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Title: Microbial Community Structure as Influenced by Season and Stand Age in a Douglas-fir (*Pseudotsuga menziesii*) Ecosystem

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Forest harvest can have significant impacts on forest ecosystems that may influence the capacity of soils to sequester carbon (C). The microbial community controls decomposition, which is a critical process in partitioning litter- and root-C between CO_2 and storage in semi-permanent soil-C pools. The objectives of this study were to determine the effect of clear-cutting and stand age on: 1) temporal dynamics of soil microbial community (SMC) structure and physiological status; and 2) shifts among microbial functional groups in taking up ^{13}C-labeled plant materials during decomposition. The experiment was conducted in Douglas-fir ecosystems within the Gifford Pinchot National Forest, Washington. We chose stands of three different ages: old-growth where trees are between 300 and 500 years old; an 8-year old stand; and a 25-year old stand. Phospholipid fatty acid (PLFA) profiling and ^{13}C-PLFA labeling techniques along with the ratio of saturated to monounsaturated PLFAs and the ratios of cyclopropyl PLFAs to their monoenoic precursors as microbial physiological stress markers were utilized.

Microbial PLFA profiles showed that SMC structure and physiological status was most affected by season and secondarily by time since clear-cutting. Total microbial biomass and bacterial and fungal biomass were significantly reduced in CC8

but not in CC25 sites relative to old-growth sites. Total microbial biomass concentration was lowest and the stress indicators were highest in August, which corresponded to low soil moisture and high temperatures.

The relative amount of ^{13}C incorporated into PLFAs was also influenced by stand age and ^{13}C source (^{13}C-labeled litter vs. ^{13}C-labeled root material). A significantly greater amount of ^{13}C was incorporated in CC8 samples compared to OG1 samples in five out of the seven sample dates. Additionally, a significantly greater proportion of ^{13}C was incorporated into soil samples containing the ^{13}C-labeled litter material relative to samples containing ^{13}C-labeled root material in four out of the seven dates. In general, 18:1ω9 and 18:2ω6,9 (common fungal biomarkers) had the greatest amount of ^{13}C incorporation throughout the study period in both clear-cut and old-growth sites, indicating the important role of fungi in the decomposition of litter and root material and translocation of C within soil layers.

Microbial Community Structure as Influenced by Season and Stand Age in a Douglas-fir (*Pseudotsuga menziesii*) Ecosystem

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CONTRIBUTION OF AUTHORS

Jennifer Moore Kucera conducted research and wrote each manuscript. Dr. Dick assisted with data collection and analysis and review of each manuscript.

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GENERAL INTRODUCTION

Microorganisms play a central role in litter decomposition and partitioning C between CO_2 evolution and sequestration of C into semi-permanent pools in soils. At the ecosystem level, forest management practices, such as clear-cutting, have been shown to influence the rates of litter accumulation and quality and micro-climatology, which could affect the microbial community structure and C sequestration processes. The Wind River Experimental Forest has a range of stand ages including original old-growth forests which provided a unique research opportunity to study management and soil microbiology interactions. The objective of this dissertation was to use phospholipid fatty acid (PLFA) profiling alone and in combination with ^{13}C-isotope tracking to determine the influence of clear-cutting and seasonal changes on the soil microbial community (SMC) composition and physiological status.

In this dissertation, microbial PLFA profiles were developed and compared for soils from sites 8 and 25 years after clear-cutting (CC8 and CC25, respectively) and in paired sites from old-growth coniferous forest stands (OG1 and OG2, respectively). The sampling protocol allowed for analysis of soil microbial community structure across all seasons which provided a unique investigation of the influence of soil moisture and temperature differences on microbial communities under natural conditions.

This dissertation is organized into four chapters. Chapter 1 contains background information on C cycling in forest soils, controls on decomposition of litter and root material, the PLFA technique, and the coupling of the stable C isotope ^{13}C with microbial community and decomposition studies. In Chapter 2, a field study was conducted to investigate the influence of clear-cutting and season on soil microbial community structure. Soil samples were collected from old-growth and

clear-cut stands at seven months under contrasting climatic conditions from November 2002 to September 2004. In Chapter 3, a pulse-labeling technique was developed to label Douglas-fir seedlings with $^{13}CO_2$ for a subsequent *in situ* soil incubation study. In Chapter 4, the ^{13}C-labeled litter or root material was added to microcosms installed in old-growth and clear-cut sites and the soil was analyzed for the relative amount of ^{13}C incorporated (^{13}C %) into PLFAs. As in Chapter 2, samples were collected over the same 22-month period in order to study the time-course ^{13}C incorporation into microbial community members during plant decomposition in forest soil from the different stand ages.

CHAPTER 1

LITERATURE REVIEW

Jennifer Moore Kucera

Carbon Cycling in Forest Ecosystems

The global carbon (C) cycle is of great interest to many including biogeochemists, oceanographers, ecologists, fossil-fuel companies, climatologists, agriculturists, foresters, environmentalists, policy makers, and the general public. Carbon is essential for all living tissue and the rate at which organic C is produced and transformed on a global level provides an index of biospheric health (Schlesinger, 1997). Energy required by most microorganisms and all humans rely heavily on the C status of our Earth.

Carbon, in the form of CO_2, is a major greenhouse gas that is released to the atmosphere as a result of anthropogenic activities. Since the industrial revolution, CO_2 concentration in the atmosphere has increased over 30% and has been associated with climatic disturbances to temperature and precipitation patterns on a global scale. The release of CO_2 from fossil fuels is estimated to be 6×10^{15} g C /yr (Schlesinger, 1997). Only 56% of this released CO_2 remains in the atmosphere (Keeling et al., 1995). Originally, scientists believed that about 33% of the CO_2 released via fossil fuels was dissolved in seawater by the enormous buffering capacity of dissolved inorganic C found in the oceans. However, recent research has suggested that a large C sink occurs in the terrestrial biosphere (Kaiser, 2000). Fisher et al. (1994) suggested that deep-rooted grasslands in South America were capable of sequestering C on a level of global importance. Conversely, Kaiser (1998) proposed that the 'C sink' was temperate North America forests and went on to say "Figuring out what's going on - whether C is spurring faster tree growth, or disappearing into soil is crucial...." Therefore, much research has been involved in determining the active pools of C within the forest ecosystem.

The largest pool of C on land is contained in soils, which store 1500×10^{15} g of C. This is more than 2.5 times the amount of C stored in land plants and twice the amount of C stored in the atmosphere (Schlesinger, 1997). In spite of this, the potential for C storage in temperate forest ecosystems has focused largely on aboveground biomass. For example, one outcome of the Kyoto protocol was that it

favored young forest stands as a means to increase the terrestrial C sink and thus reduce atmospheric CO_2 due to the notion that young trees grow faster and accumulate more CO_2 than do older trees. Recently, Schulze et al. (2000) argued that this is misguided and although there may be rapid uptake of C by young stands, this would likely be offset by high rates of C losses from soils due to respiration and lower rates of sequestration of C into stable pools. As stands progress towards old-growth stages, new equilibriums are attained where litter and root turnover rates results in an increase of C incorporation into stable soil pools (Buchmann and Schulze, 1999). Furthermore, Harmon (2001) suggested that complete forest C inventories are necessary to determine if the ecosystem is serving as a C source or sink. In our society, forest stands must be harvested to provide the necessary resources for building, paper, and fuel supplies, but this disturbance disrupts C dynamics at least in the short term, with long-term effects largely unknown. It therefore becomes crucial to assess the impacts of forest harvest on belowground C dynamics to determine the degree and duration that these changes will have on the global C cycle and for predicting global climate change.

Litter and Root Decomposition Controls

Belowground C inputs to forest systems largely come from the decomposition of litter and root material as well as direct inputs via rhizodeposition. The rate of decomposition of plant material is dependent on numerous biotic and abiotic factors including soil temperature and moisture content, the composition of the decomposer community, and the 'quality' of the litter (Swift et al., 1979). Litter quality is often defined by biochemical properties and the physical nature of the litter in relation to its ability to serve as a C, energy, and nutrient source to the decomposer. Decreased decomposition rates are often reported with increased lignin:N ratios (Melillo et al., 1982; Harmon et al., 1990), C:N ratios (Taylor et al., 1989), and increased polyphenol contents such as tannin concentrations (Hattenschwiler and Vitousek, 2000; Valachovic et al., 2004).

At the center of plant material decomposition are the soil organisms. Macro- and meso-fauna are involved in the breakdown of large pieces of plant material into smaller fragments, which increases the surface area available for microbial attack. A general model of organic matter breakdown is the conversion of macromolecules such as cellulose, hemicellulose, proteins and lignin into smaller units of sugars, amino acids, proteins, phenols and quinones, which may then be assimilated by plants and microorganisms or recombined to form more recalcitrant materials (Sylvia et al., 1998). The more recalcitrant compounds and products such as lignin and phenolics can become part of the active, protected organic matter and eventually be converted to "old" organic matter (Sylvia et al., 1998).

The rate of decomposition is often described by a first order reaction that suggests that the rate of decomposition is proportional to substrate concentration and that other factors (e.g. biomass) are not limiting. The rate constant, k, can be described mathematically where $-dA/dt = kA$ where A is the amount of substrate and t is time. The turnover time of a substrate is then described as $1/k$. The rate constant for Douglas-fir needles from sites in the Pacific Northwest have been reported to range from 0.27 to 0.39 (Harmon et al., 1990; Valachovic et al., 2004) and vary largely as a result of changes in precipitation patterns.

In general, bacteria dominate the microbial community during the early stages of decomposition with fungi becoming more important during the latter stages (Griffiths et al., 1999). Bacteria are considered to be the primary decomposers of labile constituents such as simple carbohydrates, organic acids, and amino acids, whereas soil fungi are responsible for the decomposition of recalcitrant compounds such as lignin (Dix and Webster, 1995; Sylvia et al., 1998). Fungi are particularly good at breaking down lignin because they have esterase, phenolase and peroxidase enzymes needed to degrade this complex polymer. Collectively, these fungi are referred to as white rots and are of the Basidiomycotina order. However, decomposition of plant materials is a continuous process with both fresh materials and old sources being degraded at the same time. Therefore, the entire microbial community works in concert as the products of one stage of decomposition are used as

C sources for microorganisms during subsequent stages (Kirk and Fenn, 1982; Fontaine et al., 2003). Although most studies report decomposition rate constants using a single-exponential model, there are other studies that suggest a two-stage decomposition process is more accurate; the first stage is associated with rapid mass loss followed by a later stage of slower mass loss (Berg, 1986; Chen et al., 2002).

A large proportion of forest photosynthate is allocated belowground. Waring and Running (1998) have estimated that between 10 and 60% of net primary productivity is consumed by fine-root and mycorrhizal production. The amount of C allocated to roots is often dependent upon the N status of the soil, with more C allocated belowground under N-limited conditions (Vogt et al., 1987; McDowell et al., 2001; Tingey et al., 2005). McDowell et al. (2001) reported that 59 and 47% of the total belowground C was allocated to fine-root and mycorrhizal production in N-fertilized and unfertilized sites, respectively. Using ^{13}C-depleted CO_2 in a 4-year study examining the effects of elevated CO_2 on Douglas-fir ecosystems, Hobbie et al. (2002) showed that about 75% of C allocated to fine roots originated from current year photosynthate. In a Douglas-fir ecosystem, Sulzman et al. (2005) estimated that over 70% of total soil CO_2 efflux came from belowground sources. All of these studies reveal the importance of root and mycorrhizal production and turnover to C budgets in forested ecosystems. For example, Vogt et al. (1986) have proposed that the turnover of these C sources is the largest C input to forest soils.

Similar to litter decomposition rates, Chen et al. (2002) showed that the decomposition of Douglas-fir fine roots was controlled by a two-stage decomposition process. They suggested that there was an initial stage of rapid mass loss that was largely associated with labile C sources, such as initial water-soluble extractives, followed by a slower second stage associated with recalcitrant C, such as high lignin content.

In addition to the direct turnover of roots, higher plants release a variety of C and energy sources for microbial uptake including sugars, amino acids and amines, aliphatic and aromatic acids, fatty acids, sterols, enzymes, vitamins and phenolics (Grayston et al., 1997). The constant flow of this rhizodeposition results in biological

'hot spots' where soil microbial communities and their activities vary dramatically in relation to bulk soil samples. In undisturbed forest systems, new roots are continuously exploring the soil for nutrients. This, combined with the effects of mycorrhizal colonization, likely leaves only a small volume of the bulk soil unaffected (Hernesmaa et al., 2005). However, disturbances such as through clear-cut harvesting will remove this flow of C from tree leaves or needles to the roots and thus impact the functioning of the microbial system. The degree and duration of this impact will vary depending upon forest type, harvesting technique, regeneration efforts, climate, and time since harvest.

Effects of Clear-cutting on Forest Soils

Forest management practices, such as clear-cutting, have been shown to affect many soil properties, processes, and biological populations. When the canopy is removed via harvest, the soil microclimate is immediately disturbed. For example, radiation levels that reach the soil surface increase thus increasing average soil temperatures; in addition, there are generally fluctuations in soil moisture and temperature over diurnal and seasonal periods (Keenan and Kimmins, 1993; Holmes and Zak, 1999). Increased trafficking by skidders often resulted in increased bulk density (Dick et al., 1988; Startsev et al., 1998; Saetre and Bååth, 2000; Lindo and Visser, 2003) and the relocation of organic material either in slash piles or mixed into mineral layers (Johnson et al., 1991; Houston et al., 1998).

Clear-cut harvesting also alters plant community composition and structure (Strong et al., 1995). Annual litter input and root biomass is dramatically reduced with tree removal, which often leads to indirect effects on biogeochemical cycles. For example, clear cutting has been associated with decreased levels of total N and C, NH_4^+-N, NO_3^--N, and available P in forest floors from coniferous stands (Schmidt et al., 1996; Lindo and Visser, 2003). In contrast, increased soil NO_3^--N concentrations often follow immediately after clearing in deciduous forests (Lindo and Visser, 2003)

but the NO_3^--N may be quickly lost from the system by leaching (Holmes and Zak, 1999).

Nutrient cycling is largely controlled by the microbial community, which relies on litter and root exudates for nutrients and as a C energy source and are equally susceptible to disturbances resulting from the harvest. Microbial C and N mineralization rates have been shown to increase (Frazer et al., 1990), decrease (Bauhus, 1996), and to fluctuate more widely with clear-cutting (Marra and Edmonds, 1998). The effects of clear-cutting on the soil microbial system are most pronounced immediately following the harvest. Microbial biomass C as measured by chloroform fumigation extraction was reported by Chang et al. (1995) to be reduced in the order: old growth > 10 year-old plantations > 3-year-old plantations. More specifically, immediate clear-cut effects include decreased fungal biomass (Bååth, 1980) and an initial increase in bacterial biomass that lasted between 2 and 13 years after clear-cutting (Niemela and Sundman, 1977; Sundman et al., 1978; Lundgren, 1982). However, similar fungal community and microbial process were measured in uncut forests seven to nine years after clear-cutting in mixed-wood forests of northwestern Ontario (Houston et al., 1998). Additionally, no significant long-term changes in basal respiration and microbial biomass were reported by Seastedt and Crossley (1981) in forests of southern Appalachia.

Phospholipid Fatty Acids (PLFAs)

Phospholipid fatty acids are fatty acids that are essential components of all living cells. They are organic molecules that contain many C-H bonds that store energy and are comprised of a glycerol, two fatty acids, and one phosphate group. In contrast to whole cell fatty acids such as those extracted using the fatty acid methyl ester (FAME) method, PLFAs are not found in storage products or dead cells because upon cell death, the phosphate group is quickly consumed (Tollefson and McKercher, 1983). These authors found that 50% of labeled phosphatidyl choline was decomposed in soil within eight days with no further major changes observed. Other

studies have shown a strong correlation between total PLFA content and other methods that measure microbial biomass including the substrate induced respiration technique, ATP concentrations extracted from soils, and the chloroform fumigation method (Zelles, 1999). Therefore, PLFAs are considered to represent the viable microbial biomass in a system and for this reason are preferred over extraction of all fatty acids (Zelles, 1999).

The PLFA extraction method is similar to the FAME extraction method in that the final products are methyl esters of fatty acids (FAMEs) but it is unique in that the PLFA extraction allows the separation of lipids based on polarity. Three fractions of lipids are separated using solid-phase extraction columns and solvents of varying polarity: the neutral lipids are removed first with chloroform; the glycolipids are removed with acetone; and the phospholipids are removed last with methanol. Typically, only the phospholipids are further analyzed because they are considered to be the most sensitive and useful chemical measures of microbial community structure (Tunlid and White, 1992). The PLFAs account for up to 90% of the total extractable lipids in bacteria, and thus make up a relatively constant proportion of the microbial biomass (Lechevalier, 1989). However, recent work by Bååth and Anderson (2003) showed that the neutral lipid fraction can be used to assess the nutritional status of fungi because only eukaryotic organisms use these lipids for storage purposes. However, in prokaryotic organisms, they can indicate the relative proportion of dead to live bacteria because upon cell death, the phosphate group is quickly consumed, leaving only a neutral lipid behind.

Nomenclature

Standard nomenclature is used to describe the FAMEs produced from PLFA extraction. They are designated by the total number of C atoms:number of double bonds, followed by the position of the double bond from the methyl (aliphatic) end (ω) or from the carboxyl (Δ) end of the molecule. Cis and trans geometry are indicated by the suffixes "c" and "t", respectively. Because the majority of unsaturated bonds are in the cis configuration, the "c" is often omitted (Vestal and White, 1989). The

prefixes "a" and "i" refer to anteiso- and iso-branched fatty acids. The prefix, 10Me indicates a methyl group on the tenth C atom from the carboxyl end of the molecule, "OH" indicates a hydroxyl group, and "cy" indicates cyclopropyl fatty acids. Table 1.1 lists the indicator fatty acid associated with five microbial groups.

Table 1.1. Taxonomic microbial groups with the PLFA group and specific PLFA markers used to designate such groups.

Taxonomic group	PLFA group	Specific PLFA markers	Reference
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	Frostegard and Bååth, 1996
Gm +	branched PLFAs	sum of i15:0, a15:0, i16:0, i17:0, and a17:0	O'Leary, 1988
Gm -	cyclopropyl and mono PLFAs	sum of cy17:0, 16:1ω7, 18:1ω7, and 17:1ω9	Wilkinson, 1988
Actinos	10Me-PLFAs	sum of 10Me16:0, 10Me17:0, and 10Me18:0	Kroppenstedt, 1985
Fungi	polyunsaturated PLFAs	18:2ω6,9	Federle et al., 1986; Frostegard and Bååth, 1996
F:B ratio	multiple groups	Fungi/Bacteria	Federle et al., 1986; Frostegard and Bååth, 1996
Protozoan	polyunsaturated PLFAs	sum of 20:2ω6 and 20:4ω6	Ringelberg et al., 1997

Gm + = Gram-positive bacteria
Gm - = Gram-negative bacteria
Actinos = actinomycetes

PLFAs and their application in soil microbial ecology

The practice of using the fatty acid composition of bacterial strains or pathogens has been used for years as an identification procedure under culture conditions. The data have revealed the predominance of specific fatty acids for a given taxon; information that has been extrapolated for use in environmental samples where the entire microbial community is evaluated under unaltered conditions (Bååth and Anderson, 2003; Tornberg et al., 2003). For example, bacteria and fungi can be distinguished based on the fact that bacteria do not usually synthesize polyunsaturated fatty acids. All fungi contain unsaturated 18C fatty acids with many fungi containing a high proportion of the polyunsaturated fatty acid, linoleic acid (18:2ω6,9)

(Lechevalier and Lechevalier, 1988; Stahl and Klug, 1996). Good correlations between this fatty acid and another fungal biomarker, ergosterol, have been reported (Klamer and Bååth, 2004). Bacteria may further be separated into Gram negative and Gram positive bacterial groups because the fatty acid composition of such organisms correlates well with the Gram stain (Harwood and Russel, 1984). Odd-numbered branched-chain *iso* and *anteiso* fatty acids are the major fatty acids of Gram-positive bacteria, whereas Gram-negative bacteria predominantly have even-numbered straight chain and cyclopropane fatty acids. Examples of these and other signature fatty acid markers and their associated microorganisms are presented in Table 1.1.

The PLFA pattern, together with multivariate statistical techniques, can be used to study shifts in the structure of the microbial community resulting from management or environmental changes. Other advantages of this method include: the fatty acid composition is stable and is not dependent upon plasmids, mutations or damaged cells; it is quantitative, relatively cheap, and is highly reproducible; results from the analysis provide a fingerprint of microbial diversity at the time of sampling and requires no alteration of the environment.

Although the composition of fatty acids is widely used for determining the community composition of microorganisms in ecological studies, there are several important limitations. First, the use of specific biomarkers for interpretation of community profiles comes from a database derived mostly from information on isolated, pure cultures of microorganisms. It is well established that only a minor fraction of bacteria (less than 10%) have been identified (Amann et al., 1995). Therefore, individual fatty acids may be misinterpreted to belong to a specific microorganism or group of microorganisms but possibly represent unknown microorganisms. However, with advances in molecular studies, the database is rapidly growing and adding new species each day.

Second, PLFA analysis extracts only ester-linked fatty acids. Therefore, the Archaea with ether-linked fatty acids are not included and may not be assessed. Archaea are especially important in extreme environments that are very cold, hot, or saline. Third, growth conditions and environmental stresses result in the production of

different amounts and types of PLFAs (Haack et al., 1994; Zogg et al., 1997). The PLFAs normally associated with a particular group of microorganisms may not necessarily be unique to only that group under all conditions. For example, Haack et al. (1994) explained that although the cyclopropyl fatty acids, cy17:0 and cy19:0 have been found to be characteristic for anaerobic bacteria, they are not exclusive to these groups and have been shown to be found in relatively high proportions for *Pseudomonas* species and in bacterial rhizosphere communities. Moreover, it has been shown that the relative proportion of these fatty acids increase under conditions of nutritional or environmental stress in Gram-negative bacteria (Kieft et al., 1994; Petersen and Klug, 1994). A second example involves use of the branched fatty acid, i15:0 as a biomarker for Gram-positive bacteria (Haack et al., 1994). This fatty acid also is characteristic of certain Gram-positive bacteria of the genus *Cytophaga* and related bacteria in the genus *Flavobacterium* (Haack et al., 1994). It also has been shown to increase under increased incubation temperature relative to the anteiso-15:0 fatty acid (Kaneda, 1991).

Microorganisms typically change the degree of unsaturation and the amount and type of branching with changes in temperature. Lower temperatures have been associated with decreases in fatty acid saturation and conversely, higher temperatures often result in an increase in saturation (Petersen and Klug, 1994). Changes in microbial fatty acid composition in response to environmental or nutritional stresses does make interpretation of environmental samples more challenging, but it also presents a unique ability to test the influences of these stresses under natural conditions.

Lastly, linoleic acid (18:2ω6,9), the PLFA commonly used as a fungal biomarker, also is a major plant cell lipid (Harwood and Russel, 1984). Therefore, when working with complex media such as forest soils, it is important to ensure all plant material is removed from the sample.

Regardless of these limitations, PLFA analysis has proven very useful in characterizing the response of microbial communities to environmental stress and disturbances caused by both natural and anthropogenic forces. Studies have used

PLFA profiles from a variety of environments including sediments (Findlay et al., 1989), subsurface environments (Kieft et al., 1997; Ringelberg et al., 1997), soils (Bååth, 1980; Zelles and Bai, 1993; Wander et al., 1995; Pennanen et al., 2001), humus samples (Pennanen et al., 1999; Pennanen et al., 2001), rice fields (Murata et al., 2002), stream biofilms (Jahnke et al., 2001), peatlands (Sundh et al., 1997), polluted aquifers (Ludvigsen et al., 1997), and plant roots (Tunlid et al., 1989).

Examples of PLFA analysis in forest research

The use of PLFA analysis in forest research has proven to be a useful biological assay to compare microbial community structures under a variety of systems such as sites exposed to heavy metals (Pennanen et al., 1998; Pennanen, 2001; Hinojosa et al., 2005), across pH (Pennanen et al., 2001; Bååth and Anderson, 2003; Hackl et al., 2005) and nitrogen availability (Leckie et al., 2004) gradients. Other PLFA studies on forests have shown effects of forest types (Hackl et al., 2005) and long-term C input manipulations (Brant, 2005) on soil microbial community structure. Ponder and Tadros (2002) compared PLFA profiles of forest soils four years after harvest that had two levels of soil compaction. Although they found no significant differences in the sum of bacterial biomarkers, multivariate analysis of PLFA profiles revealed structural differences between sites. In a study investigating the structure of the soil microbial community in forest sites that differed in fertility and development stage, Pennanen et al. (1999) found that the relative abundance of fungi decreased and that of bacteria increased with increasing fertility but found no differences in PLFA profiles based on forest age. Similarly, Leckie et al. (2004) investigated the soil microbial communities in forest floors of two forest types that differed substantially in nitrogen availability. They used denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), and PLFA techniques. Using DGGE and RISA, no differences were detected in the bacterial communities but the RISA technique revealed that fungal communities changed between forest types. The PLFA analysis detected subtle changes in overall community composition between forest types, as well as in fungal, bacteria, and Gram-positive bacteria biomarkers.

Differences were also distinguished between different humus layers. This shows one of the potential advantages of lipids over nucleic acid profiling; lipid extractions are fairly quantitative and give some idea of the biomass of each functional group. Although specificity is much higher and detection limits are low for nucleic acids, this information may not be useful for detecting field based microbial responses because detection and amplification (PCR) of nucleic acids provides little information on the size or relative importance of a community member under different management systems for many nucleic acid procedures (exceptions being the more difficult real-time PCR and LH-PCR).

The ratio of fungal:bacterial PLFAs (F:B ratio) has been used as an index of the potential of soils to store C due to the differences in C assimilation efficiencies between fungi and bacteria (Frostegard and Bååth, 1996). Bacteria have C assimilation efficiencies that range between 20 and 40%, whereas fungi have efficiencies up to 70% (Holland and Coleman, 1987). Therefore, enhanced soil C storage is favored by management activities or site characteristics that favor fungi (Bailey et al., 2002). Disturbances often lower the F:B ratio because fungal hyphae are destroyed. Understanding the relative dynamics of fungi and bacteria in soil relative to environmental factors and changes may allow better prediction of the C storage potential of soils, the fate of added substrates to soils, and the longevity of stored C in soils.

Stable Carbon Isotope: ^{13}C

Stable isotopes are atoms of the same element (i.e., same number of protons and electrons) but contain different number of neutrons. About 98.89 % of all C is ^{12}C; ^{13}C comprises about 1.11% of the terrestrial pool (Boutton, 1991). However, isotopic fractionation leads to deviations in the isotopic ratios of natural materials and thus provides insight into C cycling processes in soil, plant, water, and microbial systems. For example, plants discriminate against the heavier ^{13}C, but how much plants discriminate differs depending upon the photosynthetic pathway employed.

The C3 plants use the carboxylating enzyme ribulose bisphosphate carboxylase, and have $\delta^{13}C$ signatures between -21 and -30 ‰, whereas C4 plants utilize the phosphoenolpyruvate (PEP) carboxylase enzyme and have $\delta^{13}C$ signatures between -10 and -15 ‰ (Ehleringer et al., 2000). The differences arise due to limitations that occur during CO_2 fixation in C3 plants and stomatal diffusion in C4 plants (O'Leary, 1988). Additional but smaller ^{13}C fractionation occurs during secondary metabolism such as in the production of lipids and lignin which are typically depleted by 3-6 ‰ (Wedin et al., 1995). In contrast, sucrose, starch, cellulose, and hemicellulose tend to be slightly enriched (Balesdent and Mariotti, 1996).

The $\delta^{13}C$ signature of soil organic matter largely reflects the $\delta^{13}C$ signature of the vegetation but varies slightly as a result of decomposition processes (Balesdent and Mariotti, 1996; Ehleringer et al., 2000). In particular, soils under C3 vegetation show a progressive enrichment in $\delta^{13}C$ with depth (Balesdent et al., 1993; Buchmann et al., 1997; Fessenden and Ehleringer, 2002). Ehleringer et al. (2000) proposed that the enrichment is due to changes in the $\delta^{13}C$ ratios of atmospheric CO_2 over time (Seuss effect) as well as the mixing of new and older SOM components during soil C loss. The Suess effect is associated with the burning of fossil fuels. Since the Industrial Revolution, the $\delta^{13}CO_2$ of the atmosphere has been decreasing (becoming lighter) as more ^{13}C-depleted fossil fuels are used. The $\delta^{13}CO_2$ of the atmosphere was about -6.5 ‰ between 300 to 1000 years ago and today it is about 1.5 ‰ lighter at -8.0 ‰. The older SOM at depth probably originated at a time when the $\delta^{13}C$ values of atmospheric CO_2 were more positive. It is estimated that this effect accounts for about 1.5 ‰ difference between surface and deeper soils. Additional enrichment results from the mixing of microbial C residues that are thought to be ^{13}C enriched (Ehleringer et al., 2000). The authors assumed that the $\delta^{13}C$ signature of soil microorganisms would be enriched relative to their food source. These microbes are the driving force in the production of stable SOM and have such high turnover times that with time the older (i.e., deeper) SOM pool will become more enriched relative to the new SOM pool at the surface.

δ¹³C of Soil Microorganisms

The C isotopic signature of an organism is controlled primarily by the isotopic composition of the C source and the mechanism by which that C is assimilated (Pancost and Damste, 2003) and has been shown to reflect within 3‰ of the C isotope ratios of the C substrate (Blair et al., 1985; Coffin et al., 1989). In contrast to the isotopic signature of bacterial biomass, lipids tend to be depleted in ^{13}C relative to the substrate on which the organisms have been growing (DeNiro and Epstein, 1977; Abraham et al., 1998; Abraham and Hesse, 2003). Additional controls on the C signature of lipids include the biological mechanism of C assimilation (van der Meer et al., 1998; van der Meer et al., 2003) and pathways of lipid biosynthesis (Abraham et al., 1998).

Biosynthesis of fatty acids begins with the multienzyme complex fatty acid synthase and ends with palmitic acid (16:0) from which all longer chained fatty acids and some unsaturated fatty acids are synthesized (Abraham et al., 1998). Fractionation of C occurs during one of three stages of polar lipid biosynthesis: (1) transport of substrate into the cell and its degradation to acetate, (2) synthesis of palmitic acid, and (3) transportation to the membrane and esterification to the polar lipids (Abraham et al., 1998). Given a particular bacterial strain Abraham et al. (1998) found that discrimination varied within the different fatty acids. For example, myristic acid (14:0) was depleted relative to 16:0 whereas stearic acid (18:0) was enriched. Fractionation was not a function of chain length because tetracosanoic acid (24:0) was more depleted in ^{13}C relative to 16:0. Overall, the isotope ratios in fatty acids were independent of the growth stage of microorganisms and the overall isotope fractionation for different bacteria was within 3 parts per thousand or per mil (‰) (Pelz et al., 1997; Abraham et al., 1998). In a similar study, the C isotope ratios of fatty acids in fungi were studied and found to have a similar fractionation range of about 2.5‰ (Abraham and Hesse, 2003). Interestingly, stearic acid (18:0) was depleted in ^{13}C relative to 16:0 in all fungal strains whereas the opposite pattern was observed in bacteria (Abraham et al., 1998). The production of linolic acid (18:2ω6,9) is formed from two desaturation steps beginning with 18:0. The first step, 18:0 to

oleic acid (18:1ω9) resulted in a very low isotopic fractionation but the second desaturation step to 18:2ω6,9 resulted in a ^{13}C enrichment ranging from 3.6 to 4.4‰ in Zygomycotina and Ascomycotina, respectively (Abraham et al., 2003). Henn and Chapela (2000) investigated the effects of C_3- and C_4-derived sugars on the isotopic discrimination in basidomycete fungi. They found that the fungi grown on C_3-sucrose were enriched in ^{13}C, while no ^{13}C discrimination was observed when fungi were grown on C_4-sucrose. They conclude that although discrimination effects may be masked at the whole organism level, species specific fractionation by fungi does occur and is controlled by physiological processing, substrate effects, and micro-environmental conditions. Their findings become most important in C flow investigations where land use changes between C_3 and C_4 ecosystems are studied and involve fungal transfer of material between trophic levels.

PLFA Analysis Coupled with Stable-isotope Probing

It is difficult to link PLFA markers with community function because the overall PLFA profile is a consortia of all organisms present at sampling time (Ludvigsen et al., 1997) and any given microbial process may be carried out by diverse taxa (Hill et al., 2000). Stable isotope probing (SIP) with ^{13}C of specific biomarkers, such as PLFAs, is a promising new tool to directly link specific microbial processes with the organisms responsible, thus shedding valuable insight into the biogeochemical transformations in natural environments mediated by microorganisms (Boschker and Middelburg, 2002).

Stable isotope ^{13}C label may be applied to a system as either ^{13}C-bicarbonate or ^{13}CO$_2$ to trace photosynthetically fixed ^{13}C through the microorganisms in the rhizosphere (Butler et al., 2003; Treonis et al., 2004) or as ^{13}CH$_4$ to identify methane-oxidizing bacteria (Boschker et al., 1998; Bull et al., 2000). Other studies involve the addition of ^{13}C by input as natural organic substrates such as plant material (Malosso et al., 2004; Waldrop and Firestone, 2004), glucose, glycerol, mannose, and lactose (Abraham et al., 1998), acetate (Arao, 1999), or as organic pollutants such as toluene

(Hanson et al., 1999) or phenanthrene (Johnsen et al., 2002). As the ^{13}C-labeled compound is assimilated, it will be incorporated into macromolecules such as PLFAs, amino acids, and nucleic acids.

One means to minimize the problems involving interpretation of natural isotopic data with fractionation effects is to employ tracer studies (Schimel, 1993). Tracer studies involve the addition of material with a ^{13}C value significantly different from natural background levels to a system (plant, soil, etc.). The fate of this material can be tracked by analyzing the material for its isotopic composition after a given amount of time.

