AN ABSTRACT OF THE DISSERTATION OF

Marcelo Ferreira Fernandes for the degree of Doctor of Philosophy in Soil Science presented on June 13, 2006.

Title: Fatty Acid Profiling of Soil Microbial Communities: A Comparison of Extraction Methods and Temporal Dynamics in Plant Residue Amended Soils.

Abstract approved:

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A method based on fatty acid (FA) analysis is used to profile microbial community structure (MCS). Various extraction protocols are available, which alter the types of FAs extracted from soils. The more time consuming but widely used protocol extracts only FAs from phospholipids (PLFA). This technique is desirable because PLFAs are largely of microbial origin and from viable cells, since they rapidly degrade upon microbial death. This stands in contrast to other more rapid methods that directly extract FAs but may extract FAs of non-microbial origin. In this thesis, two such methods of FA extraction (EL-FAME and MIDI) were compared to PLFA extracts for detecting shifts and interpreting profiles of MSC. Soil samples from a wide array of vegetation and climatic conditions were extracted by these methods, and their FA composition analyzed by gas chromatography. MIDI extracts contained major plant-specific FAs. Ordination multivariate analysis showed that separation of MCS among samples was driven mostly by these FAs, rather than by microbial FA markers. The degree of similarity between EL-FAME and PLFA results was affected by the environmental conditions. The major

differences among methods were in the general fungal and arbuscular-mycorrhizal fungal markers that were related to the vegetation type where soils were found. Nevertheless, cross-sample relative differences in the amounts of prokaryote FAs were not impacted by EL-FAME relative to PLFA.

PLFA also was used to track shifts in MCS during the decomposition of two residues of contrasting chemical composition (maize stover, MS; and coconut husk, CH), under two N rates in a Brazilian soil. Microbial biomass-C (MBC), respiration rates and activity of C-cycle enzymes were concomitantly evaluated. A laboratory incubation was conducted for 425 d that included a simulated drought period between days 160 and 290. MS significantly impacted MCS and increased respiration rates, MBC, and enzyme activities; while changes due to CH were more subtle or non-existent. In MS, N reduced the CO_2-C losses from soil, but caused no change in MBC. Higher respiration rates under low N were associated with a more bacterial-dominated community, and a higher laccase activity.

Fatty Acid Profiling of Soil Microbial Communities: A Comparison of Extraction Methods and Temporal Dynamics in Plant Residue Amended Soils

by
Marcelo Ferreira Fernandes

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Marcelo Ferreira Fernandes, Author

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CONTRIBUTION OF AUTHORS

Marcelo Ferreira Fernandes conducted research and wrote each manuscript. Dr. Richard P. Dick has analyzed each manuscript.

TABLE OF CONTENTS

	Page
Introduction	1
Chapter 1. Interpretation and Comparison of Soil Microbial Community Based on Whole-Cell Fatty Acid (MIDI) or Phospholipid Fatty Acid (PLFA) Profiles …….	5
Abstract……..……..……..…….	6
Introduction..……..…….	7
Materials and Methods...……..…….	9
Results and Discussion……..…….	18
Conclusions……..…….	36
Acknowledgements……..…….	37
References……	37
Chapter 2. Comparison of Soil Microbial Community Profiling with PLFA and EL-FAME Methods ……...…….	43
Abstract……..……..……..…….	44
Introduction..……..…….	45
Materials and Methods...……..…….	47
Results……..……..…….	57
Discussion……..…….	68
Conclusions……..…….	76
Acknowledgements……..…….	77
References……	78

TABLE OF CONTENTS (Continued)

Page

Chapter 3. Nitrogen and Soil Moisture Controls on Succession of Soil Microbial Communities and Enzyme Activities during the Decomposition of Coconut and Maize Residues…… 82

Abstract…… 83

Introduction …… 84

Materials and Methods ...…… 86

Results …… 95

Discussion …… 109

Summary……. …… 121

Acknowledgements …… 122

References …… 122

General Conclusions …… 128

Bibliography …… 132

Appendices …… 144

LIST OF FIGURES

Figure		Page
1.1.	NMS representation of sample distances based on the abundance (pmol g^{-1} soil) of 11 plant-associated peaks. ...……..……..	24
1.2.	NMS representation of sample distances according the PLFA composition (pmol FAME $pmol^{-1}$ 16:0). ……...……...	28
1.3.	NMS representation of sample distances according the MIDI-FA composition (pmol FAME $pmol^{-1}$ 16:0). ……...……...	29
1.4.	Relationship between within-set average sample Sørensen dissimilarities based on both microbial group signature profile (MG) and plant FAME signature profile (PP) vs. overall FAME profile, for PLFA (a) and MIDI (b).	31
1.5.	MIDI:PLFA ratios based on the absolute amounts of FAMEs as a function of soil TOC.	34
2.1.	Non-metric multidimensional scaling of the EL-FAME:PLFA ratio of 28 peaks extracted from 29 soil samples. …….	67
3.1.	Accumulation of CO_2-C respired from soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. ……	96
3.2.	Microbial respiration rates (μg CO_2-C g^{-1} d^{-1}) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation ……..……	97
3.3.	(a) Mineral N content in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days (b) Sum of squares univariate tree model representing factors associated with the variability of mineral N content in soil. ……..	99
3.4.	Soil pH in samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. ……...	101
3.5.	Microbial biomass-C in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days. ……	102

LIST OF FIGURES (Continued)

Figure		Page
3.6.	Soil microbial community composition assessed by PLFA as affected by residue (NR, no residue = squares; MS, maize stover = triangles; and CH, coconut husk = circles), N rates (LN, low N = empty symbols; and HN, high N = solid symbols) and time (see color legend).	103
3.7.	Values for the 19cy to 18:1ω7c ratio (a) and EL to PL ratio for 18:2ω6c (b) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days. ……...	106
3.8.	Activities of invertase (a), cellulase (b), xylanase (c), and laccase (d) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. .....…..	107

LIST OF TABLES

Table		Page
1.1.	Taxonomy and selected soil properties of sites included in this study ……	10
1.2.	Comparison of the major steps for FAMEs production from soil samples according to PLFA, and MIDI procedures ……..	12
1.3.	FAMEs used as biomarkers for different groups of organisms ……...	14
1.4.	Relative abundance, relative frequency, indicator value, and across sample average mol percent of MIDI preferentially extracted peaks in samples extracted by MIDI or PLFA procedures ……..	19
1.5.	Relative abundance, relative frequency, indicator value, and across sample average mol percent of PLFA preferentially extracted peaks in samples extracted by PLFA or MIDI procedures ……..	22
1.6.	Regression between relative recovery of microbial FAMEs by MIDI and TOC	36
2.1.	Taxonomy and selected soil properties of sites included in this study ……	48
2.2.	Comparison of the major steps for FAMEs production from soil samples according to PLFA, and EL-FAME procedures ……..	49
2.3.	FAMEs used in this study as biomarkers for different groups of organisms …..	52
2.4.	EL-FAME vs. MIDI and EL-FAME vs. PLFA comparisons regarding the relative abundance (RA), relative frequency (RF), indicator value (IV)†, and across sample average mol percent (Avg %) of plant-specific FAMEs.	58
2.5.	Multiplicative changes† (exp ß) on FAME amount (pmol g^{-1} soil) associated with method of extraction (EL-FAME vs. PLFA), C from particulate and non-particulate organic matter (POM-C and nPOM-C), pH, vegetation [herbaceous (Herb) or forests vs. no vegetation], and interactions between the terms. ……..	61
2.6.	Multiplicative changes† (exp ß) on FAME amount (pmol g^{-1} soil) associated with lipid sources (neutral lipids vs. phospholipids; and glycolipids vs. phospholipids), and interactions between source and vegetation. ……..	64
3.1.	Chemical composition of maize stover and coconut husk fiber used to amend soil ……	88

LIST OF APPENDICES

Appendix		Page
A.	Regression tree for ammonium-N contents on residue treatment (res); N rate (N); and sampling days (d). ……..	145
B.	Regression tree for nitrate-N contents on residue treatment (res); N rate (N); and sampling days (d). …….	146
C.	Regression tree for soil pH on residue treatment (res); N rate (N); and sampling days (d). ……	147
D.	Regression tree for microbial biomass-C on residue treatment (res); N rate (N); and sampling days (d). ……..	148
E.	Regression tree for the 19:0cy to 18:1ω7c ratio on residue treatment (res); N rate (N); and sampling days (d). ……..	149
F.	Regression tree for the ratio between the contents of 18:2ω6c in EL-FAME and PLFA pools on residue treatment (res); N rate (N); and sampling days (d). ……	150
G.	Regression tree for invertase activity on residue treatment (res); N rate (N); and sampling days (d). ……..……	151
H.	Regression tree for cellulase activity on residue treatment (res); N rate (N); and sampling days (d). …….	152
I.	Regression tree for xylanase activity on residue treatment (res); N rate (N); and sampling days (d). …….	153
J.	Regression tree for laccase activity on residue treatment (res); N rate (N); and sampling days (d). ……...…….	154

FATTY ACID PROFILING OF SOIL MICROBIAL COMMUNITIES: A COMPARISON OF EXTRACTION METHODS AND TEMPORAL DYNAMICS IN PLANT RESIDUE AMENDED SOILS

GENERAL INTRODUCTION

Earlier methods to study microbial composition relied on the extraction of microbes from environmental samples and their cultivation in different types of culture media. The limitation of these methods to portray a comprehensive picture of the microbial structures in the environmental samples was highlighted after the discovery that only a very small fraction of microbes are cultivable. In the last decades, many methods that preclude the need for cultivation have been developed for studying differences in the structure of microbial communities in the environment. These methods are based on the analysis of cell components directly extracted from samples. The diversity of cell components is assessed by separating them according with differences in their physical-chemical properties. If different chemical forms of a given cell component are derived from taxonomically distinct microbial groups, then this link between chemical and microbial diversity can be used as a tool to describe microbial structure in the environmental samples.

Fatty acids with different chain structure have been ascribed to be typical of distinct microbial groups, as fungi, Gram-positive and Gram-negative bacteria, actinomycetes, etc., and therefore can be used as biomarkers for these groups. Fatty acids can be obtained from soil samples by different extraction protocols, which in turn, vary in their abilities to access different lipid pools in soil samples. In common, all of them produce fatty acid methyl esters (FAME), a

chemical substance more volatile than the original fatty acids and, therefore, more amenable for the standard analysis by gas chromatography.

An extraction method that produces FAMEs specifically from the phospholipid fraction (PL-FAME) has been considered the standard procedure of obtaining fatty acid profiles from environmental samples. Thus, this method requires, in addition to the extraction of the whole lipid content from soil, the fractionation of this extract into neutral, glycolipids and phospholipid fractions through silica columns eluted with solvents of increasing polarity. The phospholipid fraction is then subjected to a mild alkaline step aimed to convert ester-bonded fatty acids in FAMEs. The advantage of this method is that it specifically describes the structure of the living microbiota, as phospholipids are rapidly converted into neutral lipids upon microbial death.

PL-FAME, though, is more laborious than other alternative methods that employ direct extraction of FAMEs from soil samples. Two such methods exist that differ basically in the intensity of the extraction procedure and in the types of fatty acids converted into FAMEs. For instance, the ester-linked FAME method (EL-FAME), employs the same mild alkaline reaction for FAME production as used in PL-FAME, which extracts only ester-bonded types of fatty acids from soil samples. However, because the extraction procedure is performed directly in contact with soil samples, with no previous separation of lipid fractions, FAMEs are formed from ester-linked fatty acids from all lipid molecules rather than just from phospholipds. Among these lipid sources, the neutral lipids, used as storage by eukaryotes and present in dead cells, and glycolipids, would be the major potential interferences in the evaluation of microbial structure.

Another method available, adapted from the commercial protocol used by a bacterial identification system (MIDI), also extracts lipids directly from soil samples. This protocol relies

on a sequence of strong alkaline saponification and acid methylation, done at high temperature, to produce FAMEs from the whole cell content. In addition to the ester-linked fatty acids, the extracts also include FAMEs derived from free fatty acids as well as from lipids containing amide- and ether-bonded fatty acids. Some of these extra FAMEs may be derived from microbial components not included by the two former methods and, for this reason, could potentially provide a more comprehensive picture of microbial communities. However, this drastic direct method would also increase the chances of contamination of the FAME profiles by non-microbial products.

The two first chapters of this thesis investigated how the use of these direct extraction protocols would interfere in the discrimination of soil samples based on FAME profiles and in the interpretation of the results of microbial communities compared with the standard PL-FAME procedure.

In Chapter 1, we compared EL-FAME and PLFA profiles obtained from 29 soil samples collected from several sites across the State of Oregon. These samples included soil types with a wide range of vegetation cover, soil organic matter, texture, and pH. Specifically, we have investigated whether the degree of agreement between the results obtained from the two methods would depend on the soil and vegetation characteristics of the samples under investigation. Moreover, we evaluated whether method-related interferences would affect the quantification of all the FAMEs, irrespective to their microbial origin. If FAMEs are identified whose relative changes between different soil conditions are not impacted by the method of extraction, EL-FAME could be used to track shifts in the abundance of their specific microbial groups.

In Chapter 2, we compared MIDI and PL-FAME profiles obtained from the same 29 samples used in the chapter one. In this study, in addition to the usual analysis of FAMEs by gas

chromatography, we employed mass spectrometry to help identify major peaks present in the MIDI profiles to which no microbial fatty acid identification could be assigned by the MIDI identification library. Also, during the analysis, it was realized that the efficiency of FAME extraction by MIDI was strongly reduced in some soils. Therefore, we investigated if changes in the soil to extractant volume ratio could impact the FAME extraction efficiency by this method.

In the third chapter, we used PL-FAME method to track long-term changes in microbial composition in soils amended with two residues of different chemical recalcitrance (coconut husk and maize stover) and with different N rates, under laboratory conditions. Coconut husk was chosen for this purpose for its importance as both an amendment for soils with low water-holding capacity and as a waste of great impact in areas of production and consumption of the coconut fruits. Despite that importance, little information on the impact of coconut husk in the soil microbial community and related processes is available. Corn stover was used as a reference of a residue which decomposition process is well known. Along the incubation time, we have simulated a period of drought, with intensity and duration roughly similar to that found in the Brazilian Coastal Tableland, a region where coconut has been traditionally grown. In addition to the PL-FAME profiles, other variables known to be strongly affected during decomposition of plant residues were evaluated, including: microbial respiration, biomass-C, physiological markers based on FAME ratios, activity of microbial enzymes related to the C cycling, mineral N contents, and pH. This study was conducted over the course of 425 days to assess the influence of residue quality, N rates and soil moisture content on microbial properties and related processes during the decomposition.

Chapter 1

Interpretation and Comparison of Soil Microbial Community Based on Whole-Cell Fatty Acid (MIDI) or Phospholipid Fatty Acid (PLFA) Profiles

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Abstract

Fatty acid (FA) profiling is increasingly used to determine the microbial diversity of soils. This technique is based on the assumption that changes in the overall FA profile are due to changes in the microbial community. However, it is possible that the type of lipid extraction method influences the outcome of lipid profiling. Two common methods of FA extraction from soil are the phospholipid FA procedure (PLFA) and the whole cell lipid content extraction, which is based on MIDI procedure. Despite their divergent extractant chemistry and lipid separation procedures, these methods have shown a similar ability to discriminate soils from different soil management and vegetation systems. In this study we obtained PLFA and MIDI-FA profiles from 29 soil samples collected across Oregon under a wide variety of soil conditions. In addition to the identification based on chromatographic properties of the compounds, we used GC-MS to confirm some of those identifications. Omega hydroxy FAMEs and dicarboxylic acids associated with plants were identified in MIDI extracts. Plant-associated compounds were quantitatively related to particulate organic matter-C (POM-C) and qualitatively associated to the standing vegetation at sampling. Abundance of these peaks was negatively correlated to the respiration rate normalized to POM-C ($Resp_{POM}$), which increased in systems under more intensive management. We hypothesized that possible changes in the MIDI-FA profiles, in principle considered to represent responses of microbial composition to these factors, could reflect concurrent changes in the composition of non-microbial sources. Unlike PLFA profile, overall MIDI-FA diversity was highly associated with the diversity of the plant-derived peaks rather than that of microbial signature FAs. Moreover, results from regression analysis indicated a strong negative association between MIDI-FA : PLFA ratios and total organic carbon (TOC). This was further investigated to determine whether the reagents

limited the recovery of MIDI-FAs from high soil organic matter (SOM) soils. This showed that recovery of MIDI-FA microbial signatures sharply decreased with increasing soil : extractant ratios. We concluded that the MIDI method should be used with caution while interpreting changes in FA profiles as being due to shifts in microbial communities.

Introduction

Determination of fatty acid (FAs) patterns in environmental samples has become increasingly popular during the last decade as a quantitative means to assess microbial community structure. Evaluating changes in the relative composition of the overall FA profile in soil samples has been used as a strategy to compare changes in microbial communities in soils under different management and vegetation systems (Buyer and Kaufman, 1997; Drijber et al., 2000; Hackl et al., 2005; Petersen et al., 2002; Schutter and Dick, 2002). The FA profiling is based on the assumption that changes in the overall FAs are mainly driven by changes in the microbial composition. Some of these FAs have been shown to be typical signatures for different groups of microorganisms (Tunlid and White, 1992) and may thus be used to interpret these changes.

Phospholipid-derived fatty acids (PLFA) is the most widely used method for profiling microbial communities based on FA. Phospholipids represent the viable microbial community because they are rapidly decomposed after microorganism death (King et al., 1977). The PLFA extraction produces fatty acid methyl esters (FAMEs) after methylation of the phospholipid fraction, that have been separated from neutral and glycolipids by solid phase extraction in

silica columns. The mild alkaline methylation step commonly used for this technique is efficient to generate FAMEs from ester-linked fatty acids (Zelles, 1999).

The MIDI procedure is quicker and easier than the PLFA method and has also been used as a FA fingerprint of microbial communities (Cavigelli et al., 1995; Haack et al., 1994; Ibekwe and Kennedy, 1999). MIDI extraction can also be advantageous relative to PLFA when sample size is limiting, as it requires a smaller amount of soil than PLFA to maintain a reliable microbial community fingerprint (Drenovsky et al., 2004). This method, originally developed to identify bacterial strains in pure culture, is intended to extract whole-cell fatty acids and uses a strong extractant. It directly extracts FAs from soil without any previous separation of lipid fractions. The protocol encompasses a saponification step under high temperature and strong alkalinity and an acid methanolysis step. In addition to ester-linked fatty acids, those amide- and ether-linked (e.g. plasmalogens, lipopolysaccharides, sphingolipids), as well as free fatty acids are converted in FAMEs by saponification/acid methanolysis reactions (Mayberry et al., 1973; Zelles, 1999). It has been suggested that the main disadvantage of extending this method to investigate microbial community structure in soils is that a portion of the FAs may be derived from non-living organic matter (Drenovsky et al., 2004; Macalady et al., 1998; Petersen et al., 2002). Despite these reports, there are no unequivocal data showing peaks of non-microbial origin to be present in the MIDI extracts. Another confounding factor for comparing MIDI and PLFA methods is that the recovery efficiency and the effect on the chemical integrity of some fatty acids may be method dependent (Drenovsky et al., 2004; Petersen et al., 2002; Zelles, 1999).

Despite the large differences in their extraction chemistry and effects on specific biomarkers, MIDI-FA and PLFA profiles have shown similar abilities to discriminate among soils under different management or vegetation systems (Drenovsky et al., 2004). Schutter and

Dick (2000) have also reported that similarity between MIDI-FA and ester-linked fatty acids (EL-FA) profiles, but pointed out that differences become more apparent with increasing SOM content.

It is known that the composition of both microbial (Bossio et al., 1998; Hackl et al., 2005; Zelles et al., 1992) and non-microbial (Jandl et al., 2005; Nierop et al., 2001) lipids extracted from soil vary with vegetation, amount of plant biomass input, and soil management. Thus, we hypothesized that since most studies of field management are ultimately comparing some sort of plant residue treatments, either in amount, types or differentiated decomposition rates (conservation), MIDI may give the same results as PLFA but by a different mechanism.

The objective of this paper was to compare MIDI and PLFA regarding (a) their ability to extract fatty acids, both qualitatively and quantitatively, and (b) their ability to discriminate soil samples based on FAME microbial signatures.

Materials and Methods

Soil sampling and sites

Twenty-nine composite soil samples (0-10 cm) were collected from six soil series of varying texture, total organic carbon (TOC), and site history across Oregon from September to October 2003 (Table 1.1). When more than one sample was collected under the same ecosystem or management, replicates were separated at least by 30-m intervals. Field-moist soil samples were passed through a 2-mm sieve and stored at 4^{o}C for no longer than 3 days before lipid extraction.

Table 1.1. Taxonomy and selected soil properties of sites included in this study.

Sites[†]	Soil Type	TOC	POM C[‡]	nPOM C[‡]	MB C[‡]	pH	Standing vegetation	Site History	Location
		--------- g kg^{-1} dry soil ----------							
CO (1)	Dayton silt loam Vertic Albaqualf	14	3.1	11.1	0.52	5.4	no	grass field, plowed before sampling	Linn County
HY (1)	Woodburn silt loam Aquultic Argixeroll	15	0.5	15.3	0.21	5.5	no	summer vegetable/ winter fallow	Corvallis
VF (3)	Chehalis silt loam Cumulic Ultic Haploxeroll	12	1.1	10.9	0.30	6.1	corn, grasses	summer vegetable/ winter fallow	Corvallis
RC (3)	Saturn Variant silt loam Fluventic Halumbrept	26	2.5	24.0	0.80	6.1	no	summer vegetable-winter or annual winter cropping	Scio
Pburn (1)	Walla Walla silt loam Typic Haploxeroll	9	1.7	8.2	0.19	6.5	no	winter wheat / summer fallow, burning	Pendleton
PM (3)	Walla Walla silt loam Typic Haploxeroll	11	2.8	8.6	0.45	7.0	no	winter wheat / summer fallow, manure (22 Mg ha^{-1} $2y^{-1}$)	Pendleton
PN (3)	Walla Walla silt loam Typic Haploxeroll	10	2.1	8.4	0.33	5.7	no	winter wheat / summer fallow, inorganic N	Pendleton
PP (3)	Walla Walla silt loam Typic Haploxeroll	17	5.7	11.7	0.93	7.1	pasture	pasture (>70 y)	Pendleton
HR (2)	Parkdale loam Humic Vitrixerands	11	3.2	8.6	0.44	5.7	apple trees, grasses	orchard (a) inter-; (b) in-row	Hood River
CC (3)	McKenzie River fine-loamy Typic Haplumbrept	36	17.9	18.9	0.87	5.9	Douglas fir forest, shrubs	clear cut and replanting (~10y)	Blue River
OG (3)	McKenzie River fine-loamy Typic Haplumbrept	42	27.0	15.3	1.80	5.6	Douglas fir	Old growth forest	Blue River
WL (3)	Saturn Variant silt loam Fluventic Halumbrept	29	12.6	17.1	1.20	5.6	Mixed Forest	Forest (>50 y)	Scio

[†] Values for VF, RC, PM, PN, HR, PP, CC, OG, WL sites are averages from sites under the same ecosystem. Numbers between parentheses represent the number of sites collected. Low-case letters after sites names in this paper represent subsamplings.
[‡] POMC, nPOMC, and MBC refer to C in particulate and non-particulate organic matter, and microbial biomass, respectively.

Fatty acid methyl ester extraction and analysis

Three analytical replications were done on each soil sample, by both MIDI and PLFA, using 3 g of soil (wet wt with results reported on dry wt basis). Extraction procedures and reagents were similar to those described by Schutter and Dick (2000) for MIDI, and Butler et al. (2003) for PLFA. The ratio between soil and all the reagents used in MIDI extraction was what has been typically used (Cavigelli et al., 1995; Drenovsky et al., 2004; Ibekwe and Kennedy, 1999; Petersen et al., 2002; Schutter and Dick, 2000). Both methods were modified to enable quantitative recovery of lipids from solvents during transfer to a new tube or vial by 3 washings. All 3 organic phase recoveries were pooled and dried under an N_2 atmosphere. A comparison of the major steps of the two extraction methods is shown in Table 1.2.

FAME composition in the extracts was determined with an Agilent 6890 gas chromatograph (Agilent, Inc., Palo Alto, CA) equipped with a 25-m HP Ultra-2 column (internal diameter, 0.2 mm; film thickness, 0.33 μm) and a flame ionization detector (GC-FID). The identification of FAMEs was based on comparison with chromatograms of a mixture of 37 FAMEs (FAME 37 47885-4; Supelco Inc., Bellefonte, PA), a mixture of 24 bacterial FAMEs (P-BAME 24 47080-U; Supelco, Inc.), 10-Me 16:0, and 10-Me 18:0 (Matreya), and 20:4ω6c (Supelco). A linear regression between the equivalent chain lengths (ECL) and the retention times of peaks in the authentic standards was used to determine the ECL of the peaks not included in these standards. Identification was assigned after comparing the ECLs obtained with those listed by the MIS Aerobe chromatographic programs by MIDI (Microbial ID, Inc., Newark, DE).

Tridecanoic acid methyl ester (Supelco, Inc.) standard curves were used to convert chromatographic areas into pmol FAME. Absolute amounts of individual FAMEs were expressed in pmol FAME g^{-1} dry soil. The summed masses of FAMEs reported as typical of

Table 1.2. Comparison of the major steps for FAMEs production from soil samples according to PLFA, and MIDI procedures.

Steps	PLFA	MIDI	Comments
Whole lipid extraction from soil	Bligh and Dyer (1959)		
Fractionation of lipid extract in SPE silica columns	Neutral lipids (chloroform), glycolipids (acetone), and phospholipids (methanol)		PLFA: Neutral and glycolipids fractions are discarded and phospholipid fraction used in the next step for FAME production.
Cell lysis and saponification of lipids		Direct from soil samples with 3.75 *M* NaOH in MeOH:water (1:1)	
FAME production	Mild alkaline methanolysis of individual fractions (0.2 *M* KOH in methanol)	Acid methanolysis in the soil suspension containing cell lysates and saponified lipids (6.0 *M* HCl:methanol; 1:0.85)	PLFA: Mild alkaline methanolysis: Ester-linked fatty acids from phospholipids. MIDI: produces compounds from ester-linked fatty acids, free fatty acids and originally non-ester-linked fatty acids.
Partition of aqueous and organic phases and FAME recovery	Hexane. Dry organic phase under N_2 stream.	Hexane, followed by washing of residual acidic reagents from the collected organic phase with 0.3 M NaOH. Dry organic phase under N_2 stream.	The dried fractions from both methods, which contain the FAMEs, were resupended with hexane to a known volume.

fungi, Gram-negative (GN) bacteria, Gram-positive (GP) bacteria, actinomycetes (ACT), arbuscular mycorrhyzal fungi (AMF) and microeukaryotes (EUK) were used as signatures for these microbial groups (Table 1.3).

FAME extracts produced by both methods were also analyzed by gas chromatography–mass spectrometry (GC-MS) to identify major peaks for which no name was assigned by the procedures described above. Extracts were analyzed directly and aliquots converted to trimethylsilyl derivatives by reaction with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane and pyridine for 3h at 70ºC . GC-MS analyses were performed on a Hewlett-Packard model 6890 GC coupled to a Hewlett-Packard model 5973 MSD. Separation was achieved on a fused silica capillary column coated with DB-5 (30m x 0.25 mm i.d., 0.25 μm film thickness). The GC operating conditions were as follows: temperature hold at 65˚C for 2 min, increase from 50 to 300˚C at a rate of 6˚C min^{-1} with final isothermal hold at 300˚C for 20 min. Helium was used as carrier gas. The sample was injected splitless with the injector temperature at 280˚C. The silylated extracts were diluted (1:1) with n-hexane prior to injection. The mass spectrometer was operated in the electron impact mode at 70eV and scanned from 50 to 650 daltons. Individual compounds were identified by comparison of mass spectra with literature and library data, authentic standards, and interpretation of mass spectrometric fragmentation patterns. Although some of the compounds identified by this analysis were not FAME sensu stricto, but rather fatty acid dimethyl esters and alcohols, the term FAME was used throughout this text for sake of simplicity.

Areas under the GC-FID chromatograms corresponding to peaks identified by GC-MS were converted to pmol FAME.

Table 1.3. FAMEs used as biomarkers for different groups of organisms.

Microbial Groups	FAME Markers	Comments	References
Fungi	18:2ω6c	Good correspondence with ergosterol, another fungi marker; also present as storage products in fungi	Frostegård and Bååth, 1996; Olsson, 1999
Gram+ bacteria	15:0i; 15:0a; 16:0i; 17:0i; 17:0[a]		O'Leary and Wilkinson, 1988
Gram- bacteria	18:1ω7c; 17:0cy; 19:0cy		Wilkinson, 1988
Actinomycetes	16:0 10-Me; 17:0 10-Me; 18:0 10-Me		Kroppenstedt, 1992
Arbuscular mycorrhyzal fungi (AMF)	16:1ω5c	Also present in some bacteria as *Cytophaga* / *Flexibacter*; also a major source of storage (neutral lipids) in AMF spores and vesicles.	Olsson et al., 1995; van Aarle and Olsson, 2003
Microeukaryotes	20:4ω6c	Generally used as a protozoan marker, but also present in nematodes, fungi and algae.	Vestal and White, 1989; Harwood and Russell, 1984

Basal respiration, total organic carbon and particulate organic matter

Basal respiration rates were determined as the amount of CO_2 evolved from 10 to 20 days of soil incubation (25^{o}C) to eliminate influence of sieving, wetting, and root fragments. Total soil organic matter (SOM) was determined by dry combustion (furnace at 430^{o}C for 24h). We converted SOM values to TOC assuming 50% of C in SOM. Coarse (>0.106 mm) particulate organic matter (POM) was obtained from 25 g of sieved soil samples (<2.8 mm) after dispersion in sodium metahexaphosphate (5g l^{-1}) with pH adjusted to 9. Adjustment on the dispersing solution pH was made to improve dispersion of colloids in soils with low pH. Aggregates were disrupted upon agitation of soil suspensions in a rotatory shaker (250 rpm) for 4 h. The slurry was passed through a 0.106 mm-opening sieve and the portion retained was washed with excess distilled water. The filtrate, which contains both finer mineral and organic particles, was discarded. POM was separated from mineral particles by floatation in sodium-polytungstate (d = 1.8 g cm^{-3}). After separation, POM was washed with excess distilled water, dried overnight at 50^{o}C under ventilation and weighed (±0.1 mg).

Statistical analyses

Only peaks in the region between tetradecanoic methyl ester (14:0) and arachidonic acid (20:4ω6c) were included in this analysis, unless otherwise stated. This range includes the main ester-linked FAMEs used as microbial biomarkers and at the same time excludes several shorter and longer chain FAMEs reportedly associated to plants and SOM.

Identification of peaks whose extraction was highly dependent on the method used was achieved by indicator species analysis (ISA) using the values of absolute amounts of FAMEs (pmol g^{-1} dry soil) for the analysis. Although this technique was originally designed for analysis of ecological communities (Dufrene and Legendre, 1997), it seemed adequate to our objective

to describe the association between FAMEs and methods of extraction. For each attribute (FAMEs, in this case) ISA provides an indicator value (IV), varying from 0 to 100, which expresses the degree of relationship of this attribute to each group (method of extraction, in this case). This value is the product of the FAME relative abundance (amount of FAME extracted by each method relative to its summed amount across both methods) and the relative frequency (number of samples in which the FAME was detected relative to the number of total samples within a given group). IVs were tested for statistical significance using a randomization (Monte Carlo) procedure.

Non-metric multidimensional scaling (NMS) was used to ordinate soil samples in a 2-dimension space representing the variability of 11 plant-derived peaks regarding their absolute abundance (pmol FAME g^{-1} soil) in the samples. These 11 peaks encompass major plant-related peaks we identified in the chromatographic range from 12:0 to 22:0 FAME. NMS is an iterative technique that ordinates samples in a lower-dimensional space so that the distances between objects in this space match as closely as possible the distances in the original p-dimensional space, where p equals the number of variables (i.e., the number of FAMEs in the data set) (McCune and Grace, 2002). Distance was measured as Sørensen distance, and 150 iterations were performed to ensure stress was minimized (stress is the measure of the dissimilarity between ordinations in the original *p*-dimensional space and in the reduced dimensional space). Stability of the reduced-dimensional ordination pattern was assessed by plotting values of stress vs. iteration number. Relationships between the plant peak abundances in samples and variables as POM, $Resp_{POM}$ and TOC were assessed by joint-plots, overlaying these variables' gradients on the sample ordination (McCune and Grace, 2002).

NMS was also used to verify the congruence between PLFA and MIDI-FA profiles regarding sample ordination and microbial structure interpretation. Here, the peaks were

standardized to FAME 16:0 to calculate the relative composition of FAMEs in the MIDI-FA and PLFA profiles. Standardizing peaks to FAME 16:0, which correlates well with total microbial biomass (Zelles et al., 1992), has been used to avoid having changes in the amount of a few but large FAME peaks affecting the ratios of others (Drijber et al., 2000). Therefore, the contribution of each peak was expressed as pmol FAME $pmol^{-1}$ FAME 16:0. Joint plots representing the MIDI-FA and PLFA ordinations overlaid by their microbial and plant signature gradients were used to interpret changes in microbial communities and the influence of plant-peaks on the ordination structures.

The degree of relationship between the diversity of both (a) plant-associated MIDI-preferentially extracted peaks and (b) microbial FAME signatures and the diversity of the overall FAME in the MIDI-FA and PLFA profiles was ascertained by the following approach. Twenty subsets of samples (n = 5 to 29) were created from the original 29-sample set, encompassing samples with different degrees of heterogeneity regarding SOM content, soil management, standing vegetation, and geographical origin. The average dissimilarity among samples within each subset and for each extraction method was calculated by the Sørensen proportion coefficient based on the peak compositions of different profiles containing in the range between 14:0 and 20:4ω6c: (a) all the peaks (overall profile), (b) the five major plant-associated peaks (PP profile), and (c) the fourteen signature FAMEs for the six microbial groups presented in Table 1.3 (MG profile). Proportion data for individual peaks were obtained after standardizing their absolute amounts (pmol FAME g^{-1} soil) to FAME 16:0. Average dissimilarity values from the PP and MG profiles for the 20 subsets were regressed against those obtained from the overall profile, within each method.

In order to compare the efficiency of extraction of microbial signature FAMEs by the two methods under different soil conditions, the ratio MIDI:PLFA of pooled microbial signature

FAMEs was modeled as a function of TOC, POM, and vegetation type. MIDI:PLFA ratio values were transformed by natural logarithm to improve normality and homogeneity of variance of the dataset.

PC-ORD package (MjM Software, Gleneden Beach, OR) was used for ISA and NMS analysis, and S-Plus 6 (Insightful Corporation, Seattle, WA), for regression analysis.

Results and Discussion

Peaks preferentially extracted by MIDI or PLFA

Major MIDI-preferentially extracted peaks (MPEP) were identified by ISA in our soil sample set, which included soils from a wide range of SOM, vegetation type and soil management (Table 1.4). Within the chromatographic range evaluated, 40 peaks presented an indicator value (IV) significantly higher ($p<0.05$) for MIDI than PLFA. The summed MPEPs corresponded to 51% and 6% of the whole amount of peaks (mol mol^{-1}) extracted from all the samples by MIDI and PLFA, respectively.

Among the MPEPs there was an abundant and widespread occurrence of alkanoic acids, alkenoic acids, ω-hydroxy alkanoic acids, alkan-1-ols, and α,ω-alkanedioics acids. MPEPs for which mol percent in MIDI profiles, averaged across samples, was > 1%, are presented on Table 1.4.

The three ω-hydroxy alkanoic acids detected in soil samples were identified as: 12-hydroxy dodecanoic acid (12:0 12OH), 14-hydroxy tetradecanoic acid (14:0 14OH), and 16-hydroxy hexadecanoic acid (16:0 16OH). The latter two were detected in especially high

Table 1.4. Relative abundance, relative frequency, indicator value[†], and across sample average mol percent of MIDI preferentially extracted peaks in samples extracted by MIDI or PLFA procedures.

	Relative Abundance		Relative Frequency		Indicator Value		MIDI		PLFA	
Peaks	MIDI	PLFA	MIDI	PLFA	MIDI	PLFA	mol %	SE	mol %	SE
16:0 Alcohol	100	0	100	0	100 **	0	1.62	0.25	0.00	0.00
16:0 16OH	98	2	100	45	98 **	1	6.70	0.73	0.29	0.07
14:0 2OH	100	0	93	0	93 ***	0	1.51	0.16	0.00	0.00
16:0 DCA	97	3	90	14	87 **	0	2.87	0.48	0.06	0.03
14:0	87	13	100	97	87 **	13	3.73	0.21	0.76	0.04
14:0 14OH	100	0	86	0	86 **	0	6.61	1.54	0.00	0.00
17:1 ω5c	100	0	83	0	83 **	0	1.62	0.22	0.00	0.00
18:3 ω6c	81	19	97	48	78 **	9	2.04	0.25	0.53	0.16
16:1 ω5c	72	28	100	100	72 *	28	6.37	0.75	3.34	0.13
12:0 12OH	100	0	38	0	38 **	0	1.69	0.47	0.00	0.00

[†] Relative abundance, relative frequency, indicator value were obtained from indicator species analysis (ISA) using absolute amount of FAMEs (pmol FAME g^{-1} dry soil). See text for details.

amounts, contributing 6.6 and 6.7% (mol mol^{-1}), respectively, of the peak totals in MIDI samples. Schutter and Dick (2000) reported, based on identification with MIDI software, 17:1 ω7c and 19cy ω10c as the two major MIDI-FAs extracted from one of the same forest soils we also used in our paper (OG sites from McKenzie River). However, using GC-MS in our study identified these peaks as 14:0 14OH and 16:0 16OH. The misidentification is because MIDI was designed to identify microbial FAMEs. Because non-microbial peaks could elute at retention times similar to microbial FAMEs, MIDI peak identification should be considered provisional (Macalady et al., 1998).

