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**DISSERTATION**

# MICROBIAL COMMUNITY COMPOSITION AND SOIL ORGANIC MATTER DYNAMICS IN AGROECOSYSTEMS

**Submitted by**

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**Graduate Degree Program in Ecology**

**In partial fulfillment of the requirements**

**for the degree of Doctor of Philosophy**

**Colorado State University**

**Fort Collins, Colorado**

**Spring, 1999**

**COLORADO STATE UNIVERSITY**

April 6, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SERITA DRIVER FREY ENTITLED MICROBIAL COMMUNITY COMPOSITION AND SOIL ORGANIC MATTER DYNAMICS IN AGROECOSYSTEMS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

**Committee on Graduate Work**

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## ABSTRACT OF DISSERTATION

## MICROBIAL COMMUNITY COMPOSITION AND SOIL ORGANIC MATTER DYNAMICS IN AGROECOSYSTEMS

The objective of this dissertation was to examine the effects of different agricultural management practices on microbial community composition and the influence that community structure has on the formation and stabilization of soil organic matter (SOM) in agricultural soils. My research included field observations collected from several long-term agricultural experiments located in the wheat and corn-growing regions of the U.S., as well as laboratory and field experiments designed to test specific process-level hypotheses. These studies examined (1) the effects of no-tillage (NT) and conventional tillage (CT) management on microbial community composition, (2) controls on microbial community composition, (3) nitrogen (N) translocation through fungal hyphae in a NT system, and (4) the impacts of protozoan grazing on patterns of carbon utilization by the soil microbial community.

Shifts in the relative abundances of bacteria and fungi were observed and were found to be related to soil water availability which varied locally with tillage treatment and geographically with climate. Fungal biomass and the proportion of the total biomass

composed of fungi increased in surface soil in response to reduced tillage and across a gradient of increasing soil moisture, while bacterial biomass was not strongly affected by tillage and remained relatively constant in response to changing soil moisture.

Fungal translocation of N from the soil inorganic N pool into surface residues was a significant exogenous N source for fungi associated with residues decomposing in a NT field and accounted for the observed net N immobilized by those residues. Both residue quality and soil N availability were important controls on fungal biomass associated with surface residues and rates of soil-to-residue N translocation.

Protozoan grazing significantly influenced patterns of glucose utilization by the soil microbial community. The total amount of glucose utilized did not vary with protozoan grazing intensity, but a high level of grazing increased the rate of glucose use and significantly reduced the amount of measurable microbial biomass C. Microbial biomass turnover was significantly faster in high compared to low grazing treatments.

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Dedicated to my husband,

Wilmer,

my best friend.

The lesson which life repeats and constantly enforces is "look under foot." You are always nearer the divine and the true sources of your power than you think.

–John Burroughs from "The Divine Soil"

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# CHAPTER 1

## OVERVIEW

Carbon (C) emissions from intensively cultivated soils have contributed significantly to rising atmospheric $CO_2$ levels (Houghton *et al.*, 1983). There is growing interest in evaluating the degree to which this trend can be reversed through management of agroecosystems to conserve and sequester C, thereby reducing the rate of $CO_2$ accumulation in the atmosphere (Donigian *et al.*, 1994; Paustian *et al.*, 1998). A current national policy issue centers on the feasibility of managing soil C levels by encouraging farmers to switch to management practices that promote maintenance and accumulation of soil C (Lal *et al.*, 1998). Recent assessments indicated that implementation of conservation management practices, in particular, reduced tillage regimes such as no-tillage (NT), can significantly increase soil C levels (Kern and Johnson, 1993; Donigian *et al.*, 1994; Paul *et al.*, 1997). While the potential for C sequestration in NT soils exists, the mechanisms that regulate retention and accumulation of C in these systems are not fully understood.

My overall research objective is to examine and understand the microbial mechanisms that enhance C sequestration in NT systems. In particular, I am interested in examining (1) management effects on microbial community composition, (2) factors controlling microbial community composition, and (3) the influence that microbial

community structure has on soil organic matter (SOM) dynamics in agricultural soils. This dissertation represents a start at addressing these objectives. The three sections presented below provide an overview to each of the three main chapters in this dissertation and explain how each chapter relates to the overall objectives stated above.

Microbial community composition in agroecosystems

The soil microbial biomass, although usually comprising less than 5% of the total amount of soil organic carbon (C) and nitrogen (N), is responsible for most of the organic matter decomposition that occurs in soil and serves as both a source and sink of available plant nutrients (Jenkinson and Ladd, 1981). Amounts of microbial biomass are generally highest in undisturbed systems and decline with disturbance, particularly disturbance associated with cultivation of grassland and forest soils (Doran, 1980; Gupta and Germida, 1988). Agricultural management practices differ in the type and intensity of disturbance imposed and therefore the impact on the amount and activity of the microbial biomass varies with treatment. Agricultural systems that favor increased levels of microbial biomass include those associated with increased C inputs (Schnürer *et al.*, 1985) and SOM levels (Wardle, 1992), reduced tillage (Doran, 1987), retention of crop residues rather than removal by burning (Gupta, 1994), and with integrated farming systems that combine reduced tillage with increased C inputs through organic amendments (Hassink *et al.*, 1991). In several studies comparing integrated to conventional management, the amount of microbial biomass, which in these systems is dominated by bacteria, was not different between these two treatments; however, the

biomass of bacterial grazers (amoebae and nematodes) was higher under integrated management suggesting a higher production of microbial biomass where tillage is reduced and C inputs are greater (Brussaard *et al.*, 1990; Bloem *et al.*, 1994; Zwart *et al.*, 1994).

While the amount of microbial biomass corresponds closely to nutrient release, especially N mineralization (Paul and Voroney, 1984), the composition of the microbial community, composed largely of bacteria and fungi, may also be important in determining the rate of SOM decomposition and nutrient turnover and availability (Hendrix *et al.*, 1986; Beare *et al.*, 1992; Scow, 1997). Despite the effect agricultural management may have on the structure of the microbial community and the potentially important linkages between community structure and system function, relatively few studies have characterized microbial community composition in agroecosystems. Wardle (1995), in a recent literature review, compiled the results of 106 previous studies which examined the effects of tillage on one or more functional groups of the soil community. Of these papers, only ten presented data on the microflora. The majority of papers focused on micro- or macrofauna. Those papers including data on bacteria and fungi have made comparisons between cultivated and native (Gupta and Germida, 1988) and uncultivated grass systems (Schnürer *et al.*, 1986; Andren *et al.*, 1988; Paustian *et al.*, 1990), conventional and integrated farming systems (Brussaard *et al.*, 1990; Bloem *et al.*, 1994; Zwart *et al.*, 1994) and conventional tillage (CT) and no-tillage (NT) systems (Norstadt and McCalla, 1969; Doran, 1980; Linn and Doran, 1984; Beare *et al.*, 1992, 1993, 1997). Data from the CT versus NT comparisons suggest that, in general, both

bacterial and fungal abundance increases as tillage intensity is reduced (Wardle, 1995). What is less well understood is how the relative abundances of these two groups differ under these disturbance regimes.

Beare *et al.* (1992) tested the hypothesis that CT agroecosystems contain a bacterial-dominated microbial community with faster residue decomposition and nutrient mineralization, whereas NT agroecosystems contain a fungal-dominated community with slower residue decomposition and greater nutrient retention. In a field decomposition experiment with litter bags, they observed that total microbial biomass was greater on CT buried residues compared to NT surface residues; however, the proportion of the microbial biomass (bacteria plus fungi) composed of fungi was greater on NT surface residues compared to CT buried residues. Additionally, bacteria were relatively more important in decomposition of CT buried residues, while fungi had a somewhat greater influence on decomposition of NT surface residues. Neely *et al.* (1991) also observed that fungi were the dominant decomposers of NT surface residues. Fungi were also more abundant in the surface of NT mineral soil compared to CT soil (Doran, 1980; Beare *et al.*, 1993, 1997) and in tilled soil where crop residues were experimentally placed on the soil surface rather than incorporated (Holland and Coleman, 1987). While these studies support the hypothesis that management can influence microbial community composition, in addition to absolute amounts of microbial biomass, it is not known whether the observed patterns in microbial community structure can be generalized for a wide range of NT and CT soils. Additionally, factors controlling microbial community structure in NT and CT agroecosystems have not been examined.

The objectives of the study presented in Chapter 2 were to investigate the effects of NT and CT management on bacterial and fungal abundance and biomass across a range of sites and to examine the potential controls on the relative abundances of bacteria and fungi. In particular, we wanted to assess the effects of soil moisture on the degree of fungal dominance in these systems. To address our objectives we collected soil samples from six long-term tillage experiments where soil water availability varied locally with tillage treatment and geographically with climate.

Fungal growth form and SOM dynamics

A current trend in microbial ecology is the quantification of microbial community composition and there are many methods available for this purpose (Table 1.1). Many of these methods (Biolog, FAME, PLFA, community DNA hybridization) provide a community-level profile or "fingerprint" that is subsequently used to detect temporal or spatial changes in a community, to compare communities in different systems, or to examine how a community responds to disturbance (e.g., tillage, sewage sludge application). Often, however, it is not known exactly how a community has changed and to what extent a change in community composition will impact system function.

Rather than use a broad-scale approach to the measurement of microbial community composition, we have used direct microscopy to measure bacterial and fungal biomass in order to determine the relative contributions of bacteria and fungi to the total microbial community. A shift in the relative abundances of bacteria and fungi may be an important determinant of SOM decomposition rates and nutrient availability in soil

because of the fundamental differences in growth form between bacteria and fungi. The growth form of an organism constrains how that organism interacts with its environment, in particular, how it acquires and utilizes resources. Mycelial fungi, like many plants, grow indeterminately, are often branched, exhibit morphological plasticity, and have the ability to share resources between spatially distant parts of the fungal mycelium (Andrews, 1992; Ritz, 1995). These characteristics result in the development of extensive hyphal networks which influence nutrient distribution and soil structure. Nutrients may be transported within fungal hyphae from areas of high resource concentration ("hotspots") throughout the soil as fungi "forage" for unutilized substrates (Ritz, 1995). Fungi, through hyphal entanglement and production of extracellular polysaccharides which serve as binding agents, also contribute directly to the formation and stabilization of soil aggregates within which SOM is physically protected (Beare *et al.*, 1997).

The relationship between fungal growth form and resource acquisition and allocation may have important implications for SOM dynamics in NT systems, especially at the soil surface, where fungi represent a substantial portion of the microbial community and where C-rich surface residues are spatially separated from the soil inorganic N pool. It has been hypothesized that fungi comprise a larger proportion of the microbial biomass associated with NT surface residues and, therefore, are relatively more important for the decomposition of those residues, because they can more efficiently utilize the spatially compartmentalized C and N resources than can bacteria (Hendrix *et al.*, 1986; Holland and Coleman, 1986; Beare *et al.*, 1992). By bridging the soil-litter interface and

translocating nutrients, especially N, fungi may facilitate increased rates of decomposition that might otherwise be slowed due to the high C:N ratio (low quality) of the substrate (Figure 1.1).

The study described in Chapter 3 was designed to determine (1) if fungal-mediated N flow from the mineral soil to surface residues represents a significant N flux in NT systems and (2) how surface residue N composition and availability of exogenous mineral N influence residue-associated fungal biomass and rates of N translocation.

Patterns of C utilization efficiency and SOM retention

The amount of organic material retained in soil as SOM is determined by several factors. A substantial fraction (20-40%) of SOM is present as particulate organic matter consisting largely of partially decomposed plant residues (Cambardella and Elliott, 1992). Much of this material is located within stable soil aggregates where it is physically protected from decomposition (Cambardella and Elliott, 1992; Six *et al.*, 1998). Of the material that is utilized by the microbial community, only a fraction is incorporated into biomass, the remainder being lost during respiration. The amount of microbial biomass-C produced per amount of substrate-C utilized is referred to as the growth yield (Y) or C utilization efficiency. The rate and efficiency with which microbial biomass and by-products are subsequently utilized by other microorganisms depends on the chemical composition of the substrate and the physical location of that substrate within the soil.

Information on the proportion of substrate C that is used for biomass production rather than respired as $CO_2$ is useful for analyzing the impact of the microbial community on C cycling. Therefore, we were interested in comparing the growth yield efficiencies of

the microbial communities in NT and CT soils. Holland and Coleman (1987) hypothesized that SOM retention will be higher in systems where fungi predominate since fungal growth yield efficiencies are reportedly higher than those for bacteria (Waksman, 1929). To test this hypothesis, we examined patterns of C utilization in NT and CT soils which differed in the relative contributions that fungi make to the total microbial community (Frey *et al.*, 1998). Fungal:bacterial ratios were 0.96 and 0.45 for the NT and CT soils, respectively.

Glucose amended soil was incubated at 25°C and glucose-C concentration, $CO_2$-C losses, and bacterial and fungal biomass-C were determined over a 40 hr period. Two approaches were used for estimating Y: (1) $Y_s$ = (glucose-C used - respired-C)/glucose-C used, and (2) $Y_b$ = biomass-C produced/(biomass-C + respired-C). Calculation 1 assumes that all glucose-C utilized, minus that respired, is used for biomass and metabolite production. Calculation 2 assumes that substrate use equals biomass-C plus respired-C and does not account for biomass production consumed by grazers. Values for Y were 0.68 and 0.67 using calculation 1 and 0.42 and 0.62 using calculation 2 for NT and CT, respectively. These results suggested that microbial biomass turnover was higher in NT compared to CT. We hypothesized that microbivory was responsible for this increased turnover and for observed discrepancies in our yield estimates.

The study presented in Chapter 4 examines how protozoan grazing affects estimates of microbial C utilization efficiency. We established two grazing treatments (low vs high) under which patterns of glucose utilization were examined during short-term laboratory incubations. Chapter 5 describes a method for measuring soil glucose concentrations that was developed for use in the C utilization assays.

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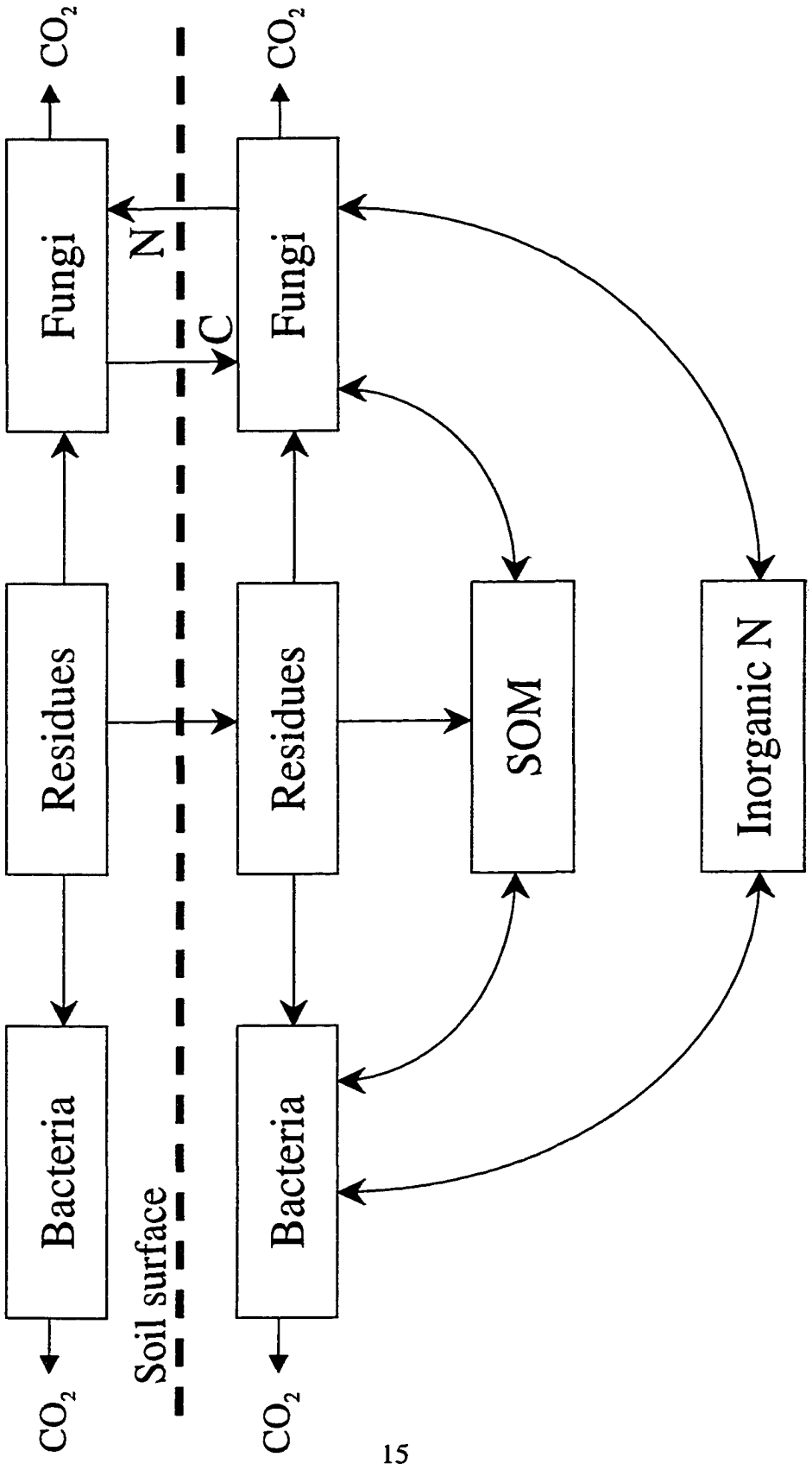
**Table 1.1**. Approaches used for the measurement of microbial community composition.

| Approach | Method |
|---|---|
| Species | Nucleic acid probes, traditional culturing techniques |
| Genetic | %G + C content, community DNA hybridization |
| Biochemical | Fatty acid profiles (FAME, PLFA) |
| Physiological | Community-level physiological profiles (Biolog) |
| Fungal:bacterial ratios | Microscopy, substrate-induced respiration with selective inhibitors, phospholipid fatty acids (PLFA) |

**Figure 1.1**. Carbon and nitrogen flows associated with residue decomposition in a no-tillage agroecosystem. Arrows depict flows of C and N, except where noted and for flows to or from inorganic components ($CO_2$ and mineral N). Populations of fungi in surface litter and in soil are depicted as interacting through translocation of N from soil to surface and C from surface to soil, while growth of bacteria in surface residue and in soil are viewed as being largely independent from each other.



# CHAPTER 2

# BACTERIAL AND FUNGAL ABUNDANCE AND BIOMASS IN CONVENTIONAL AND NO-TILLAGE AGROECOSYSTEMS ALONG TWO CLIMATIC GRADIENTS

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## ABSTRACT

Microbial community composition may be an important determinant of soil organic matter (SOM) decomposition rates and nutrient turnover and availability in agricultural soils. Soil samples were collected from six long-term tillage comparison experiments located along two climatic gradients to examine the effects of no-tillage (NT) and conventional tillage (CT) management on bacterial and fungal abundance and biomass at a wide range of sites and to examine potential controls on the relative abundances of bacteria and fungi in these two systems. Samples were divided into 0-5 and 5-20 cm depth increments and analyzed for bacterial and fungal abundance and biomass, total carbon (C) and nitrogen (N), particulate organic matter C and N (POM-C and N), soil water content, texture, pH, and water-stable aggregate distributions. Soil

moisture, which varied by tillage treatment and geographically with climate, ranged from 0.05 to 0.35 g $g^{-1}$ dry soil in the surface 0-5 cm and 0.15 to 0.28 g $g^{-1}$ dry soil at 5-20 cm. Measured organic matter C and N fractions and mean weight diameter (MWD) of water-stable aggregates were significantly higher in NT relative to CT at three of the six sites. Fungal hyphal length ranged from 19 to 292 m $g^{-1}$ soil and was 1.9 to 2.5 times higher in NT compared to CT surface soil across all sites. Few significant tillage treatment differences in soil physical and chemical properties or in fungal abundance and biomass were observed at 5-20 cm. Bacterial abundance and biomass were not consistently influenced by tillage treatment or site location at either depth. The proportion of the total biomass composed of fungi ranged from 10 to 60% and was significantly higher in NT compared to CT surface soil at five of six sites. Proportional fungal biomass was not strongly related to soil texture, pH, aggregation, or organic C and N fractions, but was positively related to soil moisture ($r = 0.67$; $P < 0.001$). The relationship between soil moisture and the degree of fungal dominance was due to the positive response of fungal biomass and the lack of response of bacterial biomass to increasing soil moisture across the range of measured soil water contents. Tillage treatment effects on fungal biomass and proportional fungal abundance were not significant when the data were analyzed by analysis of covariance with soil moisture as the covariate. These results suggest that observed tillage treatment and climate gradient effects on fungi are related to differences in soil moisture. Further research is needed, however, to determine how tillage-induced changes in the soil environment shape microbial community composition in agroecosystems.