Methodology for ^{13}C-PLFA Analysis

The ^{13}C signatures of PLFAs are determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). In general, IRMS instruments detect small variations in very small amounts of the heavier isotope in the presence of large amounts of the lighter isotope regardless if the sample is from natural abundance or enriched studies (Meier-Augenstein, 1999). Extracted PLFAs are in solution and must be separated and converted to CO_2 prior to IRMS analysis. The GC separates PLFAs followed by conversion to CO_2 by passing the sample through a quartz glass or ceramic tube filled with CuO/Pt or CuO/NiO/Pt and maintained at either 820 or 940°C (Meier-Augenstein, 1999). Water is removed from the gas sample which is then passed into the IRMS. The gas sample is ionized by electrons in the source, accelerated down a flight tube between the poles of a magnet, and are deflected in proportion to the mass-to-charge ratio, *m/z* (Barrie and Prosser, 1996). Electrical currents generated by the separated ion beams are used to calculate the relative abundances of molecules of different m/z (Barrie and Prosser, 1996). For CO_2, there are five species, $^{12}C^{16}O_2$, $^{13}C^{16}O_2$, $^{13}C^{16}O^{17}O$, $^{12}C^{16}O^{18}O$ and $^{13}C^{16}O^{18}O$, but only three molecular weights of 44, 45, and 46. The use of GC-C-IRMS instrumentation became commercially available in 1990. It provides very precise and accurate measurements of differences in isotope ratios and is most commonly used for

[13]C analyses; which have made up almost 70% of all gas isotope ratio analyses (Meier-Augenstein, 1999).

Studies involving stable isotopes at or near natural abundance levels are typically reported as delta (δ) with units in parts per thousand or per mil (‰). Delta values express relative differences in stable isotope ratios between samples and natural abundance standards assigned a δ value of zero. For C, the universally accepted standard is Pee Dee Belemnite (PDB), which is a limestone fossil of *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina (Boutton, 1991). Although this material is no longer available, the National Bureau of Standards has calibrated other material against the PDB enabling researchers to express ^{13}C values relative to PDB. The $\delta^{13}C$ therefore is calculated as follows:

$$\delta^{13}C = (R_{sample}/R_{standard} - 1)*1000 \qquad (1)$$

where R is the ratio $^{13}C/^{12}C$ measured as the mass 45 to 44 ratio. The standard ratio is the absolute ratio of the Pee Dee Belemnite standard (R_{PDB}) with a known value of 0.0112372 (Hayes, 1982).

For enrichment studies, the isotopic composition of the labeled substance added is substantially greater than natural background levels. In these situations, it is useful to express the isotopic composition in terms of atom % which is defined as,

$$\text{Atom } \% \ ^{13}C = (^{13}C/^{13}C + {}^{12}C) = 100*(R_{sample}/R_{sample} + 1), \% \qquad (2).$$

Equation 2 is most commonly used when atom % exceeds about 0.5 atom % or 500 ‰ (Dawson et al., 2002). Finally, atom % excess can be used to express the enrichment level of a sample following addition of a tracer in excess of ^{13}C background levels. It is simply the difference in atom % [13]C of the sample before the ^{13}C-labeled material is added and the atom % [13]C of the sample after the ^{13}C-labeled material is added.

PLFA-Stable Isotope Probing (SIP) applications in microbial ecology

Microbial ecology studies involving the coupling of PLFA with SIP is a recent development with the majority of studies published within the last seven years. Many studies have been conducted in freshwater and marine sediments (Boschker et al., 1998) and in ancient sediments (see review of Pancost and Damste, 2003). Results

have aided researchers to better understand the microbial controls on such biogeochemical cycles as sulphate reduction (Boschker et al., 1998; Boschker and Middelburg, 2002) and methanotrophy (Boschker et al., 1998; Bull et al., 2000; Crossman et al., 2004). Using PLFA-SIP methods Bull et al. (2000) found active populations that were related but not similar to known type II methanotrophs. Without this method, these microorganisms may not have been found because culturing of these methanotrophs has proven unsuccessful. This finding supports the notion that organisms are present in many environments but have yet to be identified.

Fractionation studies of chemolithoautotrophic and heterotrophic microorganisms have revealed the presence of new biochemical pathways such as the reversed tricarboxylic acid cycle or the 3-hydroxypropionate pathway for CO_2 fixation (van der Meer et al., 1998; van der Meer et al., 2000; van der Meer et al., 2001). The latter pathway was determined for *Chloroflexus aurantiacus*, an autotrophic microorganism that has been used to reconstruct paleoenviroments (van der Meer et al., 1998). Without the use of PLFA-SIP techniques, the importance of such microorganisms and biochemical pathways may have continued to be unrecognized.

The use of PLFA-SIP in soil systems also is expanding and has been used to study the flow of C from single source organic substances as well as complex C sources. The first study tracing the flow of C via ^{13}C-labeled acetate in soil showed that changes in the bacterial and fungal activities may be revealed by this technique (Arao, 1999). This author reported different assimilation rates for Gram-negative and Gram-positive bacteria and fungal components of the microbial community. In general, the fungal biomarker, 18:2ω6,9 was highly labeled and remained high throughout the incubation period whereas, the incorporation of ^{13}C in fatty acids characteristic of Gram-positive bacteria was constant or decreased. In contrast, fatty acids characteristic of Gram-negative bacteria in stationary growth phase had a slow assimilation rate of ^{13}C but that this value increased with time. Therefore, Arao (1999) suggested that the growth rate of soil bacteria declined after 24 h incubation but that the growth rate of the fungi increased up to 3 d after incubation. Other studies traced the flow of C into the microbial community composing the rhizosphere of soil

(Butler et al., 2003; Treonis et al., 2004) and a wetland rice soil (Lu et al., 2004). Their studies provided evidence linking the responses of the microbial community with plant photosynthesis. Therefore, PLFA-SIP techniques provide the tool to directly link microbial processes with the groups of microorganisms involved.

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CHAPTER 2

SEASONAL CONTROLS ON THE MICROBIAL COMMUNITY STRUCTURE IN DIFFERENT AGED DOUGLAS-FIR STANDS

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Abstract

Forest harvest can have significant impacts on forest ecosystems. This is of importance today because of the current interest to increase the sequestration of carbon (C) in soils. The microbial community controls decomposition which is a critical process in partitioning litter- and root-C between CO_2 via respiration and storage in semi-permanent soil-C pools. It is therefore critical to better understand how forest harvest influences the soil microorganisms and organic matter transformations under field conditions. The objective of this study was to determine the effect of clear-cutting and stand age on temporal dynamics of soil microbial community (SMC) structure and physiological status using phospholipid fatty acid (PLFA) profiling. Microbial PLFA profiles were developed and compared for soils from old-growth coniferous forest stands and sites that were clear-cut eight years ago in 1994 (CC8) and 25 years ago in 1977 (CC25). 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); increases in these ratios have been used to indicate physiological stress in microorganisms. Surface soil samples were collected during seven months under contrasting climatic conditions from November 2002 to September 2004. Soil microbial community composition was evaluated by non-metric multi-dimensional scaling plots and repeated measures ANOVAs of PLFA patterns. PLFA patterns revealed that SMC composition and physiological status were affected by time since clear-cutting but that temporal changes had an even bigger effect on microbial communities. The microbial biomass (total PLFA concentration) was significantly reduced in CC8 but not in CC25 sites relative to the paired old-growth sites. Soils from CC8 also contained lower bacterial and fungal biomass. Increased ratios of stress indicators occurred during summer sampling when the soil environment was relatively dry and warm compared to all other sampling dates. This study provides strong evidence that PLFA analysis coupled with microclimatic data may be used to assess the SMC and physiological changes at landscape levels.

Introduction

The importance of microorganisms in litter decomposition and nutrient cycling in ecosystems is well known. However, changes to the soil microbial community (SMC) structure in response to environmental factors or disturbances are poorly understood. The SMC is complex and consists of a heterogeneous group of bacterial and fungal species which individually have yet to be quantified using traditional microbiological approaches.

The use of phospholipid fatty acids (PLFA) is a community-level biochemical method that can provide information regarding shifts in the SMC structure resulting from management or environmental changes. The PLFAs are essential components of every living cell and are useful biomarkers because of their great structural diversity and are coupled with high biological specificity (Zelles, 1999). They are not found in storage products or in dead cells because upon cell death, the phosphate group is quickly consumed (Tollefson and McKercher, 1983). Therefore, PLFAs are considered to represent the viable microbial biomass in a system and for this reason are preferred over extraction of total fatty acids (Zelles, 1999). Different groups of organisms vary in their phospholipid composition and can be used as indicators of changes in specific organismal groups (Zelles, 1997; Tornberg et al., 2003). The PLFA pattern, together with multivariate statistical techniques, can be used to study changes in SMC as species composition and relative species abundance are determined. The technique is limited in that it gives no information at the species level or about microbial diversity, but it has been successful in assessing broader-scaled questions such as fungal:bacterial ratios in different systems (Bååth and Anderson, 2003).

Old-growth (OG) forests when compared to clear-cut systems provide a natural laboratory in which to assess how microbial communities respond to differences in root and litter inputs as well as the micro-climate of the forest-soil-atmosphere ecosystem. The disturbance of clear-cutting has an integrative effect on microorganisms by simultaneously altering soil microclimate (due to loss of canopy and reduction to litter layer), physical, and chemical properties. Changes in soil

moisture and temperature status influence microbial metabolism and have been shown to alter the SMC structure under laboratory conditions (Harwood and Russel, 1984; Haack et al., 1994; Petersen and Klug, 1994; Waldrop and Firestone, 2004) but have yet to be shown under field conditions. Another major outcome of clear-cutting is a change in the plant community which affects both the amount and chemistry of C inputs through litter and root exudates and thus the availability of C to microorganisms (Keenan and Kimmins, 1993). Old-growth Douglas-fir (*Pseudotsuga menziesii*) and Western Hemlock (*Tsuga heterophylla*) forests such as those found in the Pacific Northwest are composed of a heterogeneous understory with multiple layers of different shrubs and trees. Clear-cut forests replanted solely with Douglas-fir have become homogenized and consist of Douglas-fir and a ground cover of grass and ferns early after cutting until canopy closure takes place when the trees out compete the grasses for sunlight. This results in different types and quantities of litter input between these forest types and time since harvest.

Understanding the relative dynamics of bacteria and fungi in soil relative to environmental factors, and litter and root changes may allow better prediction of the C storage potential of soils, the fate of added substrates to soils, and the longevity of stored C in soils. However, the first step is to determine the relative contributions of these two groups of organisms to the metabolism of C in soil in different systems. Therefore, the objectives of this study were to determine how clear-cut forests in comparison to old-growth forests affect SMC structure relative to micro- (site or stand age) and macro- (seasonal) climatic conditions.

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. The forests are predominately Douglas-fir (*Pseudotsuga menziesii*) stands of varying ages. The area is characterized by a cool, moist climate with average annual precipitation and temperature of 22.2 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 (Shaw et al., 2004).

The soils have been classified as medial, mesic Entic Vitrands (Stabler series, www.statlab.iastate.edu/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 3,500 and 12,000 years old.

We chose forest stands of three different ages: old-growth where trees have been estimated to be between 300 and 500 years old; an 8-year old stand; and a 25-year old stand. The old-growth sites contain Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*) as the dominant tree species. Dominant understory shrub species are vine maple (*Acer circinatum*), salal (*Gaultheria shallon*), and dwarf Oregon grape (*Berberis nervosa*) and constitute about 57% of the total understory composition (Shaw et al., 2004). 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 of old-growth 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.

Stands were separated into two spatially separated study groups to account for potential differences in soil properties largely resulting from differences in elevation and microclimate. Within each study group, there were two field replicates for each stand. The elevation range for group one is between 338 and 387 m, whereas the elevation range for group two is between 500 and 536 m. The first group compared old-growth (OG1) to the 8-year-old stand (CC8). The second group compared old-growth (OG2) to the 25-year-old stand (CC25). All of the stands except for CC8 are located within the Wind River Experimental Forest (WREF) - a 4208-ha area where active forest research has been conducted since 1908 (Figure 2.1). The CC8 stands are adjacent to the WREF on USDA Forest Service land.
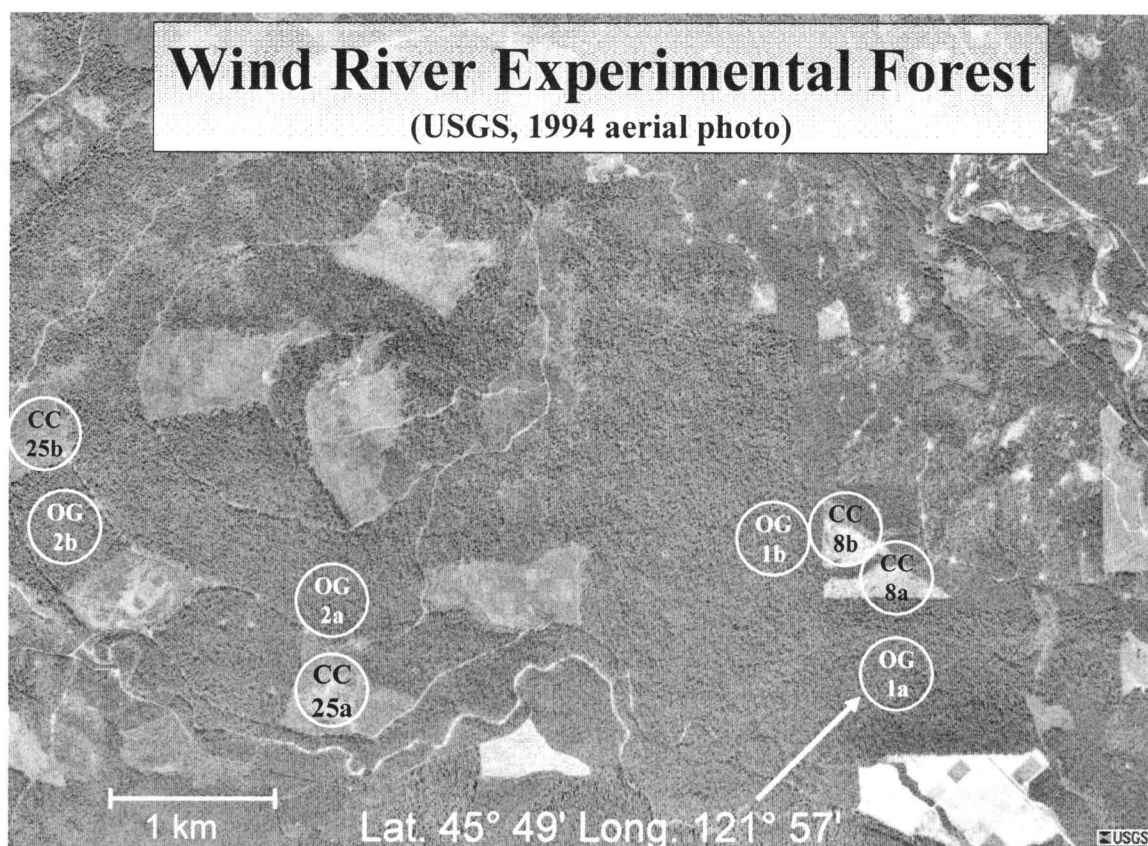
Figure 2.1. 1994 aerial photograph of the Wind River Experimental Forest in southern Washington showing the location of the 8 study sites (OG = old-growth; CC8 = stands that were clear-cut 8 years ago in 1994; CC25 = stands that were clear-cut 25 years ago in 1977; "a" and "b" represent field replicates).

Microcosm set-up and sample

Within each stand, three sub-replicate plots were established; two were used in this study and the third was used in an additional study to track the decomposition of ^{13}C-labeled litter and root materials into PLFAs (see Chapter 4). In November 2002, 14 microcosms constructed of 10-cm diameter x 12- to 16-cm long open-ended PVC columns were inserted into the top 10 cm of mineral soil at each sub-replicate plot (extra length accounts for depth of litter layer). Half of the 14 microcosms received litterbags containing ^{13}C-labeled needles or needle/stem mix and the other half received ^{13}C-labeled roots (see Chapter 4 for details). To install microcosms, a custom-made steel soil corer measuring 10-cm in diameter by 16-cm long (Scotty's

Welding, Wenatchee, WA) was used to extract the soil from the ground. Roots, when encountered, were severed with a knife. The soil corer was pounded into the ground and had a beveled edge to ensure the soil remained in the corer upon extraction. 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 in order. The top O horizon was removed, set aside, and a litterbag containing ^{13}C-labeled needle/stem mix was placed on the soil surface. The O horizon was replaced and gently repacked into the column. Columns that received ^{13}C-labeled roots were established in a similar manner except the ^{13}C-labeled roots were freely incorporated (i.e., no bags) into the top 5 cm of mineral soil before the O horizon was replaced.

Sample collection

On December 16, 2002, April 25, June 20, August 31, and November 23, 2003, and June 14 and September 1, 2004, one pair of microcosms (one containing ^{13}C-labeled needle/stem material and one containing ^{13}C-labeled root material) was randomly chosen from each sub-replicate plot for PLFA analysis. Each set consisted of two microcosms; one contained ^{13}C-labeled litter enclosed in the litterbag and the other contained ^{13}C-labeled roots. Two of the three sets were analyzed for PLFA by GC-FID (this study) and the other set was analyzed for ^{13}C-PLFA concentration as measured by GC-C-IRMS (Chapter 4). All soil samples were collected with the PVC column intact and transported to the lab within eight hours. Samples were stored at 4°C and processed within three days. Each sample was separated into litter layer, 0-5 cm soil below the litterbag or 0-5 cm mineral soil (root cores) and sieved to < 4.75 mm to remove wood pieces, roots, or stones and homogenized. Subsamples were dried at 65°C for 24 h to calculate moisture content.

Soil temperature and moisture measurements
CC8 and OG1 sites:

In April 2003, soil moisture data from the CC8 site were collected using a hand-held time-domain reflectometer (Hydrosense probe, Decagon Devices).

beginning on June 11, 2003, soil moisture data at one of the CC8 stands were collected every 15 sec and averaged hourly using water content reflectometers (model cs615; Campbell Scientific Inc.) mounted vertically through the soil profile with 30-cm rods (3.2-mm diameter and 3.2-mm spacing) and a data logger (model 23x, Campbell Scientific, Inc.). Beginning in December 2002 (prior to 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 OG1 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 time-domain reflectometers (TDR) 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 wave guides. Soil moisture and temperature data were collected every five seconds and 30-min averages were stored by a data logger (model cr10x, Campbell Scientific Inc.).

Soil temperature for OG1 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 OG1 resulted in a strong correlation ($r = 0.95$) between the two sites for data collected in 2003.

CC25 and OG2 and sites

Soil temperature at the CC25 and OG2 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 (Table 2.1). Soil samples were collected

from 0-10 cm and 10-30 cm depths after removing the litter layer. Total C and N
were determined by dry combustion using a LECO CNS-2000 Macro Analyzer.

Table 2.1. 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	$Mg\ N\ ha^{-1}$		
OG1	Litter								
		4.2	0.04	33.93	6.29	0.93	0.17	36.7	4.3
	Soil								
		0-10	0.89	3.44	30.7	0.14	1.25	24.6	5.1
		10-30	0.95	2.04	38.9	0.12	2.29	17.0	5.4
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
OG2	Litter								
		4.2	0.04	33.98	6.29	0.92	0.17	36.9	4.2
	Soil								
		0-10	0.88	4.00	35.1	0.16	1.41	25.0	4.9
		10-30	0.95	2.28	43.1	0.13	2.46	17.5	5.1
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

In order to express soil and litter total C and N values on an area basis, bulk
density measurements for soil were conducted using a Troxler nuclear density gauge
(Troxler Electronic Laboratories Inc., Research Triangle Park, NC). Bulk density
measurements for litter samples were done by the ring method. Steel rings (5-cm
diam.) were filled with litter and the depth and fresh weight were measured. Samples
were dried for 48 h at 45°C and reweighed. Bulk density is then calculated as the
weight of dry litter (g) for a given volume (cm^3). Soil and litter pH were measured by
using a combination glass electrode with a soil:water ratio of 1:2 and a litter:water
ratio of 1:10.

Soil respiration

At each of the seven sample dates, a total of eight microcosms were chosen for soil respiration measurement at each site. Soil CO_2 efflux was measured using the soda lime technique (Edwards, 1982). The CO_2 emitted from the soil was absorbed for 24 h in 5 g of pre-dried (24 h at 105°C) soda lime. The chambers were sealed by using a PVC collar attached to an inverted PVC column with a glued lid. Eight controls were used to correct for CO_2 absorption during transport and were opened briefly to simulate the time needed to place soda-lime vessels under the chamber. Flux rates were calculated from the mass increase in soda lime (after drying at 105°C for 24 h) multiplied by 1.69 to correct for the chemical release of water when soda lime reacts with CO_2 (Grogan, 1998).

PLFA analysis

PLFAs were prepared via three steps, i.e., lipid extraction using a Bligh and Dyer technique (1959), separation of phospholipids by solid-phase extraction columns, and a saponification/methylation of esterified fatty acids in the neutral and phospholipid fractions.

(i) Lipid extraction. Approximately 1 g (oven-dry equivalent) of soil was weighed into 25-mL glass Kimax centrifuge tubes equipped with Teflon-lined screw caps. Enough water and 100 mM phosphate buffer (1/2 $0.1M$ KH_2PO_4 and ½ $0.1M$ K_2HPO_4) were added to make 50 mM buffer and mixed with chloroform and methanol at a ratio of 0.8:1:2. The solution was shaken for 2 h and allowed to stand overnight. Lipids were extracted following centrifuging (7649 g for 6 min) and filtering the supernatant through Whatman no.2 filter papers. This step was repeated twice with additional chloroform and methanol additions (holding the 1:2 ratio constant). The chloroform-methanol-buffer phases were separated with addition of 7 mL of $2M$ NaCl and the chloroform phase (bottom) was removed and immediately dried under a stream of N_2 (99.99 % pure) at 37°C.

(ii) Lipid separation. The dried lipids were resuspended in chloroform and added to solid-phase extraction columns containing 500 mg of silica (Supelco, Inc., Bellefonte, Pa). Neutral lipids (NLFAs), glycolipids, and phospholipids (PLFAs)

were sequentially isolated by adding chloroform, acetone, and methanol, respectively. The phospholipid fraction was immediately dried under N_2 at 37°C and stored at -20°C (under N_2) until methylation and GC analysis.

(iii) Saponification/methylation of PLFAs. Dried PLFAs were resuspended in 1 mL of 1:1 methanol:toluene and 1 mL of 0.2M methanolic KOH, mixed and heated in a water bath at 37°C for a minimum of 15 min. After cooling to room temperature, each sample received 1 mL of hexane, 0.3 mL of 1M acetic acid, and 2 mL of water. The hexane (upper) phase containing the fatty acid methyl esters (FAMEs) was removed. This step was repeated two additional times and the hexane solution was dried under N_2 at 37°C. FAMEs were redissolved in hexane and transferred to 2-mL amber GC vials with 100 μL inserts to give a final total volume of 80 μL.

GC-FID analysis

FAMEs were separated and quantified by gas chromatography using a Hewlett Packard 5890 Series II (Palo Alto, CA) equipped with a 25-m Agilent Ultra 2 column (internal diameter, 0.20 mm; film thickness, 0.33 μm) and a flame ionization detector. The carrier gas was He, and the oven temperature was ramped from 120 to 260°C at a rate of 5°C per min with a 5-min hold at 260°C. Individual fatty acids were identified relative to several standards: 37 FAMEs mixture (FAME 37 47885-4; Supelco, Inc), 24 bacterial FAMEs mixture (P-BAME 24 47080-U; Supelco, Inc.), and MIDI standards (Microbial ID, Inc.). Quantification of FAMES was accomplished by using varying concentrations of tridecanoic FAME (Supelco, Inc.). Use of this standard allows the peak areas to be converted to a molar basis for the PLFA in question. A total of 45 fatty acids were identified but only 35 fatty acids were used in this analysis. The other 10 fatty acid peaks were deleted because they were absent in the majority of sites and those sites at which they were present made up less than 0.5 % of the total relative abundance.

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 (ω) of the molecule. Cis and trans

geometry are indicated by the suffixes "c" and "t", respectively. 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. Table 2.2 lists the indicator fatty acid associated with five microbial groups. A sum of the fatty acids indicative of Gram-positive bacteria (GM+), Gram-negative bacteria (GM-), actinomycetes (actinos), plus the five additional fatty acids listed are used as a measure of total bacterial biomass (Frostegard and Bååth, 1996). The $18:2\omega6,9$ 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 (Bååth, 2003). It is not to be confused with a measure of absolute biomass values because at the present time, no conversion factors from PLFA concentration to actual biomass have been established.

Table 2.2. Taxonomic microbial groups with the PLFA group and specific PLFA markers used to designate such groups.

Taxonomic group	PLFA group	Specific PLFA markers	Reference
Bacteria	multiple groups	sum of i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, $16:1\omega7$, $18:1\omega7$, and $17:1\omega9$	Frostegard and Bååth, 1996
Gm +	branched PLFAs	sum of i15:0, a15:0, i16:0, i17:0, and a17:0	O'Leary, 1988
Gm -	cyclopropyl and mono PLFAs	sum of cy17:0, $16:1\omega7$, $18:1\omega7$, and $17:1\omega9$	Wilkinson, 1988
Actinos	10Me-PLFAs	sum of 10Me16:0, 10Me17:0, and 10Me18:0	Kroppenstedt, 1985
Fungi	polyunsaturated PLFAs	$18:2\omega6,9$	Federle et al., 1986; Frostegard and Bååth, 1996
F:B ratio	multiple groups	Fungi/Bacteria	Federle et al., 1986; Frostegard and Bååth, 1996
Protozoan	polyunsaturated PLFAs	sum of $20:2\omega6$ and $20:4\omega6$	Ringelberg et al., 1997

Gm + = Gram-positive bacteria
Gm - = Gram-negative bacteria
Actinos = actinomycetes

Statistical Analysis

All statistical analyses were conducted using SAS (version 8) and PC-ORD (version 4) statistical software (McCune and Mefford, 1999). Each PLFA value reported is the average of four samples collected at each site (2 cores/sub-replicate plot * 2 sub-replicate plots/site). The mean of each PLFA for each site (2 field replicates per site) were used after transformation for NMS plots and as mol % for ANOVA analyses. Data used in the NMS plots were transformed using sample unit totals to represent relative abundance of each PLFA (mol % of total PLFA). A monotonic transformation (square root) of all PLFAs was also conducted in order 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 (McCune and Mefford, 1999).

The data were examined using nonmetric multidimensional scaling (NMS) with the Sorensen distance measure (Kruskal, 1964; Mather, 1976). The goal of this method is to minimize stress, or the departure from monotonicity, within the reduced ordination space. The Sorensen distance measure was chosen because it is less sensitive to outliers than other distance measures such as Chi-square distances. NMS analyses were run using the 'slow and thorough' setting in the autopilot mode. Random starting configurations were seeded by the computer's clock. Forty runs with real data were run and Monte Carlo simulations were conducted using 50 randomized runs and a stability criterion of 0.0001.

The number of dimensions chosen in the model was assessed by comparing the NMS runs with the real data to Monte Carlo simulations. The proportion of variation represented by each axis was assessed by calculating the coefficient of determination (r^2) between distances in the ordination space and Sorensen distances in the original distance matrix.

The NMS scores from each axis were then loaded into SAS (v8) using a repeated measures program to determine if stand age, sample date, or their interaction was significantly different ($p \leq 0.05$) along a given axis. In order to determine how soil microbial communities differed between stand age and the significant sample

dates, repeated measures ANOVAs were conducted on each of the 35 PLFAs, as well as the biomarkers and stress indicators listed in Tables 2.2 and 2.3. Tukey's Honestly Significantly Different (HSD) tests were used to adjust p-values for pair-wise comparisons to control for the experiment-wise error rate with significant differences defined at $p \leq 0.05$.

Table 2.3. Stress indicator categories and the PLFAs used for each. PLFAs listed are summed for each category and are based on mol % of total PLFA.

Stress indicator*	PLFAs used
SAT	14:0, 15:0, 16:0, 17:0, 18:0
MONO	i16:1, 16:1ω11, 16:1ω7, 16:1ω5, 17:1ω9, a17:1, 17:1ω8, 18:1ω9, 18:1ω7, 18:1ω5, 11Me18:1ω7
S:M ratio	SAT/MONO PLFAs
cy17:pre ratio	cy17:0/16:1ω7
cy19:pre ratio	cy19:0/18:1ω7

*SAT = saturated PLFAs; Mono = monounsaturated PLFAs; S:M = saturated/monounsaturated; cy17:0: pre = ratio of cy17:0 to it's precursor; cy19:0: pre = ratio of cy19:0 to it's precursor.

Total PLFA (PLFA$_{tot}$) concentration (nmol PLFA g^{-1} C) was used as an index of the total viable microbial biomass. The sum of PLFAs characteristic of general bacteria, GM+ bacteria, GM- bacteria, actinomycetes, fungi, and the fungal to bacteria ratio (F:B) were used as broad taxonomic microbial groupings (Table 2.2). Lastly, the sum of saturated (SAT) and monounsaturated (MONO) PLFAs as well as their ratio were used in conjunction with the ratios of cy17:0/16:1ω7 and cy19:0/18:1ω7 as indicators of physiological or nutritional stress in bacterial communities (Kieft et al., 1997; Bossio and Scow, 1998) (Table 2.3). All of these groups were also evaluated using the same statistical procedure as individual PLFAs. Total C was measured on each 0-5 cm soil sample analyzed for PLFAs on a LECO C-144 analyzer in order to express each fatty acid on a per gram C basis. Total bacteria and fungal PLFAs were calculated using both absolute (nmol g^{-1} C) and relative abundances (mol % of PLFA$_{tot}$). All other groups were calculated using mol % of PLFA$_{tot}$. Use of these broad groupings is considered a more reliable result than analysis of individual PLFAs as indicator species because they provide a more robust analysis to evaluate and

interpret SMC changes in subunit groups (Zelles, 1999). However, these groupings should be considered approximate because the use of PLFAs as biomarkers to identify specific microbial types is not always unique to that particular group, especially in environmental samples containing diverse microorganisms (Zelles, 1997).

Results

Soil temperature and moisture

Hourly soil temperature and soil moisture (v/v) data for 2003 and 2004 OG1 and CC8 sites are presented in Figures 2.2 and 2.3. In general, soil temperatures fluctuated more in soils from the CC8 site compared to soils from OG1. Samples from OG1 typically had higher soil temperatures from spring until late fall but lower temperatures during most of the summer. Soil moisture was consistently greater at OG1 compared to CC8 throughout the 22-month study period, especially in the summer months; soil moisture content fluctuated more widely in CC8 than in OG1 sites.

