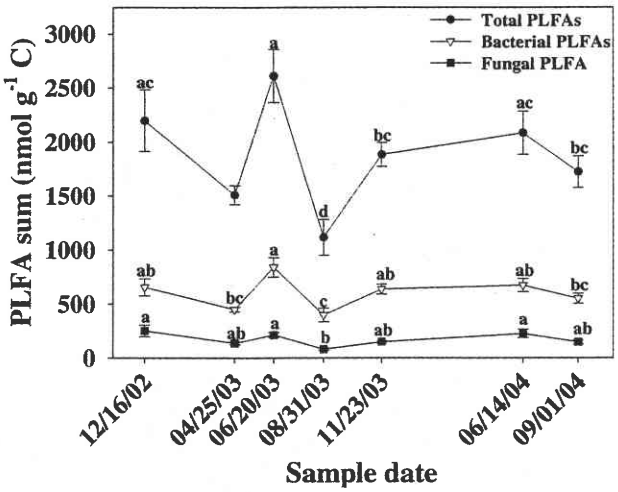

Figure 2 Total PLFA and sum of bacterial and fungal PLFA concentrations for each of the seven sample dates averaged over stand age. *Error bars* represent standard error (n=4 for OG sites and n=2 for CC sites). Letters denote significant differences ($P \leq 0.05$) between sample dates

to have higher concentrations of the fungal biomarker and a lower concentration of the actinomycetes biomarkers and lower stress indicators (lower saturated/monounsaturated [*S/M*] ratio and cy/pre ratio).

In comparison to absolute concentrations, relative concentrations (i.e., mole percent of total PLFA) of the taxonomic groupings listed in Table 2 were analyzed using repeated measures ANOVA. Although not statistically significant, there was a trend showing greater relative concentrations of GM+ bacteria and actinomycetes in CC8 samples compared to OG and CC25 samples (Table 4). In contrast, a greater relative abundance of fungi (significant at *P* and *F/B* ratio) was found for OG and CC25 samples compared to CC8 (Table 4). Although not always significant, August samples were highest in GM+ bacteria and actinomycetes biomarkers and lowest in protozoan and fungal biomarkers and *F/B* ratio relative to all other sample dates (Table 4).

Effects of Stand Age and Sample Date on Physiological Status

Physiological status was determined using the ratios of saturated to monounsaturated PLFAs (*S/M*) and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre; Fig. 4). The *S/M* ratio was significantly greater for CC8 compared to OG but not between CC25 and OG or between both clear-cuts. There also was a significant age by date interaction ($P \leq 0.01$) for both stress indicators. During August 2003, the *S/M* ratio was significantly higher in CC8 samples (0.60±0.03) than OG or CC25 samples (0.47± 0.04 and 0.48±0.02, respectively). Both clear-cut stands also had higher *S/M* ratios than OG during September 2004. In addition, the cy/pre ratio for August was higher for CC25 samples (0.99±0.08) than OG (0.81±0.02) and CC8 (0.82±0.03) samples. Within each stand age, the *S/M* (Fig. 4a) and cy/pre (Fig. 4b) ratios were highest for August samples compared to all other sample dates. Elevated levels of these indicate greater levels of microbial community stress.

Discussion

Effect of Stand Age on the SMC

Our data indicate that somewhere between 8 and 25 years after clear-cutting, the structure of the microbial community has recovered to the point that it is more similar to OG than a recent (less than 10 years) clear-cut. The recent clear-cut (CC8) had significant effects on SMC structure and total biomass and a relative reduction in bacterial and fungal biomass with clear-cutting.

Fungi are major players in decomposition and formation of organic matter [8, 9, 44]. Consequently, with clear-

Table 4 Sum of PLFAs used for taxonomic groups of the seven sample dates and stand ages

