AN ABSTRACT OF THE THESIS OF

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Title: Linking Soluble C to Microbial Community Composition and Dynamics During Decomposition of ^{13}C-Labeled Ryegrass

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Ryegrass residue consists of three main C fractions: readily available soluble C, intermediately available cellulose and hemicellulose, and slowly available lignin. Changes in chemical composition during decomposition influence rate of degradation as well as composition of the microbial community involved. Use of ^{13}C-labeled plant material coupled with analysis of phospholipid fatty acids (PLFA) by isotope ratio mass spectrometry results in a powerful tool for linking microbial community structure and C cycling processes during decomposition.

The objective was to investigate the role of soluble C in the decomposition of ryegrass straw. We wanted to determine (i) if the presence or absence of labile C in straw affects C mineralization by the microbial community, (ii) if community structure would differ based on the presence of labile C, and (iii) if community structure would shift as decomposition progressed.

Residue was added to soil microcosms at rates that reflect field loads. Treatments were unleached straw (US), leached straw (LS), and leachate (L), plus an unamended control (C). Added substrates had δ^{13}C values between 120‰ and 180‰; the native soil signature was –26‰. Respiration was measured every 4 to 6 hours for the first 5 d, and

weekly thereafter. Destructive sampling took place after 0.6, 1.6, 15, 18, 50, and 80 d of incubation and microbial biomass ^{13}C (MBC) and PLFAs were analyzed.

The soluble component of ryegrass straw strongly influenced C mineralization and assimilation, as well as microbial community composition and dynamics. CO_2 evolution rates and $\delta^{13}C$ signatures were similar in US and L during the first 3 d of incubation. Most soluble C from leachate was consumed during that time, indicated by the rapid decrease in $\delta^{13}C$ value of CO_2 evolved from L treatment. Substrate-derived C moved quickly into and through the microbial biomass.

Distinct temporal shifts occurred in community composition. Early communities in amended soils were dominated by short and branched-chain PLFAs such as 15:0a. Later samples contained more complex and longer PLFAs. 19:0cy was an indicator for late succession communities in US and L, and 18:2ω6,9 characterized late samples in LS. Soluble C affected when the temporal shift occurred; in LS and L, communities shifted earlier than in US. Lipids were differentially enriched with ^{13}C. Fungi, as indicated by 18:2ω6,9, were more effective at incorporating substrate C into cellular lipids, as this was the most highly labeled of all PLFAs.

Linking Soluble C to Microbial Community Composition and Dynamics During Decomposition of ^{13}C-Labeled Ryegrass

By Shawna K. McMahon

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Dr. Myrold and Dr. Bottomley assisted with data collection and analysis. Dr. Williams provided methodological training and advice with phospholipid fatty acid analysis.

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Introduction

This review examines literature pertaining to four main topics. First, issues surrounding plant residue decomposition will be briefly addressed, including the importance of soluble and recalcitrant components of plant residue, microbial dynamics during decomposition, microbial efficiency in carbon (C) assimilation, and the priming effect. Second, the relationship between soil microbial community diversity in relation to its function will be considered, as well as how substrate quality affects diversity. Third, basic concepts of phospholipid fatty acids (PLFAs) will be discussed, including their applications in soil microbial ecology, an examination of current methodology and some of the limitations of PLFA analysis. Finally, the application of the stable C isotope ^{13}C to microbial community and decomposition studies will be discussed, including issues pertaining to isotope fractionation and how ^{13}C is measured in biological samples.

Role of Substrate Quality in Residue Decomposition

Quality can be thought of as the ability of a substrate to provide C, energy, and nutrients to the microbes that degrade it. Substrate quality is dependent on its biochemical composition, physical nature, and the needs of the organism consuming it (Bending and Turner, 1999). Quality of C is particularly important because it constrains the supply of energy for enzyme production and growth (Fontaine *et al.*, 2003). For the purposes of decomposition, plant residue consists of three main C fractions: readily available soluble C, intermediately available cellulose and hemicellulose, and slowly available lignin (Jawson and Elliott, 1986).

Plant residues may contain up to 25% soluble, readily decomposable compounds (Swift *et al.*, 1979). This broadly defined pool of C consists of simple carbohydrates such as sucrose, fructans and glucose, and amino acids. Gunnarsson and Marstorp (2002) studied decomposition of the 18 main carbohydrates found in monocot and dicot plants. They found a wide range of turnover times ranging from a few hours for simple sugars and fructans to several weeks for cellulose; pectins and hemicelluloses had intermediate

decomposition times. Of the compounds commonly found in the soluble pool of C, sucrose, fructose, raffinose, and fructan decomposed most rapidly.

Because different components of plant residue are degraded at different rates, chemical composition changes with time (Cheshire *et al.*, 1988; Horwath and Elliott, 1996). Decomposition rate in early stages is dictated by the size of the soluble C pool and an intermediately available C fraction that is not water-soluble (Saviozzi *et al.*, 1997). Polymeric plant components, such as cellulose, must be degraded to sugars and other simple compounds by extracellular enzymes before microbes can use them (Schimel, 1995). Therefore, as decomposition progresses and remaining materials become increasingly recalcitrant, C liberation slows because acquisition of energy is slow (Fontaine *et al.*, 2003). Decomposition of recalcitrant components is limited by its chemical structure; lignin is closely associated with cell wall matrix polysaccharides, such as hemicellulose, rendering it less available to microbes (Horwath and Elliott, 1996; Kondo *et al.*, 1990).

Separation of the soluble and insoluble fractions of straw promoted more rapid decomposition of the soluble C fraction during the first 4 d of incubation in an experiment with wheat straw (Cogle *et al.*, 1989). Respiration from leachate was greater than from intact straw during this time, indicating that some of the labile C fraction is protected by structural components of straw. Decomposition is faster when microbes can access the lumen of straw without having to go through outer layers of lignin (Summerell and Burgess, 1989).

Reinertsen *et al.* (1984) observed greater CO_2-C evolution after 20 d from non-leached than from leached straw. However, Cochran *et al.* (1988) assert that this observation may be due to decomposing microbial biomass and not necessarily increased straw decomposition. This idea follows from the concept that a succession of organisms is responsible for decomposition of crop residues, resulting in concurrent mineralization and immobilization of C.

Many of the dynamics observed in microbial biomass are occurring on a millimetric or smaller scale. Therefore, decomposing particulate organic residues rapidly induce strong gradients of microbial activity in soil. Gaillard *et al.* (1999) were able to

prove these concepts by placing ^{13}C-labeled straw in soil and measuring ^{13}C-derived microbial biomass on the straw, 0-4 mm from the straw and further than 4 mm. No ^{13}C was found further than 4 mm from the straw surface.

The succession of organisms responsible for plant residue decomposition generally moves from a bacterial-dominated community early on to a fungal-dominated community during later stages (Griffiths *et al.*, 1999). Community succession can also be described as moving from r-strategists to K-strategists. r-strategists go through a short phase of rapid growth when readily available C is added to soil, then shut down metabolic activity and wait for the next input. Conversely, K-strategists grow and divide steadily over long periods, utilizing widely distributed but recalcitrant substrates (Jenkinson, 1988).

Microbial succession plays an important role in decomposition because microbial populations display enzymatic specificity for substrate degradation. Therefore, a range of organisms is required over the course of decomposition to provide all the enzymes necessary for depolymerization of plant structural components (Fontaine *et al.*, 2003).

Tester (1988) provides an alternative view of the role of microbial communities during residue decomposition. They determined that mixing wheat straw with soil had no effect on decomposition for the first 15 days compared with that of straw alone. This indicated that straw residue is populated with organisms capable of degrading readily available plant components and that soil organisms only become important later in decomposition.

Carbon Assimilation Efficiency

Substrate C acquired by soil microbes will experience one of four possible fates. First, it can be stored or incorporated among cytoplasmic constituents. Second, it can be incorporated into structural components of a cell. Third, it can be oxidized to produce energy for use in growth or maintenance. Finally, it can be released as extracellular enzymes or polysaccharides (Bremer and van Kessel, 1990).

Fungal C assimilation efficiencies range from 30 to 70%, whereas bacteria are less efficient at 20 to 40% (Holland and Coleman, 1987). These ranges indicate that

efficiency is not a static property of microbial communities. Measurements of CO_2 evolution during extended decomposition experiments indicate that growth efficiency of microbial populations decreases with time (Jawson and Elliott, 1986). Lower rates of mineralization are often observed with smaller additions of substrate, suggesting more efficient use or less turnover of microbes and their products (Bremer and Kuikman, 1994; Griffiths *et al.*, 1999). Under C starvation conditions, Morita (1988) found evidence of metabolic arrest in microbes without inhibition of C uptake. This could explain unusually high efficiencies observed in some experiments; C is accumulating without incorporation into microbial biomass.

Unbalanced growth occurs when a suitable C source is present, but one or more essential nutrients are absent (Zelles *et al.*, 1994). It can also occur in response to inadequate moisture, pH, light, or temperature (Vestal and White, 1989). It is commonly associated with the accumulation of energy reserve polymers like poly-β-hydroxybutyrate (PHB), glycogen or storage lipids (Dawes and Senior, 1973; Nickels *et al.*, 1979; Findlay and White, 1983; Tunlid and White, 1992). Thus, the ratio of PLFA to PHB, as indicators of cellular growth and C accumulation, reflects growth conditions experienced by soil microbes (Tunlid and White, 1990).

Priming Effect

The priming effect is considered to be the enhanced breakdown of native soil organic matter after the addition of easily decomposable organic substances to soil (Sauerbeck, 1966; Kuzyakov *et al.*, 2000). However, it is possible to have negative priming, where the addition of readily available C suppresses decomposition of soil organic matter (SOM) resulting in immobilization of added C (Kuzyakov *et al.*, 2000).

In a review of mechanisms responsible for causing priming, Kuzyakov *et al.* (2000) stated that the following are undisputed facts about the priming effect:

1. The priming effect arises immediately or very shortly after the addition of a specific substance;
2. Priming is greater in soils rich in C than in soils low in C; and

3. The magnitude of priming increases with the amount of added organic substance.

However, in 1966 Jenkinson observed a positive priming action in soil containing 3% organic matter and negative priming in soil containing 13% organic matter. Clearly, priming is not always greater in high-C soils.

A primary mechanism involved in the priming effect is the impact of added organic matter on the native microbial population. The addition of fresh organic matter to soil induces rapid biosynthesis and increased overall activity (Dalenberg and Jager, 1981; Fontaine *et al.*, 2003). A larger, more active population is capable of increased enzyme production, which can increase co-metabolic decomposition of SOM (Fontaine *et al.*, 2003). Small organic acids released during decomposition can enhance SOM decomposability through chemical disruption (Kuzyakov *et al.*, 2000).

The magnitude of priming depends strongly on the form of C added. Glucose has been shown to induce both a negative and positive priming effect (Jenkinson, 1966), but in general, the addition of complex compounds, such as cellulose or wheat straw, results in a larger priming effect than with additions of simple sugars (Fontaine *et al.*, 2003). Fontaine *et al.* (2003) recently attributed these observations to the relationship between microbes employing r and K strategies for growth. They suggested that r-strategists grow rapidly using readily available components of added organic matter, but are unable to use SOM C directly. On the other hand, K-strategists grow more slowly and are able to produce extracellular enzymes that are effective at degrading recalcitrant compounds in added material and SOM. Thus, the addition of complex organic substances results in a larger priming effect because increased growth is induced in the K-strategist population after the r-strategists die off following substrate depletion.

Not all observed priming effects are real. Carbon isotopes, either stable or radioactive, are necessary to distinguish between C released from added substrate or SOM (Kuzyakov *et al.*, 2000). However, non-uniform labeling of plant material or isotope fractionation in microbial processing can give inaccurate estimates of substrate- vs. soil-derived C. Plants exposed to labeled C for a short time will have a more highly labeled water-soluble fraction. Because this labile pool of C is degraded rapidly at the

beginning of decomposition, the CO_2 evolved will be more enriched than the overall plant $\delta^{13}C$ signature would suggest. The apparent priming action will be negative in this case (Jenkinson, 1966). If the soluble fraction is less highly labeled than the average plant signature, an artificial positive priming effect may be observed. During later stages of decomposition, it is difficult to be certain that CO_2 is actually derived from added substrate, as plant tissue could have been incorporated and re-mineralized from the SOM pool (Sauerbeck, 1966).

Microbial Community Diversity and Function

The composition of microbial communities affects degradation of macromolecular C compounds because only a relatively small number of populations have the enzymatic capability to degrade them. Therefore, enzymes that degrade larger substrates may be more closely related to community composition than enzymes that degrade simpler substrates (Waldrop *et al.*, 2000).

Fungi may be more important in the decomposition of surface residues because of their ability to grow hyphae into the soil to extract nutrients and their tolerance of low water potentials (Holland and Coleman, 1987). Fungi are also responsible for nearly all production of extracellular enzymes necessary to degrade recalcitrant compounds such as lignin and soil organic matter (Schimel, 1995). However, in general, bacteria are the primary decomposers of simple carbohydrates, organic acids, and amino acids (Myers *et al.*, 2001).

The impact of a lost species on community functioning depends on the functional role of that organism (Ekschmitte and Griffiths, 1998). Often we think of microbial diversity as redundancy, which may not be true given that each organism may only carry out a given process under a specific set of conditions (Schimel, 1995). This mindset may have arisen from our broad definitions of functional groups without consideration given to the conditions under which the organism is functioning (Schimel, 1995). However, the effects of species losses are likely to be more noticeable in an originally simple community than one that was more complex to start (Ekschmitte and Griffiths, 1998).

Structure is also not strictly linked to function, as illustrated by Buyer and Drinkwater (1997) who found that structurally different microbial communities were functionally similar.

Myers *et al.* (2001) studied landscape-level patterns of soil microbial composition in upland forest ecosystems with the hypothesis that chemical differences in roots and leaves could cause changes in community structure and functioning. They found that differences between communities in three forest ecosystems were partially attributable to differences in substrate chemistry. Litter transplant studies have also shown that soil microbes are adapted to native litter; transplanted litter decomposes slower than when left in its original environment (Schimel, 1995).

Griffiths *et al.* (1999) performed an experiment in which increasing levels of glucose were added as substrate to soil. The highest substrate addition had less bacterial biomass than the next lowest, potentially due to osmotic stress. At this high level of substrate addition, fungi were favored because they are more tolerant of high osmotic potential. They also observed an overall trend of increased gram-negative and gram-positive bacteria with increased substrate.

Phospholipid Fatty Acids (PLFAs)

Cellular membrane lipids (PLFAs) are comprised of two fatty acids esterified to a glycerol phosphate backbone (Guckert and White, 1986). The biosynthesis of fatty acids starts in the multienzyme complex fatty acid synthase, which combines acetate molecules to form palmitic acid (16:0) (Abraham *et al.*, 1998). Many other biosynthetic pathways are employed to modify palmitic acid forming the myriad of fatty acids found in biological cells. A PLFA profile is a phenotypic description of an organism's fatty acid biosynthetic capabilities (Guckert and White, 1986).

PLFAs are useful biomarkers of viable microbial communities because they are essential components of all living cells that degrade within hours of cell death, hence do not accumulate in soil (White *et al.*, 1997, Zelles, 1999, Burke *et al.*, 2003). PLFAs are

also confined to the lipid bilayer surrounding cells and are not found in microbial storage products (Zelles, 1999).

Nomenclature

The basis of phospholipid nomenclature is the chain length, given as the number of Cs in the fatty acid:number of double bonds. The position of double bonds can be indicated from the methyl (ω) end of the chain or from the carboxyl (Δ) end of the chain. For example, 18:2ω6,9 and 18:2Δ9,12 describe the same fatty acid, which is often inappropriately abbreviated as 18:2ω6 (Zelles, 1997). Double bonds can either be in the *cis* or *trans* configuration, which is indicated by "c" or "t" following the position of the bonds. Because almost all unsaturated bonds are in the cis configuration, the "c" is often omitted (Vestal and White, 1989). Methyl group branching is indicated by the suffixes "i," "a," or "br," for iso and anteiso, on the second and third Cs from the methyl end respectively, or branched, which indicates an unknown position. The prefix "10Me" also indicates methyl branching on the 10^{th} C from the carboxyl end. Cyclopropyl groups, which are formed by a methylene bridging between two C atoms making a double bond (Peterson and Klug, 1994), are indicated by the suffix "cy."

Applications in Soil Microbial Ecology

Communities of microbes behave differently than the sum of individual isolates; hence it is desirable to study the entire consortium of organisms living in soil (White *et al.*, 1997). However, less than 1% of all organisms in soil are culturable, and preparing pure cultures provides limited insight into interactions between community members (Tunlid and White, 1990). Methods that measure biochemical properties of microbial cells are not constrained by the same limitations as classical techniques (Wander *et al.*, 1995); lipid analysis accounts for most viable microbes present in a sample (Vestal and White, 1989).

PLFA analysis is useful in microbial ecology because microbial lipids contain a variety of fatty acids, which are used in different combinations by different organisms (Cavigelli *et al.*, 1995). Hence, the types and amounts of PLFAs present reflect the

composition of living microbial communities (Song *et al.*, 1999). However, because of overlap between PLFA profiles of microbial species, it isn't possible to pick out individual species in a community profile (Wander *et al.*, 1995). A PLFA profile is like an infrared spectrum of a complex molecule: all the information is present but it is difficult to determine which component caused which peak (White *et al.*, 1997).

Coupled with statistical pattern recognition, analysis of PLFAs has proven to be a powerful tool for examining microbial community structure (Tunlid and White, 1990; Buyer and Drinkwater, 1997). Differences in PLFA composition can be indicative of spatial or temporal variation; PLFA profiles can be used to distinguish between soils from different environments or detect changes due to environmental disturbances (Lindahl *et al.*, 1997). For example, Frostegård *et al.* (1993) observed effects on PLFA patterns when soils were exposed to heavy metals at levels that did not affect other measures of microbial health, i.e. respiration and total PLFA. Using PLFA profiles, Zelles *et al.* (1992) and Bossio *et al.* (1998) were able to distinguish between soil microbial communities that developed under different agricultural management systems. However, Wander *et al.* (1995) found that with field replication, the variability between replicates was as significant as differences between treatments and soils could not be distinguished on the basis of PLFA profiles.

Biomarkers and Ratios

Membrane lipids are good biomarkers because they are structurally diverse and display a high level of biological specificity (Zelles, 1999). These "signature" PLFAs aid in the interpretation of community fingerprints. Certain fatty acids, and conformations and ratios of fatty acids, are indicative of the structural and physiological state of the community (Bossio and Scow, 1998). Table 1.1 contains a summary of common phospholipid biomarkers and their interpretations.

Cyclopropyl fatty acids such as 19:0cy can be considered indicators of stress, as they tend to increase under conditions of high temperature, low pH, and stationary growth (Bossio and Scow, 1998). 16:0 and 18:0 are precursors to 16:1ω7 and 18:1ω7, which in turn can be converted to 17:0cy and 19:0cy (Peterson and Klug, 1994). The

ratio of precursor to product can be used to determine if increases in cyclopropyl fatty acids are due to a physiological response or the presence of organisms that regularly produce cyclopropyl groups (i.e., gram-negative bacteria (Wilkinson, 1988; O'Leary and Wilkinson, 1988)). The relationship between 17:0cy and 19:0cy is not always clear; Griffiths *et al.* (1999) observed an increase in 17:0cy with a concomitant decrease in 19:0cy with increased substrate addition, which they attributed to 19:0cy representing different gram-negative community members than 17:0cy.

Table 1.1. Common phospholipid biomarkers and their interpretations (Bossio and Scow, 1998; Ibekwe and Kennedy, 1998; Myers *et al.;* 2001, Nichols *et al.;* 1985).

Indicator	**Interpretation**
18:2ω6,9, 18:3ω6c	Fungi
10Me18:0, 10Me17:0	Actinomycetes
Branched, saturated fatty acids (15:0i, 15:0a, 17:0i, 17:0a)	Gram(+)
17:0cy, 19:0cy	Aerobic Gram(-) Anaerobic Starvation Stress
16:0	Total Microbial Biomass
16:1ω5c, 16:1ω7c, 16:1ω9c	Non-specific Bacteria
Cyclo/precursor ratio	Stress Indicator
trans/cis ratio	Stress Indicator
18:1ω8c	Type II Methanotrophs
Monounsaturated 16 C fatty acids	Type I Methanotrophs
10Me16:0	Desulfobacter

The ratio of *trans*:*cis* unsaturated fatty acids is an indicator of nutritional status, as well as temperature stress (Tunlid and White, 1992; Cronan, Jr., 2002). When nutrients become limiting, *cis* isomers are more readily metabolized compared to corresponding *trans* isomers resulting in an increased *trans*:*cis* ratio (Borga *et al.*, 1994). When *Vibrio cholerae* was starved, 16:1ω7c decreased from 39% to 11% of the membrane lipid with a concomitant increase in 16:1ω7t from 1% to 17% (Vestal and White, 1989). *Trans* configuration of double bonds allows lipids to pack more closely together in membranes, resulting in higher transition temperatures and increased membrane stability (Cronan, Jr., 2002).

Fungi produce fewer types of fatty acids than bacteria. Stahl and Klug (1996) found that four fatty acids (16:0, 18:0, 18:1ω9, and 18:2ω6,9) comprised as much as 95% of the total fatty acid content of dikaryotic fungi. Of the four, only 18:2ω6,9 is not found in bacteria, because prokaryotes do not produce polyunsaturated fatty acids (White *et al.*, 1997). In analysis of pure fungal cultures, Federle (1986) determined that on average 43% of membrane PLFA was 18:2ω6,9 . Ruess *et al.* (2002) confirmed the findings of Stahl and Klug by finding the same four fatty acids in all fungal cultures they examined. They also found results similar to Federle: 18:2ω6,9 was 34-88% of the total fungal PLFA.

The usefulness of signature phospholipids may be limited in certain environments. Bossio and Scow (1998) found that the more commonly occurring bacterial fatty acids, and not specific biomarkers, were indicative of change in agricultural soils with different C inputs. Unusual lipid markers were either below detection limits or not present at all in this system.

Association Between PLFA and Microbial Biomass

Although lipids that are characteristic of certain groups of organisms can be used to define community structure, those lipids that are produced ubiquitously can be used to estimate total microbial biomass (Borga *et al.*, 1994). Most organisms produce a variety of saturated fatty acids; the largest fatty acid fraction in all soils is the saturated fatty acids (Zelles *et al.*, 1994). This approach is based on the fact that under steady-state

conditions, phospholipids make up a relatively constant proportion of cell biomass (Ibekwe and Kennedy, 1998).

There is a lack of consensus in the literature regarding the correct conversion between PLFA and microbial biomass. Brinch-Inversion and King (1990) suggest a general conversion factor of 50 μmol of phospholipid phosphorus per gram of dry weight for natural populations of bacteria, whereas Haack *et al.* (1994) suggested 100 μmol of PLFA per gram dry weight of microbial cells. They measured a 6-fold range in PLFA associated with a unit of biomass. Although quantification of membrane lipids may be a sensitive measure of viable microbial biomass (Zelles and Bai, 1993), variation in conversion factors in the literature may arise in part because the relationship between membrane lipids and metabolically active cytoplasmic volume is not straightforward (Peterson and Klug, 1994).

More often in soil microbial ecology, we are interested in how much C is contained in the microbial biomass, rather than the actual mass of dry cells. Zelles *et al.* (1992) proposed a conversion factor of 28 μg PLFA per 100 μg biomass C and present data with r^2=0.968. Unfortunately, Bossio and Scow (1998) did not find a strong correlation between total PLFA and biomass C (r^2=0.32).

Palmitic acid (16:0) is precursor to all other fatty acids and hence is present in all biological cells (Kates, 1964). Due to the high correlation between total amounts of PLFA and the 16:0, determination of biomass can be simplified further by considering only the amount of palmitic acid in samples (Zelles *et al.*, 1992; Zelles *et al.*, 1995). Baird and White (1985) used a conversion factor of $5x10^5$ cells/10^{-12} moles 16:0 phospholipid.

Linking Community Structure and Function

To determine relationships between community composition and function, it is possible to compare PLFA profiles with measures of microbial activity. For example, Waldrop *et al.* (2000) looked at the relationship between certain PLFA biomarkers and activity of enzymes necessary for plant residue decomposition. The fungal biomarker 18:2ω6,9 was positively correlated with cellobiohydrolase and phenol oxidase.

10Me18:0, an actinomycete biomarker, was positively correlated with peroxidase and phenol oxidase. These enzymes are important for the depolymerization of the complex structure of lignin.

An organism's fatty acid composition is determined by its biosynthetic pathways, which can vary based on metabolic activity and its dietary source of C (Ruess *et al.*, 2002). From the perspective of functional diversity, it is therefore important to recover lipids found in small amounts in samples because they are likely more revealing of subtle metabolic shifts than those lipids found ubiquitously throughout the microbial community (Zelles and Bai, 1993; Song *et al.*, 1999).

Methodology

In 1959, Bligh and Dyer first described the process used by most researchers to extract PLFAs from biological samples. They used a monophasic system consisting of chloroform:methanol:water in a ratio of 1:2:0.8 to extract lipids from fish biomass. Lipids dissolve almost instantly and, in the case of microbial communities, lipid metabolism stops when solvents are added (Vestal and White, 1989). To induce phase separation, chloroform and water are added, resulting in a final ratio of 2:2:1.8 chloroform:methanol:water. More polar proteins, nucleic acids, cell walls and other cell components remain in the upper methanol-water phase or at the chloroform-water interphase, while the lipids are contained in the lower chloroform phase (Vestal and White, 1989). The chloroform phase is removed and dried under nitrogen gas to isolate bulk lipids (Bligh and Dyer, 1959).

Though the process is continuously evolving, White *et al.* (1979) made the most important modification of the original Bligh and Dyer method. They replaced water in the monophasic extraction mixture with phosphate buffer, which helps to stabilize pH during extraction. Maintaining neutral pH, in addition to processing samples quickly under cool conditions, prevents transesterification and disruption of cyclopropyl rings during extraction, allowing isolation of lipids without modification of the native form (Guckert and White, 1986). Temperatures greater than 37°C and the presence of oxygen can also break down unsaturated fatty acids (White and Ringelberg, 1998).

Bligh and Dyer (1959) were interested in extraction of bulk lipid only. For the analysis of microbial communities, it is necessary to isolate phospholipids. Silicic acid column chromatography is used to separate whole lipid extracts into neutral lipids, glycolipids, and phospholipids. Active sites on silicic acid granules called silanols contain hydroxyl groups that are directly bound to the silicon atom. These active sites interact with polar groups on the lipids; the non-polar end contributes little to separation (White and Ringelberg, 1998). Lipids are selectively eluted with increasingly polar solvents. Non-polar neutral lipids, such as di- and triglycerides, steroids, waxes, and quinones are eluted using chloroform. Acetone removes glycolipids, such as poly-β-hydroxybutyrate, from the column. Polar lipids, including phospholipids, are eluted from the column with methanol.

Zelles and Bai (1993) tested the recovery efficiency and fractionation capabilities of silicic acid solid phase extraction columns. Using pure phospholipid standards, they observed recovery efficiencies between 97% and 100%. With galactosyl diglyceride with linoleic acid as the fatty acid chain, phosphatidylethanolamine dipalmitoyl, and L-α-phosphatidylcholine dimyristoyl they were also able to confirm the column's ability to fractionate lipids into neutral, glycolipids, and phospholipids; linoleic acid was only found in the acetone fraction, whereas the two phospholipids were only detected in the methanol fraction. Therefore, solid phase extraction using silicic acid column chromatography is preferable to older methods of lipid isolation because it results in good clean-up and recovery of lipids, requires small volumes of solvent, and displays excellent selectivity for lipids (Zelles and Bai, 1993).

Mild alkaline methanolysis is used to cleave fatty acids from the phospholipid glycerol background, replacing glycerol bonds with methyl groups (White and Ringelberg, 1998). It is important that no water is present during methylation as it will compete with methanol for the fatty acids, resulting in free fatty acids rather than methyl esters (White and Ringelberg, 1998). Fatty acid methyl esters (FAMEs) can be easily volatilized, allowing identification by their retention times on polar and non-polar stationary phases during gas chromatography (White *et al.*, 1979; Cavigelli *et al.*, 1995).

Clear separation, identification and quantification of single fatty acids can be difficult because of overlap in retention times and mass spectrometry signals (Zelles and Bai, 1993). This is particularly true of cyclopropane and monounsaturated FAMEs with the same number of Cs. Identification of branched-chain saturated fatty acids is simple if the branch is located at the omega end of the molecule; mid-chain branching complicates identification (Zelles and Bai, 1993). Regardless, Zelles and Bai (1993) were able to identify as many as 162 fatty acids from the phospholipid fraction of a soil extract.

An alternative to the Bligh and Dyer methodology is MIDI (Microbial ID, Inc., Newark, Delaware). The MIDI system was developed at the University of Delaware in conjunction with Hewlett Packard to identify individual strains of bacteria based on fatty acid profiles. The database is now extensive enough to distinguish over 8000 strains of bacteria (Tunlid and White, 1990). In contrast to Bligh and Dyer, which separated lipids into neutral lipids, glycolipids, and phospholipids, MIDI extracts and considers total bulk lipids from a sample (Sasser, 1990). MIDI database profiles are based on organisms grown under pure culture conditions, which may be limiting for environmental samples (White *et al.*, 1997).

A serious drawback to using MIDI to analyze microbial community structure is that extraction of total fatty acids is not restricted to living microbial biomass (Zelles, 1999). Certain microbial storage products contain fatty acids (Dawes and Senior, 1973) and fatty acids can also be found in extracellular lipids. These extracellular lipids may be in stable forms in organic matter (Zelles, 1999) and therefore not indicative of current community composition.

Each methodology also appears to have different extraction efficiency for certain lipids. Schutter and Dick (2000) used both Bligh and Dyer and MIDI to extract duplicate samples of soils from vegetable cropping systems. They found that relative amounts of 16:1ω5, a biomarker for arbuscular mycorrhizae (Olsson *et al.*, 1998) and specific bacteria like *Cytophaga* (Frostegård *et al.*, 1993), sometimes doubled if MIDI was used. The actinomycete marker 10Me18:0 was more abundant with Bligh and Dyer, while abundance of gram-negative markers varied between methods. They concluded that

although each method was capable of distinguishing different soils, inferences regarding community structure could be influenced by the method used to generate lipid profiles.

Limitations of PLFA Analysis

A significant limitation of PLFA analysis is the lack of exclusivity of so-called signature lipids (Bossio and Scow, 1998). Interpretation of changes in patterns of PLFAs in soils in terms of changes in specific taxonomic groups is difficult because the same lipid marker may be present in membranes of organisms belonging to different taxonomic groups (Ibekwe and Kennedy, 1998). For example, 17:0cy and 19:0cy are both considered to be characteristic of anaerobic bacteria (Zelles, 1997), gram-negative bacteria (O'Leary and Wilkinson, 1988; Wilkinson, 1988), and physiological stress in any organism (Grogan and Cronan, Jr., 1997). Therefore, making a definitive statement as to the cause of observed increases or decreases in 17:0cy and 19:0cy is all but impossible.

Sometimes researchers are more interested in community function than taxonomic composition. Functionally diverse groups of organisms can have qualitatively similar fatty acid composition (Borga *et al.*, 1994). Therefore, linking PLFA markers with specific microbial processes is difficult because a myriad of organisms will contribute to the overall PLFA profile observed (Ludvugsen *et al.*, 1997). In studies employing organic matter additions, changes in PLFA profiles can be attributed to community shifts in response to increased C availability, to organisms that were colonizing the organic matter prior to addition to soil, or community response to secondary changes such as moisture, temperature, and aeration (Bossio *et al.*, 1998).

It is also difficult to interpret changes in PLFA profiles because most fatty acids we measure are common to most microorganisms and soil samples contain a huge diversity of those microbes (Zelles, 1999). In fact, about 50 fatty acids represent about 10 000 species of microorganisms and often an individual organism's profile is simple with less than 10 fatty acids (Zelles, 1997; Zelles, 1999). Ruess *et al.* (2002) detected considerable differences in lipid composition of pure fungal cultures; most contained the same fatty acids but in different proportions. Even lipids that appear to be unique to a

group of organisms, such as 18:1ω8c for Type II methanotrophs (Nichols *et al.*, 1985) are suspect. It is possible to mistakenly regard a particular fatty acid as a valid biomarker because it has not yet been investigated in other members of the same community (Zelles, 1997).