To coincide with the seven sample dates, soil moisture and temperature measurements were averaged over the previous three days (Table 2.4). Averaged across stand age, the coldest sample month was November 2003 (3.4°C) and the hottest 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 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 OG1 and is reflected in the spike in soil moisture values collected on September 1, 2004. This was unfortunate because we intended to collect samples during the same hot, dry period of the previous year (August 31, 2003) when only 2.6 mm of rain was recorded between July 14, 2003 and September 6, 2003, and soil moisture values were low. We also attempted to collect samples under the same winter conditions of the December 16, 2002 sample date. During this sampling, no snow was present but between 0 and 5 cm was on the ground
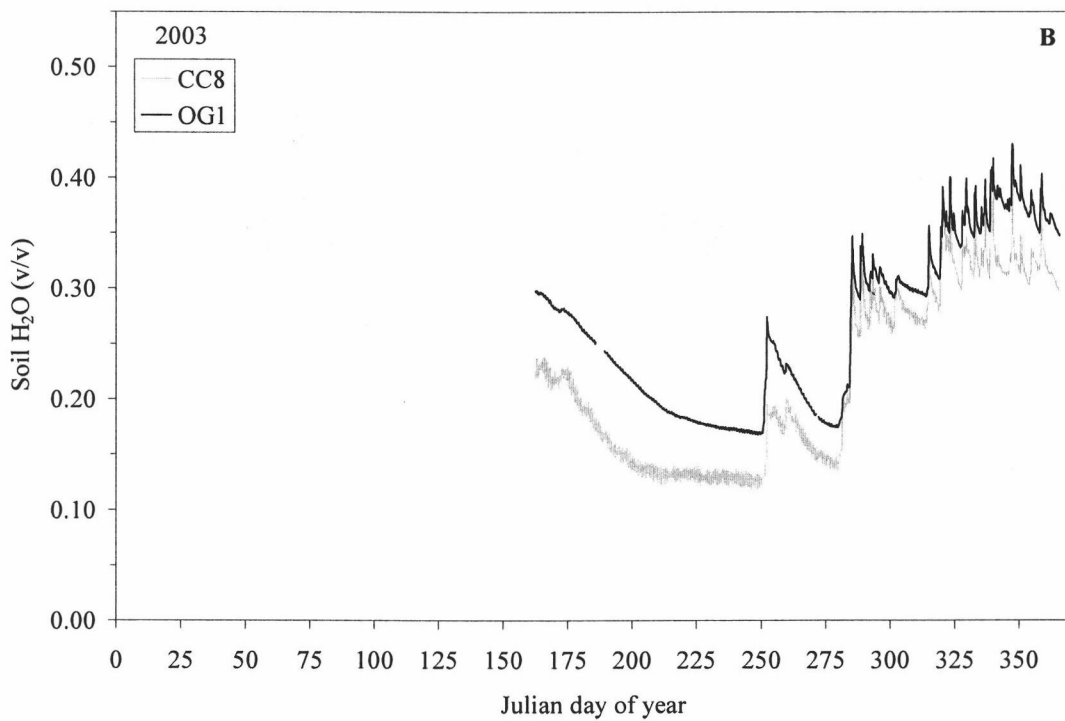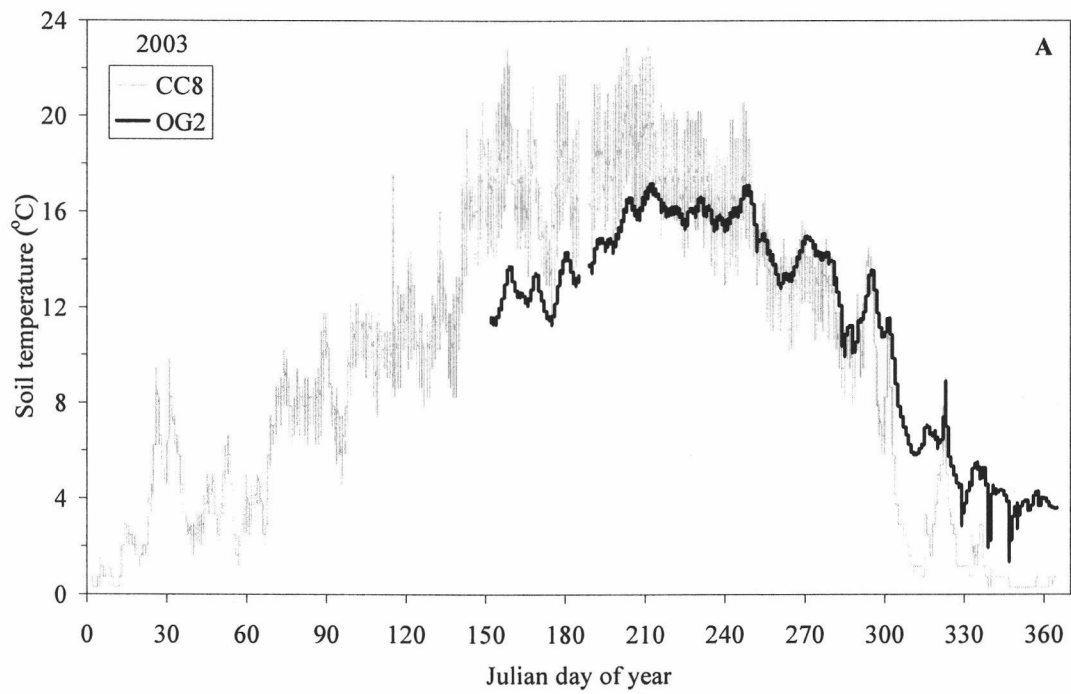
Figure 2.2. Soil temperature (°C) (A) and moisture (v/v) (B) measurements for CC8 and OG1 sites in 2003.
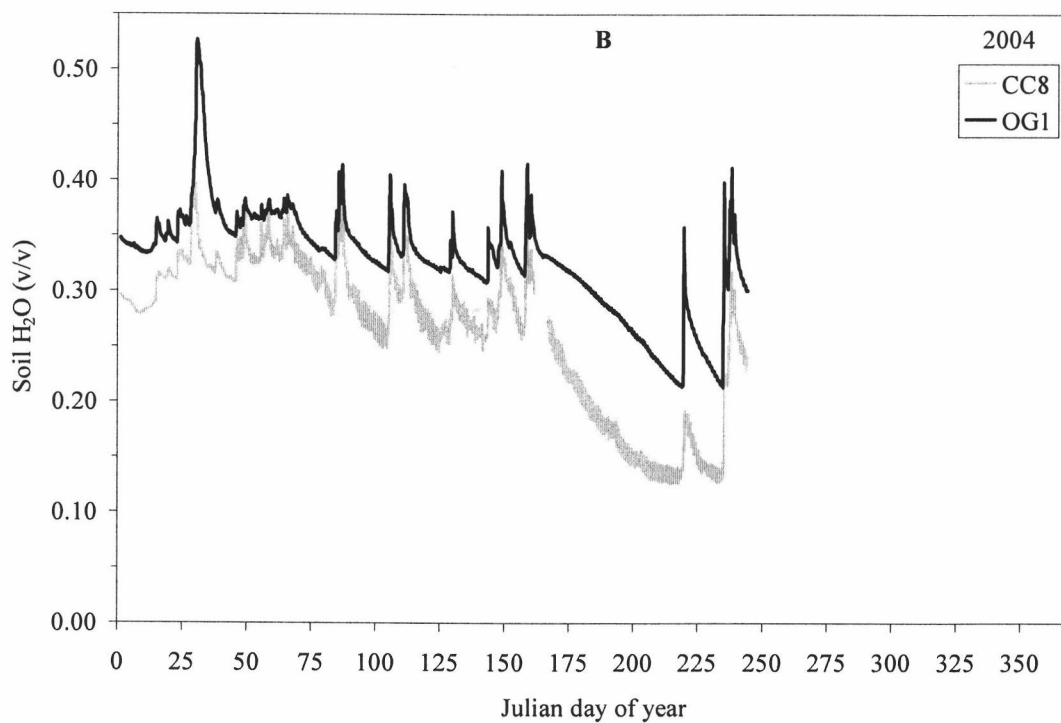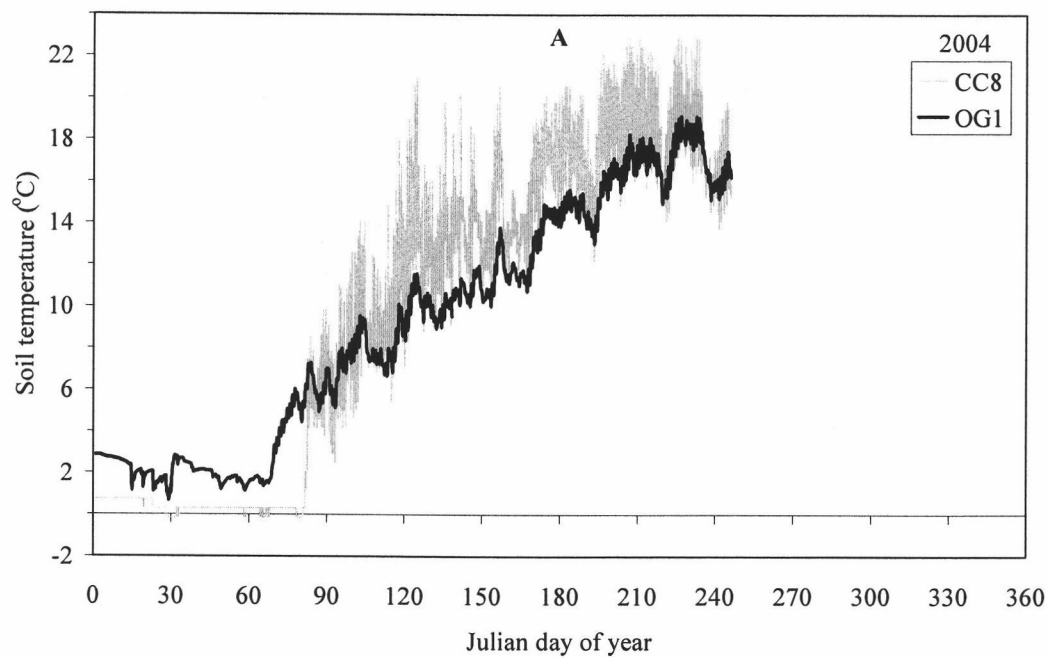
Figure 2.3. Soil temperature (°C) (A) and moisture (v/v) (B) measurements for CC8 and OG1 sites in 2004.

on the November 2003 sample period (mean snowpack for the month of December

is typically about 125 mm (Shaw et al., 2004).

Table 2.4. Soil temperature (C) and moisture (%) values averaged 3d prior to each of the sample dates for this study.

Sample date	Soil temp (°C) OG1	CC8	Soil moisture (v/v) OG1	CC8
Dec-02	7.3	4.5	n/a	n/a
Apr-03	11.6	10.2	29.4	30.2
Jun-03	13.2	16.2	28.2	21.7
Aug-03	16.2	16.0	17.1	12.7
Nov-03	4.1	2.7	34.3	30.9
Jun-04	11.4	13.3	33.4	30.6
Sep-04	16.6	16.7	30.3	24.2

Soil temperature data for 2003 OG2 and CC25 sites fluctuated less than

temperature measurements between OG1 and CC8 (Figure 2.4). Averaged across

stand age, the coldest sample months were December 2002 (4.9°C) and November

2003 (5.0°C) and the hottest month was September 2004 (14.7°C) (Table 2.5). Soils

in August 2003 were between 7.9° and 9.8°C warmer than samples collected in

December 2002 and in April and November 2003, respectively. Temperature trends

for samples from OG2 and CC25 closely match those measured for OG1 and CC8

samples (Table 2.4) although in general, OG2/CC25 sites were slightly cooler (Table

2.5).

Soil respiration rates

Averaged over the seven sample dates, soil respiration rates for OG1 and CC8

sites were 17.2 (SE ± 2.6) and 14.2 (SE ± 2.1) g CO_2 m^{-2} d^{-1}, respectively. There were

no significant differences in soil respiration rates between OG1 and CC8 (Figure 2.5).

There was a significant date effect, with December 2002 samples having significantly

lower respiration values than those collected in June 2003 and 2004, and September

2004. Respiration rates tended to increase from December 2002 (3.1 g CO_2 m^{-2} d^{-1})
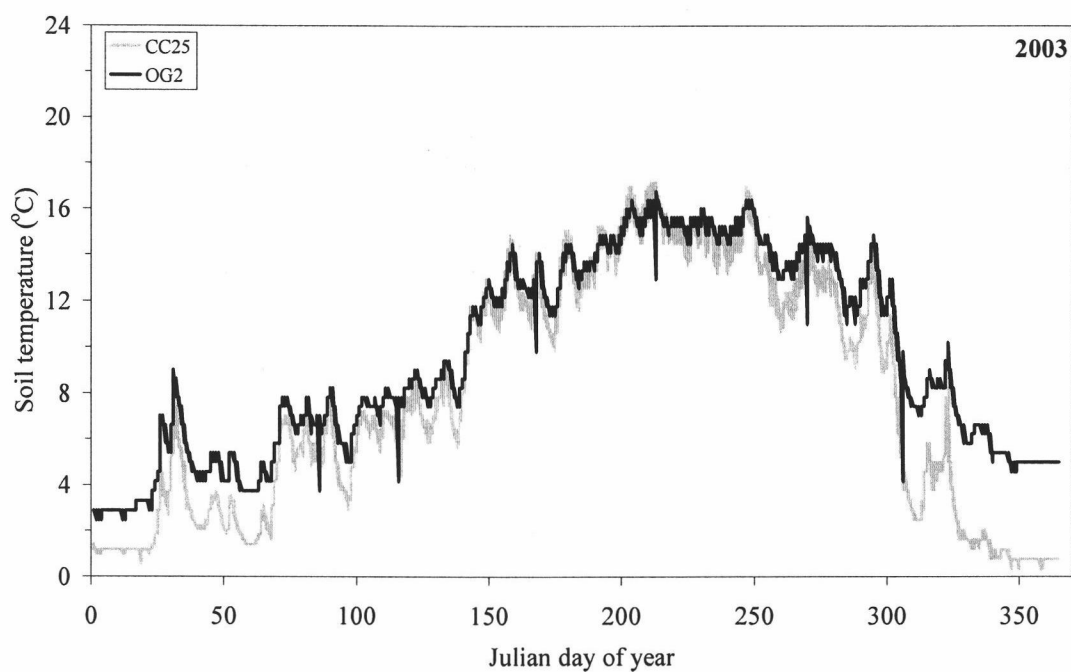
Figure 2.4. Soil temperature measurements (°C) for CC25 and OG2 sites in 2003.

Table 2.5. Soil temperature (C) and moisture (%) values for OG2 and CC25 sites averaged 3d prior to 2003 sample dates.

	Soil temp (°C)	
Sample date	OG2	CC25
Dec-02	5.5	4.3
Apr-03	7.8	6.6
Jun-03	13.1	12.4
Aug-03	15.0	14.3
Nov-03	7.3	2.7
Jun-04	N/A	N/A
Sep-04	N/A	N/A
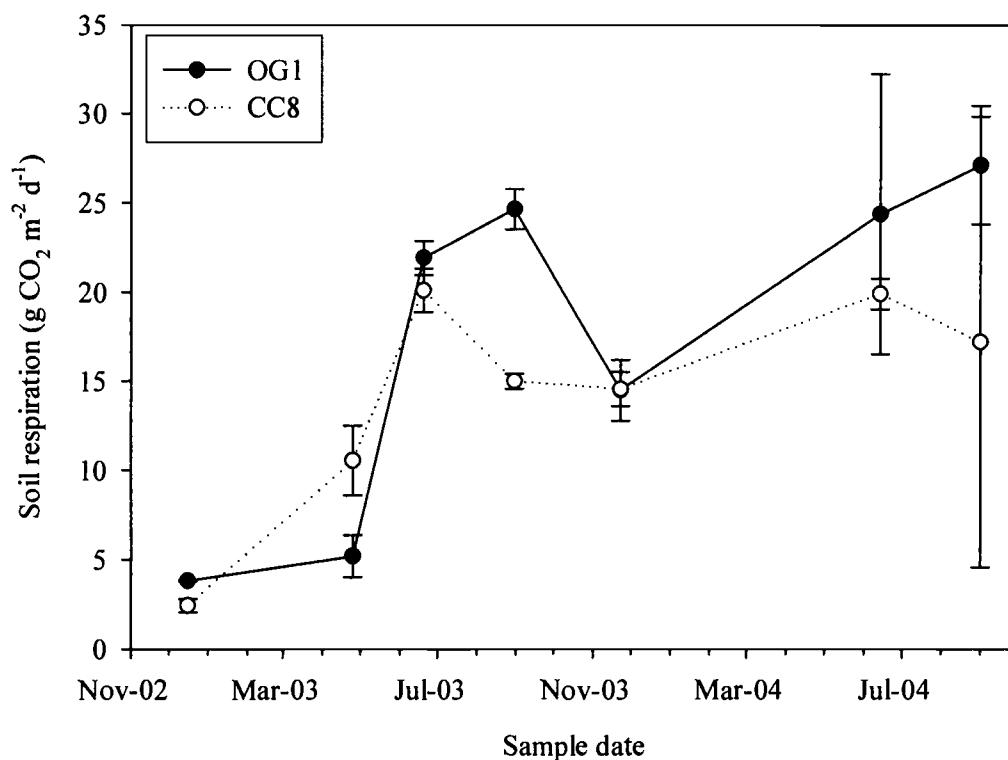
Figure 2.5. Soil respiration values (g CO_2 m^{-2} d^{-1}) for OG1 and CC8 sites over the seven sampling dates. Error bars represent standard error (n = 2).

through June 2003 (21.0 g CO_2 m^{-2} d^{-}1) and then decrease in August (19.8 g CO_2 m^{-2} d^{-1}) and November 2003 (14.5 g CO_2 m^{-2} d^{-1}). Similar rates were measured between June 2003 and 2004 as well as between August 2003 and September 2004 although September rates tended to be slightly higher (about 2 g CO_2 m^{-2} d^{-1} greater).

Averaged over the seven sample dates, soil respiration rates for OG2 and CC25 sites were 14.1 and 14.2 g CO_2 m^{-2} d^{-1}, respectively. There were no significant differences in soil respiration values between OG2 and CC25 (Figure 2.6). There was a significant date effect, with December 2002 and April 2003 samples having significantly lower respiration rates than did those collected in June and August 2003 and June and September 2004. Respiration rates tended to increase from December 2002 (3.4 g CO_2 m^{-2} d^{-1}) through August 2003 (22.0 g CO_2 m^{-2} d^{-1}) and then decrease
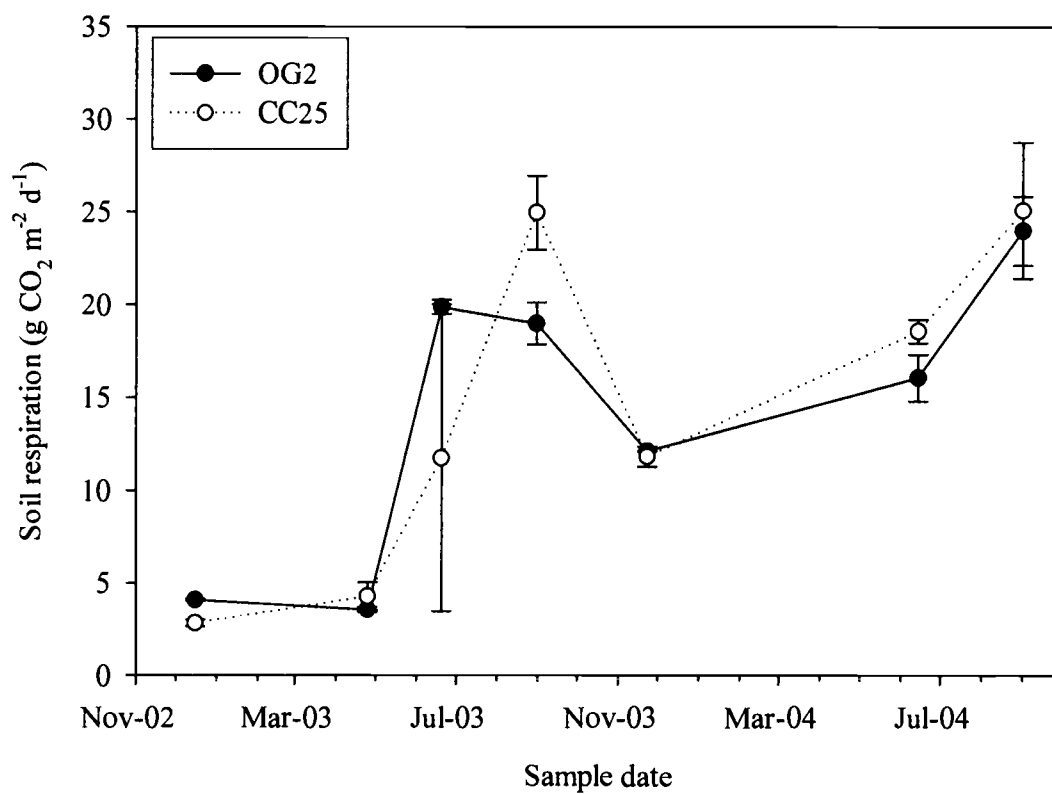
Figure 2.6. Soil respiration values (g CO_2 m^{-2} d^{-1}) for OG2 and CC25 sites over the seven sampling dates. Error bars represent standard error (n = 2).

in November 2003 (12.0 g CO_2 m^{-2} d^{-1}). Similar rates were measured between June 2003 and 2004 as well as between August 2003 and September 2004.

Total PLFA, bacterial, and fungal PLFA concentrations

        Total PLFA (PLFA$_{tot}$) is considered to be an indicator of the active living biomass and it varied throughout the 22-month sample period for both study groups (Figures 2.7 and 2.8). The lowest PLFA$_{tot}$ concentration was found for samples collected in August 2003 and highest in June 2003. Averaged across sample dates, there was a significant difference in PLFA$_{tot}$ among stand ages for OG1 (2245 nmol g^{-1} C) and CC8 (1405 nmol g^{-1} C). PLFA$_{tot}$ also significantly differed among sample
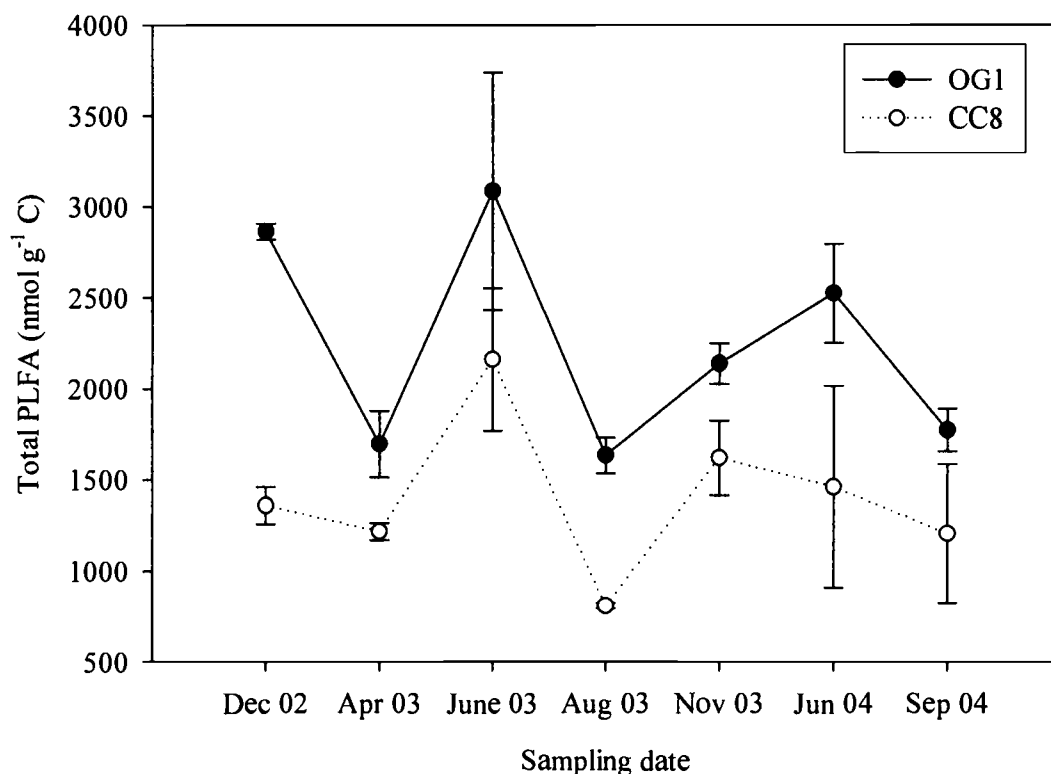
Figure 2.7. Total PLFA (nmol g^{-1} C) for OG1 and CC8 stands at each of the seven sample dates. Error bars represent standard error (n = 2).

dates when averaged across OG1 and CC8. Mean PLFA$_{tot}$ in June 2003 (2622 nmol g^{-1} C) was significantly greater than the mean amounts in April (1457 nmol g^{-1} C), August (1221 nmol g^{-1} C), and September 2004 (1491 nmol g^{-1} C).

The lowest PLFA$_{tot}$ concentration for OG2 and CC25 samples was found for samples collected in August 2003 (1010 nmol g^{-1}C) and the highest values were found in June 2003 (2601 nmol g^{-1}C). Averaged across sample dates, there was no significant difference in PLFA$_{tot}$ among stand age but PLFA$_{tot}$ was significantly different among sample dates when averaged across OG2 and CC25. Mean PLFA$_{tot}$ concentration in August (1010 nmol g^{-1} C) was significantly less than the mean concentration in December 2002 (2286 nmol g^{-1} C), June 2003 (2601 nmol g^{-1} C) and
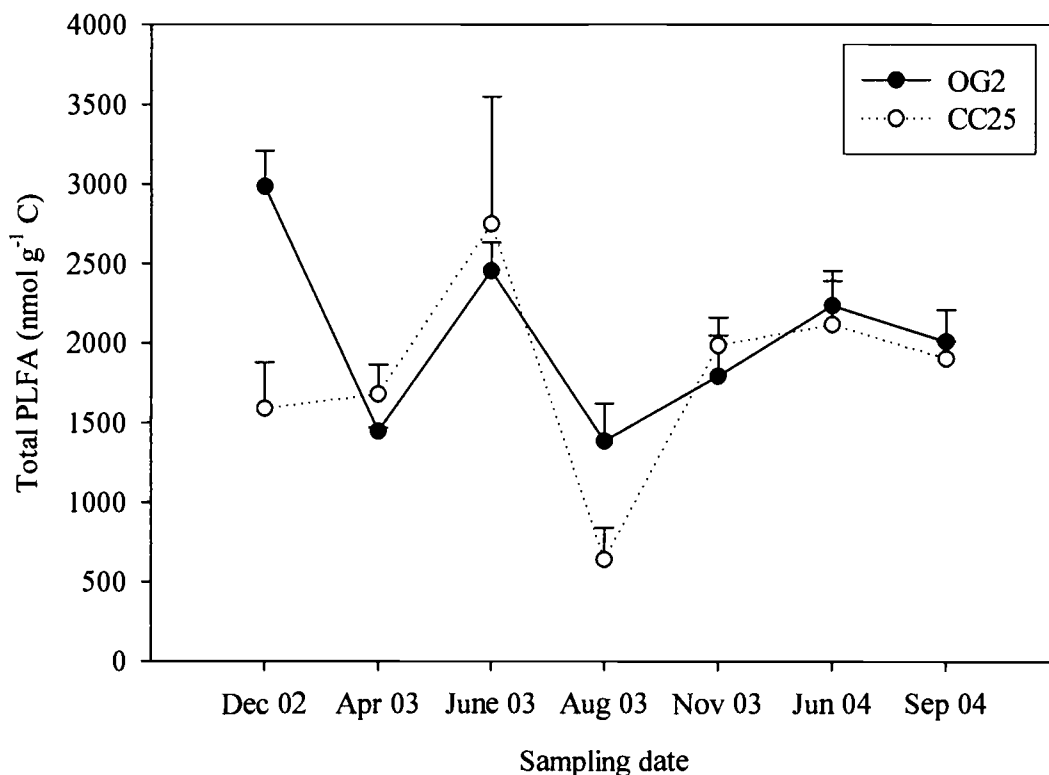
Figure 2.8. Total PLFA (nmol g^{-1} C) for OG2 and CC25 stands at each of the seven sample dates. Error bars represent standard error (n = 2).

June 2004 (2175 nmol g^{-1} C). Total PLFA measured in samples collected in June 2003 was also significantly greater than mean total amount measured in April 2003 (1561 nmol g^{-1} C).

Total absolute bacterial and fungal PLFA concentrations for OG1 and CC8 followed similar patterns as PLFA$_{tot}$ throughout the sample period. Averaged across sample dates, there was a significant difference (p = 0.036) in total bacterial PLFA among stand age for OG1 (734 nmol g^{-1} C) and CC8 (459 nmol g^{-1} C) plots (Figure 2.9). Total bacterial PLFA also significantly differed among sample dates when averaged across stand age. Mean amount of bacterial PLFA in June 2003 (860 nmol g^{-1} C) was greater than the mean amounts in August (436 nmol g^{-1} C), and September 2004 (480 nmol g^{-1} C).
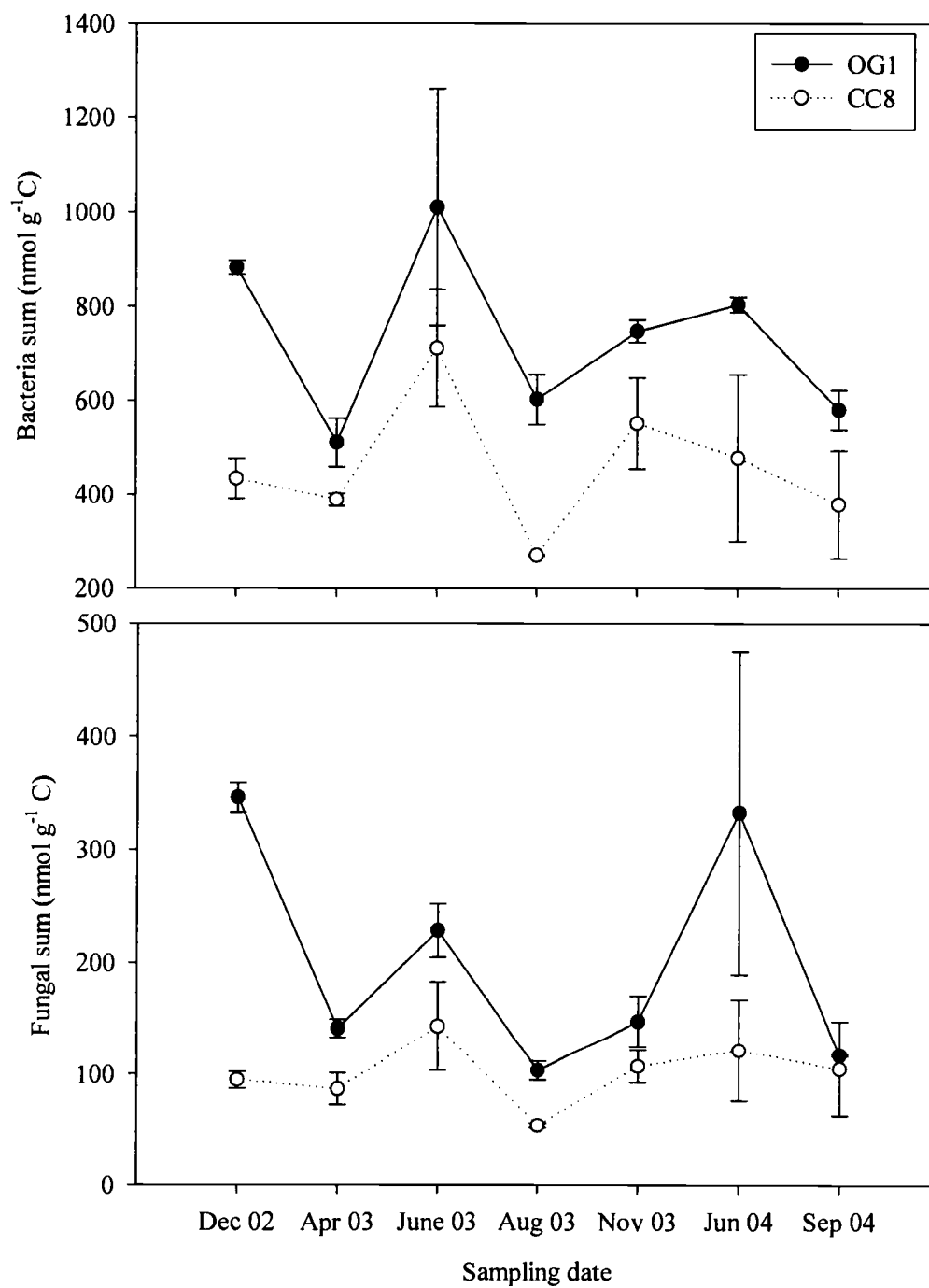
Figure 2.9. Sum of bacterial and fungal PLFAs (nmol g^{-1} C) for OG1 and CC8 stands at each of the seven sample dates. Error bars represent standard error (n = 2).

There was a significant effect of stand age ($p = 0.05$) on total fungal PLFA concentration with OG1 samples containing greater fungal biomass (202 nmol g^{-1} C) than CC8 samples (102 nmol g^{-1} C). The main effect, date, was significant at $p = 0.032$; however, pair-wise comparisons using Tukey's HSD adjustment revealed no significant effects. The F:B ratio was not significantly different between OG1 and CC8 or sample dates (data not shown).

Total absolute bacterial and fungal PLFA concentrations for OG2 and CC25 also followed similar patterns as PLFA$_{tot}$ throughout the sample period. Total bacterial PLFA concentration significantly differed among sample dates when averaged across stand age (Figure 2.10). Mean amount of bacterial PLFA in June 2003 (820 μg g^{-1} C) was significantly greater than the mean amounts in August (362 nmol g^{-1} C), and April 2003 (453 nmol g^{-1} C).

There was a significant interaction effect of age*date on total fungal PLFA concentration ($p = 0.0006$). Fungal concentrations (nmol g^{-1} C) were significantly higher in OG2 samples collected in December 2002 compared to all other sample dates. In December 2002, OG2 samples also had significantly greater fungal concentrations (436 nmol g^{-1} C) than CC25 samples (129 nmol g^{-1} C). Within the CC25 stand, April 2003 samples were significantly greater than those collected in August 2003. Similar to the OG1/CC8 results, the fungal:bacteria ratio was not significantly different between OG2 and CC25 or sample dates (data not shown).