FAMEs 12:0 12OH and 14:0 14OH were not extracted in detectable amounts by the PLFA procedure. Although 16:0 16OH was found in 45% of the PLFA-extracted samples, its relative abundance in those samples was negligible compared to when MIDI was used (Table 1.4). Hexadecan-1-ol (16:0 alcohol), 2-hydroxy tetradecanoic acid (14:0 2OH), α,ω-hexadecanedioic acid (16:0 DCA), tetradecanoic acid (14:0), 17:1ω5c and 18:3ω6c also presented high and significant IV for MIDI samples ($p<0.001$), indicating their strong relationship to this extraction method. Another peak, 16:1ω5c, which has been used as an arbuscular mycorrhizal fungi (AMF) marker, was extracted from all samples by both methods, but also showed a higher relative abundance and IV in MIDI-extracted samples ($p<0.01$).

The preferential extraction of 14:0, 16:1ω5c, and 18:3ω6c by MIDI relative to PLFA has been previously reported (Petersen et al., 2002). These three FAMEs and 16:0 alcohol were also more predominant samples extracted by MIDI than in EL-FA procedure (Schutter and Dick, 2000). However, as far as we know, this is the first report of 12:0 12OH, 14:0 14OH, 16:0 16OH, and 16:0 DCA in MIDI extracts from soil samples. These FAMEs, as well as 16:0 alcohol, were of special interest, as they have been reported to be associated with plants, being extracted mainly from cutin, suberin and their associated waxes (Hunneman and Eglinton, 1972;

Kolattukudy, 1980; Matzke and Riederer, 1991; von Rudloff, 1959; Zeier and Schreiber, 1998). Although outside of the range evaluated, other plant- and humin-associated peaks, as dihydroabietic acid (DHA), 18:1 ω9c 18OH, 18:0 18OH, 20:0 alcohol, 22:0 alcohol, 20:0 DCA and 22:0 (Conde et al., 1999; Kolattukudy and Agrawal, 1974; Nierop, 1998; Zeier and Schreiber, 1998; Zeier et al., 1999), were identified in relatively high amounts. This reinforces the large contribution of non-microbial sources to the amount and diversity of MIDI-derived peak profiles.

Nineteen peaks had an IV significantly higher ($p<0.05$) for PLFA than MIDI. The sum of these peaks' masses accounted for 41% and 8% of the whole amount of peaks (mol mol^{-1}) extracted by PLFA and MIDI, respectively, across all samples. Peaks preferentially extracted by PLFA that had mol percent, averaged across samples, > 1% in PLFA extracts are presented in Table 1.5. It is interesting to notice that, except for 16:1 2OH, all the major peaks preferentially extracted by PLFA have been commonly used as microbial markers. These included all the markers for GN (19:0cy, 17:0cy, and 18:1ω7c) and actinomycetes (16:0 10-Me, 17:0 10-Me and 18:0 10-Me), and two out of the five used here as GP markers (17:0i and 17:0a). Although in smaller amounts than in PLFA, all these peaks were extracted by MIDI from most soil samples, except for 18:0 10-Me, which was extracted from only 37% of the samples. These results are in line with those by Drenovsky et al. (2004), who reported a smaller recovery of FAME signatures for actinomycetes and bacteria by MIDI than PLFA, but in disagreement with Petersen et al. (2002). MIDI was also shown to recover significantly less of all microbial signature FAMEs presented in Table 1.5 than EL-FA (Schutter and Dick, 2000). The low recovery of these signature FAMEs by MIDI may be related to the stronger conditions of temperature and pH used in this procedure compared to PLFA and EL-FA, which employ

Table 1.5. Relative abundance, relative frequency, indicator value[†], and across sample average mol percent of PLFA preferentially extracted peaks in samples extracted by PLFA or MIDI procedures.

	Relative Abundance		Relative Frequency		Indicator Value		PLFA		MIDI	
Peaks	PLFA	MIDI	PLFA	MIDI	PLFA	MIDI	mol %	SE	mol %	SE
19:0cy ω8c	92	8	100	79	92 **	6	5.40	0.33	0.41	0.05
18:0 10-Me	86	14	100	38	86 **	5	2.03	0.08	0.53	0.10
16:1 2OH	79	21	100	59	79 **	12	1.28	0.10	0.30	0.06
18:1 ω7	79	21	100	100	79 **	21	9.80	0.46	2.14	0.13
17:0 cy	79	21	100	93	79 **	20	3.51	0.09	0.75	0.06
16:0 10-Me	76	24	100	100	76 **	24	4.66	0.11	1.14	0.09
17:0i	71	29	100	76	71 **	22	1.67	0.08	0.53	0.08
17:0a	69	31	100	100	69 **	31	1.90	0.05	0.67	0.06
17:0 10-Me	66	34	100	62	66 **	21	1.05	0.07	0.34	0.07
18:0	63	37	100	100	63 *	37	2.16	0.06	0.87	0.08

[†] Relative abundance, relative frequency, indicator value were obtained from indicator species analysis (ISA) using absolute amount of FAMEs (pmol FAME g^{-1} dry soil). See text for details.

milder conditions. For instance, cyclopropane fatty acids undergo degradation via cleavage of the cyclopropane ring in the presence of acidic methanol, a condition met in the methylation step of MIDI, forming several branched methoxylated artifacts (Vulliet et al., 1974).

Factors of MIDI-derived plant-signature FAME abundance

Absolute abundances (pmol FAME g^{-1} soil) of 11 plant-derived peaks, including the five detected in the chromatographic range usually evaluated for microbial community analysis (12:0 12OH, 14:0 14OH, 16:0 16OH, 16:0 alcohol, and 16:0 DCA), as well as seven others detected out of it (DHA, 18:1 ω9c 18OH, 18:0 18OH, 20:0 alcohol, 22:0 alcohol, and 22:0) were used to ordinate the soil samples in a 2-dimension plot by using NMS. Two major clusters of samples were observed in the NMS ordination (Figure 1.1): 1) included soil samples under forests, pastures and orchard (open symbols); and 2) samples collected from row crop sites (full symbols) except for CO, which was from a grass field site. Three peaks, 12:0 12OH, 14:0 14OH, and 22:0 alcohol, were clearly more associated to forest vegetation than to the herbaceous dominated communities (orchards, HR; and pastures, PP) in the first group. Both FAMEs 12:0 12OH and 14:0 14OH have been identified as important constituents of *Pinus sylvestris* cutin (Hunneman and Eglinton, 1972), *Picea pungens* leaf waxes (von Rudloff, 1959), *Picea abies* needles (Matzke and Riederer, 1991), and also extracted by THAM thermochemolysis from an organic horizon under a mature *Picea abies* forest (Rosenberg et al., 2003). Interestingly, we found no report that these compounds have been isolated from angiosperms or from soil dominated by this type of plants. The 22:0 alcohol has been isolated from soils under *Pinus maritima* (Jambu et al., 1993) and *Fagus-Pinus* (Nierop, 1998) forests, and from the leaf wax of *Amaranthus palmeri*, a weed of agronomical importance (Dailey et al., 1989).

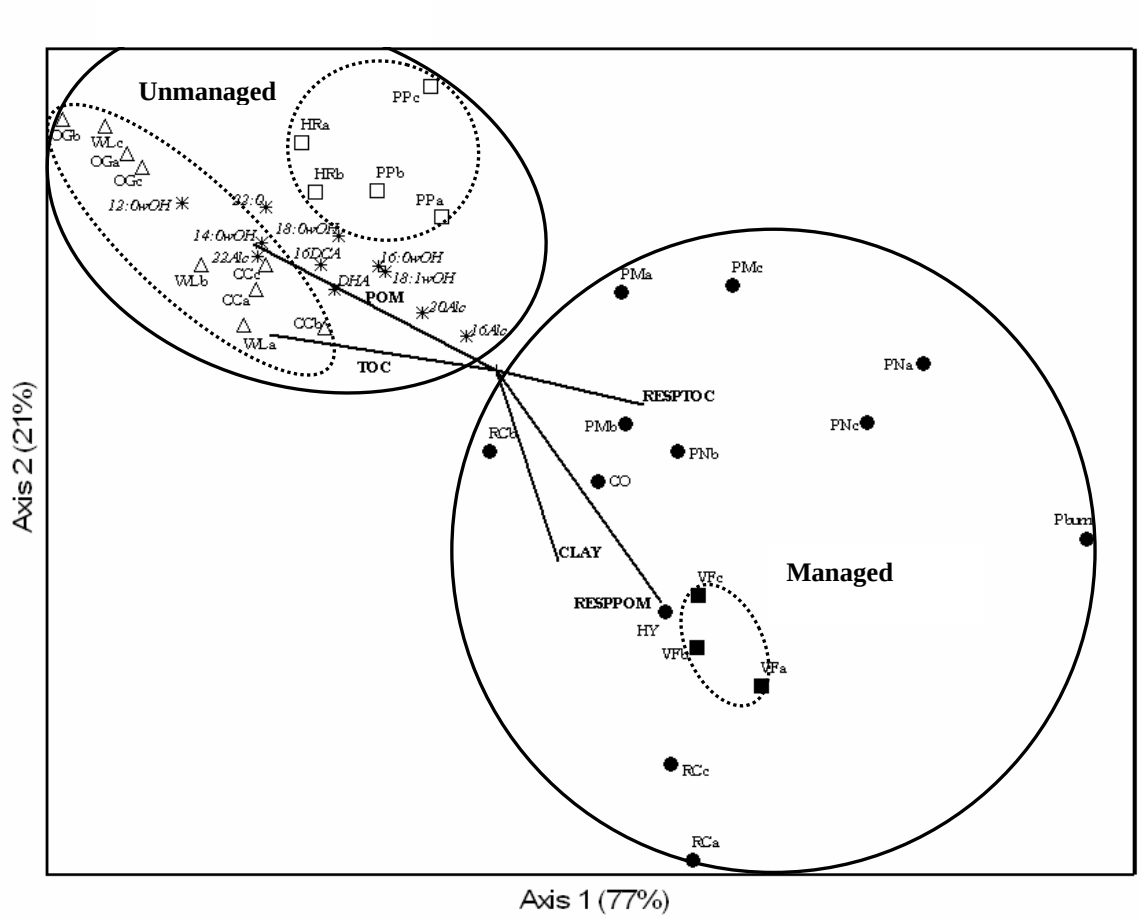

Figure 1.1. NMS representation of sample distances based on the abundance (pmol g^{-1} soil) of 11 plant-associated peaks. Continuous lines enclose management groups and dashed lines enclose groups of vegetation within management groups. Asterisks represent plant-associated peaks. Fill and open symbols represent managed and unmanaged sites, respectively. Triangles, squares and circles represent forest, herbaceous and no vegetation at the sampling time, respectively. Vectors represent intensity and direction of gradients of soil particulate organic matter (POM), soil total organic C (TOC), respiration rates normalized to POM ($Resp_{POM}$) and to TOC ($Resp_{TOC}$), and clay content. The proportion of variance explained by each axis is based on the correlation (*R*) between distance in the reduced NMS space and distance in the original space and is reported after each axis heading.

Association between the other seven peaks and the vegetation type in the first cluster was less distinct. Compounds 16:0 16OH, 16:0 DCA, 18:0 18OH, 18:1ω9c 18OH, 20:0 alcohol, and 22:0 have been reported to be isolated from suberin of several plants, including herbaceous plants and, both angiosperm and gymnosperm tree species (Holloway, 1972a; Holloway, 1972b;

Kolattukudy and Agrawal, 1974; Matzke and Riederer, 1991; Zeier and Schreiber, 1998; Zeier et al., 1999). Cutin and waxes of the aerial plant parts are also sources of 16:0 16OH, 16:0 DCA, and 22:0 (Dailey et al., 1989; del Rio and Hatcher, 1998; Hunneman and Eglinton, 1972; Loveland and Laver, 1972; von Rudloff, 1959). Moreover, all these molecules and DHA have been identified in soil and litter samples under gymnosperm or angiosperm cover by different extraction methods (Almendros and Sanz, 1991; Ambles et al., 1994; Jambu et al., 1993; Nierop, 1998; Rosenberg et al., 2003). The 16:0 alcohol has been detected as a minor component of suberin of several angiosperms (Kolattukudy and Agrawal, 1974; Zeier and Schreiber, 1998; Zeier et al., 1999) and found in a free form in the litter layer of a hydromorphic forest-podzol (Jambu et al., 1993). Hexadecanol has also been shown to be important constituents of wax of some microorganisms as *Euglena gracilis* (Kolattukudy, 1970). Therefore, although plants have been shown to produce this alcohol, its microbial origin in soil cannot be ruled out.

The second cluster contains samples from row crop sites that had no vegetation at the sampling time, except for three VF sites. None of the peaks evaluated was more associated to the row crop group than to the first group. Two peaks, 16:0 alcohol and 20:0 alcohol were relatively more important to this group than were the other peaks.

Gradients of both POM and TOC were observed toward the first group of samples, indicating a strong relationship between SOM and plant-derived FAMEs. However, all the peaks had abundances more closely associated to POM than TOC, which suggests that undecomposed and partially decomposed plant material would be the major source of these peaks in MIDI extracts.

A strong relationship was observed for $Resp_{POM}$ with both axes 1 and 2, indicating not only an increase of this variable from the first to the second cluster, but also toward the samples

in the bottom of axis 2. An underlying gradient of management intensity could be realized against the $Resp_{POM}$ gradient. Along the $Resp_{POM}$ gradient, the ascending management intensity would follow the order: old growth forest, woodland, orchards, pastures, clear-cut sites, and row crop areas. Although weaker and more aligned to axis 1 than $Resp_{POM}$, a similar trend was observed for changes in $Resp_{TOC}$. However, in contrast to $Resp_{TOC}$, the $Resp_{POM}$ gradient had subtler changes in management intensity within the row crop group along the sequence PM (manured winter wheat-summer fallow), CO (grass field), PN (winter wheat-summer fallow), and HY, VF and RC (summer vegetable-winter or annual winter cropping). From these results, there seems to be a negative relationship between soil management intensity and abundance of plant-derived peaks in MIDI extracts. This observation is in line with the reports of Schulten and Leinweber (1993) which showed a decreasing contribution of primary plant constituents at 0-10 cm depth in the following order: grassland > crop rotation > potato monoculture > bare fallow. Nierop et al. (2001) observed a much smaller contribution of alkanols and cutin/ suberin components for SOM under agricultural systems in comparison to pasture, concluding that SOM composition under undisturbed systems, such as no-till, is more similar to the composition of standing vegetation than in systems under disturbance. No-tillage systems tend to conserve organic matter at the surface as undecomposed plant material (Salinas-Garcia et al., 1997), whereas tillage increases litter decomposition rates (Cambardela and Elliott, 1993; Rovira and Greacen, 1957). Furthermore, no tillage or undisturbed systems increase undecomposed plant residues that are physically protected from microbial activity inside aggregates (Six et al., 2000).

The results above are in line with several reports from literature showing strong dependence of soil lipids composition on SOM content, vegetation type and soil management (Jandl et al., 2005; Rosenberg et al., 2003). These three factors have also been shown to

influence soil microbial composition (Drijber et al., 2000; Jackson et al., 2003; Schutter and Dick, 2002). Therefore, we hypothesized that changes in the MIDI-FA profiles may in fact be highly related to non-microbial sources coincidentally related to microbial lipid profiling when measured as PLFA.

Relationship between plant- and microbial-signature diversities with the overall PLFA and MIDI-FA profile diversity

Two-dimensional NMS representations of soil sample distances based on the overall FAME composition (pmol FAME $pmol^{-1}$ 16:0) in PLFA and MIDI extracts were presented in Figure 1.2 and Figure 1.3, respectively. Both ordinations were rotated to maximize the correlation between POM and axis 1 in an attempt to improve the comparison between the two ordinations. After this procedure, Pearson correlation coefficients (r) between POM and axis 1 were 0.86 and 0.89 for MIDI and PLFA, respectively. Similar fractions of the total variability in the original data were represented by PLFA- (93%) and MIDI-based (97%) ordinations. Linear regression between MIDI and PLFA sample ordination scores along axis 1 showed the relationship $Score_{MIDI} = Score_{PLFA} \times 1.073$ ($R^2 = 0.78$), demonstrating that the two methods were equivalent regarding the sample ordination along axis 1. However, although axis 1 alone has represented almost the entire variability in MIDI data (91%), it had only two microbial MIDI-FA signatures (fungi and GN) (Figure 1.3); whereas for PLFA, axis 1 explained 56% of the original variability but was associated with changes in the composition of four microbial PLFA signatures (fungi, GN, GP and Act) (Figure 1.2). It is also noteworthy that there was a strong association between axis 1 and three of the major plant-derived peaks in the MIDI ordination

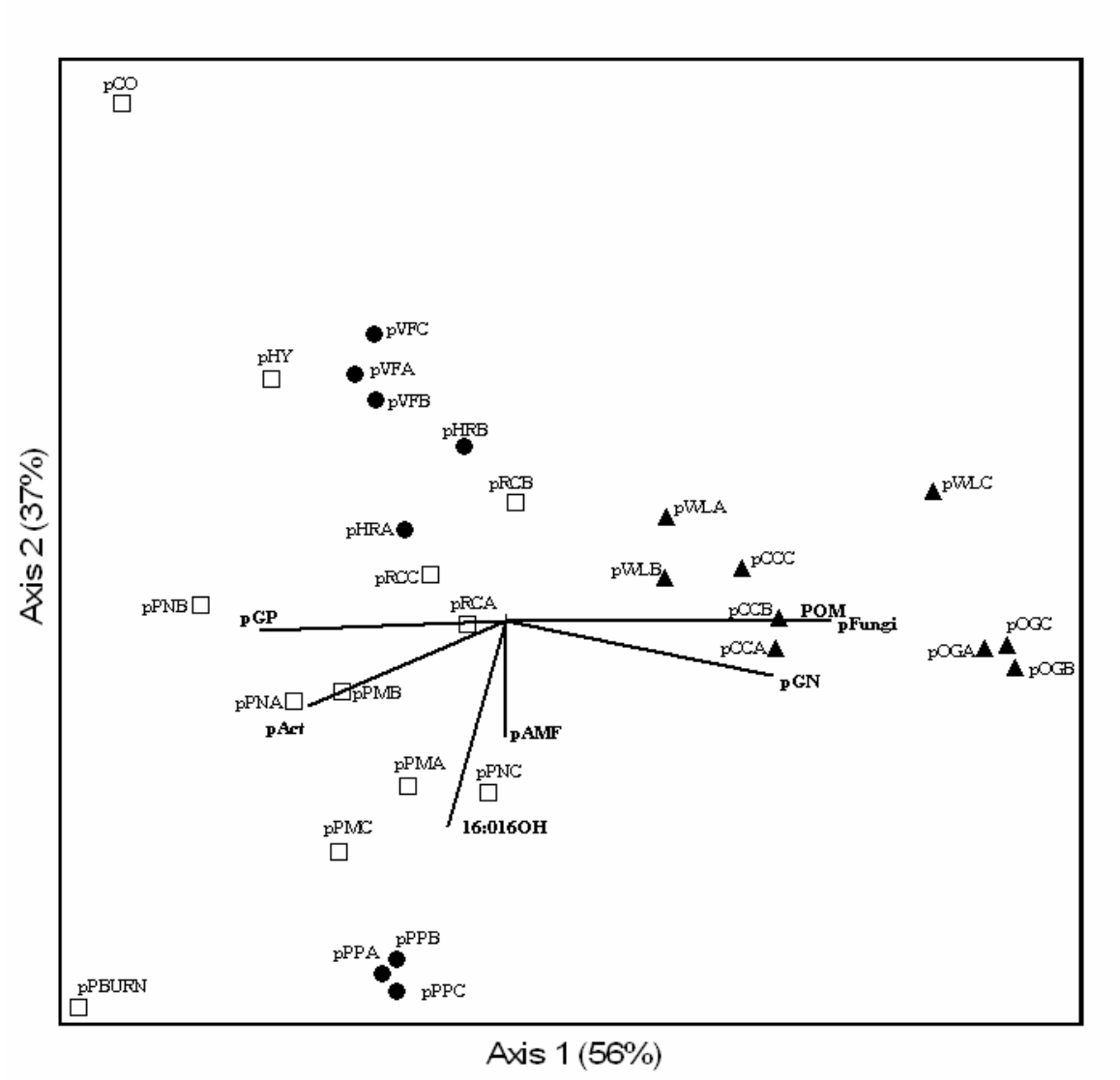

Figure 1.2. NMS representation of sample distances according the PLFA composition (pmol FAME $pmol^{-1}$ 16:0). Open squares, filled circles and filled triangles represent samples collected at sites with no vegetation, herbaceous and forest vegetation at sampling, respectively. Vectors indicate direction an intensity of compositional microbial signature FAMEs and plant-derived FAMEs gradients. pFungi, pGN, pGP, pAct, and pAMF stand for phospholipid derived FAME biomarkers for fungi, gram-negative bacteria, gram-positive bacteria, actinomycetes and arbuscular mycorrhizal fungi.

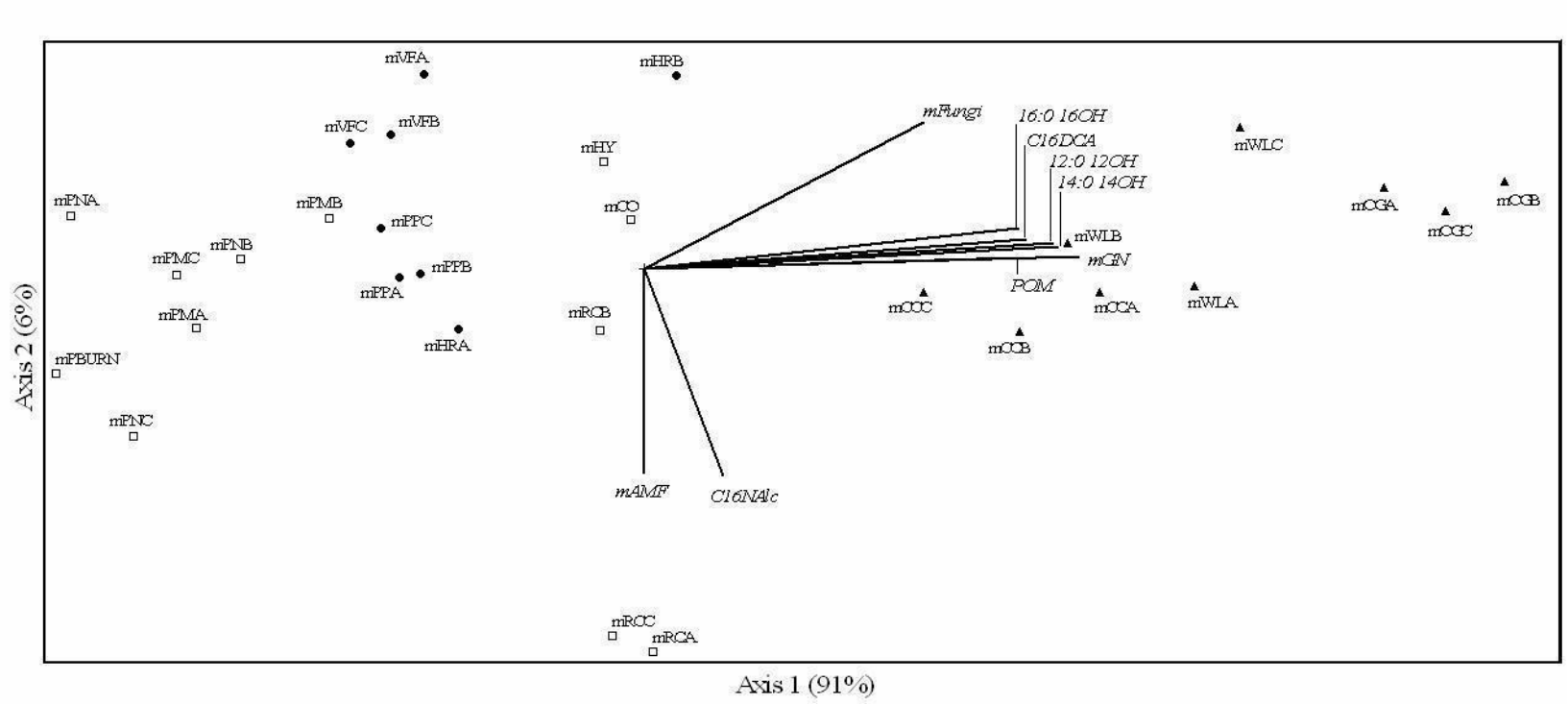

Figure 1.3. NMS representation of sample distances according the MIDI-FA composition (pmol FAME $pmol^{-1}$ 16:0). Open squares, filled circles and filled triangles represent samples collected at sites with no vegetation, under herbaceous and forest vegetation at sampling, respectively. Vectors indicate direction an intensity of compositional microbial signature FAMEs and plant-derived FAMEs gradients. mFungi, mGN, and mAMF stand for MIDI (whole-cell lipid) derived FAME biomarkers for fungi, gram-negative bacteria, and arbuscular mycorrhizal fungi.

(14:0 14OH, 16:0 16OH and 16:0 DCA). These observations suggest that, despite the similarity between the two methods for sample ordination along the axes 1, FAME microbial signatures were less important for the ordination output when MIDI rather than PLFA was used to evaluate microbial structure. Along axis 2 dissimilarities in sample distances ($score_{MIDI} = score_{PLFA}$ x 0.127 (R^2= 0.08)) as well as in the FAME gradients were more apparent between the two methods. Distribution of samples along axis 2 was associated with changes in AMF, fungi and 16:0 alcohol in MIDI ordination and, with ACT, GN, AMF and 16:0 16OH in PLFA ordination. As stated before, abundance of 16:0 16OH in PLFA extracts was negligible compared to those of microbial signatures. Comparison between FAME datasets with and without 16:0 16OH has presented a standardized Mantel statistics (r) (Sokal, 1979) greater than 0.999, indicating that this plant-specific FAME, although correlated with the gradient long axis 2, was negligibly influential for sample separation (Figure 1.2).

A standardized Mantel statistics (r) of 0.63 ($p<0.0001$) was found between the sample distance matrices from the two methods, indicating a good overall agreement between them. However, as the FAME variables determining the distribution of samples across the ordination plots were different for PLFA and MIDI, we hypothesize that a significant portion of that similarity on sample distances based on FAME composition between the methods was driven by similar conditions determining both microbial structure and accumulation of plant biomarkers in soil.

In order to verify the validity of this hypothesis, we performed a simple linear regression analysis between sample subset dissimilarities based on microbial group (MG profile) or plant-associated peaks (PP profile) against sample subset dissimilarities based on overall peak composition for MIDI and PLFA (overall profile) (Figure 1.4). This analysis allowed us to separate, in a quantitative way, the contribution of the microbial signature and plant-associated

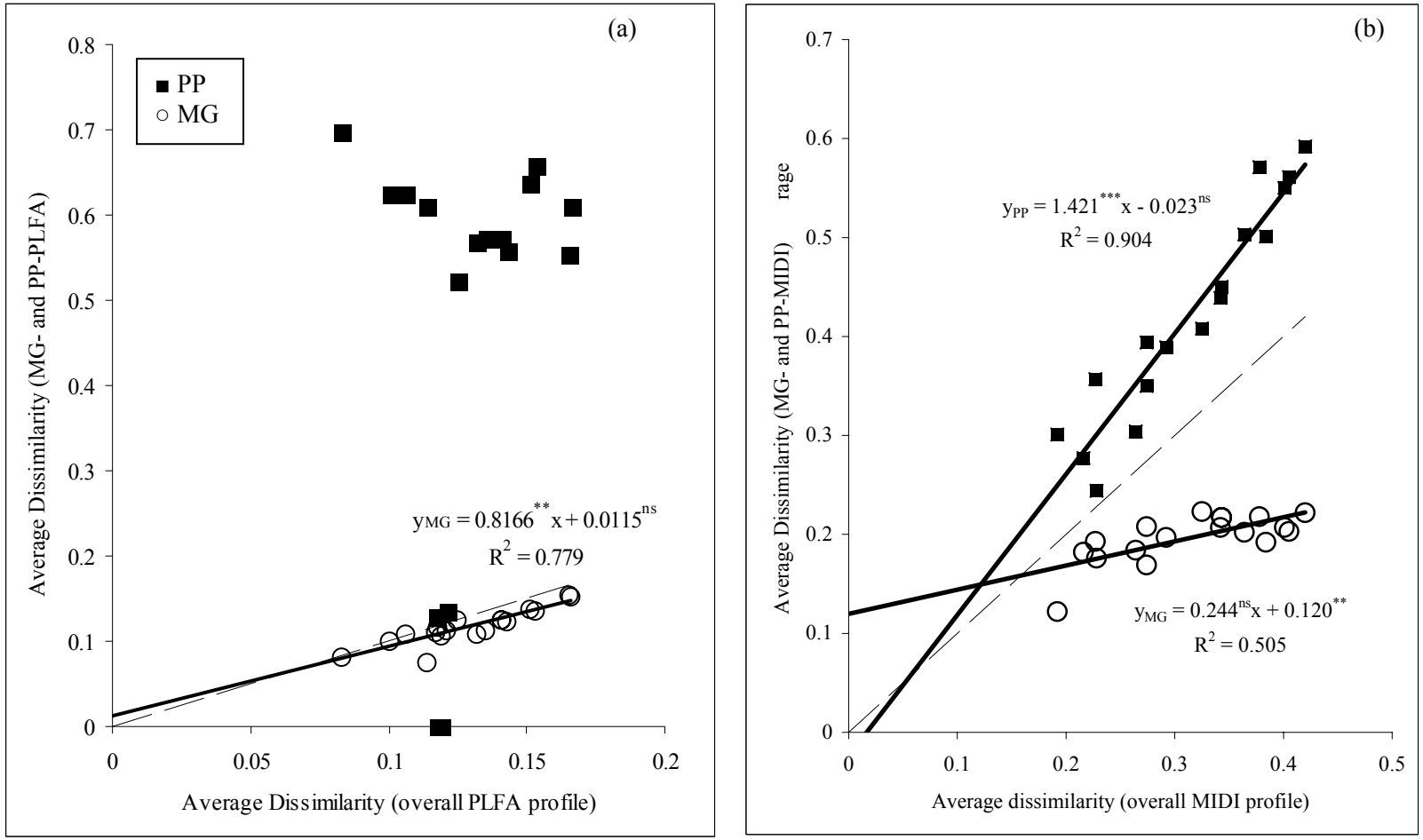

Figure 1.4. Relationship between within-set average sample Sørensen dissimilarities based on both microbial group signature profile (MG) and plant FAME signature profile (PP) vs. overall FAME profile, for PLFA (a) and MIDI (b). Slope of the dashed lines, in both figures, equals 1.

peak diversities to the overall PLFA or MIDI peak diversity. We considered that the closer the slope of the regression is from one, the more important a set of peaks (MG vs. PP) would be in determining the similarity between samples based on the overall FAME profile. For example, the average within-subset dissimilarities based on PLFA microbial group signature profiles (MG) and PLFA overall profile were strongly associated (Figure 1.4.a). Each unit change in the dissimilarity of overall peaks was associated with a change of about 0.82 in the dissimilarity of microbial signatures. However, when the same comparison was made for MIDI data, a weak association between subset average dissimilarities based on MG profile and overall MIDI profile was found (Figure 1.4.b). On the other hand, changes in the composition of plant-associated MIDI-FAs (PP profile) were highly associated to those in overall MIDI-FAs. One unit change in the dissimilarity of overall MIDI peak composition corresponded to a change of 1.4 units for the selected PP composition (Figure 1.4.b).

These results showed that sample distances based on overall MIDI-FAs were more in synchrony with plant-derived compounds than with microbial signatures, precluding the use of MIDI as a tool to infer about microbial structure in this dataset. Subsets used in this analysis included samples with varying degrees of heterogeneity regarding vegetation cover and SOM. Therefore, the low synchrony between MIDI overall profile and microbial FAME signatures was observed even considering subsets including samples where plant peaks were not very abundant. This lack of association may be related not only to the presence of plant-peaks, but also to the differential influence that MIDI extraction exerts on FAME signatures from distinct microbial groups (Table 1.5).

Association between TOC and recovery of FAMEs extracted by MIDI

In addition to the high abundance of some non-microbial peaks in many of our soil samples, we also noticed that the overall FAME recovery ratio between MIDI and PLFA (MIDI:PLFA) was highly variable across our set of samples. Lowest ratios were observed for soils under forest and with high TOC. To ascertain the soil sample conditions associated with this variation, the average of MIDI:PLFA ratios for pooled microbial signature FAMEs and 16:0 were modeled as a function of POM, TOC, and vegetation type. Regardless of the pooled microbial signatures evaluated, vegetation type and POM had no significant effect ($p>0.05$) on MIDI:PLFA ratios. Therefore, we concluded that, among the factors tested in the initial model, only TOC was strongly associated with changes in the MIDI:PLFA ratio, and choose to excluded vegetation type and POM variables as explanatory variables from the final model. Fitting curves of MIDI:PLFA ratio from different microbial signatures as a function of SOM are presented in Figure 1.5.

Interpretation of the intercepts shows that FAME signatures for eukaryotic organisms (fungi, AMF and microeukaryotes) and GP were increased by MIDI compared to PLFA; while GN and actinomycetes signatures were negatively affected by it (Figure 1.5). For eukaryotes, it seems likely that microbial storage lipids and plant-derived lipids would account for this difference (Macalady et al., 1998; Petersen et al., 2002). We compared these results to those found by Peterson et al. (2002) from soils with about 3% TOC. MIDI:PLFA recovery rates of 3.6 and 4.0 were found by those authors for 18:2ω6c and 16:1ω5c. These FAMEs were used in our paper as the only markers for fungi and AMF, respectively (Table 1.3), being thus directly comparable with their results. Models fitted for those two microbial groups showed that at 3% of TOC the MIDI:PLFA ratio was very similar to those found by Peterson et al.

(2002). However, our results showed a strong negative relationship between this ratio and TOC. The MIDI:PLFA ratio for fungi and AMF, for instance, would be <1 at TOC content of approximately 5 and 7%, respectively, while reaching much higher values in soils with low TOC. Based on the larger MIDI:PLFA ratios obtained in their work, Drenovsky et al. (2004) suggest that MIDI would be advantageous relative to PLFA when sample size is limiting, as it

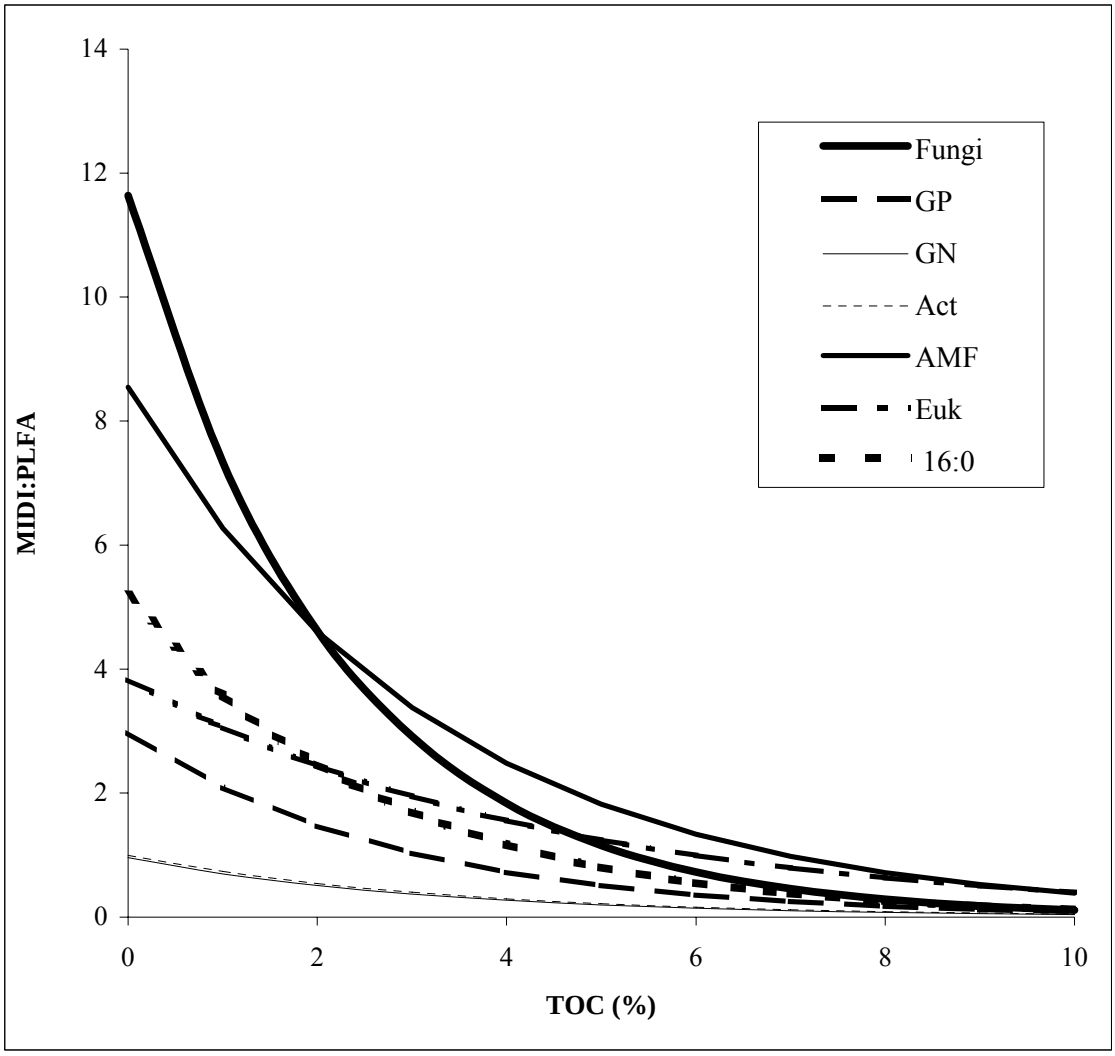

Figure 1.5. MIDI:PLFA ratios based on the absolute amounts of FAMEs as a function of soil TOC.

requires a smaller amount of soil to maintain a reliable microbial community fingerprint. According to our results, this alleged advantage would depend on the TOC in soil samples.