Much of what we know about the signature PLFAs of certain groups of microbes is based on pure culture studies. However, organisms growing in situ may have different profiles than those grown under laboratory conditions (Zelles, 1997). Ibekwe and Kennedy (1998) found that soil community profiles were dominated by straight chain fatty acids, whereas branched chain fatty acids dominated in cultured microorganisms. Also, profiles of individual taxa are known to vary quantitatively with changes in growth media (Haack *et al.*, 1994).

Taxonomic interpretation is further confounded by the ability of individual organisms to change their fatty acid composition in response to stress or temperature fluctuations (Lindahl *et al.*, 1997). Membrane fluidity is sensitive to changes in temperature, and microbes can change lipid composition to compensate for increases or decreases in temperature (Peterson and Klug, 1994). Stahl and Klug (1996) studied pure cultures of fungi and observed that though the same fatty acids were present when temperature was changed, the relative amounts differed. Groups of organisms may not respond to stress equally; Kieft *et al.* (1997) observed an increase in saturated to unsaturated, trans to cis monounsaturated, and cyclopropyl fatty acids in stressed gram-negative bacteria with little change in stressed gram-positive PLFA profiles. Therefore it is difficult to determine whether shifts in a PLFA profile are occurring due to changes in community structure or physiological changes in a community with the same taxonomic composition (Bossio and Scow, 1998).

Stable Carbon Isotope: ^{13}C

Approximately 98.88% of all C on Earth is ^{12}C; ^{13}C comprises about 1.11% of the terrestrial C pool (Boutton, 1991a). However, due to isotopic fractionation by chemical, physical and biological pathways, isotopic ratios in natural materials deviate from these

average values (Boutton, 1991a). Such deviations in isotopic ratio can provide insight into C cycling processes in soil, plant and microbial systems. The progressive change of $\delta^{13}C$ of soil organic C allows quantitative estimates of parameters such as turnover time and size of soil organic compartments (Balesdent and Mariotti, 1996). $\delta^{13}C$ in a plant is affected by environmental conditions such as soil water regime and humidity (Balesdent and Mariotti, 1996). Therefore, measurements of isotopic composition may be more accurate for describing average physiological activity of a plant than instantaneous observations of metabolic activity (Ehleringer, 1991). Also, metabolic activity of microbial communities can be estimated by measuring the rate of isotope incorporation from labeled substrates (Tunlid and White, 1992). Biologists and ecologists may be interested in differences of isotopic composition as small as 1 to 10 parts per thousand (Boutton, 1991a).

Photosynthetic pathways are responsible for conversion of inorganic forms of C, such as CO_2, into organic forms. As a result of isotopic fractionation associated with photosynthesis, organic C is generally depleted in ^{13}C (Boutton, 1991b). Plants utilizing the C_3 pathway typically have $\delta^{13}C$ signatures around –28‰, while C fixed via the C_4 pathway tend to have $\delta^{13}C$ signatures around –12‰ (O'Leary, 1988). This difference arises due to biochemical characteristics of the primary CO_2-fixing enzymes and limitations to CO_2 diffusion into the leaf (Boutton, 1991b). RuBP carboxylase, the enzyme responsible for C-fixation in the C_3 pathway, discriminates more strongly against $^{13}CO_2$ than PEP carboxylase, the enzyme employed by the C_4 pathway. PEP carboxylase is also able to maintain a steeper CO_2 diffusion gradient into the leaf, further contributing to enrichment of ^{13}C in C_4 plant tissue (O'Leary, 1988).

Though fractionation during photosynthesis is responsible for the most dramatic alterations of ^{13}C:^{12}C in biological systems, other biochemical pathways also exhibit isotopic discrimination. For example, plant material is not usually uniformly labeled with recalcitrant fractions generally being depleted by as much as 6‰ relative to the whole plant (Boutton, 1991b; Schweizer *et al.*, 1999). Cellulose and hemicellulose can be slightly enriched (1 to 2‰) relative to the bulk plant $\delta^{13}C$. These differences occur as a result of fractionation in biosynthetic pathways (Balesdent and Mariotti, 1996). Although

soil $\delta^{13}C$ usually reflects the dominant vegetation, differences in rate of decomposition of biochemical plant components can result in SOM with an isotopic composition that is slightly different from the plant material from which is was derived (Balesdent and Mariotti, 1996). However, in a long-term study at the University of Missouri (Columbia), Balesdent *et al.* (1988) found that after 100 years of continuous wheat cultivation ($\delta^{13}C$ = -27‰), the $\delta^{13}C$ of SOM had only decreased by about 4‰ from –18.6‰ to –22.9‰.

Microbial processes also affect $\delta^{13}C$ values. Soil microbial communities discriminate against ^{13}C in respiratory pathways, which alters the $\delta^{13}C$ of evolved CO_2 (Schweizer *et al.*, 1999). Though discrimination effects are often masked at the whole-organism level (Henn and Chapela, 2000), evidence can be found when individual components of the microbial cell are considered. The $\delta^{13}C$ of individual PLFAs extracted from microbial communities is variable, reflecting different isotopic fractionations associated with the various pathways responsible for producing different fatty acids (Burke *et al.*, 2003). Henn and Chapela (2000) examined the role that substrate plays in isotope discrimination by fungi. When presented with C_3-derived sucrose, the fungi generally became enriched in ^{13}C, while C_4-derived sucrose did not result in ^{13}C discrimination. They concluded that fractionation is controlled at fine scale by specific physiological processing, substrate effects, and micro-environmental conditions. However, because of the large quantity of stable isotope present, fractionation is not generally a factor in tracer studies.

^{13}C Labeling and Uses as Tracer

^{13}C is useful in biological studies because it is considered to be a more faithful tracer of ^{12}C than the radioisotope ^{14}C because of smaller mass differences (Ostle *et al.*, 2000). The use of ^{14}C also poses a risk to researchers and the environment due to its radioactive nature; ^{13}C can be used safely in a wider variety of experiments. Through the use of highly sensitive equipment, it is also possible to detect very small changes in actual ^{13}C content of biological materials.

For many applications, it is desirable to have plant material that is enriched in ^{13}C. Labeling with ^{13}C generally involves growth under continuous $^{13}CO_2$ atmosphere or pulse labeling at discrete time points during development (Meharg, 1994). Maintenance of a pure $^{13}CO_2$ headspace is expensive, but produces more uniformly labeled plants (Meharg, 1994). Pulse labeling is more cost-effective, but producing uniformly labeled material is challenging. Ostle *et al.* (2000) observed a significant decline in $\delta^{13}C$ values of both shoots and roots in 24 hours when pulse-labeling annual and perennial grassland plants. After the initial losses of ^{13}C, they observed no marked decline in $\delta^{13}C$ during the 4 weeks following labeling. However, only a small portion of pulse ^{13}C was incorporated the into biologically stable structural components of cellulose, hemicellulose and lignin.

Methodology

^{13}C is measured using isotope ratio mass spectrometry (IRMS). The first step in ^{13}C analysis is isolation of the compound of interest from the sample (e.g., microbial phospholipids). The compound must then be converted to CO_2, which is ionized by an electron beam in the ion source of the IRMS. An accelerating potential propels positive ions into a curved magnetic field (Boutton, 1991a). The path taken by ions through the magnetic field is a function of mass and energy of the ions (Boutton, 1991a). The major species of CO_2 ($^{12}C^{16}O_2$, $^{13}C^{16}O_2$, and $^{13}C^{16}O^{17}O$) are separated into three separate beams on a mass basis with molecular weights of 44, 45, and 46 respectively. As the ion beams hit Faraday cup collectors, they are neutralized generating an electrical current that is used to calculate the stable isotope ratio (Boutton, 1991a). Placing a combustion furnace in line between a flame ionization detector gas chromatograph and the IRMS allows $\delta^{13}C$ measurements on CO_2 derived from specific organic compounds.

The most common unit of measurement for expressing quantities of ^{13}C is δ-value. $\delta^{13}C$ is a differential notation useful for expressing relative differences in stable isotope ratios between samples and standards. It can be calculated by:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000, \qquad \text{where } R = \frac{^{13}C}{^{12}C},$$

which gives units of parts per thousand. The basis of the δ-value scale is the standard Pee Dee Belemnite (PDB), which has been assigned a δ-value of zero. PDB was a limestone fossil of *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina (Boutton, 1991a). Although the original supply has since been depleted, other standards have been calibrated against PDB to allow researchers to continue expressing ^{13}C values on the PDB scale.

Two other useful units in ^{13}C research are atom% and atom% excess. Atom% is a mole fraction, calculated as the proportion of ^{13}C to total (^{12}C + ^{13}C) C in a sample. Atom% is more accurate than $\delta^{13}C$ for calculations of changes in ^{13}C between samples. Atom% excess is the enrichment level of a sample following addition of a tracer in excess of ^{13}C background levels (Boutton, 1991a).

Application of ^{13}C to PLFA Analysis

Recently, the use of ^{13}C-labeled substrates has been coupled with PLFA analysis to facilitate study of C flux through microbial communities (Boschker *et al.*, 1998; Abraham *et al.*, 1998). Stable C isotopic composition in microbial markers, such as PLFAs, provides insight into the origin of C used for growth (Burke *et al.*, 2003). Generally, isotopic signatures of bacterial biomass tend to be similar to the substrate on which the organisms have been growing. In contrast, lipids tend to be depleted in ^{13}C relative to biomass as a result of enzymatic discrimination in fatty acid biosynthetic pathways (Abraham *et al.*, 1998).

Isotopic fractionation occurs during one of three stages of biosynthesis: (1) transport of substrate into the cell and its subsequent breakdown to acetate, (2) synthesis of palmitic acid, and (3) transportation to cellular membranes and esterification to polar lipids (Abraham *et al.*, 1998). Because 16:0 is synthesized via the same pathway in a given organism, at the cellular level fractionation between acetate produced from substrate degradation and palmitic acid is always the same (Abraham *et al.*, 1998). However, there is evidence that pathways responsible for producing the myriad of PLFAs found in a cell membrane discriminate differently against ^{13}C. Abraham *et al.* (1998)

found that for a given strain of bacteria, myristic acid (14:0) was usually more depleted relative to palmitic acid (16:0). Alternately, stearic acid (18:0) was usually enriched. The fractionation did not appear to arise as a result of chain length, because tetracosanoic acid (24:0) was most depleted of the lipids measured. Interestingly, they found that the same fatty acid isolated from different lipid fractions showed different isotope ratios, suggesting that it is the biosynthesis of the lipid that causes a portion of observed fractionation, in addition to fractionation originating from the synthetic pathway for each fatty acid. Glycolipid fractions are usually depleted in ^{13}C relative to phospholipid fractions.

Substrate also plays a role in determining C isotope ratios in lipids, because each compound is processed differently to produce acetate, the basic building block for fatty acids. This results in different isotopic signatures depending on what substrates were utilized in the formation of a given fatty acid. Of the substrates tested, Abraham *et al.* (1998) found that glucose resulted in the largest difference in $\delta^{13}C$ between substrate and palmitic acid. Whereas glucose resulted in depleted fatty acids, glycerol resulted in ^{13}C-enriched 16:0 in bacteria and Ascomycota.

A number of studies have employed ^{13}C-PLFA methodology to link community structure and carbon processing in soil and marine environments. Phillips *et al.* (2002) added ^{13}C-labeled cellobiose and *N*-acetylglucosamine to temperate forest soils, where they found that ^{13}C was not incorporated into all PLFAs; nine PLFAs contained 80% of excess ^{13}C recovered. Based on ratios between fungal and bacterial lipids, they were also able to determine that fungi consumed proportionally greater quantities of cellobiose than *N*-acetylglucosamine compared to bacterial populations. *N*-acetylglucosamine was used for biosynthesis more than cellobiose, as more ^{13}C was recovered in PLFAs. Hanson *et al.* (1999) linked toluene degradation with specific microbial populations in soil by adding ^{13}C-toluene. Only a small subset of soil PLFAs was enriched with ^{13}C, compared to easily degradable ^{13}C-glucose, which appeared in all PLFAs measured. Organisms capable of degrading toluene were characterized by odd-chain length PLFAs such as 15:1, 15:0, and a variety of 17-C lipids. Arao (1999) obtained data about the rate of C assimilation by various groups of organisms by measuring ^{13}C in PLFAs following

addition of ^{13}C-labeled acetate, the precursor to all lipids. Results of this experiment showed that gram-positive bacteria utilized C slower than did gram-negative bacteria. After 24 h, incorporation of ^{13}C into cyclopropyl fatty acids increased, indicating possible nutrient shortages.

Summary

Microbial processing of C during decomposition of plant materials is complex. Substrate quality plays an important role in determining the rate of decomposition as well as the organisms involved. Soluble C is readily degraded by bacterial communities characterized by r growth strategies, whereas decomposition of complex polymeric substrates, such as cellulose and lignin, is carried out primarily by fungi and K-strategist bacteria. Although it is generally accepted that microbes are less than 60% efficient in assimilating C, unbalanced growth can occur during rapid decomposition when C is plentiful but other growth factors may be limiting.

Addition of fresh organic matter, such as straw, or labile carbohydrates, such as glucose, to soil can result in a priming effect. The added substrate induces growth in the native microbial community, resulting in decomposition of soil organic matter in excess of unamended soil. Complex substrates, such as straw, tend to result in larger priming effects, as they stimulate growth in the segment of the microbial community capable of producing depolymerizing extracellular enzymes.

The use of ^{13}C-labeled plant material coupled with analysis of phospholipid fatty acids by IRMS results in a powerful tool for linking microbial community structure and C cycling during decomposition. PLFAs are useful biomarkers of active microbial communities because they are essential components of all living cells that degrade within hours of cell death, hence do not accumulate in soil. PLFAs are also only found in the lipid bilayer surrounding cells, not in storage products or other cell components. Differences in PLFA composition can be indicative of temporal or spatial variation in microbial community structure. By measuring the $\delta^{13}C$ signature of PLFAs, it is possible to determine sources of C used for lipid synthesis.

The overall objective of this thesis was to investigate the role of soluble C in the decomposition of ryegrass straw. We wanted to determine: (i) if the presence or absence of labile C in straw affects the rate at which C is mineralized by the microbial community, (ii) if community structure would differ based on the presence of labile C, (iii) if community structure would shift as decomposition progressed, and (iv) how substrate C is utilized by microbial communities. These goals were addressed through a microcosm-based incubation of unleached straw, leached straw, and leachate with soil during which measurements of $^{13}CO_2$, ^{13}C-microbial biomass, and ^{13}C-PLFAs were made.

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Chapter 2:

Microbial Dynamics During Decomposition of ^{13}C-Labeled Ryegrass Residue: Role of Soluble C

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Abstract

Ryegrass residue consists of three main C fractions: readily available soluble carbon, intermediately available cellulose and hemicellulose, and slowly available lignin. Due to variability in C availability, decomposition occurs in two phases consisting of the rapid consumption of labile C followed by a second, slower phase in which more recalcitrant compounds are degraded. Using ^{13}C-labeled ryegrass, we tracked substrate-derived C as it was processed during decomposition in a microcosm-based study. Residue was added to soil at rates that reflect field loads. Treatments were unleached straw (US), leached straw (LS), and leachate (L), plus an unamended control (CON). Added substrates had $\delta^{13}C$ values between 120‰ and 180‰; native soil signature was about –26‰. Respiration was measured every 4 to 6 h for the first 5 d, and weekly thereafter. Destructive sampling took place after 0.6, 1.6, 15, 18, 50, and 80 d of incubation and microbial biomass ^{13}C (MBC) was measured.

The soluble component of ryegrass was an important source of C during the initial stages of decomposition, indicated by similar rates of CO_2 evolution and $\delta^{13}C$ values between L and US during the first 3 d of incubation. Most soluble C from leachate was consumed during that time, indicated by the rapid decrease in $\delta^{13}C$ value of CO_2 evolved from L treatment. The addition of complex plant residues resulted in greater primed CO_2-C evolution than adding soluble, labile C in leachate. This can be attributed to induction of growth in K-strategist microbial populations, which are capable of degrading polymeric compounds such as lignin as well as soil organic matter.

Substrate-derived C moved quickly into and through the microbial biomass, illustrated by the peak and subsequent decline in substrate-derived microbial biomass C as a fraction of the total MBC. Apparent microbial growth and C assimilation efficiency was unexpectedly high after 0.6 d of incubation. Though not widely observed, this phenomenon may be attributed to the production of energy storage compounds under conditions of high C availability in the presence of some other limitation on microbial growth.

Introduction

From the perspective of a soil microbial community, ryegrass residue consists of three main C fractions. The readily available C pool consists of soluble C components such as carbohydrates and amino acids. Cellulose and hemicellulose form the intermediately available C pool, whereas lignin is slowly available due to its complex structure. Quality of these substrates, which can be thought of as the ability to provide C, energy, and nutrients to microbes, is important because it constrains the supply of energy for enzyme production and growth (Fontaine *et al.*, 2003). As a result of variability in C availability, decomposition tends to occur in two phases consisting of the rapid consumption of labile C followed by a second, slower phase in which more recalcitrant compounds are degraded.

These are fairly basic and well-understood principles of decomposition (Brady and Weil, 1999). What makes this study more interesting is the use of the stable isotope ^{13}C as a tracer for C during degradation of ryegrass. With ^{13}C–labeled plant material, it is possible to determine which pool of C, either soil-derived or substrate-derived, microbes are utilizing during decomposition and whether addition of C has induced a priming effect.

Priming refers to the extra decomposition of soil organic C that may occur following the addition of easily decomposable organic substances to soil (Kuzyakov *et al.*, 2000). Priming effects tend to be larger with large additions of C and also with the addition of complex materials such as wheat straw versus simple sugars (Jenkinson, 1966; Fontaine *et al.*, 2003). The addition of fresh organic matter induces rapid biosynthesis and increased overall activity in microbial communities, resulting in increased production of extracellular enzymes capable of SOM decomposition (Dalenberg and Jager, 1981; Fontaine *et al.*, 2003).

Because of the variety of enzymes required for depolymerization of plant structural components, succession plays an important role in decomposition due to enzymatic specificity of microbial populations (Fontaine *et al.*, 2003). The succession of organisms responsible for plant residue decomposition generally progresses from a

bacterial-dominated community early on to a fungal-dominated community during latter stages (Griffiths *et al.*, 1999). This transition can also be thought of as moving from r-strategist to K-strategist organisms. r-strategists tend to grow rapidly when readily available C is added to soil, then return to dormancy and wait for the next input. Conversely, K-strategists grow and divide steadily over long periods, utilizing widely distributed but recalcitrant substrates (Jenkinson, 1988).

Prior studies have shown that separation of soluble and insoluble fractions of straw affects C processing during decomposition. Cogle *et al.* (1989) found that respiration from leachate was greater than from intact straw during the first 4 d of decomposition, indicating that some of the labile C fraction is protected by structural components of straw. Leachate also induced rapid biomass production, as 33% of added ^{14}C was detected in the microbial biomass after 4 d. Reinertsen *et al.* (1984) observed lower respiration rates from leached straw than unleached straw over a 20-d incubation. Wu *et al.* (1993) compared biomass produced from ^{14}C-labeled glucose and ryegrass and found that after 20, 40, and 60 d, more substrate-derived biomass was produced from complex ryegrass than simple sugar.

The objectives of this study were two–fold. First, we wanted to determine if the presence or absence of the soluble component of ryegrass affects C dynamics during decomposition. Second, we wanted to follow substrate-derived C with the aid of ^{13}C as a tracer as it is processed by the microbial biomass.

Materials and Methods

Soil Description

Soil was collected from Hyslop Research Farm, located north of Corvallis, OR (N 44°38' W 123°12') in July 2002. The soil was very dry and hard at the time of sampling. In the lab, clods were broken and soil was passed through a 2-mm sieve to remove large organic debris. Following sieving, soil was air-dried and stored in buckets at 4°C until needed

The soil is classified as a Woodburn silty loam (Aquultic Argixeroll), which is naturally acidic and moderately well-drained. The climate is characterized by cool, wet winters and dry, warm summers, with 108.5 cm of annual precipitation. The surface horizon is about 25 cm deep and 1.3% organic carbon.

Labeled Ryegrass Preparation

Annual ryegrass (*Lolium multiforum* L.) was planted in Fall 2001. Plants were labeled with ^{13}C using a pulse–chase technique weekly between early April and mid-May 2002. A Plexiglas labeling chamber (60 x 60 x 75 cm) was placed over the plants and the soil-chamber interface sealed with wet mud. To generate $^{13}CO_2$, 5 mL of 4M HCl was added to 150 mg of 99 atom% $NaHCO_3$ in a sealed 120-mL serum bottle. The gas was collected in two 60-mL syringes and injected into the headspace of the labeling chamber. The 120-mL injection raised the CO_2 in the chamber by about 400 ppm. To ensure complete collection of $^{13}CO_2$, air was injected into the bottle, removed, and injected into the chamber three times. CO_2 concentration in the chamber headspace was monitored using a LI-COR 6200 CO_2 analyzer (LI-COR Inc. Lincoln, NE). When CO_2 was drawn down to 150 ppm, the $^{13}CO_2$ injection procedure was repeated a total of four times. The $^{13}CO_2$ was chased with six, 120-mL injections of unlabeled CO_2. The headspace concentration was allowed to drop to 150 ppm between chase injections.

Leaching of Straw

Straw was first cut into 1 to 2 cm lengths. To prepare leachate, 18 g of straw was placed with 300 mL of cold deionized water in a 1-L canning jar and shaken on a lateral shaker at approximately 270 rpm at 5°C for 24 h. Leachate was decanted through 250-μm mesh, centrifuged at 8000 rpm for 10 minutes, and filtered through Whatman 2 filter paper to remove fine particulate matter. All leachate was pooled and stored at 4°C for 6 d. Leached straw was collected and dried on brown paper in a forced-air oven at 60°C for 48 h.

Preliminary Analyses

Soil, unleached and leached straw, and leachate were analyzed by isotope ratio mass spectrometry (IRMS) (PDZ Europa Ltd., Crewe, Cheshire, England) to determine total C content and ^{13}C abundance. Microbial biomass carbon was measured on unamended soil to determine starting values.

Microcosm Setup

Microcosms were prepared in 1-L canning jars with 150 g (oven dry) of soil, a C source, and deionized water to bring the soil to 29% gravimetric water content. The C treatments consisted of 2.25 g unleached straw, 1.69 g leached straw, or 29.0 mL of leachate. The amount of soluble C added in leachate was equivalent to the amount of soluble C added in the intact straw. To prepare straw treatments, residue was mixed with about 120 g of soil and carefully transferred to the jar. As the soil had a fine texture and poor permeability, 24.0 mL of water was dispensed into the bottom of the jar prior to the addition of the soil–straw mixture. This allowed the water to move through the soil by capillarity, resulting in more uniform wetting. The remaining soil was poured on top to ensure burial of all plant residue. An additional 7.5 mL of water was pipetted evenly over the soil surface. Leachate treatments were prepared by dispensing 29.0 mL of leachate into the bottom of the jar, adding all the soil and pipetting an additional 2.5 mL of water on the soil surface. The control treatment had no added C and was prepared

similarly to the straw treatments without the mixing step. Table 2.1 summarizes amendments made for each treatment. Twenty-four replicates of each treatment were prepared. Jars were covered with eight layers of cheesecloth to allow adequate ventilation but slow water loss. Microcosms were placed in the incubator using a blocked design with each of four shelves representing a block. Replicates were arranged on the shelves using a Latin rectangle design.

Table 2.1. Summary of treatments, C amendments, and $\delta^{13}C$ values of soil and added C.

Treatment	Amount of C Added	$\delta^{13}C$ of Added C or Soil
Unleached Straw (US)	917 mg	+180‰
Leached Straw (LS)	675 mg	+130‰
Leachate (L)	133 mg	+120‰
Control (CON)	0 mg	–26‰

Sampling Protocol

Four randomly selected replicates (one from each block) of each treatment were destructively sampled at six times throughout the 80 d incubation. Sampling took place after 0.6, 1.6, 15, 18, 50, and 80 d. Soil was homogenized in each jar prior to subsampling. Subsamples were then taken for analysis of the water content, microbial biomass C (MBC), and KCl–extractable N.

Microbial Biomass Carbon

MBC was determined using the fumigation–direct extraction method described by Bruulsema and Duxbury (1996). Briefly, two 25-g portions of soil were weighed into 1/2-pint canning jars. One portion was fumigated with chloroform for 24 h and the other was extracted immediately. For extraction, 75 mL of 0.05 M potassium sulfate was added to the samples, which were shaken laterally for 1 h at 270 rpm. The soil

suspension was filtered through Whatman 42 filter paper and filtrate collected. Three 0.5-mL aliquots of the filtrate were dried at 50°C on acetone-rinsed 37-mm tin squares (EMAL Tech, Mason, OH). Tin squares were made into balls and analyzed by IRMS via a combustion interface. Microbial biomass was calculated as the difference between fumigated and unfumigated C corrected with K_c=0.41 (Vance *et al.*, 1987).

Respiration

Respiration was measured approximately every 6 h for the first 6 d, followed by weekly measurements for the duration of the incubation for a total of 34 measurements. Respiration was measured on four randomly selected replicates of each treatment. Initially, one set of samples was always closed providing continuous respiration data. After the first 6 d, microcosms were closed 8 to 10 h prior to the respiration sampling. Two gas samples were collected from each replicate; 15-mL and 5-mL samples were injected into 10-mL and 3-mL Vacutainers (Becton-Dickinson, Franklin Lakes, NJ). Silicone caulking was spread over the stoppers after sample injection to minimize leakage. The small sample was used for total CO_2 analysis by gas chromatography (Carle Series 100 Analytical GC, Loveland, CO); the large sample was used for $\delta^{13}C$ analysis by IRMS.

Nitrogen Mineralization

Nitrate and ammonium were extracted from 10 g of soil using 50 mL of 2 M KCl. Suspensions were shaken laterally for 1 h at 270 rpm and filtered through Whatman 2 filter paper. Filtrate was analyzed by continuous flow colorimetry using an Astoria–Pacific autoanalyzer (Clackamas, OR).

Data Analysis

$^{13}CO_2$ data from the MS was processed using GC Post Processor v.2.5 (PDZ Europa Ltd., Crewe, Cheshire, England). Peaks were integrated relative to known standards analyzed at the beginning and end of each run.

A simple mixing model was used to calculate the source (soil or added C) of C respired or incorporated into biomass:

$$Atom\%_{total} \cdot C_{total} = Atom\%_{soil} \cdot C_{soil} + Atom\%_{added} \cdot C_{added}$$

where:

$Atom\%_{total}$ = atom% calculated from $\delta^{13}C$ from whole gas or biomass sample
C_{total} = moles of C measured in whole sample
$Atom\%_{soil}$ = atom% calculated from $\delta^{13}C$ of unamended CON sample
C_{soil} = moles of C measured in unamended CON sample
$Atom\%_{added}$ = atom% calculated from $\delta^{13}C$ of added C source
C_{added} = moles of C derived from added C source

Repeated measures analysis of variance (ANOVA) was used to analyze for time and treatment effects in DOC, respiration rate, $\delta^{13}CO_2$, KCl-extractable N, and MBC using S-Plus v. 6.1 for Windows (Insightful Corporation, Seattle, WA). Where significant treatment effects were identified in variables measured on each sampling date, Tukey's honestly significant difference method for mean separation was applied. CO_2 evolution kinetics were modeled by single and double-exponential models using Scientist: Experimental Data Fitting Software (Microsoft Windows Version, MicroMath Scientific Software, Salt Lake City, UT). Equations for the two models were as follows:

Single Exponential Model: $\text{Cumulative } CO_2 = A_1\left(1 - e^{-k_1 t}\right)$

Double Exponential Model: $\text{Cumulative } CO_2 = A_1\left(1 - e^{-k_1 t}\right) + A_2\left(1 - e^{-k_2 t}\right).$

Results

Respiration

Respiration rate was maximal in all amended treatments at 0.6 d (Figure 2.1a). Maximum rates of respiration were highest in L (15.1 mg CO_2–C kg^{-1} soil h^{-1}) and US (12.5 mg CO_2–C kg^{-1} soil h^{-1}) respectively. In LS, the maximum respiration rate was about half that of US (6.98 mg CO_2–C kg^{-1} soil h^{-1}). After 0.6 d, respiration rate began to decrease in all treatments. The decline in respiration rate was more pronounced in L and at about 1 d, respiration in L was lower than in US; by 2.6 d L respiration was lower than LS. From 3 to 15 d, respiration was faster in US than LS and in L than CON, but after 15 d, the two pairs of treatments had nearly identical respiration rates (Figure 2.1b). Respiration in CON remained low and constant for the duration of the experiment. These trends were detected by repeated measures ANOVA, which showed a significant time, treatment, and time•treatment interaction (Table 2.2).

Table 2.2. Results of repeated measures ANOVA.

Response	Time	Treatment	Time•Treatment
CO_2 Rate	***	***	***
$\delta^{13}CO_2$	***	***	***
MBC	***	NS	NS
DOC	***	**	*
Nitrate	*	***	***
Ammonium	*	NS	NS

* = $p < 0.05$
** = $p < 0.001$
*** = $p < 0.0001$
NS = not significant

^{13}C dynamics was similar for all amended treatments during the first 1.6 d (Figure 2.2a). $\delta^{13}C$ increased to a maximum during the first 0.6 d. US and L both peaked at about 120‰ while the maximum $\delta^{13}C$ for LS is lower at about 90‰. After 0.6 d, $\delta^{13}C$ began to decrease in the amended treatments. At about 7 d, $\delta^{13}C$ of CO_2 from the straw treatments began to stabilize at about 60‰. CO_2 from these treatments remained enriched for the duration of the experiment, averaging about 40‰ (Figure 2.2b). $\delta^{13}C$ of leachate CO_2 continued to decline rapidly during the first week, and had reached –7‰ by 8 d of incubation; by 20 d it had stabilized at about –20‰. The δ-value of CO_2 evolved from CON was very depleted for the first 6 d of the experiment at about –40‰. After 6 d, $\delta^{13}C$ increased to –35‰, which was maintained for the duration of the incubation. $\delta^{13}CO_2$ also showed a significant time, treatment, and time•treatment interaction (Table 2.2).

Cumulative CO_2 production from US and L was very similar during the first 3 d of decomposition (Figure 2.3). Nearly 50% of CO_2–C evolved from L during the incubation was produced in the first 3 d. Cumulative CO_2 curves from 15 to 80 d were parallel in US and LS, and L and CON, reflecting similar respiration rates during this time. The curves are offset by a value equivalent to additional C respired by US and L during the first week of decomposition. Total CO_2–C evolved from US, LS, L, and CON was approximately 446, 376, 240, and 128 mg, respectively.

Sources of Respired CO_2–C

In the two straw treatments, CO_2–C is derived nearly equally from soil and substrate (Figure 2.4). In US, half of the soil-derived C is excess above CO_2–C produced by unamended CON soils. In LS, about one-third of soil-derived CO_2–C was produced from extra decomposition of soil organic matter. For leachate, the balance is shifted with 65% of CO_2–C coming from soil organic matter and only 35% from added substrate; excess soil-derived CO_2–C represented only 19% of total soil-derived C.

CO_2 Evolution Kinetics

Cumulative total CO_2, substrate-derived CO_2, and excess soil-derived CO_2 were modeled using either a single- or double-exponential function (Table 2.3). In US and L, fast and slow C pools were identified in total CO_2, whereas LS and C contained only one slow C pool. Two pools were also delineated in substrate-derived CO_2 in US; because LS and L represented the two major components of US, only one pool was identified in each model. Excess soil-derived CO_2 all originated from SOM, therefore only single exponential models were necessary to describe excess soil-derived CO_2 evolution from amended treatments.

Table 2.3. Parameters and statistics from modeling of CO_2 evolution.

CO_2 Pool	Parameter	US	LS	L	CON
Total CO_2	A_1	0.913	2.96	0.532	3.96
	k_1	0.2114	0.0187	0.534	0.0029
	A_2	7.81		4.47	
	k_2	0.0037		0.0033	
	r^2	0.9999	0.991	0.999	0.999
Substrate-Derived CO_2	A_1	0.60	1.27	0.497	
	k_1	0.24	0.0262	0.496	
	A_2	1.96			
	k_2	0.0068			
	r^2	0.9997	0.986	0.995	
Excess Soil-Derived CO_2	A_1	0.64	0.397	0.172	
	k_1	0.0607	0.0354	0.0799	
	r^2	0.992	0.988	0.993	

Microbial Biomass

MBC was very low at the start of the experiment (86.75 mg C kg^{-1} soil). By 0.6 d, extremely large increases in MBC were measured in all treatments (Figure 2.5). Unleached straw MBC was 13x larger than at Time 0, whereas CON soil had increased 8-fold. By 1.6 d, MBC had decreased to 731 mg C kg^{-1} soil in US, whereas about 420 mg

C kg^{-1} soil was measured in LS and L. CON MBC dropped back down to about 200 mg C kg^{-1} soil by 1.6 d and 130 mg C kg^{-1} soil by 15 d. MBC remained relatively stable in all treatments between 15 and 80 d.