Sample date/stand age	GM+ bacteria	GM− bacteria	Actinos	Protozoa	Fungi	*F/B*
Dec-02	9.3 (0.3)	22.6 (0.9)^{ab}	4.9 (0.2)^{b}	0.99 (0.08)^{b}	10.2 (1.3)	0.35 (0.06)
Apr-03	9.8 (0.5)	21.3 (0.4)^{ab}	4.5 (0.2)^{b}	0.97 (0.09)^{b}	9.2 (0.7)	0.30 (0.03)
Jun-03	12.6 (0.4)	19.6 (0.3)^{b}	5.8 (0.2)^{b}	0.93 (0.03)^{b}	8.2 (0.5)	0.26 (0.02)
Aug-03	12.6 (0.8)	20.4 (0.6)^{bc}	6.8 (0.2)^{a}	0.71 (0.08)^{b}	7.2 (0.5)	0.20 (0.02)
Nov-03	12.1 (0.6)	21.9 (0.5)^{ac}	5.6 (0.3)^{b}	0.93 (0.07)^{b}	8.1 (0.6)	0.24 (0.02)
Jun-04	10.4 (0.6)	21.3 (0.4)^{bc}	5.6 (0.3)^{b}	1.38 (0.04)^{a}	10.3 (1.0)	0.32 (0.04)
Sep-04	9.8 (0.5)	20.5 (0.3)^{bc}	5.8 (0.3)^{ab}	1.38 (0.03)^{a}	8.4 (0.8)	0.26 (0.02)
OG average	10.6 (0.4)	20.8 (0.3)	5.4 (0.2)	1.08 (0.06)	9.1 (0.5)	0.29 (0.02)
CC25 average	10.9 (0.5)	22.1 (0.4)	5.4 (0.3)	1.01 (0.07)	9.7 (0.5)	0.30 (0.02)
CC8 average	11.7 (0.5)	20.7 (0.5)	6.0 (0.2)	1.00 (0.08)	7.3 (0.2)	0.23 (0.01)

Values are the mean molar % of PLFAs (SE in parentheses, n=8 for sample date; for stand age averages, n=4 for OG and n=2 for CC25 and CC8). Within a given taxonomic group, letters denote significant differences (ANOVA; $P \leq 0.05$) between sample dates.