# INTRODUCTION

Tillage-induced shifts in the relative abundances of bacteria and fungi may influence the rate of soil organic matter (SOM) decomposition and nutrient availability in agroecosystems. The soil microbial community has not been well characterized in agroecosystems (Wardle, 1995), particularly in conventional tillage (CT) and no-tillage (NT) systems which are the predominant management practices in U.S. agriculture. The abundances of bacteria and fungi in NT and CT soils have been quantified using plate counts (Norstadt and McCalla, 1969; Doran, 1980; Linn and Doran, 1984). However, these data do not allow quantitative comparison of the relative amounts of bacterial and fungal biomass, a critical requirement when linking community composition to nutrient dynamics. We know of only one study site, the long-term tillage comparison experiment in Horseshore Bend, Georgia, where microbial community composition in NT and CT systems has been quantitatively examined (Hendrix *et al.*, 1986; Neely *et al.*, 1991; Beare *et al.*, 1992, 1993; Beare, 1997). At this site, the NT system contains a fungal-dominated soil community, while the CT system contains a bacterial-dominated community. Since it is not known whether this pattern is generalizable to other NT and CT systems, our primary objective was to determine the effects of NT and CT management on the relative amounts of bacterial and fungal biomass at sites representing a wide range of climatic conditions.

Our second objective was to examine potential controls on microbial community composition. Several hypotheses have been proposed to explain tillage treatment differences in relative bacterial and fungal abundance. Fungi may be favored in NT systems because (1) reduced disturbance in NT facilitates establishment and maintenance of extensive hyphal

networks (Wardle, 1995), (2) fungi, with their mycelial growth form, can bridge the soil-residue interface and utilize the spatially separated C and N resources by translocating N from the soil inorganic N pool into the C-rich surface residues (Holland and Coleman, 1987; Beare *et al.*, 1992), and (3) fungi, unlike bacteria, can maintain activity in the dry surface litter environment encountered in NT systems (Hendrix *et al.*, 1986; Holland and Coleman, 1987; Beare *et al.*, 1992). These hypotheses remain largely untested.

In this work, we examined correlations between several soil physical and chemical characteristics and relative bacterial and fungal biomass. In particular, our goal was to assess the relationship between climate- and tillage-controlled soil moisture and the degree of fungal dominance. To address our objectives we collected soil samples from six long-term tillage comparison experiments for which average soil moisture varies within each experimental site as a function of tillage treatment and across sites due to geographic differences in annual temperature and precipitation patterns.

## MATERIALS AND METHODS

### Site characteristics

Soil and residue samples were collected from NT and CT plots at six long-term field experiments in May and June, 1996 (Table 2.1). Four sites located in the Great Plains (Mandan, ND; Sidney, NE; Stratton, CO; and Bushland, TX) represent a temperature gradient. Mean annual precipitation is relatively constant (380-473 mm) across these sites while mean annual temperature ranges from 5.0°C at Mandan, ND to 12.7°C at Bushland, TX. Annual open pan evaporation ranges from 940 mm at Mandan,

ND to 1762 mm at Bushland, TX. An additional two experiments were sampled at sites located on tallgrass prairie (Manhattan, KS) and forest-derived (Lexington, KY) soils. These two sites, along with Bushland, TX, represent a precipitation gradient. Mean annual temperature at these three sites is similar (~13°C) but mean annual precipitation varies from 473 mm at Bushland, TX to 1140 mm at Lexington, KY. Our sampling schedule was such that each site was sampled at a similar time in the growing season.

Tillage treatments at all sites, except Stratton, CO, are arranged in a randomized, complete block design with three to four replications. At Stratton, CO, conventional tillage is not included as a treatment in the experiment; therefore, samples were collected from an adjacent farmer's field that has been under continuous CT cultivation for at least 50 years. There are two replications at this site. Wheat-fallow rotations were sampled at each of the Great Plains sites, while soybean-wheat and continuous corn rotations were sampled at Manhattan, KS and Lexington, KY, respectively. All samples were collected in the fallow phase of the rotation except at Manhattan, KS and Lexington, KY where cropping is continuous. In the latter two cases, samples were collected in newly planted plots. At Manhattan, KS, soybeans had not germinated at the time of sampling. At Lexington, KY, the corn plants in sampled plots were approximately two weeks old. Additional information regarding management history and current management practices at these sites can be found in the references listed in Table 2.1.

Sample collection and processing

Six 5.6-cm diameter soil cores were collected from each treatment replicate and divided into 0-5 and 5-20 cm depth increments. In addition to soil samples, surface

residues were collected from each NT plot. All samples were transported to the laboratory on ice and subsequently stored at 4°C for no longer than 72 h prior to analysis. Soil cores for each treatment replicate were composited by depth increment, gently broken apart, passed through an 8-mm sieve, thoroughly mixed and subsampled for soil water content determined gravimetrically by drying for 24 h at 105°C. A 500 g subsample was taken and used for the microbiological analyses described below. The remainder of the soil was immediately air dried. No-tillage surface residue samples were placed on a 250 $\mu$m sieve to facilitate removal of unassociated soil particles. Large rocks and soil aggregates remaining on the sieve were removed by hand.

Physical and chemical analyses

Physical and chemical analyses were conducted on air-dried samples from which crop residues, root fragments and rocks larger than 2 mm had been removed. Total organic C (TOC) and N (TON) were determined on finely ground subsamples using a Leco CHN-1000 analyzer. Soil pH was determined in a 2:1 water:soil (vol/wt) suspension. Texture and particulate organic matter C and N (POM-C and N) were determined by a modified version of the POM isolation method of Cambardella and Elliott (1992). Briefly, 30 g soil was dispersed by shaking for 18 h in 100 ml 0.5% sodium hexametaphosphate. This suspension was passed through a 53 $\mu$m sieve and the sand plus POM fraction remaining on the sieve was thoroughly rinsed with deionized water, dried at 50°C, finely ground, and analyzed for total C and N as described above. The soil suspension that passed through the sieve (silt plus clay) was transferred to a 1-L

sedimentation cylinder and the silt and clay content was determined by the hydrometer method (Gee and Bauder, 1986). Soil physical characteristics are given in Table 2.2.

The size distribution of water-stable aggregates was measured according to the method described by Cambardella and Elliott (1993). Briefly, a 100 g subsample of air-dried soil was wet sieved through a series of three sieves to obtain the following aggregate size fractions: >2000 $\mu$m, 250-2000 $\mu$m, 53-250 $\mu$m and <53 $\mu$m. Soil remaining on each sieve was backwashed into an aluminum pan, dried overnight at 50°C, and weighed. The amount of soil in each aggregate size class was used to calculate the mean weight diameter (MWD) for each treatment replicate and depth increment (Kemper and Rosenau, 1986). MWD was used as an aggregation index for comparison across treatments.

Bacterial and fungal abundance and biomass

Bacterial abundance and fungal hyphal lengths were determined on field moist subsamples from each treatment replicate and depth increment. Ten g soil was suspended in 90 ml filter (0.2 $\mu$m) sterilized water and blended in a Waring blender at high speed for 1 min (Babiuk and Paul, 1970). The blended suspension was allowed to settle for 30 sec and a 10 ml subsample was collected and used to prepare dilutions. Two sets of soil smears, one each for bacterial and fungal staining, were prepared by pipetting 10 $\mu$l of the appropriate soil dilution (1:50 or 1:100) onto microscope slides containing 6-mm diameter wells. The smears were thoroughly air-dried and heat-fixed prior to staining. Residue samples were treated similarly except that 0.5 g residue material was blended in

100 ml of filter sterilized water. After blending, 10 $\mu$l of this suspension was pipetted directly into a well on a microscope slide and dried.

Bacteria were counted from soil smears stained with DTAF [5-(4, 6-dichlorotriazin-2-yl)aminofluorescein] (Bloem *et al.*, 1995). The smears were observed under a Zeiss Axiophot epifluorescence microscope at 1000× magnification using a Zeiss filter set for blue light (BP 450-490 nm exciter filter, 510 nm beam splitter and LP 520 nm barrier filter). Bacteria were manually counted from images of each microscope field that were captured using an Optronics cooled CCD camera (model DEI-470) and Adobe Photoshop image capturing software. Camera resolution was 470 TV lines horizontal and 450 TV lines vertical (PAL). Five to twenty images were randomly collected from each smear, depending on the number of cells observed in each image. In no case were fewer than 200 individual cells enumerated. Widths and lengths of individual cells, in pixels, were manually measured on a random subset of the collected images using the mouse and IPLab Spectrum image analysis software (Signal Analytics Corporation, Vienna, Virginia). The number of pixels for each width or length measurement was converted to $\mu$m using a conversion factor obtained from measurements of an image of a stage micrometer. Twenty to thirty cells were measured for each treatment replicate and depth increment for a total of 1557 cell measurements. Bacterial biovolumes per cell (V) were calculated from average widths (W) and lengths (L) using the equation $V = \pi/4 \times W^2 \times (L-W/3)$ (Bloem *et al.*, 1995). There were no significant differences in bacterial biovolume across treatment or depth within a site; however, biovolume did vary across sites. The following biovolume values were used for conversion of bacterial abundance to

biomass: Mandan, 0.30 $\mu m^3$; Sidney, 0.28 $\mu m^3$; Stratton, 0.25 $\mu m^3$; Bushland, 0.16 $\mu m^3$; Manhattan, 0.24 $\mu m^3$; Lexington, 0.20 $\mu m^3$. Bacterial biomass C was estimated by assuming a specific carbon content of 0.22 g C $cm^{-3}$ (Bratbak and Dundas, 1984).

Fungal hyphae were stained with calcifluor M2R fluorescence brightener (Bloem *et al.*, 1995) and observed at 400× magnification using the filter set for UV illumination (BP 340-380 nm exciter filter, 400 nm beam splitter and LP 430 nm barrier filter). Thirty images were randomly collected from each smear. In a preliminary experiment, we found that collection of 30 images was necessary to obtain acceptable levels of variability in the estimation of fungal hyphal length from a given soil smear (Fig. 2.1). Fungal hyphal lengths and widths were measured as described for bacteria. The image analysis software used allows for measurement of irregularly shaped objects. Fungal hyphae, which are often curved, were measured by outlining the entire hyphal length using the mouse. There were no significant differences in hyphal width across treatment or depth, but hyphal width did vary across sites. The following width values were used for conversion of fungal length to biovolume: Mandan, 1.49 $\mu m$; Sidney, 1.27 $\mu m$; Stratton, 1.48 $\mu m$; Bushland, 1.20 $\mu m$; Manhattan, 1.27 $\mu m$; Lexington, 1.58 $\mu m$. Fungal biomass C was estimated by multiplying biovolumes by 0.33 g $cm^{-3}$ and assuming 40% C (van Veen and Paul, 1979). Our biomass estimates represent total fungal biomass as calcifluor does not differentiate between empty and cytoplasm-filled hyphae.

Statistical analyses.

Analysis of variance for determining tillage effects on soil physical, chemical and microbiological characteristics was performed using the SAS general linear models

procedure (PROC GLM, SAS Institute, 1990). Analysis of covariance was performed with soil moisture as the covariate to examine whether soil moisture could explain observed tillage differences in fungal biomass and relative fungal abundance. The assumption of analysis of covariance, that the slope of the covariate by independent variable was the same for all levels of the independent variable, was verified by testing for heterogeneity of slopes (Cody and Smith, 1997). All abundance and biomass data were log transformed and proportions were arcsine-square-root transformed prior to analysis in order to meet normality and homogeneity of variance assumptions for ANOVA and ANCOVA (Sokal and Rohlf, 1981). The SAS correlation procedure was used for determining correlation coefficients among soil physical, chemical and microbiological properties. Linear and curvilinear regressions and coefficients of determination ($r^2$) were computed using SigmaPlot for Windows 1.02 (Jandel Scientific).

## RESULTS AND DISCUSSION

### Soil physical and chemical characteristics

Intact soil cores were collected for bulk density measurements since it is often preferable to express soil C and N fractions and water content on a volumetric basis. However, it was often difficult to get intact cores in recently plowed CT plots and at sites, particularly Bushland, TX, where the surface soil was dry. Therefore, we were unable to obtain accurate bulk density values for all treatment replicates. By making volumetric calculations for the sites where intact cores were collected, we found that expression of the data on a volumetric basis did not significantly change our results. Therefore, all C

and N data are expressed on a concentration basis. Likewise, gravimetric water content is reported rather than volumetric water content or percent water-filled pore space.

The extent to which soil water content at the time of sampling reflected across-site differences in mean annual temperature and precipitation depended on the gradient along which the sites were located (Tables 2.1 and 2.2). Soil water content at 0-5 cm for sites located along the precipitation gradient was positively related to mean annual precipitation ($r = 0.90$, $n = 18$, $P < 0.001$) and negatively related to annual open pan evaporation ($r = -0.89$, $n = 18$, $P < 0.001$). Soil water content across the temperature gradient was negatively related to mean annual temperature ($r = -0.61$, $n = 22$, $P < 0.001$) and open pan evaporation ($r = -0.79$, $n = 22$, $P < 0.001$). Soil moisture was significantly higher in NT compared to CT at four of six sites for the 0-5 cm depth increment. Treatment and across-site differences for the 5-20 cm depth increment were less pronounced.

Although NT systems generally show higher levels of SOM (Doran, 1980, 1987; Paustian *et al.*, 1997) and aggregate stability (Cambardella and Elliott, 1993; Beare *et al.*, 1994), only three of the six sites showed treatment differences for these soil characteristics (Tables 2.2 and 2.3). No-till soil at the Sidney, NE, Manhattan, KS and Lexington, KY sites had significantly higher TOC and N, POM-C and N and MWD of water-stable aggregates compared to CT. Greater accumulations of microbial aminosugars (muramic acid and glucosamine) have also been observed in NT soil for these three sites (Guggenberger *et al.*, 1999). Most treatment differences were observed in the top 0-5 cm. Total organic C and N showed the greatest differences at Sidney, NE

and Lexington, KY, sites which were uncultivated prior to experiment establishment. The Nebraska site was established on virgin prairie sod, while the Kentucky site had been in bluegrass sod for at least 50 years. Our results are consistent with previous studies at these sites (Doran, 1980, 1987).

Few significant treatment differences were observed at either depth increment at the Mandan, ND, Stratton, CO, and Bushland, TX sites. These sites were established on previously cultivated and highly degraded soil. Soil organic matter accumulation coupled with a reduction in further SOM losses in the NT plots would be necessary for the development of observable treatment differences under such soil conditions. Several factors may limit SOM accumulation at these Great Plains sites. Since NT was implemented 12-15 years prior to our sampling, as opposed to the 22-26 years for the other sites, there has been less time for significant accumulation to occur. Additionally, and perhaps most importantly, C inputs are low in wheat-fallow systems, particularly in low rainfall areas (Peterson *et al.*, 1998) and decomposition rates, while potentially water-limited during the summer, can be high during the warm, moist spring (Coleman *et al.*, 1990).

Bacterial and fungal abundance and biomass

Bacterial abundance for the 0-5 cm depth increment ranged from $5.6 \times 10^8 \pm 1.4 \times 10^7$ bacteria $g^{-1}$ soil in NT at Bushland, TX to $1.6 \times 10^9 \pm 1.4 \times 10^8$ bacteria $g^{-1}$ soil in NT at Manhattan, KS for the 0-5 cm depth increment. There were no significant treatment differences at four of the six sites (Fig. 2.2A). Bacterial abundance was significantly

higher in NT relative to CT at Sidney, NE and Manhattan, KS. There was a trend of increasing bacterial abundance in CT relative to NT at 5-20 cm for the four Great Plains sites (Fig. 2.2B); however, these differences were only significant at the Mandan, ND and Stratton, CO sites. This trend was not altogether unexpected since we frequently observed a concentration of crop residues at 10-20 cm while sampling. This observation is supported by the trend toward increased levels of POM-C in CT compared to NT at 5-20 cm (Table 2.3).

Fungal abundance in surface soil (0-5 cm) was significantly higher in NT than in CT at all sites (Fig. 2.2C). Fungal hyphal length in NT ranged from 46 m $g^{-1}$ soil at Bushland, TX to 292 m $g^{-1}$ soil at Lexington, KY and was 1.9 to 2.5 times greater in NT relative to CT across all sites. Hyphal lengths in CT ranged from a low of 19 m $g^{-1}$ soil at Bushland, TX to a high of 128 m $g^{-1}$ soil at Lexington, KY. When we compared sites located along the temperature gradient, we observed that fungal hyphal length in NT increased from 72 m $g^{-1}$ soil at Mandan, ND, the coldest site on average, to 203 m $g^{-1}$ soil at Stratton, CO and declined to a low of 46 m $g^{-1}$ soil at Bushland, TX, the warmest site. Fungal abundance in surface soil at sites along the precipitation gradient increased from the dryest (Bushland, TX) to wettest (Lexington, KY) site. Fungal abundance in CT followed the same patterns, although the trends were less pronounced. No significant treatment differences in fungal hyphal length at 5-20 cm were observed; however, the across-site trends discussed above for the 0-5 cm depth were also evident at the lower depth (Fig. 2.2D). Our fungal abundance estimates are well within the wide range of 3.0 to 500 m $g^{-1}$ soil reported for other intensively cultivated systems (Holland and Coleman,

1987; Gupta and Germida, 1988; Beare *et al.*, 1993). An even wider range is obtained if the bacterially-dominated polder soils of The Netherlands (Brussaard *et al.*, 1990; Bloem *et al.*, 1994) and the fungally-dominated forest-derived soils from Sweden (Schnürer *et al.*, 1985, 1986) are considered.

Fungi were more strongly influenced by tillage than were bacteria in our study. These results are in contrast to previous studies where abundances of both bacteria and fungi in surface soil (0-7.5 cm) were observed to be higher in NT compared to CT (Doran, 1980; Linn and Doran, 1984), but are in agreement with the idea that fungi are more susceptible than are bacteria to disturbance caused by plowing (Wardle, 1995). Holland and Coleman (1987) observed significantly higher fungal hyphal lengths in the surface 0-5 cm of plowed soil when wheat residues were surface applied rather than directly incorporated into the soil, suggesting that higher fungal biomass in NT systems is related to the presence of surface residues.

To examine the relative contributions of bacteria and fungi to the microbial community, it was necessary to convert the abundance data to biomass. Since there were no significant differences in bacterial biovolumes and fungal widths between treatments within a site, absolute amounts of bacterial and fungal biomass C followed the same trends as the abundance data, except that across-site differences were more pronounced (Table 2.4). Bacterial biomass C was significantly higher in NT compared to CT at only two sites (Sidney, NE and Manhattan, KS) for the 0-5 cm depth increment. Fungal biomass C was significantly higher in NT compared to CT in surface soil for all sites. Total microbial biomass C (bacteria+fungi) in surface soil showed treatment differences

at three of the six sites (Sidney, NE; Manhattan, KS; Lexington, KY), the same sites that showed treatment differences in TOC and N, POM-C and N and in MWD of water stable aggregates (Tables 2.2 and 2.3). Total biomass was not significantly higher in NT at three of the Great Plains sites (ND, CO, TX) because significant differences in fungal biomass were offset by increases in bacterial biomass in CT. No significant treatment differences in total microbial biomass C were observed for the 5-20 cm depth increment at any of the sites.

The amount of microbial biomass present in a system is often related to SOM levels (Schnürer *et al.*, 1985; Wardle, 1992). Changes in microbial biomass may also be indicative of future changes in SOM levels since the microbial community responds rapidly to disturbance or altered residue inputs (Gupta *et al.*, 1994). In our study, total microbial biomass was higher in NT compared to CT only at those sites showing significantly higher SOM levels under NT; however, fungal biomass responded positively to NT management at all sites, suggesting that the fungal component of the microbial biomass may be a sensitive indicator of long-term change in these systems.