Substrate-derived C represented about 20% of total MBC in amended treatments at 0.6 d (Figure 2.5). A decrease in overall biomass was accompanied by an increase in substrate-derived MBC after 1.6 d. From 15 to 80 d, MBC is equally derived from soil and added C. $\delta^{13}C$ values were highly variable during the first days of incubation, though trends in all amended treatments were the same (Figure 2.7a). $\delta^{13}C$ increased to a maximum at 1.6 d and then decreased and stabilized by 18 d. Maximum MBC values were about 30‰ less than substrate $\delta^{13}C$ values. CON values were relatively constant through time and reflected the native soil $\delta^{13}C$ signature.

Dissolved Organic C

Changes in DOC were most dramatic in US and L. Less than 50% of the 887 mg DOC kg^{-1} soil added to L was recovered at 0.6 d, while about 590 mg soluble C kg^{-1} soil was extracted from US (Figure 2.6). DOC decreased significantly in both US and L by 1.6 d, with L containing about the same soluble C as LS and CON; DOC in US was about 50% lower than 0.6 d. By 15 d, DOC was less than 50 mg C kg^{-1} soil in all treatments and was almost undetectable by 18 d. At 80 d, DOC increased to 30 mg C kg^{-1} soil in straw treatments and 20 mg C kg^{-1} soil in L and CON.

DOC was most highly labeled after 0.6 d (Figure 2.7b) in all amended treatments. Straw treatments remained more enriched in ^{13}C than L, which had a similar δ-value to CON by the end of the incubation.

Nitrogen Mineralization

Prior to incubation, soil contained 0.4 mg NH_4–N kg^{-1} (Figure 2.8). Dynamics in the NH_4^+ pool were similar in all treatments: extractable NH_4^+ peaked after 0.6 d at about 1.7 mg NH_4–N kg^{-1} soil, decreased to about 1.0 mg NH_4–N kg^{-1} soil by 1.6 d and stabilized at about 0.35 mg NH_4–Nk g^{-1} soil from 15 to 50 d. On Day 80, extractable NH_4 increased to 0.7 mg NH_4–N kg^{-1} soil.

Nitrate dynamics were more variable between treatments (Figure 2.8). In CON, NO_3^- doubled from 4.0 mg NO_3–N kg^{-1} soil at 0 d to 8.0 mg NO_3–N kg^{-1} soil after 80 d. Nitrate decreased in all amended treatments between 0 and 0.6 d. Nitrate concentrations were lowest in L (0.5 mg NO_3–N kg^{-1} soil), followed by US (1.5 mg NO_3–N kg^{-1} soil), and LS (3.0 mg NO_3–N kg^{-1} soil). There was little change between 0.6 and 1.6 d. By 15 d, extractable NO_3^- was almost zero in all amended treatments. A significant increase in soil NO_3^- occurred in leachate treatments between 50 and 80 d, with only a small increase observed in US and LS during the same interval.

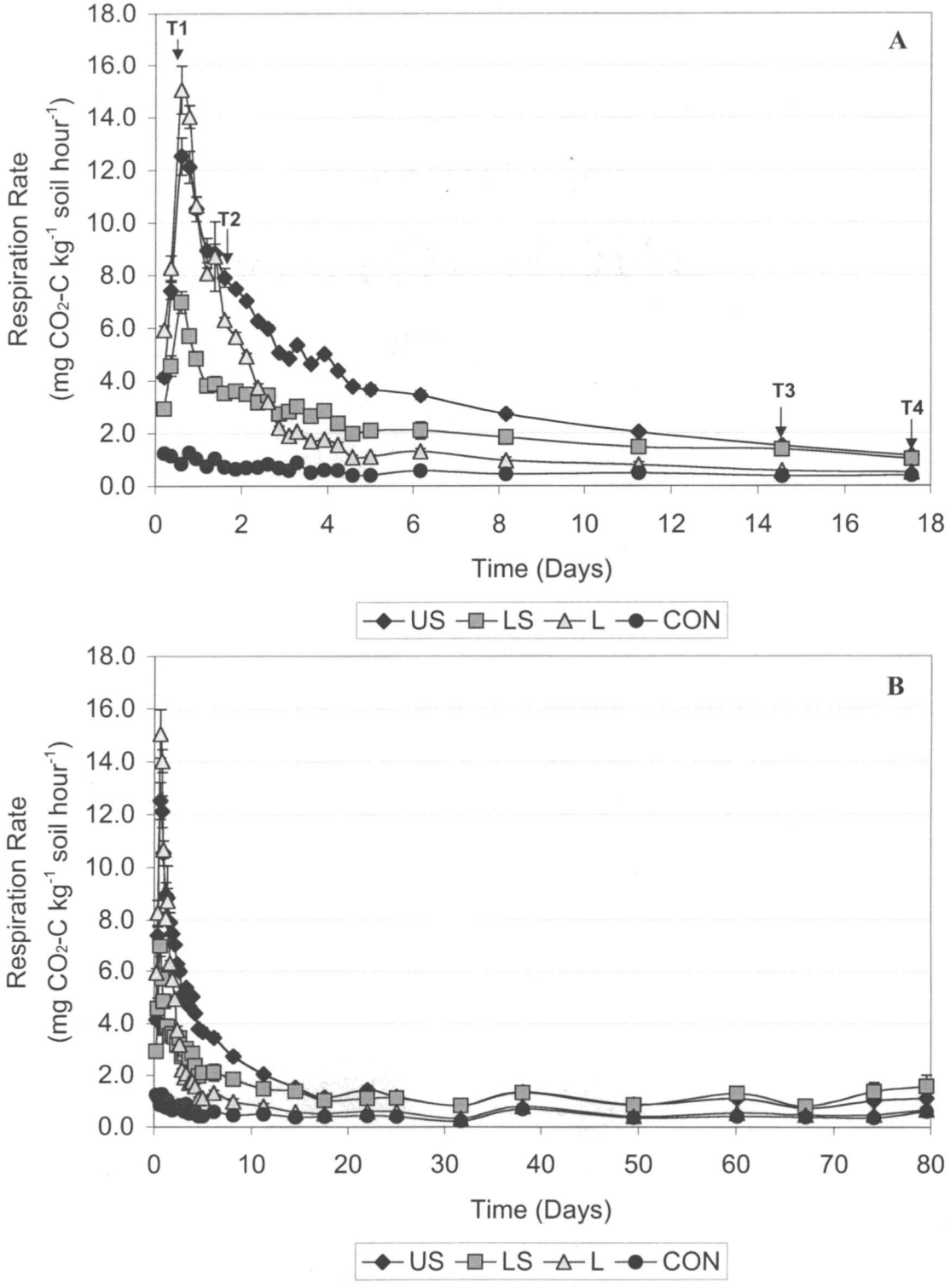

Figure 2.1. Respiration rate measured at each respiration sampling for (A) 0-18 d and (B) 0-80 d. Destructive soil sample times are indicated on (A) by T1, T2, etc. Error bars are ±1 standard error of the mean.

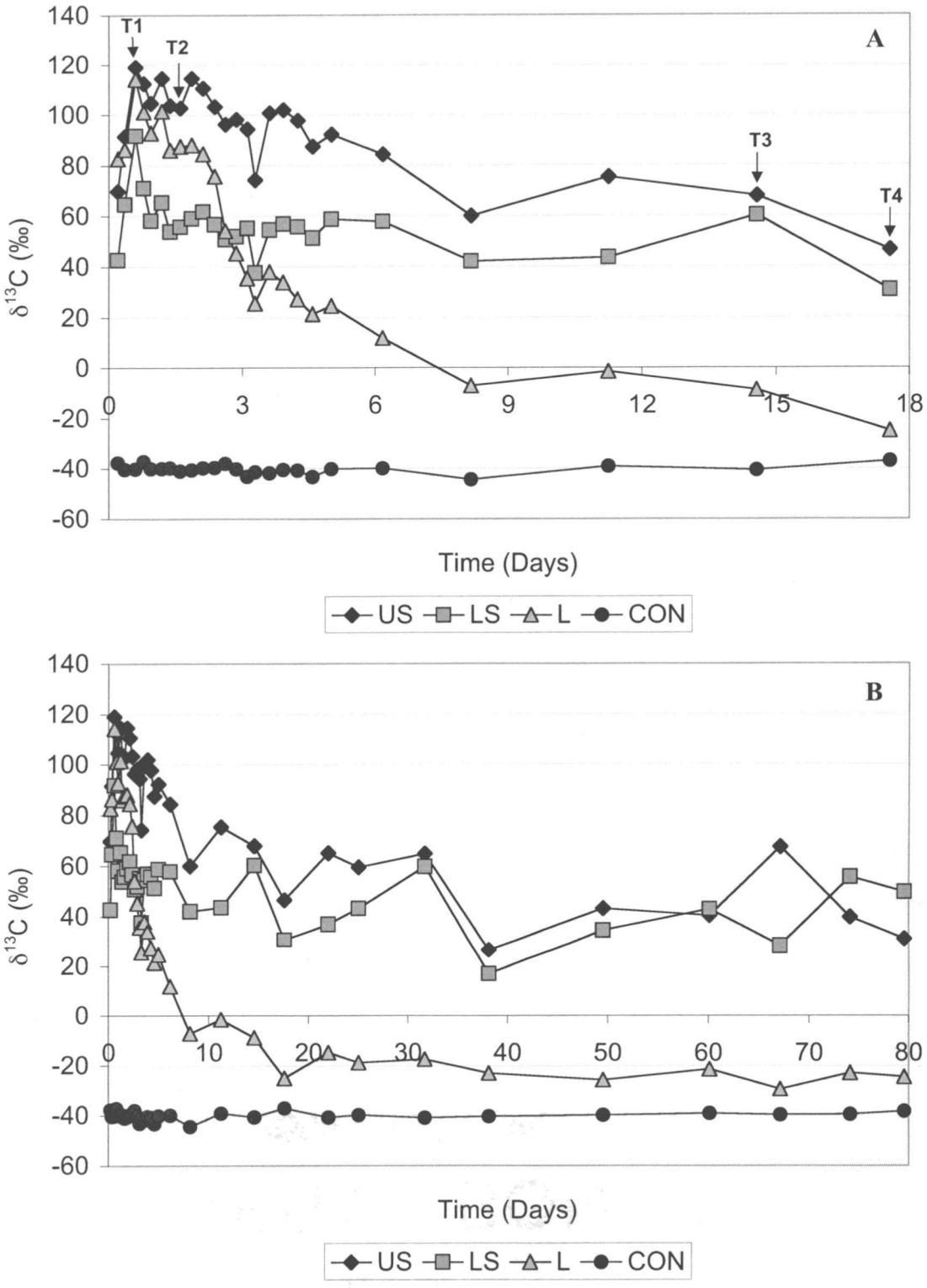

Figure 2.2. $\delta^{13}C$ of respired CO_2-C measured at each respiration sampling for (A) 0-18 d and (B) 0-80 d. Destructive soil sample times are indicated on (A) by T1, T2, etc.

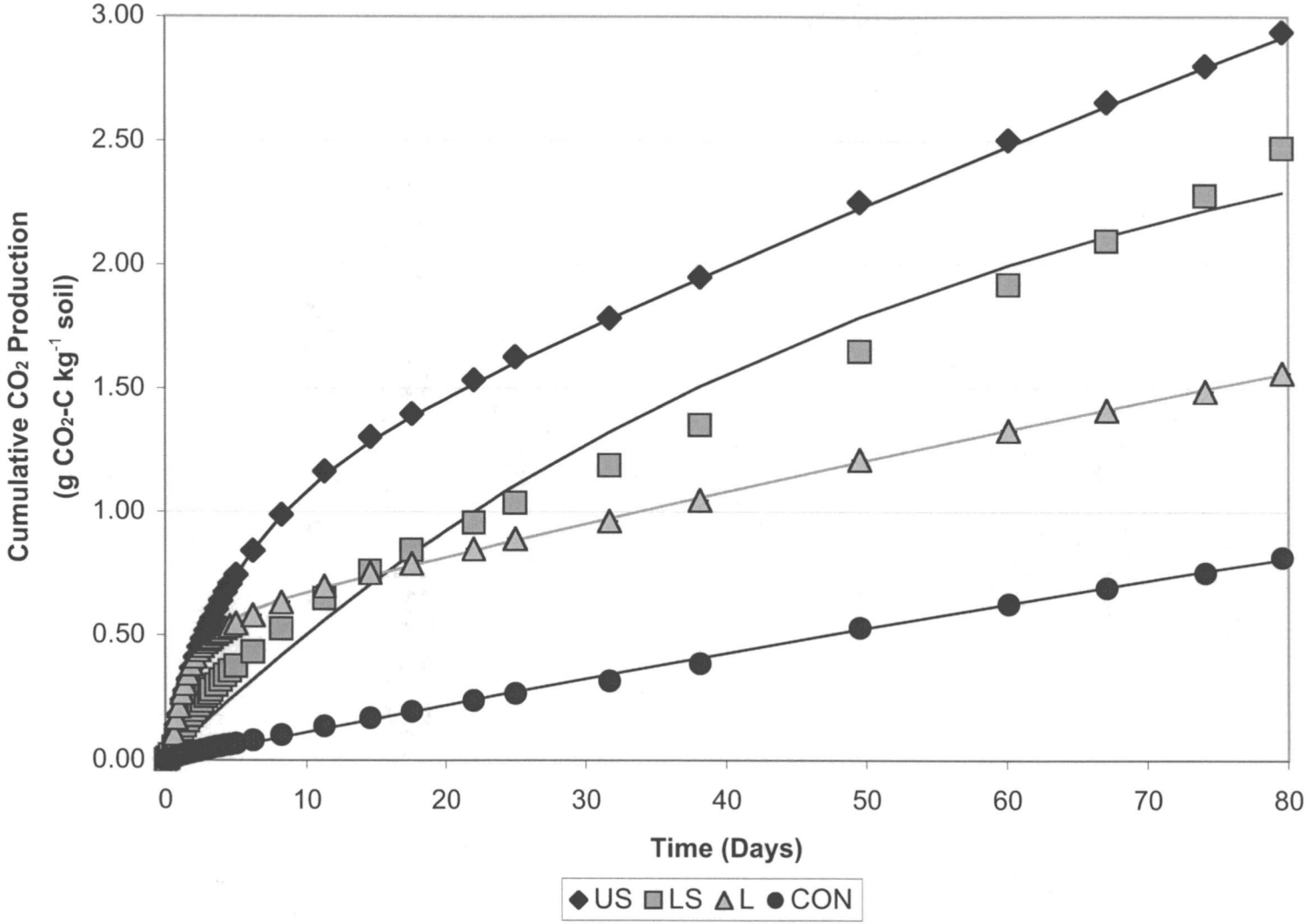

Figure 2.3. Cumulative CO_2-C production on per kilogram of soil basis. Lines are predicted values from a 1-exponential model (LS and C) or 2-exponential model (US and L). See Table 2.3 for model statistics.

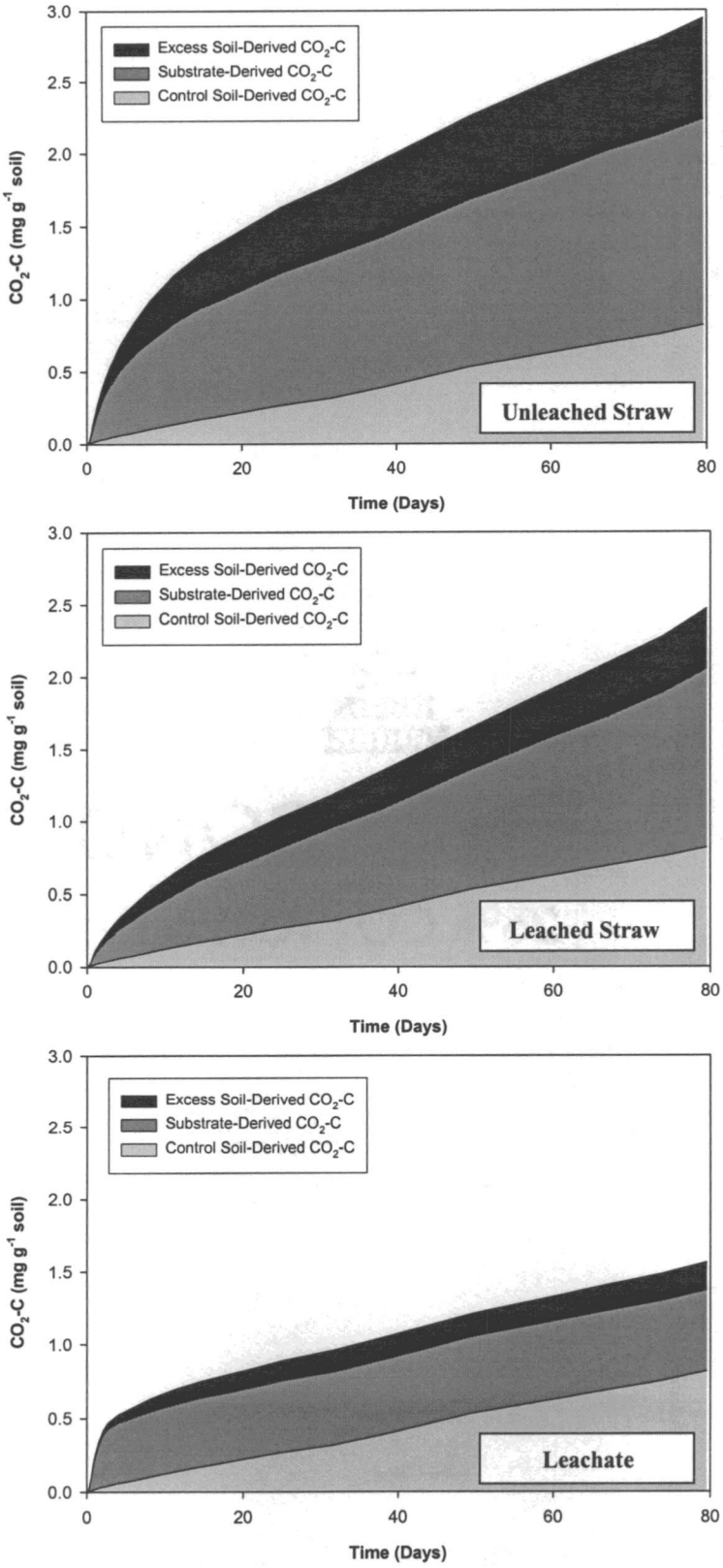

Figure 2.4. Sources of respired CO_2-C in US, LS and L. CO_2-C is separated into soil-derived CO_2-C measured from the control, and substrate-derived CO_2-C and excess soil-derived CO_2-C, which were calculated using atom% excess ^{13}C above the control.

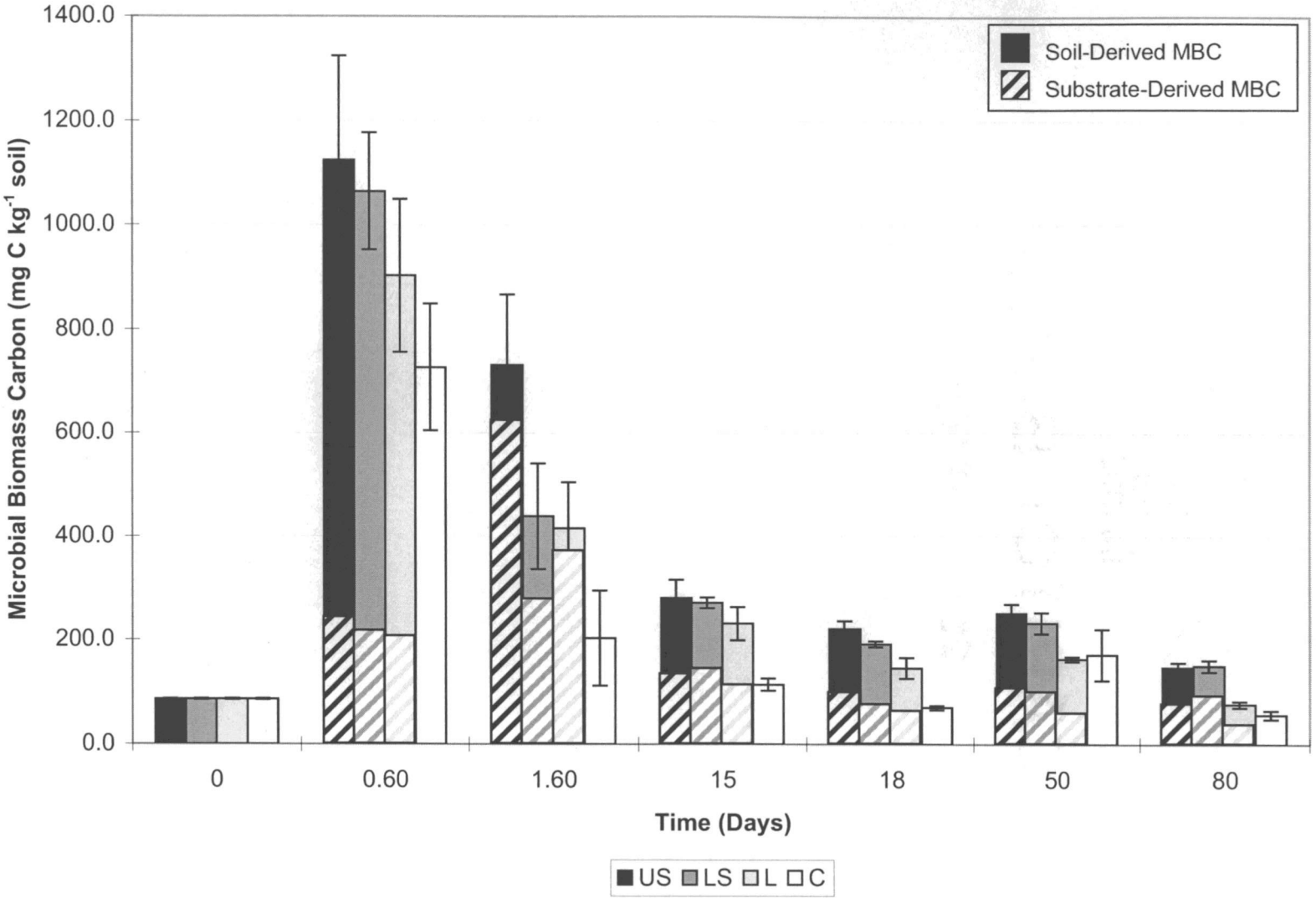

Figure 2.5. Microbial biomass carbon measured after 0.6, 1.6, 15, 18, 50, and 80 d of incubation. Bars represent total microbial biomass carbon on per gram of soil basis. Total MBC is separated into substrate-derived MBC (striped) and soil-derived MBC (solid).

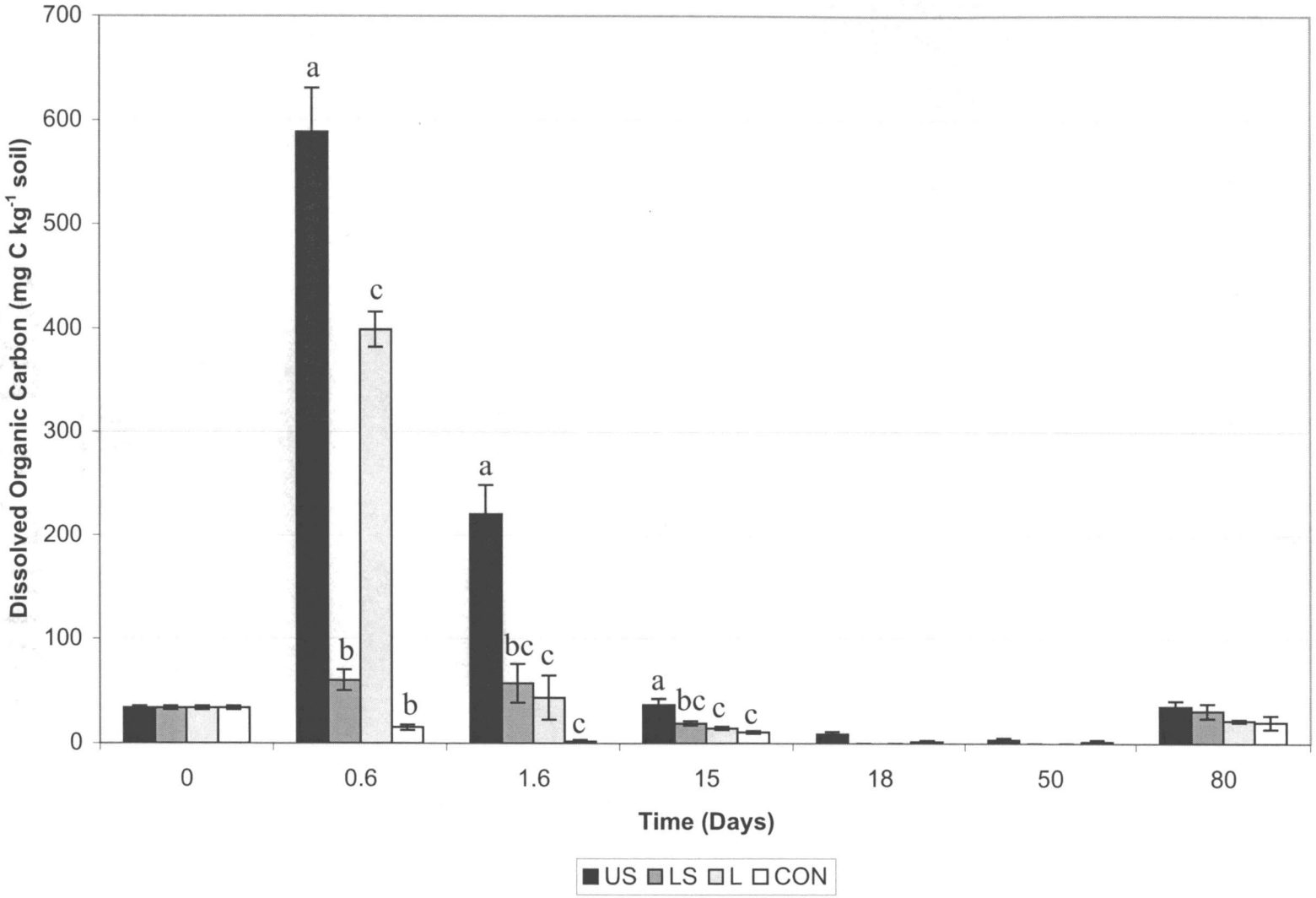

Figure 2.6. Dissolved organic carbon measured as K_2SO_4-extractable C from unfumigated soil (error bars are ±1 SE). Letters indicate significance using a Tukey test to compare treatments at each time. There were no significant differences among treatments at 0, 18, 50, or 80 d.

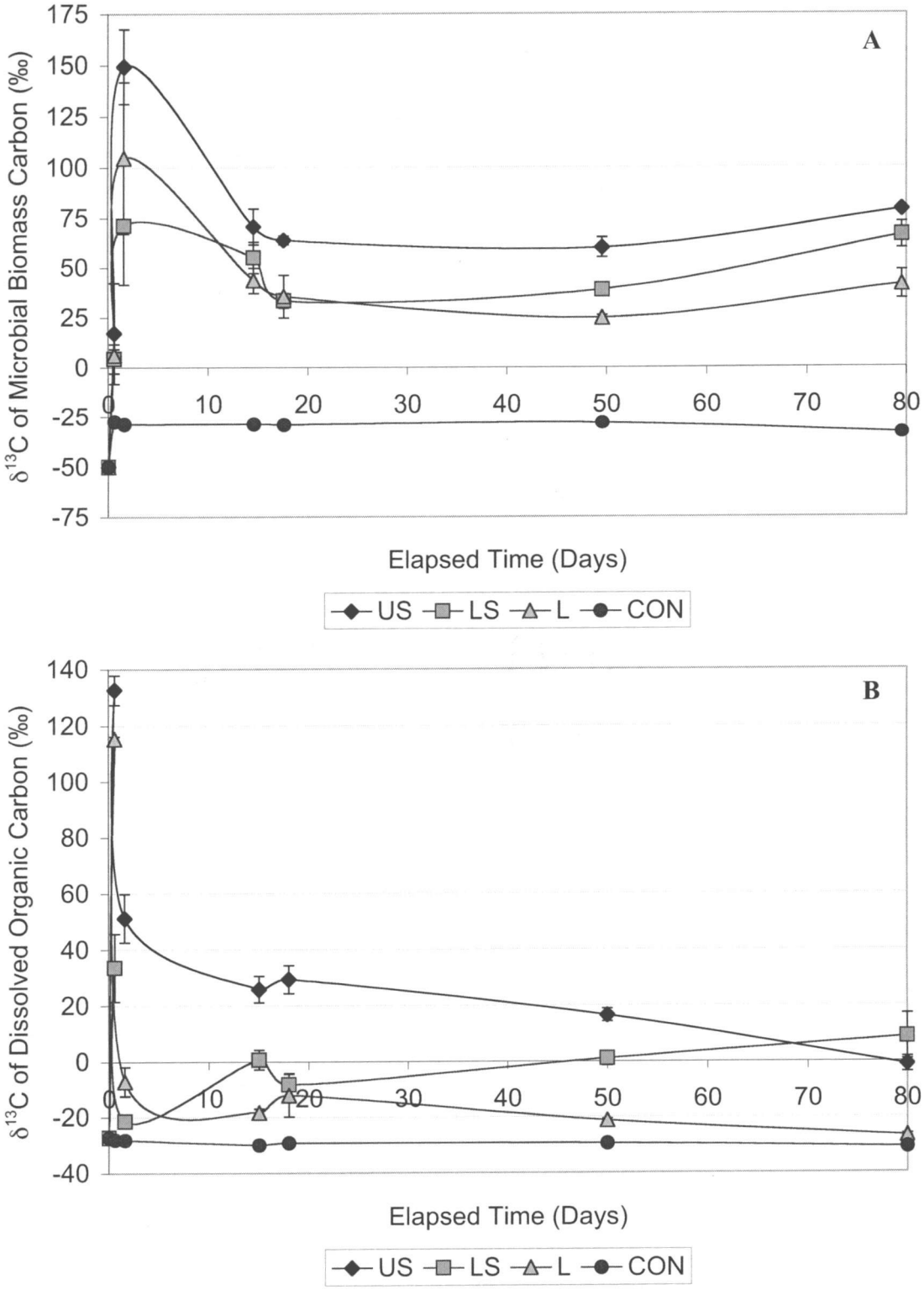

Figure 2.7. $\delta^{13}C$ of microbial biomass C (A) and dissolved organic C (B). Added carbon was labeled at 180‰, 130‰, and 120‰ for US, LS and L respectively. Native soil carbon was –26‰.