Effects of stand age and sample date on NMS scores

The NMS scores of PLFA profiles (as square root of mol %) for OG1 and CC8 samples collected at all seven sample dates were well separated on the basis of stand age (Figure 2.11) and sample date (Figure 2.12). Using repeated measures ANOVA of NMS scores for axes one and three, stand age significantly influenced NMS scores along axis one ($p = 0.016$). NMS scores were significantly separated on the basis of sample date along axis one and axis three ($p \leq 0.0001$). August samples for both stands were well separated from all other samples (Figure 2.11). A plot of PLFA samples averaged across stand age revealed a strong separation between
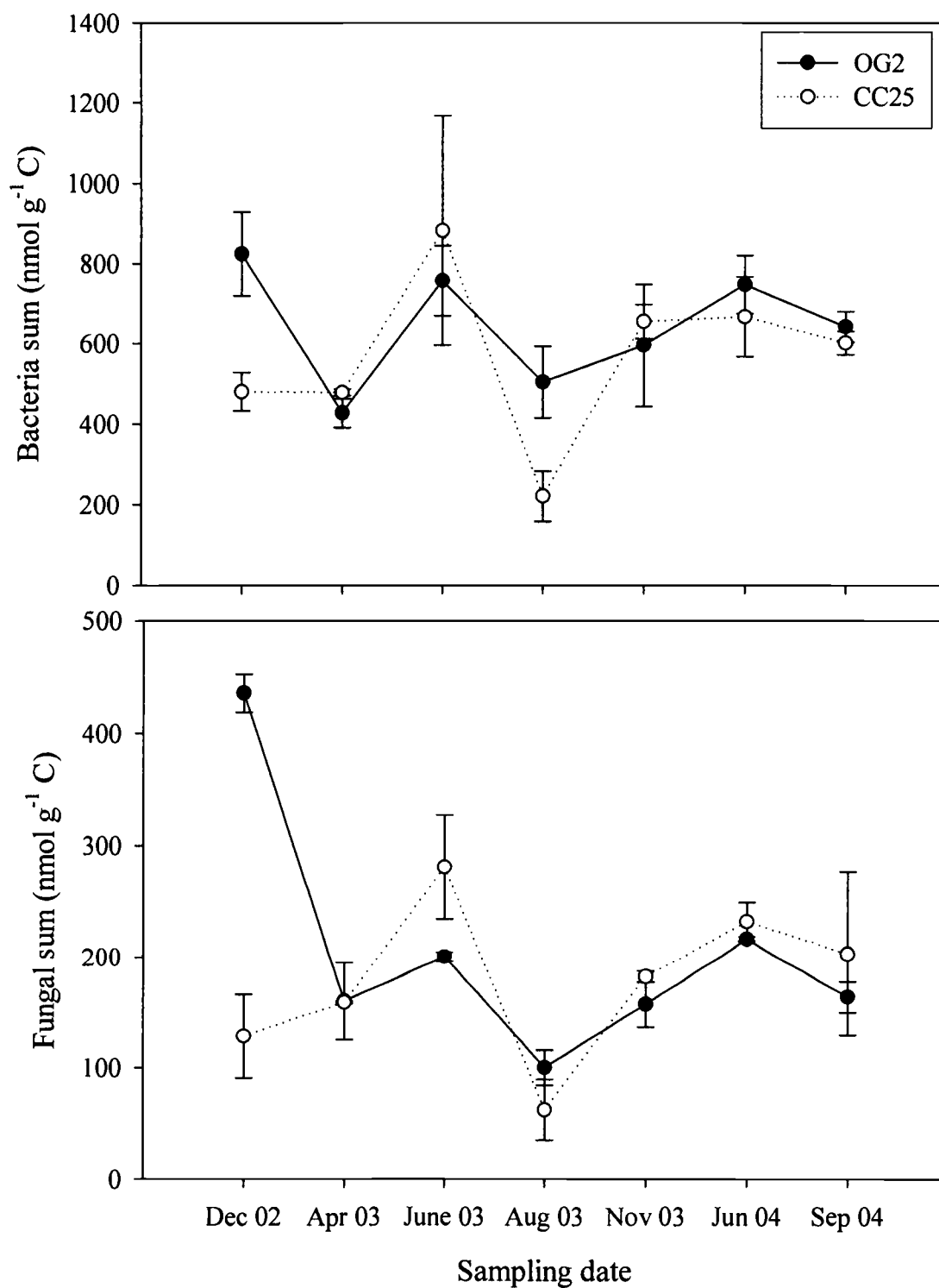
Figure 2.10. Sum of bacterial and fungal PLFAs (nmol g^{-1}C) for OG2 and CC25 stands at each of the seven sample dates. Error bars represent standard error (n = 2).
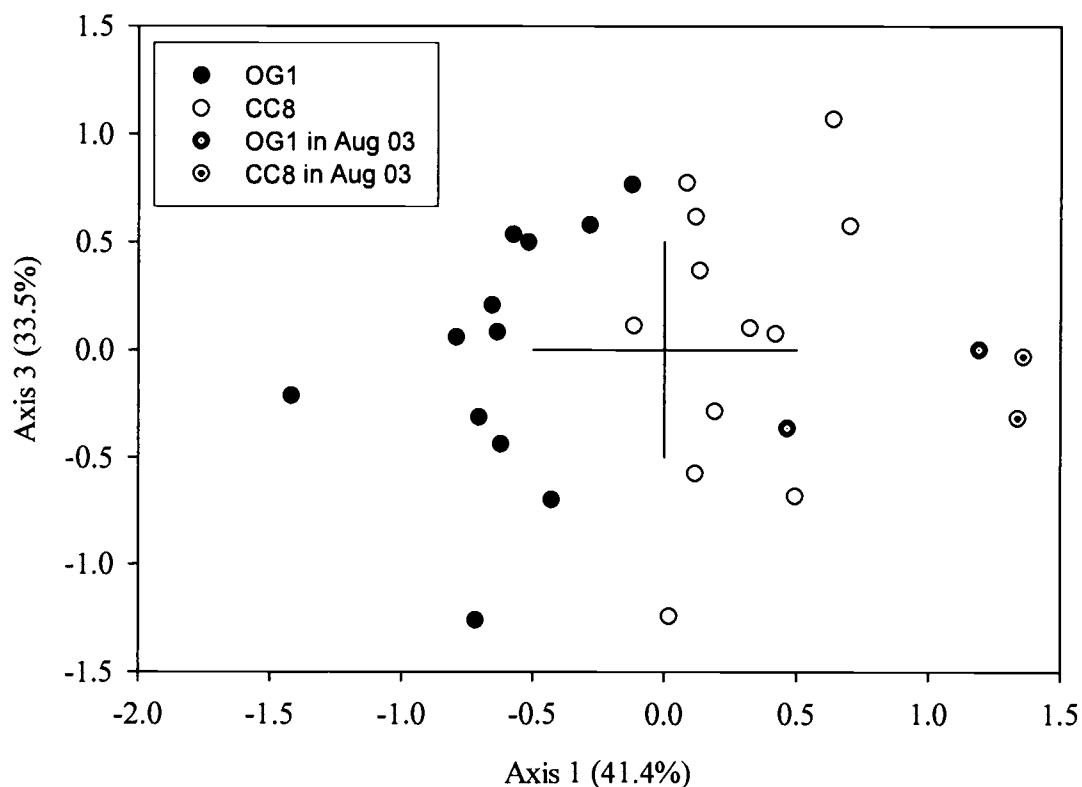
Figure 2.11. NMS plot of PLFA profiles (as square root of mol %) for OG1 and CC8 samples collected at all seven sample dates.

August samples and all other sample dates (Figure 2.12). Repeated measures ANOVA analysis indicated this separation was significant ($p \leq 0.0001$). There also was a significant interaction between age and date along axis two ($p = 0.010$). Along axis one, samples from the August 2003 sample date were significantly different from all other sample dates. Along axis three, samples from September 2004 and June 2004 were significantly different from those collected in April, June, and November 2003. Additionally, November 2003 samples were significantly different from samples collected in December 2002 and April 2003. There were no significant differences between stand age or sample date along axis two so it was not included in the figures.
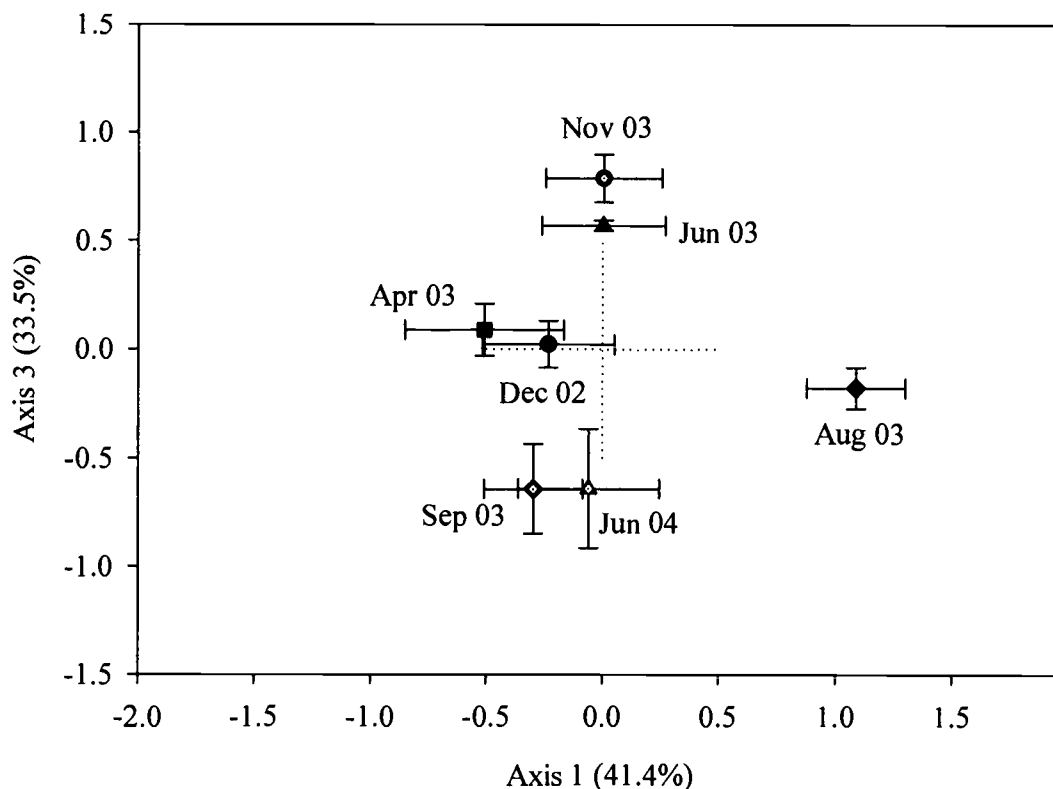
Figure 2.12. NMS plot of PLFA profiles (as square root mol %) averaged across both OG1 and CC8 stands for each of the seven sample dates. Values are the mean molar % of PLFAs. Error bars represent standard error (n = 4).

The NMS scores from OG2 and CC25 PLFA data were significantly separated along axis one on the basis date (p = 0.0008), but not based on stand age. Along axis one, samples from the August 2003 sample date were significantly different from those collected in April, June, and November 2003 (Figure 2.13). There was a significant interaction between age and date along axis two (p = 0.01) but the main effects of date or age or their interaction were not significant along axis three.
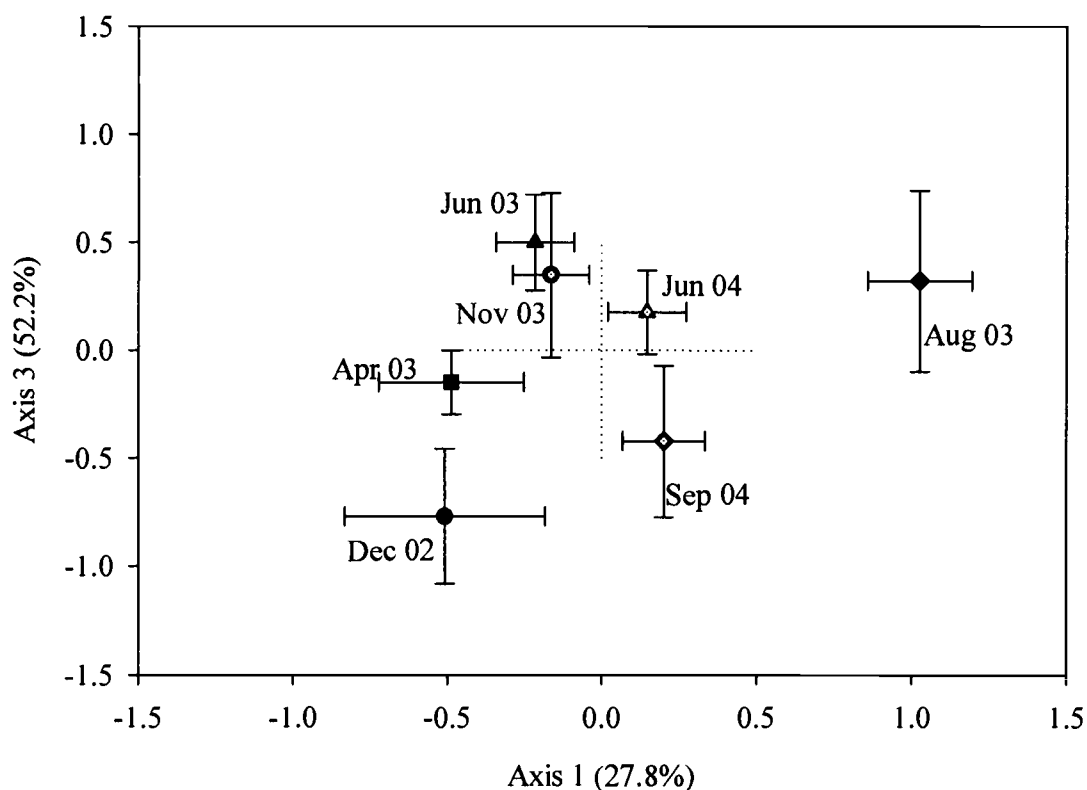
Figure 2.13. NMS plot for OG2 vs. CC25 stands averaged across stands for each of the seven sample dates. Error bars represent standard error (n = 4).

Stand age effect on individual PLFAs, biomarkers, and stress indicators

Individual PLFAs, biomarkers, and stress indicators were subjected to repeated measures ANOVA in order to determine which of these PLFAs or groups of PLFAs were different between OG1 and CC8. Samples from the CC8 stands were significantly greater in the PLFAs 16:0, 17:1ω9, a17:1, i17:0, a17:0, and 10Me16:0, whereas samples from the OG1 stands were significantly greater than CC8 samples only in the monounsaturated PLFA, 17:1ω8 (Table 2.6). None of the group biomarkers were significantly different between stand ages; however, significant differences existed for the stress indicators, SAT and S:M (Table 2.7). A significantly greater proportion of SAT PLFAs was present in the CC8 samples (15.8%) compared

to OG1 samples (14.4%). Correspondingly, a significantly greater S:M ratio was calculated for CC8 samples (0.43) compared to OG1 samples (0.38).

Table 2.6. PLFA profiles from OG1 and CC8 plots averaged over the seven sample dates. Values are the mean mol % (SE in parentheses, n = 14).

PLFA	OG1		CC8		Significance
			Stand age		
Saturated <18C					
14:0	0.98	(0.11)	0.79	(0.08)	NS
15:0	0.84	(0.05)	0.68	(0.03)	NS
16:0	10.19	(0.28)	11.72	(0.36)	**
17:0	0.55	(0.03)	0.49	(0.04)	NS
18:0	1.86	(0.08)	2.06	(0.08)	NS
Saturated >18C					
20:0	1.74	(0.09)	1.55	(0.10)	NS
21:0	0.42	(0.03)	0.51	(0.04)	NS
22:0	1.74	(0.11)	1.70	(0.08)	NS
Monounsaturated					
i16:1	0.55	(0.05)	0.73	(0.04)	NS
16:1ω11	1.34	(0.04)	1.31	(0.05)	NS
16:1ω7	6.06	(0.22)	6.01	(0.23)	NS
16:1ω5	2.19	(0.20)	2.22	(0.22)	NS
17:1ω9	1.09	(0.06)	1.52	(0.05)	**
a17:1	0.73	(0.03)	0.91	(0.03)	*
17:1ω8	4.35	(0.47)	3.16	(0.34)	*
18:1ω9	9.05	(0.23)	9.30	(0.24)	NS
18:1ω7	10.54	(0.30)	9.68	(0.32)	NS
18:1ω5	1.11	(0.05)	1.09	(0.03)	NS
18:1ω11	0.93	(0.02)	0.89	(0.02)	NS
Cyclopropyl					
cy17:0	3.46	(0.10)	3.50	(0.12)	NS
cy19:0	7.55	(0.16)	7.04	(0.23)	NS
Branched					
i15:0	4.36	(0.27)	4.59	(0.32)	NS
a15:0	1.89	(0.13)	2.20	(0.14)	NS
i16:0	2.10	(0.13)	2.40	(0.13)	NS
i17:0	0.88	(0.05)	1.13	(0.04)	*
a17:0	1.12	(0.03)	1.34	(0.04)	*
10Me					
10Me16:0	3.30	(0.15)	4.04	(0.14)	*
10Me17:0	0.57	(0.03)	0.61	(0.03)	NS
10Me18:0	1.54	(0.06)	1.39	(0.08)	NS
Hydroxy					
16:1 2OH	0.80	(0.05)	1.07	(0.04)	NS
18:0 2OH	1.38	(0.11)	1.24	(0.10)	NS
Polyunsaturated					
18:3w6	0.80	(0.04)	1.10	(0.12)	NS
18:2w6	8.93	(0.77)	7.33	(0.23)	NS
20:4w6	0.74	(0.08)	0.56	(0.08)	NS

* = $p \leq 0.05$
** = $p \leq 0.01$

Table 2.7. Sum of PLFAs used for taxonomic groups and stress indicators from OG1 and CC8 plots averaged over the seven sample dates. Values are the mean molar % of PLFAs (SE in parentheses, n = 14). Abbreviations are defined in Tables 2.2 (Taxonomic group) and 2.3 (Stress indicators).

	OG1		CC8		Significance
Taxonomic group					
Bacteria	32.71	(8.74)	32.54	(8.70)	NS
Gm +	10.34	(2.76)	11.65	(3.11)	NS
Gm -	21.15	(5.65)	20.71	(5.53)	NS
Actinos	5.41	(1.45)	6.03	(1.61)	NS
Fungi	8.93	(2.39)	7.34	(1.96)	NS
F:B ratio	0.28	(0.08)	0.23	(0.06)	NS
Protozoan	1.09	(0.29)	1.00	(0.27)	NS
Stress indicator					
SAT	14.44	(3.86)	15.75	(4.21)	*
MONO	37.93	(10.1)	36.79	(9.83)	NS
S:M ratio	0.38	(0.10)	0.43	(0.12)	*
cy17:pre ratio	0.59	(0.16)	0.60	(0.16)	NS
cy19:pre ratio	0.73	(0.19)	0.74	(0.20)	NS
Sat	14.44	(3.86)	15.75	(4.21)	*

* = p ≤ 0.05

Results for the effect of date for OG1 and CC8 samples

Averaged across OG1 and CC8, the mol % of each PLFA varied greatly over the 22-month study period (Table 2.8). The August samples had significantly greater amounts of the saturated PLFAs, 16:0 and 18:0, when compared to all other sample dates. The saturated PLFAs 15:0 and 17:0 were significantly greater in August samples compared to samples collected in June and September 2004, and June 2003 and 2004, respectively. Although not always significant, the August samples had greater PLFA concentrations for those characteristic of GM+ bacteria such as the branched PLFAs, i15:0, i16:0, i17:0, a17:0 and the 10Me-branched PLFAs 18:0 (characteristic of actinos). The PLFAs that were consistently lower in samples collected in August were 17:1ω8 and 18:0 2OH, which were lower in concentration for August samples compared to all other sample dates (significant for all dates except June 2004).

Table 2.8. PLFA profiles (mol %) from each of the seven sample dates averaged over both stand ages (SE in parentheses, n = 14).

	Sampling date												
PLFA	Dec-02		Apr-03		Jun-03		Aug-03		Nov-03		Jun-04		Sep-04	
Saturated <18C													
14:0	0.66	(0.12)	0.81	(0.04)	1.09	(0.02)	0.90	(0.10)	0.93	(0.11)	1.35	(0.28)	0.45	(0.08)
15:0	0.75	(0.12)	0.72	(0.07)	0.86	(0.08)	0.97	(0.05)	0.74	(0.06)	0.61	(0.04)	0.68	(0.06)
16:0	10.90	(0.26)	10.12	(0.32)	10.60	(0.40)	13.55	(0.69)	10.18	(0.47)	10.64	(0.46)	10.72	(0.66)
17:0	0.53	(0.03)	0.47	(0.03)	0.40	(0.07)	0.64	(0.05)	0.47	(0.01)	0.44	(0.04)	0.72	(0.03)
18:0	1.76	(0.10)	1.91	(0.07)	1.67	(0.05)	2.52	(0.09)	1.73	(0.09)	1.99	(0.08)	2.13	(0.06)
Saturated >18C													
20:0	1.60	(0.14)	1.72	(0.27)	1.65	(0.12)	1.21	(0.11)	1.84	(0.03)	1.53	(0.18)	2.00	(0.19)
21:0	0.52	(0.10)	0.44	(0.03)	0.42	(0.02)	0.36	(0.03)	0.51	(0.08)	0.40	(0.04)	0.62	(0.02)
22:0	1.67	(0.13)	1.66	(0.10)	1.74	(0.10)	1.28	(0.07)	1.78	(0.09)	1.56	(0.05)	2.34	(0.16)
Monounsaturated													
i16:1	0.55	(0.13)	0.60	(0.09)	0.79	(0.08)	0.63	(0.02)	0.83	(0.09)	0.59	(0.09)	0.51	(0.02)
16:1ω11	1.29	(0.05)	1.39	(0.07)	1.41	(0.02)	1.20	(0.05)	1.61	(0.05)	1.26	(0.07)	1.11	(0.05)
16:1ω7	7.31	(0.15)	6.26	(0.23)	6.14	(0.11)	5.32	(0.21)	6.55	(0.22)	5.62	(0.31)	5.04	(0.09)
16:1ω5	2.31	(0.09)	2.50	(0.17)	2.68	(0.09)	2.54	(0.10)	0.43	(0.08)	2.58	(0.13)	2.41	(0.13)
17:1ω9	1.35	(0.16)	1.32	(0.17)	1.14	(0.24)	1.27	(0.11)	1.58	(0.13)	1.20	(0.09)	1.26	(0.04)
a17:1	0.78	(0.10)	0.73	(0.05)	0.78	(0.11)	0.86	(0.06)	0.95	(0.06)	0.80	(0.06)	0.81	(0.04)
17:1ω8	3.40	(0.24)	5.63	(0.76)	4.90	(0.45)	1.97	(0.16)	4.20	(0.45)	1.78	(0.20)	4.40	(0.80)
18:1ω9c	9.33	(0.42)	9.17	(0.40)	8.32	(0.47)	9.01	(0.42)	9.18	(0.22)	9.98	(0.28)	9.24	(0.60)
18:1ω7	10.63	(0.65)	10.23	(0.29)	8.91	(0.16)	9.17	(0.53)	10.57	(0.91)	10.81	(0.71)	10.45	(0.27)
18:1ω5	0.94	(0.0)	1.05	(0.0)	0.99	(0.05)	1.16	(0.01)	1.26	(0.09)	1.16	(0.1)	1.16	(0.05)
18:1ω11	0.89	(0.03)	0.92	(0.02)	0.84	(0.04)	0.88	(0.06)	0.93	(0.02)	0.91	(0.03)	0.98	(0.02)

Table 2.8 (Continued).

PLFA	Dec-02		Apr-03		Jun-03		Sampling date Aug-03		Nov-03		Jun-04		Sep-04	
Cyclopropyl														
cy17:0	3.54	(0.17)	3.31	(0.02)	3.24	(0.18)	4.04	(0.16)	3.32	(0.16)	3.27	(0.20)	3.63	(0.21)
cy19:0	7.06	(0.31)	7.13	(0.27)	6.40	(0.21)	7.53	(0.42)	7.04	(0.34)	7.99	(0.41)	7.90	(0.16)
Branched														
i15:0	4.01	(0.38)	4.23	(0.35)	5.39	(0.22)	5.53	(0.28)	5.09	(0.55)	3.70	(0.60)	3.36	(0.36)
a15:0	1.87	(0.09)	1.92	(0.25)	2.49	(0.15)	2.41	(0.15)	2.51	(0.20)	1.66	(0.26)	1.46	(0.12)
i16:0	1.73	(0.07)	1.84	(0.19)	2.43	(0.19)	2.92	(0.05)	2.41	(0.22)	2.29	(0.30)	2.12	(0.11)
i17:0	0.82	(0.12)	0.81	(0.07)	0.95	(0.09)	1.19	(0.07)	1.04	(0.07)	1.10	(0.10)	1.12	(0.04)
a17:0	1.16	(0.08)	1.06	(0.06)	1.16	(0.07)	1.47	(0.08)	1.32	(0.08)	1.21	(0.04)	1.24	(0.09)
10Me														
10Me16:0	3.25	(0.30)	3.00	(0.35)	3.85	(0.14)	4.18	(0.21)	3.65	(0.21)	3.89	(0.50)	3.87	(0.26)
10Me17:0	0.58	(0.05)	0.47	(0.03)	0.56	(0.03)	0.70	(0.03)	0.56	(0.04)	0.60	(0.04)	0.66	(0.05)
10Me18:0	1.41	(0.11)	1.28	(0.05)	1.30	(0.06)	1.90	(0.07)	1.40	(0.11)	1.34	(0.13)	1.63	(0.09)
Hydroxy														
16:1 2OH	0.85	(0.15)	0.99	(0.15)	1.00	(0.10)	0.96	(0.10)	0.93	(0.08)	0.97	(0.12)	0.85	(0.10)
18:0 2OH	1.34	(0.12)	1.32	(0.06)	1.39	(0.10)	0.72	(0.06)	1.46	(0.12)	1.08	(0.10)	1.88	(0.18)
Polyunsaturated														
18:3ω6	1.43	(0.37)	1.00	(0.07)	0.85	(0.08)	0.74	(0.12)	1.00	(0.12)	0.87	(0.04)	0.79	(0.08)
18:2ω6	9.46	(1.55)	8.30	(0.51)	7.72	(0.44)	6.66	(0.50)	6.85	(0.18)	10.33	(2.10)	7.61	(0.40)
20:4ω6	0.41	(0.04)	0.67	(0.15)	0.56	(0.09)	0.35	(0.13)	0.47	(0.05)	1.10	(0.06)	0.98	(0.02)
20:2ω6	0.46	(0.04)	0.38	(0.03)	0.42	(0.06)	0.36	(0.03)	0.41	(0.07)	0.34	(0.01)	0.42	(0.05)

Results of the group biomarker and stress indicator data for OG1 and CC8 revealed similar patterns to those found with analysis of the 35 individual PLFAs (Table 2.9). The general bacteria and the actinomycetes biomarkers were only significantly greater in only August samples when compared to samples collected in December 2002 and April 2003. August samples also had a significantly greater proportion of PLFAs used as GM+ bacteria biomarkers than that calculated for all other dates except for June and November 2003.

The SAT sum stress indicator was significantly greater in samples from the August 2003 collection than that calculated for all other sample dates. Conversely, the MONO sum indicator was significantly lower in August samples compared to all other dates. This resulted in a significantly greater S:M ratio in the August samples compared to all other dates (Figure 2.14).

Increases in the cy17:0/16:1ω7 and cy19:0/18:1ω7 ratios have been reported to be stress indicators for GM- bacteria (Kieft et al., 1997; Bossio and Scow, 1998). On average, the August samples had the highest cy17:0/16:1ω7 and cy19:0/18:1ω7 values but only the cy17:0/16:1ω7 ratio was significantly higher in August samples compared to that calculated for all other sample dates except for samples collected in September (Figure 2.15).

The effect of sample date on individual PLFAs was less consistent when comparing dates other than those collected in August 2003. We found no consistent pattern in sample date and the interaction of temperature and moisture status on the presence or absence of individual PLFAs or with increases in stress indicator ratios. In order to simplify results for these date comparisons, three date grouping levels were established based on the positions from the NMS plots and ANOVA results. In group one, the saturated PLFAs 17:0, 18:0, and 22:0 were significantly greater in samples collected in September 2004 than those collected in April, June, and November 2003, whereas the monounsaturated PLFAs 16:1ω11, 16:1ω7, and 17:1ω9 were significantly less in samples from September 2004. The PLFA i15:0 was the only branched PLFA that was significantly different in September 2004 compared to samples from April, June, and November 2003 and was found to be lower for the September samples.

Table 2.9. Sum of PLFAs used for taxonomic groups and stress indicators from each of the seven sample dates averaged over OG1 and CC8. Values are the mean molar % of PLFAs (SE in parentheses, n = 4). Abbreviations are defined in Tables 2.2 (Taxonomic group) and 2.3 (stress indicators).

Taxonomic group	Dec-02		Apr-03		Jun-03		Aug-03		Nov-03		Jun-04		Sep-04	
Bacteria	31.6	(0.38)	31.2	(0.59)	31.8	(0.41)	35.2	(1.14)	34.0	(0.67)	32.6	(1.10)	32.0	(0.23)
Gm +	9.6	(0.27)	9.9	(0.90)	12.4	(0.67)	13.5	(0.46)	12.4	(0.96)	10.0	(1.17)	9.3	(0.41)
Gm -	22.8	(0.84)	21.2	(0.42)	19.4	(0.27)	19.8	(0.79)	22.0	(0.80)	20.9	(0.80)	20.4	(0.43)
Actinos	5.2	(0.30)	4.7	(0.33)	5.7	(0.13)	6.8	(0.24)	5.6	(0.16)	5.8	(0.48)	6.1	(0.37)
Fungi	9.5	(1.54)	8.3	(0.50)	7.7	(0.43)	6.7	(0.49)	6.9	(0.17)	10.4	(2.09)	7.6	(0.38)
F:B ratio	0.31	(0.05)	0.27	(0.02)	0.25	(0.02)	0.19	(0.02)	0.20	(0.00)	0.33	(0.09)	0.24	(0.01)
Protozoan	0.87	(0.05)	1.05	(0.15)	0.98	(0.04)	0.71	(0.15)	0.88	(0.09)	1.44	(0.06)	1.40	(0.03)
Stress indicator														
SAT	14.6	(0.09)	14.0	(0.32)	14.7	(0.37)	18.6	(0.72)	14.1	(0.51)	15.0	(0.31)	14.7	(0.58)
MONO	38.8	(0.84)	39.8	(0.59)	36.9	(0.24)	34.0	(0.70)	38.1	(1.29)	36.7	(0.72)	37.4	(0.62)
S:M ratio	0.38	(0.01)	0.35	(0.01)	0.40	(0.01)	0.55	(0.03)	0.37	(0.02)	0.41	(0.01)	0.40	(0.02)
cy17:pre ratio	0.49	(0.02)	0.54	(0.02)	0.54	(0.03)	0.77	(0.00)	0.51	(0.04)	0.60	(0.05)	0.73	(0.05)
cy19:pre ratio	0.68	(0.06)	0.71	(0.04)	0.74	(0.04)	0.82	(0.03)	0.69	(0.05)	0.75	(0.05)	0.77	(0.03)
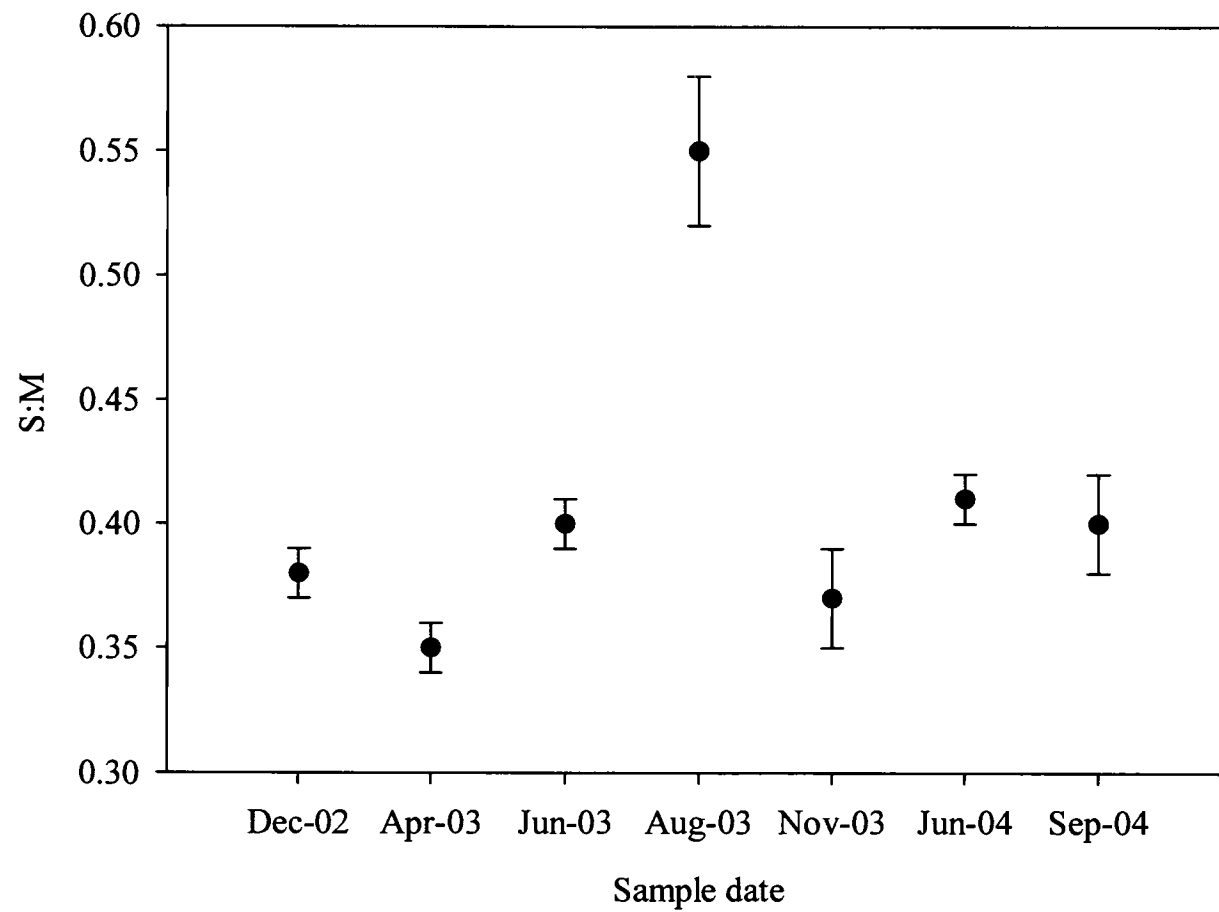
Figure 2.14. Changes in the ratio of saturated to monounsaturated PLFAs (S:M). Values are the mean molar % of PLFAs from OG1 and CC8 samples. Error bars represent standard error (n = 4).
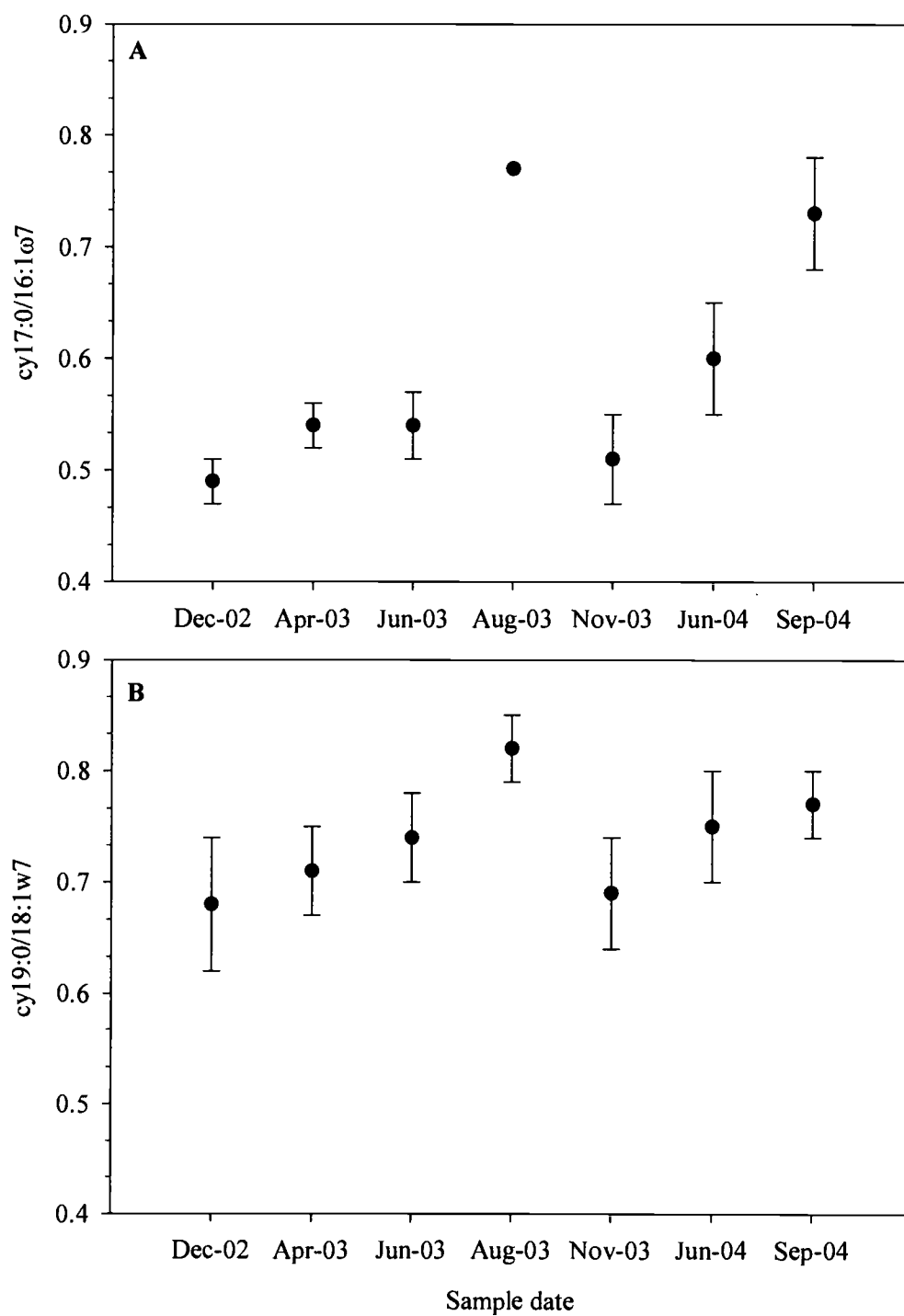
Figure 2.15. Changes in the ratio of cy17:0 to 16:1ω7 (A) and cy19:0 to 18:1ω7 (B). Values are the mean molar % of PLFAs from OG1 and CC8 samples. Error bars represent standard error (n = 4).