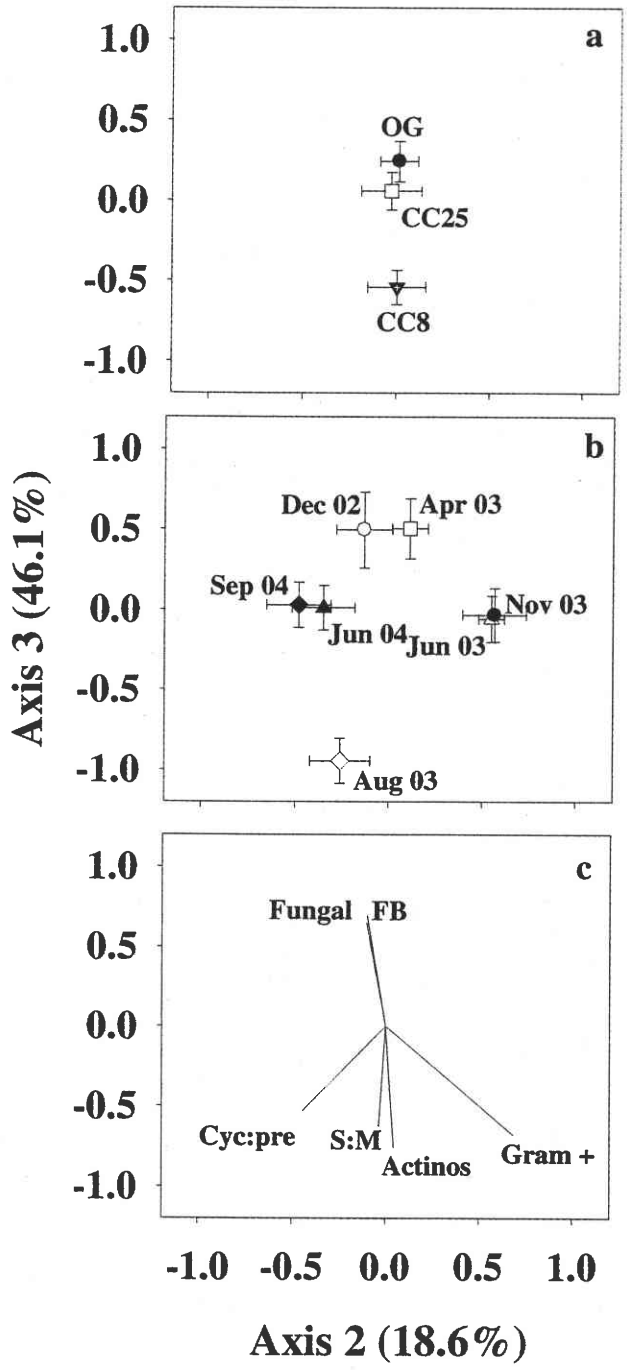

Figure 3 **a** NMS plot of PLFA profiles (as square root of mole percent) for: OG, CC25, and CC8 samples collected at all seven sample dates. *Error bars* represent standard error (n=8). **b** NMS plot of PLFA profiles (as square root of mole percent) for each of the seven sample dates averaged across stands (n=4 for OG sites and n=2 for CC sites). **c** Joint plot overlay showings vectors based on summed relative abundances of taxonomic groups and stress indicators listed in Table 2. The *angle and length of the vector* indicate the direction and strength of the variable and the NMS axis. *Axes* are arbitrary; the closer points are on the plot, the more similar they are in SMC composition. Letters denote significant differences ($P \leq 0.05$) between stand ages

cutting, there is a large reduction in litter inputs and root turnover, which would reduce the substrates. Fungi are uniquely adapted to degrade substrates, particularly lignin [8]. Furthermore, fungi are filamentous and therefore susceptible to the disturbance and compaction that occurs with clear-cutting activities. A concomitant decrease in bacteria (after 8 years from clear-cutting) is likely related to a decrease in fungal biomass/activity, as many bacteria depend on decomposition products from fungi. A reduction in bacteria also occurs because of reduced levels of fresh litter under a recent clear-cut that would otherwise provide easily decomposable organic compounds.

Although there might be an increase in the numbers of necrophilic bacteria and fungi degrading the dead root biomass, this likely would have occurred the first several years after clear-cutting. Because our clear-cut was 8 years old, the elevated activity of these necrophilic organisms would have diminished because the original roots from harvested trees would now be largely degraded.

Another potential fungal response would be negative impacts on mycorrhizal fungal biomass because the extensive root system of mature trees and associated understory was largely replaced by young, mono plantings of Douglas-fir seedlings. This results in a much lower root biomass for mycorrhizal infection. The decrease of mycorrhizal fungal hyphae also leads to a decrease of the bacteria that are directly supported by the mycorrhizal fungi as well as in the decrease of the protozoa that are dependent on mycorrhizal fungi.

The shifts on microbial communities discussed above are likely related to several conditions that clear-cutting imposes. A major factor is the reduction of C inputs because needle retention by young trees (less than 10 years old) reduces the amount of C entering the system from aboveground sources. In contrast, annual litter inputs in OG and CC25 plots have been measured at 175 and 200 g m^{-2}, respectively [25], which is similar to other studies [1, 20]. Combining this with increased rates of decomposition and loss of litter layer after clear-cutting [11], the readily available C sources that support microbial communities is reduced. This is consistent with our site where total organic C of the litter layer on an area basis was 58% lower in CC8 and 53% lower in CC25 relative to OG samples.

The shifts in SMC structure of our study could also be linked to the loss of large trees and understory plants with clear-cutting where OG Douglas-fir forests at our site contain at least 68 different vascular plants [42]. This is consistent with [19] who found a separation of microbial communities between OG and recently clear-cut forest stands. The combined effects of changes to both aboveground and belowground inputs would influence the SMC by affecting the type and chemistry of C inputs from root exudates, and litter and root materials [34]. Indeed, we found significantly lower absolute microbial biomass (likely reflecting lower