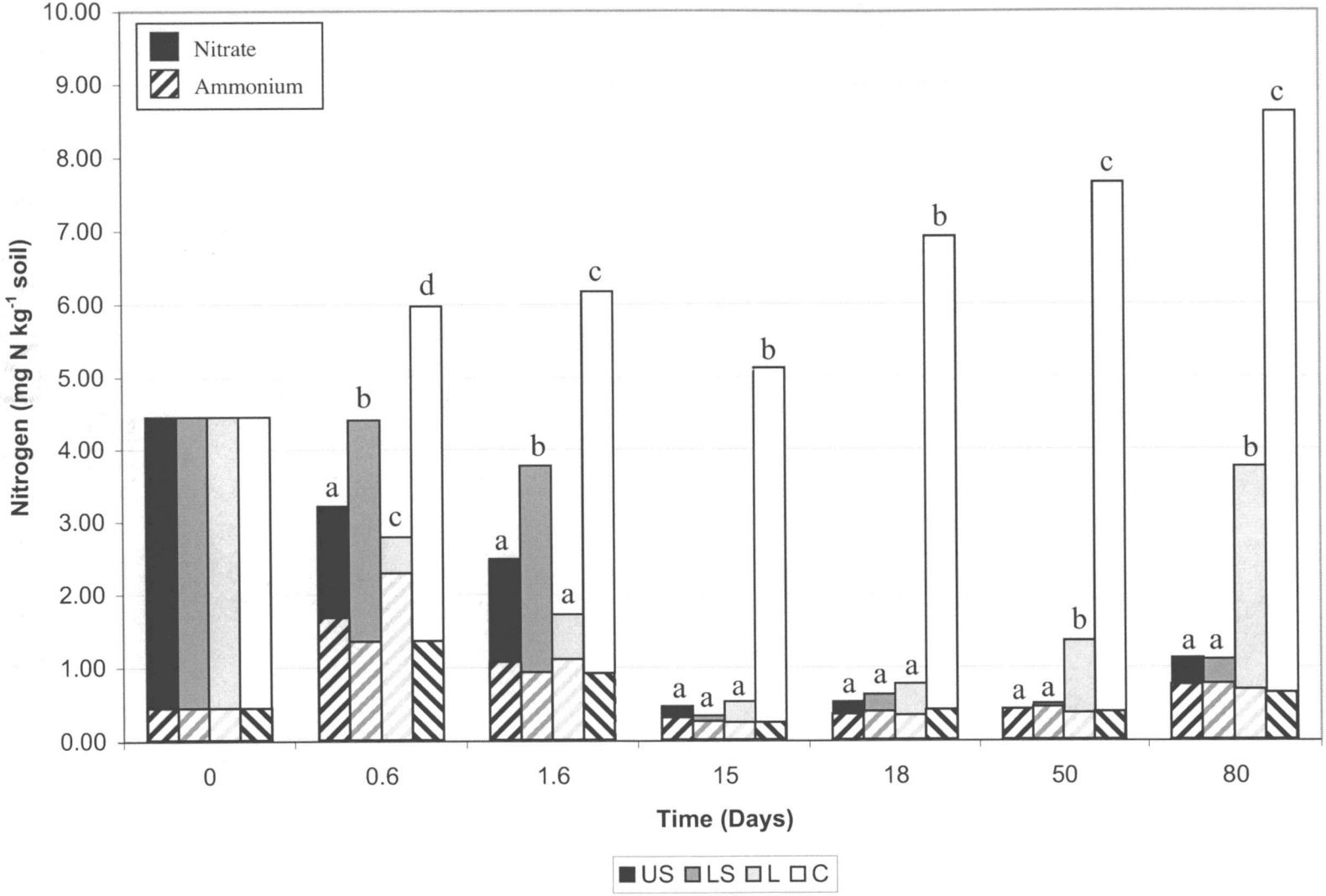

Figure 2.8. KCl-extractable nitrogen separated into ammonium (striped) and nitrate (solid). Letters above bars indicate results of Tukey test among treatments at each time point for nitrate only. Treatment had an insignificant effect on ammonium levels ($p = 0.829$).

Discussion

Role of Soluble C in Ryegrass Decomposition

Respiration data showed two distinct phases of C consumption. For the first 3 d, respiration rates in US and L were nearly identical, suggesting that soluble C is preferentially mineralized from intact straw (Knapp *et al.*, 1983). This is confirmed by $\delta^{13}C$ of CO_2 evolved from US and L, which showed microbes in both treatments produced similarly labeled CO_2–C for the first day. The rate of respiration and $\delta^{13}C$ of evolved CO_2 was initially lower in the leached straw, because the readily available and more highly labeled C had been removed by leaching, leaving more recalcitrant compounds such as cellulose and lignin. Decomposition of such compounds is slower because complex extracellular enzymes are required and energy yield is low (Schimel, 1995; Fontaine *et al.*, 2003).

After 15 d, the initial rapid flush of activity slowed as the pool of labile C was exhausted from the US and L treatments. $\delta^{13}C$ and rate of CO_2 evolution in the straw treatments began to stabilize at this time and paralleled each other for the duration of the incubation. This change in activity is indicative of a shift to focused degradation of the more recalcitrant pools of C in the straw. Following depletion of C added in leachate, the only C available to microbes in L treatments was SOM. Thus, there was little difference in respiration rate between L and CON as microbes degrade complex polymeric SOM. These results are consistent with those of Cogle *et al.* (1989), who found that soluble C from wheat straw applied separately to soil was completely consumed during the first 15 d of decomposition.

Although it is convenient to think of decomposition as discrete phases, soluble C is not being decomposed to the complete exclusion of structural C (Reinertsen *et al.*, 1984). The amount of soluble C added in leachate was equivalent to the amount of soluble C added in the intact straw. Therefore, if only soluble C was degraded initially then DOC in both treatments would be the same, which was not the case. Cogle *et al.* (1989) observed more rapid decomposition of soluble C when it was separated from intact straw, and attributed this phenomenon to physical protection of labile C by

structural components of the straw. In this experiment, protection of soluble C does appear to be important in the first 1.6 d because more soluble C was extractable from US than L (Figure 2.6). Because the respiration rate was still similar between these two treatments during the first 3 d, microbes must have been degrading structural components of intact straw in US concurrently with consumption of labile C. This is confirmed by $\delta^{13}C$ measurements of CO_2–C: after 1 d, $\delta^{13}C$ in US was higher than in L. Because the insoluble component was slightly more enriched in ^{13}C, indicated by the higher $\delta^{13}C$ of leached straw (Table 2.1), CO_2-C evolved from a combination of labile C and structural C will have a higher $\delta^{13}C$ than CO_2-C evolved from labile C alone.

Collins *et al.* (1990) found that the decomposability of wheat residues did not differ after the soluble C fractions were lost. This could account for the discrepancy in total CO_2–C evolved from US vs. LS+L; 170 mg CO_2–C was produced in excess of US from LS+L (Figure 2.3). Because labile C is only important during initial stages of decomposition, the gap between US and LS narrowed slowly during the last 50 d of incubation. The difference between US and LS+L may also reflect the effect of straw architecture on decomposition (Cogle *et al.*, 1989).

Kinetics. Exponential models of total CO_2 evolution suggest that the readily available C pool in US was nearly twice as large as in L (Table 2.3). Since all soluble C was supposedly collected in leachate, the expectation is that these pools should be similar in size. The discrepancy supports the conjecture that labile C is physically protected within straw and thus not all soluble C was extracted in leachate, resulting in a larger fast C pool in US. Although the fast C pool was larger in US than L, it was consumed at about half the rate, likely due once again to the need for degradation of structural C before the labile C can be accessed. Primed degradation of soil-derived C in US is evidenced by the fact that the slow C pool, which should be equal to LS plus C pools, is slightly larger.

Because only recalcitrant C compounds were presented to soil microbes in the LS treatment, the rate at which that slow substrate-derived C pool was utilized was an order of magnitude larger than in US. Likewise, because all substrate C in L was labile, the rate constant for the consumption of this pool was also greater than in US. Microbes in

US could simultaneously use readily available and more complex compounds, dividing the activity and lowering rate constants for both pools.

Priming Effect

Measurement of ^{13}C in respired CO_2 makes it possible to determine which pool of C microbes are utilizing for growth. In this system, there are only two available sources of C: soil organic matter or straw amendments. If there were no priming effect, the amount of soil-derived CO_2–C would be the same for all treatments. However, in the amended soils, excess soil-derived CO_2–C was produced when C was added in the form of straw or leachate (Figure 2.4).

Excess production of CO_2–C from soil-derived C was not the same in all treatments. Unleached straw induced the largest priming effect, followed by leached straw and leachate. This is consistent with the fact that priming tends to be greatest with large C additions (Jenkinson, 1996). The amount of C added in unleached straw was equivalent to 50% of native soil C, whereas leached straw represented 37% and leachate only 7.5%. However, despite LS inducing a moderate priming effect, the rate at which excess substrate-derived CO_2 was produced was an order of magnitude greater than US or L (Table 2.3).

The form of C added can also impact the magnitude of priming. Sucrose and polymers of fructose are the main carbohydrates found in annual ryegrass (Smouter *et al.*, 1995). When simple sugars such as glucose are added to soil, the response is variable. In some cases a negative priming effect is observed initially, followed by positive priming later in decomposition (Jenkinson, 1966). In other experiments, consistently positive, negative, or neutral priming effects have been reported (Kuzyakov *et al.*, 2000). In this experiment, in which soluble C consisted of about 80% glucose (data not shown), negative priming was only measured in one interval between 8.6 and 14.4 h.

Addition of easily assimilated compounds, such as glucose or fructose, to soil has a smaller effect than complex compounds like ryegrass, cellulose, or wheat straw (Fontaine *et al.*, 2003). This phenomenon can be related to the close relationship between microbial biomass and real priming effects (Kuzyakov *et al.*, 2000). During

decomposition, a succession of microorganisms occurs as substrate quality changes. Organisms responsible for decomposition can be grouped into two broad categories: r-strategists and K-strategists. r-strategists are adapted to periods of rapid growth and die or return to dormancy when added substrates are depleted (Fontaine *et al.*, 2003). These organisms consume easily degradable compounds, but generally do not produce enzymes necessary for depolymerization of complex plant components or SOM. K-strategists are continuously active because they are able to exploit widely distributed but recalcitrant substrates such as SOM or lignin (Jenkinson, 1988). Because K-strategists grow slowly, they may not be able to compete with r-strategists for readily available substrate, and thus arise after r-strategists have died off and polymerized compounds remain in soil (Fontaine *et al.*, 2003).

The induction of r-strategist but not K-strategist growth by simple compounds could explain why addition of soluble C compounds results in a smaller priming effect than addition of complex insoluble compounds (Fontaine *et al.*, 2003). In this experiment, excess soil-derived CO_2–C production was much larger with unleached straw than leachate. Because the leachate was comprised of simple, water-soluble compounds, it is likely that r-strategists were dominant in the microbial community resulting in low SOM degradation. With straw addition, K-strategists were able to take advantage of additional polymeric materials, resulting in more SOM-decomposing enzymes released into soil and a larger priming effect (Fontaine *et al.*, 2003). Wu *et al.* (1993) also observed ryegrass induced a larger priming effect than glucose when the amount of C added in each substrate was equal.

Caution must be taken when measuring and evaluating priming effects with labeled substrates. The very isotope that makes calculation of priming possible is the most frequent cause of artifacts (Kuzyakov *et al.*, 2000). In particular, non-uniformity of labeling in plant residues can result in inflated or deflated estimates of priming. If labile C is more highly labeled than cellulose and lignin, which will occur in plants exposed to ^{13}C for a short time, CO_2-C evolved during early, rapid decomposition is more enriched than the overall plant signature would suggest, resulting in an apparent negative priming effect (Jenkinson, 1966). Also, as soon as the isotope has been incorporated into SOM, it

is no longer possible to attribute $^{13}CO_2$ to substrate decomposition with absolute certainty (Sauerbeck, 1966).

Microbial Biomass Dynamics

A large water-soluble C component will support a flush of microbial growth (Summerell and Burgess, 1989). Hence, it follows that MBC would be largest in US and L, because the water-soluble component had been removed from LS. This was not the case, as there were no significant differences among treatments through time ($p = 0.129$, Table 2.2). Rather, it seems that the quantity of substrate available regulated the initial size of microbial biomass (Jawson and Elliott, 1986).

Only 20% of total microbial biomass contained substrate-derived C after 0.6 d in amended treatments, despite a large flush of microbial growth. The fact that CON soils showed an equally large increase in soil-derived microbial biomass indicates that much of the C taken up by organisms during the first 14 h of incubation was liberated during wet-up at the start of the experiment; soils were extremely dry to start and a pre-incubation was not performed.

By 1.6 d, substrate-derived MBC represented over 80% of total MBC in US and L, but only 63% in LS, suggesting that microbes were preferentially incorporating labeled labile C. When straw remained intact, total MBC was largest. However, differences in availability of soluble C did not result in differences in total MBC in LS and L treatments.

By 15 d, substrate-derived MBC represented about 50% of total MBC in all amended treatments and this trend continued for the duration of the experiment. With the exception of 1.6 d, soil-derived microbial biomass in amended treatments was similar to CON soil microbial biomass, with substrate-derived biomass contributing to an overall increase in the biomass pool. This is consistent with the results of Wu *et al.* (1993), who found that biomass formed from ryegrass simply added to the total biomass pool with unlabeled biomass remaining fairly constant. They also observed little change in labeled biomass between 20 and 40 d.

Depletion of added soluble C in leachate by 80 d resulted in MBC only marginally larger than CON soils, which had returned to pre-incubation levels. Straw treatments were able to sustain a biomass nearly twice background levels by this time.

Energy and Nutrient Dynamics

Despite a small initial microbial biomass, decomposition began immediately resulting in no measurable lag phase. This is consistent with the results of Ladd *et al.* (1995) who measured microbial decomposition of ^{14}C-lableled plant residues. During the first 1.6 d of decomposition, soluble C as measured by DOC is readily available in both unleached straw and leachate treatments. $\delta^{13}C$ data from MBC and DOC show that the release and incorporation of substrate-derived C is slightly offset; $\delta^{13}C$ peaks in DOC after 0.6 d, whereas the highest $\delta^{13}C$ value is not measured in MBC until 1.6 d. (Figure 2.7). This is consistent with current models of C processing during decomposition, where C must first move through the soluble C pool before microbial communities can use it for energy production and growth.

During the first days of incubation, there was a small increase in ammonium levels in all treatments, potentially resulting from wetting of dry soil. Rewetting of air-dry soil, as occurred in this experiment, may not only allow organisms killed during the drying process to decompose, but also release substrate in addition to dead biomass (Jenkinson, 1988). Both ammonium and nitrate levels remained low in all amended treatments until 18 d, after which time nitrate levels began to increase in L. This suggests that most N released during decomposition was being immobilized as the microbial community utilized substrate C. Increases in nitrate through time in C, and later in L likely resulted from low labile C availability, and thus reduced microbial consumption of nutrients.

Efficiency of C Assimilation

The apparent efficiency with which C was assimilated by the microbial biomass was much higher than expected (Table 2.4), as fungal efficiency ranges from 30% to 70% and bacterial efficiency ranges from 20% to 40% (Holland and Coleman, 1987).

However, other researchers have reported similarly high efficiencies. For example, van Veen *et al.* (1985) measured C assimilation efficiency greater than 70% at 0.25 and 0.5 d after substrate addition. With ^{14}C-labeled wheat straw, Bremer and van Kessel (1990) detected ^{14}C efficiencies as high as 88% after 0.25 d in treatments with 300 $\mu g\ C^{-1}\ g^{-1}$ soil; by 1 d, efficiency had declined to 70%.

Unbalanced growth, which occurs when a suitable C source is present but one or more essential nutrients or growth conditions are absent (Vestal and White, 1989; Zelles *et al.*, 1994), could be responsible for the unusually high efficiencies measured in this and other experiments. Under such conditions, microbes are able to take up C without actually utilizing it for growth or maintenance (Morita, 1988). Rather, they can store C among cytoplasmic constituents as energy reserve polymers, such as poly-β-hydroxybutyrate.

Although most researchers report MBC increases and efficiencies that conform to conventional wisdom about microbial growth and C assimilation, it is possible that enhanced C uptake occurs in other systems and simply goes undetected. By 1.6 d in this experiment, MBC levels had decreased to levels that result in reasonable efficiency values (Table 2.4). However, in many experiments (e.g. Cogle *et al.*, 1989; Wu *et al.*, 1993; Gaillard *et al.*, 1999), the first measurement of MBC is not made until two or more days into the incubation.

Table 2.4. Carbon assimilation efficiencies after 0.6 and 1.6 d reported for total carbon and ^{13}C separately.

Treatment	Day 0.6		Day 1.6	
	Total C	^{13}C	Total C	^{13}C
Unleached Straw	92%	81%	67%	74%
Leached Straw	95%	86%	69%	74%
Leachate	89%	69%	49%	56%
Control	98%	NA	81%	NA

Another possible explanation for the very large MBC values on Day 0.6 relates to the method of measurement itself. Fumigation methodologies are least reliable in situations where biomass is undergoing rapid change, such as following rewetting of air-dry soils or immediately after the addition of large quantities of plant biomass, both of which occurred at the beginning of this experiment (Jenkinson, 1988; Voroney, *pers. comm.*). Holland and Coleman (1987) found that fumigation-extraction over-estimated MBC relative to direct cell count measurements of fungal and bacterial C. They concluded that the fumigation methodology was a good measurement of the organic C pool, though not the microbial biomass itself, that was rapidly turning over during initial phases of decomposition.

Conclusion

This study confirmed that the soluble component of ryegrass straw is an important source of C during the initial stages of decomposition. We had hypothesized that the presence or absence of soluble C would affect the rate and quantity of biomass production, because not all organisms produce enzymes required to degrade all forms of plant C. In reality, microbial biomass dynamics were unaffected by substrate quality, responding primarily to the quantity of C added.

A distinct priming effect was measured in all three amended treatments, though the magnitude of excess CO_2-C production varied. Rates were also different among treatments, and rate was not correlated with magnitude, i.e. the highest rates were not associated with the greatest production. These results highlight the underlying complexity factors that drive a fairly straightforward effect.

Generally, it is thought that when microbes take in C, they use it immediately either for growth or maintenance with assimilation efficiencies between 30 and 70%. Calculated efficiencies as high as 98% in this experiment were initially considered spurious, as they appeared beyond the realm of physiological possibility. These results point to C uptake without metabolic processing, and likely the production of C storage products such as β-hydroxybutyrate. This phenomenon may occur regularly, and simply go undetected when biomass measurements are not made early enough. Further studies involving ^{13}C-labeled substrate could confirm production of β-hydroxybutyrate or other storage polymers during the most rapid phase of straw decomposition.

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Chapter 3:

Influence of Soluble C on Microbial Community Composition and Dynamics During ^{13}C-Labeled Ryegrass Straw Decomposition

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Abstract

Changes in chemical composition during straw decomposition induce succession in microbial communities. Therefore, separation of soluble C from ryegrass straw should impact decomposer community composition through time. Phospholipid fatty acid (PLFA) profiles provide insight into microbial community structure and dynamics because they are essential components of all living cells that degrade within hours of cell death. Changes in abundance of signature fatty acids are indicative of shifts in microbial community structure. Using ^{13}C-labeled ryegrass coupled with PLFA analysis by isotope-ratio mass spectrometry (IRMS), we tracked substrate-derived C as it was synthesized into microbial lipids during decomposition in a microcosm-based study. Residue was added to soil at rates that reflect field loads. Treatments were unleached straw (US), leached straw (LS), and leachate (L), plus an unamended control (CON). Added substrates had δ-values between 120‰ and 180‰; native soil signature was –26‰. Destructive sampling took place after 0.6, 1.6, 15, 18, 50, and 80 d of incubation. PLFAs were extracted from bulk soil and isolated straw (detritosphere) using a modified Bligh and Dyer method and analyzed by GC-C-IRMS. Twenty PLFAs were identified and quantified, but $\delta^{13}C$ values were highly variable.

Distinct temporal shifts occurred in PLFAs in bulk soil samples. In all amended treatments, early communities were dominated by short, branched-chain PLFAs such as 15:0a. Later samples contained more complex, longer PLFAs. 19:0cy was an indicator for late succession communities in US and L, and 18:2ω6,9 characterized late samples in LS. Soluble C affected when the temporal shift occurred; in LS and L, communities shifted earlier than in US. At all times, more ^{13}C was detected in the 16:0 and 18:2ω6,9 PLFA of the detritosphere than bulk soil. ^{13}C was slowest to appear in PLFAs from leached straw samples. 18:2ω6,9 PLFA, a fungal biomarker, was most highly labeled in all treatments. Due to the ubiquity of 16:0 in microbial cells, we cannot attribute $\delta^{13}C$ of this lipid to a single taxonomic group. However, because 18:2ω6,9 is not produced by bacteria, we conclude that fungi were more effective at incorporating substrate C into cellular lipids.

Introduction

Because different components of plant residue are degraded at different rates, chemical composition changes of detritus with time (Cheshire *et al.*, 1998; Horwath and Elliott, 1996). These changes induce a succession of organisms responsible for plant residue decomposition, with a shift from bacterial-dominated communities to fungal-dominated communities. Fungi are responsible for nearly all production of extracellular enzymes necessary to degrade structural components of straw, such as lignin and cellulose (Schimel, 1995). In general, bacteria are the primary decomposers of simple carbohydrates, organic acids, and amino acids (Myers *et al.*, 2001). It therefore follows that the separation of soluble C from ryegrass straw should impact decomposer community composition.

Phospholipid fatty acids (PLFAs) are useful biomarkers of viable microbial communities because they are essential components of all living cells that degrade within hours of cell death, hence do not accumulate in soil (White *et al.*, 1997, Zelles, 1999, Burke *et al.*, 2003). PLFA analysis is useful in microbial ecology because microbial lipids contain a variety of fatty acids, which are produced in different combinations by different organisms (Cavigelli *et al.*, 1995). Hence, the types and amounts of PLFAs present reflect the composition of living microbial communities (Song *et al.*, 1999).

Although PLFA profiling has been used extensively to characterize microbial community structure in soil, the coupling of ^{13}C-labeled substrates and lipid analysis is a relatively recent advance. Phillips *et al.* (2002) added ^{13}C-labeled cellobiose and *N*-acetylglucosamine to temperate forest soils, where they found that ^{13}C was not incorporated into all PLFAs; nine PLFAs contained 80% of excess ^{13}C recovered. Based on ratios between fungal and bacterial lipids, they were also able to determine that fungi consumed proportionally greater quantities of cellobiose than *N*-acetylglucoseamine compared to bacterial populations. *N*-acetylglucosamine was used for biosynthesis more than cellobiose, as more ^{13}C was recovered in PLFAs. Hanson *et al.* (1999) linked toluene degradation with specific microbial populations in soil by adding ^{13}C-toluene. Only a small subset of soil PLFAs was enriched with ^{13}C, compared to easily degradable

^{13}C-glucose, which appeared in all PLFAs measured. Arao (1999) obtained data about the rate of C assimilation by various groups of organisms by measuring ^{13}C in PLFAs following addition of ^{13}C-labeled acetate, the precursor to all lipids. Results of this experiment showed that gram-positive bacteria utilized C more slowly than did gram-negative bacteria.

The true power of lipid analysis of soil microbial communities is realized through the application of multivariate statistical methods to PLFA profiles. The majority of fatty acids measured in PLFA analysis are common to most microorganisms. In fact, about 50 fatty acids represent about 10 000 species of microbes and often an individual organism's profile contains less than 10 fatty acids (Zelles, 1997; Zelles, 1999). Therefore it is not necessarily which PLFAs are present in a sample that differentiates it from others, rather the combination and ratio of those lipids that may be important. Principal components analysis or other ordination techniques can be used to find the main trends in complex PLFA profiles that may not be obvious from univariate exploration of lipid data. Differences in PLFA composition can be indicative of spatial or temporal variation; PLFA profiles can be used to distinguish between soils from different environments or detect changes due to environmental disturbances (Lindahl *et al.*, 1997).

This experiment had several objectives with respect to microbial community structure and dynamics. First, we wanted to ascertain if community structure, as indicated by PLFA composition, changes over time in a given treatment. Second, we wanted to determine if the presence or absence of the soluble component of ryegrass straw would result in different communities developing in each treatment at a given time. ^{13}C-labeling provided the mechanism to address the third objective of determining whether different lipids, representing different functional groups of organisms, are enriched differently between treatments and over time.

Materials and Methods

Soil Description

Soil was collected from Hyslop Research Farm, located north of Corvallis, OR (N 44°38' W 123°12') in July 2002. The soil was very dry and hard at the time of sampling. In the lab, clods were broken and soil was passed through a 2-mm sieve to remove large organic debris. Following sieving, soil was air-dried and stored in buckets at 4°C until needed

The soil is classified as a Woodburn silty loam (Aquultic Argixeroll), which is naturally acidic and well drained. The climate is characterized by cool, wet winters and dry, warm summers, with 108.5 cm of annual precipitation. The surface horizon is about 25 cm deep and 1.3% organic carbon

Labeled Ryegrass Preparation

Annual ryegrass (*Lolium multiforum* L.) was planted in Fall 2001. Plants were labeled with ^{13}C using a pulse–chase technique weekly between early April and mid-May 2002. A Plexiglas labeling chamber (60 x 60 x 75 cm) was placed over the plants and the soil-chamber interface sealed with wet mud. To generate $^{13}CO_2$, 5 mL of 4M HCl was added to 150 mg of 99 atom% $NaHCO_3$ in a sealed 120-mL serum bottle. The gas was collected in two 60-mL syringes and injected into the headspace of the labeling chamber. The 120-mL injection raised the CO_2 in the chamber by about 400 ppm. To ensure complete collection of $^{13}CO_2$, air was injected into the bottle, removed, and injected into the chamber three times. CO_2 concentration in the chamber headspace was monitored using a LI-COR 6200 CO_2 analyzer (LI-COR Inc. Lincoln, NE). When CO_2 was drawn down to 150 ppm, the $^{13}CO_2$ injection procedure was repeated a total of four times. The $^{13}CO_2$ was chased with six, 120-mL injections of unlabeled CO_2. The headspace concentration was allowed to drop to 150 ppm between chase injections.

Leaching of Straw

Straw was first cut into 1 to 2 cm lengths. To prepare leachate, 18 g of straw was placed with 300 mL of cold deionized water in a 1-L canning jar and shaken on a lateral shaker at approximately 270 rpm at 5°C for 24 h. Leachate was decanted through 250-μm mesh, centrifuged at 11 325 x *g* for 10 minutes, and filtered through Whatman 2 filter paper to remove fine particulate matter. All leachate was pooled and stored at 4°C for 6 d. Leached straw was collected and dried on brown paper in a forced-air oven at 60°C for 48 h.

Preliminary Analyses

Soil, unleached and leached straw, and leachate were analyzed by isotope ratio mass spectrometry (IRMS) (PDZ Europa Ltd., Crewe, Cheshire, England) to determine total C content and ^{13}C abundance. Microbial biomass carbon was measured on unamended soil to determine starting values.

Microcosm Setup

Microcosms were prepared in 1-L canning jars with 150 g (oven dry) of soil, a C source, and deionized water to bring the soil to 29% gravimetric water content. The C treatments consisted of 2.25 g unleached straw, 1.69 g leached straw, or 29.0 mL of leachate. The amount of soluble C added in leachate was equivalent to the amount of soluble C added in the intact straw. To prepare straw treatments, residue was mixed with about 120 g of soil and carefully transferred to the jar. As the soil had a fine texture and poor permeability, 24.0 mL of water was dispensed into the bottom of the jar prior to the addition of the soil–straw mixture. This allowed the water to move through the soil by capillarity, resulting in more uniform wetting. The remaining soil was poured on top to ensure burial of all plant residue. An additional 7.5 mL of water was pipetted evenly over the soil surface. Leachate treatments were prepared by dispensing 29.0 mL of leachate into the bottom of the jar, adding all the soil and pipetting an additional 2.5 mL of water on the soil surface. The CON treatment had no added C and was prepared

similarly to the straw treatments without the mixing step. Table 2.1 summarizes amendments made for each treatment. Twenty-four replicates of each treatment were prepared. Jars were covered with eight layers of cheesecloth to allow adequate ventilation but slow water loss. Microcosms were placed in the incubator using a blocked design with each of four shelves representing a block. Replicates were arranged on the shelves using a Latin rectangle design.

Table 3.1. Summary of treatments, C amendments, and $\delta^{13}C$ values of soil and added C.

Treatment	Amount of C Added	$\delta^{13}C$ of Added C or Soil
Unleached Straw (US)	917 mg	+180‰
Leached Straw (LS)	675 mg	+130‰
Leachate (L)	133 mg	+120‰
Control (CON)	0 mg	–26‰

Sampling Protocol

Four randomly selected replicates (one from each block) of each treatment were destructively sampled at six times throughout the 80 d incubation. Sampling took place after 0.6, 1.6, 15, 18, 50, and 80 d. Soil was homogenized in each jar prior to subsampling. Subsamples were then taken for analysis of phospholipid fatty acids (PLFAs).

Phospholipid Fatty Acid Analysis

Straw and soil were separated for analysis of the bulk soil and detritosphere PLFAs. The method used to isolate PLFAs was based on the procedure originally proposed by Bligh and Dyer (1959) and subsequently modified by White *et al.* (1979). It consisted of a single-phase extraction of the total lipid, separation of the phospholipid fraction using solid phase extraction, and methylation by mild alkaline methanolysis.

Single-Phase Extraction. A mixture of 30 mL of methanol, 15 mL of chloroform, and 12 mL of 0.5 M potassium phosphate buffer (pH 7.1) was added to 15 g (oven dry) of soil. The suspension was shaken for 2 h at 270 rpm on an orbital shaker, and then allowed to sit for 24 h. Straw was extracted using 10 mL of methanol, 5 mL of chloroform, and 4 mL of phosphate buffer; straw samples ranged in mass from 0.5 to 1.5 g. On the second day, samples were swirled to remix, centrifuged at 20 000 x *g* for 5 minutes and poured through Whatman #1 filter paper. For the soil samples, an additional 20 mL of methanol and 10 mL of chloroform was added and samples were centrifuged again; straw samples received 7 mL of methanol and 3.5 mL of chloroform. The supernatant was poured through the same filter paper, which was then rinsed with 5 mL of chloroform. All filtrate was collected in Erlenmeyer flasks. Thirty milliliters of 3 M NaCl solution and 1.0 g Na_2SO_4 crystals were added to filtrate from soil samples to induce phase separation; straw samples received 10 mL of NaCl and 0.5 g of Na_2SO_4. Samples were allowed to sit for about 1 h until phases were clear. The chloroform phase (bottom) was transferred by pipette to a 60-mL serum bottle and dried under N_2 gas in a warm water bath (< 35°C). When dry, serum bottles were closed with a septum and crimp-top, flushed with N_2 and evacuated twice, left with positive pressure of N_2 gas, and frozen at -80°C until completion of analysis.

Phospholipid Isolation. Lipids were separated by solid phase extraction (SPE) using Supelco Supelclean LC-Si solid phase extraction tubes (Supelco Park, Bellefonte, PA). Three-milliliter tubes were used for straw residue lipids; soil lipids were separated using 6-mL tubes. Columns were prepared by adding 1 mL of chloroform to the 3-mL tubes or 2 mL to the 6-mL tubes. Detritosphere lipids were transferred from the serum bottle to the column with 3 mL of chloroform; bulk soil lipids were transferred with 4 mL of chloroform. For subsequent additions of chloroform and acetone, 2-mL aliquots were used for straw lipid samples; 4-mL aliquots were added to the soil lipid samples. Following the transfer of the lipids to the column, two additional aliquots of chloroform were added to the columns to completely remove the neutral lipid fraction. Three aliquots of acetone were used to elute the glycolipid fraction. Neither the neutral nor the glycolipid fractions were collected. Phospholipids were eluted from the column with

either two or three 2-mL aliquots of methanol for the straw or soil lipids. This fraction was collected in 15-mL test tubes and dried under N_2 gas in a warm water bath. To store dried samples in the short term, tubes were placed in 1-L Mason jars, which were sealed and flushed with N_2 for 10 minutes. Jars were frozen at –80°C.

Methylation. One milliliter of 1:1 methanol:toluene and 1 mL of 0.2 M methanolic potassium hydroxide were added to each tube. Following heating at 32°C for 15 minutes, 2 mL of 4:1 hexane:chloroform, 200 µL of 1 M acetic acid and 2 mL of deionized water were added to the tubes. The phospholipids were in the top layer consisting of hexane, toluene, and chloroform. This layer was transferred by pipette to another 15-mL tube. An additional 2 mL of 4:1 hexane:chloroform was added to the first tube, allowed to sit for 5 minutes and also transferred to the second 15-mL tube. Samples were dried under N_2 in a warm water bath. Dried, methylated phospholipids were transferred to 200-µL pulled glass inserts in 2-mL Agilent vials (Agilent Inc., Palo Alto, CA) using three 50-µL aliquots of chloroform. Chloroform was evaporated under N_2 gas at very low pressure. PLFAs were re-suspended in 40 µL of chloroform, closed with a septum screw lid, and stored at –20°C until analysis. It is important that the $\delta^{13}C$ signature of the methanol used in this step is known so that adjustments can be made for the contribution of the added methyl group on the overall $\delta^{13}C$ signature of the individual PLFAs. Methanol used for saponification had a signature of –44.6‰.

GC-C-IRMS. PLFAs were separated by capillary chromatography using an Agilent 6890 (Agilent Inc., Palo Alto, CA) capillary gas chromatograph equipped with a 30-m Hewlett Packard Innowax 2 column (0.25 mm i.d., 0.25 µm film) connected to a Europa ORCHID on-line combustion interface attached to a Europe 20-20 isotope ratio mass spectrometer (PDZ Europa Ltd., Crewe, Cheshire, England). The starting temperature was 120°C, and was ramped up to 250°C in 5°C per min increments. The run was terminated after the system had been at 250°C for 5 min. PLFAs move off the column based on boiling points and pass through the combustion unit where they are converted to CO_2 before analysis by the IRMS.