Regardless the TOC content, our models indicated that MIDI:PLFA ratio for GN and ACT were never greater than 1. Low recovery of the signature FAMEs for these two microbial groups by MIDI in comparison to PLFA was also shown previously (Drenovsky et al., 2004). FAMEs used in our paper as GN signatures were also found to have the smallest MIDI:PLFA ratio among 22 FAMEs analyzed by Petersen et al. (2002). However, the MIDI:PLFA ratio reported by those authors were always above 1. Regarding cyclopropane FAMEs, it has been shown that chemical integrity of these compounds is unpredictably affected at acidic conditions, especially in the presence of methanol (Vulliet et al., 1974). This condition is met in the methylation step used in MIDI extraction and would explain the low recovery of these FAMEs by this method.

Effect of the ratio soil: extractant on the MIDI-FA recovery

MIDI procedure was originally developed to produce FAMEs from bacterial cells aiming identification of strains based on their lipid profiles. The proportions of reagents used to extract fatty acids from 1 g of soil in the adapted method to profile microbial structure (Cavigelli et al., 1995; Drenovsky et al., 2004; Ibekwe and Kennedy, 1999; Petersen et al., 2002; Schutter and Dick, 2000); are the same proposed for 40 mg of wet bacterial cells (Paisley, 2002), according to the original purpose of MIDI. Considering that several components of SOM other than microbial lipids can also react with the reagents used in MIDI extraction, we hypothesized that the amount of TOC in samples would limit the extraction in soils with high SOM content. Three different amounts of soil (3 g, 1 g and, 0.3 g) from the OGc (8% TOC) sample were used for the extraction, keeping the amounts of reagents constant as described in the methods (n=2). In this

range of soil masses, TOC in the extraction mixture varied from 24 to 240 mg. Amounts recovered were calculated as pmol FAME g^{-1} dry soil. For each pooled FAME microbial signatures and for 16:0, these amounts were normalized to the maximum amount recovered, which was always in a replicate containing 0.3 g of soil. We found a sharp linear decrease in the recovery of all markers with increasing TOC in the extraction mixture (Table 1.6). Because we varied the amounts of soil rather than that of any specific reagents on the extraction mixtures, no conclusions can be drawn about which reagent would limit the extraction at high TOC contents. However, after each step in the MIDI extraction, aliquots from the aqueous phase of the mixtures were diluted in distilled water and the pH determined. We found no strong change in the pH of the mixtures during the saponification or methylation across different soil amount treatments (data not shown). Therefore, we suggest that likely methanol, rather than NaOH or HCl, would be likely limiting this reaction.

Table 1.6. Regression between relative recovery of microbial FAMEs by MIDI and TOC.

FAME	Equations	R^2
Fungi	$y^{\dagger} = 1.0814 - 0.00154^{***} \times TOC^{\ddagger}$	0.99
GP	$y = 1.0452 - 0.00152^{***} \times TOC$	0.99
GN	$y = 1.0070 - 0.00151^{***} \times TOC$	0.97
ACT	$y = 0.9216 - 0.00148^{**} \times TOC$	0.87
AMF	$y = 0.9287 - 0.00121^{**} \times TOC$	0.86
EUK	$y = 1.0865 - 0.00159^{***} \times TOC$	0.99
16:0	$y = 1.0079 - 0.00153^{***} \times TOC$	0.98

† Replicate value with the largest absolute amount of FAME recovered (pmol g^{-1} soil) equals 1

‡ TOC expressed in mg of soil C in the extraction mixture.

Conclusions

The diversity of overall MIDI-FA were highly associated with the diversity of the plant-derived peaks rather than with that of microbial signature FAMEs. We concluded that MIDI

method should be used with caution while interpreting changes in FA profiles as being due to shifts in microbial communities.

FAME recovery efficiency by MIDI is highly affected by the ratio soil : extractant. We suggested that consumption of reagents by the high amount of soil organic compounds other than aliphatic compounds of interest in FAME analysis might be related to this decreased recovery.

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References

Almendros, G., and J. Sanz. 1991. Structural study on the soil humin fraction-boron trifluoride-methanol transesterification of soil humin preparations. Soil Biology and Biochemistry 23:1147-1154.

Ambles, A., P. Jambu, E. Parlanti, J. Joffre, and C. Riffe. 1994. Incorporation of natural monoacids from plant residues into an hydromorphic forest podzol. European Journal of Soil Science 45:175-182.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

Bossio, D.A., K.M. Scow, N. Gunapala, and K.J. Graham. 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36:1-12.

Butler, J.L., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69:6793-6800.

Buyer, J.S., and D.D. Kaufman. 1997. Microbial diversity in the rhizosphere of corn grown under conventional and low-input systems. Applied Soil Ecology 5:21-27.

Cambardela, C.A., and E.T. Elliott. 1993. Carbon and nitrogen distribution in aggregates from cultivated and native grassland soils. Soil Science Society of America Journal 57:1071-1076.

Cavigelli, M.A., G.P. Robertson, and M.J. Klug. 1995. Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. Plant and Soil 170:99-113.

Conde, E., M.C. Garcia-Vallejo, and E. Cadahia. 1999. Waxes composition of *Quercus suber* reproduction cork from different Spanish provenances. Wood Science and Technology 33:271-283.

Dailey, O.D., R.F. Severson, and R.F. Arrendale. 1989. Nonpolar lipids of *Amaranthus palmeri* S. Wats. 1. Fatty alcohols and wax esters (saturated). Journal of Agricultural and Food Chemistry 37:1317-1321.

del Rio, J.C., and P.G. Hatcher. 1998. Analysis of aliphatic biopolymers using thermochemolysis with tetramethylammonium hydroxide (TMAH) and gas chromatography-mass spectrometry. Organic Geochemistry 29:1441-1451.

Drenovsky, R.E., G.N. Elliott, K.J. Graham, and K.M. Scow. 2004. Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. Soil Biology and Biochemistry 36:1793-1800.

Drijber, R.A., J.W. Doran, A.M. Parkhurst, and D.J. Lyon. 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biology and Biochemistry 32:1419-1430.

Dufrene, M., and P. Legendre. 1997. Species assemblages and indicator species: the need for a flexible assymetric approach. Ecological Monographs 67:345-366.

Frostegård, A., and E. Bååth. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22:59-65.

Haack, S.K., H. Garchow, D.A. Odelson, L.J. Forney, and M.J. Klug. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Applied and Environmental Microbiology 60:2483-2493.

Hackl, E., M. Pfeffer, C. Donat, G. Bachmann, and S. Zechmeister-Boltenstern. 2005. Composition of the microbial communities in the mineral soil under different types of natural forest. Soil Biology and Biochemistry 37:661-671.

Harwood, J. L., and N. J. Russell. 1984. Lipids in plants and microbes. Allen and Unwin, London.

Holloway, P.J. 1972a. The composition of suberin from the corks of *Quercus suber* L. and *Betula pendula* Roth. Chemistry and Physics of Lipids 9:158-170.

Holloway, P.J. 1972b. The suberin composition of the cork layers from some Ribes species. Chemistry and Physics of Lipids 9:171-179.

Hunneman, D.H., and G. Eglinton. 1972. The constituent acids of gymnosperm cutins. Phytochemistry 11:1989-2001.

Ibekwe, A.M., and A.C. Kennedy. 1999. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant and Soil 206:151-161.

Jackson, L.E., F.J. Calderon, K.L. Steenwerth, K.M. Scow, and D.E. Rolston. 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114:305-317.

Jambu, P., A. Ambles, J.C. Jacquesy, B. Secouet, and E. Parlanti. 1993. Incorporation of natural alcohols from plant residues in to a hydromorphic forest-podzol. Journal of Soil Science 44:135-146.

Jandl, G., P. Leinweber, H-R. Schulten, and K. Ekschmitt. 2005. Contribution of primary organic matter to the fatty acid pool in agricultural soils. Soil Biology and Biochemistry 37:1033-1041.

King, J.D., D.C. White, and C.W. Taylor. 1977. Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora. Applied and Environmental Microbiology 33:1177-1183.

Kolattukudy, P.E. 1970. Reduction of fatty acids to alcohols by cell-free preparations of *Euglena gracilis.* Biochemistry 9:1095-1102.

Kolattukudy, P.E. 1980. Biopolyester membranes of plants: cutin and suberin. Science 208:990-1000.

Kolattukudy, P.E., and V.P. Agrawal. 1974. Structure and composition of aliphatic constituents of potato tuber skin (suberin). Lipids 9:682-691.

Kroppenstedt, R. M. 1992. The genus Nocardiopsis, p. 1139-1156. In A. Balows, H. G. Trüpper, D. M., W. Harder, and K. H. Schleifer (ed.), The prokaryotes, vol. 2. Springer, Berlin.

Loveland, P.M., and M.L. Laver. 1972. Monocarboxylic and dicarboxylic acids from *Pseutotsuga menziessi* bark. Phytochemistry 11:430-432.

Macalady, J.L., M.E. Fuller, and K.M. Scow. 1998. Effects of metam sodium fumigation on soil microbial activity and community structure. Journal of Environmental Quality 27:54-63.

Matzke, K., and M. Riederer. 1991. A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst., *Quercus robur* L., and *Fagus sylvatica* L. Planta 185:233-245.

Mayberry, W.R., P.F. Smith, T.A. Langworthy, and P. Plackett. 1973. Identification of the amide-linked fatty acids of *Acholeplasma axanthum* S743 as D-(-)-3-hydroxyhexadecanoate and its homologues. Journal of Bacteriology 116:1091-1095.

McCune, B., and J.B. Grace. 2002. Analysis of Ecological Communities MjM Software Design, Gleneden Beach, OR.

Nierop, K.G.J. 1998. Origin of aliphatic compounds in a forest soil. Organic Geochemistry 29:1009-1016.

Nierop, K.G.J., M.M. Pulleman, and J.C.Y. Marinissen. 2001. Management induced organic matter differentiation in grassland and arable soil: a study using pyrolysis techniques. Soil Biology and Biochemistry 33:755-764.

O'Leary, W. M., and S. G. Wilkinson. 1988. Gram-positive bacteria, p. 117-201. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press, San Diego.

Olsson, P. A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303-310.

Olsson, P. A., E. Bååth, I. Jakobsen, and B. Söderström. 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99:623-629.

Paisley, R. 2002. Training Manual. MIS wole cell fatty acid analysis by gas chromatography MIDI, Inc, Newark, Del.

Petersen, S.O., P.S. Frohne, and A.C. Kennedy. 2002. Dynamics of a soil microbial community under spring wheat. Soil Science Society of America Journal 66:826-833.

Rosenberg, W., K.G.J. Nierop, H. Knicker, P.A. de Jager, K. Kreutzer, and T. Weiss. 2003. Liming effects on the chemical composition of the organic surface layer of a mature Norway spruce stand (*Picea abies* [L.] Karst.). Soil Biology and Biochemistry 35:155-165.

Rovira, A.D., and E.L. Greacen. 1957. The effect of aggregate disruption on the activity of microorganisms in the soil. Australian Journal of Agricultural Research 8:659-673.

Salinas-Garcia, J.R., F.M. Hons, and J.E. Matocha. 1997. Long-term effects of tillage and fertilization on soil organic matter dynamics. Soil Science Society of America Journal 61:152-159.

Schulten, H.R., and P. Leinweber. 1993. Pyrolysis-field ionization mass spectrometry of agricultural soils and humic substances: effect of cropping systems and influence of the mineral matrix. Plant and Soil 151:77-90.

Schutter, M.E., and R.P. Dick. 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Science Society of America Journal 64:1659-1668.

Schutter, M.E., and R.P. Dick. 2002. Microbial community profiles and activities among aggregates of winter fallow and cover-cropped soil. Soil Science Society of America Journal 66:142-153.

Six, J., K. Paustian, E.T. Elliott, and C. Combrink. 2000. Soil structure and organic matter: I. Distribution of aggregate-size classes and aggregate-associated carbon. Soil Science Society of America Journal 64:681-689.

Sokal, R. R. 1979. Testing statistical significance of geographic variation patterns. Systematic Zoology 28:627-632.

Tunlid, A., and D.C. White. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil, p. 229-262, *In* G. Stotzky and J. M. Bollag, eds. Soil Biochemistry, Vol. 7. Marcel Dekker, New York.

van Aarle, I. M., and P. A. Olsson. 2003. Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Applied and Environmental Microbiology 69:6762-6767.

Vestal, J. R., and D. C. White. 1989. Lipid analysis in microbial ecology. Quantitative approaches to the study of microbial communities. Bioscience 39:535-541.

von Rudloff, E. 1959. The wax of the leaves of *Picea pungens* (Colorado spruce). Canadian Journal of Chemistry 37:1038-1042.

Vulliet, P., S.P. Markey, and T.G. Tornabene. 1974. Identification of methoxyesters artifacts produced by methanolic-HCl solvolysis of the cyclopropane fatty acids of the genus *Yersinia*. Biochimica et Biophysica Acta 348:299-301.

Wilkinson, S. G. 1988. Gram-negative bacteria, p. 299-457. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press, San Diego.

Zeier, J., and L. Schreiber. 1998. Comparative investigation of primary and tertiary endodermal cell walls isolated from the roots of five monocotyledoneous species: chemical composition in relation to fine structure. Planta 206:349-361.

Zeier, J., L. Schreiber, U. Ryser, and K. Ruel. 1999. Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. Planta 209:1-12.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29:111-129.

Zelles, L., Q.Y. Bai, T. Beck, and F. Beese. 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24:317-323.

Chapter 2

Comparison of Soil Microbial Community Profiling with PLFA and EL-FAME Methods

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Abstract

The common procedure for fatty acid (FA) profiling of soil microbial structure is the extraction and analysis of phospholipids (PLFA). It is preferred because it extracts only viable FAs and eliminates storage FAs. Its drawbacks are labor-intensiveness and required use of expensive silica columns. In contrast, direct extraction of ester-linked fatty acid methyl esters (EL-FAME) is rapid and has lower costs but may have interferences from plant polymers, neutral lipids (NL), and glycolipids (GL). The objective was to determine whether major plant polymers and storage FAs would impact EL-FAME-based microbial profiling compared to that obtained by PLFA. Twenty-nine samples from a variety of soil and climatic conditions were collected across Oregon and extracted by the two methods. Small amounts of two FAs specific of plant polymers were detected by EL-FAME, mainly from forest sites, which did affect the microbial community structure interpretations. Multiple regression analysis showed that the amount of various microbial FAMEs was a function of extraction method and ecological properties such as particulate organic matter (POM), pH, and dominant standing vegetation. This analysis revealed that EL-FAME:PLFA ratios were narrower for prokaryotic than for eukaryotic FAMEs; and that standing vegetation was related to differences for fungi (18:1ω2c) and AMF (16:1ω5c) between extraction methods. These discrepancies appeared to be associated with NL and GL FAMEs that can be extracted in EL-FAME but not PLFA analysis. Non-metric multidimentional analysis revealed that EL-FAME-based community structure was clearly affected by these discrepancies when compared to PLFA. Major distortions would be reduced when samples from sites under the same vegetation are analyzed. Even for these homogeneous vegetation sample sets, a more eukaryotic-driven community structure was found for EL-FAME than for PLFA. However, across samples, relative variation in 16:0, prokaryotic FAMEs and

total FAMEs was similar between the two methods, which suggests that EL-FAME would equal PLFA as a tool for measuring the relative changes in total microbial biomass, or in these specific microbial groups' biomasses.

Introduction

The composition of fatty acid methyl esters (FAMEs) from samples has been used to track shifts in microbial diversity in soils under different management and vegetation (Bossio and Scow, 1998; Drijber et al., 2000; Hackl et al., 2005; Petersen et al., 2002; Schutter and Dick, 2002). Many FAMEs have been identified as signatures for different groups of microorganisms (Tunlid and White, 1992) and may be used to characterize those shifts.

The method of FAME extraction may impact the sources and amounts of these compounds. Phospholipid fatty acid (PLFA) analysis is thought to be the most reliable method for FAME profiling because it represents the viable microbial biomass, as phospholipids are rapidly converted into neutral lipids upon microbial death (King et al., 1977). This method includes the extraction of whole soil lipids using Bligh and Dyer (1959) extractant, the separation of phospholipids (PL) from neutral lipids (NL) and glycolipids (GL) by solid phase extraction in silica columns, and the production of FAMEs from the PL fraction by mild-alkalyne methylation. This methylation step is considered to be effective in producing FAMEs from fatty acids that are ester-linked (EL) to the complex lipids (Zelles, 1999).

The PLFA method is labor-intensive and consequently alternative methods that involve a direct FAME extraction have been developed. The total ester-linked FAMEs (EL-FAME) achieves this goal with a direct mild-alkaline methylation step on soil samples, without

separating the other lipid fractions from phospholipids. Bypassing both the extraction and the fractionation of the whole lipids makes EL-FAME an easier and much less time-consuming method, substantially increasing sample output. A potential limitation is that fatty acids from GL and NL are extracted along with those from PL. Nonetheless, EL-FAME has been equal to PLFA (Drijber et al., 2000) and LH-PCR (Ritchie et al., 2000) methods for discriminating soil management and environmental effects for microbial communities.

Co-extraction of FAMEs from NL is of major concern, as these lipids may be derived from storage products and from dead microbial biomass. Bacteria have minimal storage lipids, which likely increases the similarity between EL-FAME and PLFA methods for representing bacterial FAMEs. Eukaryotes can have significant levels of storage lipids (Bååth, 2003; Olsson et al., 1995) which may complicate EL-FAME interpretation. This was shown by Bååth (2003) who evaluated the ratio between NL fatty acids (NLFAs) and PLFAs in soils amended with glucose only (C-excess) and with glucose + N +P (no C-excess). Under excess C, this ratio was increased significantly for eukaryotes but not for bacterial FAMEs. In addition to lipids from microbial sources, those derived mainly from particulate plant residues and more humified SOM fractions could potentially contribute to the FAME profile obtained by EL-FAME and affect community interpretation. In fact, it was recently reported that dissimilarities in sample FAME compositions by MIDI, another direct extraction method, were more associated with the diversity of peaks of plant origin rather than by microbial signature FAMEs (Chapter 1).

The objectives of this study were: (a) to compare the ability of EL-FAME and PLFA to extract FAMEs associated with major plant polymers, like cutin and suberin; (b) to verify, by the use of multiple regression technique, if relative changes in the amounts of several EL-FAMEs and PLFAs are similarly associated with changes in environmental variables; (c) to investigate how eventual method-related discrepancies in these associations would affect the

sample distances and the interpretation of microbial structure determined by both methods; and (d) to investigate the potential effects that FAMEs derived from NL and GL fractions would have in the interpretation of EL-FAME-based community structures compared to that obtained by PLFA.

Material and Methods

Soil sampling

Twenty-nine composite soil samples (0-10 cm) were collected from six soil series of varying texture, total organic carbon (TOC), and site history across Oregon from September to October 2003 (Table 2.1). When more than one sample was collected under the same site, the distance between subsampling points was >30 m apart. Field-moist soil samples were passed through a 2-mm opening sieve and stored at 4^{o}C for no longer than 3 days before lipid extraction.

Fatty acid methyl ester extraction and analysis

A comparison of the major steps between the two methods is summarized in Table 2.2.

Extractions of FAMEs from soil samples were done in triplicate. Three analytical controls containing only the reagents but no soil were made for each extraction batch and protocol. For the EL-FAME procedure (Schutter and Dick, 2000), 3 g of soil (wet weight) were placed in 35-ml Teflon™-lined, screw-cap glass centrifuge tubes and mixed with 15 mL of 0.2 *M* KOH in methanol. Soils were incubated for 1 h at 37^{o}C with periodic vortexing. Next, 3 mL of 1.0 *M* acetic acid were added to neutralize the pH of the tube contents. Extracted FAMEs were

Table 2.1. Taxonomy and selected soil properties of sites included in this study.

Sites[†]	Soil Type	TOC	POM C[‡]	nPOM C[‡]	MB C[‡]	pH	Standing vegetation	Site History	Location
		--------- g kg^{-1} dry soil ----------							
CO (1)	Dayton silt loam Vertic Albaqualf	14	3.1	11.1	0.52	5.4	no	grass field, plowed before sampling	Linn County
HY (1)	Woodburn silt loam Aquultic Argixeroll	15	0.5	15.3	0.21	5.5	no	summer vegetable/ winter fallow	Corvallis
VF (3)	Chehalis silt loam Cumulic Ultic Haploxeroll	12	1.1	10.9	0.30	6.1	corn, grasses	summer vegetable/ winter fallow	Corvallis
RC (3)	Saturn Variant silt loam Fluventic Halumbrept	26	2.5	24.0	0.80	6.1	no	summer vegetable-winter or annual winter cropping	Scio
Pburn (1)	Walla Walla silt loam Typic Haploxeroll	9	1.7	8.2	0.19	6.5	no	winter wheat / summer fallow, burning	Pendleton
PM (3)	Walla Walla silt loam Typic Haploxeroll	11	2.8	8.6	0.45	7.0	no	winter wheat / summer fallow, manure (22 Mg ha^{-1} $2y^{-1}$)	Pendleton
PN (3)	Walla Walla silt loam Typic Haploxeroll	10	2.1	8.4	0.33	5.7	no	winter wheat / summer fallow, inorganic N	Pendleton
PP (3)	Walla Walla silt loam Typic Haploxeroll	17	5.7	11.7	0.93	7.1	pasture	pasture (>70 y)	Pendleton
HR (2)	Parkdale loam Humic Vitrixerands	11	3.2	8.6	0.44	5.7	apple trees, grasses	orchard (a) inter-; (b) in-row	Hood River
CC (3)	McKenzie River fine-loamy Typic Haplumbrept	36	17.9	18.9	0.87	5.9	Douglas fir forest, shrubs	clear cut and replanting (~10y)	Blue River
OG (3)	McKenzie River fine-loamy Typic Haplumbrept	42	27.0	15.3	1.80	5.6	Douglas fir	Old growth forest	Blue River
WL (3)	Saturn Variant silt loam Fluventic Halumbrept	29	12.6	17.1	1.20	5.6	Mixed Forest	Forest (>50 y)	Scio

[†] Values for VF, RC, PM, PN, HR, PP, CC, OG, WL sites are averages from sites under the same ecosystem. Numbers between parentheses represent the number of sites collected. Lower-case letters after sites names in this paper represent subsamplings.
[‡] POMC, nPOMC, and MBC refer to C in particulate and non-particulate organic matter, and microbial biomass, respectively.

Table 2.2. Comparison of the major steps for FAMEs production from soil samples according to PLFA, and EL-FAME procedures.

Step	PLFA[†]	EL-FAME	Comments
Whole lipid extraction from soil	Bligh and Dyer (1959)		
Fractionation of lipid extract in SPE silica columns	NL (chloroform), GL (acetone), and PL (methanol)		
FAME production	Mild alkaline methanolysis of individual fractions (0.2 *M* KOH in methanol)	Mild alkaline methanolysis direct from soil samples (0.2 *M* KOH in methanol)	Mild alkaline methanolysis is especially efficient in extracting ester-linked fatty acids; free fatty acids are not methylated (Zelles, 1999)
Partition of aqueous and organic phases, and FAME recovery	Hexane. Dry organic phase under N_2 stream.	Hexane. Dry organic phase under N_2 stream.	The dried fractions from all methods, which contain the FAMEs, were ressupended with hexane to a known volume.

[†] In this paper, FAMEs were produced from the three lipid factions (neutral lipids, NL; glycolipids, GL; and phospholipids, PL) after their separation in silica columns.

partitioned into an organic phase by adding 10 mL of hexane. Tubes then were centrifuged for 20 min at 500x *g* to separate organic matter from the hexane. The hexane layer was transferred to a clean glass tube with two additional 5 mL of hexane recoveries of the organic phase. The pooled hexane volumes were dried under a stream of N_2. FAMEs were resuspended in a known volume of hexane and transferred to a glass insert placed inside a 2-mL amber GC vial.

For PLFA, samples were extracted with a one-phase solvent extractant according to the procedure of Bligh and Dyer (1959). Briefly, sufficient potassium phosphate buffer (100 m*M*, pH 7) and water were added to 3 g of wet soil to achieve a final concentration of 50 m*M*

phosphate and mixed with chloroform and methanol at a ratio of 0.8:1:2 in 35-mL Teflon™-lined, screw-cap glass centrifuge tubes. This mixture was shaken for 2 h and allowed to stand overnight. Lipids were extracted the following day by centrifuging (400 x *g* for 12 min) and filtering the supernatant through Whatman no. 1 filter papers. This step was repeated twice after the remaining soil was resuspended with additional chloroform:methanol (same 1:2 ratio). Following filtration, 10 mL of 2 *M* NaCl was added to the chloroform-methanol-buffer solution in order to separate the phases (Folch et al., 1957). The bottom phase (chloroform) was removed and immediately dried under a stream of N_2. Dried lipid extracts were redissolved in chloroform and separated into neutral lipids (NL), glycolipids (GL), and phospholipids (PL) on solid-phase extraction columns containing 500 mg of silica (Supelco, Inc., Bellefonte, PA) with chloroform, acetone, and methanol, respectively (White and Ringelberg, 1998). All the fractions were immediately dried under N_2 atmosphere. Dried lipids in each one of these fractions were converted to fatty acid methyl esters (FAMEs) through mild-alkaline methanolysis by dissolving in 1 ml of 1:1 methanol:toluene and 1 mL of 0.2 *M* KOH and heating for 15 min in a water bath at 40^{o}C (White and Ringelberg, 1998). FAMEs were extracted by adding 2 mL of deionized water, 0.3 mL of 1 *M* acetic acid, and 0.5 mL of hexane (Bossio and Scow, 1998). The mixture was vigorously vortexed for 1 min and allowed to stand for 10 min. The hexane phase was transferred to a fresh test tube. The addition and recovery of hexane was repeated two times more and the volumes were pooled together with the hexane collected in the first recovery round. The pooled volume of hexane was dried under a stream of N_2. Dried samples were redissolved in a known volume of hexane and transferred to a glass insert placed inside a 2-mL amber GC vial.

FAME composition in the extracts was determined with an Agilent 6890 gas chromatograph (Agilent Inc., Palo Alto, CA) equipped with a 25–m HP Ultra-2 column

(internal diameter, 0.2 mm; film thickness, 0.33 μm) and a flame ionization detector (FID-GC). The temperature program ramped from 170 to 280^{o}C at 4^{o}C per min, with 5 min at 280^{o}C between samples to clean the column. Peaks were identified and quantified as outlined in Chapter 1.

FAME extracts produced by both methods were analyzed by GC-MS to identify major peaks to which no name was assigned by the procedures described above (Chapter 1).

The summed masses of FAMEs (pmol FAME g^{-1} soil) reported as typical of fungi, Gram-negative (GN) bacteria, Gram-positive (GP) bacteria, actinomycetes (ACT), arbuscular mycorrhyzal fungi (AMF) and microeukariotes (EUK) were used as signatures for these microbial groups (Table 2.3). FAME 18:1ω9c has also been used, in addition to 18:2ω6c, as a fungal marker by many authors (Bååth, 2003; Waldrop and Firestone, 2004). However, unless otherwise mentioned, the use of the term "fungi" in this paper refers specifically to FAME 18:2ω6c, which has been used classically to describe this group of organisms (Frostegård and Bååth, 1996; Bossio and Scow, 1998; Griffiths et al., 1999; Bååth, 2003; Bååth et al., 2004; Waldrop and Firestone, 2004). The term "eukaryotic FAMEs" was used in this paper to encompass a group of FAMEs formed by fungi, AMF and EUK signatures, rather than as a synonymous term for EUK only.

Soil carbon pools and pH

POM was obtained as described in Chapter 1. Particulate organic matter carbon (POM-C) was calculated assuming a 50% of C in this fraction. Non-particulate organic matter C (nPOM-C) was estimated by subtracting the POM-C from the soil TOC determined by gravimetry after

Table 2.3. FAMEs used in this study as biomarkers for different groups of organisms.

Microbial Groups	FAME Markers	Comments	References
Fungi	18:2ω6c	Good correspondence with ergosterol, another fungi marker; also present as storage products in fungi	Federle, 1986; Frostegård and Bååth, 1996; Olsson, 1999
Gram+ bacteria (GP)	15:0i; 15:0a; 16:0i; 17:0i; 17:0a		O'Leary and Wilkinson, 1988
Gram- bacteria (GN)	18:1ω7c; 17:0cy; 19:0cy		Wilkinson, 1988
Actinomycetes (ACT)	16:0 10-Me; 17:0 10-Me; 18:0 10-Me		Kroppenstedt, 1992
Arbuscular mycorrhyzal fungi (AMF)	16:1ω5c	Also present in some bacteria as *Cytophaga* / *Flexibacter*; also a major source of storage (neutral lipids) in AMF spores and vesicles.	Olsson et al., 1995; van Aarle and Olsson, 2003
Microeukaryotes (EUK)	20:4ω6c	Generally used as a protozoan marker, but also present in nematodes, fungi and algae.	Vestal and White, 1989; Harwood and Russell, 1984

overnight dry combustion (500^{o}C) of soil organic matter (SOM). A 50% C content also was assumed for SOM to estimate TOC.

Microbial biomass-C was determined by the chloroform fumigation-incubation method (Jenkinson and Powlson, 1976a, 1976b). After fumigation, samples were incubated for 10 days at 25^{o}C and the CO_2 evolved during this period was determined by gas chromatography. To reduce the interference of soil sieving and rewetting on the background flush of CO_2 from soils, the amount of CO_2 evolved from unfumigated samples between 10-20 days of incubation was used as the control (Jenkinson and Powlson, 1976b). A *k*c of 0.45 was used to calculate MB-C (Jenkinson, 1988). Details for this determination are described elsewhere by Schutter and Dick (2002). The pH was determined in soil:water (1:2) suspensions.

Statistical analysis

Screening FAs and Indicator Species Analysis

Only peaks in the region between tetradecanoic methyl ester (14:0) and arachidonic acid (20:4ω6c) were included in this analysis of FA from both extractions. This range includes the main ester-linked FAMEs used as microbial biomarkers and at the same time excludes several shorter- and longer-chain FAMEs reportedly associated to plants and SOM. Peaks with an area < 2% of FAME 16:0 area in each sample were disregarded. This procedure produced 35 and 30 peaks from PLFA and EL-FAME, respectively. However, only 28 peaks common to both methods were used in this study. Five minor peaks, exclusive from PLFA, were sparsely detected among the profiles, and their summed amounts varied from 1 to 3% of the total peaks within samples. Removing those peaks caused negligible impact on the determination of the community structure by PLFA (data not shown). These peaks were removed from the PLFA set because the calculation of their EL-FAME:PLFA ratio would result in division by zero, making

impossible the use of this ratio for one of the analyses included in this study and described later. The exclusion of the other two peaks has been justified in the Results.

We used indicator species analysis (ISA) to compare EL-FAME and PLFA regarding their capacity to extract five plant-associated peaks (pmol FAME g^{-1} soil) found to be present in MIDI extracts and to elute within the chromatographic range described above (Chapter 1). Because the probability of extracting FAMEs from SOM and plant residues would increase when extraction is made in direct contact with the soil sample, we also compared EL-FAME with MIDI, another direct extraction method. This comparison allowed us to verify if the milder extraction conditions in EL-FAME protocol would reduce the extraction of interfering peaks associated with plant-specific material, in comparison with MIDI. For each attribute (FAMEs, in this case), ISA provides an indicator value (IV), varying from 0 to 100, which expresses the degree of relationship of this attribute to each group (method of extraction, in this case). This value is the product of the FAME relative abundance (amount of FAME extracted by each method relative to its summed amount across both methods) and relative frequency (number of samples in which the FAME was detected relative to the number of total samples within a given group). IVs were tested for statistical significance using a randomization (Monte Carlo) procedure.

Multiple Linear Regression

Multiple regression was used to model the absolute amounts (pmol g^{-1} soil) of microbial FAME signatures and major saturated and monounsaturated FAMEs (16:0, 18:0, 16:1ω7c and 18:1ω9c) as a function of method of extraction (PLFA vs. EL-FAME), POM-C, nPOM-C, pH, and vegetation cover (no vegetation at sampling time, herbaceous and forests). Amounts of FAME signatures for GP, GN, and ACT (Table 2.3) were summed within each group and

analyzed as pooled markers for these microbial groups. All the two-way interactions between the explanatory variables were allowed in the full model, and a backward variable selection tool was used to choose the final reduced model based on an F-to-remove threshold of 4 ($p<0.05$). Whenever an interaction between two environmental variables was significant, we also checked for the significance of the three-way interaction involving these two variables and methods.

Prior to the statistical analysis, FAME data were log-transformed to fulfill requirements of normality and homogeneity of variance. Because of this transformation, and with the objective to make results more straightforward for interpretation, the coefficients ($ß_i$) for each of the terms (i) in the models were back-transformed by using $\exp(ß_i)$. The $\exp(ß_i)$ for a given term refers to the degree of association between FAME amounts and this term after taking into account the presence of the other terms in the model.

Values of $\exp(ß_i)$ for the quantitative explanatory variables (pH, nPOM-C, POM-C) represent the multiplicative changes in FAME amounts (pmol FAME g^{-1} soil) associated with a unit change in these variables. Therefore, a change of n units in either of these explanatory variables is associated with a change of $\exp(ß_i)^n$ times in the FAME amounts. In the case of categorical variables (vegetation type and method), $\exp(ßi)$ represents the multiplicative change in the amounts of FAMEs associated with one category in comparison to its control category. For the variable vegetation, three categories were used according to the type of vegetation predominant in the sites at sampling; and included: no vegetation at sampling (used as control), herbaceous, and forest cover. Exponential of the coefficients for forests ($\exp ß_{forest}$) and herbaceous ($\exp ß_{herb}$) represent the ratios between the amount of FAME extracted from sites covered with each of these vegetation types and those with no vegetation at sampling.

For method comparisons, PLFA was used as a control category for EL-FAME (EL). Therefore, in the absence of interactions between methods and other variables in the model,

exp($ß_{EL}$) may be interpreted as the ratio between the absolute amounts of FAME extracted by EL-FAME and PLFA (i.e. EL-FAME:$PLFA_{ABS}$ ratio). Comparing these ratios across microbial group signature FAMEs would allow characterizing discrepancies in the FAME profiles between the two methods, which potentially would impact the analysis of microbial communities based on FAME composition.

Occurrence of significant interactions including method and quantitative variables (e.g. exp $ß_{EL*pH}$), or method and vegetation (e.g. exp $ß_{EL*forest}$), was used as evidence that relative changes in the amount of FAME associated with these explanatory variables is method-dependent. These interactions are of special interest for this study because they will flag the occurrence of incongruent patterns of FAME extraction across different soil conditions between the two methods.

We also have investigated whether the dissimilarities between the two methods could be explained by a poor relationship between the amounts of PLFAs and FAMEs from other sources of lipid known to be extracted by EL-FAME, as neutral lipids (NL) and glycolipids (GL). For this, the same models selected for comparisons between PLFA and EL-FAME were fit for NLFA and GLFA data instead of EL-FAME, using PLFA as the control for these two lipid sources. Specifically, we were interested to observe whether the inconsistencies between EL-FAME and PLFA, expressed by interactions between method and environmental variables, also would be observed between NLFA and PLFA or GLFA and PLFA.

Non-metric multidimensional scaling (NMS)

For the following analyses, absolute amounts of individual FAMEs were relativized by FAME totals within each sample and method, as to obtain the proportion (mol%) of each of these FAMEs for the whole profile. The values of mol% for each EL-FAME, within each

sample, were divided by their PLFA counterpart, as to create a dataset with the EL-FAME : $PLFA_{mol\%}$ ratios. These ratios would simultaneously represent the discrepancies caused by the method-related preferential extractability of some peaks relative to others (EL-FAME:$PLFA_{ABS}$ ratios) and by the interactions involving methods on the final FAME composition data used to evaluate microbial structure by multivariate analysis.

Non-metric multidimensional scaling (NMS) was used to ordinate soil samples in a reduced-dimensional space based on their EL-FAME : $PLFA_{mol\%}$ ratios. Distances in the original-space were calculated according the Sørensen proportion coefficient. The objective of this analysis was to investigate how between-method inconsistencies would affect the profile composition and, in turn, the sample ordination based on EL-FAME, in comparison with PLFA.

PC-ORD package (MjM Software Design, Gleneden Beach, OR) was used for ISA, Mantel test and, NMS analysis; and S-PLUS 4.0 (Cambridge, UK), for the remainder statistical analysis.

Results

FAMEs associated with plant polymers and their effect on sample discrimination

Indicator species analysis (ISA) was used to characterize the degree of association between plant-specific FAMEs (PS-FAs) and methods of extraction. Three out of the five PS-FAs found in the MIDI extract, 12:0 12OH, 14:0 14OH, and 16:0 alcohol were not extracted in detectable amounts by either PLFA or EL-FAME (Table 2.4). These compounds also were not detected in GL or NL fractions of the Bligh and Dyer (1959) procedure. MIDI extracted greater amounts of 16:0 16OH and detected this FAME in a greater number of samples than the

Table 2.4. EL-FAME vs. MIDI and EL-FAME vs. PLFA comparisons regarding the relative abundance (RA), relative frequency (RF), indicator value (IV)[†], and across sample average mol percent (Avg %) of plant-specific FAMEs.

	EL-FAME vs. MIDI								EL-FAME vs. PLFA							
	RA		RF		IV		Avg % (SE)		RA		RF		IV		Avg % (SE)	
FAMEs	MIDI	EL	MIDI	EL	MIDI	EL	MIDI	EL	PL	EL	PL	EL	PL	EL	PL	EL
12:0 12OH	100	0	38	0	38***	0***	1.7 (0.5)	0.0 (0.0)								
14:0 14OH	100	0	86	0	86***	0***	6.6 (1.5)	0.0 (0.0)								
16:0 16OH	71	29	100	38	71***	11***	6.7 (0.7)	0.6 (0.2)	5	95	45	38	2	36^{ns}	0.3 (0.1)	0.6 (0.2)
16:0 Alcohol	100	0	100	0	100***	0***	1.6 (0.3)	0.0 (0.0)								
16:0 DCA	23	77	90	55	21***	43^{ns}	2.9 (0.5)	2.1 (0.5)	1	99	14	55	0	55***	0.1 (0.0)	2.1 (0.5)

[†] Relative abundance, relative frequency, indicator value were obtained from indicator species analysis (ISA) using absolute amount of FAMEs (pmol FAME g^{-1} dry soil). See text for details.

other two methods. Conversely, EL-FAME and PLFA generally were quite similar for the number of samples where 16:0 16OH was detected; but the abundance of this PS-FA was clearly greater in EL-FAME than in PLFA extracts. The source of this PS-FA appears to be GL rather than PL because 16:0 16OH in EL-FAME extract was highly correlated with GL ($r = 0.94$, $p<0.001$). This PS-FA was not detected in the NL fraction of any sample.