In group 2, samples collected in June 2004 were significantly different from samples collected in April, June, and November 2003 but the significant PLFAs and their directions of change were different. Only the saturated PLFA, 18:0, was significantly greater in June 2004 compared to April, June, and November 2003. The a15:0 PLFA was significantly less and i17:0 was significantly greater in June 2004 samples compared to April and November 2003 samples. The monounsaturated PLFA 17:1ω8 was consistently less in June 2004 compared to the three other sampled months. The polyunsaturated PLFA 20:4ω6 was consistently greater in samples from June 2004 and September 2004 compared to samples collected in April, June, and November 2003.

In group 3, samples collected in November 2003 were compared to those from December 2002 and April 2003. The only consistent significant differences involved the PLFAs 16:1ω5, i17:0 and a17:0. Samples from the November date had a lower abundance of 16:1ω5 and a greater abundance of i17:0 and a17:0 compared to December 2002 and April 2003 samples.

The effect of sample date on the stress indicators when comparing dates other than those collected in August 2003 are as follows (Table 2.8):

Group 1: September 2004 samples had a significantly greater cy17:0/16:1ω7 ratio compared to April, June, and November 2003 samples.

Group 2: The S:M ratio was only significantly greater for June 2004 samples than for samples from April 2003.

Group3: No significant differences were found comparing November 2003 samples and December 2002 and April 2003 samples.

Results for the effect of date for OG2 and CC25 samples

The NMS plots as well as the repeated measures ANOVA data using Tukey's HSD adjusted p-values indicate that the SMC structures changed in response to sample date (Figure 2.13 and Table 2.10). The August samples were significantly greater in the saturated PLFA, 18:0, compared to all other sample dates. The concentration of the saturated PLFA, 14:0, also was significantly less in August

Table 2.10. PLFA profiles from each of the seven sample dates averaged over OG2 and CC25 stands. Values are the mean molar % of PLFA$_{tot}$ concentration (SE in parentheses, n = 14).

PLFA	Dec-02		Apr-03		Jun-03		Aug-03		Nov-03		Jun-04		Sep-04	
Saturated <18C														
14:0	0.73	(0.12)	0.91	(0.01)	1.23	(0.06)	0.60	(0.07)	1.02	(0.09)	1.04	(0.07)	0.77	(0.13)
15:0	0.82	(0.14)	0.83	(0.08)	0.97	(0.07)	0.75	(0.11)	0.90	(0.08)	0.81	(0.03)	0.79	(0.10)
16:0	10.43	(0.39)	9.88	(0.40)	10.65	(0.66)	11.57	(0.38)	10.35	(0.37)	10.27	(0.24)	10.45	(0.60)
17:0	0.53	(0.06)	0.44	(0.01)	0.51	(0.04)	0.55	(0.04)	0.52	(0.06)	0.38	(0.07)	0.65	(0.08)
18:0	1.76	(0.16)	1.65	(0.11)	1.61	(0.08)	2.27	(0.15)	1.68	(0.09)	1.72	(0.06)	1.92	(0.12)
Saturated >18C														
20:0	2.19	(0.15)	1.82	(0.19)	1.64	(0.28)	1.33	(0.11)	1.69	(0.13)	1.70	(0.24)	2.13	(0.31)
21:0	0.58	(0.09)	0.41	(0.07)	0.36	(0.04)	0.42	(0.08)	0.44	(0.04)	0.38	(0.04)	0.61	(0.08)
22:0	2.28	(0.40)	1.71	(0.16)	1.72	(0.21)	1.43	(0.15)	1.75	(0.18)	1.62	(0.18)	2.39	(0.34)
Monounsaturated														
i16:1	0.33	(0.06)	0.55	(0.04)	0.78	(0.07)	0.57	(0.07)	0.81	(0.13)	0.62	(0.03)	0.56	(0.05)
16:1ω11	1.18	(0.15)	1.37	(0.03)	1.39	(0.04)	1.06	(0.09)	1.50	(0.06)	1.43	(0.07)	1.24	(0.04)
16:1ω7	7.63	(0.33)	7.23	(0.31)	7.11	(0.29)	5.78	(0.29)	7.14	(0.28)	6.78	(0.42)	5.92	(0.34)
16:1ω5	2.13	(0.13)	2.47	(0.09)	2.59	(0.16)	2.24	(0.27)	0.66	(0.09)	2.86	(0.16)	2.57	(0.11)
17:1ω9	1.16	(0.12)	1.13	(0.03)	1.03	(0.07)	1.04	(0.10)	1.27	(0.04)	1.16	(0.10)	1.21	(0.11)
a17:1	0.64	(0.07)	0.63	(0.05)	0.66	(0.04)	0.79	(0.07)	0.76	(0.04)	0.78	(0.07)	0.76	(0.08)
17:1ω8	3.13	(0.19)	4.59	(0.62)	3.20	(0.57)	1.93	(0.30)	2.80	(0.13)	1.83	(0.04)	3.46	(0.61)
18:1ω9	9.21	(0.43)	9.22	(0.28)	8.86	(0.24)	9.18	(0.37)	9.37	(0.25)	9.36	(0.22)	8.44	(0.15)
18:1ω7	10.15	(1.00)	9.70	(0.64)	8.32	(0.21)	9.63	(0.60)	9.98	(0.40)	10.25	(0.27)	9.81	(0.72)
18:1ω5	1.02	(0.1)	0.97	(0.1)	0.85	(0.07)	1.09	(0.08)	1.08	(0.04)	1.15	(0.1)	1.01	(0.10)
18:1ω11	0.95	(0.06)	0.88	(0.05)	0.84	(0.01)	0.94	(0.02)	0.88	(0.05)	0.82	(0.03)	0.90	(0.06)

Table 2.10 (Continued).

PLFA	Sampling date													
	Dec-02		Apr-03		Jun-03		Aug-03		Nov-03		Jun-04		Sep-04	
Cyclopropyl														
cy17:0	3.40	(0.35)	3.42	(0.30)	3.38	(0.36)	4.64	(0.53)	3.45	(0.32)	3.53	(0.36)	3.78	(0.30)
cy19:0	6.06	(0.36)	7.05	(0.36)	6.45	(0.39)	9.39	(0.39)	6.77	(0.41)	7.26	(0.46)	7.12	(0.66)
Branched														
i15:0	4.05	(0.34)	4.55	(0.20)	5.93	(0.30)	4.79	(0.77)	5.16	(0.46)	4.54	(0.40)	4.22	(0.47)
a15:0	1.58	(0.09)	1.81	(0.11)	2.35	(0.19)	1.74	(0.14)	2.21	(0.10)	2.07	(0.10)	1.85	(0.26)
i16:0	1.68	(0.12)	1.73	(0.16)	2.72	(0.30)	2.94	(0.58)	2.39	(0.34)	2.11	(0.16)	2.00	(0.22)
i17:0	0.68	(0.07)	0.69	(0.08)	0.80	(0.06)	0.95	(0.10)	0.85	(0.05)	0.96	(0.09)	1.06	(0.11)
a17:0	1.08	(0.05)	0.93	(0.08)	1.04	(0.09)	1.29	(0.08)	1.23	(0.03)	1.14	(0.07)	1.18	(0.06)
10Me														
10Me16:0	2.81	(0.30)	2.91	(0.32)	4.10	(0.52)	4.63	(0.39)	3.83	(0.41)	3.61	(0.23)	3.62	(0.44)
10Me17:0	0.46	(0.04)	0.39	(0.02)	0.57	(0.07)	0.62	(0.05)	0.53	(0.08)	0.48	(0.04)	0.46	(0.05)
10Me18:0	1.26	(0.14)	0.97	(0.09)	1.17	(0.08)	1.53	(0.10)	1.20	(0.10)	1.22	(0.17)	1.39	(0.12)
Hydroxy														
16:1 2OH	0.70	(0.07)	0.87	(0.11)	0.83	(0.14)	1.16	(0.17)	0.94	(0.25)	0.94	(0.20)	0.67	(0.07)
18:0 2OH	1.99	(0.60)	1.53	(0.19)	1.37	(0.15)	0.98	(0.15)	1.55	(0.20)	1.39	(0.12)	2.01	(0.31)
Polyunsaturated														
18:3ω6	1.54	(0.27)	1.14	(0.05)	0.91	(0.05)	0.92	(0.13)	0.94	(0.12)	1.02	(0.09)	0.78	(0.07)
18:2ω6	10.95	(2.19)	10.12	(1.21)	8.73	(0.97)	7.83	(0.78)	9.32	(0.86)	10.17	(0.65)	9.13	(1.46)
20:4ω6	0.58	(0.03)	0.59	(0.08)	0.56	(0.05)	0.35	(0.01)	0.61	(0.11)	1.05	(0.03)	0.92	(0.02)
20:2ω6	0.55	(0.12)	0.30	(0.05)	0.33	(0.02)	0.36	(0.08)	0.38	(0.07)	0.28	(0.04)	0.45	(0.07)

samples compared to samples collected in April, June, and November 2003 and June 2004. Although not significant, there was a trend where August samples had greater concentrations for the PLFAs i16:0, i17:0, a17:0, cy17:0, cy19:0 and the 10Me-branched PLFAs (Table 2.10). PLFAs which were significantly lower in samples collected in August compared to all other sample dates included the PLFAs 16:1ω7 and 17:1ω8c. August samples also were significantly lower in concentration in the PLFA 16:1ω11 compared to June and November 2003 samples.

The sum of the nine bacterial PLFAs (mol %) was significantly greater in samples from the August 2003 collection than that calculated for December 2002 and April and June 2003 sample dates (Table 2.11). The proportion of PLFAs used for GM + bacteria was also significantly affected by sample date; however, further investigation using Tukey's adjusted p-values revealed no consistent differences. August samples had a greater proportion of PLFAs used to characterize actinomycetes (10Me-PLFAs) when compared to samples collected in December 2002 and April 2003. In contrast, August samples had significantly lower concentrations of the PLFAs used as markers for protozoa compared to December 2002 and June 2004 samples.

The stress indicators, S:M and cy19:0/18:1ω7 ratios, were significantly affected by sample date (Table 2.11). The S:M ratio was significantly greater in August 2003 compared to April 2003. The ratio of cy19:0/18:1ω7 was significantly greater in August 2003 compared to samples from all other sample dates.

Discussion

Effect of stand age on soil respiration rates and the SMC

Overall there were no significant differences in soil respiration rates between old-growth sites and the clear-cut sites. However, respiration rates in August 2003

Table 2.11. Sum of PLFAs used for taxonomic groups and stress indicators from each of the seven sample dates averaged over OG1 and CC8. Values are the mean molar % of PLFAs (SE in parentheses, n = 4). Abbreviations are defined in Tables 2.2 (Taxonomic group) and 2.3 (Stress indicators).

	Sampling date												
	Dec-02		Apr-03		Jun-03		Aug-03		Nov-03		Jun-04		Sep-04	
Taxonomic group													
Bacteria	29.5	(1.55)	30.7	(0.88)	32.0	(0.74)	36.1	(0.60)	32.9	(0.85)	32.7	(0.54)	31.8	(0.54)
Gm +	9.1	(0.46)	9.7	(0.41)	12.9	(0.60)	11.7	(1.57)	11.9	(0.81)	10.8	(0.51)	10.3	(0.94)
Gm -	22.3	(1.64)	21.5	(0.73)	19.8	(0.56)	21.1	(0.79)	21.8	(0.67)	21.8	(0.29)	20.7	(0.56)
Actinos	4.5	(0.14)	4.3	(0.30)	5.8	(0.49)	6.8	(0.33)	5.6	(0.57)	5.3	(0.20)	5.5	(0.47)
Fungi	11.0	(2.18)	10.1	(1.20)	8.7	(0.97)	7.8	(0.79)	9.3	(0.86)	10.2	(0.65)	9.1	(1.45)
F:B ratio	0.40	(0.10)	0.34	(0.05)	0.27	(0.03)	0.22	(0.03)	0.29	(0.03)	0.31	(0.02)	0.29	(0.05)
Protozoan	1.12	(0.14)	0.89	(0.09)	0.89	(0.04)	0.71	(0.08)	0.99	(0.12)	1.33	(0.03)	1.37	(0.06)
Stress indicator													
SAT	14.3	(0.40)	13.7	(0.45)	15.0	(0.87)	15.7	(0.41)	14.5	(0.39)	14.2	(0.23)	14.6	(0.85)
MONO	37.5	(1.34)	38.7	(1.02)	35.6	(0.68)	34.2	(0.97)	36.3	(0.30)	37.0	(0.77)	35.9	(1.10)
S:M ratio	0.39	(0.03)	0.36	(0.02)	0.42	(0.03)	0.47	(0.02)	0.40	(0.01)	0.39	(0.01)	0.41	(0.03)
cy17:pre ratio	0.45	(0.05)	0.48	(0.06)	0.48	(0.05)	0.83	(0.14)	0.49	(0.06)	0.53	(0.07)	0.65	(0.05)
cy19:pre ratio	0.61	(0.04)	0.74	(0.06)	0.78	(0.05)	1.01	(0.05)	0.70	(0.07)	0.71	(0.04)	0.73	(0.01)

were 39.0 % greater in OG1 than in CC8 (Figure 2.5). This was the only sample date when respiration rates for the two sites did not follow similar patterns; relative to the June 2003 sample date, the respiration rate for OG1 increased in August whereas the respiration rate for CC8 declined. In another old-growth Douglas-fir forest in Oregon, Sulzman et al. (2005) also reported lower respiration rates measured in June followed by an increase through August. Although soil temperature was the same at each site (16.2°C and 16.0°C for OG1 and CC8, respectively), soil moisture was 12.7% (v/v) at CC8 and 17.1 % (v/v) at OG1. Soils from this series (Stabler) have between 12 and 15% soil moisture at permanent wilting point (-1.5 MPa) (www.statlab.iastate.edu/cgi-bin/osd/osdname.cgi). This is consistent with our data; soils contained between 10 and 16 % H_2O when held between -1 and -1.6 MPa (data not shown).

In contrast to soil respiration rates, microbial biomass, as determined by total extractable PLFA, and bacterial and fungal biomasses were found to be significantly greater in old-growth (OG1) soils compared to the most recent cut (CC8) (Figure 2.7) but not significantly different between old-growth (OG2) and the older cut (CC25) (Figure 2.8). The absolute concentration of the sum of bacterial PLFAs and the fungal PLFA, 18:2ω6,9 were significantly higher in OG1 vs. CC8 plots for all sample dates (Figure 2.9) but not when OG2 sites were compared to CC25 stands (Figure 2.10). These results suggest that clear-cutting affected the absolute concentrations of bacterial and fungal biomass eight years since clear-cutting but the impact of this affect was not observed in sites that were clear-cut 25 years ago. Changes in total microbial biomass have been reported in other studies investigating the effects of forest harvesting. Microbial biomass C as measured by chloroform fumigation extraction was reported to be reduced in the order: OG > 10 year-old plantations > 3-year-old plantations (Chang et al., 1995). However, similar fungal community and microbial processes were found in uncut forests and mixed-wood forests clear-cut seven to nine years prior to sampling (Houston et al., 1998). Fungal biomass has been shown to decrease following clear-cutting (Bååth, 1980), whereas bacterial biomass

has been shown to increase, with effects lasting between 2 and 13 years after clear-cutting (Niemela and Sundman, 1977; Sundman et al., 1978; Lundgren, 1982).

Another way to analyze PLFA data is to use relative abundances, which can provide a different perspective on the data compared to absolute PLFA concentrations values. Absolute values reflect changes in only a given microbial group (e.g. using PLFA markers for fungi or bacteria) relative to that group, whereas relative abundances (mol %) reflect the contribution of different microbial taxa to the entire SMC. Therefore, an increase in the mol % of PLFAs indicative of GM+ or GM-bacteria may occur while the absolute concentration remains the same or decreases. Although absolute concentrations of the bacterial PLFAs were higher in OG1 (Figure 2.9), the relative proportions of most bacterial PLFAs were increased in the most recent clear-cut stands (CC8) (Table 2.6). The lack of any bacterial PLFAs being greater in the OG stands (except for 17:1ω8) indicates that no one group dominated in OG stands. Conversely, samples from CC8 were significantly greater in PLFA markers commonly associated with GM+ bacteria (a17:1, i17:0, a17:0, and 10Me16:0).

Differences in the SMC structure between OG1 and the most recent cut, CC8, are likely attributed to the combined effects of soil temperature and moisture status, reduced C in the litter layer, alterations to the understory composition, and disturbances to root dynamics and belowground C flow. Environmental factors such as soil temperature and moisture status have been shown to alter the SMC structure under laboratory conditions (Harwood and Russel, 1984; Haack et al., 1994; Balkwill et al., 1998). In our study, soils from the most recent cut (CC8) had soil temperatures with greater daily fluctuations and soils were generally warmer in summer and cooler in winter compared to undisturbed OG1 sites (Figures 2.2a and 2.3a). On the other hand, soil moisture was consistently greater at the OG1 site compared to the CC8 throughout the 22-month study period, especially in the summer months, but soil moisture content fluctuated more widely in the CC8 than in OG sites (Figures 2.2b and 2.3b) which is consistent with other studies of young vs. old forest stands (Bååth, 1980; Frazer et al., 1990; Chang et al., 1995; Marra and Edmonds, 1998). It is

possible that greater temperature and moisture extremes in the CC8 sites are contributing factors for differences in the SMC structure between CC8 and OG1.

Increased soil temperature and changes in soil moisture due to tree removal have been shown to accelerate soil organic matter decomposition in the field (Covington, 1981). Total organic C concentrations in litter and mineral soil samples were not significantly different between both clear-cut sites and the associated old-growth sites. In contrast, total organic C of the litter layer on an area basis was 58% lower in CC8 and 53% lower in CC25 (Table 2.1) relative to old-growth samples. Relocation of organic material had occurred as evidenced by qualitative visual observations where the mixture of mineral soil into the organic layer or the presence of a very thin organic layer was seen (often less than 1cm deep) within the mineral soil. In a study by Johnson et al. (1991), clear-cutting resulted in the compaction of the soil organic layers and in the relocation of the organic material.

Another factor is that the understory composition and root dynamics are changed following clear-cutting which also could cause community shifts between old-growth and recently clear-cut forest stands. Grasses and ferns dominated the ground cover at the CC8 sites, whereas the understory composition of the OG sites was more heterogeneous; at least 68 vascular plants have been identified (Shaw et al., 2004). Furthermore, needles from the young re-establishing Douglas-fir trees in the CC8 sites remained on the trees and none were found at the soil surface (personal observation). Following clear-cutting, litter inputs are drastically altered if not completely removed; instead, large amounts of nutrients in slash piles are left on the forest floor (Bååth, 1980; Houston et al., 1998). Increases to aboveground litter inputs have been shown to increase the dissolved organic matter fluxes in the litter layer (Lajtha et al., 2005) but were shown to have a minor influence on the SMC structure (Brant, 2005); instead root C inputs appeared to be the major influence. In our study, root dynamics were likely changed via the removal of large trees and through changes to the plant community composition and structure, which (Strong et al., 1995). 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. Indeed, we found significant differences in absolute microbial biomass (likely reflecting total C inputs) as well as community shifts with clear-cutting (perhaps due to changes in type of C inputs). A significant increase in the relative concentration of the GM + bacterial markers was found in the CC8 sites, indicating that although overall biomass was lower, a shift in the community structure was present eight to 10 years after the cut. However, the same pattern was not found when comparing OG2 to the older cut (CC25). There were no significant differences in respiration rates, overall SMC structure, PLFA$_{tot}$ concentration, or in total bacterial and fungal biomass.

Our data suggest that clear-cutting has affected the soil microclimate, plant community composition (qualitative observations), the distribution of organic C (R.P, Dick, unpublished data), and the SMC composition. But after 25 years these effects had disappeared.

Temporal dynamics and SMC

Seasonal differences significantly influenced the SMC and resulted in both structural and physiological changes. The reduction of tree growth during summer drought conditions (Shaw et al., 2004) is likely coupled to reduced C outflow through the roots (i.e., root exudation) thus further limiting soil microorganisms. Brant (2005) showed that belowground C inputs were more influential on the SMC than were aboveground inputs in three very different forest ecosystems. Therefore, even minor changes to amount and quantity of C reaching the microbes via root exudates during the slow-growth period in summer would likely have a major impact on functioning of the SMC. The dramatic changes in soil temperature and water potential during this time would likely result in different members of the SMC becoming more or less physiologically active. In general, soil bacteria thrive under moist (-0.01 to -0.1 MPa), warm, well-aerated soils with an abundant supply of high-quality organic matter. The greatest total microbial biomass concentration (PLFA$_{tot}$ concentration) was recorded for the June 2003 sample date. The soil environmental measurements at this time would indicate ideal conditions for microbial activity, with an average soil

temperature and moisture content of 14.7°C and 25.0% (-0.2 MPa), respectively. In contrast, the lowest total microbial biomass concentrations occurred in August 2003. Based on OG1 and CC8 soil environmental conditions, soils collected for this sampling approached levels equivalent to the permanent wilting point (on average < 15 % soil moisture, < -1.0 MPa) and relatively hot (> 16°C).

The most water-stress tolerant groups of soil microorganisms include the filamentous fungi (particularly the *Ascomycotina*) and the GM+, filamentous bacteria known as the actinomycetes. Fungi are able to bridge the air gaps between the thin water films that occur in soil pore spaces under conditions of soil desiccation, thus enabling them to continue to function under these extreme conditions (Killham, 1994). The actinomycetes combat the plasmolytic effect of cell dehydration under water stress by accumulating compatible solutes constituently and by inducing the production of compatible solutes (e.g. proline) (Killham, 1994). Although fungal concentration (nmol $18:2\omega6,9$ g^{-1} C) was significantly higher in OG1 relative to CC8, the relative concentration (mol %) of the fungal PLFA marker, $18:2\omega6$, and the F:B ratio were not significantly different between samples collected in August and any other sample date, nor was there a significant effect of stand age. These findings were unexpected and it is unclear why the fungal biomarker did not respond to either sample date or clear-cutting. In a study investigating the effects of long-term manipulation of the nature and quantity of C inputs in different forest ecosystems, Brant (2005) concluded that the differences in the bacterial community were more responsible for any differences in the SMC between treatments compared to differences in the fungal community. It is possible that the fungal community, relative to the total microbial community, was not impacted by the temperature and moisture stress imposed during the month of August. However, it is more likely that using relative abundance values (i.e., mol %) in the analyses may have masked any effects of the fungal biomarker because interpretation of the fungal contribution is based solely on one of the 35 PLFAs used in this analysis whereas 10 PLFAs are used for bacteria.

Increased temperatures and decreased moisture content may require microorganisms to modify their membranes. Under laboratory conditions, the physiological status of the SMC has been evaluated by using the ratios of various stress biomarkers such as the degree of saturation and the ratio of cyclopropyl PLFAs, namely cy17:0 and cy19:0 to their monoenoic precursors (Kieft et al., 1994). Increases in the degree of saturation have been found with increased soil temperature (Petersen and Klug, 1994) and soil desiccation (Kieft et al., 1994). Thermophilic bacteria have a higher proportion of saturated fatty acids compared to unsaturated fatty acids. Increased saturation allows their membranes to form a stronger hydrophobic environment thus enabling their membranes to remain stable and functional at high temperatures. Changes to the lipid composition such as the degree of saturation occur as a result of the biosynthesis of new lipids and requires a large input of energy (Petersen and Klug, 1994) that must come from the microbial biomass or root exudates. The reduction of tree growth during summer drought conditions (Shaw et al., 2004) is likely coupled to reduced C outflow through the roots thus further limiting soil microorganisms. The OG1 and CC8 sites on average were warmest (16.1°C) and driest (14.9% H_2O) in August. Similarly high temperatures were observed for both June 2004 and August 2004, but only samples in August were found to be extremely dry (< 15% H_2O content). The saturated:monounsataruated ratio (S:M) was found to be significantly higher in August samples compared to all other sampling dates. This result suggests that soil moisture status in combination with high soil temperatures is a more important factor for increased S:M ratios than soil temperature alone. This finding should be consistent for all sites including CC25, but only the 16:0 and 18:0 saturated PLFAs were significantly greater in August compared to the April sampling date.

Temperature data for OG2 and CC25 were only available for 2003. In general, OG2 and CC25 were slightly cooler than OG1 and CC8, respectively (Tables 2.5 and 2.6). It is possible that the increased elevation (approximately 183 m) found for the OG2 and matching CC25 sites may have accounted for the slightly lower soil temperatures. Unfortunately, the lack of soil moisture data makes it impossible to

assess whether the CC25 sites are behaving differently or whether soil moisture content did not reach the critical levels recorded in the OG1 and CC8 sites that are located at a lower elevation. Reduced soil temperatures correspondingly would result in increased soil moisture for the CC25 sites as a result of reduced evapotranspiration. Further studies investigating soil moisture and temperature changes throughout the year will help validate or disprove this hypothesis.

A second indicator of stress is an increase in the cyclopropyl PLFAs (cy17:0 and cy19:0) and the ratios of cyclopropyl PLFAs to their precursors (16:1w7 and 18:1w7). An increase for this ratio has been reported to be associated with cell age, nutrient deprivation, desiccation, and/or anaerobiosis (Kieft et al., 1994). Our results revealed a significant increase in the cy17:0/16:1ω7 ratio for August samples but not for the cy19:0/18:1ω7 ratio when averaged over the OG1 and CC8 sites (Figure 2.15). Averaged across OG2 and CC25 samples, the ratio of cy19:0/18:1ω7 was significantly greater in August 2003 compared to samples from all other sample dates.

Studies where stress has been induced under laboratory conditions typically create greater temperature (often exceeding 30°C) and moisture (-15 MPa) extremes as well as complete starvation conditions for microbes. Our results, using the same physiological stress indicators, suggest that comparatively mild conditions of relatively high soil temperature (14.7°C to 16.0°C) and low moisture status (approximately -1.5 MPa) experienced in the field can induce similar stress responses. We hypothesize that stress during the warm, dry summer months is likely induced not only by changes in soil temperature and moisture status but also indirectly by reduced rhizodeposition via decreased tree growth and thus C limitations or other nutrient-limitations resulting from decreased mass flow and diffusion of nutrients to microbes.

Perspectives

Our data suggest that clear-cutting has affected the SMC structure soil but time since clearing was important with effects being most pronounced eight to ten years post clearing, but after 25 years these effects had disappeared. Additionally, seasonal climatic conditions were more influential than the effect of stand age. Soil

temperature and moisture conditions were shown to influence the SMC structure and physiological status in both old-growth and clear-cut forests. We hypothesize that these environmental changes also altered temporal patterns of root-derived substrate inputs to the soil. The combined effect of soil moisture and temperature stress in August samples was reflected in changes to the SMC structure and physiological status. Soil water stress and high temperatures experienced in August likely resulted in the modification of microbial membranes as indicated by increases in the ratios of the cyclopropyl PLFAs to their monoenoic precursors and in the ratio of saturated to monounsaturated PLFAs. Increases in the degree of saturation require a large amount of energy that is presumed to come from root exudation or dead microbes (Petersen and Klug, 1994). If tree growth is dramatically reduced during the summer drought, it is reasonable to assume that the needed energy requirements are satisfied via the rapid turnover of lipids due to the induced metabolic stress and subsequent death of microorganisms. The latter effect resulted in the overall reduction in PLFA$_{tot}$ content (or active microbial biomass). This study has provided strong evidence that PLFA analysis coupled with microclimatic data may be used to assess SMC and physiological changes at an ecological level.

Acknowledgements

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CHAPTER 3

A PULSE-CHASE METHOD FOR ^{13}C-LABELING DOUGLAS-FIR
SEEDLINGS FOR DECOMPOSITION STUDIES

Jennifer Moore Kucera and Richard P. Dick

Abstract

Tracking litter decomposition and root carbon flow via stable isotopes such as ^{13}C is gaining popularity with the advent of commercial isotopic materials. Although model ^{13}C-labeled compounds such as glucose or acetate are available commercially, there is a need for large quantities of ^{13}C-labeled plant materials to realistically study decomposition, C cycling, and biogeochemical processes *in situ* or in the laboratory. We present a pulse-labeling technique to label Douglas-fir seedlings with $^{13}CO_2$ for subsequent *in situ* soil incubation. For nine weeks, 670 Douglas-fir seedlings were exposed weekly to $^{13}CO_2$ in a closed chamber. The $^{13}CO_2$ (99 atom %) was injected into the chambers at a rate proportional to the photosynthetic rate for a total $^{13}CO_2$ volume of approximately 3 L. Over 1.5 kg of seedling dry matter was accumulated with a relatively uniform distribution of ^{13}C among seedling parts: needles = 1.57, stems = 1.45, and root = 1.36 atom %. These ^{13}C abundance values are well above background levels and can be easily detected in environmental samples. The method and chamber design described provides a procedure to accumulate large quantities of ^{13}C-labeled plant material that can then be used to trace its decomposition in the field or in large-scale laboratory experiments.

Introduction

Terrestrial ecosystems and organic C stored in soils are important components in regulating atmospheric CO_2 and global climate change. In particular, temperate forest ecosystems have been implicated in holding a great potential for storing C. Most estimates focus on aboveground biomass however soils, which contain about 1500×10^{15} g of C, store more than 2.5 times the amount of C stored in land plants and twice the amount of C stored in the atmosphere (Schlesinger, 1997). For example, the upper bound on C storage for forest ecosystems in the highly productive region of the Pacific Northwest, USA was estimated to be up to 882.6 Mg C ha^{-1} yr^{-1}

(Smithwick et al., 2002). Of this total ecosystem C storage potential, the soil pool accounted for an average of 21.6% but ranged up to 39.5%.

The major pathway for long-term soil C storage is via decomposition of plant materials into recalcitrant soil organic matter fractions. Soil microorganisms are the driving force behind plant residue decomposition and are directly involved in the formation of soil organic matter (SOM). Numerous studies have investigated the reaction rates of SOM formation, litter mass losses, litter quality, and biochemical and enzymatic pathways (Stevenson, 1994; Swift, 1996; Heal et al., 1997). However, uncertainties remain regarding the direct pathways of C movement between plants, roots, microorganisms and soils (Bromand et al., 2001). Isotopic techniques are promising tools to study C cycling in soil-plant systems and have recently been used to track C allocation and remobilization in plants (Mordacq et al., 1986; Svejcar et al., 1990; Thompson, 1996) and the fate of particular C compounds in soil in laboratory studies (Hu and van Bruggen, 1997; Abraham et al., 1998; Chotte et al., 1998; Arao, 1999; Ekblad and Hogberg, 2000; Ekblad et al., 2002; Burke et al., 2003; Butler et al., 2003; Malosso et al., 2004) but none to date has used labeled litter material in the forest ecosystems to track its decomposition *in situ*. In this paper, we outline a simple procedure to label Douglas-fir (*Pseudotsuga menziesii*) seedlings with ^{13}C applied as CO_2 gas for subsequent use in field incubation studies.

Isotopic techniques employed in the past used ^{14}C because it was readily available and provided a high level of sensitivity. This material however, is radioactive, poses serious environmental and operator hazards, has high handling and disposal costs, and therefore has been limited to laboratory studies. Recent advances in the use of stable isotopes have eliminated these drawbacks and plants have been labeled with $^{13}CO_2$ for a variety of reasons including studies on plant physiology, CO_2 fixation, and tracking ^{13}C into rhizosphere microbial communities (Mordacq et al., 1986; Svejcar et al., 1990; Berg et al., 1991; Thompson, 1996; Bromand et al., 2001; Loya et al., 2002; Butler et al., 2003). Moreover, ^{13}C is considered superior to ^{14}C in tracer studies because photosynthetic and metabolic discrimination is much less with ^{13}C than with ^{14}C (van Norman and Brown, 1952; Svejcar et al., 1990). However,

there is no information on pulse labeling of woody species for subsequent use in decomposition studies.