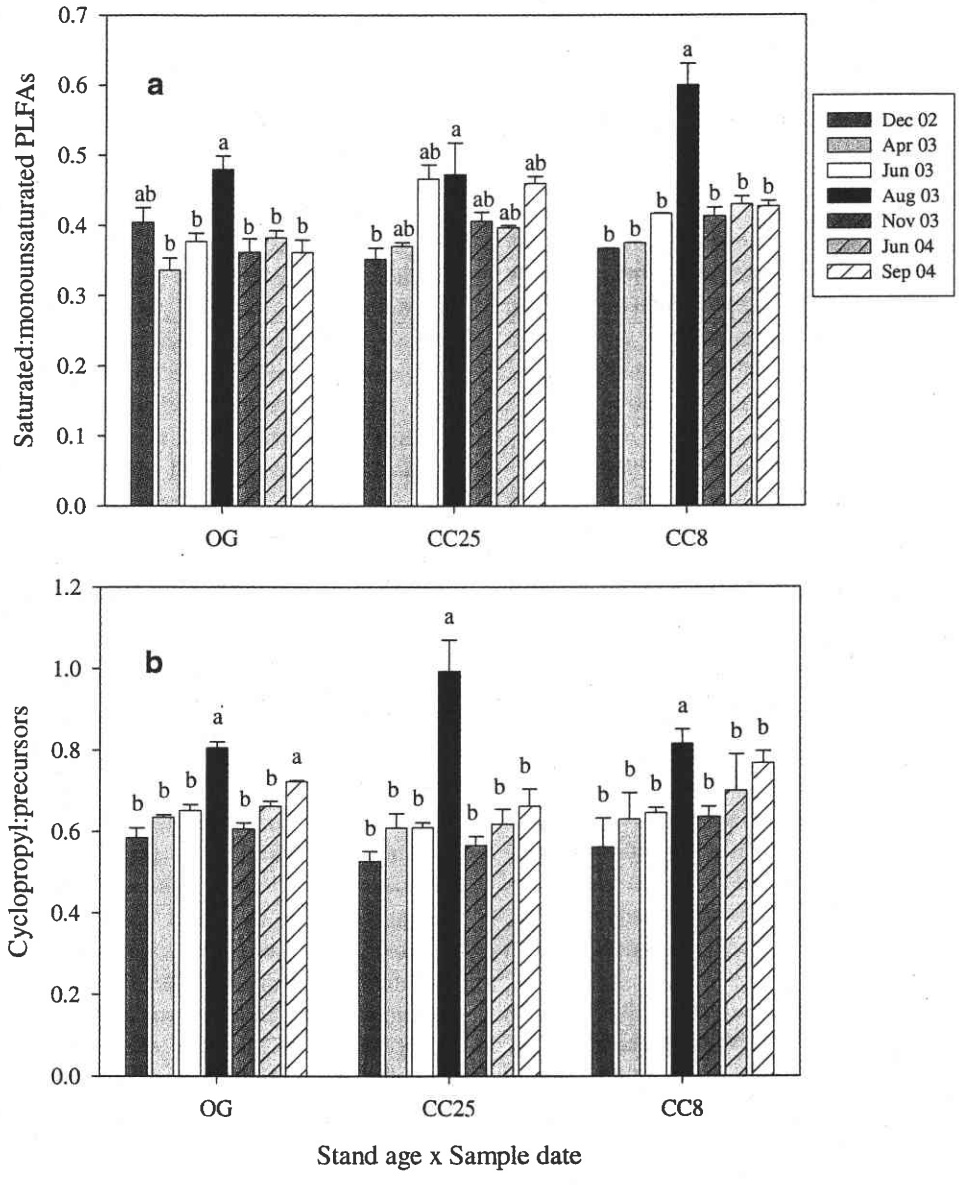

Figure 4 Changes in the ratio of saturated/monounsaturated PLFAs (**a**) and cyclopropyl PLFAs to their precursors (**b**) for each stand age at the seven sample dates. *Values* are the mean molar % of PLFAs. *Error bars* represent standard error (n=4 for OG and n=2 for CC)

total C inputs) as well as community shifts (predominantly reduced fungal biomass) with clear-cutting (perhaps because of changes in type of C inputs) over OG.

Lastly, microclimatic differences between the OG and CC8 sites are another contributing factor to our observed SMC shifts between these two systems where CC8 had significantly greater quantities of PLFAs associated with GM+ bacteria and actinomycetes [24]. These organisms are known for their ability to withstand environmental stress. Soil temperature and moisture impacts have been shown to alter the SMC structure under laboratory conditions [4, 17, 18]. Although soil temperature was well below levels observed in the laboratory to induce microbial composition changes, greater temperature and moisture extremes in the CC8 sites relative to OG are likely contributing factors for differences in the SMC structure between these forest ages. Increased fluctuations in soil temperature and moisture are consistent with other studies of young vs old forest stands and have resulted in altered microbial functioning [1, 10, 14, 30].