Significant differences between EL-FAME and PLFA were found only for 16:0 DCA; a non-significant IV was obtained for the comparison between EL-FAME and MIDI ($p=0.52$). In the same way as observed for 16:0 16OH, amounts of 16:0 DCA from GL and EL-FAME were strongly correlated ($r = 0.98$, $p<0.001$), whereas no detectable amount of this PS-FA has been observed in NL fractions.

Comparison between MIDI and PLFA methods in terms of the interference of plant polymer FAMEs on the microbial community structure has been reported elsewhere (Chapter 1). In this paper, we have focused on comparing EL-FAME and PLFA regarding this same aspect. Despite that 16:0 16OH and 16:0 DCA have been detected in the PLFA extracts of some samples, they have accounted for < 1 mol% of the FAME composition of those, likely a minor interference in the microbial community analysis. The low RFs for 16:0 16OH and 16:0 DCA extracted by EL-FAME indicate that these PS-FAs were undetected in many samples. In fact, their occurrence in EL-FAME extracts was mostly in soils from older forests (OG and WL samples) (avg. mol% = 2.0 ± 0.11 SE, and 2.8 ± 0.32 SE, for 16:0 16OH and 16:0 DCA, respectively), than in the other sites (avg. mol% = 0.5 ± 0.06 SE, and 0.2 ± 0.10 SE, for 16:0 16OH and 16:0 DCA). This vegetation effect would potentially affect multivariate samples' distances when those sites were included in the analysis, resulting in an over estimation of population separations that was biassed by plant lipids, in the same way as observed for MIDI (Chapter 1).

To evaluate the impact of those peaks on the statistical separation of populations, NMS ordinations were obtained from datasets that had these two peaks either removed or kept in before relativization of FAMEs by their totals (mol%). Within each method, Pearson correlation coefficients were determined for sample scores along axes 1 and 2 between the two datasets. Plant-FAs were more influential for the EL-FAME ($r = 0.94$ for axis 1; and 0.92, for axis 2) than PLFA ordination ($r > 0.99$ for both axes). The NMS solution for EL-FAME dataset with plant-associated peaks showed a better discrimination of forest sites from the remaining sites along axes 1 and 2. This resulted in a sample ordination similar, in this aspect, to that of PLFA (data not shown). Because of this plant-driven influence, these two peaks were removed from the data sets of both methods before obtaining the peaks mol % values for the other analysis based on FAME composition.

Influence of extraction method and environmental factors

The exp $ß_{EL}$ term was used as a proxy for EL-FAME:$PLFA_{ABS}$ ratio (Table 2.5). This is valid only for those FAMEs that showed no significant interactions between method and other variables. Considering the total amount of FAMEs, this ratio was equal to 4.8. Ratios greater than that were found for EUK, 16:0, 18:0, and 18:1ω9c, the opposite being true for the bacterial FAMEs, especially GN and ACT (Table 2.5). Fungi and 16:1ω7c EL-FAME:$PLFA_{ABS}$ ratios were dependent on the standing vegetation; this ratio for AMF varied with both vegetation and pH. For the fungal marker, EL-FAME:$PLFA_{ABS}$ equaled 10 in soils without vegetation or under herbaceous plants, and 4.5 under forest soils (exp $ß_{EL\ vs.\ forest} = 0.45$). FAME 16:1ω7c presented an EL-FAME:$PLFA_{ABS}$ ratio wider than the total FAMEs ratio only in soils under no-vegetation. For AMF, presence of herbaceous plants, but not forests, at sampling time resulted in a narrowing of

Table 2.5. Multiplicative changes[†] (exp ß) on FAME amount (pmol g^{-1} soil) associated with method of extraction (EL-FAME vs. PLFA), C from particulate and non-particulate organic matter (POM-C and nPOM-C), pH, vegetation [herbaceous (Herb) or forests vs. no vegetation], and interactions between the terms.

			Organic C			Vegetation[¶]		Interactions				
FAMEs[‡]	Intercept	EL[§]	POM	nPOM	pH	Herb	Forest	EL vs. pH[#]	EL vs. Forest	EL vs. Herb	POM-C vs. Forest	R^2
FUNGI	534***	10.0***	11.6***				6.6***		0.45***		0.14***	0.92
GP	2335***	4.1***	14.5***	1.09***			3.4***				0.07***	0.92
GN	1806***	2.5***	19.3***	1.10***			4.0***				0.07***	0.91
ACT	1049***	3.1***	14.8***				3.9***				0.08***	0.91
AMF	299***	0.4^{ns}	19.8***		1.0^{ns}	1.6*	6.5***	1.68**		0.44**	0.05***	0.90
EUK	90***	7.4***	4.3***			2.0***	4.6***				0.27***	0.93
16:0	1817***	7.9***	12.5***				4.1***				0.10***	0.95
18:0	353***	7.1***	11.4***				3.9***				0.10***	0.94
16:1ω7c	932***	5.5***	20.0***			1.2^{ns}	4.0***		0.57**	0.63**	0.06***	0.92
18:1ω9c	1208***	8.2***	18.1***				3.7***				0.07***	0.94
Total	14485***	4.8***	17.2***				4.0***				0.07***	0.93

[†] Refers to the exponential of the coefficients obtained from models with log transformed response variable (FAME, pmol g^{-1} soil). For POM-C (%), nPOM-C (%), and pH, values refer to the relative changes in FAME amount associated with each unit change in these quantitative variables.

[‡] GP: Gram-positive; GN: Gram-negative; AMF: Arbuscular mycorrhizal fungi; ACT: Actinomycetes; EUK: Eukaryotes. For details, see Table 2.3. Total refers to the sum of all 28 peaks considered in this study.

[§] Unless interactions with EL were present, values refer to the EL-FAME:$PLFA_{ABS}$ ratio for FAME recovery.

[¶] Unless interactions with Herb or Forest were present, values refer to the ratio of FAME recovery between these categories and non vegetation.

[#] For non-vegetated sites, estimated EL-FAME:PLFA for AMF signature in the sample average pH (pH = 6.1) equals 9.5. Along the range of pH values, this ratio would vary from 6.25 (pH = 5.4) to 17.6 (pH = 7.4).

***, **, * : $p < 0.001$, 0.01 and 0.05, respectively, for coefficients in the original log models.

the EL-FAME:$PLFA_{ABS}$ ratio (exp ßEL vs. Herb = 0.44) compared to non- vegetated sites AMF EL-FAME:$PLFA_{ABS}$ ratio also increased with pH (exp $ß_{EL\ vs.\ pH}$ = 1.68). Estimated ratios for this FAME varied from 6.25 (pH = 5.4) to 17.6 (pH = 7.4) under no-vegetation sites. No other significant interactions were found between method and the remainder explanatory variables.

GP, GN, and 16:0 had averages increases of 1.08 to 1.10 times with each unit increment in nPOM-C (%). Overall, positive association between FAME amounts and POM-C was stronger and more widespread across FAMEs than with nPOM-C. It is noteworthy that there appears to be a common vegetation-dependent and method-independent relationship between all the FAME types and POM-C. According to this pattern, smaller relative increases in FAME amounts have occurred in association with POM-C in soils from forest than in the other vegetation categories. In terms of FAME totals, each unit increment in POM-C (%) would correspond to an increase of 1.2 times in the amount of FAMEs in forests, and of 17.2 times under sites with herbaceous or non-vegetation.

This same pattern of interaction between vegetation and POM-C was observed for microbial biomass-C as follows:

$$MB\text{-}C = 0.013 \times 11.08^{POM\text{-}C} \times 1.23^{nPOM\text{-}C} \times 2.18^{FOREST} \times 0.11^{POM\text{-}C*FOREST}\ (r^2=0.85);$$

where all the C pools were expressed in g C 100 g^{-1} dry soil. According to this model, MB-C varied 11.08 and 1.22 (from the product of 1.23 x 0.11) times with each percent change in the POM-C content of soils from non-forested and forested sites, respectively. Original MB-C data were treated and interpreted in the same way as for FAME amounts. The coefficients for all the terms in the original log model were significant at $p<0.01$.

With the exception of AMF and EUK, no difference was found in the average amount of FAMEs extracted from herbaceous sites and those without vegetation. Extraction of AMF by PLFA, but not by EL-FAME, increased under herbaceous plants relative to non-vegetated sites.

For PLFA, the recovery of AMF under herbaceous plants was, on average, 60% greater than under non-vegetated sites at the time of sampling (exp $ß_{herb} = 1.58$, $p<0.05$). However, there was an interaction between method and vegetation (exp $ß_{EL\ vs.\ herb} = 0.43$, $p<0.01$), with the predicted amount of AMF extracted by EL-FAME from herbaceous sites being 68% of that from sites that had no-vegetation at sampling. To verify if the EL-FAME AMF in soils under herbaceous plants would be significantly smaller than under non-vegetated sites, we modeled the amount of AMF derived only from EL-FAME as a function of vegetation, pH, POM-C and the interaction between POM-C and forest, i.e., the same model for AMF in Table 2.5 but suppressing the method term. This analysis has resulted in a non-significant difference ($p = 0.10$, for herbaceous term) between EL-FAME AMF under herbaceous and non-vegetated sites nat the time of sampling. Extraction of EUK signature was doubled by both methods under herbaceous plants compared to non-vegetated sites.

Associations between FAME amounts and forests were highly positive and, except for the fungal FAME, independent of the method. Fungal FAME extraction from forest soils was 6.6 and 3.0 ($ß_{EL\ vs.\ forest} = 0.45$; $p<0.01$) times greater than non-vegetated sites when PLFA and EL-FAME were used as method of extraction, respectively.

Potential sources of interference for EL-FAME

Because models for NLFA, GLFA, and EL-FAME were indirectly related to the use of a common control (PLFA), coefficients for environmental variables that did not interact with methods were quite similar across those models and, therefore, were not shown in Table 2.6.

NLFA:PLFA and GLFA:PLFA ratios for the sum of the 28 peaks considered in this study had values of 1.02 and 0.32, respectively. However, when individual FAMEs or pooled signature FAMEs were considered, NL was found to be a minor source of prokaryote FAMEs,

relative to PLFA; but it may be equally or even more important than PLFA for eukaryotes signatures and saturated FAMEs (16:0, and 18:0). This was especially true for AMF, which had,

Table 2.6. Multiplicative changes[†] (exp ß) on FAME amount (pmol g^{-1} soil) associated with lipid sources (neutral lipids vs. phospholipids; and glycolipids vs. phospholipids), and interactions between source and vegetation.

	Lipid Source	Interactions NL vs.			Lipid Source	Interactions GL vs.		
FAMEs[‡]	NL [§]	Forest	Herb	Model R^2	GL [§]	Forest	Herb	Model R^2
Fungi	2.27^{***}	0.56^{*}		0.83	0.28^{***}			0.84
GP	0.31^{***}			0.82	0.23^{***}			0.89
GN	0.19^{***}			0.86	0.09^{***}			0.94
Act	0.17^{***}			0.90	0.19^{***}			0.92
AMF	8.65^{***}		0.32^{**}	0.71	0.31^{***}		0.46^{***}	0.81
Euk	1.95^{***}			0.80	$0.00^{¶**}$			
16:0	1.52^{***}			0.65	0.65^{***}			0.84
18:0	0.78^{ns}			0.76	0.69^{***}			0.81
16.1ω7c	2.95^{***}	0.45^{*}	0.31^{**}	0.59	0.19^{***}		0.63^{***}	0.90
18.1ω9c	2.19^{***}			0.75	0.34^{***}			0.86
Total	1.02^{ns}			0.70	0.32^{***}			0.89

[†] Multiplicative change values were obtained from multiple regression models including all the explanatory variables used for comparisons between EL-FAME and PLFA (see text and Table 2.5 for details). However, all the other terms and interactions in the fitted models were omitted, because their coefficient values were similar to those obtained for EL-FAME vs. PLFA models (Table 2.5). Thus, only values for lipid fraction association, and interactions that represent divergent patterns of association of FAMEs from neutral lipids (NL) and glycolipids (GL) comparatively to phospholipids (PL) are shown. Model R^2 refers to the full model fitted for each of the FAME.

[‡] See Table 2.5 for details.

[§] Values correspond to the association between amount of FAMEs and lipid fraction using PL as a control category for both NL and GL (see text for details). Therefore, values are interpreted as NLFA:PLFA and GLFA:PLFA ratios, unless interactions including NL or GL were significant in the models for each FAME.

[¶] Euk marker (20:4ω6c) was not detected in glycolipid fraction.

in average, 8.6 times more FAME extracted from NL than PL under both soil that were non-vegetated at sampling and forest sites, and 2.9 times from sites with herbaceous plants at the time of sampling. Nevertheless, in the same way as observed for EL-FAME, the importance of

NL relative to PL for fungal and AMF signatures, and 16:1ω7c also varied with the type of vegetation, as reported later in this section.

Although GL was relatively less important than PL as a source for most of the FAMEs, this was not valid for 16:0 and 18:0, as those GLFAs accounted for about 65 to 70% of their counterpart PLFAs in the Bligh and Dyer (1959) extract.

It is noteworthy that the sum of PL, GL and NL derived FAMEs did not account for the amount of FAMEs extracted by EL-FAME. For total FAMEs, for instance, the sum of the three Bligh and Dyer fractions was equal to 2.3 times the amount of PLFA extracted (Table 2.6), while the amount extracted by EL-FAME equaled 4.8 times that of PLFA (Table 2.4).

Interactions between lipid sources and either POM-C or nPOM-C were not significant, similar to that observed for EL-FAME vs. PLFA models (Table 2.6). Interactions observed between type of vegetation and method of extraction (EL-FAME vs. PLFA) for fungi, AMF and 16:1ω7c (Table 2.5) were consistently found between vegetation and source of lipids in models contrasting PLFA and NLFA (Table 2.6). Compared to the non-vegetated sites at the time of sampling, both EL-FAME:PLFA and NLFA:PLFA ratios were narrower for fungi under forests; for AMF under herbaceous; and for 16:1ω7c extracted from soils under herbaceous plants and forest sites. The patterns of extraction of AMF and 16:1ω7c GLFAs from samples under herbaceous sites at the time of sampling were also similar to that of their EL-FAME counterparts. However, the pH-dependent divergence in the amounts of AMF recovered between the two methods could not be associated either with NL or GL FAME sources.

Characterization of distortions in the EL-FAME : PLFA mol percent ratio

The NMS plot from EL-FAME:$PLFA_{mol\%}$ ratio data represents the discrimination of samples based on the method-related discrepancies in the peaks' ratios, rather than variations in the composition of microbial community associated with natural factors (Figure 2.1).

The separation of samples in the three vegetation categories was evident from this plot. Soil under forest and herbaceous plants were significantly different from non-vegetated sites along axis 1 ($p<0.01$), while discrimination of forests from the remainder sites was observed along axis 2 ($p<0.01$).

Axis 1 was also strongly correlated with the AMF ratio ($r = -0.81$, $p<0.001$), which increased toward the non-vegetated sites. According to the models based on the absolute amounts of FAMEs, AMF extraction by EL-FAME relative to PLFA increased with increase of pH. However, no significant correlation between pH and the axis 1 ($r = -0.32$, $p>0.05$) was observed.

Fungal ratio was negatively correlated with axis 2, with higher ratios being observed on sites with no-vegetation and herbaceous plants at sampling. This pattern is also in line with results obtained from the model describing the variability in fungal FAME.

Toward the top of the plot (Figure 2.1), a strong gradient for the GP EL-FAME:$PLFA_{mol\%}$ ratio was observed. However, no interaction between method and environmental variables was detected in the model for absolute amounts of GP. This apparent incongruence, along with the observation that GP and fungal ratios were strongly opposed along axis 2, suggests that the GP ratio could be indirectly derived from a dilution effect of GP signatures caused by the method-related enrichment of fungal EL-FAME in soil from non-forested sites.

In fact, when the same analysis was performed using an EL-FAME:$PLFA_{mol\%}$ ratio data set calculated after disregarding fungi and AMF from both PLFA and EL-FAME, absolute

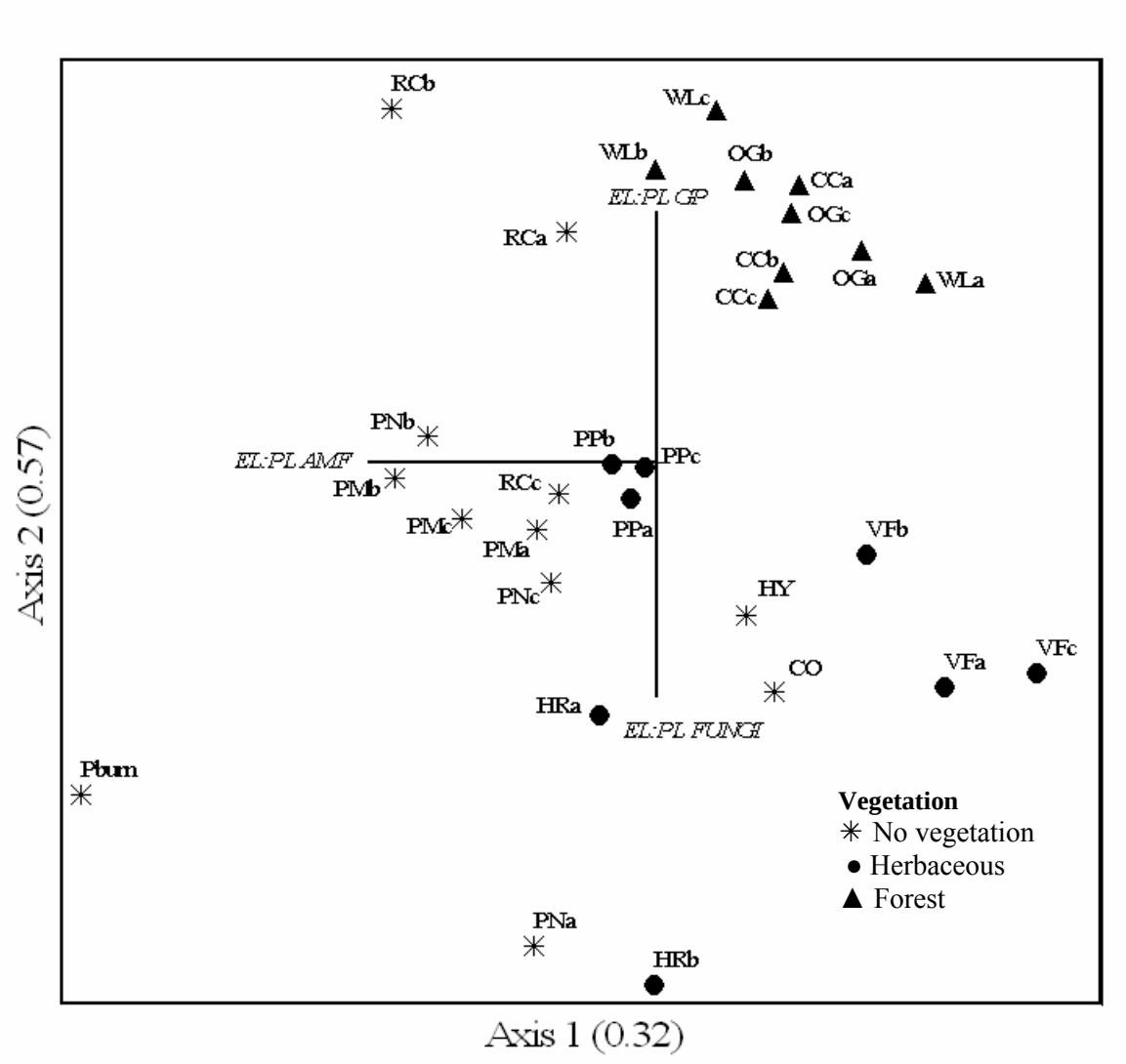

Figure 2.1. Non-metric multidimensional scaling of the EL-FAME:PLFA ratio of 28 peaks extracted from 29 soil samples. The ratio has been based on the mol percent contribution of each peak to the total FAME profile from each method. Distances between symbols are approximately proportional to the dissimilarities in the ratios between the samples. The angles and length of the overlaid radiating lines indicate the direction and strength of relationships between microbial signature ratios (Table 2.3) and the ordination scores. Only microbial ratios significantly related (Pearson correlation coefficient, $p<0.05$) to either axes 1 or 2 were shown. For details on sample identification, see Table 2.1. The proportion of variance explained by each axis is based on the correlation (*R*) between distance in the reduced NMS space and distance in the original space and is reported after each axis heading.

amounts of FAMEs resulted in no significant separation according to standing vegetation at the time of sampling (data not shown).

These results indicate that vegetation-associated divergences in the recoveries of absolute amounts of fungi and AMF between methods were high enough to still influence the relativized mol% data, and likely to affect other microbial group signatures ratios, as well.

Discussion

Extractability of plant polymer FAMEs by different methods

Because EL-FAME and MIDI extraction procedures are carried out in direct contact with soil samples, both methods are likely to produce FAMEs from a more diverse array of soil lipids than does PLFA. In addition to the microbial lipids, other lipids derived mainly from particulate plant residues and more humified SOM fractions (del Rio et al., 1998; Nierop, 1998) could contribute with the overall FAME profile obtained by EL-FAME and MIDI. In Chapter 1, five peaks related to the plant substances cutin and suberin were highly abundant and widespread in MIDI extracts but were almost absent from PLFA extracts.

Overall, the extractability of FAMEs markers for plant polymers were based on the following ascending order of extraction methods: PLFA<EL-FAME<MIDI. Milder extraction conditions of temperature and pH during EL-FAME extraction may have resulted in reduced recovery of plant-derived substances. Indeed, visual inspection of the tube contents after EL-FAME alkaline methanolysis and MIDI acid hydrolysis revealed a much darker-colored extractant and a greater disruption of the physical integrity of particulate organic material in the latter. Two plant polymer FAMEs, 16:0 16OH and 16:0 DCA, were detected in samples from

both methods. Glycolipids, or other compounds eluting in acetone fraction during fractionation in silica columns, seem to be the major sources of both of these plant polymers. Despite EL-FAME being less efficient than MIDI in extracting these peaks, the amounts extracted still caused some interference for EL-FAME-based microbial community profiling.

Relationship between microbial FAMEs in EL-FAME and in Bligh and Dyer fractions

As expected, EL-FAME was more efficient than was PLFA to recover not only plant polymer FAMEs, but all the others as well. Part of this difference may be attributed to the production of FAMEs from fatty acids ester-linked to NL and GL by EL-FAME, in addition to those derived from PL. However, summing the signatures derived from the chloroform, acetone and methanol eluates did not account for the differences in FAMEs recovered by EL-FAME and Bligh and Dyer extraction methods. Losses during the fractionation of Bligh and Dyer extract through silica columns could have accounted for some of these differences. Frostegård and co-workers (1991) have found that about 10% (organic soil) to 30% (sandy loam soil) of the lipid phosphate may be lost during the elution of the soil lipid extracts during this procedure. These loss estimates were specific for lipids containing phosphate, as phosphoglycerolipids and phosphoglycoglycerolipids. We have found no estimates in the literature for the losses in GLs and NLs during silica column fractionation.

The discriminatory effect of biphasic solvent systems (e.g. Bligh and Dyer, 1959) toward acidic PLs, which are partially retained in the aqueous phase (Kolarovic and Fournier, 1986), also would account for some of this difference. Using rat heart microsomal lipid extracts, Kolarovic and Fournier (1986) demonstrated that Bligh and Dyer biphasic solvent system extracted about 40 to 60% of acidic phospholipids (e.g. phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and phosphatidic acid) when compared to the

monophasic extraction proposed by Christiansen (1975). However, no losses were found between the two solvent systems regarding the recovery of less acidic phospholipids (phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine) and non-polar lipids such as triglycerides and monoglycerides. In the EL-FAME procedure, partitioning of the extract components into two phases would rely on the FAME chemistry rather than in the whole lipid chemistry, as in the Bligh and Dyer extraction. In the EL-FAME procedure, fatty acids are rendered highly non-polar (FAMEs) by transesterification in the KOH-methanol mixture. This step precedes the partitioning of FAMEs by the addition of hexane, which would recovery the already non-polar FAMEs. However, the partitioning of the Bligh and Dyer extract with a NaCl solution (Folch et al., 1957), as used in our study, would have mitigated this loss of phospholipids. As stated by Folch and colleagues (1957), the presence of chloride salt in the upper phase of the binary solvent system would reduce the losses of lipids from the lower organic phase.

Between-method congruence in describing a POM-C vs. vegetation interaction

Because this is an observational study in which, with exception of method, no factors describing the changes in FAME were independently varied from the others, the following considerations about the association between FAME abundance and ecological properties should be regarded as hypotheses rather than explanations for the FAME variability. Nevertheless, for the objective of this work it was more important to use those models to detect differences between the two methods and characterize conditions associated with these differences in our data set than it was to describe factors causing the variation in FAMEs.

Regardless of the method of extraction, POM-C rather than nPOM-C were strongly and positively correlated with changes in FAME markers from all the microbial groups considered

in this study. However, the correlation of FAME amounts with POM-C was more intense in soils under either no-vegetation or herbaceous plants than forested sites. A possible explanation is that the PLFA phosphate buffer decreases the PL extraction efficiency as C content of soil increases (Frostegård et al., 1991). Some of our results have indicated that this explanation might not stand in our case. Samples from non-vegetated sites at the time of sampling (RCa, RCb, and RCc) and forested sites (WLa, WLb, and WLc) were collected in the same region under similar climatic and soil characteristics (Table 2.1). Despite the similarity in the TOC contents between RC and WL sites, correlation between PLFA amounts and POM-C for RC samples was more similar to that of other non-forest, low TOC soils than with forested sites.

Moreover, this same interaction has been observed for FAMEs extracted by EL-FAME, as well as for MB-C by the fumigation incubation method. These similar results among three methods, which are based on different principles of biomass extraction from soil samples, make it unlikely that PLFA extracts have artifacts in soils with high TOC.

An alternative explanation could be due to soil type where POM-C in forests is of lower quality to support microbial growth, because plant inputs are higher in lignin and resins, than those from non-forested soils. Although some soil microorganisms, especially the basidiomycetes (Carlile and Watkinson, 1994), have been shown to decompose lignin, the efficiency of this C source for microbial growth may be very small (Voroney et al., 1981).

Potential of EL-FAMEs as proxies for PLFAs as viable biomass markers

We found no interactions between methods and any of the other variables that made a significant contribution to the models for GN, GP, ACT, EUK, 16:0, 18:0, 18:1ω9c and total FAMEs. Potential sources of interference in the EL-FAME extract, such as NL- and GL-derived FAMEs were detected for all of those FAMEs. However, similar to what we observed for EL-

FAME, they covaried with the PLFA pool, regardless of the ecological properties of the sites sampled.

Similar to FAMEs that were pooled according to microbial groups, individual GN, GP, and ACT FAME signatures had no significant interactions among methods (EL-FAME vs. PLFA) or sources of lipids (NL or GL vs. PL) (data not shown). This indicates that EL-FAME would equal PLFA in terms of quantifying relative changes in these FAMEs among samples.

Of special interest for quantifying these relative changes would be the possibility of using 18:1ω9c as a fungal marker instead of 18:2ω6c because 18:1ω9c was not impacted by interactions between method of extraction and the environmental variables. However, this should be tested on a wider range of soil types and environments to confirm these results. It is noteworthy that 18:1ω9c, as well as 18:2ω6c, had a NLFA:PLFA ratio greater than 10 when glucose was added to a forest soil sample (Bååth, 2003). However, our results showed a lower and more constant NLFA:PLFA ratios for 18:1ω9c under a very diverse set of soil samples. In the same paper, Bååth (2003) also found that NLFA:PLFA ratios for this FAME in soils across diverse environments (forest mineral soil, forest humus, row crop fields, and grasslands) to be much less variable than those found for soils with glucose added. Despite the agreement between our results and those found by Bååth (2003), our ratios were about 4.6 times greater than those found by him. However, values for NLFA:PLFA ratios for most of the other FAMEs were similar between this paper and that author.

Method-related interferences of FAME extractions

From the models describing FAME amounts as functions of method and environmental properties, two seemingly important sources of interference regarding comparison between the two methods were predicted: a) a microbial group-dependent EL:FAME:$PLFA_{ABS}$ ratio, and (b)

interactions between method and environmental variables. Both of these interferences, especially the latter, impacted the comparison of community structures between EL-FAME and PLFA.

Bacterial FAMEs had EL-FAME:$PLFA_{ABS}$ ratios smaller than those observed for the overall community, while FAMEs related to eukaryotes (18:1ω9c, EUK) and major saturated FAMEs (16:0, and 18:0), had a higher ratio than the average of the entire community. Saturated FAMEs, although present as components of the cell membranes of both prokaryotes and eukaryotes, also have been reported as major components of storage lipids (triacylglycerols) for eukaryotes (Harwood and Russell, 1984; Bååth, 2003; van Aarle and Olsson, 2003). For some soils under vegetative cover, AMF, fungi, and 16:1ω7c also had higher EL-FAME:$PLFA_{ABS}$ than the average of the entire microbial community. Similar trends were verified for the ratio between NLFA:PLFA and GLFA:PLFA ratios for each of those FAMEs. Our results for the increased importance of NL relative to PL for eukaryotic and storage FAMEs are also in agreement with those found Bååth (2003) in soils from diverse ecosystems. The increased contribution of eukaryotic storage lipid for the FAME profiles can cause dissimilarities in the microbial composition between samples to be mainly eukaryotic-drive. This appeared to be the case in a previous study by Drijber et al. (2000). They reported that separation of microbial communities in soil samples from different managements and wheat cropping phases was driven mainly by 16:1ω5c, and 20:4ω6c when EL-FAME was used. In this same study, PLFA had the same ability in discriminating among those plots, but shifts in bacterial community became more important than in EL-FAME.

Because the abundance of NL-FAMEs was generally greater than that of GLFAs, it is very likely that NL would be important factor for differences in microbial profiling outcomes between PLFA and EL-FAME.

Interactions between methods and vegetation were found for fungi, AMF and 16:1ω7c. Under forests, the EL-FAME:PLFA ratio for fungal FAME was narrower than under the other vegetation categories. This vegetation-dependent disagreement in fungal FAME extraction between the methods was similar to what we found between NL and PL. This suggests that NLFA would be the source of interference for fungal FAMEs when the two methods are compared. Bååth (2003) has associated high NL:PL ratios with the excess availablility of C relative to other nutrients. According to this author, when synthesis of new biomass is limited by N deprivation, excess of C would be accumulated as storage lipids (triglycerols). Therefore, the narrow fungal NL:PL ratio in soils from forests over other sites could be based on the following: 1) narrow bioavailable C:N ratio of forest residues (Paul and Clark, 1996); 2) the fungal community, particularly ectomycorrhizal fungi, are more adpated to scavage N without immobilizing excess of C (Näsholm et al., 1998); or, alternatively, 3) a smaller N requirement per unit of C available for fungal growth, as it could be the case also for ectomycorrhizal fungi, which use part of its C to carry out functions related with the symbiosis.

Modeling of AMF amounts also involved an interaction between method and vegetation. The PLFA extracted AMF marker was 60% ($\exp(ß_{herb}) = 1.6$) greater for soil from soil of herbaceous than non-vegetation sites at the time of sampling. However, for EL-FAME extractions, the amount of AMF in soil under herbaceous plants was not significantly different than sites that were not under vegetatation at the time of sampling. The same pattern of correlation found for EL-FAME regarding vegetation was found for both NL and GL fractions which suggests these sources of AMF varied differently from their PL counterpart and are likely to have contributed to the dissimilarities between PLFA and EL-FAME. Although the EL-FAME, but not PLFA, AMF pool increased with pH, we were not able to associate this between-method dissimilar pattern with variations in NL or GL pools. AM fungi are very rich in

lipids, especially NLs (Olsson et al., 1995). NL in vesicles and spores are the main source of storage products for those fungi (Jabaji-Hare et al., 1984; Bago et al., 2000). Among the FAMEs found linked to NL, 16:1ω5c has been shown to be especially abundant, and is a good marker for AMF storage. It also has been described as present in glycolipid fractions of vesicles (Jabaji-Hare et al., 1984) and lipid extracts from soil samples (Drijber et al., 2000). Despite our results showed that both NL and GL had a similar disparate pattern of 16:1ω5c abundance relative to PL, the lower abundance of AMF-GLFA than NLFA suggests that NL would be far more important than GL as a source of interference for EL-FAME.

PLFA 16:1ω5c is a component of membrane lipids and, within the same species, has a relatively constant amount per unit of mycelium biomass (Olsson and Johansen, 2000) and does not change drastically after mycelium is disconnected from shoots (Olsson et al., 1995). However, Olsson et al. (1997) have shown that, unlike PLFA, NLFA 16:1ω5c can vary with the length of the hyphae, and suggested that this variation may be related with spore formation or with the nutritional status of the mycelium. This last consideration is supported by observations that P fertilization is more impacting on the vesicle formation (intraradical fungi storage structures) than on intraradical hyphae growth of *Glomus fasciculatum* (Abbott et al., 1984). NLFA 16:1ω5c has also been shown to be more sensitive than PLFA in respect to impacts like separation of the fungi from its host (Olsson et al., 1995), tillage intensity (Drijber et al., 2000), and, according to our results, to soil pH. We suggest that the great diversity of soil managements, fertilization rates, and plant cycles stages encompassed in our study may have contributed to a highly variable amount of NLFA AMF, making separation of soil samples according to vegetation more unlikely for PLFA than NLFA.

EL-FAME has been shown to equal PLFA in separating microbial communities by PCA from winter wheat-fallow system samples regarding rotation phase (fallow vs. winter), and

tillage treatments (Drijber et al., 2000). In this paper, samples were collected four weeks after the wheat harvest in the cropped phase of the rotation, i.e., sampling plots from both treatments had no plant cover. Despite the overall similarity between the two methods in discriminating samples, AMF abundance in PLFA and EL-FAME seemed to be impacted to some extent by different factors. PLFA AMF proportion was quite similar across management, either within fallow or plots recently cropped to wheat; while cropping cycle seems to have been a major factor, with AMF values being higher and closer to sod for recent wheat than fallow (Drijber et al., 2000). These results would be in line with our observed increase in AMF PLFA in the presence of the host.

However, results from those same authors showed that EL-FAME, AMF values in sod were about one order of magnitude greater than in the other plots, but this difference decreased with decreasing management intensity, indicating that management intensity was been important in determining EL-FAME AMF abundance. The great variability of NLFA 16:1ω5c due to other factors described earlier, would likely make it more difficult to separate samples populations using ordination multivariate techniques by a unique factor such as vegetation categories. A high variability in such a large pool of FAMEs, as it is the case for NLFA 16:1ω5c, would have a major impact on microbial structure and make interpretation more difficult when EL-FAME rather than PLFA is used.

Conclusions

EL-FAME is a milder extractant than the MIDI method but still extracted two plant polymer FAs (16:0 16OH and 16:0 DCA) which, if present in a soil sample, would confound

the outcomes of EL-FAME profiling. We found these two markers to be most evident in forest soils.

Two other major interferences related to EL-FAME in comparison with PLFA were detected: 1) eukaryotic signature and storage FAMEs; and 2) the type of vegetation at sampling. These divergences were observed for AMF and fungi, and are likely related with conditions leading to distinct accumulation of NL and PL in those organisms.

These drawbacks would be less important in studies on microbial community changes within soils that had the same vegetative cover. For instance, according to our results, one should be cautious in using EL-FAME to compare continuous cropping systems with those that have a fallow stage. In this case, sampling during the fallow phase when no plants are present vs. the continuous cropped plots might lead to confounding interpretations.

However, several FAMEs, including those from bacteria, major saturated, and unsaturated FAMEs, have shown similar pattern of extraction from both methods, regardless the environmental conditions from where the soil was sampled. Therefore, we conclude that when the goal is to determine relative changes on the abundances of these FAMEs due to field or lab treatments, rather than evaluating shifts in overall microbial composition, EL-FAME seems to equal PLFA in representing these changes.

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References

Abbott, L. K., A. D. Robson, and G. de Boer. 1984. The effect of phosphorus on the formation of hyphae in soil by the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatum*. New Phytologist 97:437-446.

Bååth, E. 2003. The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. Microbial Ecology 45:373-383.

Bååth, E., L. O. Nilsson, H. Goransson, and H. Wallander. 2004. Can the extent of degradation of soil fungal mycelium during soil incubation be used to estimate ectomycorrhizal biomass in soil? Soil Biology and Biochemistry 36:2105-2109.

Bago, B., P. E. Pfeffer, and Y. Shachar-Hill. 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology 124:949-957.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

Bossio, D. A., and K. M. Scow. 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35:265-278.

Carlile, M. J., and S. C. Watkinson. 1994. The Fungi. Academic Press, San Diego.

Christiansen, K. 1975. Lipid extraction procedure for in vitro studies of glyceride synthesis with labeled fatty acids. Analytical Biochemistry 26:93-99.

del Rio, J. C., D. E. McKinney, H. Knicker, M. A. Nanny, R. D. Minard, and P. G. Hatcher. 1998. Structural characterization of bio- and geo-macromolecules by off-line thermochemolysis with tetramethylammonium hydroxide. Journal of Chromatography A 823:433-448.

Drijber, R. A., J. W. Doran, A. M. Parkhurst, and D. J. Lyon. 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biology and Biochemistry 32:1419-1430.

Federle, T. W. 1986. Microbial distribution in soil - new techniques, p. 493-498. *In* F. Megusar and M. Gantar (ed.), Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497-509.

Frostegård, A., and E. Bååth. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22:59-65.

Frostegård, A., A. Tunlid, and E. Bååth. 1991. Microbial biomass measured as total phosphate in soils of different organic content. Journal of Microbiological Methods 14:151-163.

Griffiths, B. S., K. Ritz, N. Ebblewhite, and G. Dobson. 1999. Soil microbial community structure: Effects of substrate loading rates. Soil Biology and Biochemistry 31:145-153.