The relative contributions of bacteria and fungi to the total microbial biomass were significantly influenced by tillage (Fig. 2.3), especially at 0-5 cm. Fungal biomass made up a significantly higher proportion of the total biomass in surface soil of NT compared to CT at five of six sites even though bacterial biomass was greater than fungal biomass under both tillage treatments in most cases (Fig. 2.3A). Our data are in agreement with the work of Beare *et al.* (1992) and Beare (1997) who observed higher fungal to bacterial ratios in NT compared to CT residues and soil at their site in

Horseshoe Bend, GA. Our results indicate that fungi are a relatively more important component of the microbial biomass in NT compared to CT surface soil across a wide range of climates.

In general, proportional fungal biomass, when calculated over the whole plow layer (0-20 cm), was higher in NT compared to CT (data not shown). However, most significant treatment differences in fungal biomass and proportional fungal biomass were observed in the 0-5 cm depth (Fig. 2.2 and Fig. 2.3). Thus a shift in microbial community composition in NT systems will be most critical for residue decomposition and nutrient cycling processes occurring near the soil surface.

Expressed as a percentage of TOC, total microbial biomass (bacterial+fungal C) ranged from 0.3 to 0.8%. There were no differences between CT and NT soils for this variable. The observed values are at the low end of the range of 0.3 to 3.1% reported for bacterial and fungal biomass estimates made by direct counts (Schnürer *et al.*, 1985; Paustian *et al.*, 1990; Bloem *et al.*, 1994) and are lower than most estimates using microbial biomass C values as measured by chloroform-fumigation methods (Hassink *et al.*, 1991; Gupta *et al.*, 1994). Discrepancies between the two methods for microbial biomass estimation have been reported (Holland and Coleman, 1987; Ingham and Horton, 1987; Bloem *et al.*, 1994). In particular, microbial biomass C estimates obtained by chloroform fumigation are often significantly higher than those obtained by direct microscopic enumeration.

While chloroform fumigation methods may result in overestimation of microbial biomass C (Ingham and Horton, 1987; Bloem *et al.*, 1994), direct count methods may

give underestimates. Reasons cited for this underestimation include (1) masking of microbes, especially bacteria, by soil particles, (2) weakly stained bacterial cells and non-fluorescent hyphae (Scheu and Parkinson, 1994), (3) difficulty in detaching microorganisms, especially fungi, from plant residues, and (4) inaccurately assumed or measured biovolumes. Additionally, a wide range of factors are reported for conversion of biovolume to biomass, especially for bacteria. To minimize these problems, we conducted preliminary experiments to determine the level of dilution required to minimize masking and maximize cell counts. We also made careful biovolume measurements rather than assume a literature value for all cells. Our bacterial biovolume (0.16-0.30 $\mu m^3$) and hyphal width (1.20-1.49 $\mu m$) estimates appear reasonable when compared with reported values. As for conversion factors, we selected the most widely used and accepted conversion factor for fungal biomass (van Veen and Paul, 1979) and selected an intermediate value from the published range for conversion of bacterial biovolume to biomass (Bratbak and Dundas, 1984). Although our direct count methods may have underestimated bacterial and fungal biomass, the relative differences between treatments and sites were not affected.

Relationships among soil characteristics and bacterial and fungal biomass

Bacterial and fungal biomass in surface soil of NT and CT were not strongly related to soil texture or pH but were positively correlated with measured soil C and N fractions (Table 2.5). Cause and effect relationships are difficult to assess, however, since these fractions, like bacterial and fungal biomass C, were also positively related to

soil water content with correlation coefficients of 0.74, 0.73, 0.71, and 0.64 for TOC, TON, POM-C and POM-N, respectively ($n = 34$, $P < 0.001$ for all comparisons). Mean weight diameter was significantly correlated with fungal biomass ($r = 0.66$, $P < 0.001$); however, this relationship was due to the high MWD values observed at the Lexington, KY site. When the KY data were excluded from the analysis, this relationship was no longer significant ($r = 0.34$, $P > 0.05$). There were no significant correlations between sand, silt or clay content and soil moisture. Correlation analysis for the 5-20 cm depth increment is not presented since few differences in soil physical, chemical or microbiological characteristics were observed. Bacterial and fungal biomass and proportional fungal abundance associated with NT surface residues showed no significant correlations with residue or soil moisture (data not shown).

Soil water content measured at the time of sampling accounted for the highest proportion of the variation in both bacterial and fungal biomass data (Table 2.5, Fig. 2.4A). Both bacterial and fungal biomass were positively related to water content. A curvilinear function best described the relationship for bacteria ($r^2 = 0.62$, $n = 34$, $P < 0.001$). Bacterial biomass increased linearly from water contents of 0.05 g $g^{-1}$ dry soil to approximately 0.15 g $g^{-1}$ dry soil and then remained relatively constant above that value. Fungal biomass was linearly related to soil moisture across the entire range of water contents measured ($r^2 = 0.72$, $n = 34$, $P < 0.001$). Due to the differential response of bacteria and fungi to soil moisture, proportional fungal abundance was curvilinearly related to soil moisture (Fig. 2.4B; $r^2 = 0.54$, $n = 34$, $P < 0.001$). Since bacterial and fungal biomass responded similarly to water contents below 0.15 g $g^{-1}$ dry soil,

proportional fungal abundance was not related to water content below this value. Above 0.15 g $g^{-1}$ dry soil, however, fungi composed an increasing percentage of the total biomass as water content increased, ranging from approximately 20% at 0.15 g $g^{-1}$ dry soil to 60% at 0.35 g $g^{-1}$ dry soil.

Soil moisture as a control on microbial community composition.

Our results suggest that soil moisture may be an important control on microbial community structure, not only across sites varying in soil moisture as a result of varying precipitation and temperature regimes, but also between tillage treatments within a site. This idea is supported further by our examination of the data by analysis of covariance using soil moisture as the covariate (Sokal and Rohlf, 1981). This analysis allowed us to determine whether treatment differences in our measured dependent variables would exist if soil moisture were held constant across tillage treatments. When the data were adjusted for soil moisture, significant tillage treatment effects on fungal biomass and the proportion of the microbial biomass composed of fungi were not observed (Table 2.6). We also ran the covariance analysis of variance with MWD, TOC, and POM-C as covariates and found that when the data were adjusted for these variables the tillage effects on fungal parameters were still significant (data not shown). These results lead us to hypothesize that treatment-induced differences in soil moisture account, at least in part, for the observed shifts in microbial community structure in NT agroecosystems.

Other hypotheses have been proposed to explain tillage treatment differences in microbial community composition. It has been suggested that fungi are relatively more abundant than bacteria in NT compared to CT systems because they are physiologically

capable of growth and activity at much lower water potentials than are bacteria and thus have an advantage over bacteria in the dry environment encountered in NT surface residues (Hendrix *et al.*, 1986; Holland and Coleman, 1987; Beare *et al.*, 1992). This hypothesis, based on studies in which the responses of bacteria and fungi to drying have been examined (Griffin, 1972; Wilson and Griffin, 1975; Schnürer et al., 1986), predicts that the degree of fungal dominance in NT surface residues will be inversely related to soil moisture (Hendrix et al., 1986). While total microbial biomass, tends to decline in response to drying (Wardle, 1992), there is often a differential effect of drying on the biomass and activity of bacteria and fungi. Griffin (1972, 1981) reviewed the data on microbial growth over a range of soil water potentials. Bacterial abundance and activity decline rapidly as soil water content falls below field capacity and bacterial respiration and bacterially-mediated transformations are negligible at potentials below -1.5 MPa (Wilson and Griffin, 1975; Griffin, 1981). Many fungi are active at considerably lower water potentials. Schnürer *et al.* (1986) found that both bacterial numbers and active fungal hyphae declined rapidly as a field soil dried; however, active hyphal dynamics paralleled $O_2$ consumption and thus the majority of microbial respiration was attributed to fungi.

The above explanation, though widely accepted, cannot account for the increased fungal abundance observed in NT soils. Firstly, previous studies have shown that fungi are the dominant decomposers of NT surface residues (Neely *et al.*, 1991; Beare *et al.*, 1992). However, we estimate, based on measurements of bacterial and fungal biomass on surface residues and average residue loading data obtained from long-term site records

(Peterson *et al.*, 1998), that bacterial and fungal biomass associated with NT surface residues represents less than 1% of the total microbial biomass present in the system to a soil depth of 20 cm. Therefore, relative fungal dominance in NT surface residues cannot explain the increased fungal abundance in NT systems as a whole. Secondly, our data do not support the prediction that relative fungal dominance is inversely related to soil moisture since we found no correlation between our residue biomass estimates and soil water content. Finally, if we applied to soil the argument that fungi have an advantage over bacteria under dry conditions, we would predict higher relative fungal abundance in CT rather than NT since CT soil, in general, is drier than adjacent NT soil where the presence of surface residues reduces evaporative water loss.

While many fungi are physiologically capable of growth under dry conditions that otherwise would limit bacterial activity, this trait does not explain our observation of increasing proportional fungal abundance with increasing soil moisture. Although bacteria and fungi have different low-end water potential limits for growth, most microorganisms attain maximum growth rates at or above -0.1 MPa and therefore under moister conditions, relative differences in bacteria and fungi are likely due to factors other than direct effects of water potential on growth, such as pH, aeration, nutrient availability and microbivory (Cook and Baker, 1983). In our study, fungal biomass increased linearly, while bacterial biomass was relatively constant across most of the observed range in soil water content. Schnürer *et al.* (1986) observed, in irrigated field plots kept continuously moist, that both bacterial and fungal biomass were relatively constant; however, observations of cell sizes and populations of grazers suggested that fungal

growth was promoted to a greater degree than bacterial growth. Schnürer *et al.* (1986) suggested that bacteria become substrate limited under continuously moist conditions as available labile C sources are utilized, while fungi remain active by utilizing more recalcitrant SOM. Alternatively, the germination and proliferation of "sugar fungi" may be promoted by the soluble compounds released when dry soil is wetted following an irrigation or precipitation event.

In summary, different agricultural tillage practices can strongly influence the abundance and biomass of soil microorganisms. In this study, fungal biomass and the proportion of the total biomass composed of fungi increased in surface soil in response to reduced tillage and across a gradient of increasing soil moisture, while bacterial biomass was not strongly affected by tillage and remained relatively constant in response to changing soil moisture above a water content of approximately 0.15 g $g^{-1}$ dry soil. Soil moisture may differentially influence bacteria and fungi either by directly affecting survival and growth or indirectly through shifts in substrate availability or microbivore populations. We caution, however, that altering the tillage regime impacts the soil environment in complex, interactive ways. No-till soils, in addition to being moister, tend to have higher SOM levels, higher bulk densities, and lower temperatures. Further research, especially controlled experimentation, is needed to elucidate the role that tillage-induced changes in the soil environment play in shaping microbial community composition.

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**Table 2.1.** Site characteristics.

| Site[1] | Experiment duration (years) | Previous management | Soil classification | MAP[c] (mm) | MAT (°C) | OPE (mm) | Sampling date (1996) |
|---|---|---|---|---|---|---|---|
| Mandan, ND[1] | 12 | >60 yr CT wheat-fallow[s] | fine-loamy, mixed, Typic Argiborolls | 402 | 5.0 | 940[v] | June 24 |
| Sidney, NE[2] | 26 | uncultivated native sod | fine-silty, mixed, mesic Pachic Haplustoll | 380 | 8.5 | 1120[v] | June 17 |
| Stratton, CO[3] | 11 | 50+ yr CT wheat-fallow | fine-silty, mixed, mesic Aridic Argiustoll | 410 | 10.7 | 1270 | June 3 |
| Bushland, TX[4] | 15 | 30+ yr CT wheat-fallow | fine, mixed, thermic Torrertic Paleustoll | 473 | 12.7 | 1762 | May 13 |
| Manhattan, KS[5] | 22 | 60+ yr CT annual cropping | fine-silty, mixed, mesic Cumulic Haplustoll | 835 | 12.8 | 1200[v] | May 20 |
| Lexington, KY[6] | 26 | 50 yr bluegrass sod | fine, mixed, mesic Typic Paleudalfs | 1140 | 13 | 899[a] | June 10 |

[1]Numbers refer to references from which site information was obtained: [1]Black and Tanaka (1997), [2]Lyon et al. (1997), [3]Peterson and Westfall (1997), [4]Jones et al. (1997), [5]Havlin and Kissel (1997), [6]Frye and Blevins (1997).

[s]Ardell Halvorson (pers. comm.).

[c]Abbreviations: MAP = mean annual precipitation, MAT = mean annual temperature, OPE = open pan evaporation for the growing season, April-September.

[v]Data obtained from the National Climate Data Center database.

[a]Data obtained from Farnsworth and Thompson (1982).

**Table 2.2.** Soil physical characteristics.

| | Depth increment | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0-5 cm | | | | | 5-20 cm | | | | |
| Site[§] | Sand content (g kg$^{-1}$) | Clay content (g kg$^{-1}$) | pH | Water content (g g$^{-1}$) | MWD[¶] (mm) | Sand content (g kg$^{-1}$) | Clay content (g kg$^{-1}$) | pH | Water content (g g$^{-1}$) | MWD (mm) |
| Mandan, ND | | | | | | | | | | |
| NT | 180 | 260 | 6.2 | 0.23 | 0.29 | 180 | 280 | 6.3 | 0.23 | 0.29 |
| CT | 200 | 280 | 6.2 | 0.20 | 0.23 | 190 | 310 | 6.4 | 0.23 | 0.32 |
| Sidney, NE | | | | | | | | | | |
| NT | 150* | 310* | 6.5* | 0.31* | 0.49* | 350 | 210 | 7.0 | 0.23* | 0.54* |
| CT | 360 | 210 | 6.8 | 0.16 | 0.32 | 370 | 210 | 6.8 | 0.20 | 0.32 |
| Stratton, CO | | | | | | | | | | |
| NT | 220 | 330 | 7.4* | 0.27* | 0.22 | 190 | 360 | 7.9 | 0.26* | 0.37 |
| CT | 280 | 240 | 8.1 | 0.19 | 0.26 | 270 | 260 | 8.3 | 0.22 | 0.35 |
| Bushland, TX | | | | | | | | | | |
| NT | 170 | 330 | 5.6 | 0.06 | 0.25 | 140 | 380 | 6.5 | 0.18* | 0.40 |
| CT | 170 | 310 | 5.8 | 0.05 | 0.20 | 140 | 380 | 6.6 | 0.15 | 0.36 |
| Manhattan, KS | | | | | | | | | | |
| NT | 60 | 210 | 5.2 | 0.23* | 0.28* | 50 | 270 | 5.5 | 0.22 | 0.24 |
| CT | 60 | 210 | 5.2 | 0.14 | 0.16 | 50 | 260 | 5.6 | 0.21 | 0.22 |
| Lexington, KY | | | | | | | | | | |
| NT | 80 | 300 | 6.1 | 0.34* | 1.86* | 80 | 310 | 6.6 | 0.28* | 1.03* |
| CT | 70 | 320 | 6.0 | 0.23 | 0.61 | 70 | 320 | 6.3 | 0.25 | 0.66 |

[§]The sites are listed according to the climatic gradient along which they are located, with Mandan, ND, Sidney, NE, Stratton, CO and Bushland, TX located along the north-south temperature gradient and Bushland, TX, Manhattan, KS and Lexington, KY located from west to east along the precipitation gradient.

[¶]MWD = mean weight diameter of water-stable aggregates.

*Treatment means within a site and depth increment are significantly different at the 0.05 probability level.

**Table 2.3**. Total organic and particulate organic matter (POM) C and N.

| Site | Depth increment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0-5 cm | | | | 5-20 cm | | | |
| | Total organic C | Total organic N | POM-C | POM-N | Total organic C | Total organic N | POM-C | POM-N |
| | g kg$^{-1}$ soil | | | | | | | |
| Mandan, ND | | | | | | | | |
| NT | 19.5 | 2.00 | 2.55 | 0.19 | 18.3* | 1.77* | 0.98 | 0.08 |
| CT | 22.1 | 2.00 | 2.63 | 0.18 | 21.5 | 2.07 | 1.29 | 0.08 |
| Sidney, NE | | | | | | | | |
| NT | 23.0* | 2.33* | 4.36* | 0.38* | 13.1 | 1.40 | 1.06 | 0.12 |
| CT | 12.7 | 1.33 | 1.42 | 0.12 | 13.0 | 1.33 | 1.29 | 0.10 |
| Stratton, CO | | | | | | | | |
| NT | 10.5 | 1.10 | 2.66 | 0.19 | 8.1 | 0.75 | 0.68* | 0.05 |
| CT | 9.9 | 1.10 | 2.09 | 0.18 | 9.5 | 0.90 | 1.22 | 0.07 |
| Bushland, TX | | | | | | | | |
| NT | 9.7 | 1.17 | 1.28 | 0.10 | 8.0 | 0.87 | 0.40 | 0.04 |
| CT | 10.1 | 1.07 | 1.32 | 0.09 | 8.8 | 1.00 | 0.50 | 0.04 |
| Manhattan, KS | | | | | | | | |
| NT | 18.2* | 1.87* | 4.00* | 0.33* | 13.9 | 1.20 | 0.81 | 0.06 |
| CT | 11.7 | 1.17 | 2.27 | 0.17 | 11.7 | 1.17 | 1.08 | 0.08 |
| Lexington, KY | | | | | | | | |
| NT | 24.9* | 2.73* | 3.19* | 0.21* | 13.8 | 1.60 | 1.55 | 0.11 |
| CT | 12.1 | 1.20 | 1.74 | 0.13 | 12.4 | 1.47 | 1.61 | 0.12 |

*Treatment means within a site and depth increment are significantly different at the 0.05 probability level.

**Table 2.4.** Bacterial, fungal and total microbial biomass C.

| | Microbial Biomass C | | | | | |
|---|---|---|---|---|---|---|
| | 0-5 cm | | | 5-20 cm | | |
| Site | Bacteria | Fungi | Total biomass[§] | Bacteria | Fungi | Total biomass |
| | $\mu$g C g$^{-1}$ soil | | | | | |
| Mandan, ND | | | | | | |
| NT | 39.7 | 16.3* | 56.0 | 31.5* | 11.3 | 42.8 |
| CT | 44.8 | 8.5 | 54.9 | 50.9 | 7.5 | 58.4 |
| Sidney, NE | | | | | | |
| NT | 50.8* | 20.4* | 71.2* | 35.3 | 15.7 | 50.9 |
| CT | 35.5 | 8.0 | 43.5 | 45.1 | 19.8 | 64.9 |
| Stratton, CO | | | | | | |
| NT | 46.6 | 42.5* | 89.1 | 28.5* | 59.3 | 87.7 |
| CT | 60.2 | 19.6 | 79.8 | 60.3 | 56.7 | 117.0 |
| Bushland, TX | | | | | | |
| NT | 19.9 | 6.8* | 26.7 | 24.3 | 9.7 | 34.0 |
| CT | 23.4 | 2.8 | 26.2 | 35.9 | 8.0 | 43.9 |
| Manhattan, KS | | | | | | |
| NT | 82.6* | 19.8* | 102.0* | 47.1 | 14.7 | 61.8 |
| CT | 41.2 | 7.9 | 49.1 | 47.6 | 12.1 | 59.7 |
| Lexington, KY | | | | | | |
| NT | 48.3 | 74.3* | 123.0* | 47.2 | 38.9 | 86.1 |
| CT | 42.2 | 32.7 | 74.9 | 39.4 | 39.6 | 78.9 |

[§]Total microbial biomass is calculated here as bacterial biomass C plus fungal biomass C.
*Treatment means within a site and depth increment are significantly different at the 0.05 probability level.