Our findings of only the most recent clear-cut (CC8) to be substantially different than OG forests are consistent with other studies where elevated levels of bacterial biomass lasted between 2 and 13 years after clear-cutting [29, 35, 37, 45]. Similar to our study, it has been shown that

fungal biomass was reduced after clear-cutting [1]. Conversely, similar levels of fungal biomass were found by [20] between uncut mixed species and 7- to 9-year old clear-cut forests.

Temporal Dynamics and SMC

Seasonal changes in the SMC were more pronounced than the effects of clear-cutting and are likely linked to the availability of nutrients and water throughout the year. The largest shift on microbial community occurred in August, which was a response to an environmental stress, evidenced by elevated levels of the stress ratios *S/M* and cy/pre [22, 40].

These stress markers reflect the degree to which microorganisms are affected by temperature, nutrient, or water stress. Because temperatures were below levels shown to result in increased stress ratios (around 25°C; [40]), it is not likely that temperature was a major stress factor in August. In terms of nutrient stress, we have no empirical data of nutrient availability to determine whether the elevated stress indicators in August could be related to nutrient stress. A large amount of energy is required to produce saturated fatty acids. This energy must come from the turnover of microbial biomass because the release of root exudates is assumed to be substantially reduced during the dry summer months. We found an overall reduction in total microbial biomass ($PLFA_{tot}$) in August coupled with an increase in the amount of saturated fatty acids relative to the monounsaturated fatty acids (*S/M* ratio) that would support this hypothesis.

We do have soil moisture data supporting the idea that the microbial community was under water stress in August. At that time, the CC8 and OG sites had water potentials of −1.3 to −0.9 MPa. These are lower water potentials than what other studies have shown to significantly reduce respiration when soil water potentials reached −0.3 to −0.6 MPa [49]. Similarly, [13] found that respiration rates in surface soils (0–15 cm) decreased between 60 and 70% when soil moisture was decreased from −0.5 to −1.5 MPa. Based on this limited information from the literature, water potentials in August were low enough to inhibit microbial communities.

The shift in populations in August was reflected most in an increase in mole percent of the actinomycete PLFA markers. Actinomycetes are known to be adapted to water stress by resisting plasmolysis and maintaining cell turgor during low water potential by accumulating compatible solutes (e.g., proline and glycerol) [24]. In addition, they are filamentous, enabling them to bridge air gaps between the thin water films that occur in soil pore spaces under conditions of soil desiccation [24].

Fungi also are filamentous and therefore would be expected to better withstand the August water stress period over bacteria. However, this was not the case, as there was no significant increase in the relative concentration (mole percent) of the fungal PLFA marker, 18:2ω6, or in the *F/B* ratio of August over other dates, nor because of stand age. In fact, the relative concentration of the fungal marker was lowest during the month of August compared to the other sample dates (Table 4).

With the soil temperature and water contents of the soils varying between 4 and 17°C, and 13 and 35%, respectively, over a year, perhaps, it is not surprising that season had a large affect on microbial community structure. However, given the negative impact that clear-cutting has on plant community structure and C inputs, it was somewhat surprising that the seasonal effect was so much stronger than clear-cutting. The differences in the degree of impact between season and clear-cutting on community structure may be more a result of very strong seasonal drivers whereby temperature, water, and photosynthetic C inputs work in concert to impact community structure in many of the same ways that clear-cutting does on a somewhat smaller and less obvious way. These results are consistent with studies that have reported significant seasonal shifts in SMC in forest systems [6, 7, 34, 48].

In summary, this study has provided strong evidence that PLFA analysis coupled with microclimatic data may be used to assess SMC and physiological changes at an ecosystem level. The results for this site suggest that, although canopy and litter input effects are still quite different for a 25-year clear-cut compared to virgin OG, the microbial communities after 25 years have recovered sufficiently to be much more similar to OG forests than a recent clear-cut. This study also emphasizes the temporal variability of the SMC in these ecosystems, and thus, it is recommended that samples be collected over multiple seasons to best assess management impacts on microbial dynamics for a given landscape.

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