In order to successfully track C during decomposition, the plant material in question must be uniformly labeled and have a $\delta^{13}C$ signature that after incubation will be significantly different from the background signature of soil and other plant residues. Continuous exposure to $^{13}CO_2$ is one means to accomplish this goal but requires expensive, sophisticated incubation chambers with controls for all growth factors (e.g. $[CO_2]$, $[O_2]$, temperature, light, moisture). A more economical method is to employ a pulse-labeling technique whereby plants are periodically exposed to $^{13}CO_2$ for short periods and the remainder of the time the plants are grown at standard ambient CO_2 and under more easily managed conditions. The challenge then becomes to ensure homogenous labeling throughout plant components (i.e., leaves or needles, stems, and roots). Bromand et al. (2001) successfully labeled wheat plants (*Triticum aestivum* L. cv. 'Katepwa') under the premise that applying label in proportion to the photosynthetic rate at regular intervals throughout the growing season would result in a uniform distribution of ^{13}C in the plant. We took a similar approach and present a method for labeling tree seedlings for subsequent use in microcosms to track the fate of ^{13}C in soil. Furthermore, we present data showing the significant enrichment of soil after field incubations of these labeled Douglas-fir seedlings.

Materials and Methods

In June 2002, a total of 670 Douglas-fir seedlings were obtained from a local nursery (Plum Creek Timber, Oregon). Seeds were planted into "Superblock™ 60/250ml" styrofoam blocks in a peat/perlite growth medium. The seedlings were between 5 and 7 cm high at the beginning of the labeling period. Each block contained up to 60 seedlings grown in individual cylinders that were open at the bottom to allow adequate drainage. A total of 12 blocks were obtained from the nursery, 8 of which were labeled and the remaining 4 served as unlabeled controls. Trees were irrigated regularly (every 2-3 d) and fertilized as needed with 1250 µg

N/ml, 643 μg P/ml, and 1042 μg K/ml. Blocks were stored outside under the shelter of a clear plastic roof to allow sunlight and a shade cloth on the south side to reduce the sun's intensity.

Two chambers were constructed of a wood frame (0.61 m x 0.65 m x 0.90 m) with Teflon-sheeting "windows" on 4 out of 5 sides (Figure 3.1). Two 12VDC cooling fans (no. 273-238, 10-cm diam., 4.2W, 85 cfm, RadioShack, USA) were installed in the upper opposite corners of the chambers to ensure proper circulation. Fans were powered by 6V alkaline lantern batteries (no. 23-560, RadioShack, USA) that were mounted outside of the chamber. Ice packs were placed inside the chambers to minimize excessive heating and to condense excess humidity. Gas leakage from the chambers was evaluated prior to the labeling experiment by pumping in a known concentration of CO_2 gas and monitoring for losses over a 3 h period with a LI-COR 6200 infrared gas analyzer equipped with a LI-6250 CO_2 analyzer (LI-COR, Inc., Lincoln, Nebraska).
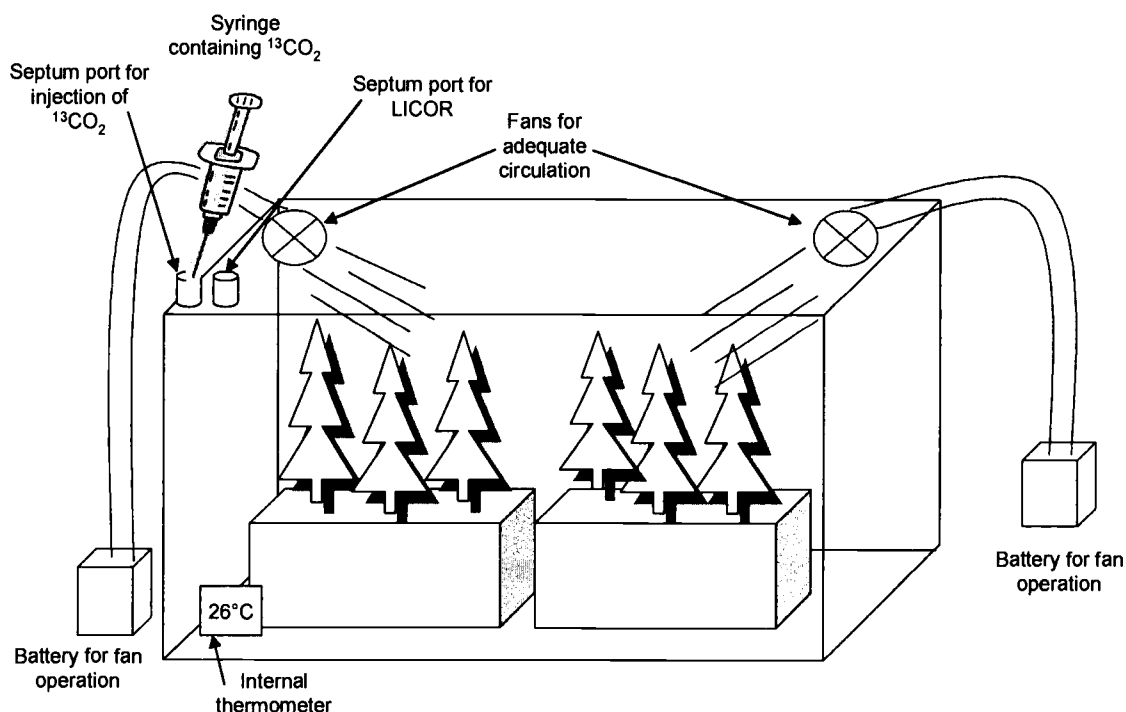

Figure 3.1. Schematic drawing of $^{13}CO_2$ labeling chamber (see text for details).

Labeling with $^{13}CO_2$ (99 atom % $^{13}CO_2$, Cambridge Isotope Company, Cambridge, Massachusetts) occurred once a week for nine weeks between the months of June and September 2002. Each week, four blocks of Douglas-fir seedlings were placed inside the chamber and sealed (two blocks per chamber) and repeated the following day for the remaining four blocks. Although [CO_2] inside the chamber was continuously monitored with the LI-COR CO_2 analyzer, this instrument provides only a semi-quantitative measure of the CO_2 concentration when $^{13}CO_2$ is used as a result of differing wavelengths for maximum absorption of $^{13}CO_2$ (2270 cm^{-1} λ) and $^{12}CO_2$ (2360 cm^{-1} λ) (Svejcar et al., 1990). Therefore, it was necessary to determine an approximate CO_2 uptake rate to be used as a guide when injecting $^{13}CO_2$ which was done each labeling day before labeling was initiated. The time necessary for trees to consume about 200 μg $^{12}CO_2$-C m^{-3} was determined by allowing the initial CO_2 concentration inside the chambers to naturally decline to about 200 μg CO_2-C m^{-3}. $^{12}CO_2$ from a tank was injected into each chamber to bring the concentration to > 150 μg CO_2-C m^{-3} above the initial concentration (usually between 60-120 mL $^{12}CO_2$). Injections were made using a 60-mL syringe and a septum installed in the top frame of the chamber (Figure 3.1). The time required for the $^{12}CO_2$ levels to reach the starting concentration was used to schedule injections with the ^{13}C-labeled CO_2.

The seedlings within the labeling chamber were pulse-labeled three times by injecting up to 120 mL 99 atom % $^{13}CO_2$ followed by a chase period with $^{12}CO_2$ (three injections of 120 mL each). At the end of the 9-week labeling period, the total quantity of 99 atom % $^{13}CO_2$ for each block ranged between 2450 and 3000 mL.

In October 2002, seedlings were harvested, rinsed with deionized water to remove the peat and perlite material, dried at 45°C, and separated into needle, stem, and root parts. The seedling fractions were composited by block and a subsample was ground to pass a 0.42-mm sieve and analyzed for ^{13}C abundance with a PDZ Europa 20-20 isotope ratio mass spectrometer interfaced with a Europa Roboprep elemental analyzer. The $\delta^{13}C$ is calculated according to the following equation:

$$\delta^{13}C = (R_{sample}/R_{standard} -1)*1000 \qquad (1)$$

where R is the ratio $^{13}C/^{12}C$ measured as the mass 45 to 44 ratio. The standard ratio is the absolute ratio of the Pee Dee Belemnite standard (R_{PDB}) with a known value of 0.0112372 (Hayes, 1982). Results are expressed as parts per thousand (‰).

The amount of ^{13}C in seedling parts was calculated after conversion of $\delta^{13}C$ values to absolute ratios (R), and then to fractional abundances (F) according to the following relationships:

$$R_{sample} = {}^{13}C/{}^{12}C = [\delta^{13}C/1000 + 1]*R_{PDB} \tag{2}$$

$$F = {}^{13}C/({}^{12}C+{}^{13}C) = R_{sample}/(R+1) \tag{3}$$

where $\delta^{13}C$ and R_{PDB} values are calculated as described above. The fractional abundance of each seedling component is then multiplied by the C content of a given component (e.g., needles) to yield the quantity of ^{13}C in that component. Atom percent excess (APE) of ^{13}C as a result of the labeling experiment is the difference between total ^{13}C (%) in the labeled sample and ^{13}C (%) in the unlabeled sample.

Results and Discussion

Approximately 752 g, 400 g, and 435 g of oven-dry (45°C) labeled needles, stems, and roots were produced, respectively (Table 3.1). The average ^{13}C signature relative to the PDB standard was 415.4 ‰ for needles, 295.1 ‰ for stems, and 228.4 ‰ for roots. This was a significant enrichment relative to the control samples which had an average isotopic signature of -20.5 ‰ for needles, -19.1 ‰ for stems, and -24.0 ‰ for roots. These values represent a total ^{13}C incorporation of 1.68 g for needles, 0.671 g for stems, and 0.563 g for roots, and a total above-ground uptake efficiency of 20.9% (excluding roots). Overall, 25.7% of added ^{13}C was incorporated into the seedlings, with a distribution of 42.8, 32.5, and 24.7% incorporated into needles, stems, and roots, respectively (Table 3.1). The remaining 74.3% was assumed lost to respiration or fixed in the perlite/peat mixture. Although this loss is substantial, our values are consistent with values reported in the literature (Svejcar et al., 1990; Berg et al., 1991).

Table 3.1. Total plant biomass, plant C, ^{13}C (%) for plant parts and control values, amount ^{13}C gained and % ^{13}C allocated over the 9-week labeling period.

	Needles	Stems	Roots	Whole seedling
Dry matter (g)	751.6	399.8	434.8	1586.2
Total C (g)	349.9	184.3	203.1	737.2
^{13}C (atom %)	1.5656	1.4507	1.3615	
Control natural ^{13}C (%)	1.0854	1.0866	1.0843	
^{13}C gain (atom % excess)	0.4802	0.3641	0.2772	1.1215
Total ^{13}C gain (g)	1.6801	0.6708	0.5629	2.9139
^{13}C allocation (%)	42.8	32.5	24.7	100.0

Analysis of variance for seedling part and block revealed strong evidence of an interaction between block and plant part ($F_{2,11}$ = 10.4, p = 0.003). The average δ^{13}C values for needles, roots, and woody parts in blocks 1-4 were found to be significantly lower than corresponding values in samples from blocks 5-8 (p ≤ 0.005) (Figure 3.2). These differences are likely the result of a difference of total ^{13}CO$_2$ added to the blocks. Blocks 1-4 received approximately 2.5 L of ^{13}CO$_2$ while blocks 5-8 received 3 L of ^{13}CO$_2$ (99.5% ^{13}C) over the 9-week labeling period. Furthermore, the δ^{13}C value of the woody parts for blocks 1-4 were not significantly different from the δ^{13}C value of the roots from the same block set, whereas significant differences were observed for woody vs. root material in blocks 5-8. It is likely that this difference is due to blocks 1-4 not receiving the last pulse-labeling (week 8), which would reduce the allocation of the new C into woody parts for winter storage.

These ^{13}C-labeled materials meet the requirements of being uniformly labeled at a level substantially different from background soil and plant levels. Soils from a representative Douglas-fir ecosystem in southwestern Washington have ^{13}C signatures of -25.6 to -26.6 ‰ (data not shown) and needle samples range from -25.6 to -30.9 ‰ (Fessenden and Ehleringer, 2002). If 2 g of needles that were 1.56558 atom % ^{13}C and 47.9 % C are added to 1 kg of soil (3.75% C), then the average enrichment of the soil C pool after 90 % of the material had decomposed in 1 year (assuming 50 % will be
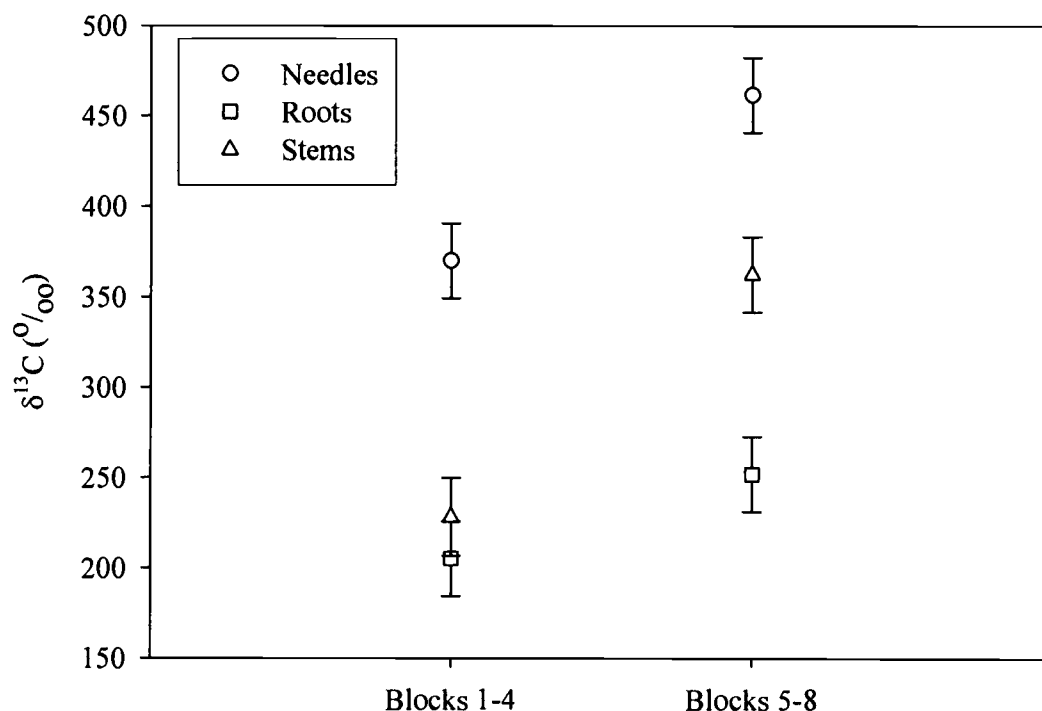
Figure 3.2. Comparison of $\delta^{13}C$ from needles roots and stems averaged over labeling groups (blocks 1-4 and blocks 5-8). Error bars indicate standard error (n = 4).

lost to the atmosphere as CO_2) would be about 0.0055 atom % above background levels. This difference is easily detected by mass spectrometry and will provide an adequate signal to track the decomposition of this material into soil C pools.

As an example, we present unpublished data from a field incubation that utilized the same litter material reported above. The Douglas-fir seedling needle/stem mix of material was added at the rate of 175 g m^{-2} (a rate similar to annual litter inputs) and incubated in a microcosm placed in old-growth forest soil at Wind River Experimental Forest (SW WA). The unamended soil had a $\delta^{13}C$ value of -26.0 (2 % CV) and the amended mineral soil (0-5 cm depth) beneath the labeled litter bag , 3 of the 4 stand ages ranged from -15.2 to -23.8 after 2 months field incubation. Further fractionation of the soils showed the humin fraction (recalcitrant and insoluble in strong acid or base) showed soil beneath labeled litter bags to range from -22.9 to

-24.3 compared to the unmeneded control that was -25.0 (1 % CV). Again, these differences illustrate the ability of this material to be readily tracked in soils.

Perspectives

In summary, the pulse-labeling technique presented here has useful applications in C cycling studies where large quantities of 'natural' materials are necessary for both laboratory and *in situ* experiments. The method is simple, safe, and inexpensive relative to continuous labeling chambers. The chamber design can be easily constructed with materials readily available. The quantity of material generated may be increased by constructing more chambers or by labeling additional blocks on subsequent days. The time investment during the growth and labeling period averaged nine person hours per week. It would also be applicable for other plant types where greater biomass would accumulate in a much smaller space (e.g., herbaceous species).

Acknowledgements

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CHAPTER 4

APPLICATION OF ^{13}C-LABELED LITTER AND ROOT MATERIALS
FOR *IN SITU* DECOMPOSITION STUDIES USING PHOSPHOLIPID
FATTY ACIDS

Jennifer Moore Kucera and Richard P. Dick

Abstract

Microorganisms play a central role in litter decomposition and partitioning C between CO_2 evolution and sequestration of C into semi-permanent pools in soils. At the ecosystem level, forest stand age influences rates of litter accumulation and quality and micro-climatology which could affect the microbial community structure and C sequestration processes. Although numerous laboratory experiments have studied the decomposition of individually ^{13}C-labeled materials, few studies have verified their findings under field conditions. The objective of this study was to track the decomposition of ^{13}C-labeled Douglas-fir (*Pseudotsuga menziesii*) materials into the microbial community using [13]C- phospholipids fatty acid (PLFA) analysis in forest soil of old-growth stands (>500 yrs) compared to stands clear-cut in either 1994 or 1977. We used *in situ* microcosms that contained either ^{13}C-labeled litter or root material and analyzed the soil for relative amounts of ^{13}C incorporated ([13]C %) into PLFAs. Microcosms were collected seven times over a 22-month incubation period. A significantly greater amount of [13]C % was incorporated in CC8 samples compared to OG1 samples in samples collected in five out of the seven sample dates. Additionally, a significantly greater proportion of ^{13}C was incorporated into soil samples containing the ^{13}C-labeled litter material ("L") relative to that found in samples containing ^{13}C-labeled root material ("R") in April, June, August, and November 2003. In general, 18:1ω9 and 18:2ω6,9 (common fungal biomarkers) had the greatest amount of ^{13}C incorporation throughout the study period in both clear-cut and old-growth sites, especially in plots with ^{13}C-labeled litter. This suggests that fungi have grown up into the litterbag and translocated C into the soil layers. Our results confirm the use of ^{13}C-labeled materials in field studies for assessing microbial community dynamics using ^{13}C-PLFA analysis.

Introduction

Soil microbial community structure is a function of the interactions between litter quality and quantity, root deposition, and the microclimate of the soil. This

community is responsible for the decomposition of litter, the cycling of nutrients for plant growth and the formation of soil organic matter. Forest management practices, such as clear-cutting, have been shown to disrupt the soil microbial ecosystem via removal of organic matter, reduction in litter input, changes in root dynamics, alteration of nutrient cycles (Schmidt et al., 1996; Lindo and Visser, 2003) and increased temperature and moisture extremes (Keenan and Kimmins, 1993; Holmes and Zak, 1999). Although considerable progress has been made in understanding the factors affecting the rates of litter decomposition and formation of soil organic matter (Swift et al., 1979; Stevenson, 1994; Heal et al., 1997), most of what is known about the role of microbial functional groups during decomposition/C sequestration in soils is based on indirect methods that use inferences such as activity measurements, biomass C, or respiration to study the "black box" of decomposition.

The composition and structure of the microbial community may affect degradation rates of organic substances. For example, communities with relatively high proportions of fungi have advantages over communities dominated by bacteria because fungi are able to extend hyphae into the soil to extract nutrients and water (Holland and Coleman, 1987) whereas bacteria are limited in movement. Furthermore, fungi produce the necessary extracellular enzymes needed to degrade recalcitrant material such as lignin (Hammel et al., 1993). However, structurally different microbial communities may still function at similar levels (Buyer and Drinkwater, 1997).

The ability to identify specific groups of microorganisms responsible during the decomposition of organic material will provide needed information regarding the direct pathways of C movement between plants, roots, microorganisms and soils (Bromand et al., 2001; Loya et al., 2002). Traditional methods such as culturing and identification, and direct counts through microscopy are limited because a high percentage of organisms are not culturable and identification through morphological characteristics can be difficult. Plus, all these methods are extremely labor intensive and non-quantitative, making field-based studies of soil microbial ecology/diversity impractical. The use of phospholipid fatty acids (PLFA) is a community-level

biochemical method that can provide information regarding shifts in the microbial community structure resulting from management or environmental changes. It is difficult, however, to link PLFA markers with community function because the overall PLFA profile is a consortia of all organisms present at sampling time (Ludvigsen et al., 1997) and any given microbial process may be carried out by diverse taxa (Hill et al., 2000).

Stable isotope probing (SIP) with ^{13}C of specific biomarkers, such as PLFAs, is a promising new tool to directly link specific microbial processes with the organisms responsible; thus shedding valuable insight into the biogeochemical transformations in natural environments that are mediated by microorganisms (Boschker and Middelburg, 2002). Isotopic techniques have recently been used to track C allocation and remobilization in plants (Mordacq et al., 1986; Svejcar et al., 1990; Thompson, 1996) and the fate of particular C compounds in soil in laboratory studies (Hu and van Bruggen, 1997; Abraham et al., 1998; Chotte et al., 1998; Arao, 1999; Ekblad and Hogberg, 2000; Ekblad et al., 2002; Abraham and Hesse, 2003; Burke et al., 2003; Butler et al., 2003; Malosso et al., 2004) but none to date has used labeled litter material in forest ecosystems to track its decomposition *in situ*. The objective of this study was to track the decomposition of ^{13}C-labeled Douglas-fir (*Pseudotsuga menziesii*) materials into the microbial community using ^{13}C-phospholipids fatty acid (PLFA) analysis in forest soil of old-growth stands (>500 yrs) compared to stands clear-cut in either 1994 or 1977.

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. The forests are predominately Douglas-fir (*Pseudotsuga menziesii*) stands of varying ages. The area is characterized by a cool, moist climate with average annual precipitation and temperature of 22.2 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 (Shaw et al., 2004).

The soils have been classified as medial, mesic Entic Vitrands (Stabler series, www.statlab.iastate.edu/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 12000 years old.

We chose forest stands of three different ages: old-growth where trees have been estimated to be between 300 and 500 years old; an 8-year-old stand; and a 25-year-old stand. These stands were separated into two spatially separated study groups to account for potential differences in soil properties largely resulting from differences in elevation and microclimate. Within each study group, there were two field replicates for each stand. The elevation range for group one is between 338 and 387 m, whereas the elevation range for group two is between 500 and 536 m. The first group compared old-growth (OG1) to the 8-year-old stand (CC8). The second group compared old-growth (OG2) to the 25-year-old stand (CC25). The O horizon of old-growth 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. All of the stands except for CC8 are located within the Wind River Experimental Forest (WREF) - a 4208-ha area where active forest research has been conducted since 1908 (Figure 4.1). The CC8 stands are adjacent to the WREF on USDA Forest Service land. Within each stand, 3 sub-replicate plots were established to assess local spatial variation.

^{13}C-labeled Douglas-fir material

A pulse-chase technique was used to label Douglas-fir seedlings with ^{13}C (see Chapter 3 for full details). For nine weeks, about 670 Douglas-fir seedlings were exposed weekly to ^{13}CO$_2$ (99 atom % ^{13}CO$_2$, Cambridge Isotope Company, Cambridge, Massachusetts) in a closed chamber. The seedlings within the labeling chamber were pulse-labeled three times by injecting up to 120 cm^3 (roughly equivalent to 216 mg of ^{13}CO$_2$) 99 atom % ^{13}CO$_2$ followed by a chase period with ^{12}CO$_2$ (three injections of 120 cm^3 each). This amount of CO$_2$ was added at a rate
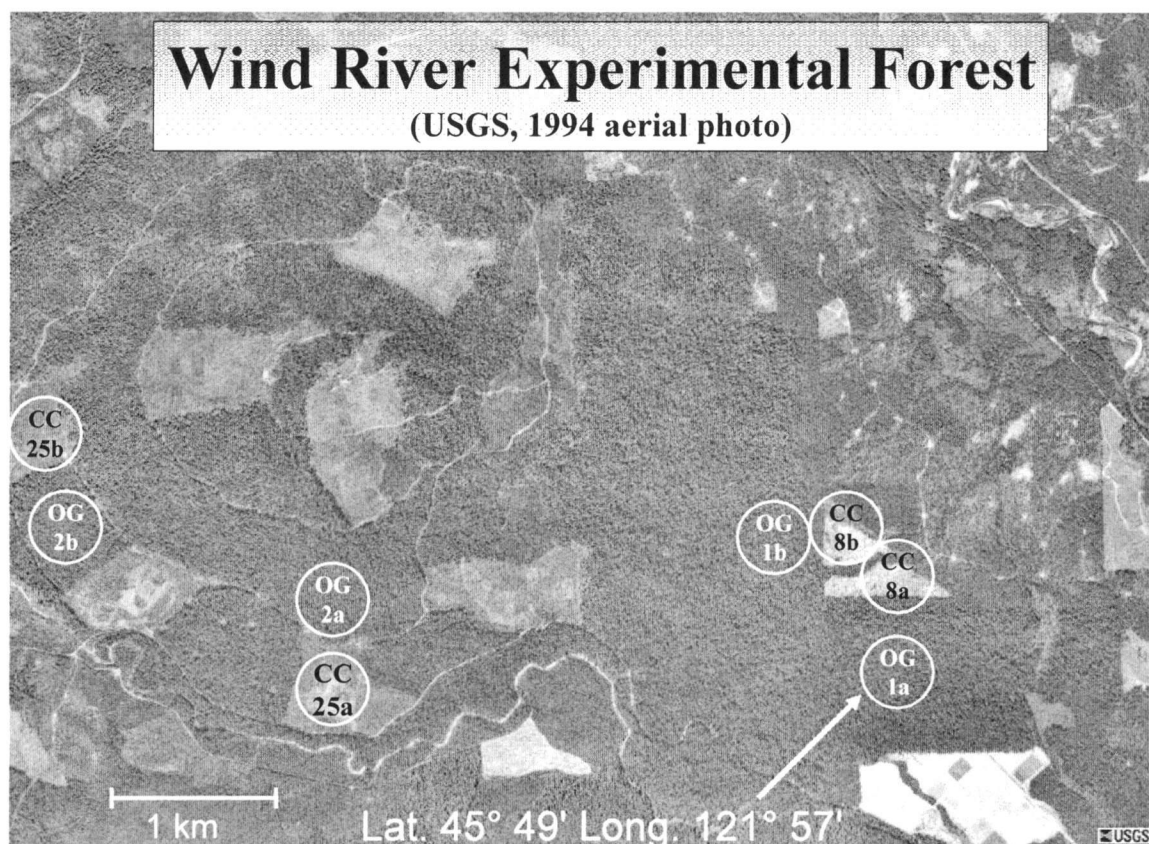
Figure 4.1. 1994 aerial photograph of the Wind River Experimental Forest in southern Washington showing the location of the 8 study sites (OG = old-growth; CC8 = stands that were clear-cut 8 years ago in 1994; CC25 = stands that were clear-cut 25 years ago in 1977; "a" and "b" represent field replicates).

proportional to the photosynthetic rate as determined prior to labeling by monitoring the time it took for 120 cm^3 of $^{12}CO_2$ (tank source) to be consumed by the seedlings (average of three times). The CO_2 concentration was monitored with a LI-COR 6200 infrared gas analyzer equipped with a LI-6250 CO_2 analyzer (LI-COR, Inc., Lincoln, Nebraska). At the end of the 9-week labeling period, the total quantity of 99 atom % $^{13}CO_2$ for each block (a block contained about 60 seedlings) ranged between 1225 and 1500 cm^3. In October 2002, seedlings were harvested, rinsed with deionized water to remove the peat and perlite material, dried at 45°C, and separated into needle, stem, and root parts. Each fraction was combined to obtain a homogenous mix. Over 1.5 kg of seedling dry matter was accumulated with a relatively uniform distribution of ^{13}C

among seedling parts: the average ^{13}C signature relative to the PDB standard was 415.4, 295.1, and 228.4 ‰ for needles, stems, and roots, respectively. This was a significant enrichment relative to the control samples (i.e., seedlings grown at the same time without enrichment) which had an average isotopic signature of -20.5 ‰ for needles, -19.1 ‰ for stems, and -24.0 ‰ for roots. Needle samples from Douglas-fir trees from WREF were reported to have similar ^{13}C signatures that ranged from -25.6 to -30.9 ‰ (Fessenden and Ehleringer, 2002).

Microcosm set-up

In November 2002, 14 microcosms constructed of 10-cm diameter x 12- to 16-cm long open-ended PVC columns were inserted into the top 10 cm of mineral soil at each of the three sub-replicate plots in each stand (extra length accounts for depth of litter layer). Half of the 14 microcosms received litterbags containing the ^{13}C-labeled needles or needle/stem mix and the other half received ^{13}C-labeled roots.

The litterbags (7.2 cm^2) were constructed of a polyester-mesh layer (1.0 mm) on top and a nylon-mesh layer (0.5 mm) on the bottom. These mesh sizes were selected to allow entry of soil fauna on the top and mycorrhizal mycelium through the bottom while minimizing the loss of seedling material as well as the intrusion of soil particles into the bag. The ^{13}C-labeled needles were added at the rate of 175, 200, and 100 g m^{-2} for both OG sites (OG1 and OG2), CC25, and CC8, respectively; these rates were similar to annual litter inputs reported within the WREF (Klopatek, 2002). Thus, 1.4, 1.6, and 1.0 g of ^{13}C-labeled needles were placed into litterbags and sewn closed with nylon thread. In addition to needles, litterbags for the OG1 and OG2 sites also contained 1.0 g of stem material mixed with the needles. Root material was added at the rate of 388, 173 g m^{-2} for OG, CC25 plots, respectively; rates similar to annual root production within the WREF (Klopatek, 2002). The rate of root production for the CC8 was assumed to be 75% that of the CC25 site. Thus, root material was added at the rate of 130 g m^{-2} for these sites. These rates correspond to the addition of 1.2, 0.5, and 0.4 g of ^{13}C-labeled root material to the soils within OG, CC25, and CC8 plots, respectively.

To install microcosms, a custom-made steel soil corer measuring 10-cm in diameter by 16-cm long (Scotty's Welding, Wenatchee, WA) was used to extract the soil from the ground. Roots, when encountered, were severed with a knife. The soil corer was pounded into the ground and had a beveled edge to ensure the soil remained in the corer upon extraction. 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 in order. The top O horizon was removed, set aside, and a litterbag containing the ^{13}C-labeled needle/stem mix was placed on the soil surface. The O horizon was replaced and gently repacked into the column. Columns that received ^{13}C-labeled roots were established in a similar manner except the ^{13}C-labeled roots were freely incorporated (i.e., no bags) into the top 5 cm of mineral soil before the O horizon was replaced.

Sample collection

On December 16, 2002, April 25, June 20, August 31, and November 23, 2003, and June 14 and September 1, 2004, one pair of microcosms (one containing ^{13}C-labeled needle/stem material and one containing ^{13}C-labeled root material) was randomly chosen from each sub-replicate plot for PLFA analysis. Each set consisted of two microcosms; one contained ^{13}C-labeled litter enclosed in the litterbag and the other contained ^{13}C-labeled roots. One of the three sets was analyzed for PLFA concentration and the ^{13}C value for each PLFA by GC-C-IRMS (this study) and the other two sets were analyzed only for PLFA concentration as measured by GC-FID (results reported in Chapter 2). All soil samples were collected with the PVC column intact and transported to the lab within eight hours. Samples were stored at 4°C and processed within three days. Each sample was separated into litter layer, 0-5 cm soil below the litterbag or 0-5 cm mineral soil (root cores) and sieved to < 4.75 mm to remove wood pieces, roots, or stones and homogenized. Subsamples were dried at 65°C for 24 h to calculate moisture content.

Sample collection for ^{13}C-PLFA natural abundance

Two sets of soil samples were collected to determine ^{13}C-PLFA natural abundance. The first set was collected in April 2003. Five soil samples (0-5cm) were collected using a soil corer and bulked to give one composite sample for each site. The second set consisted of one microcosm installed in April 2003 at each site that contained no ^{13}C-labeled material. This microcosm was collected at the end of the study (September 2004) and soil (0-5cm) was analyzed to determine the natural ^{13}C abundance of individual PLFAs. Results were averaged between the two sample dates and served as the background δ^{13}C values for individual PLFAs.

Total C and N Analysis and pH

Soil and litter samples were collected in June 2002 to obtain total C and N values (Table 4.1). Soil samples were collected from 0-10 cm and 10-30 cm depths after removing and collecting the litter layer. Total C and N were determined by dry combustion using a LECO CNS-2000 Macro Analyzer.

In order to express soil and litter total C and N values on an area basis, bulk density measurements for soil were conducted using a Troxler nuclear density gauge (Troxler Electronic Laboratories Inc., Research Triangle Park, NC). Bulk density measurements for litter samples were done by the ring method. Steele rings (5-cm diam.) were filled with litter and the depth and fresh weight were measured. Samples were dried for 48 h at 45°C and reweighed. Bulk density is then calculated as the weight of dry litter (g) for a given volume (cm^3). Soil and litter pH were measured by using a combination glass electrode with a soil:water ratio of 1:2 and a litter:water ratio of 1:10 (Table 4.1).

PLFA analysis

PLFAs were prepared via three steps: lipid extraction using a Bligh and Dyer technique (1959), separation of phospholipids by solid-phase extraction columns, and saponification/methylation of esterified fatty acids in the neutral and phospholipid fractions.

Table 4.1. 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 %C	Carbon Mg C ha^{-1}	Nitrogen % N	Nitrogen Mg N ha^{-1}	C/N	pH
OG1	Litter								
		4.2	0.04	33.93	6.29	0.93	0.17	36.7	4.3
	Soil								
		0-10	0.89	3.44	30.7	0.14	1.25	24.6	5.1
		10-30	0.95	2.04	38.9	0.12	2.29	17.0	5.4
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
OG2	Litter								
		4.2	0.04	33.98	6.29	0.92	0.17	36.9	4.2
	Soil								
		0-10	0.88	4.00	35.1	0.16	1.41	25.0	4.9
		10-30	0.95	2.28	43.1	0.13	2.46	17.5	5.1
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

(i) Lipid extraction. Approximately 1 g (oven-dry equivalent) of soil was weighed into each 25-mL glass Kimax centrifuge tube equipped with a Teflon-lined screw cap. Enough water and 100 mM phosphate buffer (1/2 0.1 M KH_2PO_4 and ½ 0.1M K_2HPO_4) were added to make 50 mM buffer and mixed with chloroform and methanol at a ratio of 0.8:1:2. The solution was shaken for 2 h and allowed to stand overnight. Lipids were extracted following centrifuging (7649 g for 6 min) and filtering the supernatant through Whatman no.2 filter papers. This step was repeated twice with additional chloroform and methanol additions (holding the 1:2 ratio constant). The chloroform-methanol-buffer phases were separated with addition of 7 mL of 2 M NaCl and the chloroform phase (bottom) was removed and immediately dried under a stream of N_2 (99.99 % pure) at 37°C.