**Table 2.5.** Correlation coefficients among soil physical and chemical characteristics, bacterial biomass, fungal biomass, and the proportion of total biomass composed of fungi (%Fungi) for the 0-5 cm depth increment.

| Soil characteristic | Bacterial biomass C ($\mu$g g$^{-1}$ soil) | Fungal biomass C ($\mu$g g$^{-1}$ soil) | %Fungi |
|---|---|---|---|
| Sand, g kg$^{-1}$ | NS | NS | NS |
| Silt, g kg$^{-1}$ | 0.42* | NS | NS |
| Clay, g kg$^{-1}$ | -0.39* | NS | 0.40* |
| pH | NS | NS | NS |
| Water content, g g$^{-1}$ dry soil | 0.67*** | 0.85*** | 0.67*** |
| Total organic C, mg g$^{-1}$ soil | 0.44** | 0.45** | NS |
| Total organic N, mg g$^{-1}$ soil | 0.40** | 0.51* | 0.42* |
| Particulate organic matter C, mg g$^{-1}$ soil | 0.65*** | 0.49** | NS |
| Particulate organic matter N, mg g$^{-1}$ soil | 0.65*** | 0.41* | NS |

*, **, ***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively; NS = not significant.

**Table 2.6.** Results of analysis of variance (ANOVA) and analysis of covariance (ANCOVA) showing the F value and level of significance for each variation source associated with total fungal biomass and the proportion of the total biomass composed of fungi (n = 34).

| Analysis<br>Dependent variable | Sources of variation[§]<br>Tillage | <br>Moisture |
|---|---|---|
| ANOVA | | |
| Total fungal biomass | 8.97** | N/A |
| Fungi as proportion of total biomass | 8.72** | N/A |
| ANCOVA with moisture as covariate | | |
| Total fungal biomass | 1.09 NS | 59.78*** |
| Fungi as proportion of total biomass | 1.93 NS | 16.78*** |

[§]N/A = not applicable, NS = not significant, ** and *** indicate significance at the 0.01 and 0.001 probability level, respectively.

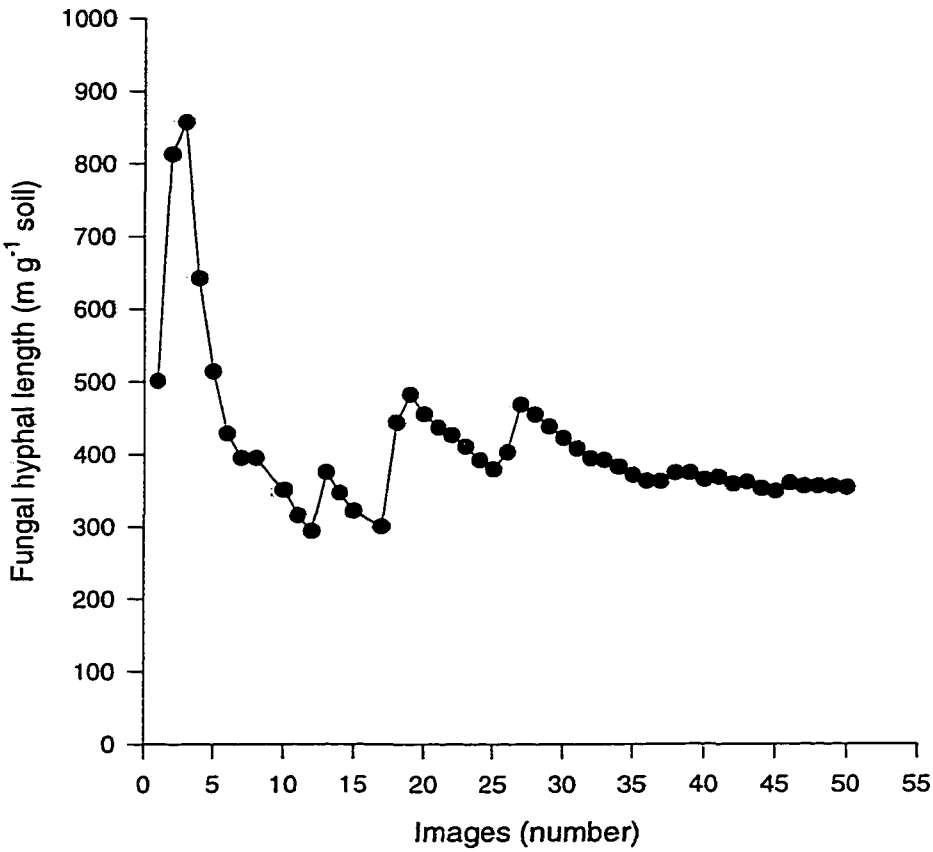
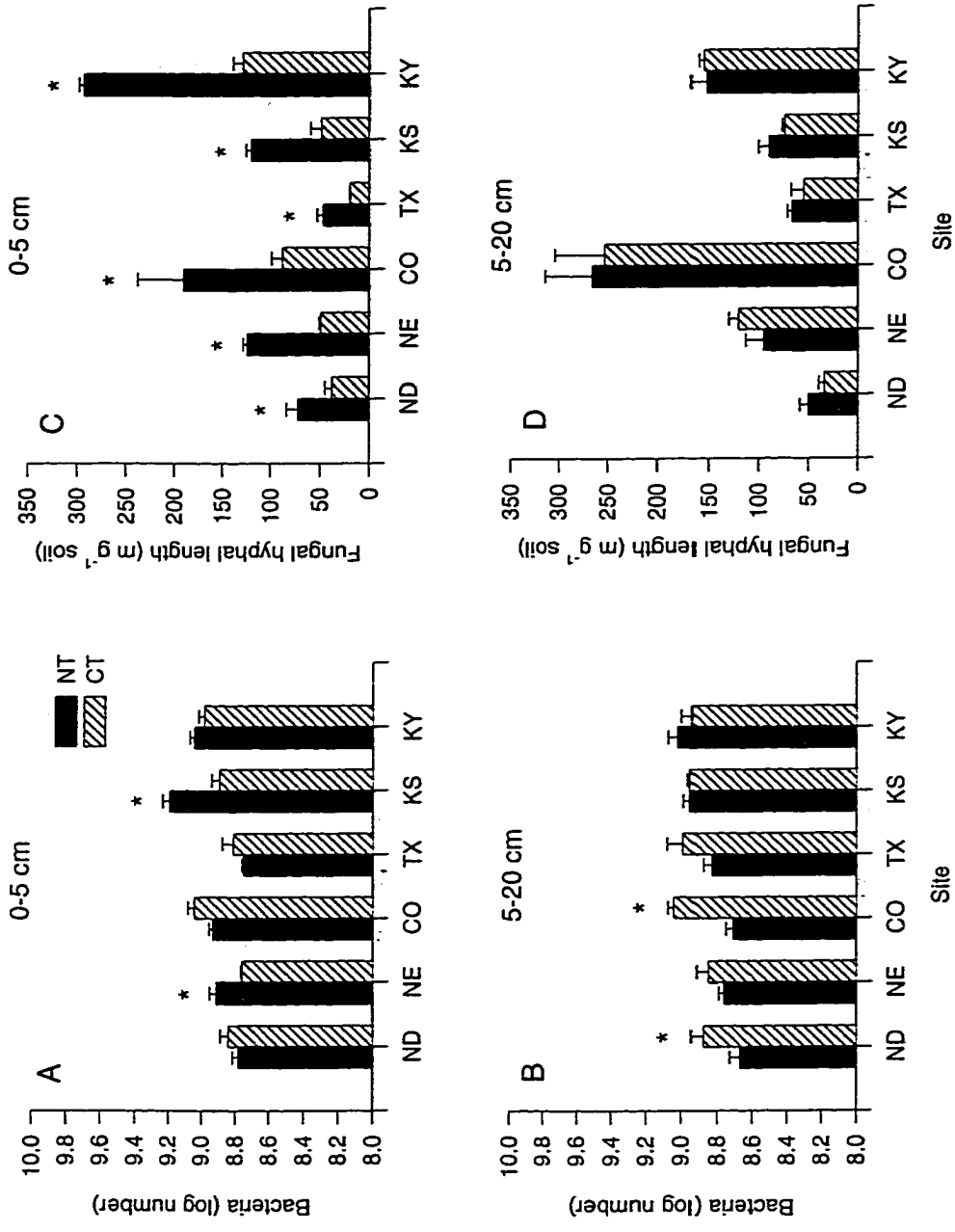



**Figure 2.1.** Fungal abundance as a function of the number of microscope fields from which fungal hyphal length measurements were made.

**Figure 2.2.** Average abundances for bacteria (A and B) and fungi (C and D) in the 0-5 cm and 5-20 cm depth increment, respectively. Site abbreviations are: ND = Mandan, ND; NE = Sidney, NE; CO = Stratton, CO; TX = Bushland, TX; KS = Manhattan, KS; KY = Lexington, KY. Sites are arranged according to their location along gradients of increasing temperature (ND to TX) and precipitation (TX to KY). An * indicates a statistically significant difference ($P \leq 0.05$) between tillage treatments within a site and depth increment. Bars represent standard errors.



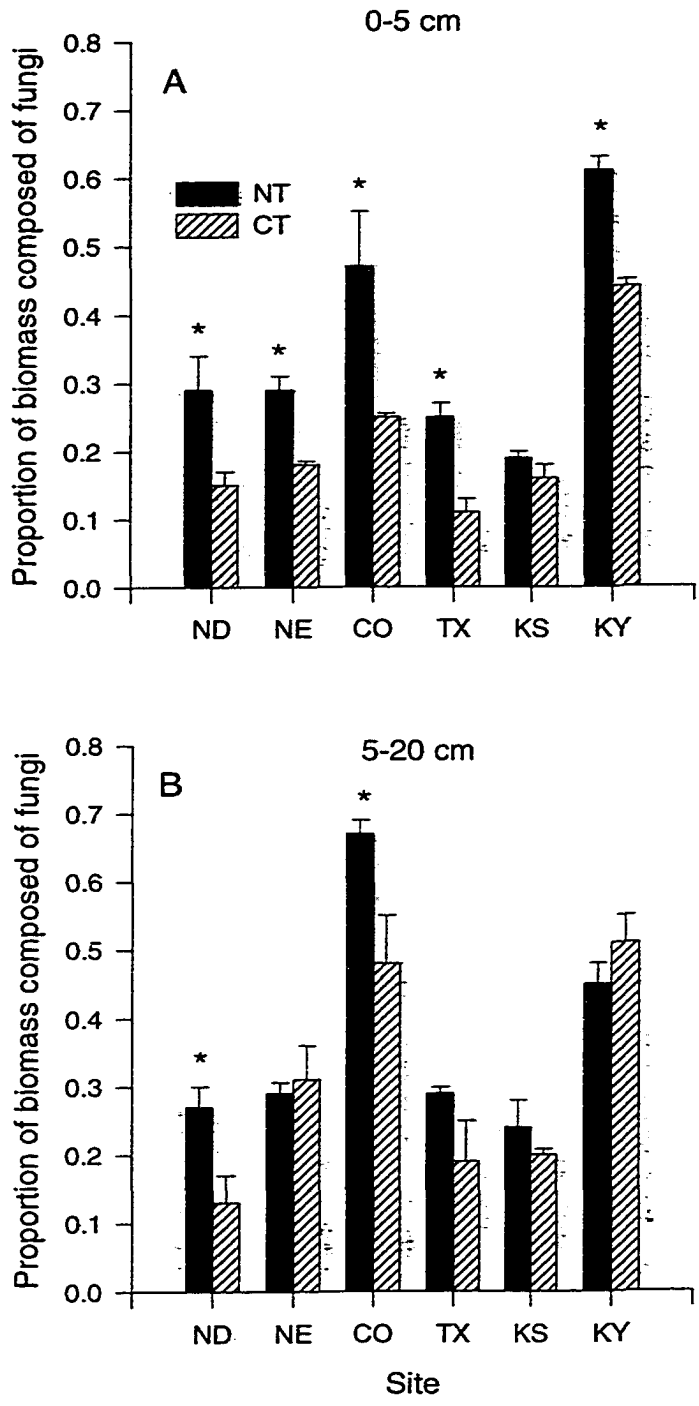



**Figure 2.3.** The proportion of total microbial biomass C composed of fungi for the 0-5 cm (A) and 5-20 cm (B) depth increments. Abbreviations and symbols are as in Fig. 2.2.

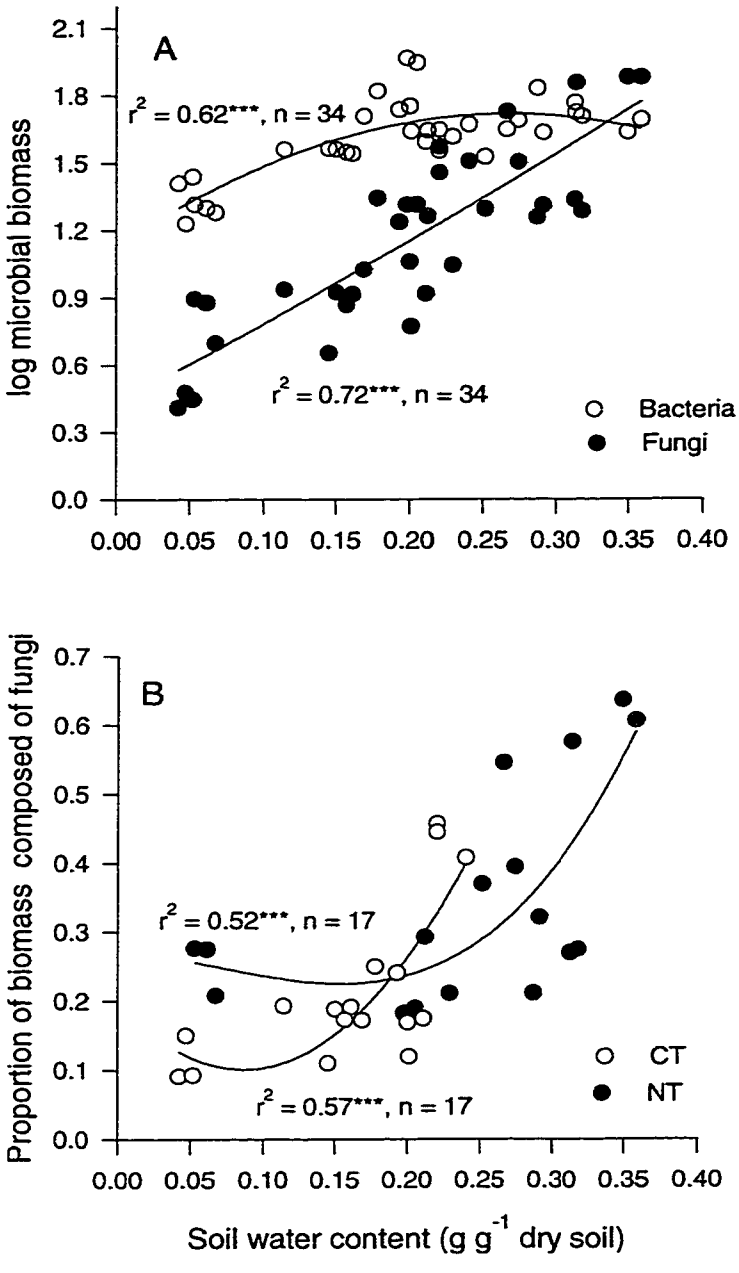



**Figure 2.4.** Relationships between soil water content of the 0-5 cm depth increment and (A) bacterial and fungal biomasss C and (B) the proportion of total microbial biomass composed of fungi; data from all sites, both tillage treatments (A only) and all field replicates are combined.

# CHAPTER 3

# FUNGAL TRANSLOCATION AS A MECHANISM OF EXOGENOUS NITROGEN INPUTS TO DECOMPOSING SURFACE RESIDUES IN A NO-TILLAGE AGROECOSYSTEM

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## ABSTRACT

Additions of $(^{15}NH_4)_2SO_4$ to the soil inorganic nitrogen (N) pool were used to measure rates of N flux from the mineral soil to surface-applied wheat straw decomposing in intact soil cores collected from a no-tillage (NT) field. Half of the soil cores were treated with a fungicide (Captan) to reduce fungal populations. Fungicide application significantly reduced fungal biomass, decomposition rates, and net N immobilization in surface residues. Net N immobilization over the study period was estimated to be 1.5 and 0.9 g N $m^{-2}$ for untreated and fungicide-treated residues, respectively. The rate of $^{15}N$ transfer averaged 13.4 $\mu$g $^{15}N$ $g^{-1}$ residue $d^{-1}$ for untreated wheat straw. Fungal inhibition reduced $^{15}N$ flux by 59-78%, reductions of similar

magnitude to those observed for fungal biomass. Nitrogen transfer in sterilized soil cores accounted for only 7.8 % of the total upward N transport in control cores, indicating that abiotic processes did not contribute substantially to N flux. We estimate a total annual fungal-mediated N flux of 2.4 g $m^{-2}$, which is nearly equivalent to the N immobilization potential predicted, based on initial N and lignin content, for the wheat straw used in this study. We conclude that fungal N translocation is a significant exogenous N input and can account for the observed net N immobilized by surface residues decomposing in the field. Both residue quality and N availability appear to be important controls on fungal biomass associated with surface residues and rates of soil-to-residue N translocation.

## INTRODUCTION

Net immobilization of exogenous nitrogen (N) by the microbial community associated with decomposing surface plant litter is well documented for forests (Bocock, 1964; Gosz *et al.*, 1973; Melillo *et al.*, 1982; Blair *et al.*, 1992), grasslands (Pastor *et al.*, 1987; Seastedt *et al.*, 1992), and no-tillage (NT) agricultural systems where crop residues remain as a mulch layer on the soil surface (Holland and Coleman, 1987; Beare *et al.*, 1992). Potential external N sources include abiotic inputs through capillary flow, $NH_3$ volatilization-sorption, throughfall, and wet or dry atmospheric N deposition, and biota-mediated inputs via asymbiotic $N_2$-fixation, import of insect biomass and frass, and fungal N translocation. Few studies have been done to quantify the relative contributions of these various mechanisms; however, several observations suggest that filamentous fungi contribute significantly to the N flux. Tracer studies with $^{15}N$-labeled inorganic N

have confirmed both the lateral and upward movement of significant quantities of $^{15}N$ from mineral soil to decomposing residues in forest and grassland ecosystems (Schimel and Firestone, 1989; Hart and Firestone, 1991; Hart *et al.*, 1993). Wessen and Berg (1986) observed that the N immobilization stage of decomposing barley straw coincided with the start of ingrowth of fungal hyphae into litter bags. Nitrogen immobilization by rye straw decomposing on the surface of NT plots was significantly reduced by fungicide application (Beare *et al.*, 1992; Beare, 1997).

Fungi are well-adapted to colonization of nutrient-poor and recalcitrant substrates due to their wide-ranging enzymatic capabilities, their efficient accumulation and concentration of nutrients into vegetative and reproductive tissues, and their ability to recycle and reallocate nutrients from older hyphae to regions of active growth (Paustian and Schnürer, 1987). Filamentous fungi can also translocate nutrients between spatially-separated resources over considerable distances (Frankland *et al.*, 1990). Large fluxes and rapid rates of translocation of C and mineral nutrients through hyphae have been demonstrated in mycorrhizal and wood-decaying, cord-forming fungi (reviewed by Frankland *et al.*, 1990). It has been hypothesized that the ability of fungi to bridge the soil-litter interface in NT systems and translocate N from the soil inorganic N pool into surface residues allows them to proliferate on and more readily decompose an otherwise C-rich, but N-poor substrate (Hendrix *et al.*, 1986; Holland and Coleman, 1987; Beare *et al.*, 1992).

Rates of fungal N translocation in agricultural systems are unknown; therefore, our main objective was to quantify fungal-mediated N flow from the soil inorganic N

pool into surface residues decomposing in a NT field. We established a biotic exclusion experiment in which a fungicide was used to inhibit fungi from intact soil cores, and inorganic $^{15}N$, applied to surface soil, was used to follow movement of N from the mineral soil into control or fungicide-treated surface residues. We predicted that if fungal translocation is a significant source of exogenous N, then inhibition of fungi should result in significantly reduced rates of N flow to surface residues. To better understand factors controlling fungal translocation, we conducted a laboratory incubation to examine the effects of residue composition, particularly initial N concentrations, and availability of external inorganic N on residue-associated fungal biomass and rates of N translocation. We predicted that the highest rates of N flux would occur when surface residues with a low initial N concentration were incubated in the presence of high soil inorganic N levels.