PLFA data from the MS was processed using GC Post Processor v.2.5 (PDZ Europa Ltd., Crewe, Cheshire, England). Peaks were identified based on comparison

with a known lipid profile generated by GC-MS analysis of PLFAs from similar soil. Twenty peaks were identified and were analyzed on a mol% basis (Table 3.2). $\delta^{13}C$ values were determined based on CO_2 pulse standards at the beginning of each run.

Using a pulse of CO_2 with a known $\delta^{13}C$ as the standard for $\delta^{13}C$ determination in PLFA analysis has two limitations. First, the CO_2 does not pass through the gas chromatograph and combustion unit and is therefore not subjected to the same potential modifications as the PLFAs. Second, it is difficult to get reliable $\delta^{13}C$ values for small peaks, because the shortest possible CO_2 pulse of 1 s produces a peak as much as 5x larger than lipid peaks. Peaks of interest are often clearly identifiable on the chromatogram with intensities of $5x10^{-12}$; however, reliable $\delta^{13}C$ values require intensity as high as $1.5x10^{-11}$. PLFAs were excluded from $\delta^{13}C$ analysis if the standard error between replicates was greater than 20‰ or if only one replicate contained the lipid.

Table 3.2. Identity and taxonomic classification of PLFAs analyzed in community profiles.

PLFA	Taxonomic Classification
15:0i, 15:0a, 16:0i, 17:0i, 17:0a	Gram-Positive Bacteria
14:0, 15:0, 17:0, 17:0cy, 19:0cy	Gram-Negative Bacteria, Stress Indicator
16:0, 18:0, 16:1ω7, 18:1ω7	Non-specific Bacteria
18:1ω9, 18:2ω6,9	Fungi
10Me16:0, 10Me17:0	Actinomycetes
14:0i, 17:1	Undefined

Nomenclature. PLFAs were named using the following convention: number of Cs in the chain:number of double bonds, followed by the position of the double bonds from the methyl (omega = ω) end of the chain (e.g., 18:2ω6,9). The suffixes "i" and "a" indicate the iso and anteiso forms of the PLFA (e.g., 15:0i). The prefix 10Me indicates a methyl group on the tenth C from the carboxyl end of the chain (e.g. 10Me17:0). Cyclopropane groups are indicated by the suffix "cy" (e.g., 19:0cy).

Data Analysis

It was not always possible to identify all 20 peaks, due to poor extraction efficiency resulting in small peaks dropping below detection limits. This produced incomplete lipid profiles for some replicates. If a sample was missing more than four peaks relative to the maximum number of peaks observed in other replicates of the same treatment, it was excluded from analysis. The absence of more than four peaks masked more important trends in peaks that were actually present. Twenty-four of 144 samples were either lost during extraction or excluded due to weak profiles.

Ratios were calculated between fungal and bacterial PLFAs. All PLFAs except 16:0, 18:0, and those listed as fungal (Table 3.2) were counted as bacterial (Frostegård and Bååth, 1996). 16:0 and 18:0 are ubiquitously produced by both bacteria and fungi, so are excluded from ratio calculations. Ratios were calculated based on total mol%.

All multivariate data analysis was conducted using PC-ORD v. 4.0 (McCune and Mefford, 1999). PLFA profiles were analyzed using principal components analysis (PCA) on a correlation matrix of mol% values. Data were determined to meet linearity and normality assumptions through examination of summary statistics of each PLFA. A multi-response permutation procedure (MRPP) was used to test the hypotheses of no difference between treatments at a given time point and no difference between times for a given treatment. MRPP is a nonparametric method for testing group differences that is not constrained by distributional assumptions (McCune and Grace, 2002). MRPP provides a measure of effect size (A), which describes with-in group homogeneity; perfect homogeneity produces A = 1. Significance of A is tested using a randomization test. Euclidean distance measure was used for MRPP to be compatible with PCA.

When significant treatment or time differences were identified by MRPP, groups were chosen from PCA ordination plots for indicator species analysis (ISA). The strength of an indicator is determined by its faithfulness or omnipresence in a group and its exclusiveness to that group. A perfect indicator is always present in the group for which it is an indicator and occurs in no other group (McCune and Grace, 2002). The indicator value for a given species, or PLFA in this case, is the product of proportional abundance of the species in a group relative to the abundance of that species in all groups

and the proportional frequency of the species in each group (McCune and Grace, 2002). Statistical significance of an indicator value was tested by a Monte Carlo method with 1000 randomizations of samples among groups. PLFAs were considered indicators if they were significant with $p = 0.001$, except in leached straw through time, where the sample size was too small to attain $p < 0.002$.

Repeated measures analysis of variance (ANOVA) was used to test for time and treatment effects on $\delta^{13}C$ PLFA using S-Plus v. 6.1 for Windows (Insightful Corporation, Seattle, WA). Where significant time•treatment effects were identified, Tukey's honestly significant difference method for mean separation was applied.

Results

Individual Treatments Through Time

In all cases, PCA captured the dominant trend of interest, i.e., temporal shift in PLFA profiles, on principal component one (PC 1). Hence, further discussion of results will focus only on PLFAs that were correlated with PC 1, and thus descriptive of the differences among groups of points separated along the first ordination axis.

Bulk Soil. In the unleached straw treatment (US), two distinct groups consisting of samples from 0.6, 1.6, and 15 d, and from 18, 50, and 80 d were produced by PCA (Figure 3.1(A)). These groups were separated primarily by PC 1, which accounted for 51% of variation in the data. In the leached straw (LS) and leachate (L), PCA also produced two groups of samples: 0.6 and 1.6 d, and 15, 18, 50, and 80 d (Figure 3.1 (B) and (C)). These groups were also separated by PC 1, which summarized 44% and 65% of the variation in LS and L, respectively. No distinct groups were observed in the unamended control. PCA was only able to capture 42% of variation in CON samples on the first two principal components. MRPP results indicate that groups in the amended treatments were strongly significant (Figure 3.1).

Table 3.1 summarizes the PLFAs that were correlated with the first principal component at $r^2 \geq 0.6$. 15:0 and 15:0a were positively correlated with PC 1 in all three amended treatments. 14:0 and 17:0 were positively correlated with PC 1 in US and L, whereas 14:0i and 10Me16:0 were positively correlated in LS and L. PLFAs that were positively associated with the first PCA axis had high mol% values in early samples and decreased with time (Figure 3.2). Indicator species analysis (ISA) results show that 15:0a is a strong indicator of microbial communities at early samplings ($p \leq 0.002$) in all amended treatments (Table 3.3). 10Me16:0 is also a significant indicator of early samples in LS treatments.

PLFAs that were negatively correlated with PC 1 were important in distinguishing samples taken later in the incubation. In this case, US and L samples were very similar, with 16:1, 17:0i, 17:1, 18:0, and 19:0cy correlating with PC 1 in both ordinations. With the exception of 17:1 in US, all were significant according to ISA. In contrast with

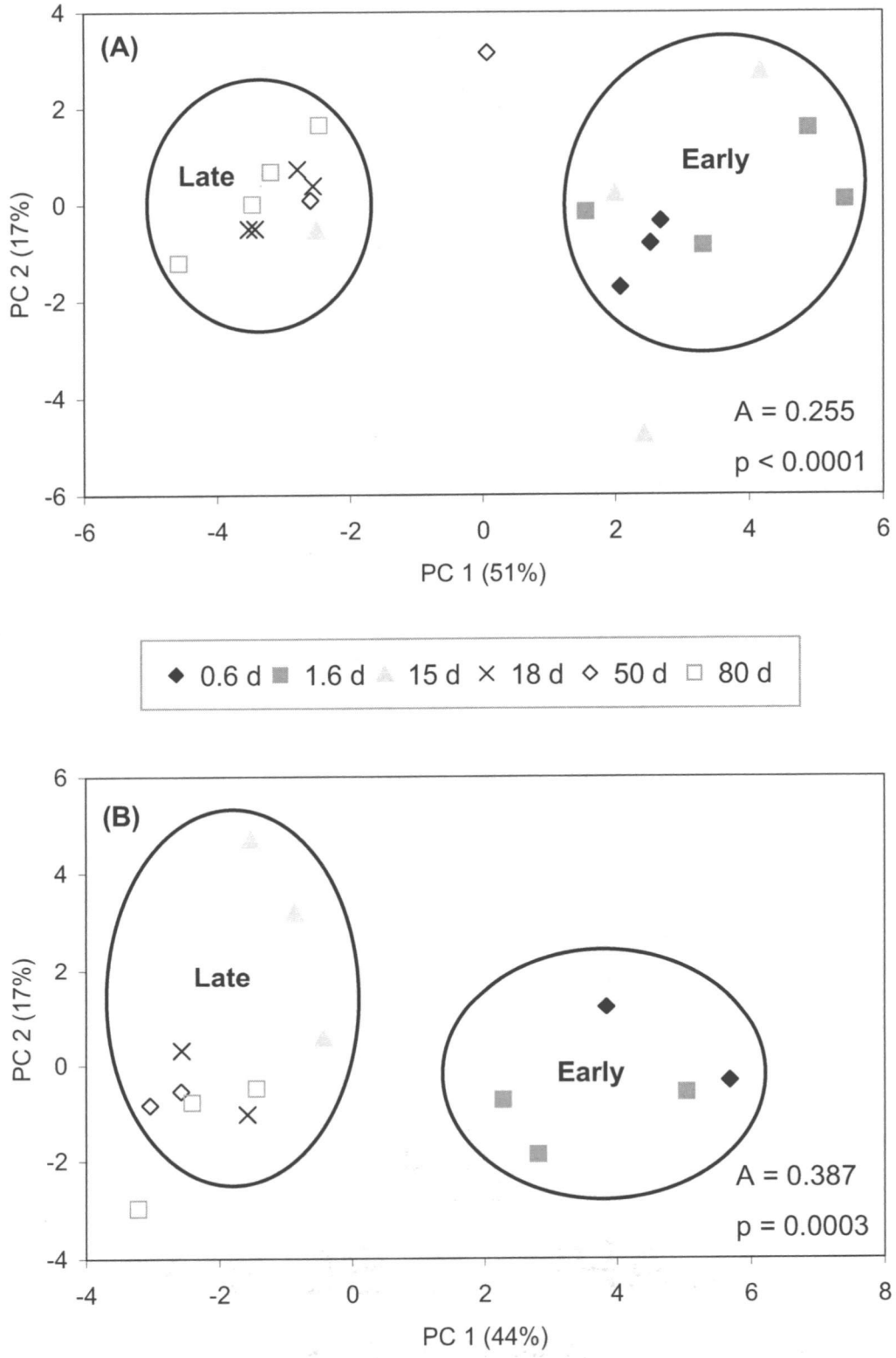

Figure 3.1. PCA for soil treatments through time. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. A = US, B = LS.

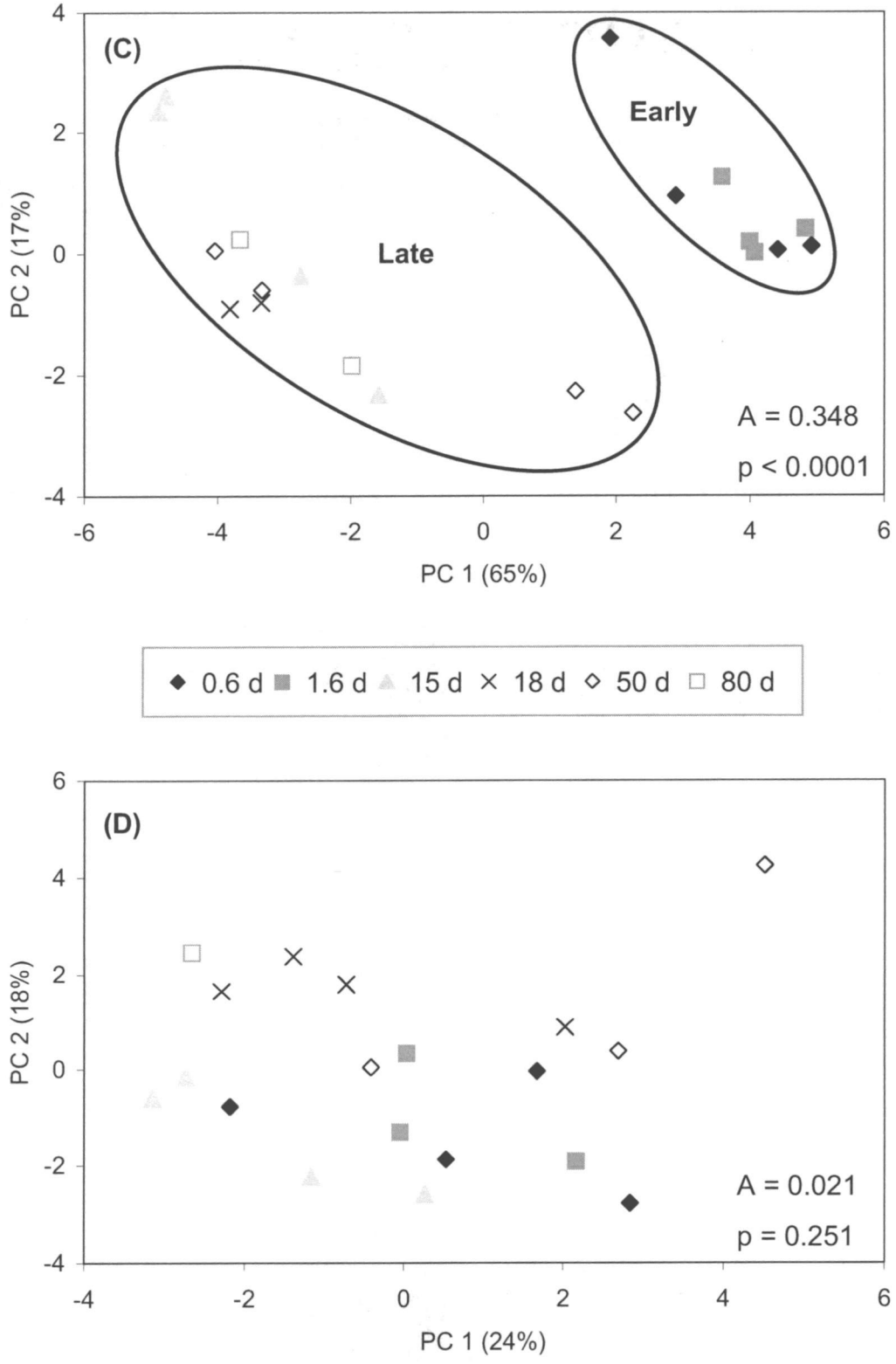

Figure 3.1 (Continued). PCA for soil treatments through time. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. C = L, D = CON.

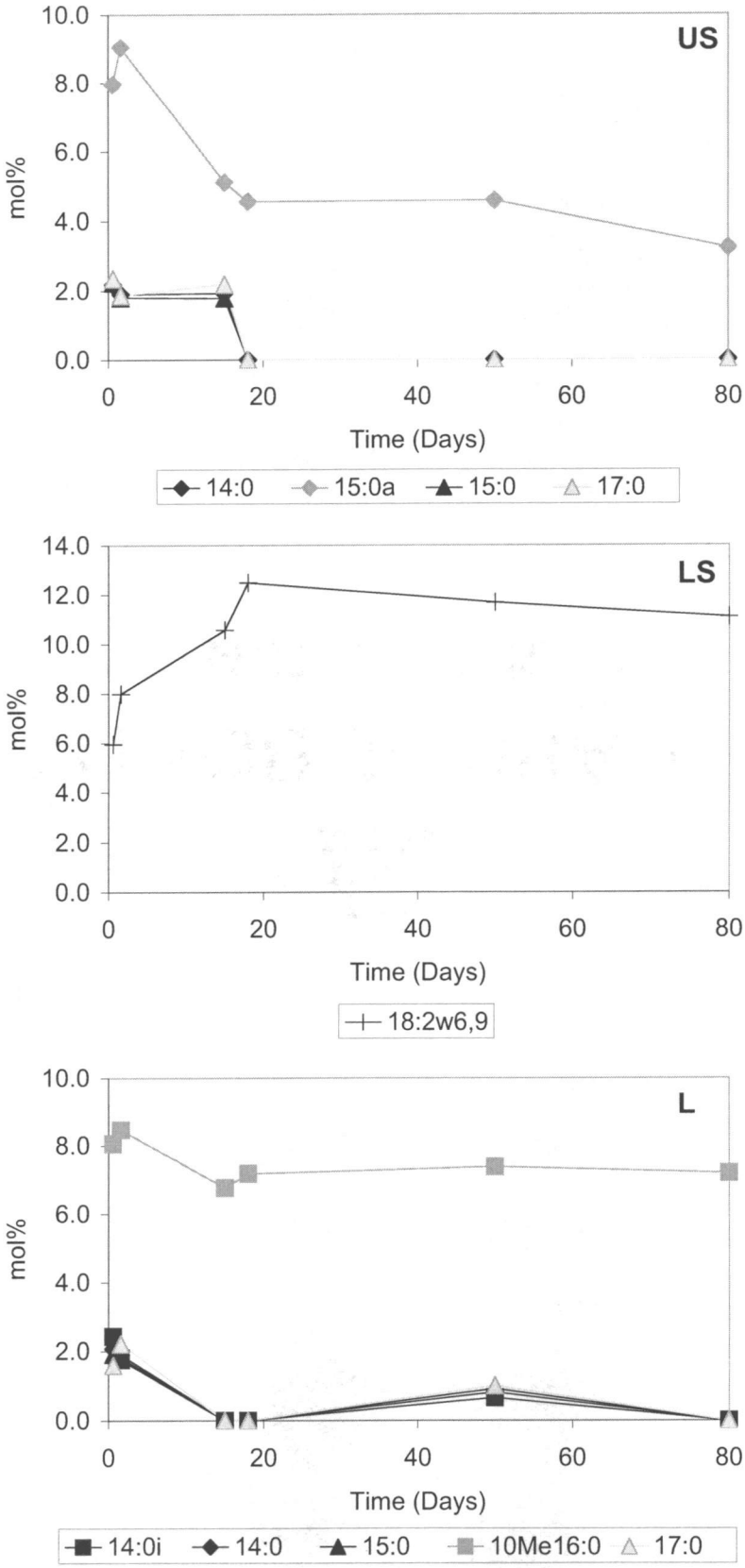

Figure 3.2. PLFAs positively correlated with PC 1 for US, LS and L at all times.

PLFAs associated with early samples, these PLFAs remained at fairly constant mol% through time (Figure 3.3). Only 18:2ω6,9 had a strong negative correlation with PC 1 in LS; 18:2ω6,9 was also a significant indicator of late samples according to ISA.

Due to the lack of temporal pattern in CON samples, only two PLFAs were correlated with PC 1 (16:0 (+) and 17:0a (-)) and neither were significant indicators.

Table 3.3. PLFAs correlated with first principal component in PCA of individual bulk soil treatments through time. Significant indicators from ISA are bolded (p = 0.001 unless otherwise noted).

Correlation	US	LS	L	CON
Positive	14:0	14:0i	14:0	16:0
	15:0	15:0	14:0i	
	15:0a	15:0i	15:0	
	17:0	**15:0a***	**15:0a**	
		10Me16:0*	10Me16:0	
			17:0	
Negative	**16:1**	**18:2ω6,9***	**16:1**	17:0a
	17:0		**17:0i**	
	17:1		**17:1**	
	18:0		17:0cy	
	19:0cy		10Me17:0	
			18:0	
			19:0cy	

* p = 0.002

Detritosphere. Despite MRPP indicating that samples from each sampling time produce statistically significant groups, temporal trends in detritosphere samples are not as obvious as bulk soil samples (Figure 3.4). In unleached straw detritosphere (USS), two groups consisting of 0.6, 18, and 50 d, and 1.6, 15, and 80 d can be distinguished. However, the non-sequential nature of these groups suggests that community compositional change was not a linear process and may have oscillated several times during the incubation. These two groups were also separated on PC 1, with 49% of variation explained. Leached straw detritosphere (LSS) PCA structure was dominated by the difference between 50 d and all other sample times. The trend captured by PC 1 is the absence of 10 PLFAs from all samples except those from 50 d (see Table 3.4 –

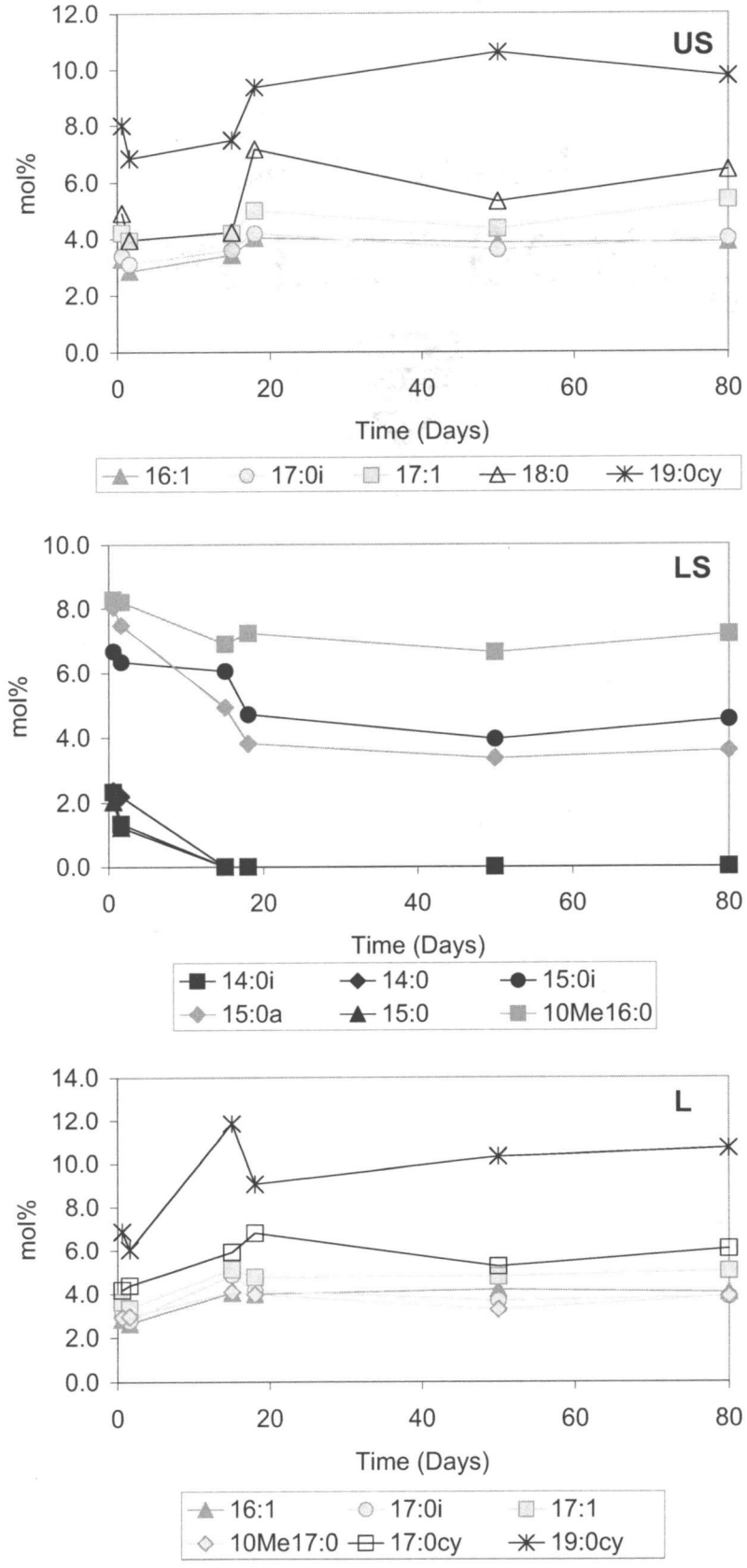

Figure 3.3. PLFAs negatively correlated with PC 1 for US, LS and L at all times.

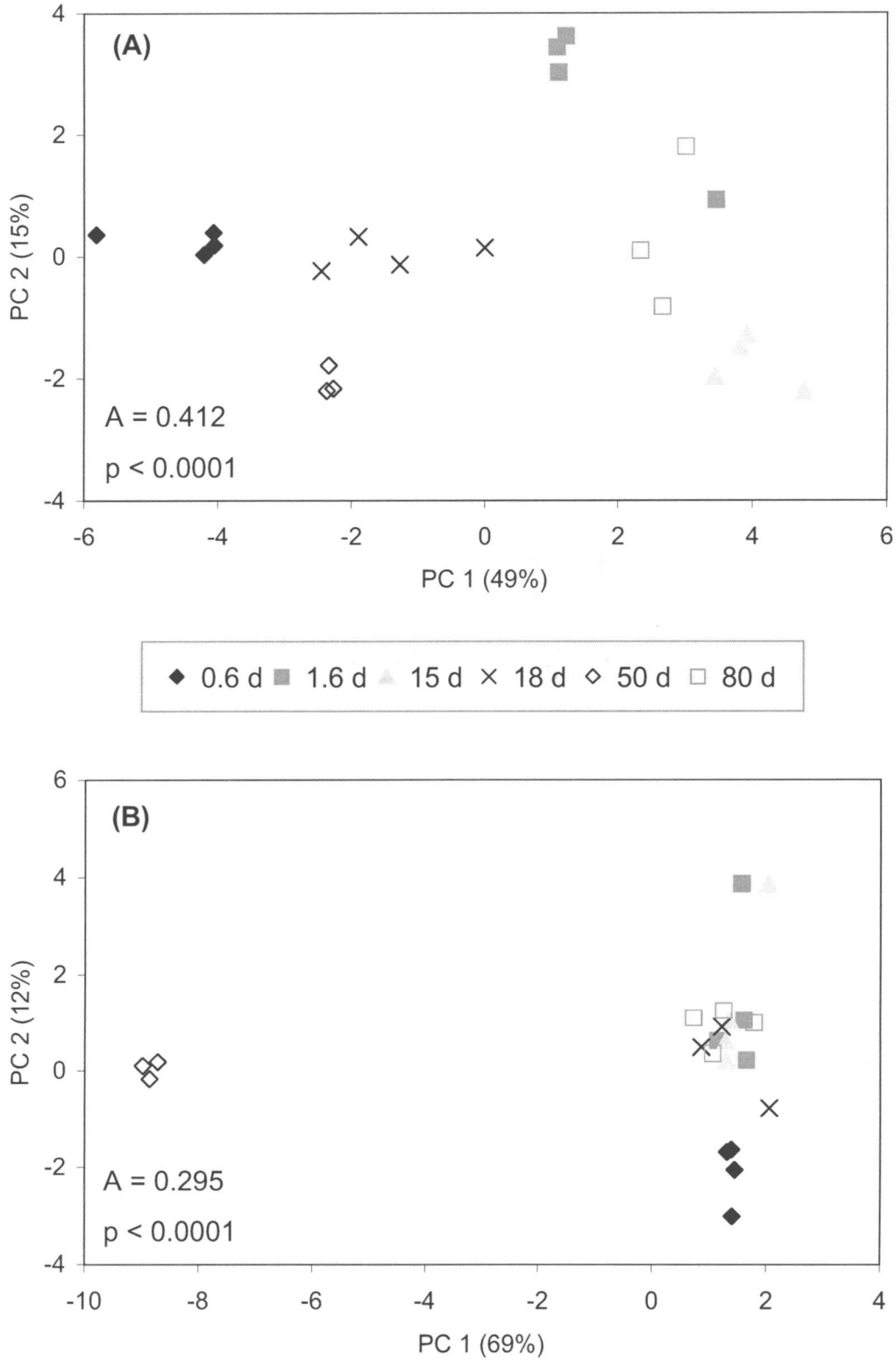

Figure 3.4. PCA for detritosphere treatments through time. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. A = USS, B = LSS.

Negative Correlation). Sixty-nine percent of the variation in this data were due to this disparity.

A suite of PLFAs, consisting of 15:0, 16:1, 17:0, 17:0i, 17:0a, 17:1, and 10Me17:0 were negatively correlated with PC 1 of both USS and LSS; 15:0i, 15:0a, and 16:0i were also negatively associated with PC 1 of LSS (Table 3.4). In USS, these PLFAs were present at fairly constant mol% in 0.6, 18, and 50 d, but were absent from 1.6, 15, and 80 d (Figure 3.5), resulting in the temporal trend observed on the ordination diagram. In LSS, the correlated PLFAs were only present in 50 d samples. No PLFAs were positively correlated with PC 1 in LSS, but 18:0, 18:1ω7, and 18:1ω9 were positively correlated in USS. These PLFAs were present at fairly constant mol% at all times except T3, when they doubled in mol% (Figure 3.5). None of the PLFAs correlated with PCA axes were identified as significant indicators by ISA.

Table 3.4. PLFAs correlated with first principal component in PCA of individual detritosphere treatments through time.

Correlation	**USS**	**LSS**
Positive	18:0	None
	18:1ω7	
	18:1ω9	
Negative	15:0	15:0
	16:1	15:0i
	17:0	15:0a
	17:0i	16:0i
	17:0a	16:1
	17:1	17:0
	10Me17:0	17:0i
		17:0a
		17:1
		10Me17:0

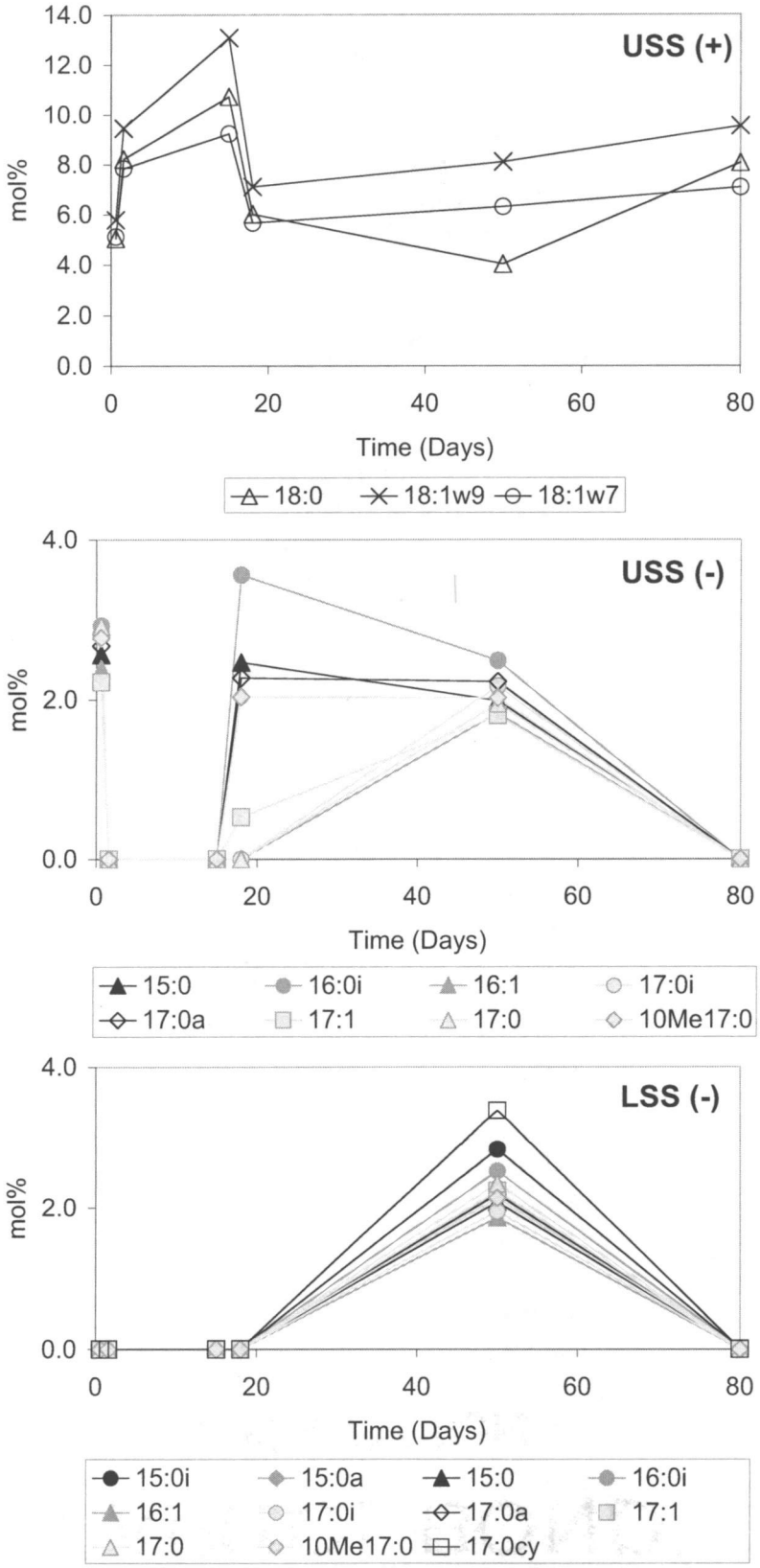

Figure 3.5. PLFAs positively and negatively correlated with PC 1 for USS and LSS at all times.