(ii) Lipid separation. The dried lipids were resuspended in chloroform and added to solid-phase extraction columns containing 500 mg of silica (Supelco, Inc., Bellefonte, Pa). Neutral lipids (NLFAs), glycolipids, and phospholipids (PLFAs) were sequentially isolated by adding chloroform, acetone, and methanol, respectively. The phospholipid fraction was immediately dried under N_2 at 37°C and stored at -20°C (under N_2) until methylation and GC analysis.

(iii) Saponification/methylation of PLFAs. Dried PLFAs were resuspended in 1 mL of 1:1 methanol:toluene and 1 mL of 0.2M methanolic KOH, mixed and heated in a water bath at 37°C for a minimum of 15 min. After cooling to room temperature each sample received 1 mL of hexane, 0.3 mL of 1 M acetic acid, and 2 mL of water. The hexane (upper) phase containing the fatty acid methyl esters (FAMEs) was removed. This step was repeated two additional times and the hexane solution was dried under N_2 at 37°C. FAMEs were redissolved in hexane and transferred to 2-mL amber GC vials with 100 μl inserts to give a final total volume of 80 μl.

GC-C-IRMS analysis

The PLFAs were separated and quantified by gas chromatography (Agilent 6890) equipped with a 30-m Hewlett Packard Innowax 2 column (0.25 mm internal diameter; 0.25 μm film thickness) connected to a Europa ORCHID on-line combustion interface attached to a Europa 20-20 mass spectrometer. The carrier gas was He, and the oven temperature was ramped from 120 to 260°C at a rate of 5°C per min with a 5-min hold at 260°C. The PLFAs are converted to CO_2 before analysis by the isotope ratio mass spectrometer (IRMS). Carbon dioxide of known isotopic composition was injected at the beginning and end of each run. Individual fatty acids were identified relative to several standards: 37 FAMEs mixture (FAME 37 47885-4; Supelco, Inc), 24 bacterial FAMEs mixture (P-BAME 24 47080-U; Supelco, Inc.), and MIDI standards (Microbial ID, Inc.). Quantification of FAMES was accomplished by using varying concentrations of tridecanoic FAME (Supelco, Inc.). A total of 23 fatty acids were identified but only 11 fatty acids were used in this analysis. The other 13 fatty acid peaks were deleted because of limitations encountered when using pulses of

CO_2 with a known $\delta^{13}C$ value as a standard. The shortest possible CO_2 pulse of 1 s produces a peak as much as five times larger than PLFA peaks. Although the PLFA peaks are clearly identifiable, reliable $\delta^{13}C$ values require intensity values as high as 1.5×10^{-11}. Therefore, detection of only nine peaks could be used for ^{13}C analysis even though 23 peaks could positively be identified. The peaks labeled 16:1ω7 and 18:1ω7 co-eluted with PLFAs 10Me16:0 and 10Me18:0 and hence are designated as 16:1ω7+ and 18:1ω7+, respectively.

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 (ω) of the molecule. Cis and trans geometry are indicated by the suffixes "c" and "t", respectively. 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.

PLFA $\delta^{13}C$ values were corrected for the addition of the extra C atom introduced during methylation using the equation of Pelz et al. (1997):

$$\delta^{13}C_{FA} = (C_{FAME} \times \delta^{13}C_{FAME}) - (C_{ME} + \delta^{13}_{ME})/C_{FA}, \tag{1}$$

where C_{FAME} is the number of carbons from the FAME; C_{ME} was the number of carbons from the methyl group; and C_{FA} was the number of carbons from the fatty acid. The amount of ^{13}C incorporated into each PLFA was calculated as the ^{13}C of PLFAs in the labeled samples in excess of those in the non-labeled controls:

$$^{13}C\text{-PLFA} = [(\text{atom } ^{13}C \% _{PLFA\text{-labeled}}) - (\text{atom } ^{13}C \% _{PLFA\text{-unlabeled}})] \times \text{PLFA}, \tag{2}$$

where ^{13}C-PLFA was the ^{13}C amount incorporated into the individual PLFA (μg ^{13}C kg^{-1} soil). The (atom $^{13}C \% _{PLFA\text{-labeled}}$) and (atom $^{13}C \% _{PLFA\text{-unlabeled}}$) were atom $^{13}C\%$ of individual PLFAs in labeled and unlabeled samples, respectively. PLFA was the content of individual PLFAs in the labeled sample (μg C kg^{-1} soil). Values were calculated based on the mean atom $^{13}C \%$ of unlabeled PLFAs as well as plus one standard error of the mean. Using the higher value, any value that was negative was assumed to be zero (reflecting no incorporation of ^{13}C into that particular PLFA) so

that small positive values that fell within the error of the PLFA would be more accurately represented. The amount of ^{13}C-PLFA incorporated relative to the total amount of ^{13}C added was calculated as

$$^{13}C \% = {}^{13}C\text{-PLFA}/{}^{13}C\text{-added material} * 100\% * 1 \times 10^3, \quad (3)$$

where ^{13}C-PLFA is defined in equation (2) and ^{13}C-added material is the amount of ^{13}C ($\mu g\ {}^{13}C\ kg^{-1}$ soil) in the total amount of litter or root material added to each microcosm.

Statistical Analysis

All statistical analyses were conducted using SAS (version 8) and PC-ORD (version 4) statistical software (McCune and Mefford, 1999). The relative amount of ^{13}C incorporation data (^{13}C %) also were examined using nonmetric multidimensional scaling (NMS) with the Sorensen distance measure (Kruskal, 1964; Mather, 1976). The goal of this method is to minimize stress, or the departure from monotonicity, within the reduced ordination space. The Sorensen distance measure was chosen because it is less sensitive to outliers unlike other distance measures such as Chi-square distances (McCune and Grace, 2002). The NMS analyses were run using the 'slow and thorough' setting in the autopilot mode. Random starting configurations were seeded by the computer's clock. Forty runs with real data were run and Monte Carlo simulations were conducted using 50 randomized runs and a stability criterion of 0.0001.

The number of dimensions chosen in the model was assessed by comparing the NMS runs with the real data to Monte Carlo simulations. The proportion of variation represented by each axis was assessed by calculating the coefficient of determination (r^2) between distances in the ordination space and Sorensen distances in the original distance matrix.

NMS scores from each axis were then loaded into SAS (v8) to determine if stand age, ^{13}C source, or their interaction was significantly different ($p \leq 0.05$) along a given axis. In order to determine how soil microbial communities differed between stand age and the significant sample dates, analysis of variance (ANOVA) was used to

analyze for stand age (OG1 vs. CC8 and OG2 vs. CC25) and ^{13}C-source effects using the relative amount of ^{13}C incorporated in each of the 9 PLFAs.

Results

Needle decomposition rates

The differences in mass loss of needles between December 2002 and June 2004 were significantly greater in samples from old-growth sites compared to those from the clear-cut sites (Figure 4.2). In September 2004, there was little to no litter remaining in any of the samples making precise weight measurements difficult as a result of encroachment of soil into the bags, a common limitation in litterbag studies. There were no significant differences in the percent needle mass loss between OG1 and OG2 nor were there significant differences between CC8 and CC25.
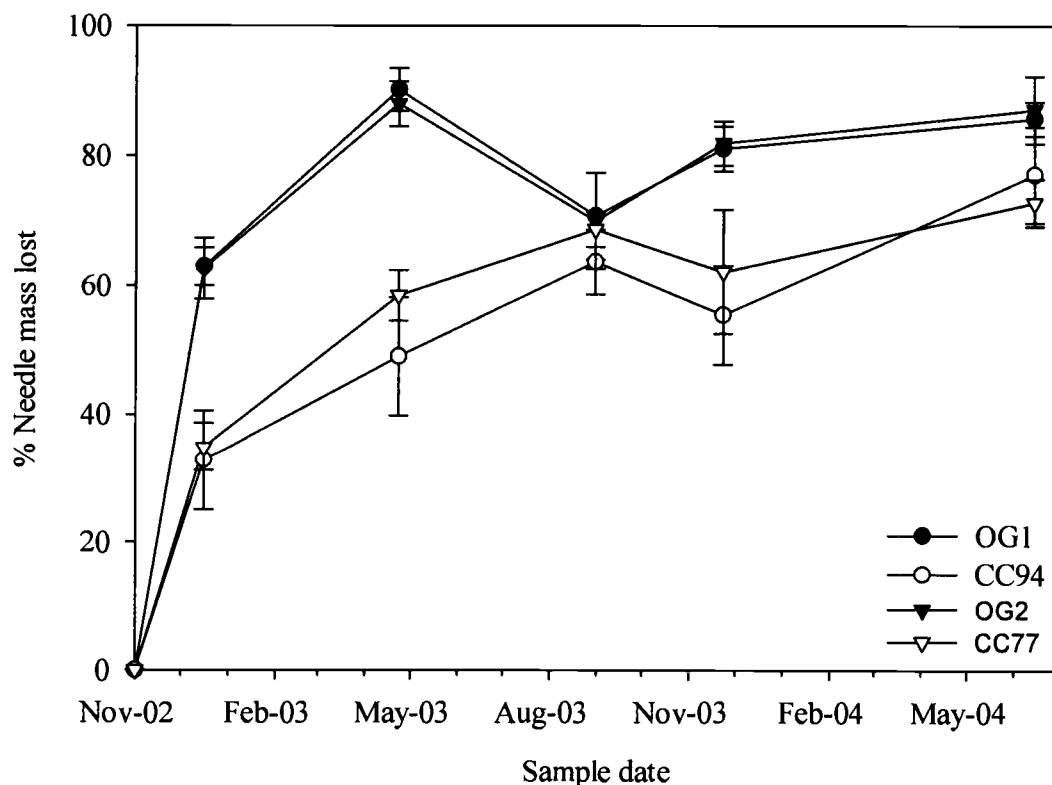

Figure 4.2. Needle mass lost (%) for samples from OG1, CC8, OG2, and CC25 sites from study initiation (Nov 2002) through Jun 2004.

δ¹³C values of control PLFA samples

The average $\delta^{13}C$ values (‰) for individual PLFAs on control soils collected in April 2003 and September 2004 (control samples) are shown in Figure 4.3. There was a significant difference between sample dates for PLFAs 16:0, 16:1ω7, 18:2ω6,9, and cy19:0. All PLFAs (except for i15:0, 16:1ω5, and cy17:0) were significantly depleted of ^{13}C in comparison to the corresponding $\delta^{13}C$ values of total soil C (-26.0 ‰) (represented by the solid line in Figure 4.2). Because our samples were collected over both spring (April and June) and late summer sample periods (August 2003 and September 2004) as well as two early winter samplings (December 2002 and November 2003), we felt that averaging both control dates would best represent the $\delta^{13}C$ value of individual PLFAs (see discussion for further details).
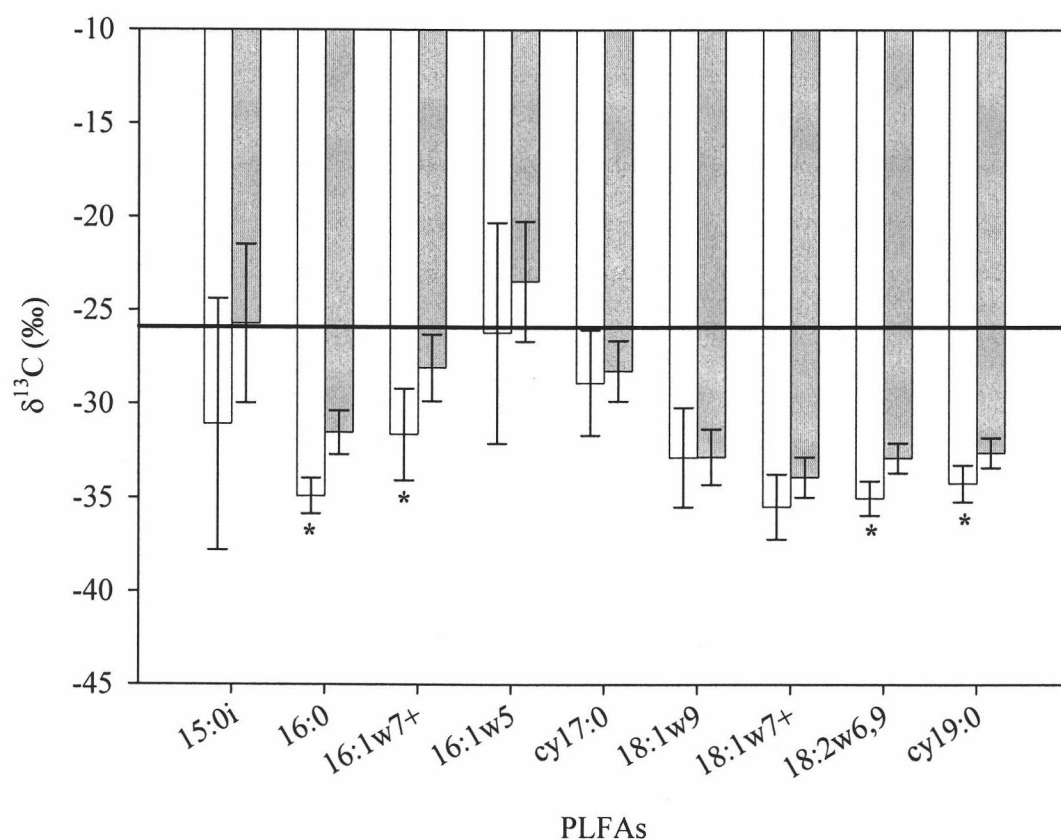

Figure 4.3. $\delta^{13}C$ values (‰) for individual PLFAs collected in April 2003 (open bars) and September 2004 (filled bars). Error bars are standard error. Bars with an "*" denotes a significant difference (p ≤ 0.05) in $\delta^{13}C$ value between the two sample dates. The solid line represents the average $\delta^{13}C$ value of SOC for all sites.

It is unclear what proportion of the ^{13}C isotope ratio from the peaks labeled 16:1ω7+ and 18:1ω7+ was solely from these PLFAs because they co-eluted with PLFAs 10Me16:0 and 10Me18:0, respectively. In a separate study investigating seasonal and stand age effects on PLFA composition from the same stands, we were able to determine that on average, 66% of the 16:1ω7 + 10Me16:0 peak was attributed to 16:1ω7. Similarly, 18:1ω7 made up about 89% of the 18:1ω7 + 10Me18:0 peak concentration. Although we were able to account for mass differences, we were not able to determine if 66 and 89% of the ^{13}C signature is due to 16:1ω7 and 18:1ω7, respectively because a large signature can come from a peak with a small area. Unfortunately for interpretation purposes, these peaks represent two different microbial groups and thus cannot be used together as a general taxonomic category. The PLFAs 16:1ω7 and 18:1ω7 are Gram-negative biomarkers whereas the 10Me16:0 and 10Me18:0 are characteristic of the actinomycetes, a specialized group of Gram-positive biomarkers (Zelles and Bai, 1994; Zelles, 1999).

^{13}C incorporation into PLFAs for OG1 and CC8 sites

The ANOVA analysis for the NMS scores revealed a significant effect of age and source along both axes (p ≤ 0.05), however the effect of ^{13}C source is more obvious with the majority of root samples (R) clustered to the right of center along axis one and the majority of litter samples (L) clustered to the left (Figure 4.4). All seven sample dates were included to assess if overall differences could be detected. In order to explain these differences it was necessary to separate results on a date-by-date basis. The ^{13}C % incorporation into PLFAs from the OG1 and CC8 soils varied from 0 % (ex., multiple PLFAs in December 2002) to 25 % (18:2ω6,9 for CC8 L in June 2003) (Figures 4.5-4.9). A significantly greater amount of ^{13}C % was incorporated in CC8 samples compared to OG1 samples in samples collected in December 2002 and April, June, August, and November 2003 (Tables 4.2-4.6). There was no significant effect of stand age for the last two dates, June 2004 and September 2004. A significantly greater proportion of ^{13}C was incorporated into soil samples containing the ^{13}C-labeled litter material ("L") relative to that found in samples containing ^{13}C-

labeled root material ("R") in April, June, August, and November 2003 (Tables 4.3-4.6). A significant age*source effect was found in samples collected in April, June, August, and November 2003. Results from the four pre-planned contrasts revealed that a greater proportion of ^{13}C was incorporated into PLFAs from the CC8L samples compared to those from the OG1L samples collected in April, June, August, and November 2003 (Tables 4.1-4.5). Additionally, CC8L and OG1L samples contained more ^{13}C than did CC8R and OG1R samples, respectively in samples from April, June, August, and November 2003.
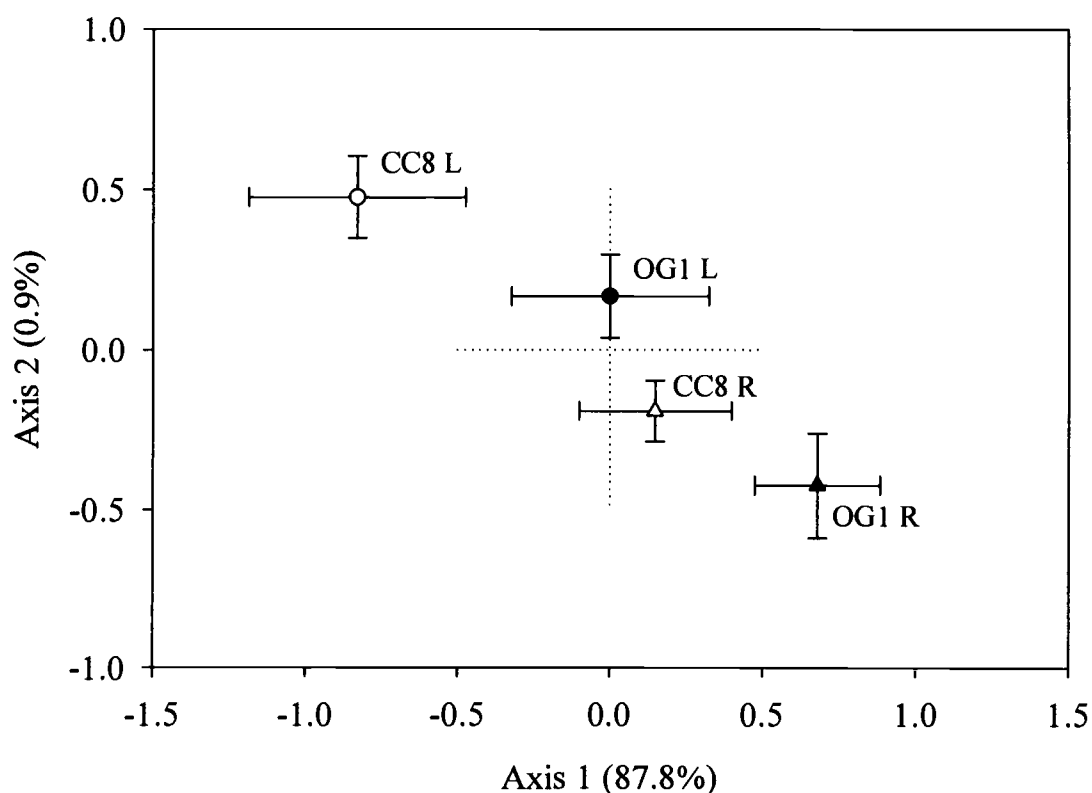

Figure 4.4. NMS plot of relative ^{13}C incorporation (% x 10^{-3}) for PLFAs collected at all seven sampling dates for OG1 and CC8 sites containing either ^{13}C-labeled litter (L) or root (R) material. ANOVA analysis revealed a significant effect of age and source along both axes (p ≤ 0.05).
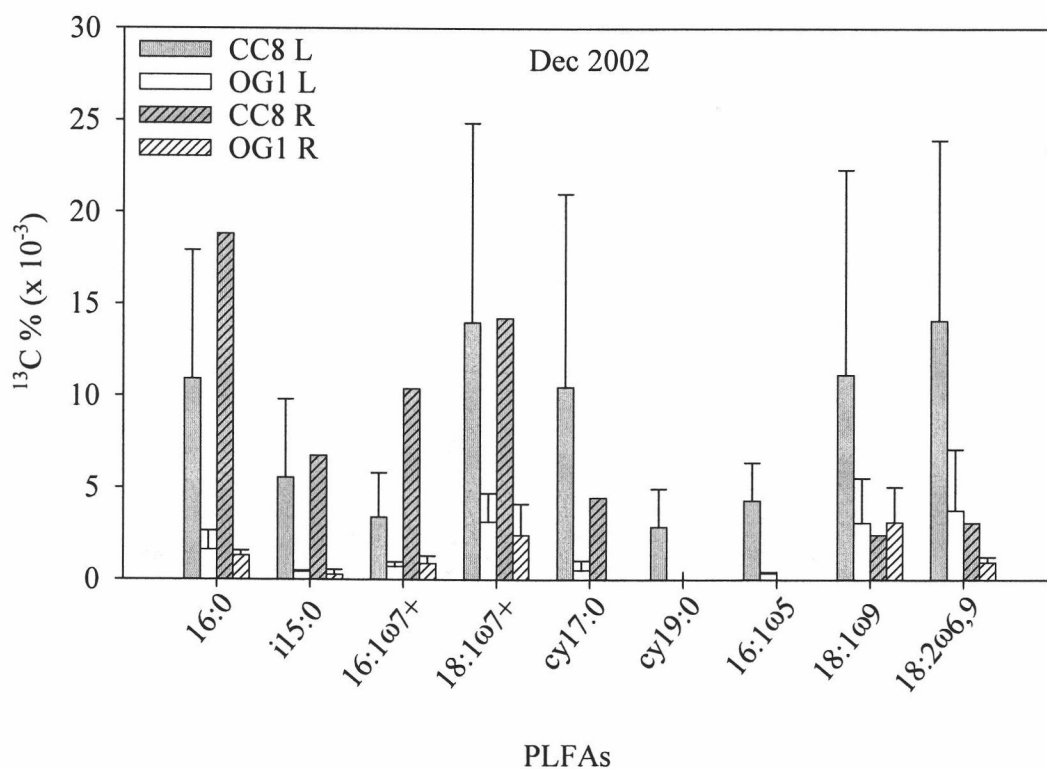
Figure 4.5. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source (litterbag (L) and root (R) samples) for Dec 2002. Values are means +/- standard error (n = 2 except for CC8 R where n = 1).

Table 4.2. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Dec 2002. *, **, and *** indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively. NS = not significant.

|	Source of variation		
PLFA	Age	Source	Age*Source
16:0	NS	NS	NS
i15:0	NS	NS	NS
16:1ω7+	NS	NS	NS
18:1ω7+	*	NS	NS
cy17:0	NS	NS	NS
cy19:0	NS	NS	NS
16:1ω5	NS	NS	NS
18:1ω9	NS	NS	NS
18:2ω6,9	NS	NS	NS

Figure 4.6. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source (litterbag (L) and root (R) samples) for Apr 2003. Values are means +/- standard error (n = 2).

Table 4.3. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Apr 2003. Contrasts are defined as: A_L= CC8L vs. OG1L; A_R = CC8R vs. OG1R; S_8 = CC8L vs. CC8R; S_{OG1}= OG1L vs. OG1 R. *, **, and *** indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively. NS = not significant.

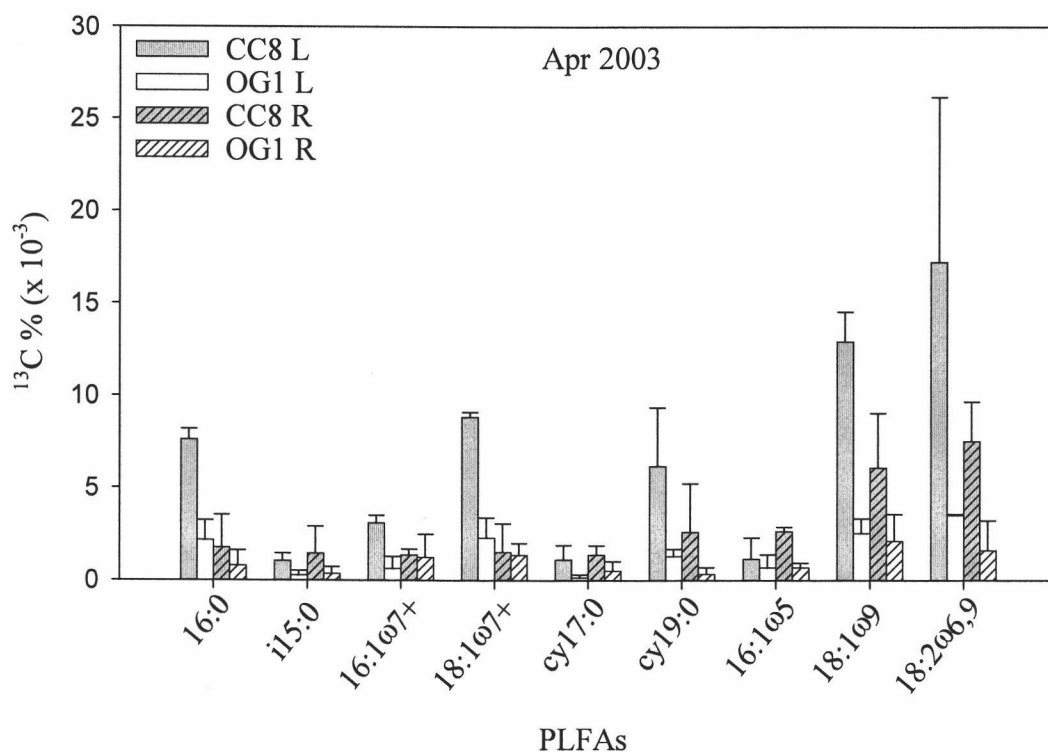| PLFA | Source of variation | | | Contrasts | | | |
	Age	Source	Age*Source	A_L	A_R	S_8	S_{OG1}
16:0	*	*	NS				
i15:0	NS	NS	NS				
16:1ω7+	NS	NS	NS				
18:1ω7+	*	*	*	**	NS	**	NS
cy17:0	NS	NS	NS				
cy19:0	NS	NS	NS				
16:1ω5	NS	NS	NS				
18:1ω9	*	NS	NS				
18:2ω6,9	NS	NS	NS				

Figure 4.7. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source (litterbag (L) and root (R) samples) for Jun 2003. Values are means +/- standard error (n = 2).

Table 4.4. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Jun 2003. The abbreviations are defined in Table 4.3.

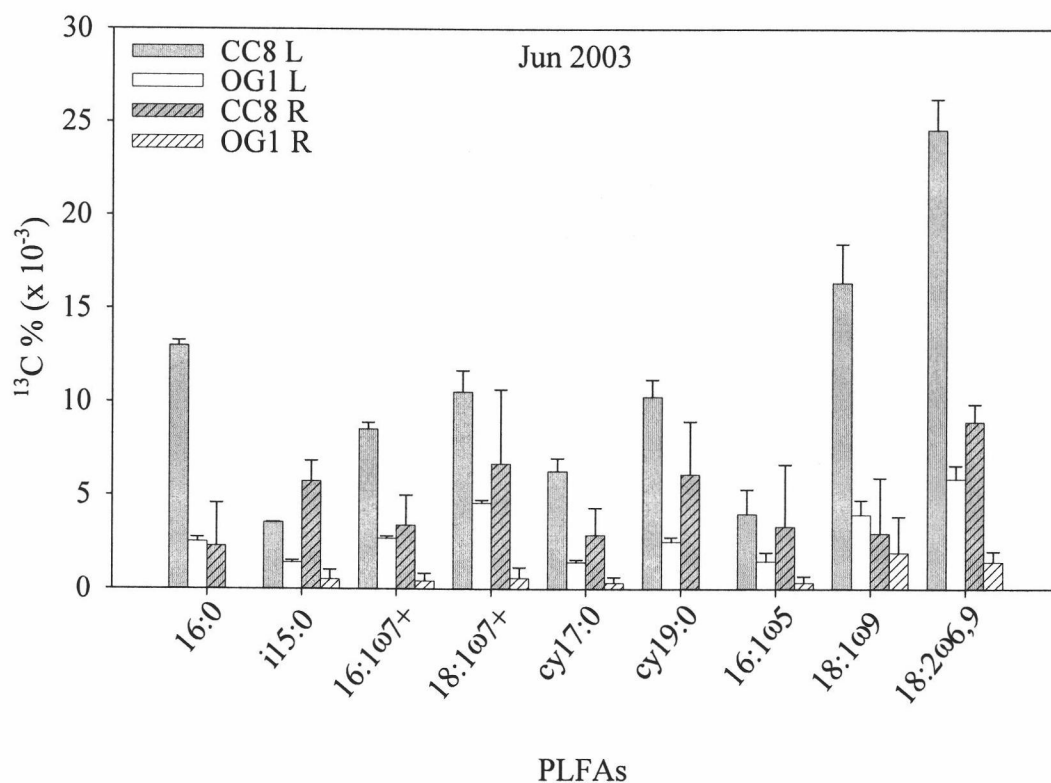| PLFA	Source of variation			Contrasts			
	Age	Source	Age*Source	A_L	A_R	S_8	S_{OG1}
16:0	**	**	*	**	NS	**	NS
i15:0	**	NS	NS				
16:1ω7+	**	*	NS				
18:1ω7+	*	NS	NS				
cy17:0	*	*	NS				
cy19:0	**	NS	NS				
16:1ω5	NS	NS	NS				
18:1ω9	*	*	*	**	**	NS	NS
18:2ω6,9	***	***	**	***	**	***	*
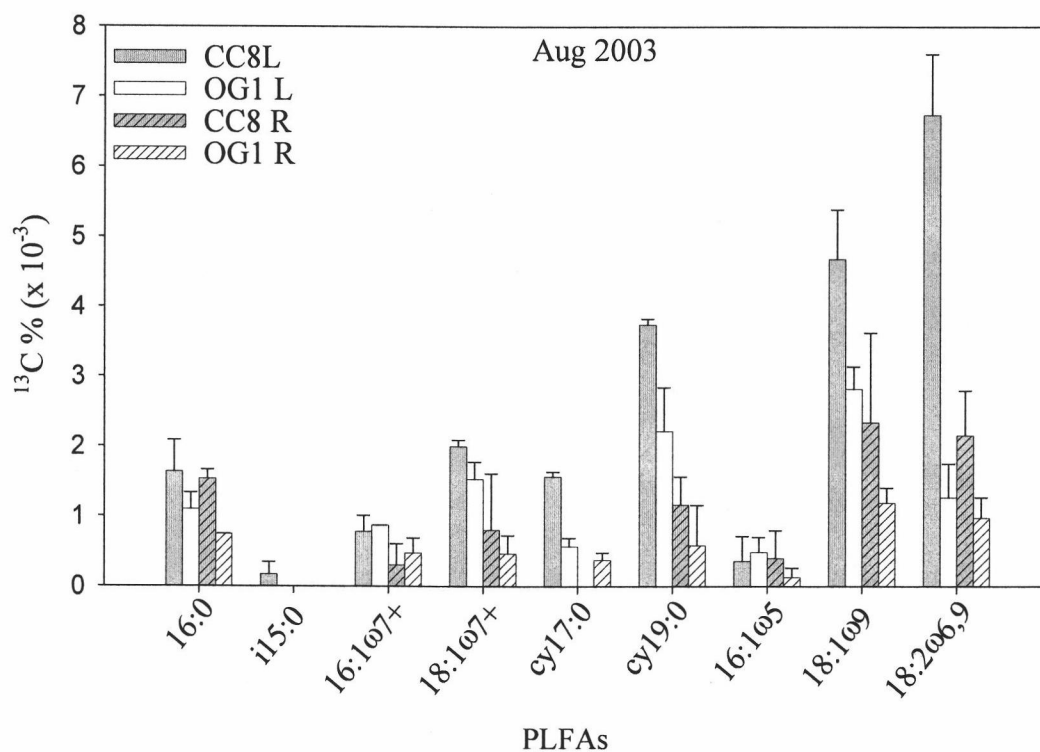
Figure 4.8. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source (litterbag (L) and root (R) samples) for Aug 2003. Values are means +/- standard error (n = 2).

Table 4.5. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Aug 2003. The abbreviations are defined in Table 4.3.

| PLFA | Source of variation | | | Contrasts | | | |
	Age	Source	Age*Source	A_L	A_R	S_8	S_{OG1}
16:0	NS	NS	NS				
i15:0	NS	NS	NS				
16:1ω7+	NS	NS	NS				
18:1ω7+	NS	NS	NS				
cy17:0	*	***	***	***	*	***	NS
cy19:0	NS	**	NS				
16:1ω5	NS	NS	NS				
18:1ω9	NS	NS	NS				
18:2ω6,9	**	*	*	**	NS	**	NS

Figure 4.9. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source (litterbag (L) and root (R) samples) for Nov 2003. Values are means +/- standard error (n = 2).

Table 4.6. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Nov 2003. The abbreviations are defined in Table 4.3.

PLFA	Source of variation			Contrasts			
	Age	Source	Age*Source	A_L	A_R	S_8	S_{OG1}
16:0	***	NS	*	*	***	NS	*
i15:0	NS	NS	NS				
16:1ω7+	NS	NS	NS				
18:1ω7+	***	**	NS				
cy17:0	***	***	***	***	NS	***	*
cy19:0	***	*	NS				
16:1ω5	**	NS	NS				
18:1ω9	NS	NS	NS				
18:2ω6,9	**	*	NS				

The PLFA 16:0 is a general biomarker found in all organisms. The relative amount of ^{13}C incorporated in 16:0 was found to be greater in samples from the CC8L site than OGL and OGR sites for April, June, and November 2003 (Figure 4.10). A similar trend was found for 18:1ω9 and 18:2ω6,9 (common fungal biomarkers) (Figure 4.11). In general, these PLFAs also had the greatest amount of ^{13}C incorporation throughout the study period (Figures 4.5 thru 4.9).