## MATERIALS AND METHODS

### Fungal biomass and N dynamics in intact soil cores collected from a NT field

Our field study was installed on April 22, 1998 in the fallow phase of a wheat-corn-fallow rotation at the Dryland Agroecosystem Project experimental site located near Stratton, Colorado, USA (Peterson and Westfall, 1997; Peterson *et al.*, 1997). No-tillage management of this site began in 1985 after more than 50 yr of continuous wheat-fallow, moldboard plow cultivation. Native vegetation was shortgrass prairie. The soil is classified as a fine-silty, mixed, mesic Aridic Argiustoll with 22% sand, 45% silt and 33% clay. Nitrogen fertilizer is surface-applied annually at planting. Fertilizer N application rates are based on the $NO_3^-$-N content of the soil profile to 180 cm and

typically range from 56-78 kg N $ha^{-1}$ for wheat and 100-112 kg N $ha^{-1}$ for corn. Total organic carbon (TOC) and nitrogen (TON) in the upper 20 cm of mineral soil are 2250 ± 55 and 225 ± 16 g $m^{-2}$, respectively. A stratification of organic matter occurs in the NT plots at Stratton with TOC and N accumulating at the soil surface, a common observation in many NT systems. Total organic C and N in the surface 0-2.5 cm is 393 ± 25 and 35 ± 3 g $m^{-2}$ soil, respectively, and represents 17.5% and 15.6% of the TOC and N to a depth of 20 cm. Total inorganic N concentration in the surface 0-2.5 cm has ranged from 2.8 to 37 $\mu$g $g^{-1}$ soil since 1986, with an average of 13.9 ± 2.0 $\mu$g $g^{-1}$ soil (Peterson and Westfall, 1997; Peterson *et al.*, 1997). Mean annual temperature, precipitation and open pan evaporation are 10.7°C, 410 mm and 1270 mm, respectively. Above-average rainfall was recorded for July and August of the study period (Fig. 3.1). Precipitation was below-average for June and September and near average for April, May and October 1998. Additional information regarding management history, current management practices, and the climatic regime at this site can be found in Peterson and Westfall (1997) and Peterson *et al.* (1997).

Forty steel cylinders (10 cm dia., 18 cm deep) were driven to a depth of approximately 15 cm within a 1.5 $m^2$ area that had been cleared of all surface residues. The cylinders were arranged into three blocks with each block located between rows of standing corn stubble. Winter wheat (*Triticum aestivum*) straw, collected from the Colorado State University Agricultural Research Development and Education Center, was defoliated, cut into approximately 2 cm pieces, and surface applied to each intact soil core. Initial N concentration and C:N and lignin:N ratios are given in Table 3.1. The rate

of straw addition (3.5 g $core^{-1}$) was equivalent to 450 g $m^{-2}$ and was somewhat higher than the average wheat residue loading rate (370 g $m^{-2}$) for this site and rotation. The higher residue amounts were necessary to ensure sufficient material for analysis throughout the growing season. A circular piece (10 cm dia.) of nylon mesh (3 x 5 mm openings) was placed between the soil surface and applied residues to facilitate residue removal when sampling. Additional nylon mesh was wired to the top of each cylinder to keep the residue from blowing away.

Two treatments, fungicide and no fungicide (control), were randomly assigned to cores within each experimental block. Fungicide selection and determination of field application rates were based on preliminary studies with the goal of selecting a fungicide that would significantly reduce fungal populations while having minimal impacts on nontarget organism groups (Ingham and Coleman, 1984; Ingham *et al.*, 1986; Beare *et al.*, 1992). Captan (50W, wettable powder, 48.7% active ingredient, 2.1%N) was applied in aqueous suspension (236 mg in 50 ml water) and distributed uniformly over the surface of each core. This application rate was equivalent to 15 g active ingredient $m^{-2}$. Control cores received 50 ml water only. Treatments were applied at monthly intervals throughout the growing season (April-October).

On 28 May, 25 August, and 24 October (36, 125 and 185 days after experiment initiation), two cores were randomly selected from each treatment replicate and returned to the laboratory. Surface residues from one set of cores (one core from each treatment replicate) were removed and analyzed for mass loss, gravimetric moisture content, ash content, fungal biomass, and total C and N. Soil from the surface 0-2.5 cm was removed

and analyzed for moisture content, fungal biomass, total C and N, and inorganic N in the extractable soil solution. The second set of cores were incubated in the presence of $^{15}N$ to determine the potential for nitrogen translocation. An $^{15}N$-enriched $(NH_4)_2SO_4$ solution (99.9 atom%) containing 7.8 mg N was injected into each soil core, giving an N addition rate of 1 g $m^{-2}$ (Hart *et al.*, 1993). The $^{15}N$ solution was injected in 0.1 ml increments at 25 points in a horizontal plane 1 cm below the soil surface using a 15 cm, 18-gauge spinal needle through an injection port drilled into the side of each cylinder. Preliminary tests indicated that this method of injection was preferable to vertical injection through the residue layer since it eliminated the risk of surface residue contamination. Residues removed immediately following injection using the side port showed no signs of $^{15}N$ contamination (data not shown).

Immediately following $^{15}N$ labeling, each core was sealed inside a 2.5 gal bucket containing a leur lock fitting in the lid to allow for headspace gas analysis. The cores were incubated at 25°C for 6 d at which time the residues were carefully removed and analyzed for $^{15}N$ enrichment. Surface soil (0-2.5 cm) was analyzed for total N and $^{15}N$ and the amount of inorganic N and $^{15}N$ in the extractable soil solution.

Three additional intact soil cores, treated with water only, were removed from the field on the 25 August sampling date, returned to the lab, and used to determine the potential for abiotic N transport from the mineral soil to surface residues. The cores were sterilized by autoclaving for 90 min at 121°C and 18 psi on three consecutive days and subsequently asceptically injected with filter-sterilized (0.2 μm) $^{15}N$-labeled $(NH_4)_2SO_4$ solution and incubated as previously described. Maintenance of sterile conditions was

monitored by measuring $CO_2$ evolution during the incubation period. No significant increases in headspace $CO_2$ concentrations were observed throughout the incubation for two of the three cores indicating that sterile conditions were maintained. Evidence of significant respiration was observed in the third core and it was therefore excluded from further analysis. At the end of the incubation period, surface residues from the two sterile cores were removed and analyzed for mass loss, moisture content, TOC and N and $^{15}N$. Surface soil samples (0-2.5 cm) were analyzed for total and inorganic N and $^{15}N$.

Effects of residue composition and N availability on fungal biomass and N translocation

The laboratory incubation was a factorial design with three initial residue N levels (0.24, 0.38, and 0.99 %N), two soil inorganic N levels (10 and 100 $\mu g$ N $g^{-1}$ soil) and three replicates per treatment. The soil used was a Weld silt loam classified as a fine, montmorillonitic, mesic aridic Paleustoll collected from the Central Great Plains Research Station at Akron, Colorado, USA (Halvorson *et al.*, 1997). This soil reportedly has a low mineralization potential (M. Vigil, pers. comm.) and was selected so that dilution of added inorganic $^{15}N$ with native unlabeled N would be minimal during the incubation period. Soil was air dried and sieved (2 mm). Soil subsamples of 150 g each were added to incubation units consisting of 9 cm diam plastic containers. This amount of soil was necessary to achieve a soil depth of 2.5 cm within the plastic containers, the depth to which soil was sampled in the field experiment. Soil was amended with 10 or 100 $\mu g$ N $g^{-1}$ soil as $(^{15}NH_4)_2SO_4$ (1.96 atom%) in sufficient deionized water to bring the soil to field capacity. The inorganic N concentration of unamended soil was $10.94 \pm 0.38$

$\mu$g N $g^{-1}$ soil. Following soil amendment, 1.5 g wheat stems (0.24%N), wheat leaves (0.99%N), or a mixture of stems and leaves (0.38%N), cut in approximately 2 cm pieces, were placed on the soil surface. The residues were pre-moistened with deionized water to minimize upward capillary movement of N following residue placement. A circular piece of nylon mesh (3 x 5 mm openings) was placed between the soil and surface residues to reduce soil contamination of residues and facilitate residue removal at the end of the incubation. Incubation units were sealed inside a 0.5 gal food jar with enough water in the bottom of the jar to maintain a humid atmosphere and reduce soil moisture loss. Every other day, the jars were opened for a few minutes to replenish oxygen levels. The incubation lasted 30 d at which time surface residues were removed and analyzed for gravimetric moisture content, mass loss, fungal biomass, total C and N concentrations, and $^{15}N$ enrichment. Soil samples were analyzed for moisture content, fungal biomass, and inorganic N and $^{15}N$ levels. Three sterile controls at the 0.24% residue N level and 100 $\mu$g $g^{-1}$ N addition rate were included to evaluate the potential for abiotic flow of N from mineral soil to surface residues. Sterilization procedures and $CO_2$ evolution measurements were as described for the field experiment. No significant increase in headspace $CO_2$ was observed throughout the 30 d incubation, indicating that sterile conditions were maintained.

Microbial and chemical analyses

Total physiologically active microbial biomass and the relative contributions of fungi to that biomass were measured using a substrate-induced respiration (SIR) method

developed for plant residues (Beare *et al.*, 1990; Beare *et al.*, 1991; Neely *et al.*, 1991). Total fungal biomass C was estimated using the equation: 231.5 + 17.3(fungal SIR), where fungal biomass C has units of $\mu$g C $g^{-1}$ residue dry weight and fungal SIR has units of $\mu$g $CO_2$-C $g^{-1}$ residue dry weight $h^{-1}$ (Beare *et al.*, 1991). Fungal biomass in soil samples was determined by direct microscopy and computer-assisted image analysis (Frey *et al.*, 1999) since attempts to optimize the SIR method for soil were unsuccessful. Microbial analyses were made on field-moist subsamples. All remaining residues and soils were dried at 60°C and 105°C, respectively, and finely ground. Total organic C and N were determined on residue and soil subsamples using a Leco CHN-1000 analyzer. Lignin content was determined by proximate analysis using the methods of Goering and van Soest (1970). Residue and soil ash contents were determined by dry combustion in a muffle furnace at 500°C for 4 h. Residue mass loss and N data were corrected for soil contamination using the soil correction equation of Blair and Crossley (1988). Decay constants (k) were calculated using soil-corrected mass data and the single negative exponential decay function: $\log_e$[remaining mass/initial mass] = -kt (Olson, 1963). Soil inorganic N was extracted by shaking 10 g of field moist soil in 50 ml 2 M KCl on an orbital shaker for 30 min, filtering the soil suspension (Whatman No. 1), and analyzing the extractant for $NH_4^+$ and $NO_3^-$ using an Alpkem autoanalyzer. Filter papers were pre-leached with 50 ml 2 M KCl to remove any $NH_4^+$ and $NO_3^-$ contamination. Soil extracts were prepared for N isotopic mass ratio analysis using the diffusion method of Brooks *et al.* (1989) as modified by Stark and Hart (1996), except that 1-pt Mason jars were used as the reaction containers instead of specimen cups (Khan *et al.*, 1998). Filter disks

containing diffused N and residue and soil samples were analyzed for N isotope ratios using a Carlo Erba NA 1500 CN analyzer coupled to a Micromass VG Isochrom-EA mass spectrometer (Micromass UK Ltd, Manchester, UK).

Statistical analyses

Two-way analysis of variance was performed using the SAS general linear models procedure (PROC GLM, SAS Institute, 1990) to determine the significance of two biocide treatments and three sampling dates in the field study and two soil inorganic N levels and three residue materials with differing initial N concentrations in the lab incubation. A preliminary analysis of variance of the field data indicated a non-significant block effect which was then collapsed into the error term in subsequent analyses. Significance was assumed when $P \leq 0.05$. Fungal biomass data were log transformed prior to analysis in order to meet normality and homogeneity of variance assumptions for ANOVA (Sokal and Rohlf, 1981).

## RESULTS AND DISCUSSION

Fungal biomass

Fungal biomass in the field experiment was significantly greater in control than in fungicide-treated surface residues at all sampling dates (Fig. 3.2). The amount of biomass in control cores ranged from $0.68 \pm 0.10$ to $2.72 \pm 0.34$ mg fungal C $g^{-1}$ residue and fungicide application significantly reduced that biomass by 54-75%. The greatest reductions (75 and 71%) were observed at the August and October sampling dates. In

both treatments, fungal biomass was lowest at the first sampling in May, one month after experiment initiation, and increased significantly by the second sampling date in August. Fungal biomass at the October sampling date was not significantly different from that in August, although September through October was a period of low rainfall. We observed, when removing residues from the surface of intact soil cores prior to analysis, that untreated residues were matted together with visible fungal hyphae and were highly decomposed, especially those residues collected at 4 (Aug) and 6 (Oct) months after experiment initiation. No fungi were visually observed on fungicide-treated residues and the residues showed few signs of decay.

In the laboratory incubations, fungal biomass associated with decomposing surface residues differed as a function of initial residue N concentration and the availability of soil inorganic N (Fig. 3.3). Under low soil N conditions, fungal biomass increased with increasing initial residue N concentration ($r^2 = 0.97$, $p < 0.001$); whereas, when soil N availability was high, levels of fungal biomass were not as strongly related to % initial N ($r^2 = 0.81$, $P < 0.05$) and stem material, with a low N concentration, supported the same level of biomass as a mixture of stems+leaves with an overall higher N concentration. For all three residue materials, high soil N availability resulted in increased levels of fungal biomass, suggesting that fungal translocation of available soil inorganic N supported the proliferation of fungi in the overlying residues. Biomass associated with low N stem material showed the greatest response to soil N availability, with a 246% increase compared to a 46 and 40% increase for the stem+leaf mixture and leaves alone, respectively. The amount of biomass associated with stem material under

high soil N conditions, is similar to that observed by Neely *et al.* (1991) for residues decaying in the field with considerably higher (0.51-1.11%) initial N concentrations. With adequate exogenous N available, it appears that low quality residues can develop and maintain a substantial fungal population.

Ambient soil inorganic N levels in the field experiment were similar to those of the low soil N treatment in the laboratory incubation, with 12.9, 6.9, and 10.4 $\mu$g N $g^{-1}$ soil observed for the May, August and October sampling dates, respectively. The amount of fungal biomass associated with decomposing wheat stems at the May sampling date in the field experiment was similar to that for stem material incubated under low soil N conditions in the laboratory incubation. However, field biomass estimates for the August and October sampling dates were more similar to the leaf material incubated under high soil N levels in the lab, even though inorganic soil N levels in the field were low and moisture conditions were dry for the last two months of the field study. Presumably, the field residues were capable of supporting greater fungal populations as the N concentration of the material increased as decomposition progressed over the course of the growing season. Neely *et al.* (1991) examined the fungal component of the physiologically active microbial biomass using substrate induced respiration (SIR) for decaying residues from six plant species and observed that the highest levels of fungal SIR were associated with residues with the highest initial N concentrations. They also observed a negative correlation between fungal SIR and the C:N ratio of the decomposing substrates.

Residue decomposition

Patterns of residue mass loss in the field experiment reflected the observed differences in fungal biomass, with decomposition losses being greatest in the control cores (Table 3.2). Untreated residues lost 27% of their initial mass by the end of the study, while fungicide-treated residues lost only 13% of their initial mass. Decomposition in the lab incubation was correlated with initial residue N concentration ($r^2 = 0.64$ , $P < 0.05$) with about 30% mass loss for the highest quality residue (leaves) versus about 10% mass loss for stems (Table 3.3). In contrast to the results for fungal biomass, there were no significant differences in decomposition between the high and low inorganic N treatments for any of the three residue types (Table 3.3).

Residue N dynamics

Berg and Staff (1981) developed a three phase conceptual model to describe the N dynamics they observed for decomposing substrates in litter bag studies. During an initial leaching phase, labile nitrogenous compounds are rapidly released. This stage is followed by an accumulation or immobilization phase where an increase in the N concentration of the decomposing material occurs and often an increase in absolute N is also observed. The increase in litter N concentration occurs as C is lost via respiration and N is retained in the microbial biomass that becomes intimately associated with the decomposing substrate. An increase in the absolute amount of N is attributed to microbial immobilization of exogenous N. The final phase in the N dynamics model is characterized by N loss due to mineralization. Whether and to what extent each of these

three phases occurs for a given substrate depends on many factors including initial substrate composition, substrate placement (incorporated vs surface-applied), environmental constraints, the composition of the decomposer community, and the availability of exogenous N (Holland and Coleman, 1987; Pastor *et al.*, 1987; Beare *et al.*, 1992; Blair *et al.*, 1990, 1992).

Our data indicate an increase in both the relative concentration and absolute amounts of N in both untreated and fungicide-treated residues decomposing in the field (Table 3.1 and Table 3.2). Fungicide application, however, significantly reduced residue N concentration and the amount of N that accumulated in surface residues compared to untreated control residues. We calculated the amount of net N immobilization over the field study period by regressing the percentage of original residue remaining vs the percentage of residue N and using the regression parameters in the following equation: ((intercept$^2$ / -4(slope)) - 100(% initial residue N)) / 10 (Aber and Melillo, 1982). Using these calculated values and known residue loading rates for this site, we estimate a net N immobilization of 1.5 g m$^{-2}$ and 0.86 g m$^{-2}$ for control and fungicide-treated residues, respectively, for the growing season (April-October). This treatment difference represents a 43% reduction in N immobilization when fungal populations are inhibited. Our estimate for growing season N accumulation is similar to the 1.81 g N m$^{-2}$ immobilized by winter rye decomposing on the surface of NT plots in Georgia (Beare *et al.*, 1992).

Soil inorganic N flux to surface residues

The rate of $^{15}N$ transfer from amended mineral soil to untreated surface residues in

intact cores collected from the field averaged 13.4 ± 1.9 $\mu$g $^{15}N$ $g^{-1}$ residue $d^{-1}$ and did not differ significantly across sampling dates (Fig. 3.4). Less than 2% of the $^{15}N$ applied to the soil inorganic N pool was recovered in untreated surface residues (Table 3.4). However, this N flux represents a larger transfer of total N ($^{14}N+^{15}N$) over the same period since added $^{15}N$ was significantly diluted at the time of injection into the mineral soil by native soil inorganic N with an isotopic signature at background levels (i.e. natural abundance). Total N transfer is dependent on the $^{15}N$ flux and the level of $^{15}N$ enrichment of the N being transferred and was estimated according to the following equation:

Total N flux = $^{15}N$ flux/mean $^{15}N$ enrichment of soil inorganic N pool

The $^{15}N$ enrichment of the soil inorganic N pool averaged over the assay period was used since, following the initial dilution at the time of injection, no further significant dilution of added label occurred, suggesting that there was a low rate of native SOM mineralization over the six day measurement period. The above equation assumes there was no significant isotopic discrimination in the immobilization process and that the $^{15}N$ enrichment of the KCl-extractable soil solution characterized the $^{15}N$ enrichment of the immobilized N.

The pattern of estimated total N transfer from mineral soil to untreated surface wheat residues was similar to that for $^{15}N$ flux with no significant differences in total N transfer observed across sampling dates (Table 3.5). The rate of total N flux averaged 46.0 ± 3.8 $\mu$g N $g^{-1}$ residue $d^{-1}$. Fungicide application reduced total N flux by 52 to 86%, reductions of similar magnitude to those observed for fungal biomass. Translocation rates were not related to fungal biomass ($r^2 = 0.33$; $P > 0.05$) since N flux rates were

similar across sampling dates while fungal biomass increased 353% from May to August. Consequently, the amount of upward N transport per unit fungal biomass, which was similar for both control and fungicide-treated residues, was over 4 times greater in May than in August and October, suggesting a decline in microbial demand for N as the residue C:N ratio narrowed. Nitrogen transfer from sterile soil to surface residues at the August sampling date was $3.6 \pm 0.4$ $\mu$g N $g^{-1}$ residue $d^{-1}$ and accounted for only 7.8 % of the total upward N transport in control cores, indicating that abiotic processes such as capillary flow and $NH_3$ volatilization-sorption did not contribute substantially to N flux during the incubation period.