All Treatments at Each Sampling Time

The dominant trend observed when all treatments were analyzed together using PCA is that at any given time point, detritosphere and bulk soil PLFA profiles were different (Figure 3.6). This trend, captured on PC 1 of each ordination, accounted for between 60% and 73% of variation in the data. These groups were always strongly significant according to MRPP ($p \leq 0.0007$).

PLFAs correlated with PC 1 of ordinations of all treatments at each time point are summarized in Table 3.5. PLFAs that were positively correlated with PC 1 were generally present at much higher mol% in bulk soil than detritosphere samples. 15:0i, 15:0a, 16:0i, 16:1, 17:1, and 10Me17:0 were associated with bulk soil at all times, whereas 17:0a and 17:0cy were positively correlated at all but the last sample time. 17:0i became important in bulk soil by 1.6 d and persisted throughout the incubation, whereas 10Me16:0 was positively correlated with PC 1 from 18 to 80 d. Many of these PLFAs were significant indicators for separating bulk soil from detritosphere.

Negatively correlated PLFAs were more variable between sample times. However, generally 16:0, 18:0, 18:2ω6,9, 18:1ω9, and 19:0cy were important for positioning samples on the detritosphere side of the ordination. 18:2ω6,9 and 19:0cy were almost always significant indicators according to ISA.

Bulk Soil at Each Sampling Time

When considered separately from detritosphere, it was apparent that treatment had little effect on lipid composition at a given time point (Figure 3.7). At 0.6 d, amended treatments were distinctly different from CON samples, with $p = 0.005$ from MRPP, but they were not different from each other. This trend was captured on PC 1 with 48% of the variance explained. By 1.6 d, LS and CON were more similar as they separated from US and L. At 15 d, US appears to be somewhat different from the other three treatments, though the strength of this trend has weakened ($p = 0.011$). In 18, 50, and 80 d, no significant patterns are observed among the four treatments.

Weaker group structure resulted in fewer correlated PLFAs and significant indicators (Table 3.6). At 0.6 d, 14:0, 14:0i, 15:0, and 15:0a are negatively correlated

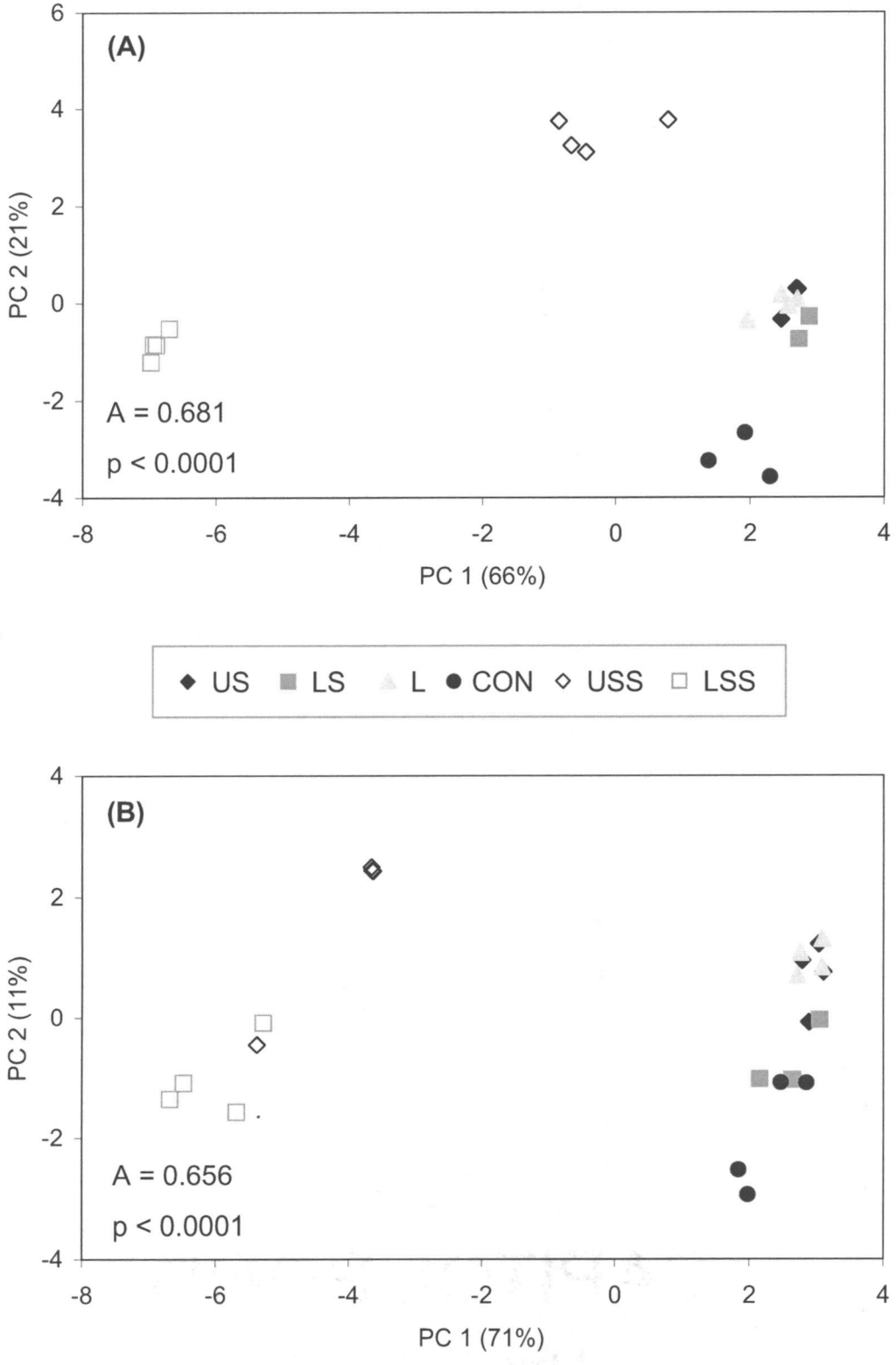

Figure 3.6. PCA for all treatments at individual sampling times. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. A = 0.6 d, B = 1.6 d

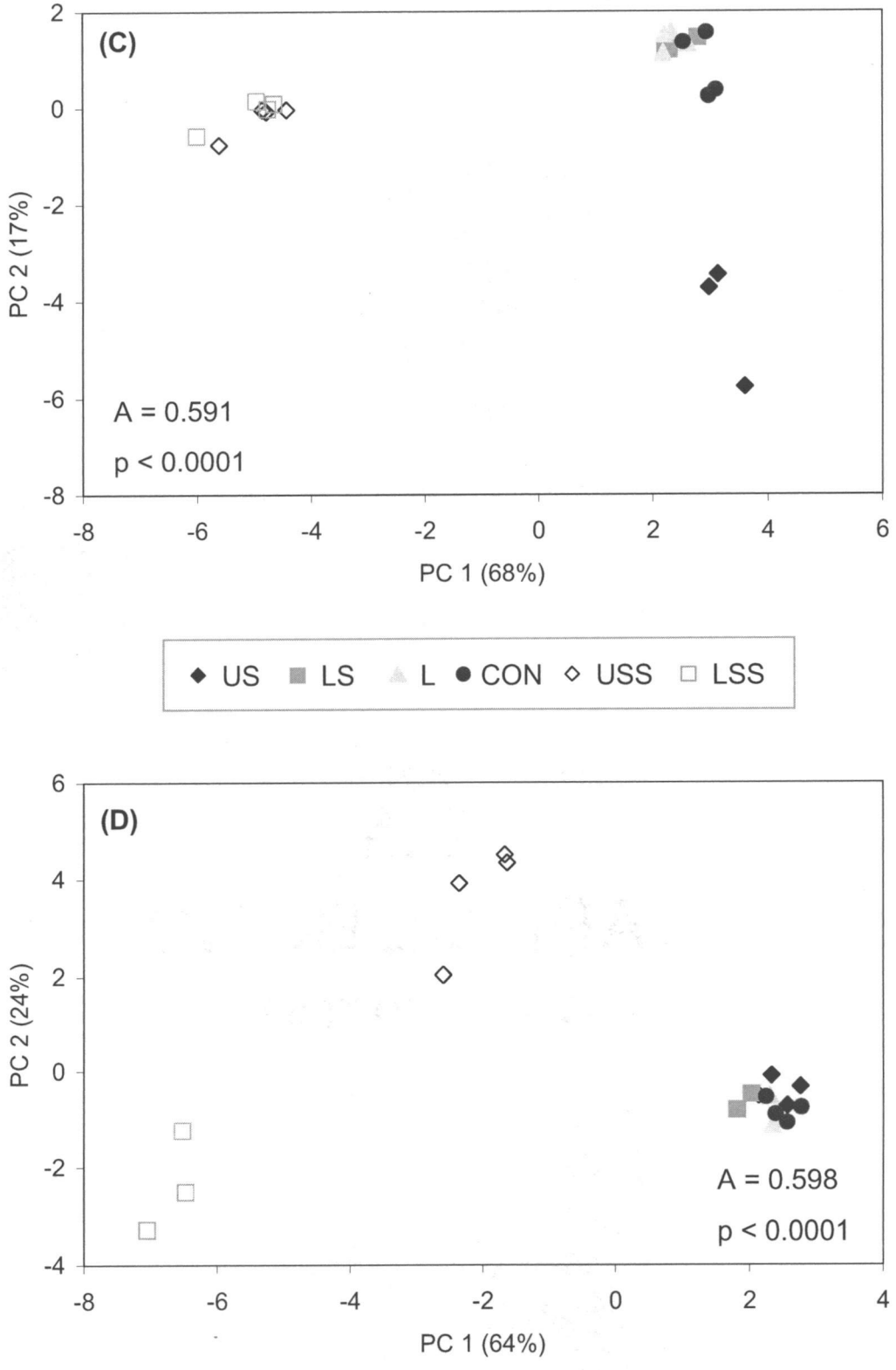

Figure 3.6 (Continued). C = 15 d, D = 18 d.

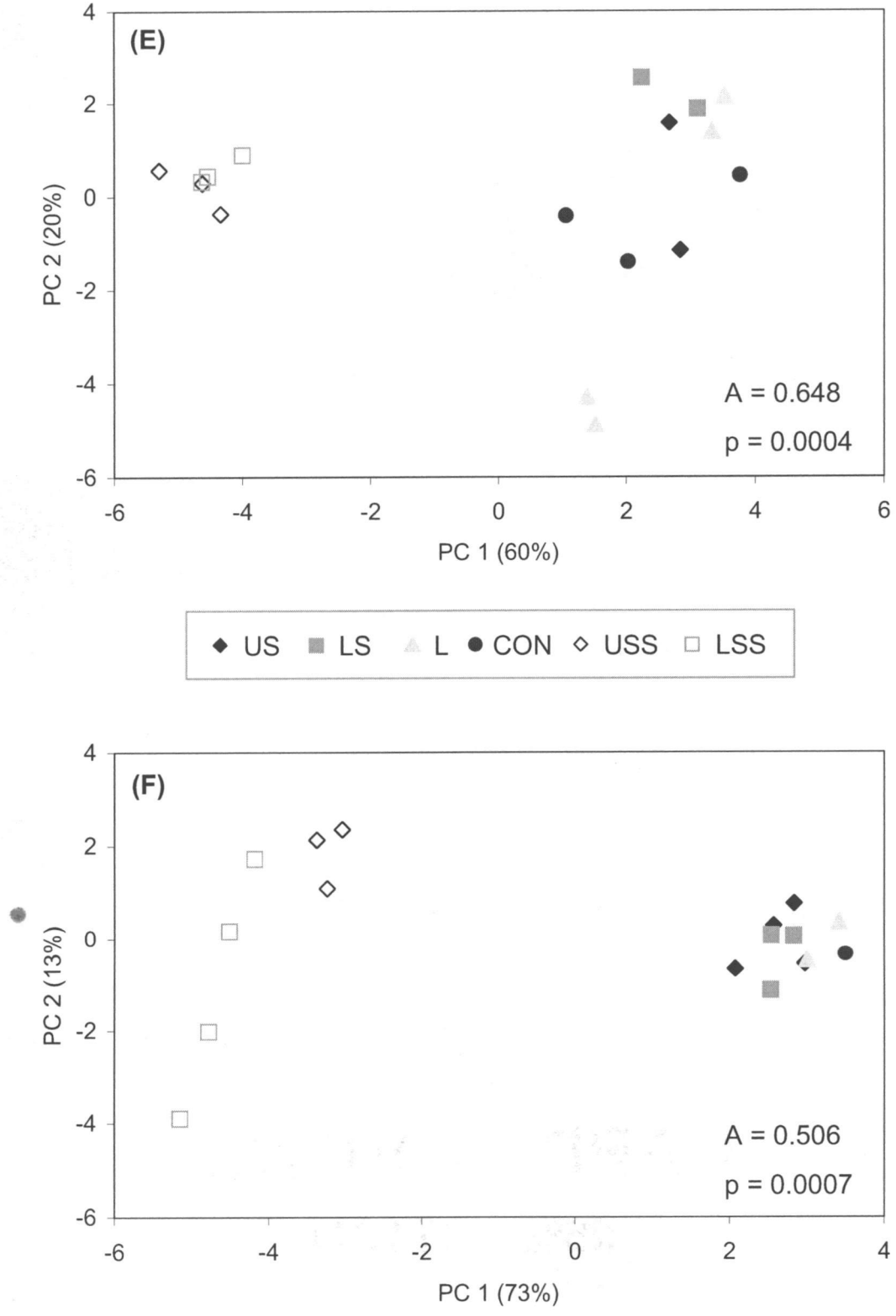

Figure 3.6 (Continued). E = 50 d, G = 80 d.

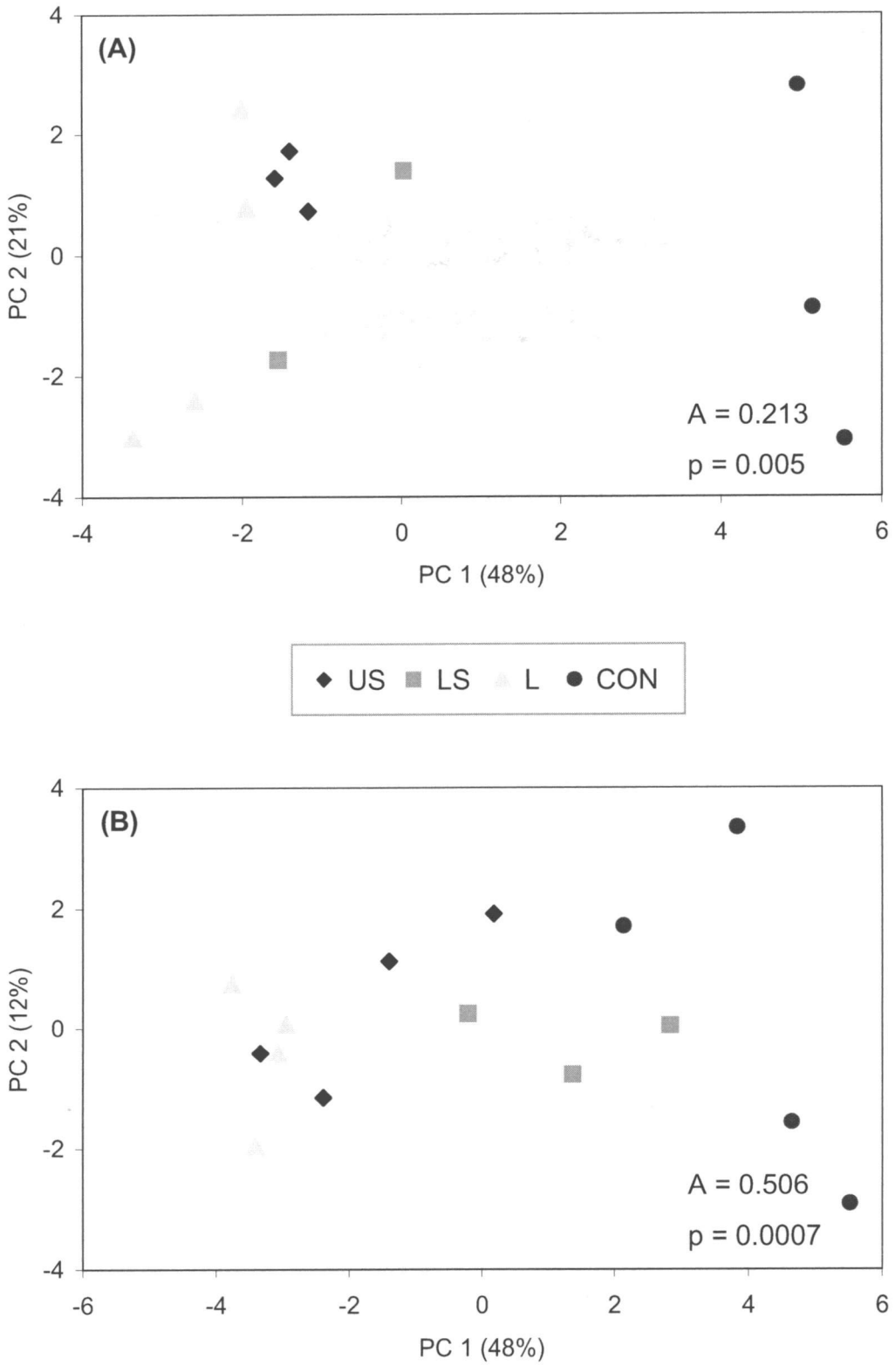

Figure 3.7. PCA for soil treatments at individual sampling times. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. A = 0.6 d, B = 1.6 d.

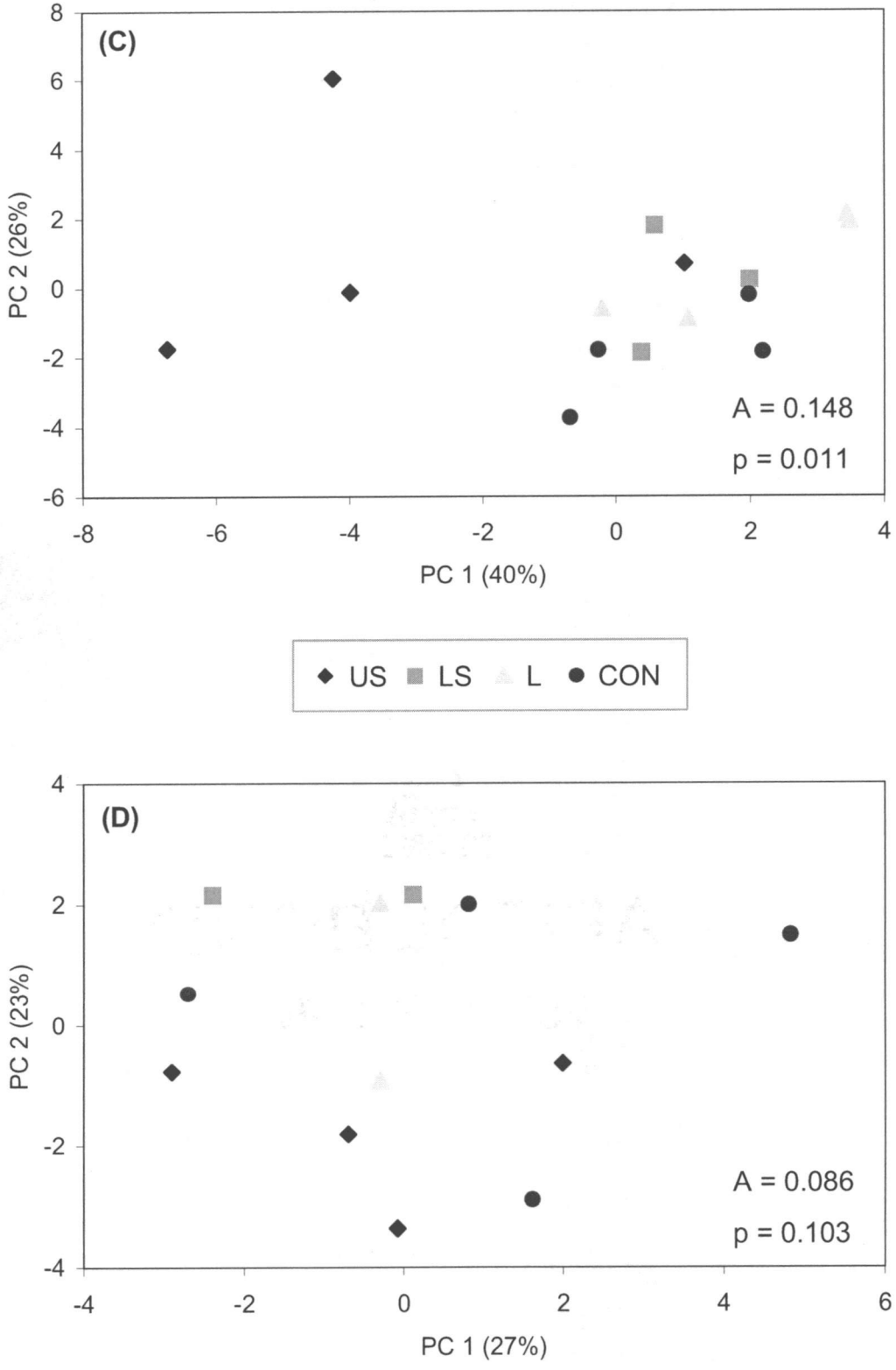

Figure 3.7 (Continued). C = 15 d, D = 18 d.

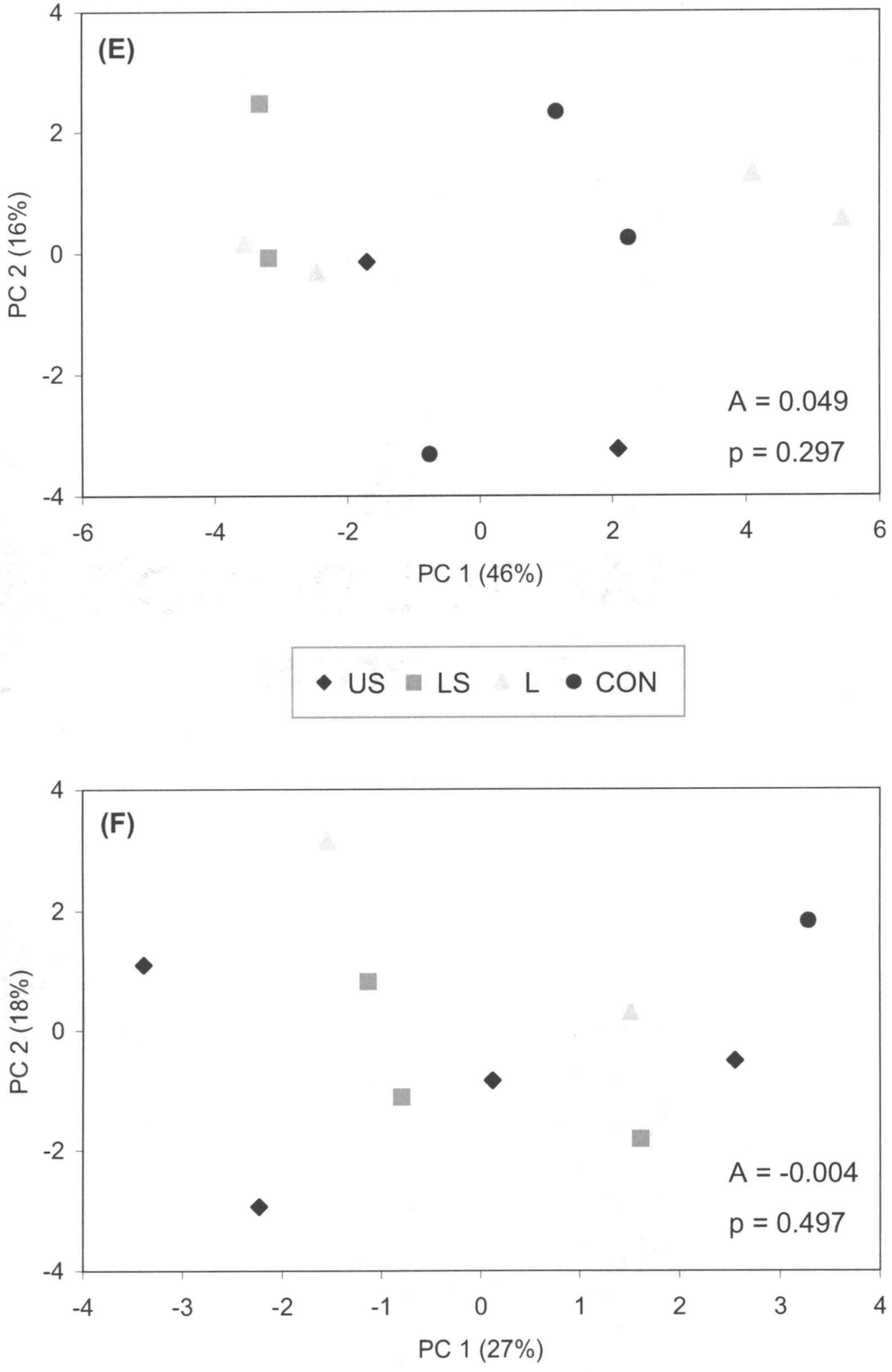

Figure 3.7 (Continued). E = 50 d, F = 80 d.

with PC 1, and thus associated with amended treatments. 15:0a, 16:0i, and 17:0 are negatively correlated at 1.6 d, whereas 14:0i, 15:0, and 17:0 are important at 15 d. Of these PLFAs, only 15:0a at 0.6 d was a significant indicator. 16:1, 17:1, and 17:0cy were positively correlated with PC 1 at 0.6 d; all were significant indicators of CON samples. At 1.6 d, 16:1, 17:0i, 17:1, 18:0, and 19:0cy were positively correlated, while at 15 d 17:0i, 10Me17:0, and 19:0cy were positively associated with PC 1. The absence of significant groups in 18, 50, and 80 d makes correlated PLFAs much less interpretable.

Table 3.5. PLFAs correlated with first principal component in PCA of all treatments (soil and detritosphere) at each sampling time. Significant indicators from ISA are bolded ($p = 0.001$).

Correlation	0.6 d	1.6 d	15 d	18 d	50 d	80 d
Positive	15:0i	**15:0i**	15:0i	**15:0i**	**15:0i**	15:0i
	15:0a	**15:0a**	15:0a	**15:0a**	**15:0a**	15:0a
	16:0i	16:0i	16:0i	**16:0i**	**16:0i**	16:0i
	16:1	16:1	16:1	16:1	**16:1**	16:1
	17:0a	17:0i	10Me16:0	**10Me16:0**	**10Me16:0**	10Me16:0
	17:1	17:0a	17:0i	17:0i	**17:0i**	17:0i
	17:0cy	17:1	17:0a	**17:0a**	**17:0a**	17:1
	10Me17:0	17:0cy	17:1	17:1	**17:1**	10Me17:0
		10Me17:0	17:0cy	17:0cy	**17:0cy**	
			10Me17:0	10Me17:0	**10Me17:0**	
Negative	16:0	16:0	16:0	16:0	**18:2ω6,9**	**16:0**
	18:0	**18:0**	**18:0**	18:2ω6,9		18:1ω9
	18:2ω6,9	**18:1ω9**	**18:1ω7**	**19:0cy**		18:2ω6,9
	19:0cy	**18:2ω6,9**	**18:1ω9**			**19:0cy**
		19:0cy	**18:2ω6,9**			
			19:0cy			

Table 3.6. PLFAs correlated with first principal component in PCA of bulk soil at each sampling time. Significant indicators from ISA are bolded (p = 0.001).

Correlation	0.6 d	1.6 d	15 d	18 d	50 d	80 d
Positive	**16:1**	16:1	17:0i	15:0i	16:0	16:1
	17:1	17:0i	10Me17:0			10Me17:0
	17:0cy	17:1	19:0cy			
		18:0				
		19:0cy				
Negative	14:0	15:0a	14:0i	None	None	16:0
	14:0i	16:0i	15:0			
	15:0	17:0	17:0			
	15:0a					

Detritosphere at Each Sampling Time

At 0.6, 1.6, and 18 d, PC 1 separated USS and LSS into significantly different groups with 90%, 59%, and 72% of variation explained respectively. However at 15, 50, and 80 d it was not possible to distinguish between the two types of detritosphere based on PCA of lipid profiles (Figure 3.8). At 0.6 d, the samples are separated based on the presence of 14:0, 15:0, 15:0i, 15:0a, 16:0i, 16:1, 17:0, 17:0i, 17:0a, 17:1, 17:0cy, and 10Me17:0 in USS but not LSS. 10Me16:0, 18:0, 18:1ω7, 18:1ω9, 18:2ω6,9, and 19:0cy were all negatively correlated with PC 1, and thus were influential in determining LSS sample ordination. A similar complement of PLFAs was correlated with PC 1 at 18 d (Table 3.7). However, the group structure at 1.6 d was weaker, with p = 0.023 from MRPP, and had fewer correlated PLFAs. 14:0, 15:0i, and 15:0a were positively associated with PC 1; 18:1ω9 and 18:1ω7 were negatively correlated.

Similar lipids were correlated with PCA axes at 18, 50, and 80 d, when USS and LSS were not significantly different. PLFAs including 18:1ω7, 18:1ω9, 18:2ω6,9, and 19:0cy were important in determining sample scores in the ordination. No significant indicators were identified for any time point.

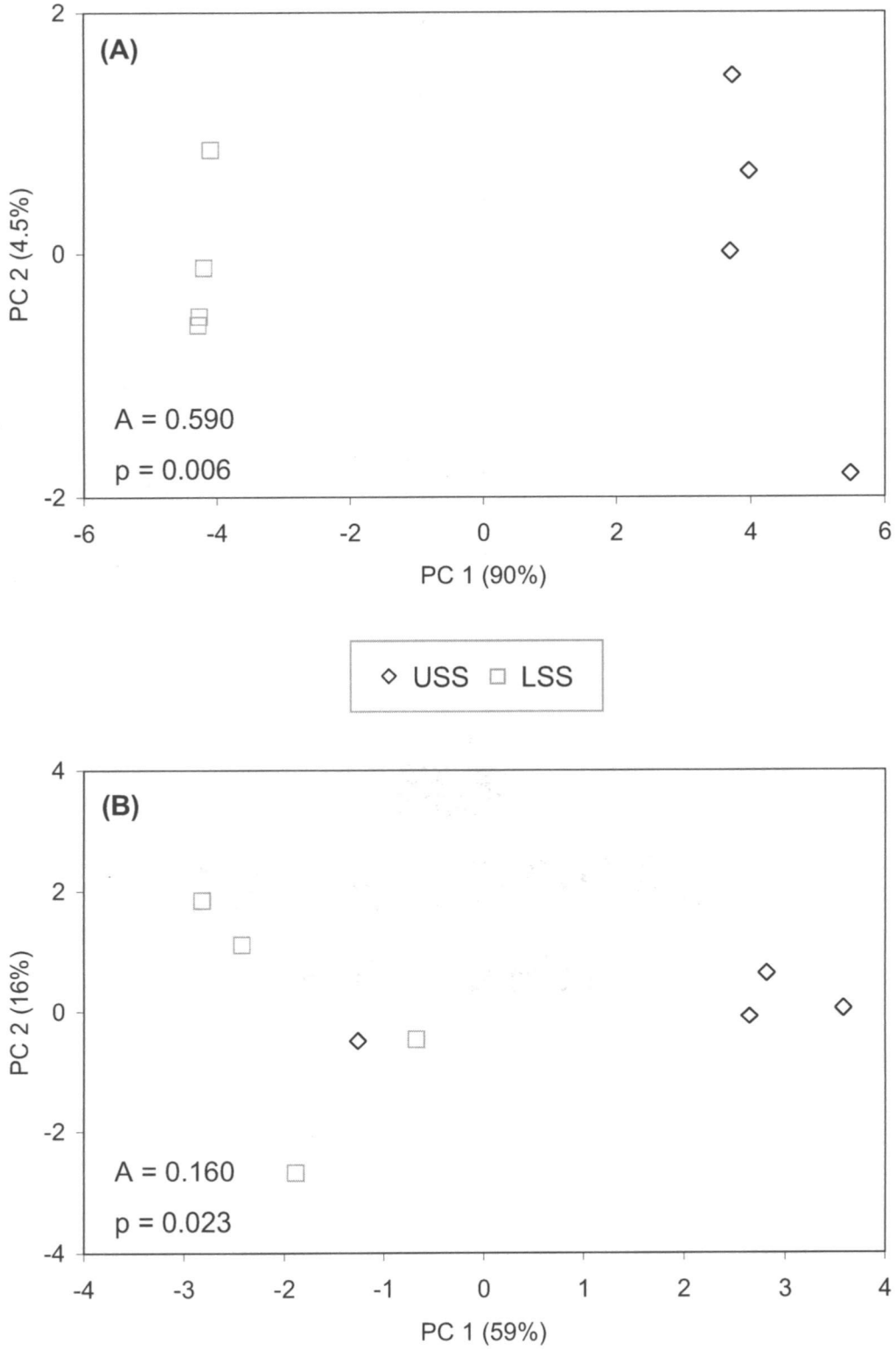

Figure 3.8. PCA for detritosphere treatments at individual sampling times. Percent of variance explained is indicated on each axis and MRPP statistics are presented on each figure. A =0.6 d, B = 1.6 d.