Hackl, E., M. Pfeffer, C. Donat, G. Bachmann, and S. Zechmeister-Boltenstern. 2005. Composition of the microbial communities in the mineral soil under different types of natural forest. Soil Biology and Biochemistry 37:661-671.

Harwood, J. L., and N. J. Russell. 1984. Lipids in plants and microbes. Allen and Unwin, London.

Jabaji-Hare, S., A. Deschene, and B. Kendrick. 1984. Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. Mycologia 76:1024-1030.

Jenkinson, D. S. 1988. Determinantion of microbial biomass carbon and nitrogen in soil, p. 368-386. *In* J. R. Wilson (ed.), Advances in nitrogen cycling in agricultural ecosystems. CAB International, Wallingford, UK.

Jenkinson, D. S., and D. S. Powlson. 1976a. The effect of biocidal treatments on metabolism in soil. I. Fumigation with chloroform. Soil Biology and Biochemistry 8:167-177.

Jenkinson, D. S., and D. S. Powlson. 1976b. The effects of biocidal treatments on metabolism in soil. V. A method for measuring soil biomass. Soil Biology and Biochemistry 8:209-213.

King, J. D., D. C. White, and C. W. Taylor. 1977. Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora. Applied and Environmental Microbiology 33:1177-1183.

Kolarovic, L., and N. C. Fournier. 1986. A comparison of extraction methods for the isolation of phospholipids from biological sources. Analytical Biochemistry 156:244-250.

Kroppenstedt, R. M. 1992. The genus *Nocardiopsis*, p. 1139-1156. *In* A. Balows, H. G. Trüpper, D. M., W. Harder, and K. H. Schleifer (ed.), The prokaryotes, vol. 2. Springer, Berlin.

Näsholm, T., A. Ekblad, A. Nordin, R. Giesler, M. Högberg, and P. Högberg. 1998. Boreal forest plants take up organic nitrogen. Nature 392:914-916.

Nierop, K. G. J. 1998. Origin of aliphatic compounds in a forest soil. Organic Geochemistry 29:1009-1016.

O'Leary, W. M., and S. G. Wilkinson. 1988. Gram-positive bacteria, p. 117-201. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press, San Diego.

Olsson, P. A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303-310.

Olsson, P. A., E. Bååth, I. Jakobsen, and B. Söderström. 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99:623-629.

Olsson, P.A., E. Bååth, and I. Jakobsen. 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Applied and Environmental Microbiology 63:3531-3538.

Olsson, P. A., and A. Johansen. 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycological Research 104:429-434.

Paul, E. A., and F. E. Clark. 1996. Soil Microbiology and Biochemistry, 2nd ed. Academic Press, San Diego.

Petersen, S. O., P. S. Frohne, and A. C. Kennedy. 2002. Dynamics of a soil microbial community under spring wheat. Soil Science Society of America Journal 66:826-833.

Ritchie, N. J., M. E. Schutter, R. P. Dick, and D. D. Myrold. 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Applied and Environmental Microbiology 66:1668-1675.

Schutter, M.E., and R.P. Dick. 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Science Society of America Journal 64:1659-1668.

Schutter, M. E., and R. P. Dick. 2002. Microbial community profiles and activities among aggregates of winter fallow and cover-cropped soil. Soil Science Society of America Journal 66:142-153.

Tunlid, A., and D. C. White. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil, p. 229-262. *In* G. Stotzky and J. M. Bollag (ed.), Soil Biochemistry, vol. 7. Marcel Dekker, New York.

van Aarle, I. M., and P. A. Olsson. 2003. Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Applied and Environmental Microbiology 69:6762-6767.

Vestal, J. R., and D. C. White. 1989. Lipid analysis in microbial ecology. Quantitative approaches to the study of microbial communities. Bioscience 39:535-541.

Voroney, R. P., J. A. van Veen, and E. A. Paul. 1981. Organic C dynamics in grassland soils. II. Model validation and simulation of long term effects of cultivation and rainfall erosion. Canadian Journal of Soil Science 61:211-224.

Waldrop, M. P., and M. K. Firestone. 2004. Microbial community utilization of recalcitrant and simple carbon compounds: impact of oak-woodland plant communities. Oecologia 138:275-284.

White, D. C., and D. B. Ringelberg. 1998. Signature lipid biomarker analysis, p. 255-272. *In* R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (ed.), Techniques in microbial ecology. Oxford University Press, New York.

Wilkinson, S. G. 1988. Gram-negative bacteria, p. 299-457. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids, vol. 1. Academic Press, San Diego.

Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biology and Fertility of Soils 29:111-129.

Chapter 3

Nitrogen and Soil Moisture Controls on Succession of Soil Microbial Communities and Enzyme Activities during the Decomposition of Coconut and Maize Residues

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Abstract

Coconut husks (CH) are widely found in the tropics but are typically discarded in landfills or burned. CH could be used as an amendment to improve degraded tropical soils but information is lacking on the biological responses and controls on CH decomposition. The objective was to investigate microbial and C-cycle enzyme activity responses to N and soil moisture during the decomposition of CH. A control (soil only), along with CH and maize stover (MS) were amended (4 mg C g^{-1} soil) to a Brazilian soil in combination with two N rates (10 [LN], or 100 [HN] μg N g^{-1} soil) and incubated (24^{o}C) for 130 d under optimal moisture (-0.12 MPa). This was followed by a simulated seasonal drought from 130 to 290 d (-1.5 MPa) with a return to optimal moisture from 290 to 425 d. Seven destructive samples were taken (12, 33, 54, 112, 212, 350 and 425 d) and analyzed for respiration, total biomass-C, PLFA-based community structure and C-cycle enzyme activities (invertase, cellulase, xylanase and laccase). Residue chemical composition was an important factor in determining the composition of microbial community structure and enzyme activity with the recalcitrant CH having subtle or non-existent responses to N or to changes in moisture regimes. In contrast, MS amended soil became fungal-dominated and had increased rates of enzyme activity. In MS soil, the higher N rate widened fungal-to-bacteria ratio, increased the activity of hydrolytic enzymes, and decreased respiration rates and the activity of laccase. After about 50 d, respiration rates were significantly lower in the high than in the low N rate of MS-amended soils. This divergence in respiration between high and low N coincided with a shift in microbial structure and elevated laccase activity. At this point the fungal FAME marker (18:2ω6c) ratio between neutral lipid (NL) and glycolipids (PL) pools increased in MS-LN over MS-HN suggesting that N could have limited fungal community in this period. We hypothesize that the increased losses of C by

respiration in MS-LN could be related with the lower conservation of soil C by bacterial-dominated communities and/or with an increased activity of laccase under LN. Moreover, soil moisture content has also significantly impacted microbial community structure. A temporary bacterial-dominated community developed during the dry period in the MS-LN, which would also be in line with a second, short-lived period of higher respiration rates in this treatment compared with MS-HN upon rewetting. Finally, drought has also resulted in a more stressed gram-negative community in NR-HN, but the same was not valid for NR-LN. Microbial community structure in CH was not affected by changes in moisture content.

Introduction

Management of organic inputs is becoming increasingly important with the interest in developing more sustainable agricultural systems. Crop residues left in the field after harvest and other organic inputs are the raw material for humus formation and may impact the mineral nutrition of subsequent crops and affect soil quality. Therefore, knowledge about decomposition dynamics and the microbial community that controls this process is essential for management of agroecosystems.

The chemical composition of plant residue (substrate quality) is one of the major factors controlling their decomposition rates (Martens, 2000; Melillo et al., 1982). Some indexes of substrate quality, such as the lignin content, C:N ratio, and lignin:N have been proposed. Increases in these properties have been reported to slow the decomposition rates of plant material in the soil (Melillo et al., 1982; Melillo et al., 1989; Quemada and Cabrera, 1995; Taylor et al., 1989; Tian et al., 1992). Because dead plant residues from row crops are primarily cell wall material (carbohydrates and aromatic C compounds), they generally have a wide C:N

ratio (60 to 150). The C:N ratio of decomposers (5 to 15) is generally much lower than that, and this discrepancy may result in N limitation for microbial growth and activity, which consequently could decrease the rates of decomposition of residues added to the soil (Recous et al., 1995; Sakala et al., 2000).

It has been long recognized that N plays an important role in determining the rate at which organic matter is decomposed (Aber and Melillo, 1982; Chandra and Bollen, 1960). Most agricultural crop residues have a high C:N ratio of, and would be expected to have higher rates of decomposition when put in N-deficient soils amended with N fertilizers to enable growth of decomposer microorganisms. However, this has not been a consistent outcome of N inputs which have led to increases (Recous et al., 1995; Sakala et al., 2000), decreases (Sjoberg et al., 2004), or no change (Pinck et al., 1950) in the observed decomposition rates of crop residues. It has been suggested N mineralization rates of both litter and SOM is dependent on the chemical composition of the substrate (Fog, 1988; Neff et al., 2002). As a result, N additions could increase decomposition rates of some C substrates while, simultaneously, decreasing rates of others (Berg and Matzner, 1997; Fog, 1988).

Changes in soil enzyme activities (Carreiro et al., 2000; Henriksen and Breland, 1999; Keyser et al., 1978; Sinsabaugh et al., 2002) and in microbial biomass, activity and composition (Agren et al., 2001; Clay and Clapp, 1990; Lilleskov et al., 2001; Sinsabaugh et al., 2002) due to N availability have been observed or suggested but are not well understood.

Soil water content also has been shown to play an important role in determining the rates of decomposition of organic residues (Appel, 1998; Davidson et al., 2006). Dormancy or spore formation in soil microorganisms (Harris, 1981) are adaptations to low water potential that can result in substantial reductions in respiration per unit biomass or reductions in total respiratory biomass. Moreover, microbial respiration can be limited by access to carbon

substrates because of low water content and the resulting decrease in diffusion of carbon substrates, extracellular enzymes, and microbial mobility (Grant and Rochette, 1994).

Wet-dry cycles are short-term perturbations that can affect the availability of both C and N substrates in soils (Kieft et al., 1987) as well as elicit dynamic responses from soil microbes in terms of activity, biomass (Lundquist et al., 1999b) and composition (Frey et al., 1999; Halverson et al., 2000). The increased availability of organic compounds in response to rapid changes in soil water potential has been associated to the release of microbial intracellular solutes induced by osmotic shock (Halverson et al., 2000) or cell lysis (Bottner, 1985), and to the degradation of soil aggregates, which exposes protected organic matter (Lundquist et al., 1999a).

In this study we have compared how the decomposition of two materials of contrasting quality (corn stover and coconut husk) are affected by N rates and variation in soil moisture with time, as well as the concurrent changes in the microbial community and enzyme profiles during the incubation period. We were interested in understanding microbial controls on coconut husks (CH) it is a crop residue produced in large amounts in the tropics. This residue is either buried in landfills or burned in the field. Because soil from producing areas are, in general, coarse-textured and with low SOM content, amending soil with CH could improve soil quality and improve water-holding capacity of the soils that is critical for semi-arid tropical regions for optimal crop growth.

Material and Methods

Experimental Design

Soil (fine-loamy kaolinitic isohyperthermic Typic Fragiudults) was collected (0-20 cm) from the Umbaúba Experimental Station (Brazilian Agency for Agricultural Research,

Embrapa), located in a coastal tableland ecosystem in Sergipe State, Northeastern Brazil (11°16' S, 37°26', 105 m altitude). This soil had a pH of 5.3, was 1.30% organic C, 0.08% N, 5 mg P kg^{-1}, 72% sand, 10% silt, and 18% clay. The sample was sieved (<4 mm), air dried and stored. Soil was limed ($CaCO_3$:$MgCO_3$, 4:1 molar ratio) to about pH 6.0, fertilized with P (20 mg P kg^{-1} soil, as triple super phosphate), K (30 mg K kg^{-1} soil, as K_2SO_4) and S (12 mg S kg^{-1} soil, as K_2SO_4), and given a conditioning incubation at 25°C for 45 days (-0.12 MPa). After that period, soil samples were dried to approximately –1.0 MPa at room temperature and divided into 200 g (dry weight basis) portions for the incorporation of N fertilizer and amendment with organic residues.

Four replications of a control (soil only), along with coconut (CH) and maize stover (MS) were amended (4 mg C g^{-1} soil) to a Brazilian soil in combination with two N rates (10 [LN], or 100 [HN] μg N g^{-1} soil) and incubated (24°C) for 130 d under optimal moisture (-0.12 MPa). This was followed by a simulated seasonal drought from 130 to 290 d (-1.5 MPa) with a return to optimal moisture from 290 to 425 d. Seven destructive samples were taken (12, 33, 54, 112, 212, 350 and 425 d) and analyzed for respiration, total biomass-C, PLFA-based community structure and C-cycle enzyme activities (invertase, cellulase, xylanase and laccase). Control soil also was mixed to mimic the disturbance experienced by treated soils. Total C, total N, C:N ratio and the proportions of cellulose, lignin and ash fractions in the MS and CH amendments are shown in Table 3.1. Acid-detergent fiber contents were determined according to the Goering and van Soest (1970) method adapted for Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NY). The mass of residue lost after the acid-detergent treatment was regarded as the amount of acid detergent solubles, and includes hemicellulose and water-soluble fractions. ADF residue would include cellulose, lignin and ash. Cellulose content was calculated as the difference between the ADF residue before and after treatment with sulfuric acid (72% by

weight). The remaining material was burned in an oven at 550^{o}C in order to determine the ash content. Lignin content was calculated as the mass of sulfuric acid-treated residue corrected for ash.

Soil moisture was initially adjusted to -0.12 MPa and then corrected weekly gravimetrically. To reduce variation in soil moisture between watering, each microcosm was covered with a plastic sheet with 10-15 holes of about 1 mm of diameter. The moisture regimen was maintained during the incubation to simulate the average soil moisture (0-20 cm) regimes that occurs annually in the Brazilian coastal tableland region from where the soil was sampled. The soil water content was maintained at -0.12 MPa from 0 to 130 d; followed by slow drying to -1.5 MPa, at d 160 and held at -1.5 MPa till 290 d. At day 318, soil moisture was slowly returned to -0.12, and maintained till day 425. During the drying or rewetting phases, the soil water content

Table 3.1. Chemical composition of maize stover (MS) and coconut husk fiber (CH) used to amend soil.

	C:N	Total C[†]	Total N	AD soluble[‡]	Lignin	Cellulose	Ash[¶]
		---------- % ----------					
MS	140	42	0.3	56	3	36	5
CH	110	44	0.4	15	45	31	9

[†] Dry combustion
[‡] Acid-detergent soluble fraction; include hemicellulose and water soluble fraction
[¶] Ash determined by the ignition of the material remaining after sulfuric acid treatment

was lowered or raised, respectively, by about 4% every week until the desired moisture level for that phase was reached. For the drying phase the cover was removed until the desired moisture was reached for a given week and this moisture level was maintained gravimetrically the remainder of that week. Treatments were destructively sampled for laboratory analyses at days 12, 33, 54, 112, 212, 350 and 425 of the period of incubation. One set of samples was placed inside Mason™ jars (1 L) during each sampling interval for monitoring of rates of CO_2 evolved.

Incubation conditions for microcosms outside and inside the jars were identical, except that, during periods of CO_2 measurements, jars were covered with a lid equipped with a rubber septum. To avoid anaerobic conditions, Mason™ jars were kept closed for accumulation of CO_2 for periods of 24 h during the first two weeks, 48 h from weeks 3 through 5, and 72 h for the remainder period. At each sampling, microcosms within the Mason™ jars were prepared for laboratory analysis, and a new set of microcosms placed in the Mason™ jars to continue the respiration analysis until the next sampling.

Laboratory analyses

The CO_2 content of the headspace was analyzed by gas chromatography (Carle Series 100 AGC, Loveland, CO) and expressed as μg C-CO_2 g^{-1} soil d^{-1}.

At each of the seven sampling dates the following analyses were performed. For the analysis of community PLFA profiles, we have followed the protocol described in Butler et al. (2003). This includes a one-phase chloroform, methanol and phosphate buffer extraction of total lipids (Bligh and Dyer, 1959), the fractionation of the lipids into neutral, glyco- and phospholipids (White and Ringelberg, 1998), and the mild alkaline methylation of the phospholipid fraction to obtain the fatty acid methyl esters (FAME) (White and Ringelberg, 1998). FAME composition in the extracts was determined with an Agilent 6890 gas chromatograph (Agilent Inc., Palo Alto, CA) equipped with a 25–m HP Ultra-2 column (internal diameter, 0.2 mm; film thickness, 0.33 μm) and a flame ionization detector (FID-GC). The temperature program ramped from 170 to 280^{o}C at 4^{o}C per min, with 5 min at 280^{o}C between samples to clean the column.

Individual fatty acids were identified relative to several standards: 37 FAMEs mixture (FAME 37 47885-4; Supelco Inc., Bellefonte, PA), 24 bacterial FAMEs mixture (P-BAME 24

47080-U; Supelco, Inc.), and MIDI standards (Microbial ID Inc., Newark, DE). Quantification of FAMEs was accomplished by using varying concentrations of tridecanoic FAME (Supelco, Inc.) and allowed peak areas to be converted to a molar basis.

Standard nomenclature is used to describe FAMEs. They are designated by the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the methyl (aliphatic) end (ω) of the molecule. Cis geometry is indicated by the suffix "c". The suffixes "a" and "i" refer to anteiso- and iso-branched fatty acids. The suffix 10-Me indicates a methyl group on the tenth carbon atom from the carboxyl end of the molecule; "OH" indicates a hydroxyl group, and "cy" indicates cyclopropane fatty acids.

The summed masses of FAMEs (pmol FAME g^{-1} soil) reported as typical of fungi (18:2ω6c, 18:1ω9c), Gram-negative (GN) bacteria (18:1ω7c, 17:0cy, 19:0cy), Gram-positive (GP) bacteria (15:0i, 15:0a, 16:0i, 17:0i, 17:0a), and actinomycetes (ACT) (16:0 10-Me, 17:0 10-Me, 18:0 10-Me) were used as signatures for these microbial groups. The ratio 19:0cy to its precursor 18:1ω7c (19cy to 18:1ω7c) was used as an indicator of nutritional and desiccation stress on GN communities.

A parallel extraction of FAMEs by EL-FAME, a method producing FAMEs from phospholipid (PL), neutral lipids (NL) and glycolipids (GL), was carried out from all samples. The amount of 18:2ω6c in the NL fractions has been used as a measurement of lipid storage (Bååth, 2003; Olsson et al., 1995). Calculations from data in Chapter 1 have shown that the amount of 18:2ω6c in the GL fraction accounts for only 3 to 12% of the sum of this FAME in PL, NL and GL fractions. Therefore, the ratio between the absolute amounts of fungal marker 18:2ω6c in EL-FAME and PLFA extracts ($EL:PL_{18:2\omega6c}$) was used as a proxy for the ratio of this fungal marker between NL and PL fractions. The NL:PL ratio has been shown to increase in conditions where the supply of C is in excess relative to that of N (Bååth, 2003).

Activities of four enzymes related to the C cycle (laccase, xylanase, cellulase, and invertase) were determined in soil samples stored for 3 to 5 days at 4^{o}C at the moisture content of the corresponding sampling date. Activities of the hydrolytic enzymes xylanase, cellulase and invertase (Schinner and von Mersi, 1990) were determined after incubating 1 g of soil (moist basis) (50^{o}C, 24 h) with 10 ml of 2*M* acetate buffer (pH 5.5) containing as respective substrates, xylan from oat spelts (1.2% w/v), carboxymethyl cellulose sodium salt (0.7% w/v) or saccharose (1.2% w/v). Reducing sugars released during incubation reduced alkaline potassium hexacyanoferrate (III) to potassium hexacyanoferrate (II), which was measured spectrophotometrically at 690 nm. Analytical triplicates were used for each soil sample and enzyme. For each sample, a blank mixture with soil and buffer, but no substrate, was incubated in the same conditions as above and analyzed to discount the background of reducing sugars in the soil. Results were calculated as μg glucose released g^{-1} soil h^{-1}.

Laccase was extracted by a modified method of Lang et al. (1997). Soil samples (1 g) were placed in an Eppendorf tube and mixed with 2 ml of 50 m*M* phosphate buffer (pH 7.0). The tubes were shaken (200 rpm) for 1 h at room temperature and, next, centrifuged at 3000 x *g* for 5 min. Supernatant was carefully decanted into a new Eppendorf tube and centrifuged at 5000 x *g* for 15 min. Aliquots of 200 μl were carefully taken from the supernatant in tubes from the second centrifugation round and used for quantification of laccase. Samples were extracted in duplicates. Laccase activity was measured by the oxidation of ABTS (2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid)) (Niku-Paavola et al., 1990). The extract aliquots were mixed with 3 ml 25 m*M* succinate buffer (pH 4.5) containing ABTS (2.75 mg ml^{-1}), and incubated at room temperature (Niku-Paavola et al., 1990). Rates of ABTS oxidation were quantified by measuring the absorbance (436 nm) at every 5 min. (from 5 to 45 min.) after mixing the extract with the reagents. A blank reaction containing 3 ml of ABTS-succinate

buffer mixture and 200 µl of extraction phosphate buffer was carried out and measured in the same way. The rate of increase in absorbance (Δ Abs min^{-1}) was converted into the rate of ABTS oxidation using an $\varepsilon = 29300\ M^{-1}\ cm^{-1}$ for the ABTS-azine cation radical (Niku-Paavola et al., 1990), and results were calculated as nmol of ABTS-azine cation produced g^{-1} soil h^{-1}.

Microbial biomass-C (MBC) was determined by the fumigation-extraction method (Vance et al., 1987) using a k_C of 0.45 (Jenkinson, 1988). For each sample, three fumigated and two non-fumigated replicates were evaluated. The $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ contents were determined by titration of the steam-distilled soil extracts (2*M* KCl), according with Bremner and Keeney (1965). A soil suspension (1:2, soil:water) was used for pH measurements.

Statistical Analysis

A two-way ANOVA was used to test the effect of residues and N rates on the cumulative CO_2-C released at each headspace sampling date. Significant differences from ANOVA ($p<0.05$) were further analyzed using the Fisher LSD test. A three-way ANOVA was used to analyze log-transformed data to determine effects of N rate, residue, and time on respiration rates. Because a significant triple interaction between time, N and residue was found, we analyzed the double interaction between time and N, within each residue. For the residue treatments presenting a significant two-way interaction, respiration rate between N treatments were compared by the Fisher LSD test within each sampling time. This approach enabled us to determine periods of time when an N effect on respiration rates took place.

Shifts in the composition of PLFA profiles were analyzed by non-metric multidimensional scaling (NMS) using the PC-ORD package (MjM Software Design, Gleneden Beach, OR). Before the analysis, PLFA data were converted to mol percent of peak totals.

Correlations between PLFA mol percent concentrations and NMS coordinates were calculated to identify PLFAs which gradients were associated with axes 1 and 2.

Nitrogen, microbial biomass C, enzyme activities and PLFA-derived physiological ratios (19cy to 18:1ω7c and EL:$PL_{18:2\omega6c}$) were analyzed using sums of squares univariate regression tree models (SS-URT) (De'ath and Fabricius, 2000), with time, residues, and N rate treated as categorical explanatory variables. For these response variables, tree regression models were chosen instead of ANOVA because of the general occurrence of significant high order interactions between the explanatory variables, and because response variable data in general have not met the assumptions required for the use of parametric methods.

SS-URT explains the variation of a single numeric response variable using explanatory variables that may be numerical and/or categorical (Breiman et al., 1984; De'ath and Fabricius, 2000). Starting with all the data represented by a single node at the top of the tree, the tree is grown by repeated binary splitting of the data. Each split is defined by a simple rule, based on a single explanatory variable. The explanatory variable used in each split is chosen among all the others for being the one resulting in the smallest sums of squares within the two nodes (impurity) formed by the split. Splitting procedure is continued until an overlarge tree is grown, which is then pruned back to the desired size (number of leaves). A series of 20, 10-fold cross-validations (Breiman et al., 1984) was run in this study to choose the most frequently occurring (modal) tree size with minimum error rate (De'ath and Fabricius, 2000). In general, these trees were too large to interpret. As an alternative to this drawback, Breiman et al. (1984) suggested the 1-SE rule whereby the best tree is taken as the smallest tree such that its estimated error rate is within one standard error of the minimum. Use of 1-SE rule can result in a smaller tree than the suggested by the minimum cross-validated-error, but with minimal increase in the estimated error rate (at most <1-SE). The minimum number of observations per group was set as three.

Tree graphs depict all the split rules used for the group separation. The length of the branches in the tree is proportional to the variability explained by the independent variable used in the split. The terminal nodes, also called leaves, are labeled with the response variable means between all the samples included in them.

Although averages from two terminal nodes directly derived from the same parental node are different, this may not be true between averages across different parental nodes. Therefore, as a complimentary analysis to tree models, we compared averages among all the leaves of the selected tree using Bonferroni procedure ($p<0.05$). A library of S-Plus functions for tree routines (RPART: Recursive partitioning), developed by T. Therneau (unpublished data), was used for all SS-URT. S-Plus (version 4.0) statistical software (Statistical Science, 1999) was used for these analyses.

PLFA composition, a variable with multivariate responses, was analyzed by sums of squares multivariate regression tree (SS-MRT) models (De'ath, 2002). SS-MRT is a natural extension of SS-URT, and the concepts described above for the univariate analysis also form the basis for SS-MRT. For SS-MRT, impurity of a node can be redefined as the sum of squares about the multivariate mean. Geometrically, this is simply the sum of squared Euclidean distances of samples about the node centroid. Analogously to SS-URT, each split minimizes the sums of squared distances (SSD) of samples from the centroids of the nodes to which they belong. Pruning and selection of the tree size was preceded as explained in the SS-URT. For those analyses, we have used a library of SS-URT routines (T. Therneau, unpublished data) extended by the inclusion of additional C routines to fit multivariate regression trees (De'ath, 2002). S-Plus (version 4.0) was used for all the SS-MRT analyses. Although in this paper we have shown tree model graphs for only two of the variables studied (mineral N content and PLFA), the description of the results in terms of differences between treatments was based in

those models. The tree model pictures for all the other variables are displayed as supplemental material in the Appendix of this thesis.

Results

Microbial respiration

At all sampling dates, cumulative CO_2-C was significantly ($p<0.01$) affected by residue treatments in the order: MS > CH > NR (Figure 3.1). Amending soil with CH residue caused an increase of 43% in the CO_2-C released after 425 d, regardless of the N rate. This CO_2-C loss, when expressed on a percentage of the amount of C added by each amendment, (CO_2-C $_{amended}$ − CO_2-C $_{control}$)/(C $_{amendment}$), equaled 13% of the C in CH. In contrast to NR and CH, the cumulative CO_2-C at the end of the incubation for MS was clearly affected by N rates ($p<0.01$), with losses being 18% greater from LN than HN samples. Compared with NR, MS-LN and MS-HN lost 3.4 and 2.9 times more C, respectively. The respired C of the MS amendment C was calculated as 72% for LN and 57% for HN.

Analysis of variance showed there was a significant triple interaction involving sampling time, residue, and N ($p<0.0001$). Therefore, we analyzed the double interaction between time and N, within each residue. For NR, this interaction was not significant ($p>0.10$), but time, as a single effect was significant ($p<0.01$). The double interaction was significant for CH ($p<0.05$) and for MS ($p<0.001$). In order to determine periods of time when the N effect was occurring in MS or CH, we compared respiration rate between N treatments, within each residue and sampling time (Figure 3.2). Differences between N rates within CH were found in 4 out of the 34 samplings ($0.01< p <0.05$). Those differences occurred sparsely rather than in consecutive

dates, indicating that no long-lasting effects of N have taken place during the period of incubation of CH samples.

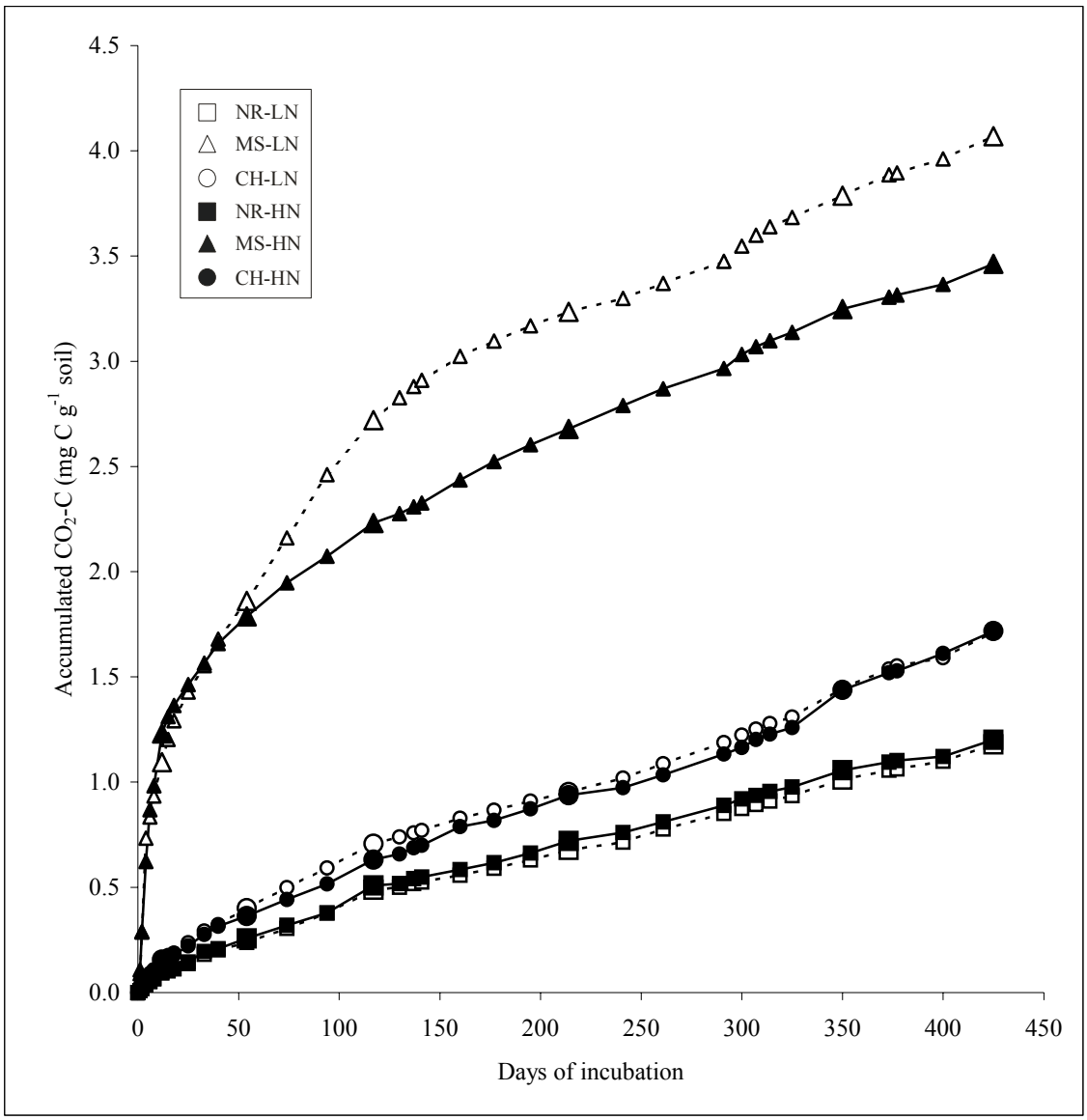

Figure 3.1. Accumulation of CO_2-C respired from soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. Larger symbols refer to the times of destructive sampling. Accumulated CO_2-C averages differed between MS-HN and MS-LN at 54 and 74 days at 5% of probability, and after 74 days at 1% of probability.

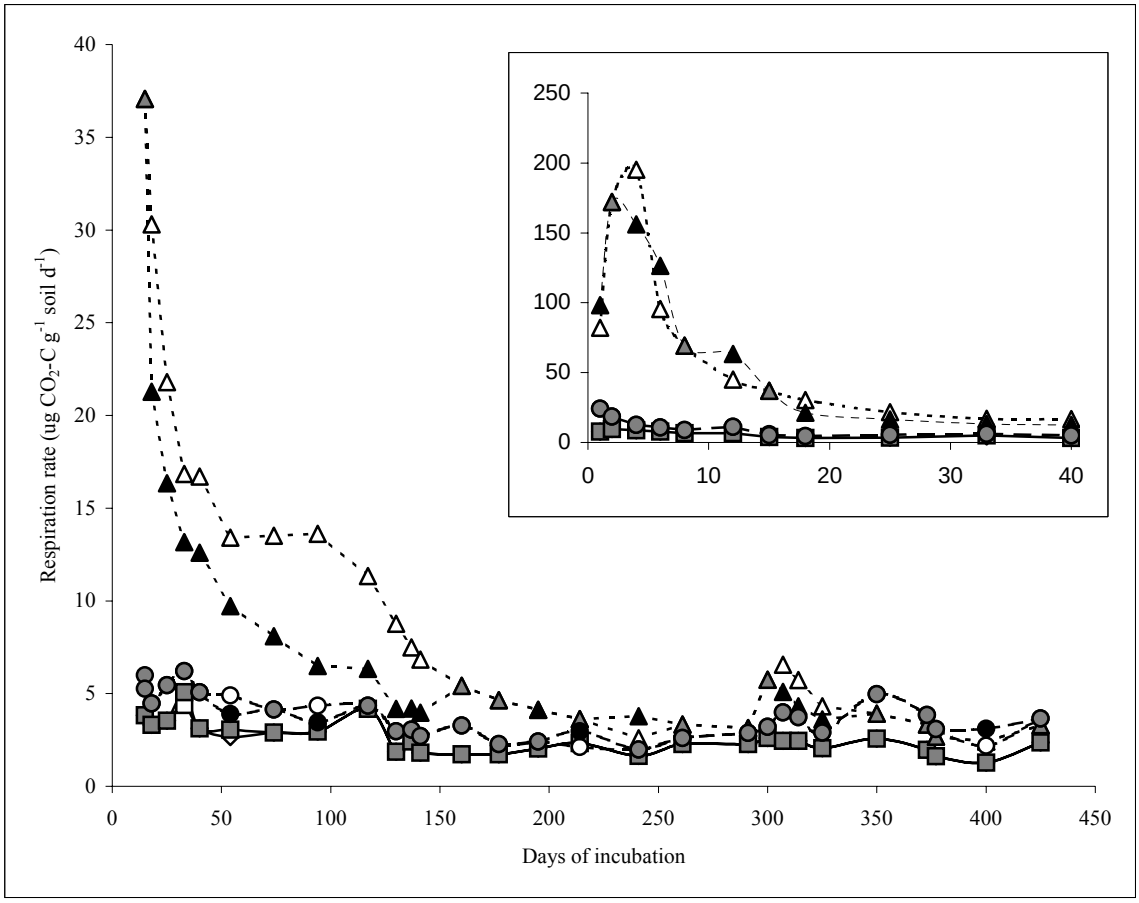

Figure 3.2. Microbial respiration rates (μg CO_2-C g^{-1} d^{-1}) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. Insert depicts a more detailed curve of microbial respiration rates between days 0 and 40. Squares, triangles and circles represent NR, MS and CH, respectively. Grey symbols represent averages between LN and HN, within each residue and sampling time, whenever the N rate effect was not significant ($p>0.05$). Black and white symbols represent HN and LN averages, respectively, when differences in N rate were significant ($p>0.05$), within each combination of time and residue.

A more prominent N effect on respiration rates was found in MS. Through the first 15 days, the effect was not consistent, with higher rates oscillating between LH and HN. However, in all the 11 samplings between days 18 and 141, respiration rates in LN were always significantly higher than for HN ($p<0.01$ in all dates, except days 25 and 54, when $p<0.05$). This

N effect disappeared as the moisture content reached its minimum (160 d). During the dry period, except for a higher respiration rate from HN than LN samples observed in MS at date 241, the two N rates had very similar CO_2-C emission rates. Upon rewetting, an increased respiration rate was again observed for LN in comparison with HN (day 307 to 325), but this time, it was weaker ($p<0.05$, for all samplings) and short-lived than that observed for the pre-drought period. After that, N rates did not significantly affect respiration rates in MS samples.

Accumulated CO_2-C in LN-MS, from day 40 to 141 MS exceeded HN-MS by 0.56 mg CO_2-C g^{-1} soil (Figure 3.2). This amount accounted for 93% of the difference between LN and HN samples in the CO_2-C lost during the entire incubation compared to the 307 to 325 d period which evolved only 2.5% of the total CO_2-C.

Mineral Nitrogen

The selected 8-leaf tree regression model describes mineral nitrogen (min-N) dynamics in soil samples (Figures 3.3.a and 3.3.b). The model explained 86% of the variation in min-N; with time, residue and N explaining 42, 25 and 19% of that variability, respectively. Data were split in two temporal clusters: up to 212 d and later dates (Figure 3.3.b). Both temporal clusters were further split according to the residue treatment, with MS samples having smaller min-N contents than their CH or NR counterparts. All the four resulting clusters were then divided according to N rates. Within each combination between N rate and residue treatment, min-N was quite constant or increased slightly up to 212 d, but then had a steep increase afterward. During the period up to 212 d, N was strongly immobilized in the MS-LN samples (Figures 3.3.a and 3.3.b), while MS- HN had a less restrictive N availability, with values similar to the obtained for CH and NR amended with LN. The highest min-N contents have been observed in NR-HN and CH-HN.