A substantial amount (50-66%) of the $^{15}N$ initially applied to the soil inorganic N pool was recovered in the non-extractable soil N pool at the end of the six day incubation (Table 3.4). This pool includes N immobilized by the microbial biomass and by-products and $NH_4^+$ abiotically fixed in a non-extractable form by SOM or clay minerals. Hart *et al.* (1993) also observed a larger than expected amount of $^{15}N$ in the SOM pool 1 month after $^{15}NH_4^+$ addition to an annual grassland and estimated that as much as half of the $^{15}N$ recovered in the SOM pool may have been abiotically fixed. Davidson *et al.* (1991) found that over 30% of added $^{15}NH_4^+$ was non-extractable with concentrated salt solution 15 min after addition to autoclaved, intact soil cores. That rapid, abiotic $^{15}NH_4^+$ fixation occurred in the present study is supported by the significantly lower than expected $^{15}N$ enrichment of the soil inorganic N pool immediately following injection into soil cores (data not shown). Approximately 30% of added $^{15}N$ was recovered in the KCl-extractable inorganic N pool at the end of the assay period.

In addition to estimating rates of N translocation in a NT system, we examined the effects of residue quality and soil N availability on patterns of N flux independently of abiotic contraints using laboratory incubations. We hypothesized that (1) for a given residue type, N flux would increase under high soil N conditions due to greater N availability and (2) for a given N level, N flux would increase with decreasing initial residue N concentration due to increasing microbial N demand. We therefore predicted that N flux would be greatest for wheat stems decomposing under high soil N conditions.

For all three residue types, N flux was significantly greater at the high compared to the low soil inorganic N level, supporting our first hypothesis (Fig. 3.5 and Table 3.6). Holland and Coleman (1987) observed higher levels of fungal biomass and greater N immobilization associated with surface-applied wheat straw decomposing in a fertilized compared to an unfertilized agricultural field. Nitrogen immobilization rates in grass litter decomposing in an old field were also found to be correlated with N fertilizer additions (Pastor *et al.*, 1987).

The atom% $^{15}N$ enrichment of surface residues incubated on soil amended with 100 ppm N was inversely related to initial residue N concentration (Fig. 3.5); however, $^{15}N$ enrichment does not accurately reflect $^{15}N$ flux since, in calculating $^{15}N$ flux, both the $^{15}N$ enrichment and the total N concentration of the analyzed material must be taken into consideration. The leaf material had the lowest $^{15}N$ enrichment and the highest total N concentration which resulted in an $^{15}N$ flux that was not significantly different from the stem material which had the highest $^{15}N$ enrichment and the lowest total N concentration

(Fig. 3.5). The highest amounts of $^{15}N$ transfer were observed for the stem plus leaf mixture with an intermediate $^{15}N$ enrichment and residue N concentration. Nitrogen flux to leaf material was lower than for the stem+leaf mixture, most likely due to its high residue N concentration and ability to support a substantial fungal population even in the absence of exogenous N (Fig. 3.2). Mixing some leaf material with stems provided a rich substrate for initial fungal establishment and likely increased the time over which N accumulation took place. We suspect that the lower than expected N flux associated with stem material is a result of the short duration of the lab incubation and does not accurately reflect the N translocation potential of this material. Fungal establishment was likely delayed due to the low quality of this substrate and, while N translocation rates may have been high during the latter stages of the incubation, the total amount of N transfer observed after only 30 d was, therefore, lower than anticipated.

Fungal translocation as a mechanism for N immobilization in decomposing residues

Aber and Melillo (1982) developed a set of equations, based on data for the decomposition and N dynamics of leaf litter for six northern hardwood tree species (Melillo *et al.*, 1982), that allow the quantitative prediction of the "nitrogen factor", the total amount of N that will be immobilized per gram original weight during decomposition of any substrate, as a function of initial N and lignin content. Using their approach, we predict an N immobilization potential for the wheat straw used in this study of 6.1 mg N $g^{-1}$ initial residue, equivalent to 2.3 g N $m^{-2}$. Given the rates of N translocation observed in the field study, the average residue loading rate at our study site,

the annual decay rate observed for control residues, and taking into account the amount of N transferred abiotically in sterile soil cores, we estimate a fungal N translocation potential of 2.4 g N $m^{-2}$. This estimate indicates that fungal translocation of soil N into decomposing surface wheat residues could provide all of the necessary exogenous N to meet the predicted N immobilization potential for this substrate. Our fungal translocation estimate is greater than the actual net N immobilization observed for residues decomposing in the field (1.5 g N $m^{-2}$); however, observed net N accumulation (the balance between N mineralization and immobilization processes which often occur simultaneously) may be considerably less than gross N accumulation.

Our estimate of fungal translocation is also greater than the 0.29 and 0.9 g N $m^{-2}$ $yr^{-1}$ attributed to fungal translocation in an annual grassland (Hart *et al.*, 1993) and an old-growth forest (Hart and Firestone, 1991), possibly due to differences between the systems in residue quality, the amount of residue inputs, and the availability of exogenous N. The fluxes reported by Hart *et al.* (1993) and Hart and Firestone (1991) may also underestimate gross N translocation in the systems they studied since inorganic $^{15}N$ was injected in the field and measurement of $^{15}N$ recovery in decomposing surface litter was not made until 4-5 mo following $^{15}N$ addition, allowing time for some of the $^{15}N$ translocated to surface residues to be returned to the mineral soil due to mineralization and/or leaching.

The N immobilized by residues decomposing in the field may derive from a variety of sources including atmospheric N deposition, asymbiotic N fixation, abiotic transfer, and fungal translocation of available soil N. Surface-applied fertilizer N may

represent another potentially important exogenous N source for residue decomposers in some NT systems; however, it did not contribute to N immobilization in our study since we conducted our experiment in the fallow phase of the rotation which is unfertilized. Inorganic N inputs from wet and dry deposition in eastern Colorado average 0.24 ± 0.02 g N $m^{-2}$ $yr^{-1}$ (National Acid Deposition Program, 1999). Given that microbial acquisition of simulated N deposition is reportedly low (Seastedt and Crossley, 1983; Downs *et al.*, 1996), this source of external N cannot account for more than a small percentage of the net N immobilization observed in our field study. Although we know of no studies where asymbiotic N fixation in decomposing wheat residues has been examined, results from other systems indicate that rates of N fixation in surface litter range from 0-0.1 g $m^{-2}$ $y^{-1}$ and represent less than 10% of total N immobilization (Berg and Söderström, 1979; Fahey et al., 1985; Russell and Vitousek, 1997; Thompson and Vitousek, 1997).

The results of our study indicate that fungal N translocation is an important exogenous N source and can account for net N immobilization observed for surface residues decomposing in the field.

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**Table 3.1.** Initial N concentration and the C:N and lignin:N ratios for wheat residues used in this study. Stems were used for both the field experiment and lab incubation.

| Wheat residue type | Nitrogen (%) | C:N | Lignin:N |
|---|---|---|---|
| Stems | 0.24 | 187 | 47.9 |
| Stems + leaves | 0.38 | 109 | 35.5 |
| Leaves | 0.99 | 41 | 10.2 |

**Table 3.2.** Percent mass remaining and relative and absolute N at the end of the field study (185 days) and decay rate constants (k) for control and fungicide-treated surface residues[1].

| | Control | Fungicide |
|---|---|---|
| Residue remaining (%) | 73.4 ± 1.1* | 86.9 ± 0.6 |
| k ($yr^{-1}$) | 0.61 ± 0.03* | 0.28 ± 0.01 |
| N concentration (%) | 0.58 ± 0.03* | 0.40 ± 0.01 |
| C:N | 59.4 ± 10.5* | 117.6 ± 5.4 |
| N remaining (%) | 180.2 ± 9.9* | 144.9 ± 4.3 |

[1]Mean ± standard error. An * indicates a statistically significant difference ($P \leq 0.05$) between treatments.

**Table 3.3**. Percent mass remaining and N concentration of surface residues after 30 d of lab incubation on soil amended with 100 or 10 ppm inorganic N[1].

| | Initial residue N | | |
|---|---|---|---|
| | Low | Intermediate | High |
| Residue remaining (%) | | | |
| 100 ppm N | 86.6 ± 3.5 | 76.3 ± 1.7 | 66.2 ± 3.2 |
| 10 ppm N | 89.7 ± 1.5 | 75.9 ± 1.6 | 69.0 ± 4.9 |
| N concentration (%) | | | |
| 100 ppm N | 0.30 ± 0.03 | 0.72 ± 0.02* | 1.36 ± 0.05* |
| 10 ppm N | 0.25[2] | 0.51 ± 0.04 | 1.13 ± 0.05 |

[1]Mean ± standard error. An * indicates a statistically significant difference ($P \leq 0.05$) between treatments within a residue type.
[2]Only one replicate.

**Table 3.4.** Percentage of applied $^{15}N$ recovered in each treatment combination for the 24 October sampling date[1].

| N sink | Control | Fungicide |
|---|---|---|
| Soil organic N[2] | 49.5 ± 5.9 | 66.3 ± 7.9 |
| Soil inorganic N | 31.3 ± 4.6 | 29.7 ± 2.0 |
| Surface residues | 1.3 ± 0.1 | 0.1 ± 0.1 |
| Total | 82.1 ± 10.6 | 95.9 ± 9.8 |

[1]Mean ± standard error.
[2]Excludes $^{15}N$ recovered in the inorganic N pool.

**Table 3.5.** Estimated total N flux from the soil mineral N pool into decomposing surface residues in intact soil cores collected from a NT agroecosystem[1].

| Sampling day | Control | Fungicide |
|---|---|---|
| | ----μg N $g^{-1}$ residue $d^{-1}$ ---- | |
| 28 May | 43.7 ± 7.0* | 21.1 ± 4.4 |
| 25 Aug | 45.8 ± 8.7* | 12.1 ± 5.4 |
| 24 Oct | 49.9 ± 3.8* | 6.5 ± 3.3 |

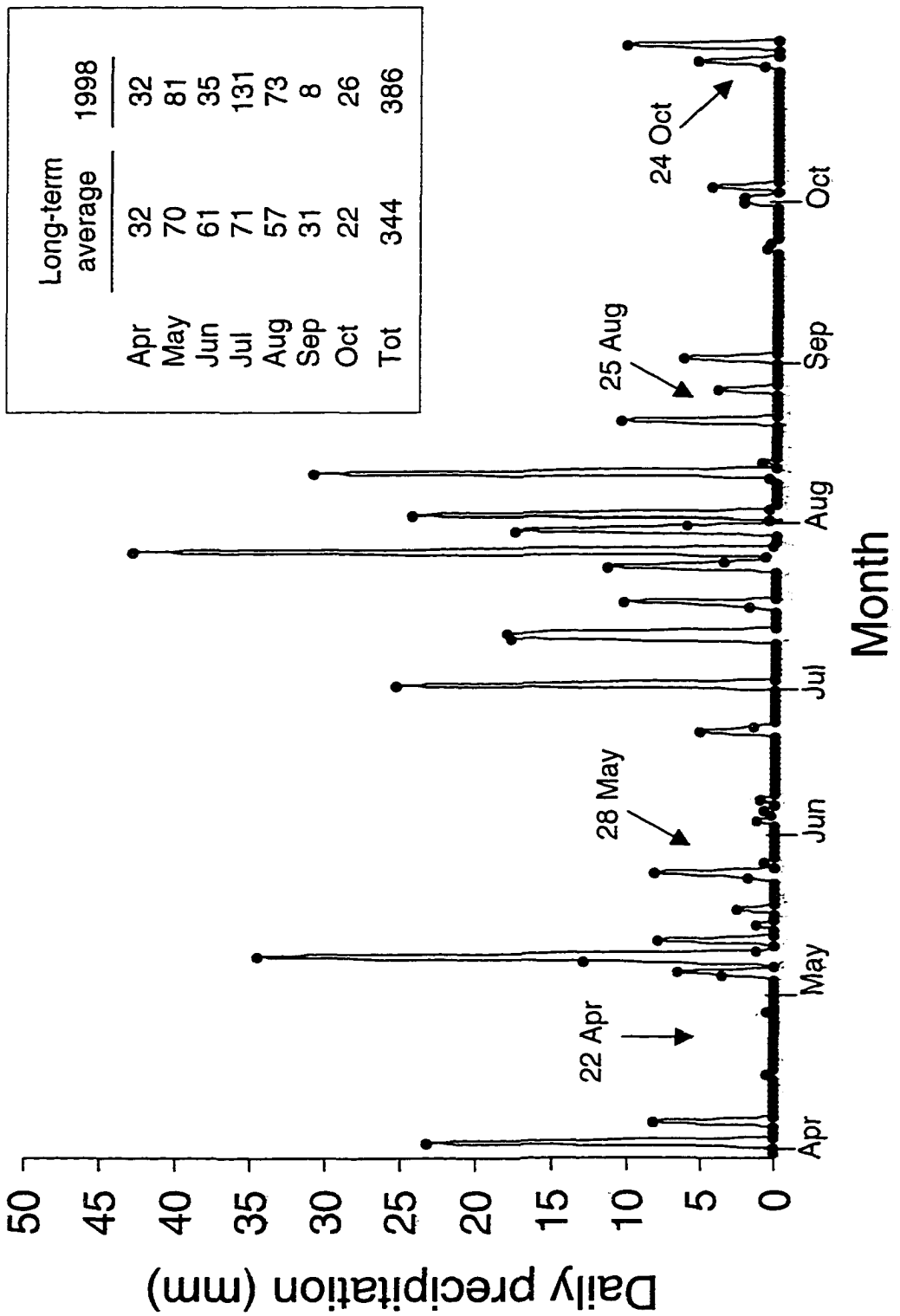
[1]Mean ± standard error. An * indicates a statistically significant difference ($P \leq 0.05$) between treatments within a sampling date.

**Table 3.6.** Estimated total N transfer from soil inorganic N pool into surface residues in 30 d lab incubation[1].

| Initial Residue N | 100 ppm N | 10 ppm N |
|---|---|---|
| | -------mg N $g^{-1}$ residue------- | |
| Low | 3.44 ± 0.18* | 0.89 ± 0.08 |
| Intermediate | 5.05 ± 0.39* | 1.21 ± 0.33 |
| High | 3.04 ± 0.63* | 0.54 ± 0.23 |

[1]Mean ± standard error. An * indicates a statistically significant difference ($P \leq 0.05$) between N levels. Analysis of variance indicated that the first-order interaction between residue type and soil N level was not significant ($P > 0.05$).

**Figure 3.1**. Daily precipitation for the study period (April-October 1998) at Stratton, Colorado. Dates of experiment initiation (22 Apr) and sampling (28 May, 25 Aug, 24 Oct) are shown. Long-term (1949-1992) and 1998 average monthly precipitation (mm) for the growing season are shown in the inset table.



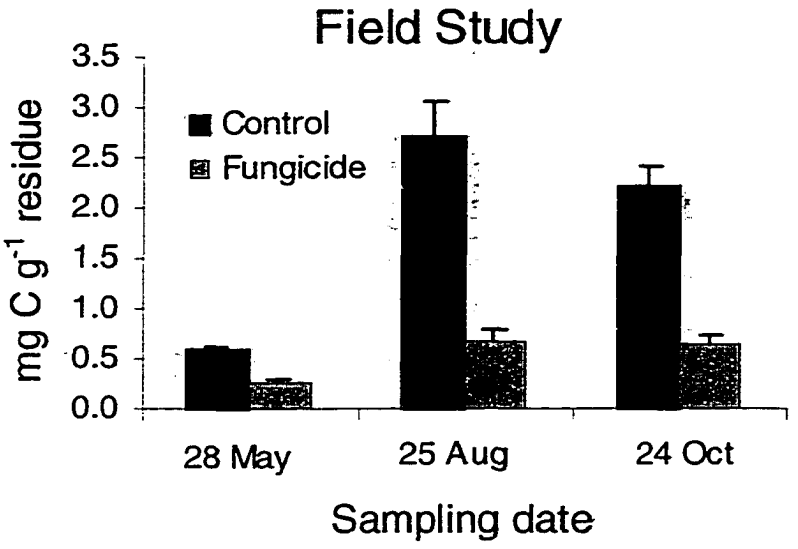



**Figure 3.2.** Fungal biomass C associated with control and fungicide-treated surface straw from intact soil cores collected from a NT field. Values are means ± 1 standard error.

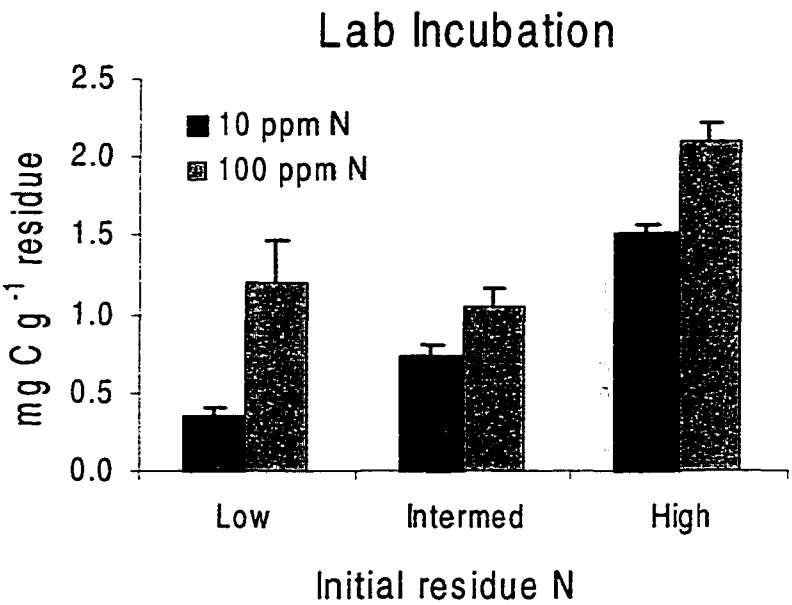



**Figure 3.3.** Fungal biomass C in residues of differing initial N concentration incubated on soil amended with 10 or 100 ppm inorganic N. Values are means ± 1 standard error.

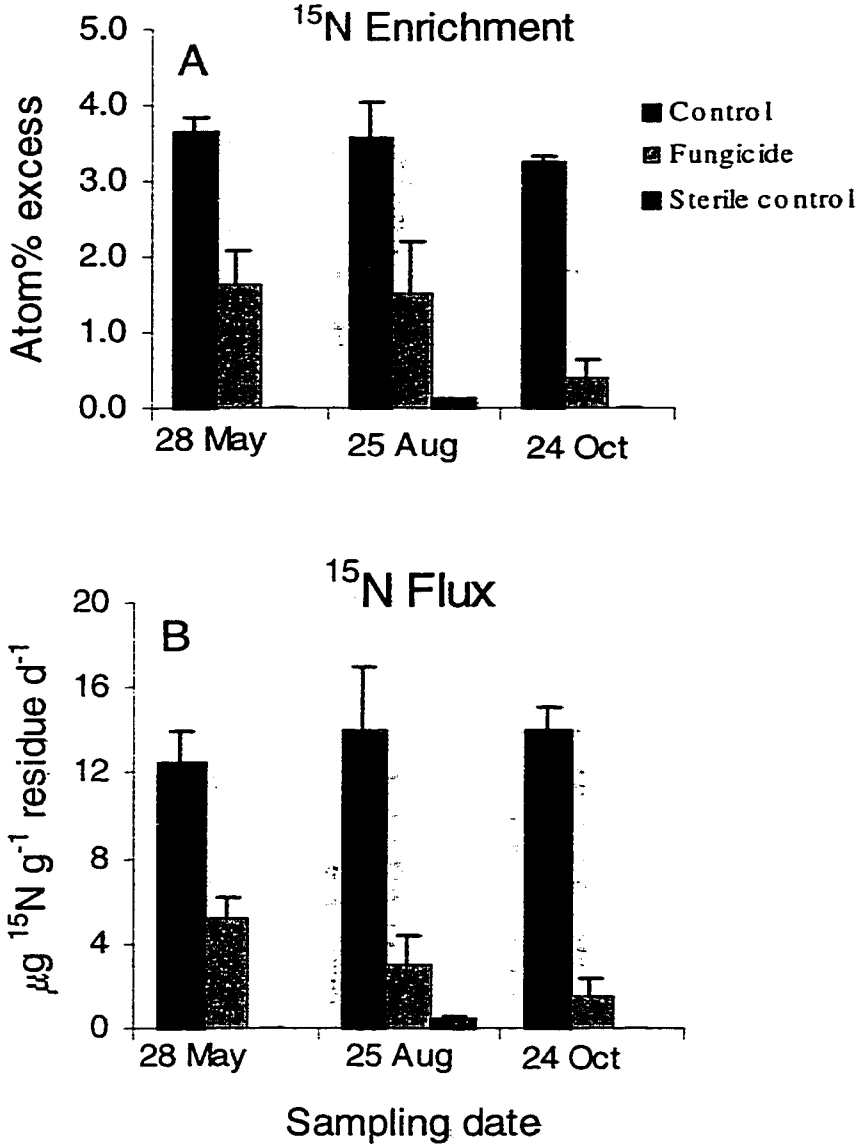



**Figure 3.4.** Atom % $^{15}N$ enrichment (A) and $^{15}N$ flux (B) for control, fungicide-treated, and sterilized surface residues from intact soil cores collected from a NT field and incubated for six days in the presence of inorganic soil $^{15}N$. Values are means ± 1 standard error.