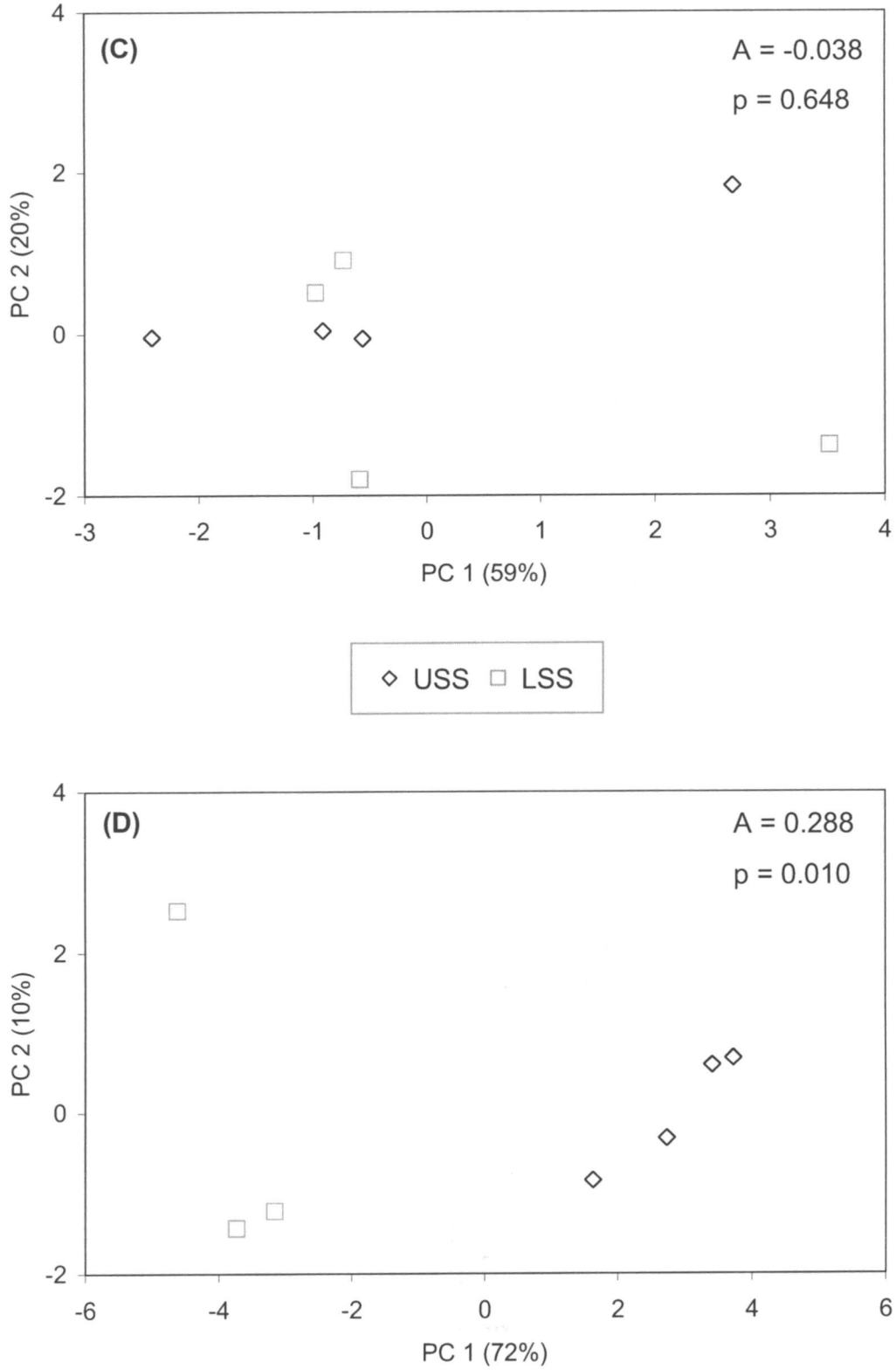

Figure 3.8 (Continued). C = 15 d, D = 18 d.

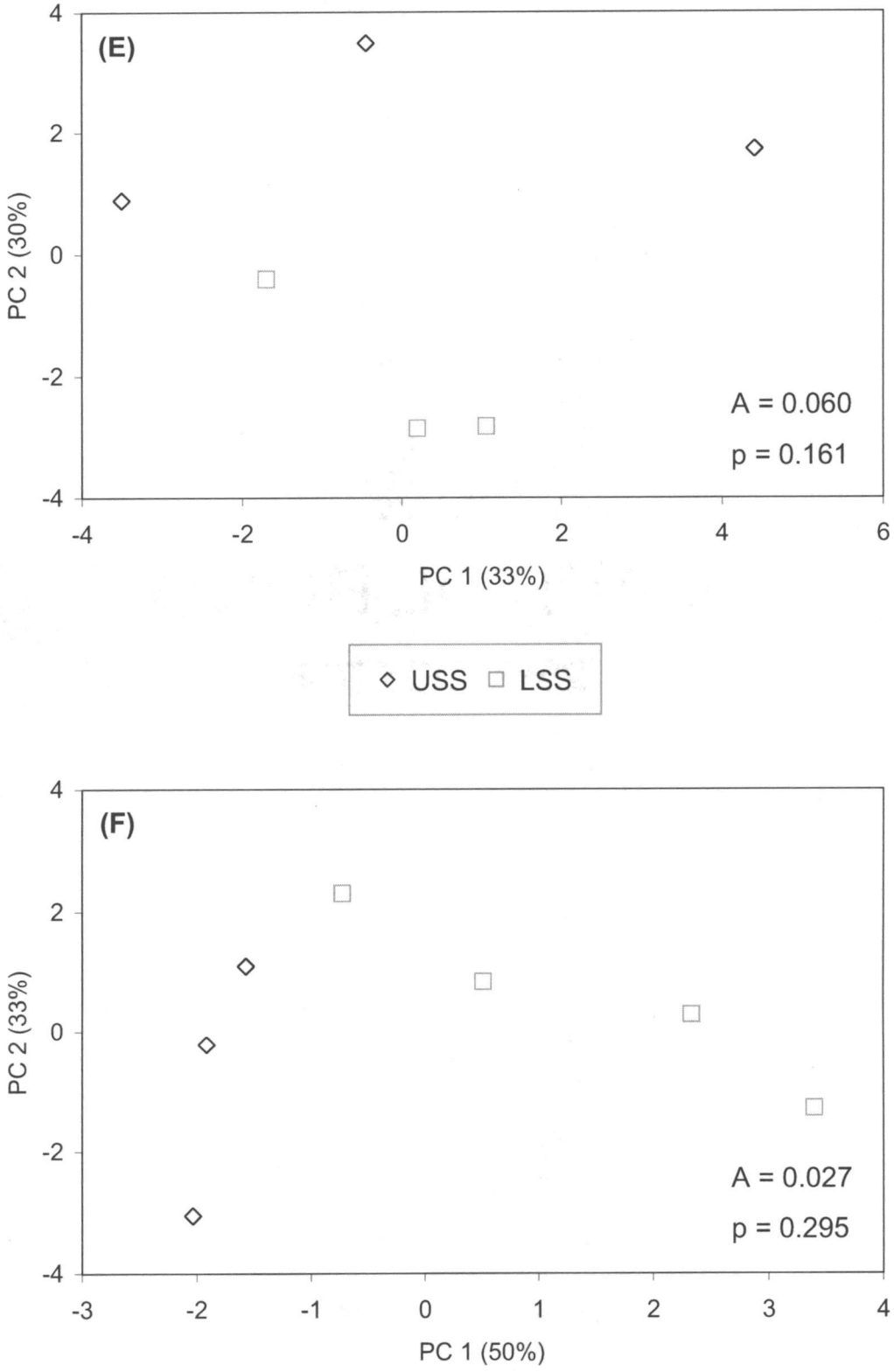

Figure 3.8 (Continued). E = 50 d, F = 80 d.

Table 3.7. PLFAs correlated with first principal component in PCA of detritosphere at each sampling time. There were no significant indicators.

Correlation	0.6 d	1.6 d	15 d	18 d	50 d	80 d
Positive	14:0 15:0 15:0i 15:0a 16:0i 16:1 17:0 17:0i 17:0a 17:1 17:0cy 10Me17:0	14:0 15:0i 15:0a	18:0 18:1ω9	14:0 15:0 15:0i 15:0a 16:0i 10Me16:0 17:0a 17:0cy 10Me17:0	16:0 16:0i 16:1 18:1ω7	10Me16:0 17:0cy
Negative	10Me16:0 18:0 18:1ω7 18:1ω9 18:2ω6,9 19:0cy	18:1ω9	19:0cy	18:0 18:1ω7 18:1ω9 19:0cy	18:2ω6,9	18:1ω9 18:1ω7

Fungal:Bacterial PLFA Ratios

Table 3.8 summarizes fungal:bacterial PLFA ratios. Ratios for LS were slightly higher than other bulk soil samples, particularly at 18 and 50 d. However, overall bulk soil ratios were quite similar and constant over time. Detritosphere ratios were much more variable and, without exception, much larger than bulk soil ratios.

Table 3.8. Mean ratio (±SE) of fungal to bacterial PLFAs, calculated on a mol% basis for all treatments through time.

Time	US	LS	L	CON	USS	LSS
0.6 d	0.20±0.01	0.17±0.00	0.22±0.03	0.20±0.01	0.43±0.02	0.91±0.04
1.6 d	0.20±0.01	0.24±0.01	0.22±0.00	0.19±0.01	0.85±0.06	1.16±0.16
15 d	0.24±0.01	0.26±0.01	0.25±0.01	0.20±0.01	2.95±0.93	2.17±0.60
18 d	0.26±0.02	0.33±0.00	0.26±0.01	0.23±0.01	0.86±0.06	1.61±0.32
50 d	0.26±0.00	0.31±0.01	0.24±0.01	0.21±0.01	0.92±0.04	0.81±0.02
80 d	0.32±0.01	0.30±0.02	0.24±0.01	0.25*	1.24±0.39	1.95±0.37

* n = 1

$\delta^{13}C$ of Selected PLFA

Figure 3.9 shows plots of $\delta^{13}C$ in all treatments through time for 16:0 and 18:2ω6,9 PLFA. Detritosphere samples were more highly enriched than bulk soil samples for both PLFAs. In USS and LSS, $\delta^{13}C$ increased with time to a maximum of about +120‰ in 16:0 and +140‰ in 18:2ω6,9. 16:0 PLFA in bulk soil was less highly labeled than 18:2ω6,9 at about +40‰ versus +100‰. ^{13}C moved quickly into both PLFAs in US and L as $\delta^{13}C$ values had increased to +20‰ in 16:0 and +60‰ in 18:2ω6,9 by 0.6 d. LS was enriched more slowly, with $\delta^{13}C$ values around 0‰ in both PLFAs after the same interval. However, US and LS in both lipids were equally enriched by the end of the incubation, whereas $\delta^{13}C$ in L bulk soil had begun to decline. CON $\delta^{13}C$ values were depleted for all PLFAs and reflected the $\delta^{13}C$ of native soil organic matter. Thus, CON points are plotted as the overall average for all lipids analyzed for $\delta^{13}C$.

Five additional PLFAs were evaluated for $\delta^{13}C$ focusing on bulk soil only: 15:0i, 15:0a, 10Me16:0, 18:1ω7, and 19:0cy. Each of these lipids was enriched differently during the incubation (Figure 3.10). In 15:0i, all amended treatments have similar $\delta^{13}C$ values on 0.6 and 1.6 d, around 0‰. By 15 and 18 d, more ^{13}C had been incorporated into US and L, whereas LS remains at a fairly constant $\delta^{13}C$. In contrast, 15:0a is most highly enriched on 0.6 and 1.6 d in all three treatments, although higher $\delta^{13}C$ values were

measured in US and L. By 15 d, these values decreased by about 40‰ and remained fairly constant for the duration of the experiment. Initial trends in 18:1ω7 and 10Me16:0 are similar to 15:0i with gradual enrichment in all amended treatments. US and L were generally more highly labeled than LS, and values remained fairly constant over time. 19:0cy was least enriched of the five PLFAs considered at 0.6 and 1.6 d, when US and L $\delta^{13}C$ values were the same. L reached a maximum of about +60‰ at 15 d and then decreased with time, whereas US continued to increase slowly with time to a maximum of +50‰ at 80 d. Trends in $\delta^{13}C$ from LS were similar to US until 80 d, where 19:0cy was only labeled at about +10‰.

Repeated measures ANOVA identified significant time and treatment effects for all PLFAs considered. Time•treatment interactions were only significant in 16:0 (all treatments), 16:0 (bulk soil), 15:0a, and 10Me16:0 (Table 3.9). Tukey's honestly significant difference method was applied to these lipids, and results are summarized in Table 3.10.

Table 3.9. Results of repeated measures ANOVA.

PLFA	Time	Treatment	Time•Treatment
16:0 (All Treatments)	***	***	***
18:2ω6,9 (All Treatments)	***	***	NS
16:0 (Bulk Soil)	***	***	**
18:2ω6,9 (Bulk Soil)	*	***	NS
15:0i	*	***	NS
15:0a	*	***	*
10Me16:0	*	***	**
18:1ω7	*	***	NS
19:0cy	*	***	NS

* = $p < 0.05$
** = $p < 0.001$
*** = $p < 0.0001$
NS = not significant

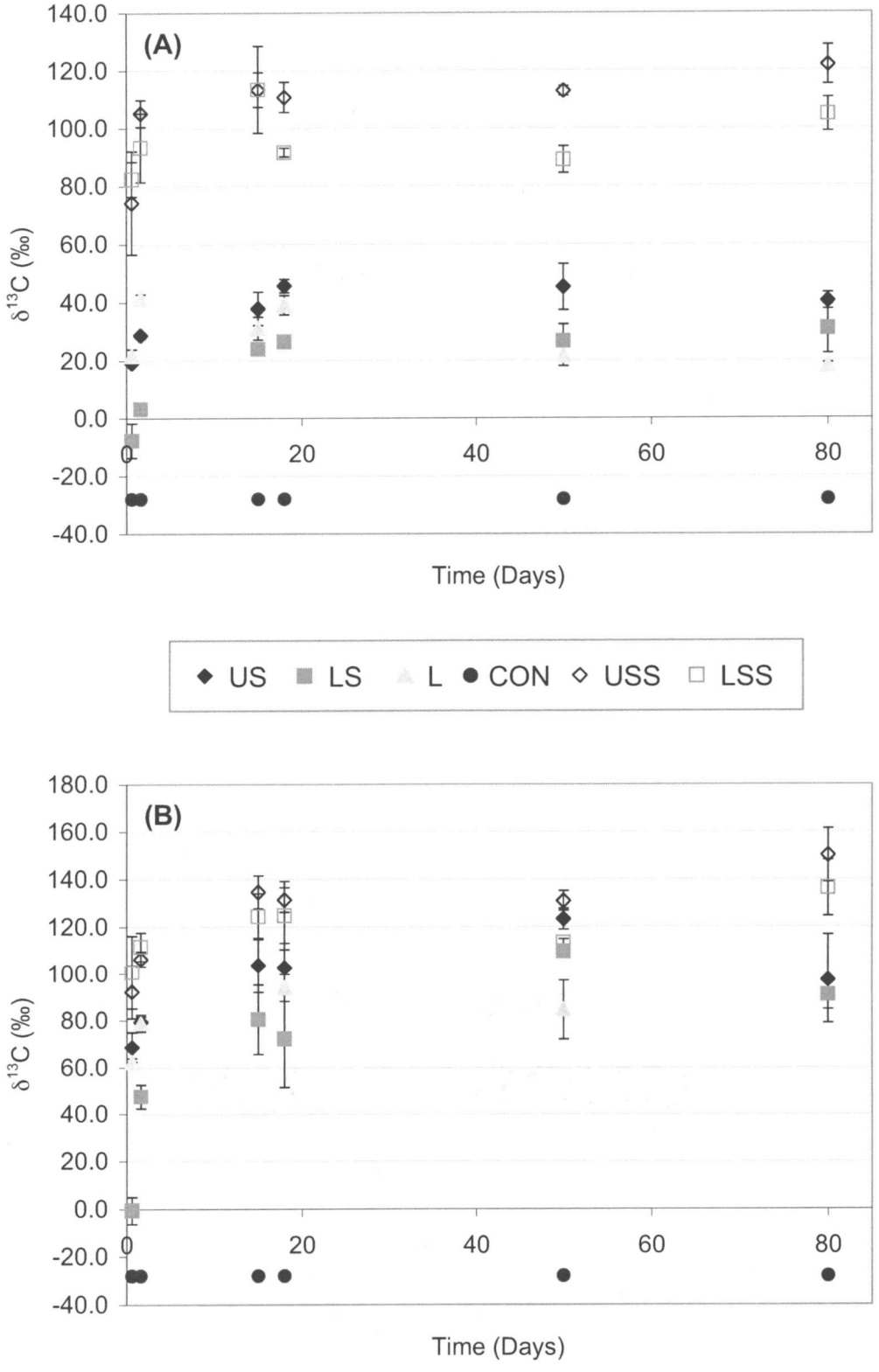

Figure 3.9. $\delta^{13}C$ of 16:0 PLFA (A) and 18:2ω6,9 (B) in all treatments through time.

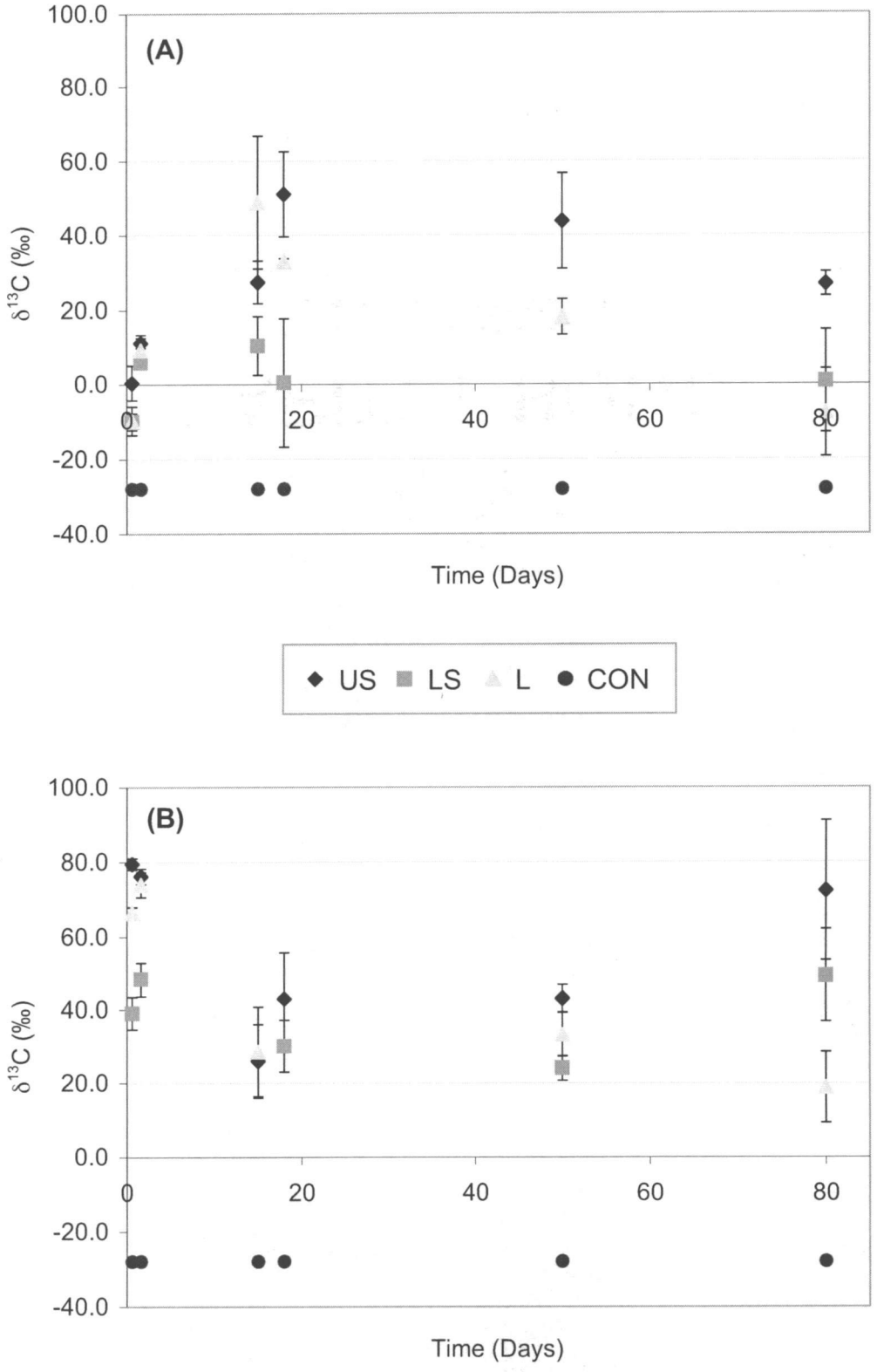

Figure 3.10. $\delta^{13}C$ of 15:0i PLFA (A) and 15:0a PLFA (B) in bulk soil through time.

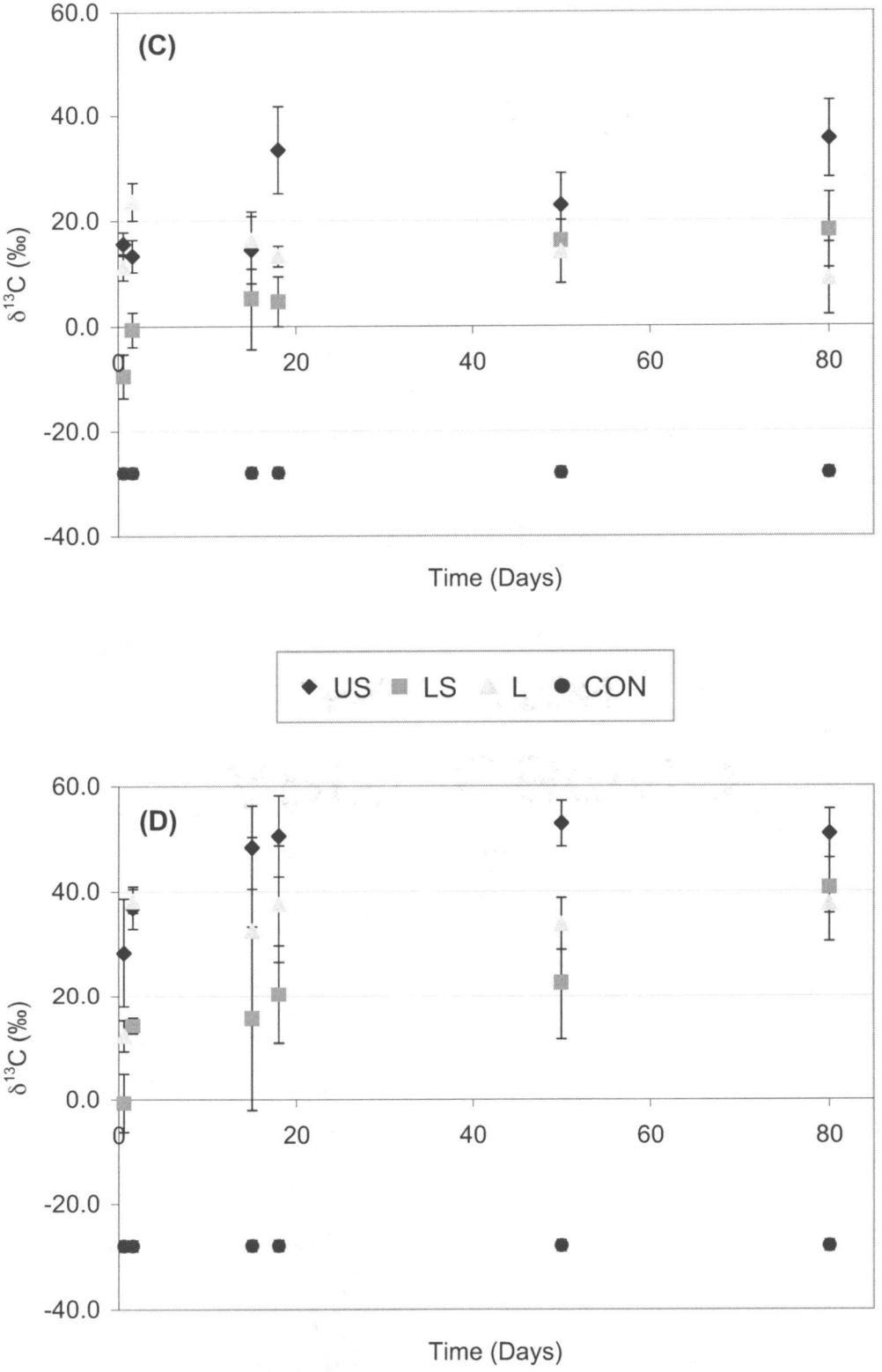

Figure 3.10 (Continued). $\delta^{13}C$ of 10Me16:0 PLFA (C) and 18:1ω7 PLFA (D) in bulk soil through time.

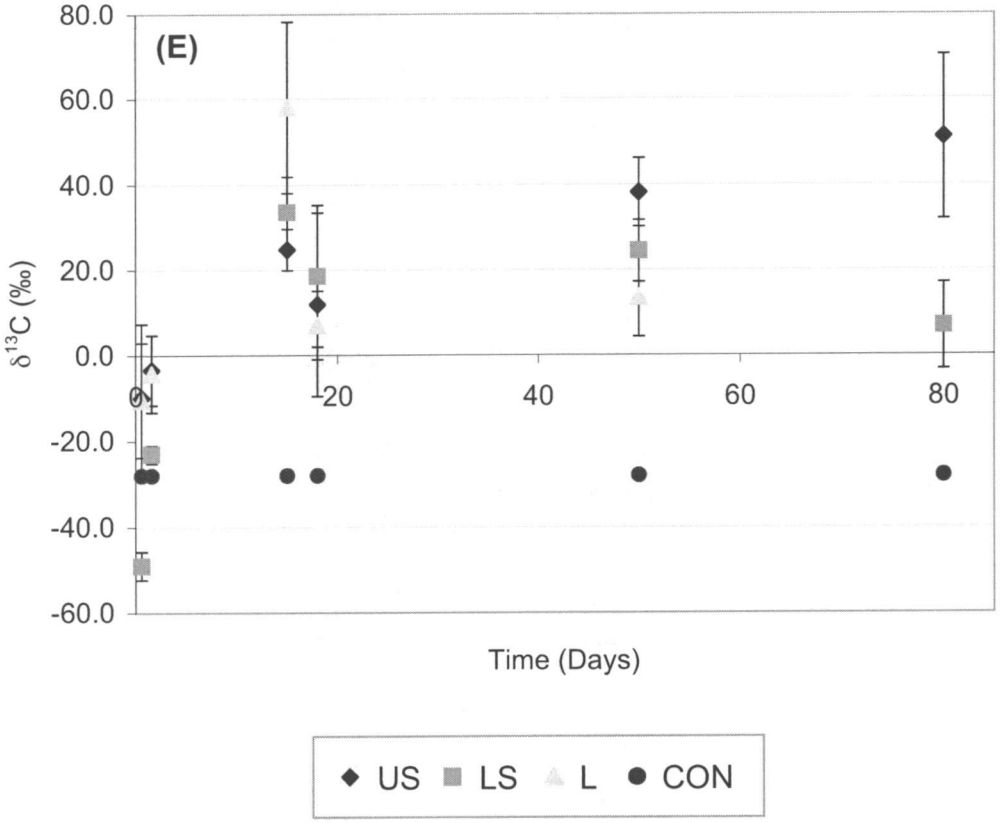

Figure 3.10 (Continued). $\delta^{13}C$ of 19:0cy PLFA (E) in bulk soil through time.

Table 3.10. Results of Tukey's test of PLFAs where a significant time•treatment interaction was identified. Within a PLFA, the same letter designates no significant difference.

PLFA	Treatment	0.6 d	1.6 d	15 d	18 d	50 d	80 d
16:0 (All Treatments)	US	ab	ab	a	a	a	a
	LS	ab	a	a	b	ab	a
	L	a	b	a	ab	b	a
	Control	b	c	b	c	c	b
	USS	c	d	c	d	d	c
	LSS	c	d	c	e	e	c
16:0 (Bulk Soil)	US	a	a	a	a	a	a
	LS	b	b	a	b	ab	ab
	L	a	c	a	a	b	b
	Control	c	d	b	c	c	c
15:0a	US	a	a	a	a	a	a
	LS	b	b	ab	a	a	a
	L	c	a	a	ab	a	ab
	Control	d	c	b	b	b	b
10Me16:0	US	a	a	a	a	a	a
	LS	b	b	a	b	a	a
	L	a	a	a	b	a	a
	Control	c	c	b	c	b	b

Discussion

Community Composition Through Time

Bulk Soil. During early decomposition, microbial communities in bulk soil from all amended treatments were similar, being primarily characterized by higher mol% of 14- and 15-C PLFAs. Bossio *et al.* (1998) also saw increases in these short, branched-chain fatty acids in plots receiving large straw inputs. However, some PLFAs were important in US and L, but not LS treatment, suggesting that to a certain extent, the L community was a subset of the US community. Overlap also occurred between LS and L, with 14:0i and 10Me16:0 characterizing these two communities to a greater extent than US. Ruess *et al.* (2002) found that on average, fungi prepared in pure culture contain less than 1% PLFAs with chain length less than 15 C, therefore early communities were strongly dominated by bacteria. It seems that the addition of C in general, and not the quality of the C source, was driving community composition early in the incubation, because all three amended treatments had similar indicator PLFAs during this time.

A distinct shift occurred in PLFA composition of the bulk soil communities towards longer and more complex PLFAs later in the incubation (Figure 3.1). This shift occurred between 1.6 and 15 d for LS and L, and between 15 and 18 d for US. This could be attributed to more available C in intact straw versus leached straw and leachate. Another possibility is that C source complexity had a strong impact on community temporal dynamics. Either simple soluble C or recalcitrant structural C in isolation was only able to support early succession degrader communities for between 1.6 and 15 d, while both together prolonged survival of this community to 18 d.

Interestingly, soluble C seemed to be a driving factor determining the final composition, regardless of when the shift occurred. US and L communities were distinguished by increasing mol% of 16- and 17-C PLFAs. These lipids tend to be associated primarily with bacterial-dominated communities, which play an important role in the degradation of the simple C compounds, which were more abundant in US and L.

Fungi were most important for distinguishing later samples in LS, as 18:2ω6,9 was the only indicator for these samples.

Cyclopropyl PLFAs have been attributed to stress conditions (Bossio and Scow, 1998). Because the addition of readily available C induces rapid biosynthesis and increased overall activity (Dalenberg and Jager, 1981; Fontaine *et al.*, 2003), it is possible that a larger bacterial community was produced in US and L that subsequently crashed when easily degradable substrate was depleted, resulting in more 19:0cy production. With soluble C removed from LS, microbial growth may have been steadier, dominated by slower-growing K-strategist bacteria and fungi. The microbial community in LS later in decomposition was in fact characterized by high mol% of 18:2ω6,9, the PLFA most commonly associated with fungi. There was no detectable shift in CON soil communities, thus leading to the conclusion that changes in other treatments are attributable to added C.

Analysis of bulk soil treatments together at each sampling time confirms results of individual treatments through time (Figure 3.7). Particularly at 0.6 d, PCA shows that the amended samples were characterized by different PLFAs than CON soil. Interestingly, some of the same PLFAs that differentiated early from late samples on individual treatments also contributed to the separation of amended from CON soil samples. This suggests that as decomposition progresses, communities tend to shift back towards native microbial populations prior to amendment. The delay in community shift in US until 15 d is captured by PCA of all bulk soil treatments. At 15 d, LS and L group with CON, separately from US. From 18 d through the end of the incubation, there was no significant difference between amended treatments and control.