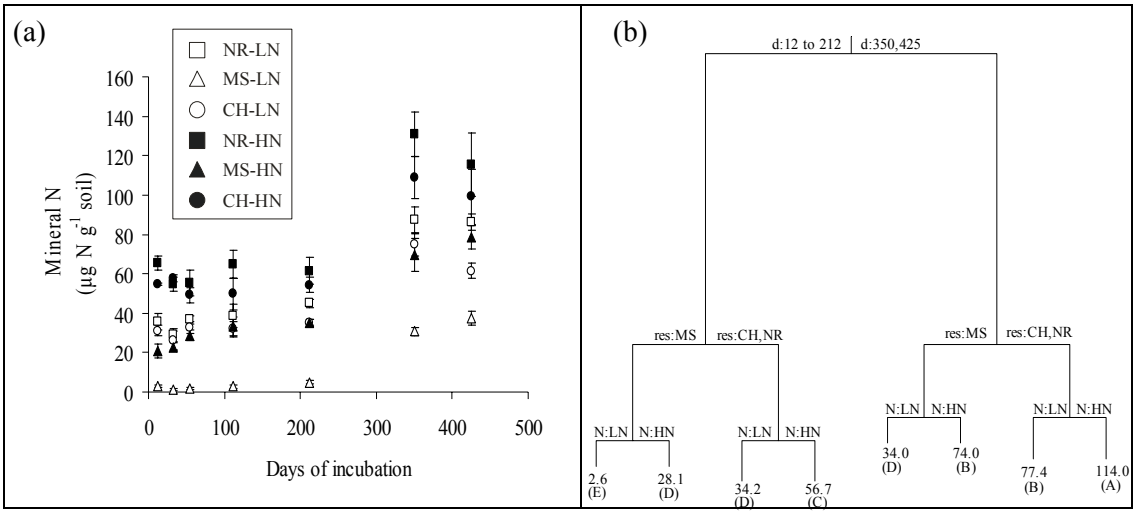

Figure 3.3. (a) Mineral N content in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 µg N g^{-1}; and HN, high N = 100 µg N g^{-1}) during 425 days. Analyses were done in samples collected at days 12, 33, 56, 112, 212, 350, and 425 after the start of the incubation. Sampling at 212 d was taken approximately in the middle of a simulated dry period (water potential = -1.5MPa). Vertical bars represent ±1 SE (n=4). (b) Sum of squares univariate tree model representing factors associated with the variability of mineral N content in soil. Branch length is proportional to the variability explained by each split. Explanatory variables associated with the decrease in the node sum of squares are shown at the sides of each intermediate node; and their values indicate which groups of treatments or sampling time are included in each cluster formed after the split. Cluster averages are represented at the tip of the terminal nodes (leaves). Averages followed by the same letters are not significantly different ($p>0.05$) according with Bonferroni test. For details about aspects related to the tree growing and pruning, see Material and Methods.

When data from all the samples were analyzed together, a strong correlation was detected between min-N and NO_3^--N contents (min-N = 0.98 NO_3^--N + 5.54, R^2=0.95). The "close to the unit" slope in this equation indicates that the min-N pool in soil at the sampling dates was composed primarily by NO_3^--N. NH_4^+-N contributed little to the min-N pool, except in the first sampling for CH and NR treatments, when this N form was quantitatively as important as NO_3^- -N. Therefore, the sharp increase in min-N contents after the dry period (>212 d, Figure 3.3.a) was derived from a high net mineralization of N and intensive nitrifying activity.

Soil pH

A 7-leaf tree model represented soil pH changes, and explained 91% of the total variability. Partially explained variability was due to residue, N and time were 42, 26 and 23%, respectively.

The NR and CH samples, regardless of the N rate, and MS-HN samples had a similar pH at 12 d (Figure 3.4). Final pH values followed the order CH-HN = NR-HN < CH-LN = NR-LH < MS-HN. In MS-LN, pH was highly buffered at 5.68 from d 1 to 212, but, after soil rewetting, pH dropped to 5.31. In summary, pH decreased faster at HN than LN, within each residue treatment, and this decrease was more accentuated in NR and CH than MS.

Microbial biomass C

A 3-leaf model explained 89% of the total variability for MB-C. Residue had the largest effect on MB-C, explaining 87% of the total variability, followed by time (2%). As expected, there was a strong residue effect (Figure 3.5). This was a long-lasting effect, which spanned the incubation period. MB-C in the MS soil was rather constant, except for a decrease observed between dates up to 112 d and the later samplings. Unlike MS, CH did not show any difference from NR for MB-C. N rates not affect MB-C, regardless of the residue treatment or time of sampling.

Microbial community structure

A 2-dimensional NMS ordination of PLFA profiles (Figure 3.6.a) depicts changes in microbial structure (stress = 7.0; final instability = 0.0004). This ordination represented 98% of the variability in the total PLFA composition of samples; with 9% and 89% of this variability

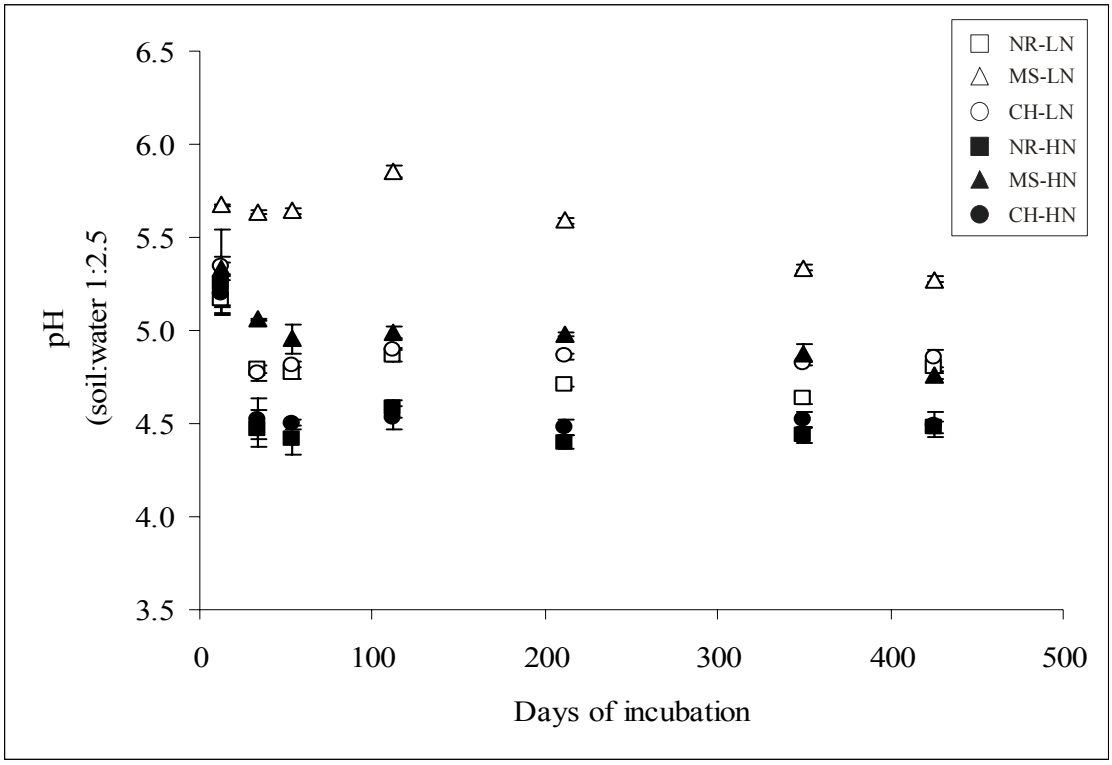

Figure 3.4. Soil pH in samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. Analyses were done in samples collected at days 12, 33, 56, 112, 212, 350, and 425 after the start of the incubation. Sampling at 212 d was taken approximately in the middle of a simulated dry period (water potential = -1.5MPa). Vertical bars represent ±1 SE (n=4).

distributed along Axes 1 and 2, respectively. Variations in PLFA profiles along Axis 1 were significantly ($p<0.01$) and negatively associated with three monosaturated compounds, 16:1ω5c, 16:1ω7c and 18:1ω7c (Figure 3.6.b). Changes in profile composition along Axis 2 were positively associated with PLFA markers for fungi ($p<0.01$), and negatively associated with GN, GP, and actinomycetes markers ($p<0.01$). The fungal:bacterial ratio was used as an integrative variable to summarize the results from all these individual microbial groups. This variable was significantly and negatively associated with NMS scores along Axis 2 ($r = -0.97^{***}$).

To help determine which factors were associated with the variation in PLFA composition, the NMS scores along both axes were simultaneously modeled as a function of residue, N rates,

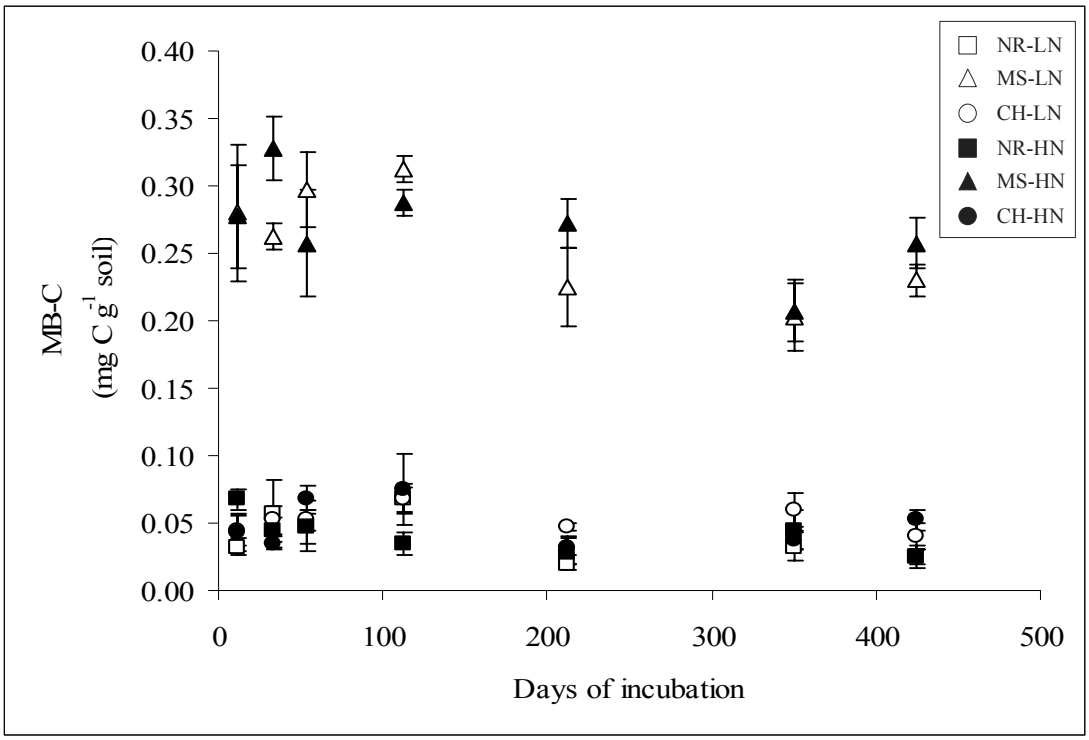

Figure 3.5. Microbial biomass-C in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days. Analyses were done in samples collected at days 12, 33, 56, 112, 212, 350, and 425 after the start of the incubation. Sampling at 212 d was taken approximately in the middle of a simulated dry period (water potential = -1.5MPa). Vertical bars represent ±1 SE (n=4).

and time, using the multivariate tree regression technique. With this procedure, an eleven-leaf tree was found that represented the major differences in microbial structure (Figure 3.6.c). This model explained 97% of the total variability in the dataset, with partial contributions of 85, 10, and 2% from residue, time and N, respectively. This strong residue effect can be observed mostly along NMS Axis 2, and resulted in a clear separation between MS samples and those from NR and CH (Figure 3.6.a). These results, along with those from the NMS ordination, showed that communities developed in MS amended soils became relatively more enriched in fungi and depleted in bacteria than those from the CH and NR treatments.

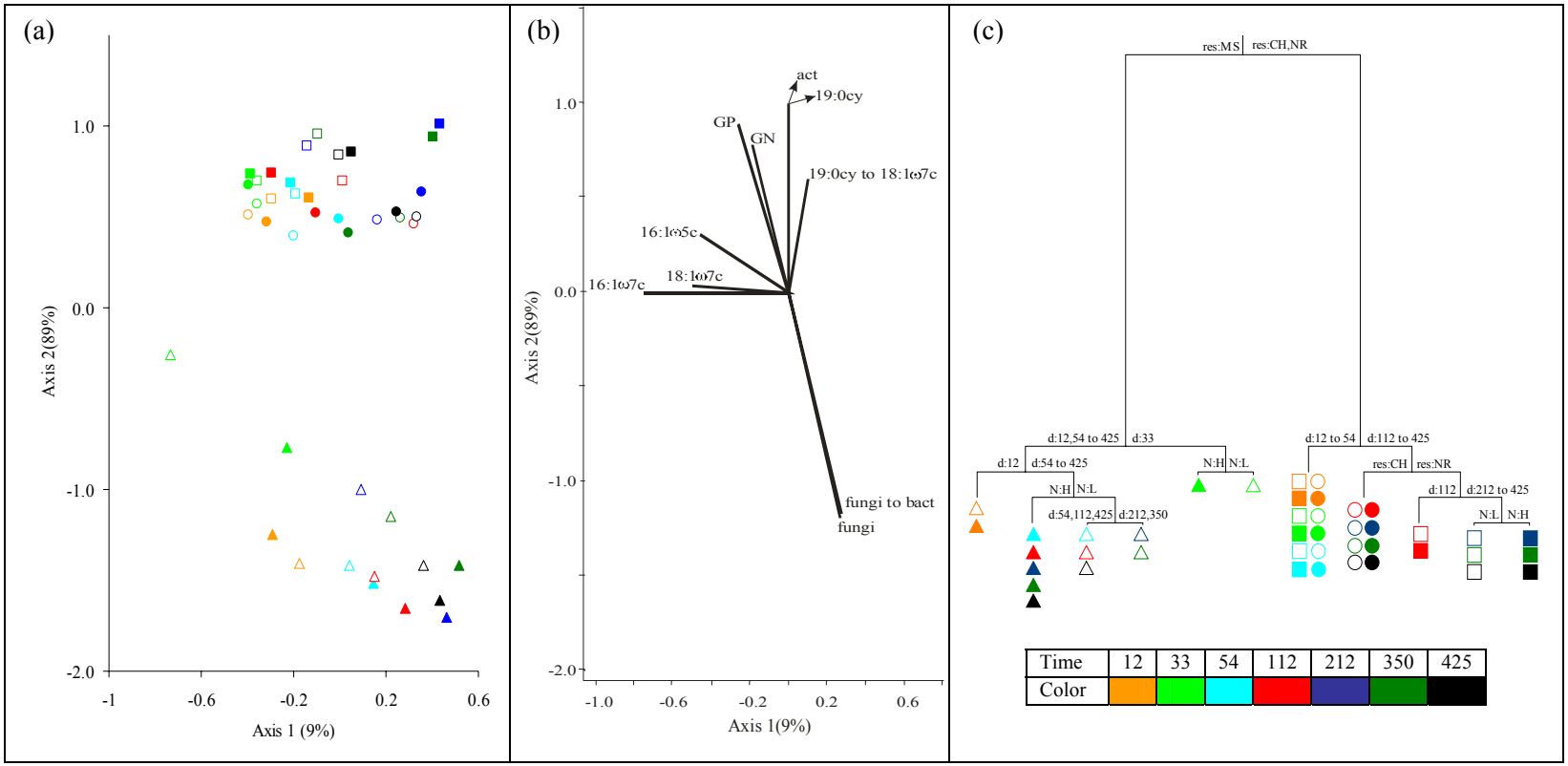

Figure 3.6. Soil microbial community composition assessed by PLFA as affected by residue (NR, no residue = squares; MS, maize stover = triangles; and CH, coconut husk = circles), N rates (LN, low N = empty symbols; and HN, high N = solid symbols) and time (see color legend). NMS plot (a), joint plot of microbial FAME correlated with NMS axes 1 and 2 (Pearson coefficient >0.3) (b), and multivariate regression tree (c). Communities represented by symbols under the same terminal node (c) were not separated by the tree model selected. For details on tree model, see text and Figure 3. The proportion of variance represented by each NMS axis is based on the correlation (*R*) between distance in the reduced NMS space and distance in the original space and is reported after each axis heading.

Within the MS branch, a temporary community, relatively more enriched in bacteria, was established at 33 d, as the PLFA composition from 12 d and ≥ 54 d were more similar to each other than with that at 33 d (Figures 3.6.a and 3.6.c). Nevertheless, the magnitude of this shift from 12 to 33 d was dependent on N rate, as LN samples had a stronger change toward a more bacterial-dominated community than HN. Although this trend persisted for the rest of the incubation period, the N effect was accentuated by the onset of the dry period. During this period, a further narrowing of the fungi:bacteria ratio was observed in the LN community. However, once the soil of these samples was rewetted, a progressive return toward the pre-drought condition of the PLFA composition was observed. In contrast to LN-MS, microbial community structure for MS-HN amended soil was not affected by moisture content.

CH had much less of an impact on the soil microbial community structure than MS. Indeed, no major differences were observed between communities from CH and NR during the three first sampling dates (12, 33 and 54 d) (Figures 3.6.a and 3.6.c). From 112 d onwards, NR samples shifted towards a more bacterial composition, while CH samples supported a relatively higher fungal community until the end of the incubation. Finally, in NR samples at ≥212 d PLFA composition was affected by N rates. Separation between samples at ≥212 d and earlier samples was subtler for LN-NR than for HN-NR. This N effect was expressed along Axis 1, with HN-NR having lower relative contents of three monosaturated FAMEs (16:1ω5c, 16:1ω7c and 18:1ω7c) than LN-NR.

Physiological Status Markers

An eight-leaf tree accounted for 91% of the variability in the stress marker (19cy:18:1ω7c ratio). Residue type explained 51% of the variation and was the major factor impacting the 19:0cy:18:1ω7c ratio, followed by time (38%) and N (2%).

MS had less stressed ratios than did NR and CH samples (Figure 3.7.a). Within MS, the 19cy:18:1ω7c ratio was fairly constant from 12 to 112 days, after which it increased and remained constant throughout the rest of the incubation period. It is interesting to notice that, although the increase in 19cy:18:1ω7c ratio coincided with the onset of the dry period, this ratio did not decrease to the pre-drought condition values upon soil rewetting. Temporal changes in 19cy:18:1ω7c ratio were better defined in the CH and NR cluster, following a monotonic pattern, with averages increasing in the order: 12 d = 33 d = 54 d < 112 d < 212 d = 350 d < 425 d. Finally, in the last three samplings, N impacted this ratio, with higher values being observed for HN than LN.

For the EL:$PL_{18:2\omega6c}$ ratio, a 5-leaf tree accounted for 64% of variability of the data. Time explained 47% of the variability in EL:$PL_{18:2\omega6c}$, whereas residue, and N rate contributed nearly 9% each. Samples at 33 d had a higher ratio than other dates (Figure 3.7.b). For samples from all the dates, except at d 33, a temporal separation in the EL:$PL_{18:2\omega6c}$ was observed, but that did not follow a linear pattern, as intermediate dates (54 through 350 d) had ratios narrower than either the first or last samplings. The 33 d samples were split into two residue clusters, one with MS and another comprising both CH and NR samples. At 33 d, ratios from MS samples were wider than in NR and CH, but only MS-LN significantly differed from these later. EL:$PL_{18:2\omega6c}$ at 33 d was 3.8 for LN-MS and 2.2 for HN-MS; what corresponded to an average 1.8 fold increase in the ratio under the more restrictive N situation.

Enzyme Activity

Invertase was affected by all the three explanatory variables (residue, N, and time) (Figure 3.8.a). A tree model with 9 leaves explained 80% of the data variability with time, residue and N explaining 44, 31 and 6% of the variation in invertase activity. Invertase activity was increased

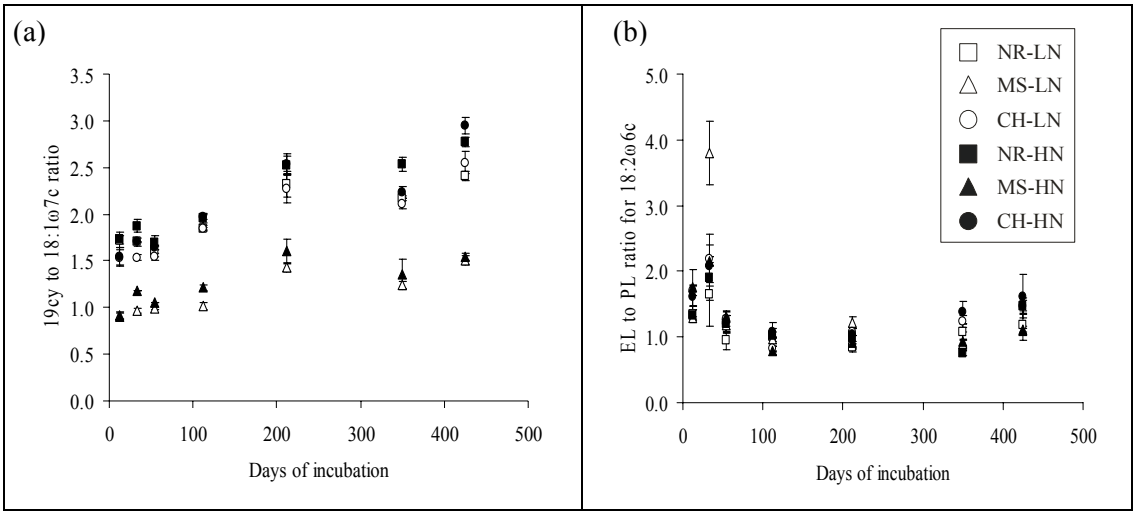

Figure 3.7. Values for the 19cy to 18:1ω7c ratio (a) and EL to PL ratio for 18:2ω6c (b) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days. Analyses were done in samples collected at days 12, 33, 56, 112, 212, 350, and 425 after the start of the incubation. Sampling at 212 d was taken approximately in the middle of a simulated dry period (water potential = -1.5MPa). Vertical bars represent ±1 SE (n=4).

remarkably right after the addition of MS, but was unresponsive to CH. In MS samples, a continuous and sharp decrease took place between d 12 to 112 d. From 12 to 54 d samplings, N was positively correlated with invertase in MS samples, but subsequently this effect either decreased or disappeared. After reaching a minimum at 112 d, invertase in MS peaked during the dry period and returned to the pre-drought condition after soil rewetting. For CH and NR, invertase activity was quite constant from 12 to 54 d, and, as observed for MS, reached a minimum at 112 d, and showed a transient peak at the dry period sampling. Nitrogen did not impact the activity of invertase in NR and CH samples.

A 13-leaf model represented about 90% of the variation in the cellulase activity. Residue, time and N explained 51, 31 and 8% of that variability, respectively. Samples with CH and NR, irrespective to the N rate, and MS-HN peaked at 33 d, decreased to a minimum at 112 d and

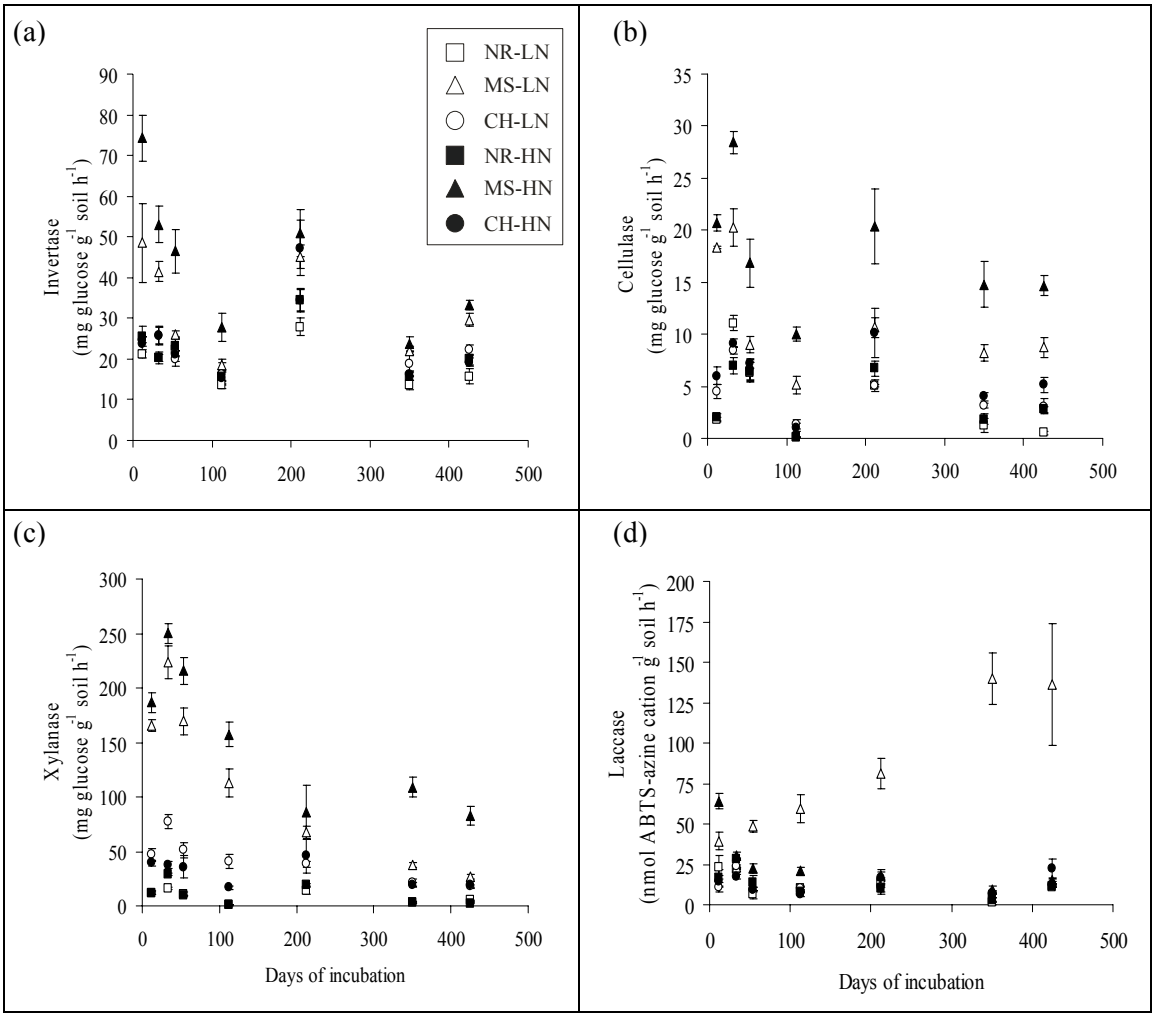

Figure 3.8. Activities of invertase (a), cellulase (b), xylanase (c), and laccase (d) in soil samples as affected by residue amendment (NR, no residue; MS, maize stover; CH, coconut husk) and N rates (LN, low N = 10 μg N g^{-1}; and HN, high N = 100 μg N g^{-1}) during 425 days of incubation. Analyses were done in samples collected at days 12, 33, 56, 112, 212, 350, and 425 after the start of the incubation. Sampling at 212 d was taken approximately in the middle of a simulated dry period (water potential = -1.5MPa). Vertical bars represent ±1 SE (n=4).

showed a temporary peak during the dry period (Figure 3.8.b). MS-LN had similar activity at 12 and 33 d, and then decreased to a relatively constant low value thereafter. Nitrogen had a consistent and positive impact on the cellulase activity in MS, but not for NR and CH.

An 11-leaf tree explained 94% of the xylanase variability. Residue contributed 61% of that variability, while time and N have accounted for 30 and 3% of the variability, respectively.

It is interesting to notice that of the enzymes evaluated, xylanase was showed the most separation among residue treatments (Figure 3.8.c). As opposed to invertase, cellulase and laccase, xylanase activity which were more consistently responsive to the addition of CH; as at all the sampling dates, CH samples had higher values than did NR. Levels of xylanase in NR were relatively low and quite constant throughout the experiment. CH activity decreased over time, with a small transient peak during the drought period. Xylanase activity was not affected by N rates, but in general was higher in MS-HN than MS-LN samples. Regardless of the N rates, xylanase peaked at 33 days in MS samples, and then decreased.

Eighty-one percent of the variability in laccase activity was explained by a 6-leaf tree model, with partial contribution of explanatory variables decreasing in the following order: time (31%) > residue (30%) > N (19%). These results indicated that N was relatively more important in controlling the activity of laccase than it was for the three hydrolytic enzymes. Despite the high content of lignin in CH, this residue did not increase laccase activity relative to NR (Figure 3.8.d). Activity for both residues was low and rather constant throughout the incubation period. The effect of MS addition on this enzyme was highly dependent on N rates, being strongly inhibited at the high rate almost the entire incubation period. MS-HN had a laccase peak at 12 d, but after which it was not significantly different than NR and CH. On the other hand, laccase activity in MS-LN sharply increased from day 12 until day 350, leveling off for the remainder of the period of this study.

Discussion

Overall Impact of Residue Quality on the Biological and Chemical Variables

MS caused a major microbial respiration and biomass response. Conversely, microbial responses to CH were small or non-existent. These differential microbial responses to MS and CH amendments are likely related to their chemical composition. Although the two residues had similar C, N and cellulose contents, they strongly differed in the composition of the other fractions. Acid detergent-soluble compounds, which are more bioavailable than cellulose, were a major component of MS. Lignin was the most abundant fraction in CH.

These results are consistent with those from other studies using model compounds. For example, lignin has been shown to cause no effect on MBC, Biolog activities, and microbial structure community (Schutter and Dick, 2001). Although a group of soil microorganisms can degrade lignin, this polymer has been thought to contribute little or no C for energy to heterotrophic microbiota (Kirk et al., 1976). This could be part of the explanation for the results observed in samples amended with CH.

In contrast with lignin, cellulose and components of the soluble fractions have been reported to strongly impact microbial biomass and respiration (Hu and van Bruggen, 1997; Schutter and Dick, 2001; Fontaine et al., 2004; McMahon et al., 2005). This is in line with the clear microbial response to MS. Nevertheless, due to the relatively high cellulose content in CH, we would have expected a greater response of microbial biomass to CH than what was observed.

Although the major impact of residue composition on microbial responses appears to be related to the degree of easily available C (acid soluble C), there could be a contribution of the physical arrangement of lignin and cellulose in CH that limits cellulose availability. It is known

that plant cells with secondary walls are impregnated with lignin, which covers and shields cellulose and hemicellulose from microbial decay (Hall et al., 1982). The lignocellulose index (LCI, ratio of lignin concentration in plant litter to the concentration of lignin plus cellulose in plant litter) is a measure of how accessible plant fiber polysaccharides are to enzymatic hydrolysis (Melillo et al., 1989). According to the authors, LCI increases as the decomposition proceeds, reaching values about mid-0.6 when resistant fractions of litter become the major substrate for the process which is largely lignin-encrusted cellulose. This residue has a low degradation rate because it needs to be delignified before cellulose can be hydrolyzed. The CH used in our work had an initial LCI of 0.59, and that would be an indication that a large part of the cellulose is shielded by lignin limiting its potential as a source of C for microbial growth.

Residue type caused a shift in microbial communities, with MS amended soils being strongly dominated by fungi relative to the other residue treatments. CH had a higher fungal community than NR, but differences in this variable were more subtle and occurred much later than for MS.

In general, the response of the activity of the three hydrolytic enzymes to the residue quality conformed to the presumed bioavailability of their substrates. Invertase, cellulase and xylanase, which are associated with the decomposition of soluble C compounds, cellulose and hemicellulose, respectively, were higher in MS than in CH. Moreover, invertase peaked at 12 d in MS, which suggests there was a synchrony between this enzyme activity and the availability of more soluble substances in this residue. Cellulase and xylanase had their highest activities in the second sampling date, after the respiration rates decreased. Overall, this temporal variation in the activity of these two latter hydrolytic enzymes was observed irrespective of the residue treatment.

NR samples had a small peak of microbial activity right after the beginning of the incubation with lower levels thereafter. Since no residues were added, the initial response to disturbance in preparing the microcosms that exposed particulate substrates protected inside aggregates.

Addition of CH increased the activity of cellulase at day 12 and at the two latest samplings (350 and 425 days). This enzyme activity at d 12 may be related to a higher initial availability of lignin-unshielded cellulose, while the stimulation late in the incubation may be related to the release of some cellulose from the lignocellulose complex.

Some deviation from the relationship between enzyme activity and presumed availability of the respective substrate was observed for hemicellulose in CH. Although this structural polymer has been reported to be a minor component of CH (0.25%) (Nathanael, 1965), we found that xylanase was the enzyme in which activity was more clearly stimulated by this residue. The explanation for this seemingly contradictory result may be related to the cross-induction of xylanase synthesis by cellulose, which has been reported to take place in several organisms (Dobozi et al., 1992; Herzog et al., 1991).

Laccase activity did not respond to the residues according to substrate chemistry. While this enzyme is involved with the breakdown of lignin, it did not respond to the addition of the lignin-rich CH, but instead to the addition of MS. This result is in accordance with the report of Keyser et al. (1978) who have shown that lignin exerts no positive control on the synthesis of ligninolitic enzymes by the fungi *Phanerochaete chrysosoporium* and *Coriolus versicolor.* Also, according to Kirk et al. (1976), lignin does not serve as a sufficient carbon and energy source for *Phanaerochaete chrysosporium*. Lignin degradation by this fungus has only been observed when an additional growth substrate such as cellulose, glucose or succinate is provided.

Soil N levels were affected by the residue chemical composition. Although the C:N ratio for MS and CH was about the same, addition of MS caused a significant decrease in the soil min-N; whereas CH did not affect this variable. Because the main process involved in residue-induced N immobilization in agricultural soils is a biological one (Frey et al., 2000), the availability of organic C to decompose soil microorganisms is thought to determine the N immobilization capacity. Thus, the lower contents of soluble components and the high recalcitrance of the structural C in CH would cause the bioavailable C:N ratio of the fractions decomposing at any time to be narrower, decreasing the demand of soil N by the microorganisms. On the other hand, the high contents of readily available C in MS fractions stimulated microbial growth and resulted in a higher demand of soil N to complement microbial N requirements, that ultimately resulted in higher net N immobilization. In our work, levels of min-N were nearly undetectable during the first 112 to 212 d in the MS-LN, while higher amounts were present in the other treatments.

Soil acidification was attenuated in MS in relation to CH and NR. Buffering of pH as a result of the addition of plant residues has been observed (Xu et al., 2006). These authors showed that the effect of plant residues on the soil pH depends on the alkalinity of the residues and the balance between processes that cause soil pH to increase (decarboxylation of organic anions and ammonification) or decrease (nitrification). Although the amount of ashes in the two residues is about the same, we do not have information on the alkalinity of these materials. However, the higher rates of decarboxylation of organic anions, supposedly associated with the higher decomposition rates, and the higher immobilization of N in MS would have promoted buffering of soil pH, the opposite being true for NR and CH. It is noteworthy that the higher respiration rates and the low availability of N for nitrification in MS-LN relative to MS-HN would also be related to differences in the pH of these treatments.

Microbial Response to N Additions during Maize Stover Decomposition

Temporal changes and a N effect on the variables were more evident in MS-amended samples than in CH or NR treatments. Consequently, MS treatment is discussed in detail in this section.

High microbial respiration rates were observed during the first 18 days of MS decomposition for both N rates. Respiration rates peaked between the second and the fourth days in MS samples (Figure 3.2). For both MS-LN and MS-HN, rates started to stabilize at lower values at about 18 days. By day 18, MBC and accumulated CO_2-C in MS were about 0.25 and 1.25 mg C g^{-1} soil greater than NR. We estimated that the sum of these two values (1.5 mg of C g^{-1}) should correspond to the minimum amount of C derived from MS, as some microbial components and microbial-derived C not accounted for in the chloroform-extractable fraction of biomass could even increase this value. This amount corresponded to about 40% of the total C added in this residue. Because the acid-soluble fraction (water soluble + hemicellulose) corresponds to about 60% of the MS residue, it is likely that from 18 days onwards, cellulose would be the most important residue fraction driving microbial activity.

Fractions more soluble than cellulose (acid soluble) seemed to be the major sources of C and energy driving the decomposition process during this time. Respiration rates peaked between the second and the fourth days in MS samples (Figure 3.2). For both MS-LN and MS-HN, rates started to stabilize at lower values at about 18 days. This period of high microbial activity would be expected to be dominated by the decomposition of fractions more soluble than cellulose. By day 18, MBC and accumulated CO_2-C in MS were about 0.25 and 1.25 mg C g^{-1} soil greater than NR. We estimated that the sum of these two values (1.5 mg of C g^{-1}) should correspond to the minimum amount of C derived from MS, as some microbial components and

microbial-derived C not accounted for in the chloroform-extractable fraction of biomass could even increase this value. This amount corresponded to about 40% of the total C added in this residue. Because the acid-soluble fraction (water soluble + hemicellulose) corresponds to about 60% of the MS residue, it is likely that from 18 days onwards, cellulose would be the most important residue fraction driving microbial activity.

In the first 18 days, the N effect on microbial respiration rates was variable but after this, HN had lower respiration rates than the LN. This was likely because after 18 d, soluble C had been consumed and cellulose dominated as a source of C in MS amended soils. Henriksen and Breland (1999) also showed that decomposition of cellulose was reduced with the addition of N.

A strong stimulation of MBC was observed right after the addition of MS. Despite the fact that respiration rates sharply decreased between the first and third weeks, MBC was nearly constant for 112 d. This suggests that a large part of the biomass derived from MS soon became inactive, probably in response to a reduction in the C supply (Cochran et al., 1988; Knapp et al., 1983). According to Cochran et al. (1988), the large increase in microbial populations right after the addition of straw to the soil would first be due to fast-growing microbes which thrive in the easily decomposable pool of residues. With the exhaustion of this C pool, this population becomes dormant. Moreover, part of this population increase could occur due to the diffusion of the more soluble fractions compounds into C deprived aggregates (Ladd et al., 1995). This would increase protection of dormant cells from predators and, in turn, the mean residence time of this biomass (Wright et al., 1995).

As observed for respiration rates and MBC, microbial community structure was unaffected by N rates at day 12. The lack of response of all these microbial variables to N fertilizer early in the period of decomposition seems contradictory, since MS is a residue with a wide C:N ratio and that a large portion of its C is present in the soluble fraction. While the C:N

ratio of the whole residue may be wide, its soluble components may be quite narrow. The C:N ratios of the whole wheat residues used by Gaillard et al. (2003) and Bremer et al. (1991) were about 167 and 80, respectively, whereas the corresponding values for the more soluble fraction of these residues equaled 49 and 23. Therefore, the soluble fraction released from these residues could be less restrictive in N for the microbial growth than the structural components decomposed later (Bremer et al., 1991). Moreover, most of the readily available N fertilizer added in the beginning of the incubation would primarily alleviate the competition for N among soil organisms using this soluble fraction.