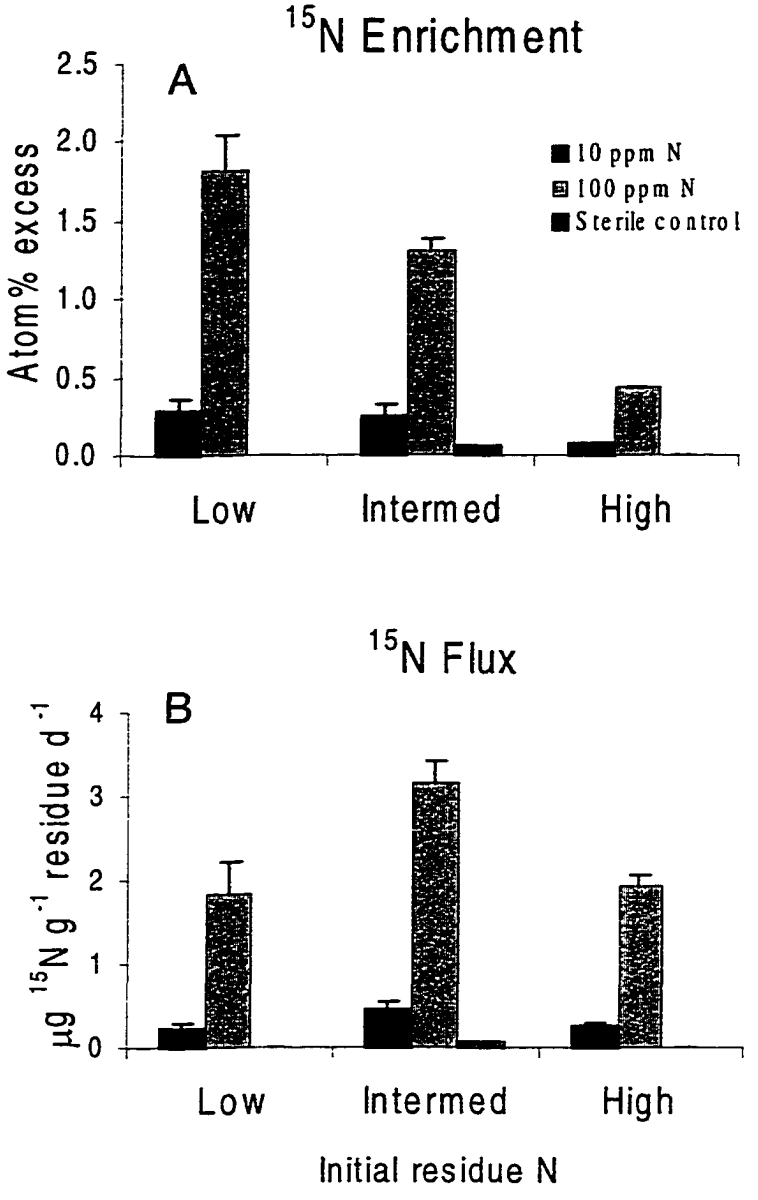



**Figure 3.5.** Atom % $^{15}N$ enrichment (A) and $^{15}N$ flux (B) for surface residues of differing initial N concentration incubated for 30 d on soil amended with 10 or 100 ppm inorganic soil $^{15}N$. The $^{15}N$ flux in the sterile control accounted for 2.7% of the $^{15}N$ transfer into stem material decomposing under high soil N conditions. Values are means ± 1 standard error.

# CHAPTER 4

# PROTOZOAN GRAZING AFFECTS ESTIMATES OF CARBON UTILIZATION EFFICIENCY OF THE SOIL MICROBIAL COMMUNITY

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## ABSTRACT

Reliable estimates of carbon (C) assimilation efficiency (Y = microbial production / substrate utilization) are needed to quantify and predict soil C dynamics. We examined patterns of C assimilation in two soils, a Paleustoll (USA) and Rhodoxeralf (Australia), under two levels of protozoan grazing (low vs high) when substrate was not limiting. Soil, either amended with unlabeled or $^{14}C$-labeled glucose was incubated at 25°C and glucose-C concentration, $CO_2$-C evolution, and microbial biomass-C were determined over a 12-20 h period. Three approaches were used for estimating Y: $Y_s = (dS_C - \Sigma CO_2\text{-}C) / dS_C$, $Y_b = dB_C / (dB_C + \Sigma CO_2\text{-}C)$, and $Y_c = dB_C / dS_c$ where $dS_C$ is the amount of substrate utilized, $\Sigma CO_2$-C the cumulative amount of $CO_2$-C evolved, and $dB_C$ the amount of biomass produced. Calculation of $Y_s$ assumes that all substrate-C utilized, minus that

respired, is used for biomass and metabolite production. Calculation of $Y_b$ assumes that substrate use equals biomass-C plus respired-C and does not account for biomass production consumed by grazers. Under low grazing, the three estimates of Y were similar with an average value of 0.58 and 0.55 for the Paleustoll and Rhodoxeralf, respectively. Under high grazing, the value of Y varied depending on the calculation used, with values of $Y_b$ (0.44) and $Y_c$ (0.26) being significantly lower than $Y_s$ (0.67). The total amount of glucose utilized did not vary with protozoan grazing intensity, but a high level of grazing increased the rate of glucose use and significantly reduced the amount of measurable biomass C. Substrate-based yield ($Y_s$) provided the most reliable C assimilation efficiency estimate under both grazing treatments.

## INTRODUCTION

Microbial growth yield efficiency (Y) determines the fraction of substrate carbon (C) assimilated that is allocated for biomass and metabolite production, the balance being used for energy generation. Since the efficiency of C utilization is an important control on SOM formation, reliable yield estimates for the soil microbial community are needed for input into soil organic matter (SOM) budgets and simulation models. Carbon assimilation efficiency, under optimal batch or continuous culture conditions, has been shown to be near 0.60 for a wide range of aerobic heterotrophs, including both bacterial and fungal species (Payne, 1970). This value has also been obtained for individual bacterial species inoculated into sterile soil amended with glucose (Anderson *et al.*, 1981; Elliott *et al.*, 1983); however, a wide range of values exists for glucose-amended, non-

sterile soil containing a mixed microbial population (Table 1). Variability in yield estimates has been attributed to differences in substrate concentration (Bremer and Kuikman, 1994; Shen and Bartha, 1996), pH (Seto and Noda, 1982), and temperature (Seto and Misawa 1982). Chapman and Gray (1986) suggested that values of Y are often underestimated because maintenance requirements are ignored or because experiments are not terminated before substrate recycling begins to occur.

Some of the variability in yield estimates obtained for non-sterile soil may also be due to the fact that several approaches, based on different assumptions, have been used for estimating Y:

$$Y_s = (dS_C - \Sigma CO_2\text{-}C) / dS_C \quad (1)$$

$$Y_b = dB_C / (dB_C + \Sigma CO_2\text{-}C) \quad (2)$$

$$Y_c = dB_C / dS_c \quad (3)$$

$$Y_r = 1 - (\Sigma CO_2\text{-}C / S_{tot}) \quad (4)$$

where $dS_C$ is the amount of added substrate C that is utilized, $\Sigma CO_2$-C is the cumulative C lost during respiration, $dB_C$ is the change in microbial biomass C, and $S_{tot}$ is the total amount of substrate C added. Calculation of $Y_s$ assumes that all substrate C utilized minus that lost during respiration is assimilated into microbial biomass and intra- and extracellular products. Calculation of $Y_b$, biomass-based yield, assumes that biomass-C plus cumulative respired C equals the amount of substrate C utilized and does not account for metabolite production or for biomass production that is consumed by grazers. Calculation of $Y_r$ assumes that all added substrate is utilized over the study period and that cryptic growth (i.e. substrate recycling) is negligible.

In a previous study of C utilization patterns in a non-sterile, agricultural soil, calculated values for Y were 0.68, 0.42 and 0.21 for $Y_s$, $Y_b$, and $Y_c$, respectively (Frey *et al.*, 1998). We hypothesized that microbivory, especially by soil protozoa, resulted in an underestimate of $Y_b$ and $Y_c$ since microbial biomass production consumed by grazers during the experimental period was unaccounted for by our biomass measurements. The present study was designed to test this hypothesis. We predicted that under high grazing intensity, the substrate-based approach would give a higher estimate of Y than would the two biomass-based approaches ($Y_s > Y_b > Y_c$), while under low grazing intensity the three methods of calculation would result in similar yield estimates ($Y_s \cong Y_b \cong Y_c$).

## MATERIALS AND METHODS

Our study consisted of two steps. In the first step, we established two grazing intensity treatments (low vs high). In the second step, we examined the effects of grazing on patterns of substrate utilization in short-term (12-20 hr) glucose amendment assays.

### Soils

Surface soil (0-5 cm) was collected from two locations: a Weld silt loam (clay, 23%; organic C, 0.5%) classified as a fine, montmorillonitic, mesic aridic Paleustoll was collected from a wheat-fallow rotation (fallow phase) at the Central Great Plains Research Station at Akron, Colorado, USA (Halvorson *et al.*, 1997); an Urrbrae fine sandy loam (clay, 18%; organic C, 1.85%) classified as a Rhodoxeralf was collected from the wheat-pasture rotation (wheat phase) in the permanent rotation trial at the Waite

Agricultural Research Institute in South Australia (Grace *et al.*, 1995). Soil at both locations was collected from a number of places in the field plots at a time when soil moisture levels were very low.

Establishment of protozoan grazing levels

It is difficult to manipulate active protozoan densities in non-sterile soils without causing significant effects on soil physical properties and microfloral populations; therefore, we used our knowledge of the natural dynamics in protozoan populations to establish grazing treatments of different intensity. During field research on the dynamics of different groups of protozoa (ciliates, amoebae, and flagellates) following rainfall or after irrigation events, it was observed that populations of protozoa were highest 5-10 days after a wetting event (Elliott and Coleman, 1977; Clarholm, 1981; Gupta and Germida, 1989; Gupta and Roper, 1996). Increases in protozoan numbers are generally preceded by an increase in bacterial abundance (Clarholm, 1981). Using this information, preliminary laboratory incubations were designed in which dry soil was wetted and incubated for 12-14 days, over which time protozoan population densities were monitored. It was anticipated that soil from early in the incubation could be used as a low grazing treatment, while soil from later in the incubation could be used as a high grazing treatment.

*Paleustoll (USA).* The soil was sieved (<2 mm) and air-dried. Winter wheat (*Triticum aestivum*) straw was ground with a Wiley mill to pass a 1-mm screen and used to amend 1500 g soil at a rate of 1500 $\mu$g wheat-C $g^{-1}$ soil. The soil was amended to

stimulate microbial activity and encourage the development of a large protozoan population. The soil was then brought to field capacity (0.23 g $g^{-1}$) with inorganic nutrient solution containing $(NH_4)_2SO_4$, $KH_2PO_4$ and $Na_2HPO_4$ in sufficient quantities to achieve soil C:N and C:P ratios of 10 and 100, respectively. Ritz and Griffiths (1987) observed that protozoan numbers were significantly greater following glucose addition when the soil C:N ratio was 10:1 compared to 100:1. The soil was sealed in a 2.5 gal bucket and incubated for 12 d at 25°C. Subsamples were removed every two days for determination of $CO_2$-C evolution rates and protozoan abundance. Respiration measurements, used as an indicator of microbial activity, were made on three replicate 25 g subsamples. Each sample was sealed in a 125 mL erlenmeyer flask with a luer lock fitting in the stopper to allow for headspace gas analysis using a LI-COR infrared gas analyzer (model LI-6252) following a 2-8 hr incubation period. Total protozoan numbers were estimated on three replicate 10 g subsamples by a modification of the most probable number (MPN) method using 5-fold dilutions in 24-well tissue culture plates with 6-8 MPN replications per treatment replicate. A 1:20 soil extract:physiological saline solution was used as diluent and *Aerobacter faecalis* served as the bacterial food source for the protozoa. *Aerobacter* is considered to be readily palatable to protozoa which are known for their selective grazing habits (Stout and Heal, 1967; Gupta *et al.* 1998b). Protozoa were enumerated after incubation at 25°C in a moist chamber for 5 d. Preliminary tests were undertaken to determine the most suitable protozoan culture method for our experimental conditions.

*Rhodoxeralf (Australia).* The soil was sieved (<2.34 mm) and large pieces of plant material removed. Care was taken to avoid breaking macroaggregates during sieving. Prepared soil was left undisturbed for stabilization for 7 days prior to use in the laboratory experiments. Triplicate 200 g subsamples, with moisture adjusted to 55% water-filled pore space, were incubated at 25 °C in closed jars with 0.5M NaOH used as a $CO_2$ trap. At regular intervals (days 3, 5, 7, 10 and 14), subsamples were analyzed for total and cyst populations of different groups of protozoa (ciliates, flagellates and amoebae) according to Gupta *et al.* (1998a), using a *Rhizobium* sp. as a bacterial food source. NaOH traps were analyzed for $CO_2$-C absorbed using the double end-point titration method of Tiessen et al. (1982).

Effects of grazing on patterns of substrate utilization

Based on the preliminary experiments described above, sampling days for "low" (day 2 for the Paleustoll; days 1 and 3 for the Rhodoxeralf) and "high" (day 8 for the Paleustoll; day 7 for the Rhodoxeralf) grazing treatments were established for the main experiment. Soil collection and preparation was as described for the preliminary incubations.

*Paleustoll (USA).* Following amendment with wheat straw and adjustment of soil moisture, the soil was incubated at 25°C for 12 d. Subsamples were removed at 2, 4, 8 and 11 d for determination of $CO_2$-C evolution rates and protozoan abundance as previously described for this soil. To determine glucose use efficiency (Y) on days 2 (low grazing) and 8 (high grazing), 225 g soil was subsampled and glucose was added in

aqueous suspension at a target concentrations of 2000 mg glucose $g^{-1}$ soil, equivalent to 800 $\mu$g C $g^{-1}$ soil. Actual soil glucose concentrations, measured immediately after glucose addition, were 982 and 813 µg glucose-C $g^{-1}$ soil for the low and high grazing treatment, respectively. It is unlikely that this difference had a significant effect on patterns of glucose utilization for these two treatments. Yield coefficients did not vary over a wide range of soil glucose concentrations (288-2304 µg C $g^{-1}$ soil) for two agricultural soils (Bremer and Kuikman, 1994). Seto and Alexander (1985) obtained similar results for a heterogenous bacterial mixture grown in liquid culture with glucose concentrations ranging from 43 pg to 100 µg $ml^{-1}$.

After glucose addition, the soil was divided into nine 25 g aliquots, placed in sealed 125-ml erlenmeyer flasks, and incubated at 25°C for 20 and 13 hr for the low and high grazing intensity treatment, respectively. The length of incubation following glucose amendment was determined by preliminary tests (data not shown) and varied according to treatment since our goal was to terminate each incubation just prior to the point at which the respiration rate began to decline, an indication that substrate concentrations were becoming limiting. Anderson and Domsch (1986) observed that substrate exhaustion was always accompanied by an immediate decline in $CO_2$-C evolution rates. Respiration rates were measured at 1-4 hr intervals throughout the incubation, with more frequent measurements made during the first several hr. Residual soil glucose concentrations and microbial biomass were measured on three randomly selected samples at incubation initiation (0 h), 6 h, and incubation termination (20 or 13 h). Soil glucose concentrations were measured using the hexokinase/glucose-6-phosphate dehydrogenase (HK)

enzymatic assay (Frey *et al.*, 1999a). Total bacterial and fungal biomass were determined by direct microscopy and computer-assisted image analysis (Frey *et al.*, 1999b). Total microbial biomass C was calculated as bacterial biomass C plus fungal biomass C.

*Rhodoxeralf (Australia).* Following adjustment of soil moisture, triplicate 500 g samples were weighed into glass jars and incubated at 25 °C. A 25 ml aliquot of 1M NaOH was used to trap evolved $CO_2$. Subsamples of soil were taken from the main samples on days 0, 1, 3, 5, 7, and 14 to determine total and cyst populations of different groups of protozoa and microbial biomass C measured by chloroform fumigation-extraction using 0.5M $K_2SO_4$ (Sparling *et al.*, 1993; Sparling and Zhu, 1993). To determine glucose use efficiency (Y) on days 1 and 3 (low grazing) and 7 (high grazing), $^{14}C$-labeled glucose was added in aqueous solution (1000 μg C $g^{-1}$ soil and 0.841 KBq $^{14}C$ $g^{-1}$ soil), mixed thoroughly, and incubated at 25 °C. with 0.5M NaOH as a $CO_2$-C ($^{14}C$ and $^{12}C$) trap. During the 22h incubation assay, NaOH traps were replaced at regular intervals (1, 2, 6, 8, 12, 18 and 22 h) and residual glucose and microbial biomass-$^{14}C$ were measured at 1, 6, 12, 18 and 22 h. Residual glucose was measured using a diagnostic kit (Glucose Tinder Procedure No. 315, Sigma Aldrich Pty Ltd). Labeled $^{14}C$ in the $CO_2$-evolved and in microbial biomass was measured using 0.5 and 1 ml aliquots after mixing with 10 ml of Hiphase III scintillation cocktail (LKB scintillation products, England). Loss of $CO_2$-C during the frequent change over of NaOH traps was tested in a separate experiment and found to account for <1% of the evolved $CO_2$-C. Although the length of incubation for the glucose amendment experiments was the same for both grazing treatments in the Rhodoxeralf, yield calculations were made over the time period (18 h

for low and 12 h for high grazing) when >85% of added glucose was utilized leaving greater than 10% of added glucose still available. This was done to avoid changes in the pattern of carbon utilization due to substrate limitation.

Statistical analyses

Analysis of variance was performed using the SAS general linear models procedure (PROC GLM, SAS Institute, 1990). Significance was assumed when $P \leq 0.05$. Differences across more than two pairs of means were determined using the Student-Newman-Kuels (SNK) means separation test (Sokal and Rohlf, 1981).

## RESULTS AND DISCUSSION

Establishment of protozoan grazing levels

Our results support the use of soil incubated for a short period (1-3d) for a low grazing intensity treatment and soil incubated for a longer period (7-8d) for a high grazing intensity treatment. Protozoan population dynamics and soil $CO_2$-C evolution rates in the main experiments (Fig. 4.1) were similar to those observed in preliminary experiments (data not shown) and were expected based on previous field and laboratory studies where protozoan numbers were observed to peak 5-10 days after dry soil was wetted by rainfall or irrigation (Elliott and Coleman, 1977; Clarholm, 1981; Gupta and Roper, 1996). Protozoan numbers in our study were low for the first 3-4 days and increased significantly 7-8 days after water addition. Naked amoebae were the most abundant protozoa in the Rhodoxeralf, representing 57-84% of the total population. At day 7 (high grazing

treatment), amoebae accounted for 84% of the total protozoan numbers. Flagellates and ciliates were less numerous throughout the incubation, representing 16-41 and 0.3-3.7% of the total, respectively. Using conversion factors reported by Beare (1997) and Beare *et al.*, (1992), we estimate a total protozoan biomass for the high grazing treatment of 95 μg C $g^{-1}$ soil, with amoebae accounting for 95% of the total. Individual protozoan groups were not enumerated for the Paleustoll; however, amoebae are often reported to be the most common type of protozoa found in soil (Elliott and Coleman, 1977; Clarholm, 1981). Protozoan numbers were an order of magnitude lower in the Paleustoll compared to the Rhodoxeralf for the 4-8 d period. Protozoan densities in the Paleustoll ranged from 1.7 x $10^3$ to 26.6 x $10^3$ cells $g^{-1}$ soil and were similar to estimates obtained by Elliott and Coleman (1977) for a grassland soil incubated for 22 d following a wetting event.