Detritosphere. Community composition in the detritosphere did not follow such straightforward patterns. In USS, 0.6 d was characterized by 15-, 16-, and 17-C PLFAs (Figure 3.5). By 1.6 and 15 d, these lipids dropped off to be replaced by higher mol% of 18-C PLFAs commonly associated with fungi. Stahl and Klug (1996) found that four fatty acids, 16:0, 18:0, 18:1ω9, and 18:2ω6,9, comprised as much as 95% of the total fatty acid content of dikaryotic fungi. Ruess *et al.* (2002) confirmed these results by finding the same four fatty acids in all fungal cultures they examined. Because fungi are

the primary producers of depolymerization enzymes, they would have played a very important role on the surface of straw residue. 18 and 50 d saw a flush of bacterial-associated lipids once again, perhaps in response to nutrients liberated by fungal activity between 1.6 and 18 d. By 80 d, these lipids dropped below detection limits again.

LSS PLFA profiles followed a different trajectory through time. At all times communities were characterized primarily by 16:0, 10Me16:0, 18:1ω7, 18:1ω9, 18:2ω6,9, and 19:0cy (Appendix I). Tester (1988) found that straw residue is populated with bacteria capable of degrading soluble components of straw even in the absence of soil. Leaching would have removed surface bacteria, thus relying more heavily on colonization of straw by actinomycetes and fungi from soil before significant degradation could occur. The absence of soluble C would have further slowed bacterial attack. Hence, it is not surprising that evidence of bacterial growth only occurs at 50 d in leached straw detritosphere; release of nutrients from leached straw would be slower than from unleached straw. These lipids dropped below detection limits in LSS by 80 d also.

Comparison of USS and LSS at individual times confirms these trends (Figure 3.8). At 0.6 and 18 d, when USS communities were characterized by more bacterial lipids than LSS, MRPP results were significant. At all other times, USS and LSS were not significantly different, either as a result of mutual absence or presence of 15-, 16-, and 17-C bacterial lipids.

Bulk Soil vs. Detritosphere. Despite clear temporal trends in bulk soil and detritosphere PLFAs when considered in isolation, analysis of all six lipid profiles revealed only one clear pattern: detritosphere and bulk soil communities were different (Figure 3.6). The addition of readily available substrate results in strong spatial gradients and redistribution of microbes in soil. While decomposing straw continues to release soluble C, diffusion only occurs up to 4 mm from the residue surface (Gaillard *et al.*, 1999). Thus, microbes living in the bulk soil would have been more C limited than those colonizing the detritosphere. Because certain organisms are better adapted to energy and nutrient shortages, the community composition as measured by PLFA analysis was different.

Indicator Species Analysis

Not all PLFAs measured, nor all those correlated with PC 1 in PCA, were useful for differentiating communities at different sample times using ISA. Widmer *et al.* (2001) had a similar outcome, with only 6 of 73 PLFAs useful for distinguishing soils with different textures, even though the first principal component was associated with 17 PLFAs. Bossio and Scow (1998) also found lipids common to most microorganisms were indicative of change in response to changes in energy and aeration status, more so than unusual lipid biomarkers, which are not commonly found in agricultural systems.

Generally, where groups were very clear on the PCA ordination, at least one significant indicator was found. Indicators also varied from group to group. When all sample times were considered for bulk soil samples separately, 15:0a was the best indicator for early times, while 16:1, 17:0, 18:0, 19:0cy, and 18:2ω6,9 were the best for later times. However, when all treatments were considered on individual sampling dates, 16:1 and 17:0 were indicative of bulk soil samples regardless of time, and detritosphere was indicated by complex 18-C lipids and 19:0cy.

Microbial Community Processing of Substrate C

Though only seven peaks were suitable for $\delta^{13}C$ analysis, five of the seven (15:0i, 15:0a, 16:0, 18:1ω7, and 18:2ω6,9) along with 16:0i, 16:1ω7, 18:0, and 18:1ω9 were found to contain 80% of excess ^{13}C recovered by Phillips *et al.* (2002) after incubating soil with ^{13}C-cellobiose and ^{13}C-N-acetylglucosamine.

With the exception of leached straw 19:0cy on 0.6 and 1.6 d, all PLFAs were enriched by at least 20‰ relative to unamended CON. Such an increase in $\delta^{13}C$ can only be attributed to labeled substrate degradation, because PLFAs closely reflect the signature of substrates used during synthesis (Abraham *et al.*, 1998). These results are consistent with Hanson *et al.* (1999), who found ^{13}C in all PLFAs after adding ^{13}C-labeled glucose to soil. Generally, the more highly labeled the substrate, the more highly labeled the PLFAs; US and L were more enriched than LS until later time points when most of the leachate had been consumed.

Though both 15:0i and 15:0a are generally associated with gram-positive bacteria, 15:0a became labeled more quickly and to a larger extent (Figure 3.10 (A) and (B)) in bulk soil than 15:0i. Several explanations are possible for this phenomenon. First, gram-positive bacteria are a highly diverse group of organisms containing such genera as aerobic *Bacillus* and anaerobic *Clostridium* (Paul and Clark, 1996). Thus, organisms in included in this group have very different life strategies and, while they may all produce 15:0i and 15:0a, they may do so in radically different proportions. Enrichment of 15:0a but not 15:0i may indicate that a subset of the gram-positive bacteria that have higher proportions of 15:0a in their cell membranes were active during early decomposition. Second, it is possible that the same organism produces both 15:0i and 15:0a in equal proportions, but at different times during its lifecycle or under various growth conditions. In this case, the high $\delta^{13}C$ value seen in 15:0a could merely indicate that 15:0a is produced preferentially during periods of rapid growth.

10Me16:0, which is considered to be an indicator of actinomycetes, was fairly uniformly labeled in L throughout the experiment and increased in US and LS through time (Figure 3.10 (C)). Actinomycetes are a diverse group of gram-positive bacteria capable of producing depolymerization enzymes for recalcitrant compounds, such as lignin and chitin (Paul and Clark, 1996). Thus, they play an important role in the decomposition of straw resulting in more ^{13}C incorporation into PLFAs than with leachate alone.

Of the lipids analyzed for ^{13}C, 19:0cy was the most variable among treatments over time. Cyclopropyl fatty acids have been shown to increase in organisms under physiological stress (Bossio and Scow, 1998). 17:0cy and 19:0cy have also been attributed to gram-negative bacteria (O'Leary and Wilkinson, 1988) and anaerobic bacteria (Zelles, 1997). Because the microcosms were maintained at or below 30% gravimetric water content significant anaerobic activity is unlikely. However, the state of rapid microbial growth and activity early in the incubation may have resulted in more gram-negative bacteria and/or nutrient shortages that induced production of 19:0cy by many groups of organisms. Because nutrient dynamics affecting individual bacteria are

occurring on a millimetric or smaller scale, microsites of stress could have produced the highly variable $\delta^{13}C$ values for 19:0cy.

The importance of spatial scales and C diffusion through soil is made clear through the comparison of $\delta^{13}C$ values from bulk soil and the detritosphere. In both 16:0, a general indicator of microbial biomass, and 18:2ω6,9, the fungal marker, detritosphere $\delta^{13}C$ values were higher than that of bulk soil. The disparity is smaller for 18:2ω6,9 than 16:0, potentially due to proliferation of fungal hyphae from the straw surface out into bulk soil. Evidence from Gaillard *et al.* (1999) suggested that C from straw does not move more than 4 mm from the straw surface.

Bacteria vs. Fungi in Ryegrass Decomposition

Ruess *et al.* (2002) found that fungi produce small quantities of short and branched-chain PLFAs. Therefore, PLFAs 15-C and shorter can be attributed almost exclusively to bacteria. In US and L, both 18:2ω6,9 and 15:0a were highly labeled immediately, indicating that both fungi and bacteria were rapidly assimilating substrate carbon. However, ^{13}C dynamics in these two lipids did not continue to mirror each other. Fungi appeared to play a continual role in substrate decomposition, as the $\delta^{13}C$ of 18:2ω6,9 continued to increase to a maximum between 18 and 50 d; 18:2ω6,9 was almost always the most highly labeled PLFA in US. Activity of bacteria appeared to be somewhat cyclic in straw treatments, as $\delta^{13}C$ of 15:0a peaked at 1.6 d, decreased through 50 d and increased again at 80 d.

The removal of soluble C from straw had a greater influence on C dynamics in fungi than bacteria. $\delta^{13}C$ in 15:0a from LS followed a similar temporal trend to US; however, 18:2ω6,9 was much slower to be labeled in this treatment.

High fungal:bacterial PLFA ratios (> 1.0) suggest fungal dominance in a microbial community (Bailey *et al.*, 2002). Ratios calculated for bulk soil were very low, between 0.17 and 0.33, suggesting strong bacterial dominance. A wide range of fungal:bacterial ratios has been reported in the literature, with values as low as 0.01 (Bååth and Anderson, 2003) and as high as 5.0 (Bailey *et al.*, 2002), both in prairie systems. Bardgett and McAlister (1999) were also interested in fungal vs. bacterial

dominance in grassland systems under different types of management, and reported a much narrower range of ratio, from 0.92 to 2.32, where the highest ratio was observed under moderate management. Thus, it is apparent that these ratios may be sensitive indicators for differences among ecosystems.

In this experiment, fungal:bacterial ratios were only greater than 1.0 in detritosphere samples, showing that fungi comprised a much larger portion of the microbial community closer to the straw surface. However, despite higher fungal contributions to detritosphere communities, 18:2ω6,9 was not significantly more enriched in ^{13}C in detritosphere than in bulk soil. Because fungi form long hyphae, they are able to access nutrients over larger spatial scales; therefore, those found in the bulk soil may still have been incorporating substrate C that was not available to bacteria, which operate at a much finer scale.

PLFAs measured in this experiment were not enriched equally with ^{13}C. Substrate quality influenced both the rate at which ^{13}C was incorporated into PLFAs and which PLFAs were labeled first. Because discrimination in biosynthetic pathways is generally quite small, on the order of 5‰, the wide range in $\delta^{13}C$ signatures measured in PLFAs likely due to differential contribution of taxonomic groups to a given lipid pool. With the exception of 18:2ω6,9, most soil microbes can produce the seven lipids considered in $\delta^{13}C$ analysis. However, on an individual basis, organisms produce unique combinations of PLFAs, and groups of microbes tend to contain higher proportions of certain PLFAs than others. For example, 15:0i and 15:0a are more abundant in the cell membranes of gram-positive bacteria than other taxonomic groups. Therefore, changes in $\delta^{13}C$ of one PLFA not observed in another can be attributed to activity of microbes that produce higher than average quantities of the lipid in question.

Conclusion

Combining ^{13}C and PLFA analysis provides clearer insight into microbial community structure and functioning when unique lipid biomarkers are not present in the soil. Fractionation can only account for small variations in $\delta^{13}C$ signatures in PLFAs, therefore if large shifts in $\delta^{13}C$ are measured, even in lipids produced by most soil organisms, attributing those shifts to broad taxonomic groups becomes easier. Information about the spatial scale upon which decomposition occurs was also gained through the use of ^{13}C; PLFAs extracted from detritosphere communities were much more highly labeled than those from bulk soil communities.

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Chapter 4:
General Conclusion

Shawna K. McMahon

Soluble Carbon and Ryegrass Straw Decomposition

Although soluble C did not influence the overall size of microbial biomass produced during decomposition, the composition of those communities were clearly different, as was the rate at which substrate C was processed. CO_2 evolution was fastest from US and L, where soluble C was present. Once this C source had been depleted, CO_2 evolution dynamics in L were similar to the unamended CON, and US resembled LS. PLFAs extracted from LS were always slowest to become labeled, even if they reached similar enrichment levels to the other treatments later in the incubation. This indicates that soluble C drives early C decomposition dynamics, but does not necessarily influence the overall outcome.

Quality of added substrate may also influence the so-called priming effect, as priming was greatest in US where all forms of C were present, and least in L where only soluble C was present. Even when equal amounts of C are added, more complex forms tend to induce a larger priming effect by stimulating a more complex community.

^{13}C Phospholipid Fatty Acid Analysis

Results of PLFA analysis from soil must be considered carefully, particularly due to the lack of exclusivity of so-called signature lipids (Bossio and Scow, 1998). A given lipid marker may be present in membranes of organisms belonging to different taxonomic groups, making interpretation of changes in PLFA profiles in soils difficult (Ibekwe and Kennedy, 1998). Taxonomic interpretation is further confounded by the ability of individual organisms to change their fatty acid composition in response to stress or temperature fluctuations (Lindahl *et al.*, 1997). Therefore a soil's PLFA profile may shift if conditions experienced by the microbial community change without an actual change in community composition.

^{13}C-labeled substrates enhance the interpretability of PLFA analysis results. Fractionation does not differ greatly among lipid synthesis pathways within a single organism. Therefore, if large differences in $\delta^{13}C$ are observed between PLFAs, the supposition that a specific group of organisms is more actively utilizing substrate than another group is more believable.

Future Research

Straw decomposition is a very well studied process from the perspective of C dynamics, and methodologies have not changed significantly in the last 50 years. However, the drive to understand microbial community composition has led to the application of pure culture methods like PLFA analysis to complex soil systems. In order to interpret PLFA results, certain PLFAs that tend to be associated with certain taxonomic groups in pure culture are assigned as biomarkers to those groups in soils. There are two major issues that I see with this practice that must be addressed to retain PLFA analysis as a viable soil community analysis method. First, better detection of less common PLFAs would help to refine biomarker interpretation. Most soil microbes produce most PLFAs currently measured on our and other researchers' systems. The ability to quantify PLFAs present in very small quantities may be more informative about community differences than shifts in ubiquitous lipids. Second, it may be necessary to redefine microbial taxonomic groups all together in order to effectively apply PLFA analysis to complex communities. Groupings such as gram-positive and gram-negative were developed based on culturable organisms before more precise methods were available. Genetic methods are continuously revealing inconsistencies and inaccuracies in the current taxonomic paradigm, and by linking those methods with PLFA analysis, better interpretations of both types of data may be possible.

PLFA methods are continuously modified and refined to fit different situations. However, in our quest for data, quality control is sometimes overlooked, specifically the need for internal standards. In the case of ^{13}C-PLFA analysis, internal standards become doubly important because they allow for accurate quantification of each lipid by providing an estimate of extraction efficiency, and also provide a more comparable standard for ^{13}C determination than other options such as CO_2 pulses. Unfortunately, internal standards can be difficult to recover for unknown reasons, or may interfere with lipids extracted from the sample. Therefore, it is necessary to find a standard that is stable, can be consistently detected, and has a different retention time than sample lipids.

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Appendix

Shawna K. McMahon

Appendix. Table of mean ± standard error mol% PLFA for all treatments at all times.

Time (d)	Treatment	Phospholipid Fatty Acid						
		14:0i	14:0	15:0i	15:0a	15:0	16:0i	16:0
0.6	US	2.14 ± 0.20	2.18 ± 0.17	5.50 ± 0.13	7.97 ± 0.23	2.23 ± 0.15	4.83 ± 0.10	8.45 ± 0.17
	LS	2.33 ± 0.06	2.38 ± 0.07	6.68 ± 0.16	8.06 ± 0.85	2.03 ± 0.03	4.87 ± 0.05	9.52 ± 0.55
	L	2.43 ± 0.08	2.07 ± 0.16	5.65 ± 0.14	9.29 ± 0.60	1.92 ± 0.07	5.04 ± 0.35	10.10 ± 0.61
	C	0.00 ± 0.00	0.00 ± 0.00	7.22 ± 0.63	5.10 ± 0.46	0.00 ± 0.00	4.86 ± 0.11	10.35 ± 0.97
	USS	0.97 ± 0.62	2.00 ± 0.22	2.76 ± 0.07	4.17 ± 0.21	2.48 ± 0.27	2.84 ± 0.04	17.77 ± 0.74
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	21.27 ± 1.54
1.6	US	1.89 ± 0.15	2.08 ± 0.04	6.10 ± 0.27	9.35 ± 0.89	1.85 ± 0.29	5.32 ± 0.17	10.32 ± 0.72
	LS	1.34 ± 0.68	2.19 ± 0.21	6.34 ± 0.32	7.48 ± 0.63	1.23 ± 0.63	4.91 ± 0.18	9.02 ± 0.40
	L	1.81 ± 0.18	1.90 ± 0.05	6.00 ± 0.32	9.46 ± 0.61	1.76 ± 0.07	5.56 ± 0.13	10.84 ± 0.54
	C	0.00 ± 0.00	1.16 ± 0.67	6.68 ± 0.17	5.10 ± 0.34	1.18 ± 0.68	4.82 ± 0.10	10.17 ± 0.19
	USS	0.00 ± 0.00	2.63 ± 0.88	2.83 ± 0.95	4.01 ± 1.35	0.00 ± 0.00	0.00 ± 0.00	18.51 ± 0.78
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.62 ± 0.62
15	US	1.60 ± 0.55	1.92 ± 0.65	6.33 ± 0.63	5.12 ± 0.49	1.78 ± 0.72	5.29 ± 0.45	9.59 ± 1.24
	LS	0.00 ± 0.00	0.00 ± 0.00	6.06 ± 0.21	4.93 ± 0.15	0.00 ± 0.00	5.11 ± 0.15	9.00 ± 0.54
	L	0.00 ± 0.00	0.00 ± 0.00	4.93 ± 0.58	4.50 ± 0.18	0.00 ± 0.00	4.92 ± 0.29	8.21 ± 0.99
	C	0.00 ± 0.00	1.36 ± 0.79	6.74 ± 0.48	4.98 ± 0.14	0.00 ± 0.00	4.67 ± 0.08	9.05 ± 0.66
	USS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.87 ± 1.89
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.28 ± 1.18
18	US	0.00 ± 0.00	0.00 ± 0.00	5.08 ± 0.26	4.56 ± 0.18	0.00 ± 0.00	4.54 ± 0.16	10.14 ± 0.63
	LS	0.00 ± 0.00	0.00 ± 0.00	4.71 ± 0.55	3.81 ± 0.39	0.00 ± 0.00	3.92 ± 0.08	9.40 ± 0.92
	L	0.00 ± 0.00	0.00 ± 0.00	4.83 ± 0.39	4.01 ± 0.36	0.00 ± 0.00	4.20 ± 0.29	10.30 ± 0.09
	C	0.00 ± 0.00	0.00 ± 0.00	5.40 ± 0.47	4.35 ± 0.13	0.00 ± 0.00	4.40 ± 0.11	10.16 ± 0.62
	USS	0.00 ± 0.00	1.59 ± 0.53	3.44 ± 0.21	3.52 ± 0.07	2.46 ± 0.10	3.56 ± 0.29	15.65 ± 0.26
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	19.42 ± 3.43
50	US	0.00 ± 0.00	0.00 ± 0.00	6.17 ± 0.70	4.58 ± 0.50	0.00 ± 0.00	4.74 ± 0.90	11.19 ± 1.01
	LS	0.00 ± 0.00	0.00 ± 0.00	3.95 ± 0.37	3.36 ± 0.13	0.00 ± 0.00	4.08 ± 0.05	9.00 ± 0.58
	L	0.64 ± 0.37	0.79 ± 0.46	5.40 ± 0.64	4.09 ± 0.44	0.90 ± 0.52	4.91 ± 0.30	10.57 ± 0.76
	C	0.00 ± 0.00	0.00 ± 0.00	5.59 ± 0.23	4.16 ± 0.44	1.33 ± 0.68	4.54 ± 0.44	11.32 ± 0.85
	USS	0.00 ± 0.00	0.00 ± 0.00	2.56 ± 0.22	2.22 ± 0.08	1.98 ± 0.06	2.49 ± 0.10	12.78 ± 0.78
	LSS	0.00 ± 0.00	0.00 ± 0.00	2.83 ± 0.29	2.21 ± 0.19	2.08 ± 0.08	2.52 ± 0.07	11.94 ± 0.23
80	US	0.00 ± 0.00	0.00 ± 0.00	4.51 ± 0.43	3.23 ± 0.27	0.00 ± 0.00	3.72 ± 0.28	10.13 ± 0.63
	LS	0.00 ± 0.00	0.00 ± 0.00	4.56 ± 0.64	3.59 ± 0.39	0.00 ± 0.00	3.86 ± 0.10	10.37 ± 0.15
	L	0.00 ± 0.00	0.00 ± 0.00	5.53 ± 0.94	4.20 ± 0.27	0.00 ± 0.00	4.62 ± 0.66	9.80 ± 0.78
	C	0.00 ± 0.00	0.00 ± 0.00	4.22 ±	3.44 ±	0.00 ± 0.00	4.58 ±	9.54 ±
	USS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	21.06 ± 3.97
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.29 ± 1.76

Appendix cont'd.

Time (d)	Treatment	Phospholipid Fatty Acid						
		10Me16:0	16:1	17:0i	17:0a	17:1	17:0	10Me17:0
0.6	US	7.72 ± 0.30	3.29 ± 0.26	3.40 ± 0.25	3.32 ± 0.26	4.22 ± 0.17	2.35 ± 0.22	3.12 ± 0.11
	LS	8.29 ± 0.37	3.49 ± 0.23	3.20 ± 0.22	3.06 ± 0.21	4.30 ± 0.03	1.30 ± 1.30	3.39 ± 0.32
	L	8.06 ± 0.33	2.84 ± 0.13	3.10 ± 0.24	3.16 ± 0.06	3.62 ± 0.19	1.60 ± 0.55	2.91 ± 0.21
	C	9.09 ± 0.63	4.43 ± 0.30	3.97 ± 0.41	3.51 ± 0.28	5.60 ± 0.31	0.00 ± 0.00	3.69 ± 0.10
	USS	4.37 ± 0.15	2.31 ± 0.19	2.74 ± 0.20	2.59 ± 0.16	2.15 ± 0.11	2.83 ± 0.29	2.69 ± 0.09
	LSS	8.32 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.6	US	7.95 ± 0.28	2.94 ± 0.20	3.22 ± 0.11	3.26 ± 0.08	4.07 ± 0.24	1.95 ± 0.66	3.14 ± 0.16
	LS	8.21 ± 0.33	3.49 ± 0.16	3.17 ± 0.19	3.61 ± 0.10	5.02 ± 0.33	0.00 ± 0.00	3.52 ± 0.23
	L	8.45 ± 0.25	2.64 ± 0.05	2.77 ± 0.13	3.37 ± 0.11	3.33 ± 0.14	2.22 ± 0.23	2.95 ± 0.12
	C	7.66 ± 0.22	4.07 ± 0.13	4.11 ± 0.11	3.41 ± 0.22	5.38 ± 0.07	0.00 ± 0.00	3.58 ± 0.36
	USS	6.94 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	LSS	3.66 ± 2.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15	US	7.10 ± 0.59	3.43 ± 0.26	3.60 ± 0.46	3.29 ± 0.18	4.19 ± 0.30	2.19 ± 0.83	3.36 ± 0.23
	LS	6.90 ± 0.23	3.98 ± 0.28	4.19 ± 0.39	4.48 ± 0.51	5.19 ± 0.39	0.00 ± 0.00	3.90 ± 0.11
	L	6.77 ± 0.50	4.05 ± 0.08	4.85 ± 0.64	3.99 ± 0.21	5.13 ± 0.17	0.00 ± 0.00	4.08 ± 0.23
	C	7.46 ± 0.39	4.10 ± 0.12	4.32 ± 0.19	4.03 ± 0.16	5.91 ± 0.37	0.00 ± 0.00	4.49 ± 0.38
	USS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18	US	6.95 ± 0.32	4.04 ± 0.16	4.20 ± 0.17	4.45 ± 0.15	5.00 ± 0.38	0.00 ± 0.00	4.30 ± 0.18
	LS	7.23 ± 0.22	4.25 ± 0.14	3.70 ± 0.21	3.86 ± 0.21	4.63 ± 0.07	0.00 ± 0.00	3.93 ± 0.23
	L	7.18 ± 0.49	3.98 ± 0.13	4.14 ± 0.01	3.96 ± 0.00	4.77 ± 0.35	0.00 ± 0.00	3.96 ± 0.05
	C	7.46 ± 0.42	4.27 ± 0.11	4.18 ± 0.13	3.64 ± 0.20	5.87 ± 0.29	0.00 ± 0.00	4.01 ± 0.40
	USS	3.49 ± 0.28	0.00 ± 0.00	0.00 ± 0.00	2.27 ± 0.78	0.53 ± 0.53	0.00 ± 0.00	2.03 ± 0.68
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
50	US	7.62 ± 0.88	3.86 ± 0.03	3.61 ± 0.01	3.42 ± 0.41	4.35 ± 0.07	0.00 ± 0.00	3.25 ± 0.47
	LS	6.64 ± 0.36	3.90 ± 0.15	3.53 ± 0.45	3.02 ± 0.34	4.88 ± 0.36	0.00 ± 0.00	4.14 ± 0.01
	L	7.27 ± 0.29	4.14 ± 0.12	3.64 ± 0.15	3.47 ± 0.33	4.73 ± 0.11	0.98 ± 0.57	3.24 ± 0.19
	C	7.90 ± 0.16	3.74 ± 0.07	3.97 ± 0.25	3.25 ± 0.28	5.23 ± 0.25	1.67 ± 0.86	3.52 ± 0.16
	USS	2.91 ± 0.14	1.83 ± 0.12	2.17 ± 0.10	2.22 ± 0.13	1.80 ± 0.06	1.95 ± 0.06	2.02 ± 0.07
	LSS	2.87 ± 0.19	1.86 ± 0.05	1.95 ± 0.03	2.19 ± 0.09	2.24 ± 0.12	2.34 ± 0.16	2.14 ± 0.07
80	US	7.04 ± 0.24	3.88 ± 0.17	4.01 ± 0.06	3.75 ± 0.34	5.37 ± 0.29	0.00 ± 0.00	3.44 ± 0.14
	LS	7.19 ± 0.09	4.05 ± 0.27	3.50 ± 0.26	3.45 ± 0.13	5.51 ± 0.25	0.00 ± 0.00	3.55 ± 0.33
	L	7.20 ± 0.13	4.08 ± 0.05	3.88 ± 0.03	3.60 ± 0.37	5.07 ± 0.24	0.00 ± 0.00	3.90 ± 0.01
	C	7.48 ±	4.87 ±	4.43 ±	4.42 ±	6.58 ±	0.00 ± 0.00	4.50 ±
	USS	5.49 ± 0.38	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	LSS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Appendix cont'd.

Time (d)	Treatment	Phospholipid Fatty Acid					
		17:0cy	18:0	18:1ω9	18:1ω7	18:2ω6,9	19:0cy
0.6	US	4.18 ± 0.35	4.92 ± 0.10	6.83 ± 0.34	8.02 ± 0.33	7.34 ± 0.20	8.00 ± 0.55
	LS	4.27 ± 0.09	4.31 ± 0.43	6.74 ± 0.31	8.25 ± 0.04	5.95 ± 0.16	7.59 ± 1.79
	L	4.17 ± 0.15	4.10 ± 0.25	6.77 ± 0.24	7.73 ± 0.20	8.57 ± 1.46	6.87 ± 0.75
	C	5.50 ± 0.24	5.40 ± 0.31	7.69 ± 0.34	8.06 ± 0.13	6.52 ± 0.75	9.02 ± 1.84
	USS	2.13 ± 0.11	4.90 ± 0.10	5.62 ± 0.11	4.97 ± 0.31	17.65 ± 0.67	12.09 ± 0.71
	LSS	0.00 ± 0.00	11.42 ± 0.58	10.46 ± 0.59	8.48 ± 0.19	21.67 ± 0.75	18.38 ± 0.43
1.6	US	3.71 ± 0.12	4.06 ± 0.40	7.15 ± 0.16	7.63 ± 0.15	6.96 ± 0.28	7.03 ± 0.67
	LS	4.47 ± 0.24	4.76 ± 0.46	7.37 ± 0.15	8.35 ± 0.25	7.53 ± 0.20	7.99 ± 0.45
	L	4.37 ± 0.11	3.57 ± 0.09	6.98 ± 0.20	7.51 ± 0.18	8.47 ± 0.26	6.03 ± 0.35
	C	5.27 ± 0.29	5.47 ± 0.29	7.39 ± 0.30	8.03 ± 0.41	7.06 ± 0.29	9.46 ± 0.26
	USS	0.00 ± 0.00	8.21 ± 0.98	9.45 ± 0.61	7.83 ± 0.48	24.13 ± 1.19	15.46 ± 1.19
	LSS	0.00 ± 0.00	9.42 ± 1.50	12.47 ± 1.03	10.55 ± 1.00	26.04 ± 1.20	20.23 ± 1.51
15	US	5.61 ± 0.41	4.24 ± 0.23	7.20 ± 0.49	7.48 ± 0.50	9.18 ± 0.78	7.47 ± 1.06
	LS	5.34 ± 0.39	5.23 ± 0.26	7.08 ± 0.30	7.81 ± 0.55	10.58 ± 0.63	10.21 ± 0.62
	L	5.93 ± 0.15	5.69 ± 0.33	7.92 ± 0.17	7.69 ± 0.30	9.50 ± 0.70	11.86 ± 1.12
	C	5.20 ± 0.41	6.33 ± 0.49	6.87 ± 0.16	6.93 ± 0.10	7.42 ± 0.42	10.13 ± 0.95
	USS	0.00 ± 0.00	10.73 ± 1.24	13.08 ± 2.03	9.23 ± 0.45	37.51 ± 2.17	11.58 ± 3.87
	LSS	0.00 ± 0.00	11.16 ± 2.59	13.57 ± 1.89	10.76 ± 1.02	33.09 ± 0.37	14.14 ± 4.74
18	US	5.71 ± 0.23	7.16 ± 0.88	8.61 ± 0.35	7.59 ± 0.25	8.32 ± 0.99	9.37 ± 0.32
	LS	5.09 ± 0.35	6.65 ± 0.18	8.21 ± 0.21	8.66 ± 0.07	12.49 ± 0.21	9.45 ± 1.23
	L	6.81 ± 0.02	6.72 ± 0.90	8.71 ± 0.38	8.98 ± 0.77	8.39 ± 0.28	9.07 ± 0.60
	C	5.54 ± 0.23	6.96 ± 1.14	8.47 ± 0.21	8.59 ± 0.58	7.18 ± 0.83	9.52 ± 0.95
	USS	4.17 ± 0.36	6.01 ± 0.80	7.10 ± 0.71	5.67 ± 0.27	28.97 ± 1.01	9.53 ± 0.46
	LSS	0.00 ± 0.00	14.40 ± 2.09	11.02 ± 0.32	8.63 ± 0.82	29.40 ± 5.95	17.12 ± 1.27
50	US	5.56 ± 0.08	5.32 ± 0.19	8.09 ± 0.15	8.53 ± 0.10	9.12 ± 0.09	10.61 ± 2.99
	LS	5.64 ± 0.79	5.97 ± 0.18	8.31 ± 0.32	8.85 ± 0.05	11.70 ± 1.02	13.03 ± 1.64
	L	5.21 ± 0.51	5.48 ± 0.41	8.46 ± 0.15	7.82 ± 0.61	8.03 ± 0.75	10.22 ± 1.03
	C	5.29 ± 0.32	5.63 ± 0.08	7.84 ± 0.34	8.50 ± 0.62	6.67 ± 1.20	9.87 ± 0.77
	USS	2.90 ± 0.04	4.03 ± 0.25	8.10 ± 0.51	6.31 ± 0.37	31.73 ± 1.91	9.98 ± 0.59
	LSS	3.39 ± 0.12	4.63 ± 0.29	7.77 ± 0.46	6.05 ± 0.15	29.63 ± 0.76	11.37 ± 0.33
80	US	5.80 ± 0.32	6.42 ± 0.25	8.76 ± 0.41	8.58 ± 0.42	11.62 ± 0.75	9.75 ± 0.71
	LS	5.16 ± 0.23	6.00 ± 0.18	8.39 ± 0.33	9.31 ± 0.63	11.11 ± 1.07	10.42 ± 0.16
	L	6.08 ± 0.09	6.09 ± 0.11	7.96 ± 0.37	8.68 ± 0.88	8.58 ± 0.07	10.73 ± 1.89
	C	5.13 ±	6.21 ±	8.53 ±	8.71 ±	8.14 ±	9.20 ±
	USS	5.99 ± 1.13	8.05 ± 1.30	9.53 ± 0.88	7.08 ± 0.74	28.42 ± 7.71	14.38 ± 1.95
	LSS	0.00 ± 0.00	9.48 ± 2.19	11.67 ± 0.86	9.28 ± 1.02	35.75 ± 4.05	16.53 ± 1.72