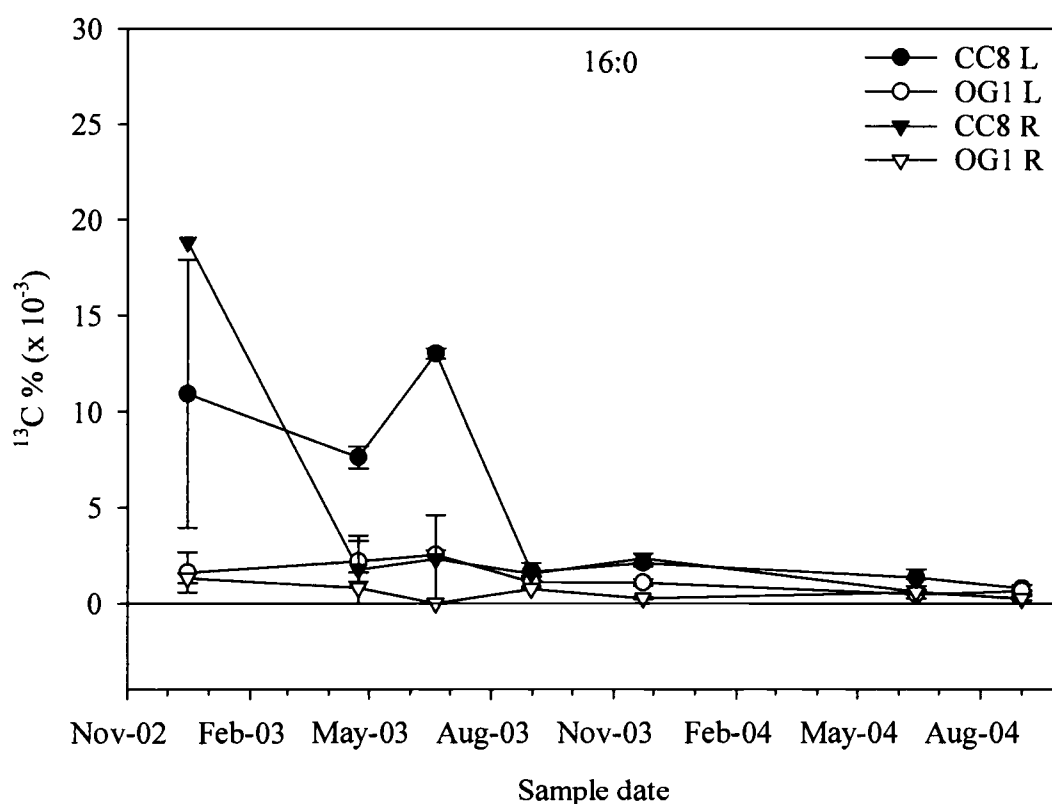Figure 4.10. Relative amount of ^{13}C (% x 10^{-3}) incorporated in 16:0 in OG1 (open symbols) and CC8 (closed symbols) for litterbag (L) and root (R) samples over the 22-month incubation.

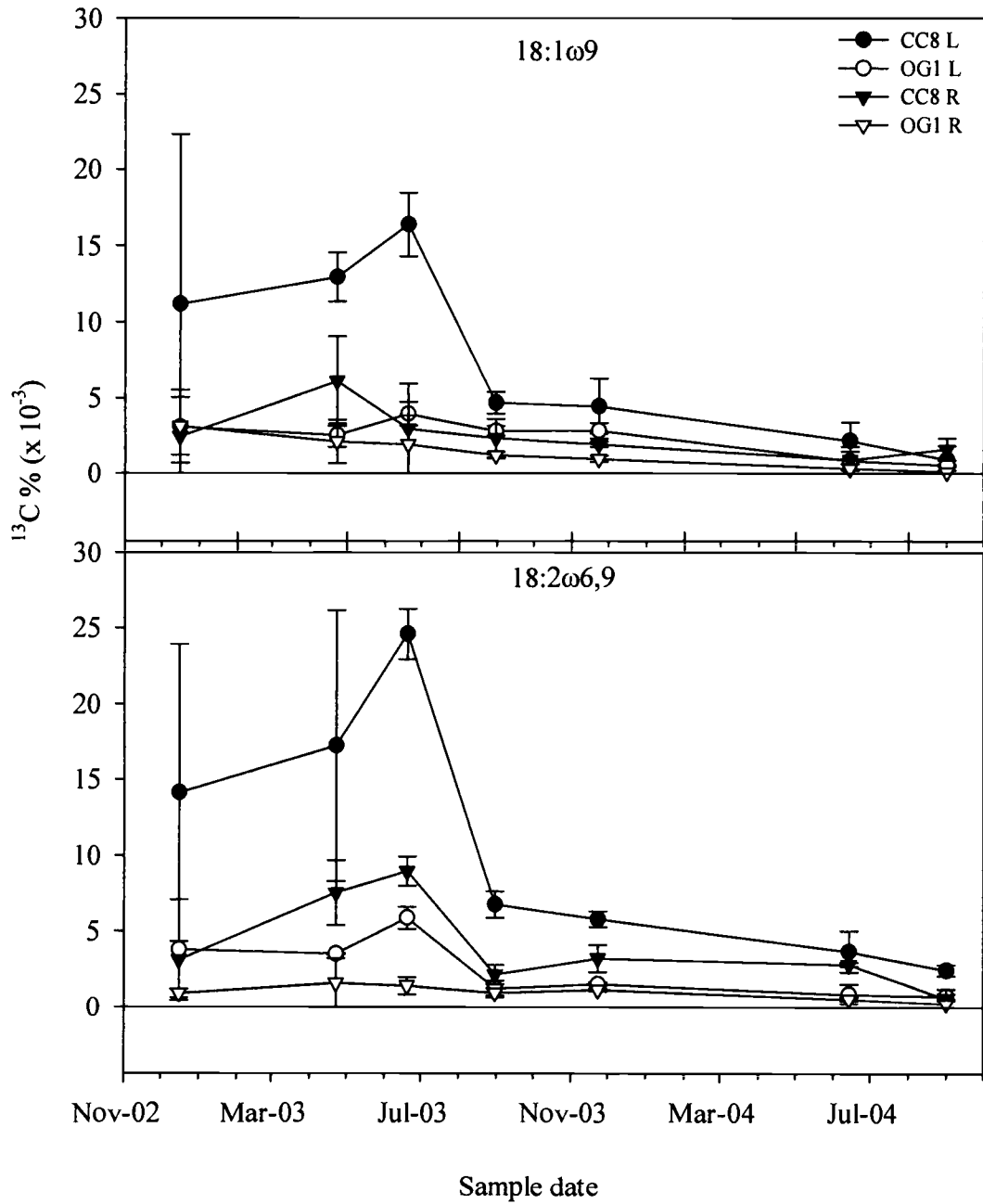Figure 4.11. Relative amount of ^{13}C (% x 10^{-3}) incorporated in 18:1ω7 (top) and 18:2ω6,9 (bottom) in OG1 (open symbols) and CC8 (closed symbols) for litterbag (L) and root (R) samples over the 22-month incubation.

^{13}C incorporation into PLFAs for OG2 and CC25 sites

An NMS plot was constructed for the relative ^{13}C incorporation (% x 10^{-3}) for PLFAs collected at all seven sampling dates for OG2 and CC25 sites. A two-dimensional solution was recommended with a final stress of 10.8 and instability of 0.00001. The ANOVA analysis, however, revealed no significant effect of either age or source along axis 1 or 2 (data not shown). Further data analysis on a date-by-date basis showed no significant effects of age, source or their interaction for samples collected in April and November 2003 and June and September 2004. Significant effects were found for samples collected in December 2002, and June and August 2004 (Figures 4.12-4.14 and Tables 4.7-4.9). Similar to the OG1 vs. CC8 results, the CC25 samples tended to have a greater amount of ^{13}C incorporated into the PLFAs as compared to the OG2 samples. Additionally, when there was a main effect of source, PLFAs from samples containing the ^{13}C-labeled litter had greater amounts of ^{13}C incorporated than did PLFAs from samples containing ^{13}C-labeled roots.

In general, 18:1ω9 and 18:2ω6,9 (common fungal biomarkers) had the greatest amount of ^{13}C incorporation throughout the study period ranging from 9.48x10^{-3} % in April 2003 to 1.06 x10^{-3} % in June 2004. A significant age*source interaction effect was present for the December 2002 samples (Table 4.7) for both PLFAs but only source was significant for 18:1ω9 for June 2003 samples (Table 4.8). In June and September 2004, the cyclopropyl PLFAs were also among the PLFAs with the greatest relative amount of ^{13}C incorporation.

Discussion

Effect of sample date on δ^{13}C-PLFA values

The δ^{13}C values (‰) for the control PLFAs collected in April 2003 and September 2004 are shown in Figure 4.1. Initially, we hypothesized that δ^{13}C signatures of PLFAs would not differ over time because one of the major C sources for the microbial biomass, soil organic C (SOC) was found to have a consistent δ^{13}C
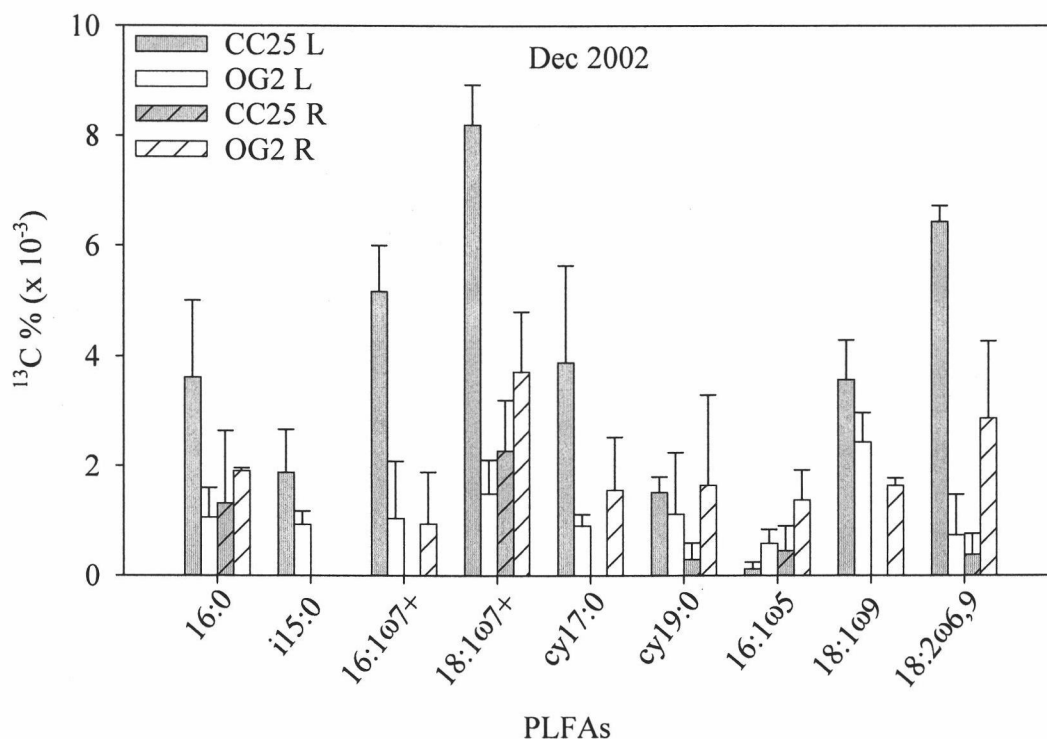
Figure 4.12. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source for Dec 2002. Values are means +/- standard error (n = 2).

Table 4.7. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Dec 2002. Contrasts are defined as: A_L= CC25L vs. OG2L; A_R = CC25R vs. OG2R; S_{25} = CC25L vs. CC25R; S_{OG2}= OG2L vs. OG2 R. *, **, and *** indicate significance at p ≤ 0.05, 0.01, and 0.001, respectively. NS = not significant.

	Source of variation			Contrasts			
PLFA	Age	Source	Age*Source	A_L	A_R	S_{25}	S_{OG2}
16:0	NS	NS	NS				
i15:0	NS	*	NS				
16:1ω7+	NS	*	*	*	NS	**	NS
18:1ω7+	*	NS	**	**	NS	**	NS
cy17:0	NS	NS	NS				
cy19:0	NS	NS	NS				
16:1ω5	NS	NS	NS				
18:1ω9	NS	**	*	NS	NS	**	NS
18:2ω6,9	NS	NS	**	**	NS	**	NS

Figure 4.13. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source for Jun 2003. Values are means +/- standard error (n = 2).

Table 4.8. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Jun 2003. *, **, and *** indicate significance at p ≤ 0.05, 0.01, and 0.001, respectively. NS = not significant.

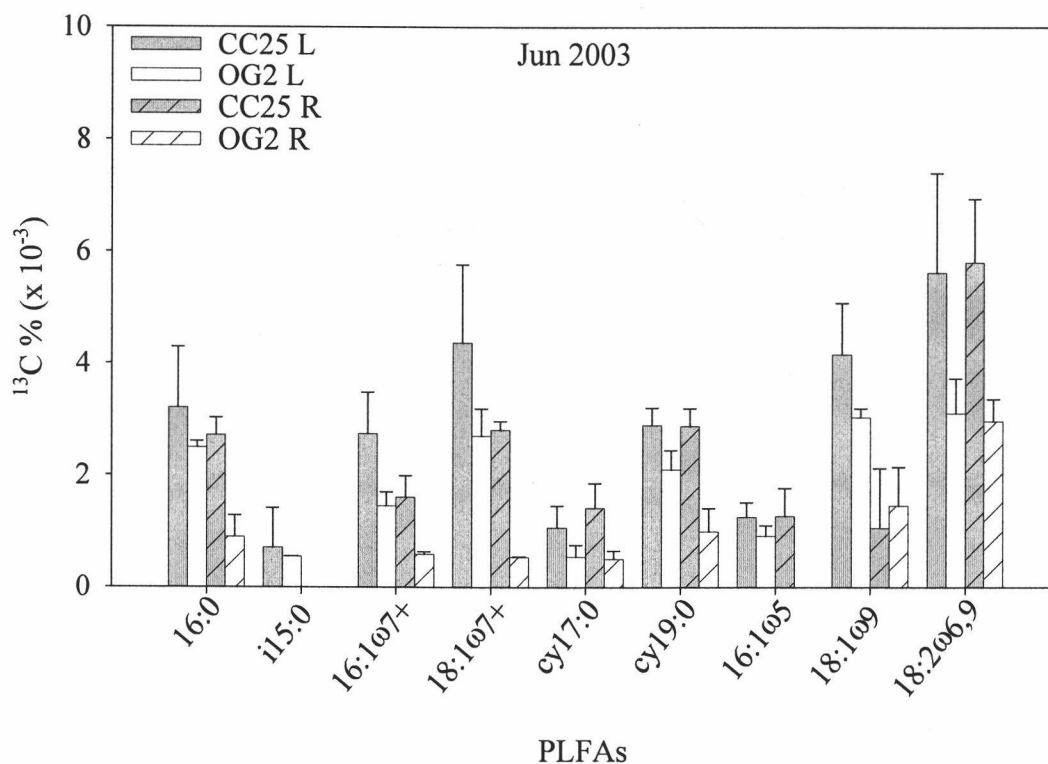| PLFA | Source of variation | | |
	Age	Source	Age*Source
16:0	NS	NS	NS
i15:0	NS	NS	NS
16:1ω7+	NS	NS	NS
18:1ω7+	NS	NS	NS
cy17:0	NS	NS	NS
cy19:0	NS	NS	NS
16:1ω5	*	NS	NS
18:1ω9	NS	*	NS
18:2ω6,9	NS	NS	NS

Figure 4.14. Relative amount of ^{13}C (% x 10^{-3}) incorporated into PLFAs as a function of stand age and ^{13}C source for Aug 2003. Values are means +/- standard error (n = 2).

Table 4.9. Levels of significance from ANOVAs for the relative amount of ^{13}C incorporated into PLFAs as a function of stand age and ^{13}C source for Aug 2003. Abbreviations are defined in Table 4.7.

PLFA	Age	Source	Age*Source	A_L	A_R	S_{25}	S_{OG2}
		Source of variation			Contrasts		
16:0	NS	NS	NS				
i15:0	NS	NS	NS				
16:1ω7+	NS	NS	*	NS	*	NS	NS
18:1ω7+	*	***	***	***	NS	***	*
cy17:0	NS	NS	NS				
cy19:0	NS	NS	NS				
16:1ω5	NS	NS	NS				
18:1ω9	NS	NS	NS				
18:2ω6,9	NS	NS	NS				

value within a forest stand over time (Fessenden and Ehleringer, 2002) and because a microbe's isotopic signature should reflect microbial C sources (Blair et al., 1985; Coffin et al., 1990; Abraham et al., 1998; Boschker et al., 1999). The average $\delta^{13}C$ values (‰) of SOC collected at 0-10 cm for OG1, CC8, OG2 and CC25 were not significantly different: -25.5, -26.3, -25.6, and -26.6, respectively (data not shown). These values are similar to those found by Fessenden and Ehleringer (2002) who collected samples from some of the same sites. Overall, the $\delta^{13}C$ value of PLFAs collected in April 2003 and September 2004 were -32.3 and -27.3 ‰, respectively. Similar variation in the $\delta^{13}C$ values of PLFAs were also shown to vary depending upon sample date in a study by (Pelz et al., 2005). Additionally, they also found that the majority of the PLFA values for C3 soils were ^{13}C depleted.

Not only were the PLFAs more depleted in ^{13}C relative to SOC values, but there was a significant difference in $\delta^{13}C$ value of four of the PLFAs collected between the two sample dates (Figure 4.1). The carbon isotopic signature of an organism has been shown to reflect (within 3-6 ‰) the C isotope ratios of their C substrate (Blair et al., 1985; Coffin et al., 1990; Abraham et al., 1998; Boschker et al., 1999; Abraham and Hesse, 2003) and is largely controlled by the biological mechanism of C assimilation and pathways of lipid biosynthesis (Abraham et al., 1998; van der Meer et al., 1998; Abraham and Hesse, 2003; van der Meer et al., 2003). One of the main sources of C for microorganisms is soil organic matter, which typically is found in higher concentrations near the surface of a soil profile and decreases with depth. At our sample depth of 5 cm below the litter layer, four out of the nine PLFAs were found to be significantly more positive in samples collected in September 2004 than those collected in April 2003. It is possible that these differences are attributed to the response of the microbial community to conditions of stress such as the relatively high soil temperatures that occur during the late summer. The September 2004 sample date is characterized by an average soil temperature of 16.7°C (typical temperature for summer) and soil moisture content of 27% (v/v) (Chapter 2). The soil moisture content was atypically moist for this time of year but was preceded by a period of no precipitation and soil moisture contents falling below

15%. High soil temperatures and extreme moisture contents have been associated with a shift in the proportion of certain PLFAs produced by the microbial biomass such as increased degree of saturation and increased ratios of cy17:0 and cy19:0 to their precursors (Kieft et al., 1994; Zogg et al., 1997). In an additional study involving the same soil samples collected for the present study, we found significant increases in the ratios indicative of stress during the hot and dry soil environment of August (Chapter 2). Abraham et al. (1998) found that the C isotope ratio was independent of growth stage but they did not assess the C isotope ratio under stressed conditions such as temperature or moisture extremes.

It is not possible from our limited sampling protocol to extract whether the slightly enriched ^{13}C values measured in PLFAs collected in September 2004 are the result of reduced discrimination against the heavier ^{13}C during this time or if other factors are responsible. In particular, ^{13}C values of lipids and biomass measurements from fungi vary depending upon C source (Henn and Chapela, 2000; Hobbie et al., 2004) and type of fungi (ectomycorrhizal fungi are typically enriched in ^{13}C compared to saprotrophic fungi) (Henn and Chapela, 2001; Hobbie et al., 2004; Trudell et al., 2004). Stahl and Klug (1996) found that the temperature at which a fungus was cultured affected its fatty acid composition but the degree of change was dependent upon the fungus. They also indicated that temperature differences did not affect the types of fatty acids present but rather their relative amounts. For example, stearic acid (18:0) was found to be depleted in ^{13}C relative to 16:0 in all fungal strains whereas the opposite pattern was observed in bacteria (Abraham et al., 1998). These findings, coupled with the variability in ^{13}C signatures found during our two sampling dates, are compelling evidence for the need for further *in situ* sampling over different seasons. Without this information, interpretation of what is taking place under natural conditions with only one sample point may be misleading.

Effect of stand age on $\delta^{13}C$-PLFA values

A greater amount of [13]C % was incorporated in some of the PLFAs from CC8 samples compared to OG1 samples in samples collected in five out of the seven

sample dates. This finding may be related to differences in the fungal communities between the two groups. We found a greater amount of ^{13}C was incorporated into the PLFAs 18:1ω9 and 18:2ω6,9 in samples from CC sites when compared to OG sites. These PLFAs are commonly used as fungal biomarkers although 18:1ω9 has also been found in bacteria (Zelles, 1997). A strong correlation (R = 0.96) was found between 18:1ω9 and 18:2ω6,9 in the amount of ^{13}C incorporated in each (Figure 4.15) suggesting that the ^{13}C signatures of both PLFAs are associated with the fungal community.
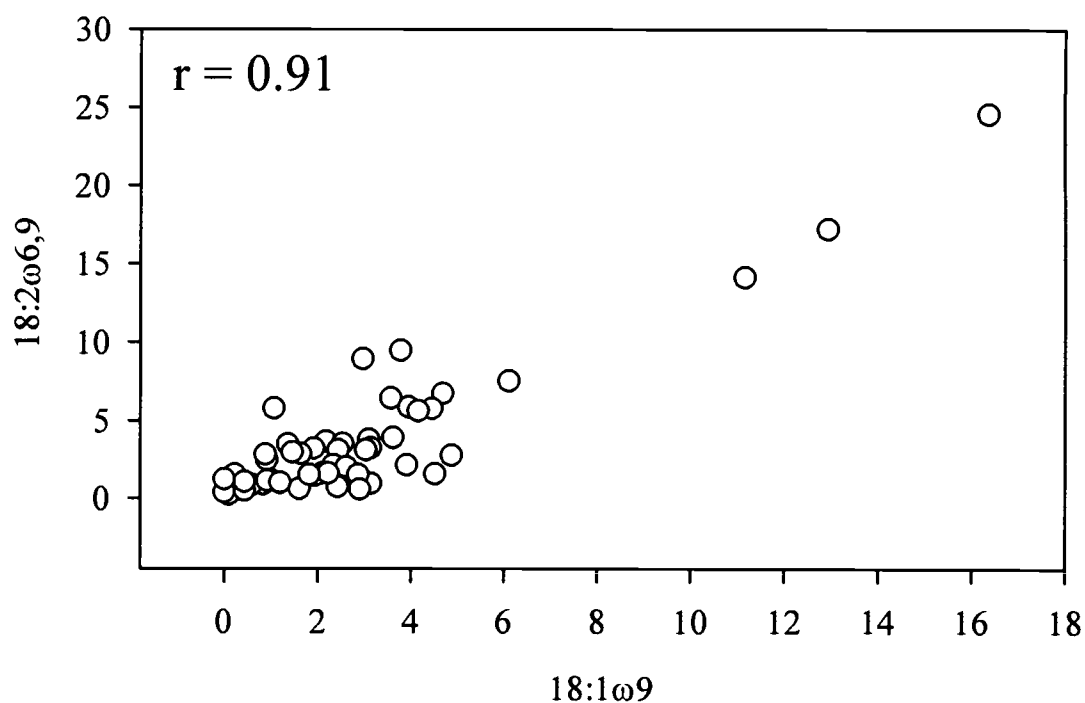
Figure 4.15. Correlation between the PLFAs 18:1ω9 and 18:2ω6,9 for all samples.

In forest ecosystems, mycorrhizal and saprophytic fungi are important drivers of C dynamics. In the litter layer alone, fungi can account for 10-60% of the total biomass (Newell, 1992). Fungi are considered to be the primary decomposers of the more recalcitrant C compounds such as lignin and are commonly found in forest soils (Hammel et al., 1993). Ectomycorrhizal fungi can increase the effective root surface of trees for nutrient and water uptake through mycorrhizal associations (Fogel and

Hunt, 1983; Killham, 1994). Fungal biomass has been shown to decrease following clear-cut harvesting (Bååth, 1980), likely because of disturbance to the mycorrhizal associations between fungi and trees. Because $\delta^{13}C$ values of PLFAs represent values of metabolically-active populations within the microbial community (Pelz et al., 2005), we hypothesized that fungi would be less active (i.e., incorporate less ^{13}C) in samples from the CC sites compared to those from the OG sites. However, our results suggest the opposite. One possible explanation involves a limitation of the ^{13}C-PLFA technique; the PLFA analysis does not distinguish between microbial species.

The major fatty acids found in ectomycorrhizal and saprophytic fungal species are 18:2ω6,9, 18:1ω9, and 16:0; which together can make up over 90% of the total fatty acid content (Stahl and Klug, 1996; Sancholle et al., 2001). The biomarker most commonly used for fungi is 18:2ω6,9, however 18:1ω9 is sometimes used as well (Federle et al., 1986; Phillips et al., 2002). We are using the relative amount of ^{13}C incorporated into the PLFAs as a proxy for the active microorganisms that decompose ^{13}C-labeled litter and root material. Ectomycorrhizal fungi get the majority of their C directly from photo-assimilated C from the host, whereas saprotrophic fungi must rely on decomposition processes. About 15% of net primary production was consumed by mycorrhizal fungi in a mature stand of Pacific silver fir (*Abies amabilis*) (Vogt et al., 1982). Therefore, it is possible that the ^{13}C signal coming from the fungal biomarkers in the OG samples is largely from unlabeled C originating from photosynthetic sources. In contrast, the ^{13}C signal coming from the fungal biomarkers in the CC samples may be largely from the decomposition of the labeled material by saprophytic fungi. In both sites, fungi are present and active, however the signal we obtain may be coming from different types of fungi utilizing different C sources.

The high $\delta^{13}C$ variability of all soil PLFAs suggests that the microbial community is simultaneously utilizing different C sources. It would be expected under field conditions that microorganisms not only assimilate the new organic material that we added but also would use, to a large extent, old organic material (Pelz

et al., 2005). Further testing under controlled laboratory conditions is needed to separate these complex decomposition pathways and microbial C assimilation.

The turnover rate of Douglas-fir litter has been shown to range between 0.27 to 0.37 year^{-1} (Harmon et al., 1990; Valachovic et al., 2004), whereas fine-roots turnover more slowly, at 0.197 year^{-1} (Chen et al., 2002). It was beyond the scope of this study to determine decomposition rate constants. We placed our litter bags at the soil/litter layer interface to track decomposition into the soil microbial community whereas traditional litter decomposition studies place them at the forest floor surface. We also did not use root bags to enclose our ^{13}C-labeled roots because we wanted to ensure adequate soil contact to reflect the natural soil environment.

Regardless, our ^{13}C-PLFA results suggest that needles were decomposed faster than was root material because, for a given stand age, generally less ^{13}C was incorporated in root samples relative to litter samples. This trend appeared to be most prevalent in CC8 samples and especially for the fungal PLFAs (18:2ω6, 9 and 18:1ω9). This relatively high amount of ^{13}C incorporated in the fungal biomarkers, especially in the samples containing ^{13}C-labeled litter, suggests that fungi have grown up into the litterbag and translocated C into the soil layers. We did not account for movement of ^{13}C as a result of soluble C sources that easily move through the soil. It is possible that some of the ^{13}C moved out of our sampling area (0-5 cm soil) in the root samples as the roots were placed within the top 5 cm of mineral soil. If leaching from the litterbags occurred, it is likely that the microorganisms consumed the C within the 5 cm below the litterbag and that we were able to detect it. Because water-soluble C is a relatively labile C source, it would seem reasonable that the ^{13}C would have been incorporated into the bacterial biomarkers more than the fungal biomarkers, especially Gram-bacteria, because biomarkers for these bacteria have been shown to increase with the availability of organic substrates (Bossio and Scow, 1998; Burke et al., 2003).

Perspectives

In this study we tracked ^{13}C from labeled Douglas-fir litter or root material into the microbial phospholipid fatty acids during decomposition. This is a first step to extend our previous work on the soil microbial community structure as affected by stand age of Douglas-fir as well as seasonal influences (Chapter 2). Background levels of the control PLFA ^{13}C signatures varied significantly between the two sample dates but these temporal differences may be small depending upon the level of ^{13}C enrichment of lipids from labeled litter amended soils. Enriched ^{13}C levels were significantly greater than background levels in some of the PLFAs after one month's field incubation of labeled microcosms. Subsequent destructive sampling over a 22-month period showed there was seasonal variation in the degree of ^{13}C enrichment of individual PLFAs. The seasonal variability of C acquisition by microorganisms showed the importance of capturing seasonal dynamics to understand the fate of C and to correctly identify the microbial players. This supports the previous work of Lipson and coworkers (2002) and Brant (2005) who showed strong changes in microbial communities, especially of fungi, on a seasonal basis. The rapid loss of litter and labeling of lipids after only one month suggests that more frequent time intervals are needed immediately after ^{13}C-labeled materials are added to soils even under field conditions.

We applied the material in late fall thinking the cool temperatures and snow cover would suppress rapid decomposition. However, recent studies by Lipson et al. (2002) and Schadt et al. (2003) have shown high microbial biomass and activity during the winter, especially involving soil fungi. Regardless, we were able to detect enriched levels of ^{13}C in the fungal biomarker at the end of the 22-month period, especially in the clear-cut sites. Our work suggests that the ^{13}C-PLFA method is powerful and robust and can substantially aid soil ecologists at the field scale. Additional recommendations are that when litter is placed within the surface litter layer and above the mineral soil, sampling increments should be 0 to 2.5 and 2.5 to 5.0 cm below the litter layer, rather than 0 to 5 cm depth, because it seems likely that the highest level of enrichment was in the 0-2.5 cm depth. We also found high variability

of ^{13}C lipids that was much higher than other non-isotopic microbial assays we have utilized before. We attribute this to high small-scale variability of the isotope technique relative to microbial uptake of C. From an experimental perspective this would suggest there is a need to have smaller litter pieces that are highly homogenized if incorporated in soils or evenly distributed in the litter layer for litter layer placement of labeled materials. These modifications as well as advances in GC-C-IRMS systems and analytical software will likely prove extremely useful for further interpretation into microbial community structure and function *in situ.*

Acknowledgements

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GENERAL CONCLUSION

Microorganisms are the driving force behind litter decomposition and nutrient cycling and are affected by differences in litter quality, temperature, and moisture. Although considerable progress has been made in understanding the factors affecting the rates of litter decomposition and formation of soil organic matter, most of what is known about the role of microbial functional groups during decomposition/C sequestration in soils is based on indirect methods that use inferences such as activity measurements, biomass C, or respiration to study the "black box" of decomposition. The goal of this dissertation was to determine how season and clear-cutting influenced the soil microbial community (SMC) structure. In particular, we wished to investigate the use of the stable isotope ^{13}C coupled with phospholipid fatty acid (PLFA) analysis in the field to track decomposition of ^{13}C-labeled materials.

In Chapter 2, we investigated the influence of clear-cutting and season on soil microbial community structure using PLFA profiles from soils collected in old-growth and clear-cut Douglas-fir ecosystems. The major findings were:

- SMC structure and physiological status were affected by time since clear-cutting; the most recently cut stand had the lowest total microbial biomass as well as the lowest bacterial and fungal biomass concentrations.

- Seasonal changes were more influential than clear-cutting in determining the soil microbial community structure. More specifically, the combined effect of soil moisture and temperature stress in August samples was reflected in changes to the SMC structure and physiological status.

In Chapter 3, we presented a pulse-labeling technique to label Douglas-fir seedlings with ^{13}CO$_2$. We successfully labeled more than 1.5 kg of Douglas-fir seedling dry matter with ^{13}C at a relatively uniform distribution of ^{13}C among seedling parts: needles = 1.57, stems = 1.45, and root = 1.36 atom %. These ^{13}C abundance values were well above background levels and were well suited for the final objective of use in field incubation studies. The method and chamber design described could be

adapted to label other plant species at enrichment levels for multiple field or laboratory experiments.

In Chapter 4, we used the ^{13}C-labeled litter or root material in microcosms installed in old-growth and clear-cut sites and the soil was analyzed for the relative amount of ^{13}C incorporated (^{13}C %) into PLFAs. The key findings from this experiment were:

- We tracked the decomposition of this material into the PLFAs of microorganisms that were directly involved. The greatest enrichment was found in PLFAs from clear-cut samples compared to old-growth samples, especially in PLFAs used as fungal biomarkers.

- A significantly greater amount of ^{13}C % was incorporated in CC8 samples compared to OG1 samples in samples collected in five out of the seven sample dates.

- A significantly greater proportion of ^{13}C was incorporated into soil samples containing the ^{13}C-labeled litter material ("L") relative to that found in samples containing ^{13}C-labeled root material ("R") in April, June, August, and November 2003.

- Fungi appeared to be key players in the decomposition of the ^{13}C-labeled material, especially in clear-cut sites.

Terrestrial ecosystems and organic C stored in soils are important components in regulating atmospheric CO_2 and global climate change. In particular, temperate forest ecosystems have been implicated in holding a great potential for storing C. Most estimates focus on aboveground biomass however soils, which contain about 1500×10^{15} g of C, store more than 2.5 times the amount of C stored in land plants and twice the amount of C stored in the atmosphere. In order to make accurate predictions in global climate change models, it is critical to understand what controls soil microbial community functions such as decomposition of organic material. The intricate web of climate, plant inputs, and microbial processes is poorly understood in soil systems but with advances in soil microbiology and analytical methods, researchers are beginning to realize the important role that soils may play in both

moderating and accelerating the release of greenhouse gases into the atmosphere. The results presented in this dissertation are but a small step in understanding the complex world of microbes and how forest management practices and seasonal dynamics influence their community and C cycling. Future research using an integrative approach that investigates the response of the entire ecosystem from belowground to above the canopy is necessary in balancing the C budget and addressing the needs of climate change research.

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