A strong shift in the microbial community structure toward less fungal biomass was observed from day 12 to 33, for both N rates. In our experiment, this shift took place mainly due to a decrease in the fungal PLFAs, rather than an increase in the bacterial ones. This was a temporary effect; by day 54, communities returned to a structure more similar to that observed on day 12. A behavior similar to that also was observed between days 15 and 50 during the decomposition of wheat straw under lab conditions (McMahon et al., 2005). Cochran et al. (1988) postulated that after the consumption of the soluble compounds, a new community emerges which is more adapted to decompose the structural components of straw. Although communities at days 12 and 54 were more similar regarding the fungal dominance than those at day 33, it is likely that the newly established communities were composed of fungal types different from those found at day 12. Variation in the fungal composition related to differences in resource quality at particular stages of decomposition of wheat straw (Broder and Wagner, 1988) and wheat leaves and internodes (Robinson et al., 1994) have been observed in culturing-based studies.

At 33 days and later samplings, N significantly impacted microbial community structure. It is interesting to note that the onset of the N effects on respiration rates coincided with changes

in microbial composition between MS-LN and MS-HN. Samples with HN had a relatively more fungal-dominated community than the LN. We would expect that since the C:N ratio of bacteria is narrower than for fungi, bacteria would have a higher demand for N per unit of biomass-C. Also, fungi are known to be adapted for N deprivation (Frey et al., 2000; Paustian and Schnurer, 1987). In spite of that, Henriksen and Breland (1999) support our observations. They found that the faster growth of bacteria on easily decomposable material, with concomitant immobilization of the initially available N, imposed a greater restriction of N on the succeeding fungal community. Following this rationale, we suggest that a possible N restriction to fungal communities would have been intensified at 33 days by the slow turnover of the large dormant biomass which had immobilized most of the N during its earlier growth using the more soluble residue fractions. Also, a large part of the fungal biomass would grow in close contact with the remaining N-poor, structural fraction of the residue, dominated largely by cellulose, which would increase the demand for N.

The wider EL:$PL_{18:2\omega6c}$ ratio observed in MS-LN than MS-HN provided evidence that the fungal community was N-limited at 33 days. This ratio was used here as a proxy for the ratio between the contents of 18:2ω6c in the neutral lipid and phospholipid pools (NL:$PL_{18:2\omega6c}$). According to Bååth (2003), an increase in the NL:$PL_{18:2\ \omega6c}$ would indicate there was an accumulation of lipid storage products in response to N starvation. Another explanation for this increased ratio would be that upon microbial death, phospholipids are converted into neutral, diacyl-glycerol lipids. Therefore, rather than indicating accumulation of lipid storage, the increased ratio could reflect a higher rate of fungal death around 33 days.

The above discussion assumes only direct effects of N on microbial properties. The addition of inorganic N acidifies soils, which could favor fungi over bacteria (Blagodatskaya and Anderson, 1998).

The observation that communities with wider fungi-to-bacteria ratios are more conservative in the use of soil C (Blagodatskaya and Anderson, 1998) would provide some support to the hypothesis that changes in microbial community structure in our work are related with the N effect on the respiration rates.

Nitrogen positively impacted the activity of all three hydrolytic enzymes in MS samples, but had no effect on these enzymes' activities in the other residue treatments. Activities of xylanase and cellulose have responded to N addiction during the decomposition of plant litters (Carreiro et al., 2000; Henriksen and Breland, 1999). Carreiro et al. (2000) pointed out that this N-related response in cellulase activity was inversely proportional to the residue recalcitrance which corresponds to a lack of a N effect on cellulose activity for the CH amendment.

The response of N on the respiration rates and activity of hydrolytic enzymes was inversely related with in the MS treatment. This may indicate that these enzymes are not associated with the causes of this N effect on the respiration rates. Additions of nitrate to the soil have reduced the flow of C through the microbial community by increasing the amounts of soil organic C (DeForest et al., 2004a). This would be particularly likely in the presence of fungal-dominated communities, as fungi, especially microscopic fungi of the Imperfecti group, play a significant role in the synthesis of humic substance in soils. These fungi degrade lignin and cellulose and in the process synthesize appreciable amounts of humic acid-like polymers. For example, Martin et al. (1972) showed that one-third of the substance formed by *Hendersonula toruloidea*, including biomass is humic acid. The MS-HN was more fungal dominated so it maybe that although this treatment had high hydrolytic enzyme activity (suggesting high rates of decomposition), the low observed respiration rates may mean that fungi in the MS-HN treatment are shunting more C into humic substances that reduces CO_2 losses, whereas just the opposite may be happening in MS-LN.

Unlike the hydrolytic enzymes, laccase activity was strongly inhibited in HN, compared with LN. Moreover, a leveling-off of laccase activity was observed in LN with a coinciding increase in min-N in the soil. Inhibition of ligninolitic enzymes such as laccases and peroxidases by N added to the soil has been commonly reported in the literature (Carreiro et al., 2000; DeForest et al., 2004a; DeForest et al., 2004b; Sinsabaugh et al., 2002). The synthesis of ligninolytic enzymes and the rates of lignin decomposition by *Phanerochaete chrysosoporium* have been shown to be triggered in response to N starvation (Keyser et al., 1978). This pattern of response of laccase is in agreement with the N effect on the respiration rates.

Due to the structural similarity between lignin and SOM, it is likely that ligninolytic enzymes also attack SOM. This has been confirmed by Fog (1998), who has shown that the N effect occurs not only in connection with lignin degradation, but also on soil organic matter (humus). Indeed, these enzymes have been known to break down phenolic compounds by oxidation, making polyphenol-bound soil proteins accessible to fungal proteases (Ramstedt and Soderhall, 1983). Therefore, it may be possible that part of the extra C loss by respiration in MS-LN was derived from SOM by soil microorganisms while scavenging for N produced ligninolytic enzymes to release N and at the same time making cellulose and other C compounds bioavailable.

Soil Drought and Rewetting Effects

We varied soil moisture to mimic typical seasonal patterns in NE Brazil in order to more realistically determine the biological response to CH amendments. A positive and transient effect of soil rewetting was observed on the respiration rates of MS samples. Soil rewetting has been shown to cause a flush of microbial respiration, and has been attributed to the exposure of organic sources such as lysed cells (Bottner, 1985; Halverson et al., 2000) and the intra-

aggregate particulate organic matter, released by the rupture of the aggregates (Lundquist et al., 1999a).

Soil min-N contents had a sharp positive response to soil rewetting. This response was mostly associated with increases in NO_3^--N, rather than NH_4^+-N. A flush of compounds from inactive cells would increase gross N mineralization rates, and at that late time, when the pools of easily available C are not large enough, net re-immobilization by heterotrophic microorganisms becomes less likely. The larger concentrations of ammonium left in the soil could then be effectively converted in nitrate by nitrifiers.

Overall, the activity of hydrolytic enzymes had a second peak during the dry period, regardless of the residue treatment, except for xylanase (MS-LN and MS-HN) and cellulase (MS-LN). These results were not supported by previous studies, which show activity of these enzymes to decrease in response to the soil drought (Ross et al., 1984; Sardans and Peñuelas, 2005).

Finally, two major changes in community structure that were dependent on N rates were observed during the dry periods. First, NR-HN communities differed from their LN counterparts by having a lower content in monosaturated FAMEs, which have been used by many authors as markers for GN bacteria. This was a temporary shift; upon soil rewetting, differences between N rates tended to disappear. Interestingly, the ratio between 19cy to 18:1ω7c, used as an indicator of desiccation stress in GN bacteria, was significantly higher in the NR-HN than in NR-LN during the dry period and later samplings. Therefore, it seems that this microbial shift was a response to a combination of two stress factors, drought and HN, on the GN population.

Another temporary and N-related shift in microbial community was observed in response to drought in MS samples. Conversely to NR, the shift in the MS community structure has occurred in LN samples. In this case, a decrease in fungal PLFA markers was the main

cause of that shift. Frey et al. (1999) have determined that moisture was the major factor determining microbial composition in soil under different management along two climatic gradients. In agreement with our findings, those authors reported that the degree of dominance of fungi over bacteria increased with the decrease of soil moisture, and that was related to a positive response of fungal biomass to water in the soil, rather than to a negative response of bacterial populations. It is noteworthy that the second, short-lived peak of greater respiration rates of MS-LN over MS-HN occurred right after soil rewetting. Therefore, this observation would be in line with the same pattern between bacterial-dominated community and higher respiration rates observed in the first period of N effect on the respiration rates.

From these observations, we suggested that N constituted an extra stress factor for microbial populations in NR and MS samples under drought. However, this N-related extra stress would come from different mechanisms. Evidences for this come from the observations that: 1) the combined effect of N and drought has affected different populations, depending on the residue treatment; and 2) opposing N treatments (LN vs. HN) are related with the shifts in the dry period in NR and MS. While in LN an excess of N (HN) seems to have caused populations in LN to be more stressed; while in MS, a more reduced availability of N (LN) may have led to a more stressing condition.

Summary

Residue chemical composition was an important factor in determining the composition of microbial community structure and enzyme activity with the recalcitrant CH having subtle or non-existent responses to N or to changes in moisture regimes.

In contrast, MS-amended soil became fungal dominated and had increased rates of enzyme activity. In MS soil, the higher N rate widened fungal-to-bacteria ratio, increased the activity of hydrolytic enzymes, and decreased respiration rates and the activity of laccase.

At 33 d, the fungal FAME marker (18:2ω6c) ratio between neutral lipid (NL) and PL pools increased in MS-LN over MS-HN suggesting that N could have limited fungal community. This time coincides with a strong shift in the microbial community structure.

We suggest that the increased losses of C by respiration in MS-LN could be related with the lower conservation of soil C by bacterial dominated communities and/or with an increased activity of laccase under LN.

Moreover, soil moisture content has also significantly impacted microbial community structure. A temporary bacterial-dominated community has developed during the dry period in the MS-LN, which would also be in line with a second, short-lived period of higher respiration rates in this treatment compared with MS-HN upon rewetting. Finally, drought has also resulted in a more stressed Gram-negative community in NR-HN, but the same was not valid for NR-LH. Microbial community structure in CH has not been affected by changes in moisture content.

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References

Aber, J.D., and J.M. Melillo. 1982. Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. Canadian Journal of Botany 60:2263-2269.

Agren, G.I., E. Bosatta, and A.H. Magill. 2001. Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. Oecologia 128:94-98.

Appel, T. 1998. Non-biomass soil organic N - the substrate for N mineralization flushes following soil drying-rewetting and for organic N rendered $CaCl_2$- extractable upon soil drying. Soil Biology and Biochemistry 30:1445-1456.

Bååth, E. 2003. The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. Microbial Ecology 45:373-383.

Berg, B., and E. Matzner. 1997. Effect of N deposition on decomposition of plant litter and soil organic matter in forest systems. Environmental Reviews 5:1-25.

Blagodatskaya, E.V., and T.-H. Anderson. 1998. Interactive effect of pH and substrate quality on the fungal-to-bacterial ratio and qCO_2 of microbial communities in forest soils. Soil Biology and Biochemistry 30:1269-1274.

Bligh, E.G., and W.J. Dyer. 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

Bottner, P. 1985. Response of microbial biomass to alternate moist and dry conditions in a soil incubated with ^{14}C and ^{15}N plant material. Soil Biology and Biochemistry 17:329-337.

Breiman, L., J.H. Friedman, R.A. Olshen, and C.G. Stone. 1984. Classification and regression tree. Wadsworth International Group, Belmont, CA, USA.

Bremer, E., W. van Houtum, and C. van Kessel. 1991. Carbon dioxide evolution from wheat and lentil residues as affected by grinding, added nitrogen, and the absence of soil. Biology and Fertility of Soils 11:221-227.

Bremner, J.M., and D.R. Keeney. 1965. Steam distillation methods for determination of ammonium, nitrate and nitrite. Analytica Chimica Acta 32:485-495.

Broder, M.W., and G.H. Wagner. 1988. Microbial colonization and decomposition of corn, wheat, and soybean residue. Soil Science Society of America Journal 52:112-117.

Butler, J.L., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69:6793-6800.

Carreiro, M.M., R.L. Sinsabaugh, D.A. Repert, and D.F. Parkhurst. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81:2359-2365.

Chandra, P., and W.B. Bollen. 1960. Effect of wheat straw, nitrogenous fertilizers, and carbon-to-nitrogen ratio on organic decomposition in a subhumid soil. Agricultural and Food Chemistry 8:19-24.

Clay, D.E., and C.E. Clapp. 1990. Mineralization of low C-to-N ratio corn residue in soils fertilized with NH_4^+ fertilizer. Soil Biology and Biochemistry 22:355-360.

Cochran, V.L., K.A. Horton, and C.V. Cole. 1988. An estimation of microbial death rate and limitation of N or C during wheat straw decomposition. Soil Biology and Biochemistry 20:293-298.

Davidson, E.A., I.A. Janssens, and Y. Luo. 2006. On the variability of respiration in terrestrial ecosystems: moving beyond Q_{10}. Global Change Biology 12:154-164.

De'ath, G. 2002. Multivariate regression trees: a new technique for modeling species-environment relationships. Ecology 83:1105-1117.

De'ath, G., and K.E. Fabricius. 2000. Classification and regression trees: a powerful yet simple technique for the analysis of complex ecological data. Ecology 81:3178-3192.

DeForest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton. 2004a. Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest. Soil Biology and Biochemistry 36:965-971.

DeForest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton. 2004b. Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forest. Soil Science Society of America Journal 68:132-138.

Dobozi, M.S., G. Szakacs, and C.V. Bruschi. 1992. Xylanase activity of *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 58:3466-3471.

Fog, K. 1988. The effect of added nitrogen on the rate of decomposition of organic matter. Biological Review 63:433-462.

Fontaine, S., G. Bardoux, D. Benest, B. Verdier, A. Mariotti, and L. Abbadie. 2004. Mechanisms of the primming effect in a savannah soil amended with cellulose. Soil Science Society of America Journal 68:125-131.

Frey, S.D., E.T. Elliott, and K. Paustian. 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biology and Biochemistry 31:573-585.

Frey, S.D., L.F. Elliot, K. Paustian, and G.A. Peterson. 2000. Fungal translocation as a mechanism for soil nitrogen inputs to surface residue decomposition in a no-tillage agroecossystem. Soil Biology and Biochemistry 32:689-698.

Gaillard, V., C. Chenu, and S. Recous. 2003. Carbon mineralisation in soil adjacent to plant residues of contrasting biochemical quality. Soil Biology and Biochemistry 35:93-99.

Goering, H.K., and P.J. van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some application) ARS-USDA, Washington, D.C.

Grant, R.F., and P. Rochette. 1994. Soil microbial respiration at different water potentials and temperatures: theory and mathematical modeling. Soil Science Society of America Journal 58:1681-1690.

Hall, J.L., T.J. Flowers, and R.M. Roberts. 1982. Plant cell structure and metabolism Longman, New York.

Halverson, L.J., T.M. Jones, and M.K. Firestone. 2000. Release of intracellular solutes by four soil bacteria exposed to dilution stress. Soil Science Society of America Journal 64:1630-1637.

Harris, R.F. 1981. Effect of water potential on microbial growth and activity, p. 23-95, In J. F. Parr, et al., eds. Water Potential Relations in Soil Microbiology. Soil Science Society of America, Madison, WI.

Henriksen, T.M., and T.A. Breland. 1999. Decomposition of crop residues in the field: evaluation of a simulation model development from microcosm studies. Soil Biology and Biochemistry 31:1423-1434.

Herzog, P., A. Torronen, A. Harkki, and C.P. Kubicek. 1991. Mechanism by which xylan and cellulose trigger the biosynthesis of endo-xylanase I by *Trichoderma reesei*. Progress in Biotechnology 7:289-293.

Hu, S., and A.H.C. van Bruggen. 1997. Microbial dynamics associated with multiphasic decomposition of ^{14}C-labeled cellulose in soil. Microbial Ecology 33:134-143.

Jenkinson, D.S. 1988. Determinantion of microbial biomass carbon and nitrogen in soil, p. 368-386, *In* J. R. Wilson, ed. Advances in nitrogen cycling in agricultural ecosystems. CAB International, Wallingford, UK.

Keyser, P., T.K. Kirk, and J.G. Zeikus. 1978. Ligninolytic enzyme system of *Phanaerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. Journal of Bacteriology 135:790-797.

Kieft, T.L., E. Soroker, and M.K. Firestone. 1987. Microbial biomass response to a rapid increase in water potential when a dry soil is wetted. Soil Biology and Biochemistry 19:119-126.

Kirk, T.K., W.J. Connors, and J.G. Zeikus. 1976. Requirement for a growth substrate during lignin decomposition by two-rotting fungi. Applied and Environmental Microbiology 32:192-194.

Knapp, E.B., L.F. Elliot, and G.S. Campbell. 1983. Microbial respiration and growth during the decomposition of wheat straw. Soil Biology and Biochemistry 15:319-323.

Ladd, J.N., M. Amato, P.R. Grace, and J.A. van Veen. 1995. Simulation of ^{14}C turnover through the microbial biomass in soils incubated with ^{14}C-labeled plant residues. Soil Biology and Biochemistry 27:777-783.

Lang, E., G. Eller, and F. Zadrazil. 1997. Lignocellulose decomposition and production of ligninolytic enzymes during interaction of white rot fungi with soil microorganisms. Microbial Ecology 34:1-10.

Lilleskov, E.A., T.J. Fahey, and G.M. Lovett. 2001. Ectomycorrhizal fungal aboveground community change over an atmospheric nitrogen deposition gradient. Ecological Applications 11:397-410.

Lundquist, E.J., L.E. Jackson, and K.M. Scow. 1999a. Wet-dry cycles affect dissolved organic carbon in two California agricultural soils. Soil Biology and Biochemistry 31:1031-1038.

Lundquist, E.J., K.M. Scow, L.E. Jackson, S.L. Uesugi, and C.R. Johnson. 1999b. Rapid response of soil microbial communities from conventional, low input, and organic farming systems to a wet/dry cycle. Soil Biology and Biochemistry 31:1661-1675.

Martens, D.A. 2000. Plant residue biochemistry regulates soil carbon cycling and carbon sequestration. Soil Biology and Biochemistry 32:361-369.

Martin, J.P., K. Haider, and D. Wolf. 1972. Synthesis of phenols and phenolic polymers by *Hendersonula toruloidea* in relation to humic acid formation. Soil Science Society of America Proceedings 36:311-315.

McMahon, S.K., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2005. Dynamics of microbial communities during decomposition of carbon-13 labeled ryegrass fractions in soil. Soil Science Society of America Journal 69:1238-1247.

Melillo, J.M., J.D. Aber, A.E. Linkins, A. Ricca, B. Fry, and K.J. Nadelhoffer. 1989. Carbon and nitrogen dynamics along the decay continuum: plant litter to soil organic matter. Plant and Soil 115:189-198.

Melillo, J.M., J.D. Aber, and J.F. Muratore. 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63:621-626.

Nathanael, W.R.N. 1965. Coconut husk as industrial raw material. Coconut Bulletin 9:111-136.

Neff, J.C., A.R. Townsend, G. Gleixner, S.J. Lehman, J. Turnbull, and W.D. Bowman. 2002. Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature:915-917.

Niku-Paavola, M.L., L. Raaska, and M. Itavaara. 1990. Detection of white-rot fungi by a non-toxic stain. Mycological Research 94:27-31.

Olsson, P.A., E. Bååth, I. Jakobsen, and B. Söderström. 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99:623-629.

Paustian, K., and J. Schnurer. 1987. Fungal growth response to carbon and nitrogen limitation: a theoretical model. Soil Biology and Biochemistry 19:613-620.

Pinck, L.A., F.E. Allison, and M.S. Sherman. 1950. Maintenance of soil organic matter. II. Losses of carbon and nitrogen from young and mature plant material during decomposition in soil. Soil Science 69:391-401.

Quemada, M., and M.L. Cabrera. 1995. Carbon and nitrogen mineralized from leaves and stems of four cover crops. Soil Science Society of America Journal 59:471-477.

Ramstedt, M., and K. Soderhall. 1983. Proteases, phenoloxidase and pectinase activities in mycorrhizal fungi. Transactions of the British Mycological Society 81:157-160.

Recous, S., D. Robin, D. Darwis, and B. Mary. 1995. Soil inorganic N availability: effect on maize residues decomposition. Soil Biology and Biochemistry 27:1529-1538.

Robinson, C.H., J. Dighton, J.C. Frankland, and J.D. Roberts. 1994. Fungal communities on decaying wheat straw of different resource qualities. Soil Biology and Biochemistry 26:1053-1058.

Ross, D.J., T.W. Speir, J.C. Cowling, and K.N. Whale. 1984. Temporal fluctuation in biochemical properties of soil under pasture. II. Nitrogen mineralization and enzyme activities. Australian Journal of Agricultural Research 22:319-330.

Sakala, W.D., G. Cadish, and K.E. Giller. 2000. Interactions between residues of maize and pigeonpea and mineral N fertilizers during decomposition and N mineralization. Soil Biology and Biochemistry 32:679-688.

Sardans, J., and J. Peñuelas. 2005. Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. Soil Biology and Biochemistry 37:455-461.

Schinner, F., and W. von Mersi. 1990. Xylanase, CM-cellulase and invertase activity in soil: an improved method. Soil Biology and Biochemistry 22:511-515.

Schutter, M., and R.P. Dick. 2001. Shift in substrate utilization potential and structure of soil microbial communities in response to carbon substrates. Soil Biology and Biochemistry 33:1481-1491.

Sinsabaugh, R.L., M.M. Carreiro, and D.A. Repert. 2002. Allocation of extracellular enzymatic activity in relation to litter decomposition, N deposition, and mass loss. Biogeochemistry 60:1-24.

Sjoberg, G., S.I. Nilsson, T. Persson, and P. Karlsson. 2004. Degradation of hemicellulose, cellulose and lignin in decomposing spruce needle litter in relation to N. Soil Biology and Biochemistry 36:1761-1768.

Taylor, B.R., D. Parkinson, and W.F.J. Parsons. 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology 70:97-104.

Tian, G., B.T. Kang, and L. Brussaard. 1992. Biological effects of plant residues with contrasting chemical composition under humid tropical conditions - decomposition and nutrient release. Soil Biology and Biochemistry 24:1051-1060.

Vance, E.D., P.C. Brookes, and D.S. Jenkinson. 1987. An extraction method for measuring soil microbial C. Soil Biology and Biochemistry 19:703-707.

White, D.C., and D.B. Ringelberg. 1998. Signature lipid biomarker analysis, p. 255-272, In R. S. Burlage, et al., eds. Techniques in microbial ecology. Oxford University Press, New York.

Wright, D.A., K. Killham, L.A. Glover, and J.I. Prosser. 1995. Role of pore size location in determining bacterial activity during predation by protozoa in soil. Applied and Environ. Micro. 61(10):3537-3543.

Xu, J.M., C. Tang, and Z.L. Chen. 2006. The role of plant residues in pH change of acid soils differeing in initial pH. Soil Biology and Biochemistry 38:709-719.

GENERAL CONCLUSIONS

Shifts in soil microbial composition can be assigned by evaluating changes in the fatty acid composition of samples. Different methods have been used to extract fatty acids from soil samples, but the choice of the method can affect the type and the amounts of fatty acids extracted. PLFA has been the most widely used method to profile changes in soil microbial community structure. Due to the complexity of this procedure, alternative methods employing direct extraction have been proposed. In this thesis we compared two simplified, direct extraction procedures (MIDI and EL-FAME) with PLFA, for their impacts on interpreting and profiling microbial community structure.

Chapter 1 dealt with comparison between MIDI (a whole-cell lipid extraction protocol) and PLFA. MIDI has presented some major drawbacks as a tool to interpreting changes in soil microbial communities:

- MIDI was able to extract high amounts of various omega hydroxy fatty acids and dicarboxylic acids, which are derived from plant polymers such as cutin and suberin. The amount of these plant-associated compounds was positively correlated with particulate organic matter and their composition in MIDI extract varied with the standing vegetation at sampling.
- Differences in MIDI fatty acid composition between samples were strongly determined by variations in these plant compounds. Although MIDI and PLFA were somewhat similar in separating samples under different soil management, we concluded that MIDI method should be used with caution to profiling microbial communities because it can extract FAs of plant origin.

- Also, recovery of microbial signature fatty acids by MIDI was sharply and negatively associated with soil organic matter contents, and also decreased with the increase in the soil : extractant ratios.

In chapter 2, the EL-FAME extraction method was tested as a possible substitute for PLFA. Both EL-FAME and PLFA employ the mild alkaline methanolysis to produce fatty acid methyl esters from soil lipids. In contrast with PLFA, no fractionation of lipid classes is made in EL-FAME. Therefore, fatty acids from neutral lipids and glycolipids are co-extracted with those from phospholipids in EL-FAME procedure. These latter sources of fatty acids are likely to interfere in the interpretation of changes in microbial community structure based on EL-FAME, relative to PLFA. Our main conclusions for the comparison of EL-FAME and PLFA are as follows:

- Although EL-FAME is a milder extractant than the MIDI method, it still extracted two plant polymer FAs (16:0 16OH and 16:0 DCA) which, if present in a soil sample, would confound the outcomes of EL-FAME profiling. We found these two markers to be most evident in forest soils.
- On a relative basis, EL-FAME extracted more eukaryote and storage-related FAMEs than PLFA extractions, which indicates that EL-FAME-based microbial community structure outcomes are more driven by changes in eukaryotic than in prokaryotic FAMEs.
- Discrepancies in the extraction of both fungal and AMF FAMEs were observed between different types of vegetation. These divergences were likely related with conditions leading to distinct accumulation of NL and PL in those organisms.
- These drawbacks would be less important in studies on microbial community structure changes within soils that had the same vegetative cover. According to our results, one

should be cautious, for instance, in using EL-FAME to compare different crop phases in a crop rotation system, or sites under different types of vegetation.

- Several FAMEs, including those from bacteria, major saturated and unsaturated FAMEs showed a similar pattern of extraction from both methods, regardless of the environmental conditions from where the soil was sampled. Therefore, we conclude that when the goal is to determine relative changes on the abundances of these FAMEs due to field or laboratory treatments, rather than evaluating shifts in overall microbial composition, EL-FAME seems to equal PLFA in representing these changes.

In chapter 3, we used PLFA to study the controls exerted by N fertilizer rates (a low N rate of 10 μg N g^{-1} soil, LN; and a high N rate of 100 μg N g^{-1} soil, HN) and soil moisture on the microbial community structure during the decomposition of coconut husk (CH) and maize stover (MS) in a tropical soil over a 425 day laboratory incubation. Parallel analyses of the activity of C-cycle enzymes, and microbial biomass and activity were also conducted. A simulated seasonal drought was included from 130 to 290 d (-1.5 MPa) with a return to optimal moisture (-0.12 MPa) from 290 to 425 d.

- Residue chemical composition was an important factor in determining microbial community structure and enzyme activity. The recalcitrant CH had subtle or non-existent responses to N or to changes in moisture regimes. In contrast MS amended soil became fungal dominated and had elevated rates of enzyme activity.
- In MS soil, the higher N rate widened fungal-to-bacteria ratio, increased the activity of hydrolytic enzymes, and decreased respiration rates and the activity of laccase.
- The onset of this difference in respiration rates between high and low N coincided with a shift in microbial structure and elevated laccase activity. Also at this time, the fungal FAME

marker (18:2ω6c) ratio between neutral lipid (NL) and PL pools increased in MS-LN over MS-HN suggesting that N could have limited the fungal community in this period.

- We suggest that the increased losses of C by respiration in MS-LN could be related with the lower conservation of soil C by bacterial dominated communities and/or with an increased activity of laccase under LN.
- Soil moisture content significantly impacted microbial community structure. A temporary bacterial-dominated community developed during the dry period in the MS-LN, which would also be in line with a second, short-lived period of higher respiration rates in this treatment compared with MS-HN upon rewetting.
- Finally, drought resulted in a more stressed environment for Gram-negative bacteria in NR-HN soil, which was not the case for NR-LN. Microbial community structure in CH was not affected by changes in moisture content.

Bibliography

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Abbott, L.K., A.D. Robson, and G. de Boer. 1984. The effect of phosphorus on the formation of hyphae in soil ny the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatum*. New Phytologist 97:437-446.

Aber, J.D., and J.M. Melillo. 1982. Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. Canadian Journal of Botany 60:2263-2269.

Agren, G.I., E. Bosatta, and A.H. Magill. 2001. Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. Oecologia 128:94-98.

Almendros, G., and J. Sanz. 1991. Structural study on the soil humin fraction-boron trifluoride-methanol transesterification of soil humin preparations. Soil Biology and Biochemistry 23:1147-1154.

Ambles, A., P. Jambu, E. Parlanti, J. Joffre, and C. Riffe. 1994. Incorporation of natural monoacids from plant residues into an hydromorphic forest podzol. European Journal of Soil Science 45:175-182.

Appel, T. 1998. Non-biomass soil organic N - the substrate for N mineralization flushes following soil drying-rewetting and for organic N rendered $CaCl_2$- extractable upon soil drying. Soil Biology and Biochemistry 30:1445-1456.

Bååth, E. 2003. The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. Microbial Ecology 45:373-383.

Bååth, E., and T.-H. Anderson. 2003. Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. Soil Biology and Biochemistry 35:955-963.

Bååth, E., L.O. Nilsson, H. Goransson, and H. Wallander. 2004. Can the extent of degradation of soil fungal mycelium during soil incubation be used to estimate ectomycorrhizal biomass in soil? Soil Biology and Biochemistry 36:2105-2109.

Bago, B., P.E. Pfeffer, and Y. Shachar-Hill. 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology 124:949-957.

Berg, B., and E. Matzner. 1997. Effect of N deposition on decomposition of plant litter and soil organic matter in forest systems. Environmental Reviews 5:1-25.

Blagodatskaya, E.V., and T.-H. Anderson. 1998. Interactive effect of pH and substrate quality on the fungal-to-bacterial ratio and qCO_2 of microbial communities in forest soils. Soil Biology and Biochemistry 30:1269-1274.

Bligh, E.G., and W.J. Dyer. 1959. A rapid method for total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37:911-917.

Bossio, D.A., and K.M. Scow. 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35:265-278.

Bossio, D.A., K.M. Scrow, N. Gunapala, and K.J. Graham. 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36:1-12.

Bottner, P. 1985. Response of microbial biomass to alternate moist and dry conditions in a soil incubated with ^{14}C and ^{15}N plant material. Soil Biology and Biochemistry 17:329-337.

Breiman, L., J.H. Friedman, R.A. Olshen, and C.G. Stone. 1984. Classification and regression tree. Wadsworth International Group, Belmont, CA, USA.

Bremer, E., W. van Houtum, and C. van Kessel. 1991. Carbon dioxide evolution from wheat and lentil residues as affected by grinding, added nitrogen, and the absence of soil. Biology and Fertility of Soils 11:221-227.

Bremner, J.M., and D.R. Keeney. 1965. Steam distillation methods for determination of ammonium, nitrate and nitrite. Analytica Chimica Acta 32:485-495.

Broder, M.W., and G.H. Wagner. 1988. Microbial colonization and decomposition of corn, wheat, and soybean residue. Soil Science Society of America Journal 52:112-117.

Butler, J.L., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69:6793-6800.

Buyer, J.S., and D.D. Kaufman. 1997. Microbial diversity in the rhizosphere of corn grown under conventional and low-input systems. Applied Soil Ecology 5:21-27.

Cambardela, C.A., and E.T. Elliott. 1993. Carbon and nitrogen distribution in aggregates from cultivated and native grassland soils. Soil Science Society of America Journal 57:1071-1076.

Carlile, M.J., and S.C. Watkinson. 1994. The Fungi. Academic Press, San Diego.

Carreiro, M.M., R.L. Sinsabaugh, D.A. Repert, and D.F. Parkhurst. 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81:2359-2365.

Cavigelli, M.A., G.P. Robertson, and M.J. Klug. 1995. Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. Plant and Soil 170:99-113.

Chandra, P., and W.B. Bollen. 1960. Effect of wheat straw, nitrogenous fertilizers, and carbon-to-nitrogen ratio on organic decomposition in a subhumid soil. Agricultural and Food Chemistry 8:19-24.

Christiansen, K. 1975. Lipid extraction procedure for in vitro studies of glyceride synthesis with labeled fatty acids. Analytical Biochemistry 26:93-99.

Clay, D.E., and C.E. Clapp. 1990. Mineralization of low C-to-N ratio corn residue in soils fertilized with NH_4^+ fertilizer. Soil Biology and Biochemistry 22:355-360.

Cochran, V.L., K.A. Horton, and C.V. Cole. 1988. An estimation of microbial death rate and limitation of N or C during wheat straw decomposition. Soil Biology and Biochemistry 20:293-298.

Conde, E., M.C. Garcia-Vallejo, and E. Cadahia. 1999. Waxes composition of *Quercus suber* reproduction cork from different Spanish provenances. Wood Science and Technology 33:271-283.

Dailey, O.D., R.F. Severson, and R.F. Arrendale. 1989. Nonpolar lipids of *Amaranthus palmeri* S. Wats. 1. Fatty alcohols and wax esters (saturated). Journal of Agricultural and Food Chemistry 37:1317-1321.

Davidson, E.A., I.A. Janssens, and Y. Luo. 2006. On the variability of respiration in terrestrial ecosystems: moving beyond Q_{10}. Global Change Biology 12:154-164.

De'ath, G. 2002. Multivariate regression trees: a new technique for modeling species-environment relationships. Ecology 83:1105-1117.

De'ath, G., and K.E. Fabricius. 2000. Classification and regression trees: a powerful yet simple technique for the analysis of complex ecological data. Ecology 81:3178-3192.

DeForest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton. 2004a. Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest. Soil Biology and Biochemistry 36:965-971.

DeForest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton. 2004b. Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forest. Soil Science Society of America Journal 68:132-138.

del Rio, J.C., and P.G. Hatcher. 1998. Analysis of aliphatic biopolymers using thermochemolysis with tetramethylammonium hydroxide (TMAH) and gas chromatography-mass spectrometry. Organic Geochemistry 29:1441-1451.

del Rio, J.C., D.E. McKinney, H. Knicker, M.A. Nanny, R.D. Minard, and P.G. Hatcher. 1998. Structural characterization of bio- and geo-macromolecules by off-line thermochemolysis with tetramethylammonium hydroxide. Journal of Chromatography A 823:433-448.

Dobozi, M.S., G. Szakacs, and C.V. Bruschi. 1992. Xylanase activity of *Phanerochaete chrysosporium*. Applied and Environmental Microbiology 58:3466-3471.

Drenovsky, R.E., G.N. Elliott, K.J. Graham, and K.M. Scow. 2004. Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. Soil Biology and Biochemistry 36:1793-1800.

Drijber, R.A., J.W. Doran, A.M. Parkhurst, and D.J. Lyon. 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biology and Biochemistry 32:1419-1430.

Dufrene, M., and P. Legendre. 1997. Species assemblages and indicator species: the need for a flexible assymetric approach. Ecological Monographs 67:345-366.

Federle, T.W. 1986. Microbial distribution in soil - new techniques, p. 493-498, *In* F. Megusar and M. Gantar, eds. Perspectives in microbial ecology. Slovene Society for Microbiology, Ljubljana, Yugoslavia.

Fog, K. 1988. The effect of added nitrogen on the rate of decomposition of organic matter. Biological Review 63:433-462.

Folch, J., M. Lees, and G.H.S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497-509.

Fontaine, S., G. Bardoux, D. Benest, B. Verdier, A. Mariotti, and L. Abbadie. 2004. Mechanisms of the primming effect in a savannah soil amended with cellulose. Soil Science Society of America Journal 68:125-131.

Frey, S.D., E.T. Elliott, and K. Paustian. 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biology and Biochemistry 31:573-585.

Frey, S.D., L.F. Elliot, K. Paustian, and G.A. Peterson. 2000. Fungal translocation as a mechanism for soil nitrogen inputs to surface residue decomposition in a no-tillage agroecossystem. Soil Biology and Biochemistry 32:689-698.

Frostegård, A., and E. Bååth. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22:59-65.

Frostegård, A., A. Tunlid, and E. Bååth. 1991. Microbial biomass measured as total phosphate in soils of different organic content. Journal of Microbiological Methods 14:151-163.

Frostegård, A., A. Tunlid, and E. Bååth. 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biology and Biochemistry 25:723-730.

Gaillard, V., C. Chenu, and S. Recous. 2003. Carbon mineralisation in soil adjacent to plant residues of contrasting biochemical quality. Soil Biology and Biochemistry 35:93-99.

Goering, H.K., and P.J. van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some application) ARS-USDA, Washington, D.C.

Grant, R.F., and P. Rochette. 1994. Soil microbial respiration at different water potentials and temperatures: theory and mathematical modeling. Soil Science Society of America Journal 58:1681-1690.

Griffiths, B.S., K. Ritz, N. Ebblewhite, and G. Dobson. 1999. Soil microbial community structure: Effects of substrate loading rates. Soil Biology and Biochemistry 31:145-153.

Haack, S.K., H. Garchow, D.A. Odelson, L.J. Forney, and M.J. Klug. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Applied and Environmental Microbiology 60:2483-2493.

Hackl, E., M. Pfeffer, C. Donat, G. Bachmann, and S. Zechmeister-Boltenstern. 2005. Composition of the microbial communities in the mineral soil under different types of natural forest. Soil Biology and Biochemistry 37:661-671.

Hall, J.L., T.J. Flowers, and R.M. Roberts. 1982. Plant cell structure and metabolism Longman, New York.

Halverson, L.J., T.M. Jones, and M.K. Firestone. 2000. Release of intracellular solutes by four soil bacteria exposed to dilution stress. Soil Science Society of America Journal 64:1630-1637.

Harris, R.F. 1981. Effect of water potential on microbial growth and activity, p. 23-95, *In* J. F. Parr, et al., eds. Water Potential Relations in Soil Microbiology. Soil Science Society of America, Madison, WI.

Harwood, J.L., and N.J. Russell. 1984. Lipids in plants and microbes Allen and Unwin, London.

Henriksen, T.M., and T.A. Breland. 1999. Decomposition of crop residues in the field: evaluation of a simulation model development from microcosm studies. Soil Biology and Biochemistry 31:1423-1434.

Herzog, P., A. Torronen, A. Harkki, and C.P. Kubicek. 1991. Mechanism by which xylan and cellulose trigger the biosynthesis of endo-xylanase I by Trichoderma reesei. Progress in Biotechnology 7:289-293.

Holloway, P.J. 1972a. The composition of suberin from the corks of *Quercus suber* L. and *Betula pendula* Roth. Chemistry and Physics of Lipids 9:158-170.

Holloway, P.J. 1972b. The suberin composition of the cork layers from some *Ribes* species. Chemistry and Physics of Lipids 9:171-179.

Hu, S., and A.H.C. van Bruggen. 1997. Microbial dynamics associated with multiphasic decomposition of ^{14}C-labeled cellulose in soil. Microbial Ecology 33:134-143.

Hunneman, D.H., and G. Eglinton. 1972. The constituent acids of gymnosperm cutins. Phytochemistry 11:1989-2001.

Ibekwe, A.M., and A.C. Kennedy. 1999. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant and Soil 206:151-161.

Jabaji-Hare, S., A. Deschene, and B. Kendrick. 1984. Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. Mycologia 76:1024-1030.

Jackson, L.E., F.J. Calderon, K.L. Steenwerth, K.M. Scow, and D.E. Rolston. 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114:305-317.

Jambu, P., A. Ambles, J.C. Jacquesy, B. Secouet, and E. Parlanti. 1993. Incorporation of natural alcohols from plant residues in to a hydromorphic forest-podzol. Journal of Soil Science 44:135-146.

Jandl, G., P. Leinweber, H.-R. Schulten, and K. Ekschmitt. 2005. Contribution of primary organic matter to the fatty acid pool in agricultural soils. Soil Biology and Biochemistry 37:1033-1041.

Jenkinson, D.S. 1988. Determinantion of microbial biomass carbon and nitrogen in soil, p. 368-386, *In* J. R. Wilson, ed. Advances in nitrogen cycling in agricultural ecosystems. CAB International, Wallingford, UK.

Jenkinson, D.S., and D.S. Powlson. 1976a. The effects of biocidal treatments on metabolism in soil. V. A method for measuring soil biomass. Soil Biology and Biochemistry 8:209-213.

Jenkinson, D.S., and D.S. Powlson. 1976b. The effect of biocidal treatments on metabolism in soil. I. Fumigation with chloroform. Soil Biology and Biochemistry 8:167-177.

Keyser, P., T.K. Kirk, and J.G. Zeikus. 1978. Ligninolytic enzyme system of *Phanaerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. Journal of Bacteriology 135:790-797.

Kieft, T.L., E. Soroker, and M.K. Firestone. 1987. Microbial biomass response to a rapid increase in water potential when a dry soil is wetted. Soil Biology and Biochemistry 19:119-126.

Kieft, T.L., D.B. Ringelberg, and D.C. White. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. Applied and Environmental Microbiology 60:3292-3299.

King, J.D., D.C. White, and C.W. Taylor. 1977. Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora. Applied and Environmental Microbiology 33:1177-1183.

Kirk, T.K., W.J. Connors, and J.G. Zeikus. 1976. Requirement for a growth substrate during lignin decomposition by two-rotting fungi. Applied and Environmental Microbiology 32:192-194.

Knapp, E.B., L.F. Elliot, and G.S. Campbell. 1983. Microbial respiration and growth during the decomposition of wheat straw. Soil Biology and Biochemistry 15:319-323.

Kolarovic, L., and N.C. Fournier. 1986. A comparison of extraction methods for the isolation of phospholipids from biological sources. Analytical Biochemistry 156:244-250.

Kolattukudy, P.E. 1970. Reduction of fatty acids to alcohols by cell-free preparations of *Euglena gracilis*. Biochemistry 9:1095-1102.

Kolattukudy, P.E. 1980. Biopolyester membranes of plants: cutin and suberin. Science 208:990-1000.

Kolattukudy, P.E., and V.P. Agrawal. 1974. Structure and composition of aliphatic constituents of potato tuber skin (suberin). Lipids 9:682-691.

Kroppenstedt, R.M. 1992. The genus *Nocardiopsis*, p. 1139-1156, *In* A. Balows, et al., eds. The prokaryotes, Vol. 2. Springer, Berlin.

Ladd, J.N., M. Amato, P.R. Grace, and J.A. van Veen. 1995. Simulation of ^{14}C turnover through the microbial biomass in soils incubated with ^{14}C-labeled plant residues. Soil Biology and Biochemistry 27:777-783.

Lang, E., G. Eller, and F. Zadrazil. 1997. Lignocellulose decomposition and production of ligninolytic enzymes during interaction of white rot fungi with soil microorganisms. Microbial Ecology 34:1-10.

Lilleskov, E.A., T.J. Fahey, and G.M. Lovett. 2001. Ectomycorrhizal fungal aboveground community change over an atmospheric nitrogen deposition gradient. Ecological Applications 11:397-410.

Loveland, P.M., and M.L. Laver. 1972. Monocarboxylic and dicarboxylic acids from *Pseutotsuga menziessi* bark. Phytochemistry 11:430-432.

Lundquist, E.J., L.E. Jackson, and K.M. Scow. 1999a. Wet-dry cycles affect dissolved organic carbon in two California agricultural soils. Soil Biology and Biochemistry 31:1031-1038.

Lundquist, E.J., K.M. Scow, L.E. Jackson, S.L. Uesugi, and C.R. Johnson. 1999b. Rapid response of soil microbial communities from conventional, low input, and organic farming systems to a wet/dry cycle. Soil Biology and Biochemistry 31:1661-1675.

Macalady, J.L., M.E. Fuller, and K.M. Scow. 1998. Effects of metam sodium fumigation on soil microbial activity and community structure. Journal of Environmental Quality 27:54-63.

Martens, D.A. 2000. Plant residue biochemistry regulates soil carbon cycling and carbon sequestration. Soil Biology and Biochemistry 32:361-369.

Martin, J.P., K. Haider, and D. Wolf. 1972. Synthesis of phenols and phenolic polymers by *Hendersonula toruloidea* in relation to humic acid formation. Soil Science Society of America Proceedings 36:311-315.

Matzke, K., and M. Riederer. 1991. A comparative study into the chemical constitution of cutins and suberins from *Picea abies* (L.) Karst., *Quercus robur* L., and *Fagus sylvatica* L. Planta 185:233-245.

Mayberry, W.R., P.F. Smith, T.A. Langworthy, and P. Plackett. 1973. Identification of the amide-linked fatty acids of *Acholeplasma axanthum* S743 as D-(-)-3-hydroxyhexadecanoate and its homologues. Journal of Bacteriology 116:1091-1095.

McCune, B., and J.B. Grace. 2002. Analysis of Ecological Communities MjM Software Design, Gleneden Beach, OR.

McMahon, S.K., M.A. Williams, P.J. Bottomley, and D.D. Myrold. 2005. Dynamics of microbial communities during decomposition of carbon-13 labeled ryegrass fractions in soil. Soil Science Society of America Journal 69:1238-1247.

Melillo, J.M., J.D. Aber, A.E. Linkins, A. Ricca, B. Fry, and K.J. Nadelhoffer. 1989. Carbon and nitrogen dynamics along the decay continuum: plant litter to soil organic matter. Plant and Soil 115:189-198.

Melillo, J.M., J.D. Aber, and J.F. Muratore. 1982. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63:621-626.

Näsholm, T., A. Ekblad, A. Nordin, R. Giesler, M. Högberg, and P. Högberg. 1998. Boreal forest plants take up organic nitrogen. Nature 392:914-916.

Nathanael, W.R.N. 1965. Coconut husk as an industrial raw material. Coconut Bulletin 9:111-136.

Neff, J.C., A.R. Townsend, G. Gleixner, S.J. Lehman, J. Turnbull, and W.D. Bowman. 2002. Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature:915-917.

Nierop, K.G.J. 1998. Origin of aliphatic compounds in a forest soil. Organic Geochemistry 29:1009-1016.

Nierop, K.G.J., M.M. Pulleman, and J.C.Y. Marinissen. 2001. Management induced organic matter differentiation in grassland and arable soil: a study using pyrolysis techniques. Soil Biology and Biochemistry 33:755-764.

Niku-Paavola, M.L., L. Raaska, and M. Itavaara. 1990. Detection of white-rot fungi by a non-toxic stain. Mycological Research 94:27-31.

O'Leary, W.M., and S.G. Wilkinson. 1988. Gram-positive bacteria, p. 117-201, *In* C. Ratledge and S. G. Wilkinson, eds. Microbial lipids, Vol. 1. Academic Press, San Diego.

Olsson, P.A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303-310.

Olsson, P.A., E. Bååth, and I. Jakobsen. 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Applied and Environmental Microbiology 63:3531-3538.

Olsson, P.A., E. Bååth, I. Jakobsen, and B. Söderström. 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99:623-629.

Olsson, P.A., and A. Johansen. 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycological Research 104:429-434.

Paisley, R. 2002. Training Manual. MIS wole cell fatty acid analysis by gas chromatography MIDI, Inc, Newark, Del.

Paul, E.A., and F.E. Clark. 1996. Soil Microbiology and Biochemistry. 2nd ed. Academic Press, San Diego.

Paustian, K., and J. Schnurer. 1987. Fungal growth response to carbon and nitrogen limitation: a theoretical model. Soil Biology and Biochemistry 19:613-620.

Petersen, S.O., P.S. Frohne, and A.C. Kennedy. 2002. Dynamics of a soil microbial community under spring wheat. Soil Science Society of America Journal 66:826-833.

Pinck, L.A., F.E. Allison, and M.S. Sherman. 1950. Maintenance of soil organic matter. II. Losses of carbon and nitrogen from young and mature plant material during decomposition in soil. Soil Science 69:391-401.

Quemada, M., and M.L. Cabrera. 1995. Carbon and nitrogen mineralized from leaves and stems of four cover crops. Soil Science Society of America Journal 59:471-477.

Ramstedt, M., and K. Soderhall. 1983. Proteases, phenoloxidase and pectinase activities in mycorrhizal fungi. Transactions of the British Mycological Society 81:157-160.

Recous, S., D. Robin, D. Darwis, and B. Mary. 1995. Soil inorganic N availability: effect on maize residues decomposition. Soil Biology and Biochemistry 27:1529-1538.

Ritchie, N.J., M.E. Schutter, R.P. Dick, and D.D. Myrold. 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. Applied and Environmental Microbiology 66:1668-1675.

Robinson, C.H., J. Dighton, J.C. Frankland, and J.D. Roberts. 1994. Fungal communities on decaying wheat straw of different resource qualities. Soil Biology and Biochemistry 26:1053-1058.

Rosenberg, W., K.G.J. Nierop, H. Knicker, P.A. de Jager, K. Kreutzer, and T. Weiss. 2003. Liming effects on the chemical composition of the organic surface layer of a mature Norway spruce stand (*Picea abies* [L.] Karst.). Soil Biology and Biochemistry 35:155-165.

Ross, D.J., T.W. Speir, J.C. Cowling, and K.N. Whale. 1984. Temporal fluctuation in biochemical properties of soil under pasture. II. Nitrogen mineralization and enzyme activities. Australian Journal of Agricultural Research 22:319-330.

Rovira, A.D., and E.L. Greacen. 1957. The effect of aggregate disruption on the activity of microorganisms in the soil. Australian Journal of Agricultural Research 8:659-673.

Sakala, W.D., G. Cadish, and K.E. Giller. 2000. Interactions between residues of maize and pigeonpea and mineral N fertilizers during decomposition and N mineralization. Soil Biology and Biochemistry 32:679-688.

Salinas-Garcia, J.R., F.M. Hons, and J.E. Matocha. 1997. Long-term effects of tillage and fertilization on soil organic matter dynamics. Soil Science Society of America Journal 61:152-159.

Sardans, J., and J. Peñuelas. 2005. Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. Soil Biology and Biochemistry 37:455-461.

Schinner, F., and W. von Mersi. 1990. Xylanase, CM-cellulase and invertase activity in soil: an improved method. Soil Biology and Biochemistry 22:511-515.

Schulten, H.R., and P. Leinweber. 1993. Pyrolysis-field ionization mass spectrometry of agricultural soils and humic substances: effect of cropping systems and influence of the mineral matrix. Plant and Soil 151:77-90.

Schutter, M., and R.P. Dick. 2001. Shift in substrate utilization potential and structure of soil microbial communities in response to carbon substrates. Soil Biology and Biochemistry 33:1481-1491.

Schutter, M.E., and R.P. Dick. 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Science Society of America Journal 64:1659-1668.

Schutter, M.E., and R.P. Dick. 2002. Microbial community profiles and activities among aggregates of winter fallow and cover-cropped soil. Soil Science Society of America Journal 66:142-153.

Schutter, M.E., R.P. Dick, and J.M. Sandeno. 2001. Seasonal, soil type, and alternative management influences on microbial communities of vegetable cropping systems. Biology and Fertility of Soils 34:397-410.

Sinsabaugh, R.L., M.M. Carreiro, and D.A. Repert. 2002. Allocation of extracellular enzymatic activity in relation to litter decomposition, N deposition, and mass loss. Biogeochemistry 60:1-24.

Six, J., K. Paustian, E.T. Elliott, and C. Combrink. 2000. Soil structure and organic matter: I. Distribution of aggregate-size classes and aggregate-associated carbon. Soil Science Society of America Journal 64:681-689.

Sjoberg, G., S.I. Nilsson, T. Persson, and P. Karlsson. 2004. Degradation of hemicellulose, cellulose and lignin in decomposing spruce needle litter in relation to N. Soil Biology and Biochemistry 36:1761-1768.

Sokal, R.R. 1979. Testing statistical significance of geographic variation patterns. Systematic Zoology 28:627-632.

Taylor, B.R., D. Parkinson, and W.F.J. Parsons. 1989. Nitrogen and lignin content as predictors of litter decay rates: a microcosm test. Ecology 70:97-104.

Tian, G., B.T. Kang, and L. Brussaard. 1992. Biological effects of plant residues with contrasting chemical composition under humid tropical conditions - decomposition and nutrient release. Soil Biology and Biochemistry 24:1051-1060.

Tunlid, A., and D.C. White. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil, p. 229-262, *In* G. Stotzky and J. M. Bollag, eds. Soil Biochemistry, Vol. 7. Marcel Dekker, New York.

van Aarle, I.M., and P.A. Olsson. 2003. Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Applied and Environmental Microbiology 69:6762-6767.

Vance, E.D., P.C. Brookes, and D.S. Jenkinson. 1987. An extraction method for measuring soil microbial C. Soil Biology and Biochemistry 19:703-707.

Vestal, J.R., and D.C. White. 1989. Lipid analysis in microbial ecology. Quantitative approaches to the study of microbial communities. Bioscience 39:535-541.

von Rudloff, E. 1959. The wax of the leaves of *Picea pungens* (Colorado spruce). Canadian Journal of Chemistry 37:1038-1042.

Voroney, R.P., J.A. van Veen, and E.A. Paul. 1981. Organic C dynamics in grassland soils. II. Model validation and simulation of long term effects of cultivation and rainfall erosion. Canadian Journal of Soil Science 61:211-224.

Vulliet, P., S.P. Markey, and T.G. Tornabene. 1974. Identification of methoxyesters artifacts produced by methanolic-HCl solvolysis of the cyclopropane fatty acids of the genus *Yersinia*. Biochimica et Biophysica Acta 348:299-301.

Waldrop, M.P., and M.K. Firestone. 2004. Microbial community utilization of recalcitrant and simple carbon compounds: impact of oak-woodland plant communities. Oecologia 138:275-284.

White, D.C., and D.B. Ringelberg. 1998. Signature lipid biomarker analysis, p. 255-272, *In* R. S. Burlage, et al., eds. Techniques in microbial ecology. Oxford University Press, New York.

Wilkinson, S.G. 1988. Gram-negative bacteria, p. 299-457, *In* C. Ratledge and S. G. Wilkinson, eds. Microbial lipids, Vol. 1. Academic Press, San Diego.

Wright, D.A., K. Killham, L.A. Glover, and J.I. Prosser. 1995. Role of pore size location in determining bacterial activity during predation by protozoa in soil. Applied and Environ. Micro. 61(10):3537-3543.

Xu, J.M., C. Tang, and Z.L. Chen. 2006. The role of plant residues in pH change of acid soils differeing in initial pH. Soil Biology and Biochemistry 38:709-719.

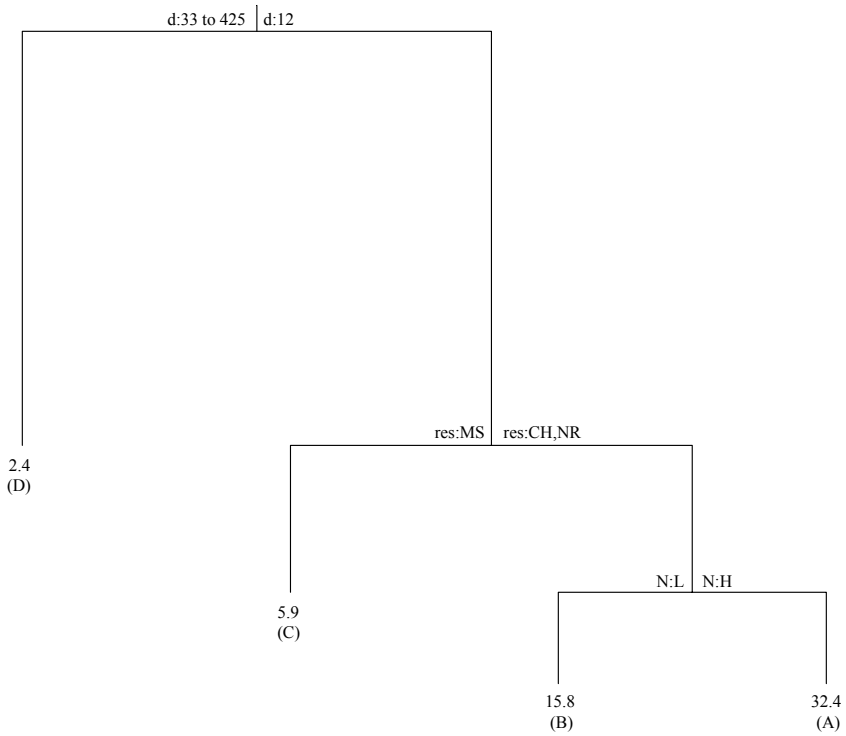
Zeier, J., and L. Schreiber. 1998. Comparative investigation of primary and tertiary endodermal cell walls isolated from the roots of five monocotyledoneous species: chemical composition in relation to fine structure. Planta 206:349-361.

Zeier, J., L. Schreiber, U. Ryser, and K. Ruel. 1999. Chemical analysis and immunolocalisation of lignin and suberin in endodermal and hypodermal/rhizodermal cell walls of developing maize (*Zea mays* L.) primary roots. Planta 209:1-12.

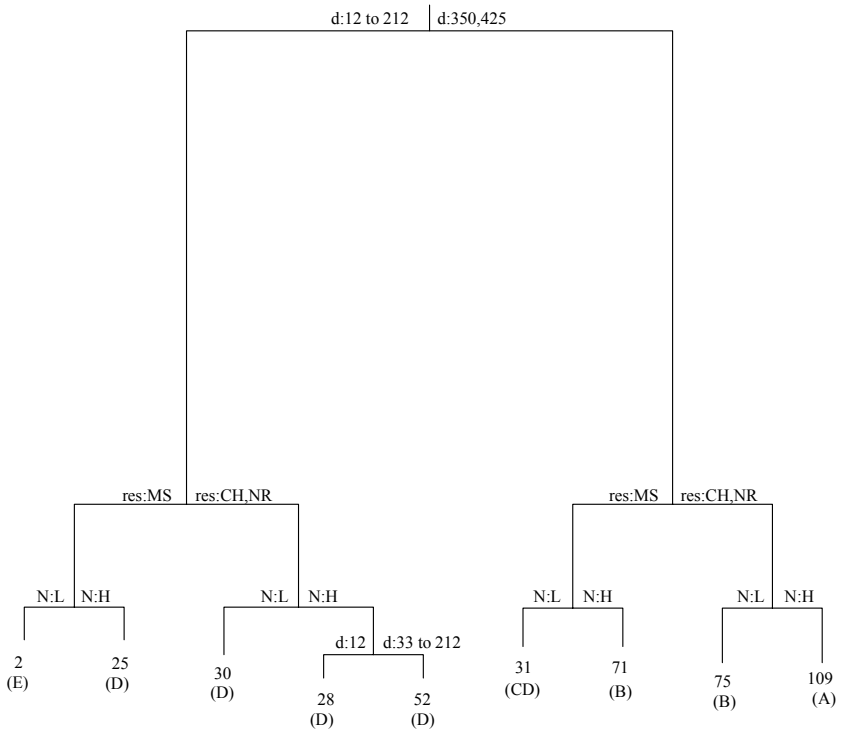
Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biology and Fertility of Soils 29:111-129.

Zelles, L., Q.Y. Bai, T. Beck, and F. Beese. 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology and Biochemistry 24:317-323.

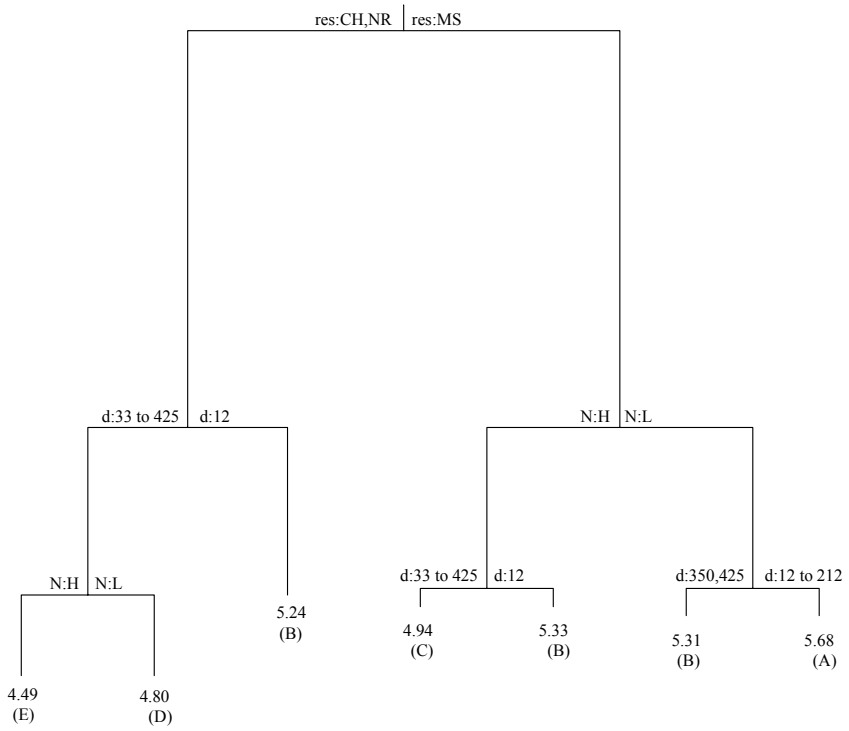
Appendices



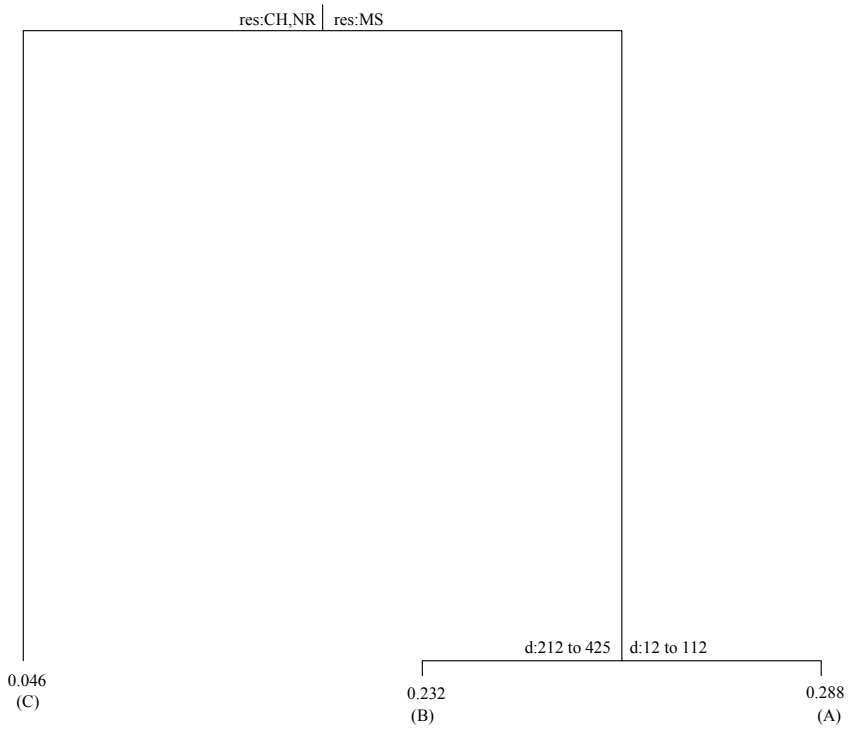
Appendix A. Regression tree for ammonium-N contents on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



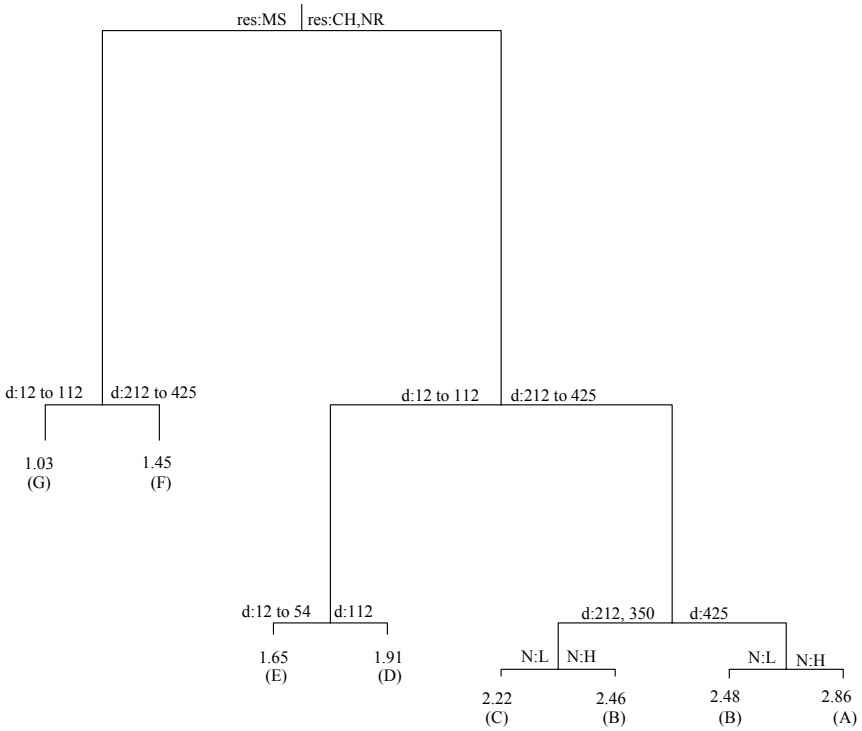
Appendix B. Regression tree for nitrate-N contents on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



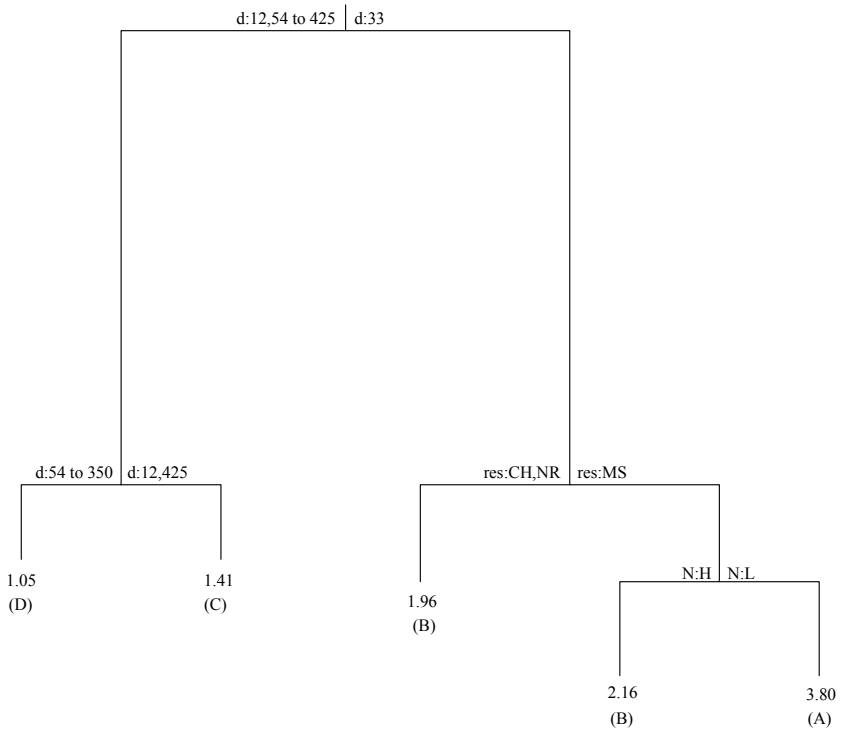
Appendix C. Regression tree for soil pH on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



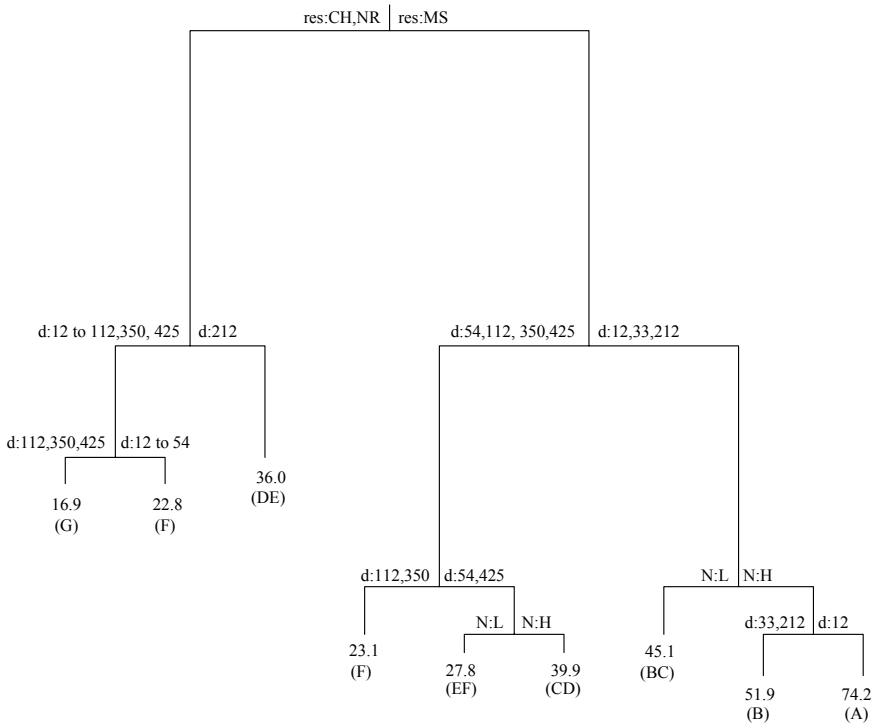
Appendix D. Regression tree for microbial biomass-C on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



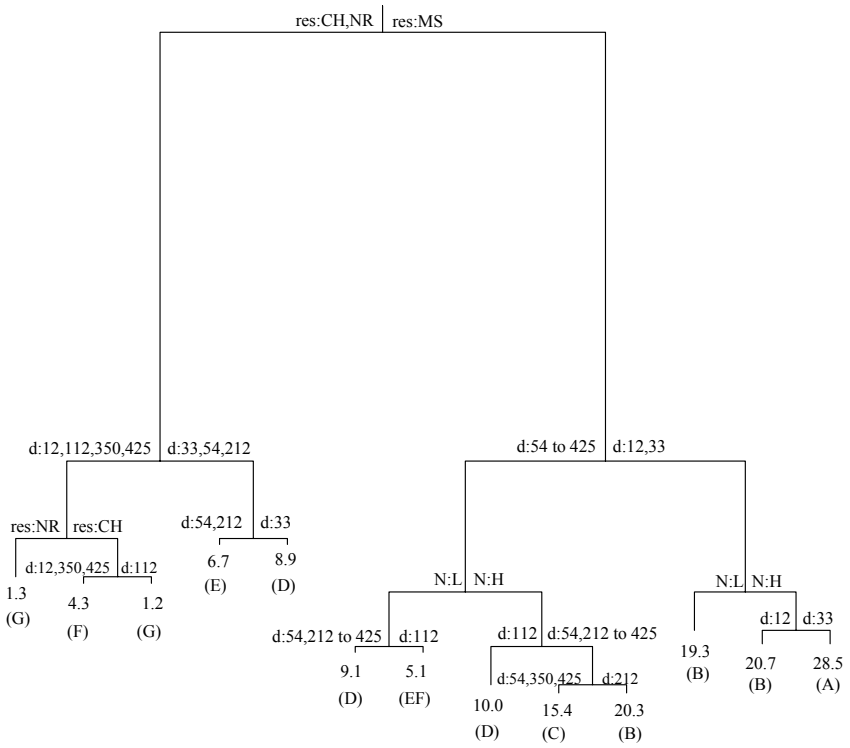
Appendix E. Regression tree for the 19:0cy to 18:1ω7c ratio on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



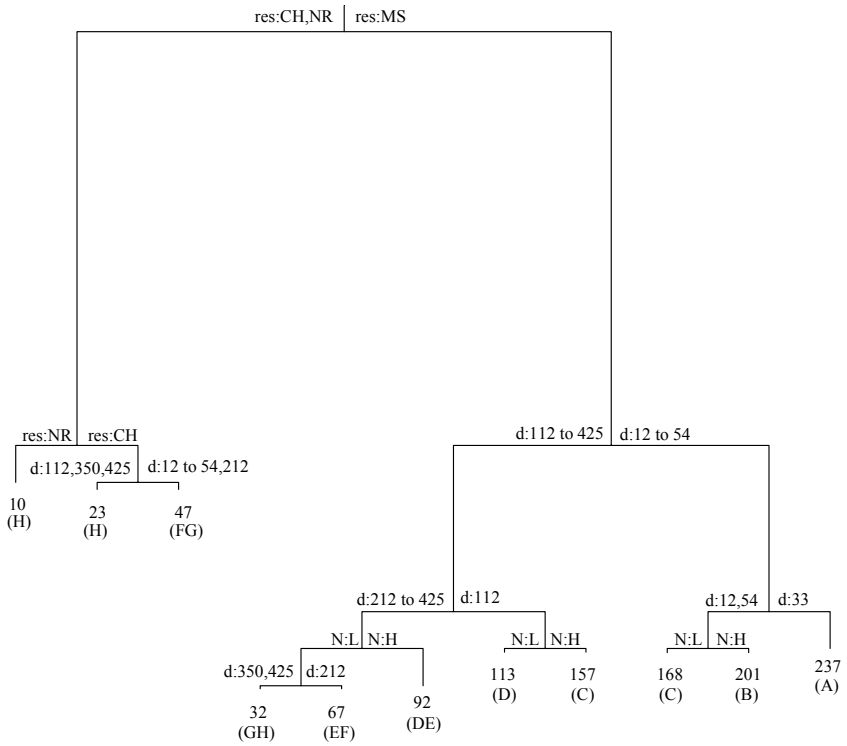
Appendix F. Regression tree for the ratio between the contents of 18:2ω6c in EL-FAME and PLFA pools on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



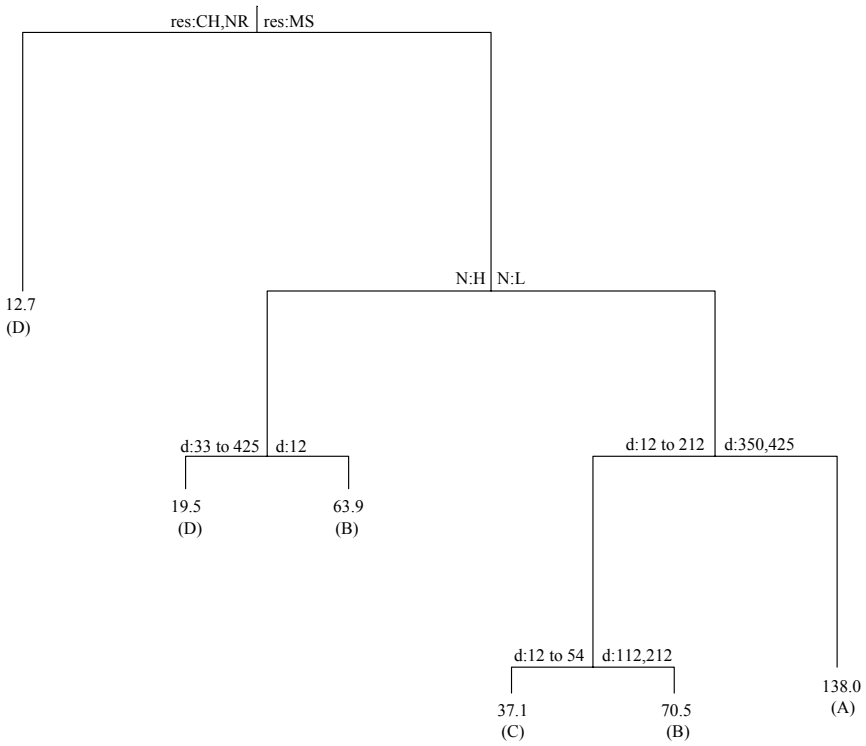
Appendix G. Regression tree for invertase activity on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



Appendix H. Regression tree for cellulase activity on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



Appendix I. Regression tree for xylanase activity on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). MS, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.



Appendix J. Regression tree for laccase activity on residue treatment (res); N rate (N); and sampling days (d). Values at terminal nodes are within-cluster averages for the response variable. Averages followed by the same letter do not differ at 5% probability (Bonferroni procedure). S, CH and NR stands for maize stover, coconut husk and no residue treatments; respectively. Low and high N rates are represented by L and H. Branch lengths are proportional to the amount of variability explained by each independent variable.