Effects of grazing on patterns of substrate utilization

Obtaining accurate estimates of C utilization efficiency requires that measurements of substrate utilization, respiration losses, and microbial biomass be made while soil substrate concentrations are non-limiting. Once all of the substrate has been utilized, cryptic growth (i.e., recycling of microbial products) results in lower yield estimates due to inflated cumulative $CO_2$-C values. Elliott *et al.* (1983) obtained a value of Y = 0.68 for *Psuedomonas cepacia* grown in glucose-amended, sterile soil for 15 h. This value dropped to Y = 0.34 after 63 h when >90% of the glucose had been utilized. Likewise, model simulations of bacterial growth in soil yielded estimates of Y = 0.65 until C substrate was exhausted at about 24 h, after which time Y dropped to 0.19 (Hunt

*et al.*, 1985). In our study, short-term (12-20 h) glucose-amendment assays were used, thereby, limiting our investigation of glucose utilization patterns to the initial period following amendment when glucose was still readily available.

This approach resulted in shorter assay times for the low (18-20 h) compared to the high (12-13 h) grazing treatments since the presence of protozoa increased rates of glucose utilization in both soils (Fig. 4.2). Glucose utilization rates were similar under both grazing regimes during the first 6 h following glucose amendment, after which time glucose disappearance was significantly greater under high compared to low grazing ($P \leq 0.05$). In the Paleustoll, approximately 70% of the added glucose was utilized in 13 and 20 h for the high and low grazing treatments, respectively. Glucose use was more rapid in the Rhodoxeralf with approximately 90% being lost from that soil in 12 h under high grazing and 18 h under low grazing.

Rates of $CO_2$-C evolution ranged from 2 to 33 μg $CO_2$-C $g^{-1}$ soil $h^{-1}$ (Fig. 4.3) and are within the range reported for glucose-amended soil during the first 10-15 h following amendment (Behera and Wagner, 1974; Elliott *et al.*, 1983). Respiration rates were similar for both grazing treatments; however, the $CO_2$-C evolution curve for the low grazing treatment lagged behind that of the high grazing treatment by about 6 h. Preliminary experiments indicated that respiration rates began to decline after 12-13 h under high grazing and 18-20 h under low grazing for both soils (data not shown).

Glucose amendment stimulated the production of microbial biomass under both grazing treatments; however, observed increases in biomass were significantly less under high compared to low grazing (Table 4.2). In the Paleustoll, a ten-fold increase in

biomass was observed under low grazing over the 20 h assay period. Although biomass measurements were not made at the 6 h sampling point for the low grazing treatment, $CO_2$-C evolution rates were relatively constant for the first 6 h (Fig. 4.3), suggesting that most of the biomass production occurred between 6-20 h. A three-fold increase in biomass was observed for the high grazing treatment with most growth taking place in the first 6 h following glucose amendment. Direct microscopy was used to estimate microbial biomass in the Paleustoll; therefore, the relative contributions of bacteria and fungi to the total microbial biomass could be determined. Additionally, observations were made regarding changes in cell size that occurred over the assay period. Bacteria accounted for >80% of the total biomass at all sampling points in both grazing treatments; therefore, most of the microbial production was due to increases in bacterial biomass. The increase in bacterial biomass was due to increases in both cell numbers and cell size. Bacterial biovolume increased from 0.26 to 0.97 $\mu m^3$ under low grazing and from 0.34 to 0.66 $\mu m^3$ under high grazing. Grazing significantly reduced the number of bacterial cells produced and kept the cells that were produced from attaining the large size observed when protozoa were low in abundance. A smaller average cell size under high grazing intensity could also be attributed to the preference of protozoa for larger cells. For example, Gonzalez *et al.* (1990) observed selective grazing by protozoa of larger bacterioplankton cells.

Chloroform fumigation-extraction was used to estimate biomass in the Rhodoxeralf and biomass production was calculated as the amount of soluble $^{14}C$ in the chloroform-labile fraction. Most of the biomass production in this soil occurred in the

first 6 h following glucose amendment for both the low and high grazing treatments (Table 4.2). Under low grazing, biomass continued to increase from 6-12 h, with little additional increase after that time. Under high grazing, biomass decreased significantly from 6-12 h indicating that some biomass recycling had started to take place.

Our glucose utilization, respiration, and biomass data are in agreement with results from other studies showing that protozoa regulate bacterial abundance in soil and stimulate bacterial activity. It is well documented that microbial abundance increases significantly in soil recently amended with a labile C source (Behera and Wagner, 1974; Anderson *et al.*, 1978; Elliott *et al.*, 1983). In field studies, declines in microbial biomass were correlated with an increase in protozoan abundance (Clarholm, 1981; Gupta and Roper, 1996). In laboratory manipulation experiments, bacterial numbers were significantly lower in treatments where grazers were present (Habte and Alexander, 1977; Anderson *et al.*, 1978; Coleman *et al.*, 1978; Gupta and Germida, 1989; Kuikman *et al.*, 1990a,b). Bacterial densities remained high in soil where protozoa were present when the eukaryotic inhibitor actidione was applied, but rapidly declined when an antibiotic-resistant protozoan strain was added (Habte and Alexander, 1977). Protozoan grazing stimulates $CO_2$ production, an indicator of metabolic activity (Coleman *et al.*, 1978; Elliott *et al.*, 1980; Hunt *et al.*, 1984; Gupta and Germida, 1989; Kuikman *et al.*, 1990a,b; Rutherford and Juma, 1992). Wright *et al.* (1995), using a luminescence-based marker system, observed a significant increase in the activity of *Psuedomonas fluorescens* in the presence of the soil ciliate *Colpoda steinii.*

Cumulative $CO_2$-C evolved and biomass-C produced accounted for 92 to 100% of the total amount of glucose utilized during the low grazing assays (Table 4.3 and Table 4.4). On average, 42% of the glucose utilized was respired, with the balance (54%) used for biomass production. Under high grazing, 59% of the glucose utilized was accounted for, with only 26% of glucose utilized going to biomass production. The remainder of the glucose (41%) presumably was present as microbial metabolites or indirectly utilized by grazers through the consumption of microbial production between 0 and 12-13 h when biomass measurements were made. This was supported by our observation of high levels of $^{14}C$ in the unfumigated extracts in the high grazing treatment for the Rhodoxeralf (data not shown). Shields *et al.* (1973) also observed that 39% of added glucose-C was unaccounted for in their respiration and biomass estimates 7 days after glucose addition when only negligible amounts of reducing sugars were still present.

The amount of C unaccounted for in the high grazing treatment was 230 and 369 $\mu g\ C\ g^{-1}$ soil for the Paleustoll and Rhodoxeralf, respectively (Table 4.3). It is possible that some glucose was consumed directly by grazers. Many soil protozoa can be cultured on dissolved organic nutrients and some flagellates (small forms) are osmotrophic (reviewed by Ekelund and Rønn, 1994). It is unlikely, however, that direct protozoan consumption can account for a significant proportion of the missing C. It is generally accepted that phagotrophy (e.g., bacterial consumption) and not osmotrophy is the main route of nutrient uptake in soil protozoa. Ekelund and Rønn (1994) suggested that the low surface to volume ratio of protozoa makes them poor competitors with bacteria for dissolved nutrients.

Reported protozoan consumption rates of bacteria range from 2,000 to 12,000 bacteria consumed per protozoan cell division for soil amoebae (Ekelund and Rønn, 1994) which comprised 95% of the total protozoan biomass in the high grazing treatment for the Rhodoxeralf. The number of amoebae enumerated in this soil ($2 \times 10^5$ cells $g^{-1}$ soil) could have consumed $4 \times 10^8$ to $2.4 \times 10^9$ bacterial cells. Assuming a specific bacterial density of 1.1 g cm-3, a dry matter content of 50%, and a C content of 50% (Bratbak and Dundas, 1984), this level of bacterial consumption is equivalent to 56 to 330 μg C $g^{-1}$ soil. Given that amoebae can multiply within hours under optimum conditions (Clarholm, 1984; Ekelund and Rønn, 1994), protozoan grazing can account for much of the missing C in the high grazing treatment. It is unlikely, though, that most of this C (230-369 μg C $g^{-1}$ soil) went toward the production of protozoan biomass. The efficiency of conversion of bacterial biomass into protozoan biomass is low (Hunt *et al*,.1984). Coleman *et al.* (1978) estimated the efficiency of amoebal production to be 0.4. A re-examination of the Coleman *et al.* (1978) data, which took cryptic growth into account, indicated an even lower conversion factor of 0.25 (Coûteaux et al., 1988 as cited by Ekelund and Rønn, 1994). It has been suggested that protozoa use the cytoplasmic contents of bacteria for growth and energy production and release undigested cell walls and other recalcitrant materials as waste (Hunt *et al.*, 1984; Rutherford and Juma, 1992). We observed that the amount of soluble, non-glucose $^{14}C$ in non-fumigated 0.5M $K_2SO_4$ soil extracts of the Rhodoxeralf increased from 16 μg C $g^{-1}$ soil in the low grazing treatment to 359 μg C $g^{-1}$ soil in the high grazing treatment, the latter value being nearly equivalent to the amount of missing C for the high grazing treatment in this soil. Parsons

and Smith (1989) also observed an accumulation of extractable, non-glucose constituents in soil extracts 12 h after glucose amendment. These results support the low conversion efficiencies previously reported for protozoa and explain why the biomass estimates for the Rhodoxeralf, obtained by chloroform fumigation-extraction and which should include protozoan biomass, accounted for a similar proportion of total glucose utilized as did the biomass estimates for the Paleustoll which were determined by direct microscopy where protozoan biomass is excluded.

Efficiency of glucose utilization

Our estimates of C utilization efficiency support our earlier predictions. When protozoan abundance was low, the three approaches to calculating Y gave similar results ($Y_s = Y_b = Y_c$), with values of Y ranging from 0.57-0.58 for the Paleustoll and 0.51-0.59 for the Rhodoxeralf (Table 4.5). When protozoa were present, estimates of Y were in the order of $Y_s > Y_b > Y_c$. The substrate-based yield estimates averaged 0.61 for the Paleustoll and 0.62 for the Rhodoxeralf and are close to those values ($Y \approx 0.60$) obtained for pure and mixed cultures of bacteria isolated from soil (Payne, 1970).

Chapman and Gray (1986) argued that the true yield coefficient is often underestimated because maintenance energy requirements are neglected. Maintenance energy represents the fraction of microbial biomass C that is used for energy production to support cellular functions other than growth, including protein and nucleic acid turnover, osmoregulation, and motility. Estimating Y using a substrate-based approach ($Y_s$) could thus underestimate the true yield since microbial respiration, which is assumed

to include both a growth and maintenance energy component, is used in the calculation of $Y_s$ to estimate microbial biomass production. Thus, the respiration rate $R_b$ ($\mu g$ $CO_2$-C $g^{-1}$ soil $h^{-1}$) can be calculated as:

$$R_b = (1 - Y) \cdot S_c + B \cdot M_b$$

where $S_c$ is total substrate-C uptake, Y is the theoretical ("true") yield, B is microbial biomass and $M_b$ is the maintenance energy rate (Hunt et al., 1984). Accurate estimates of maintenance energy costs have been hard to obtain because of the difficulty of separating respiration into its growth and maintenance energy components. Maintenance energy estimates range from 0.0003 to 0.012 $h^{-1}$ (Babiuk and Paul, 1970; Shields *et al.*, 1973; Behera and Wagner, 1974; Anderson and Domsch, 1985; Smith *et al.*, 1986), although a value of 0.001 $h^{-1}$, as recommended by Babiuk and Paul (1970), is most commonly used. Using this value, we estimate that 4.1 and 3.5 $\mu g$ C $g^{-1}$ soil was used for maintenance energy under low and high grazing, respectively, for the Paleustoll. These values represent 0.6% of the total amount of substrate-C utilized by the microbial community in these two treatments. If we subtract this amount of C from the cumulative amount of $CO_2$-C evolved, we obtain values for $Y_s$ of 0.59 and 0.66 as compared to 0.58 and 0.65 (Table 4.5) for the low and high grazing treatments, respectively. These results indicate that only a small proportion of the glucose-C consumed was used for maintenance and thus $Y_s$ is mainly determined by the energy demand for growth and any underestimate is minimal.

Values of Y determined experimentally for non-sterile soil containing a mixed microbial population vary substantially (Table 4.1) with substrate-based calculations

giving a narrower and higher range of values (Y = 0.54-0.73) compared to biomass-based calculations (Y = 0.26-0.66). Our results indicate that substrate-based yield estimates ($Y_s$) will approach the true growth yield efficiency under conditions of both low and high grazing intensity, while biomass-based yield estimates ($Y_b$ and $Y_c$) will only approach the true yield when grazer numbers are low and microbial biomass production can be determined reasonably accurately.

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**Table 4.1.** Summary of studies on microbial carbon assimilation efficiency (Y) in soil.

| Study | Glucose-C applied ($\mu g\ g^{-1}$ soil) | Incubation time (h) | Y¥ |
|---|---|---|---|
| Sterile soil inoculated with a single organism: | | | |
| Anderson *et al.*, 1981£ | 500 | 48 | 0.60[4] |
| Elliott *et al.*, 1983£ | 700 | 15-40 | 0.61[2] |
| Non-sterile soil with a mixed population: | | | |
| Shields *et al.*, 1973 | 937 | 72 | 0.63[4] |
| Behera and Wagner, 1974 | 800 | 36 | 0.39[2] |
| Adu and Oades, 1978 | 4000 | nd | 0.58[4] |
| Anderson and Domsch, 1986 | 55-2543 | 24-48 | 0.37-0.53[3] |
| Parsons and Smith, 1989 | 360 | 24 | 0.54-0.73[1] |
| Ladd *et al.*, 1992 | 1000 | 48 | 0.50-0.77[1] |
| Bremer and Kuikman, 1994 | 36-2304 | 72 | 0.58-0.70[4] |
| Shen and Bartha, 1996 | 0.4-400 | 336 | 0.47-0.66[2] |
| S. Frey, this study | 813-983 | 25 | 0.39-0.68[1,2] |
| V.V.S.R. Gupta, this study | 1000 | 72 | 0.26-0.61[1,2] |

£ Used *Pseudomonas paucimobilis,* originally described by Anderson et al., (1981) as *P. cepacia.*

¥ Method for calculating Y: (1) $Y_s = (dS_C - \Sigma CO_2\text{-}C) / dS_C$; (2) $Y_b = dB_C / (dB_C + \Sigma CO_2\text{-}C)$; (3) $Y_c = dB_C / dS_c$; (4) $Y_r = 1 - (\Sigma CO_2\text{-}C / S_{tot})$.

**Table 4.2.** Biomass C in non-sterile soil containing a mixed microbial population and different levels of protozoa.

| Soil | Sampling Time (h) | Grazing intensity[§] ----------Low---------- | | High |
|---|---|---|---|---|
| | | μg C $g^{-1}$ soil | | |
| Paleustoll (USA) | | Day 2 | --- | Day 8 |
| | 0 | $42 \pm 6^{b}$ | | $70 \pm 5^{c}$ |
| | 6 | nd | | $164 \pm 21^{b}$ |
| | 13 | nd | | $210 \pm 18^{a}$ |
| | 20 | $445 \pm 31^{a}$ | | nd |
| | | μg $^{14}C$ $g^{-1}$ soil | | |
| Rhodoxeralf (Australia) | | Day 1 | Day 3 | Day 7 |
| | 6 | $371 \pm 6^{b}$ | $388 \pm 18^{b}$ | $335 \pm 15^{a}$ |
| | 12 | $449 \pm 15^{a}$ | $463 \pm 26^{a}$ | $243 \pm 20^{b}$ |
| | 18 | $474 \pm 13^{a}$ | $439 \pm 8^{a}$ | nd |

[§]Means ± 1 standard error; nd = no determination; values followed by the same letter within a grazing treatment are not significantly different ($P \leq 0.05$).

**Table 4.3**. Carbon utilization patterns in non-sterile soil containing a mixed microbial population and different levels of protozoan grazing.

| Soil | Grazing intensity[§] ----------Low---------- | | High |
|---|---|---|---|
| | $\mu g\ C\ g^{-1}$ soil | | |
| Paleustoll (USA) | Day 2 | --- | Day 8 |
| Glucose-C used | 701 ± 69 | | 567 ± 23 |
| $CO_2$-C evolved | 289 ± 11 | | 197 ± 2 |
| MB-C produced[£] | 396 ± 34 | | 140 ± 20 |
| | $\mu g\ ^{14}C\ g^{-1}$ soil | | |
| Rhodoxeralf (Australia) | Day 1 | Day 3 | Day 7 |
| Glucose-$^{14}$C used | 880 ± 10 | 862 ± 5 | 900 ± 15 |
| $^{14}CO_2$-C evolved | 385 ± 24 | 351 ± 26 | 288 ± 11 |
| MB-$^{14}$C produced | 474 ± 13 | 439 ± 78 | 243 ± 20 |

[§]Means ± 1 standard error.
[£]Microbial biomass-C.

**Table 4.4**. Carbon balance in non-sterile soil containing a mixed microbial population and different levels of protozoan grazing.

| Soil | Grazing intensity[§] ----------Low---------- | | High |
|---|---|---|---|
| | % of glucose used | | |
| Paleustoll (USA) | Day 2 | --- | Day 8 |
| $CO_2$-C evolved | 42.4 ± 6.1[a] | | 34.8 ± 1.6[a] |
| Biomass-C | 57.1 ± 4.7[a] | | 24.4 ± 2.5[b] |
| Total[¥] | 99.6 ± 10.1[a] | | 59.2 ± 0.9[b] |
| Rhodoxeralf (Australia) | Day 1 | Day 3 | Day 7 |
| $^{14}CO_2$-C evolved | 43.7 ± 2.3[a] | 40.7 ± 3.2[a] | 32.0 ± 1.0[b] |
| Biomass-$^{14}$C | 54.0 ± 2.1[a] | 50.8 ± 1.0[a] | 26.8 ± 1.8[b] |
| Total | 97.7 ± 0.7[a] | 91.5 ± 4.2[b] | 58.8 ± 1.9[c] |

[§]Means ± 1 standard error; values followed by the same letter across grazing treatments are not significantly different ($P \leq 0.05$).

[¥]The total amount of glucose used (refer to Table 3) that was accounted for in $CO_2$-C evolution and biomass production.

[£]Microbial biomass-$^{14}$C was measured by chloroform fumigation-extraction using 0.5M $K_2SO_4$ as an extractant.

**Table 4.5**. The effect of protozoan grazing on estimates of glucose utilization efficiency (Y).

| Soil | Grazing intensity[§] ----------Low---------- | | High |
|---|---|---|---|
| Paleustoll (USA) | Day 2 | --- | Day 8 |
| $Y_s$[¥]: | 0.58 ± 0.06 [a] | | 0.65 ± 0.02 [a] |
| $Y_b$: | 0.58 ± 0.02 [a] | | 0.41 ± 0.04 [b] |
| $Y_c$: | 0.57 ± 0.05 [a] | | 0.24 ± 0.02 [c] |
| Rhodoxeralf (Australia) | Day 1 | Day 3 | Day 7 |
| $Y_s$: | 0.56 ± 0.02 [a] | 0.59 ± 0.03 [a] | 0.68 ± 0.01 [a] |
| $Y_b$: | 0.55 ± 0.02 [a] | 0.56 ± 0.01 [a] | 0.46 ± 0.02 [b] |
| $Y_c$: | 0.54 ± 0.02 [a] | 0.51 ± 0.01 [b] | 0.27 ± 0.02 [c] |

[§]Means ± 1 standard error; values followed by the same letter within a grazing treatment are not significantly different ($P \leq 0.05$).

[¥] $Y_s = (dS_C - \Sigma CO_2\text{-}C) / dS_C$; $Y_b = dB_C / (dB_C + \Sigma\ CO_2\text{-}C)$; $Y_c = dB_C / dS_c$ where $dS_C$ is the amount of added substrate C that is utilized, $\Sigma\ CO_2$-C is the cumulative C lost during respiration, and $dB_C$ is the change in microbial biomass C,.

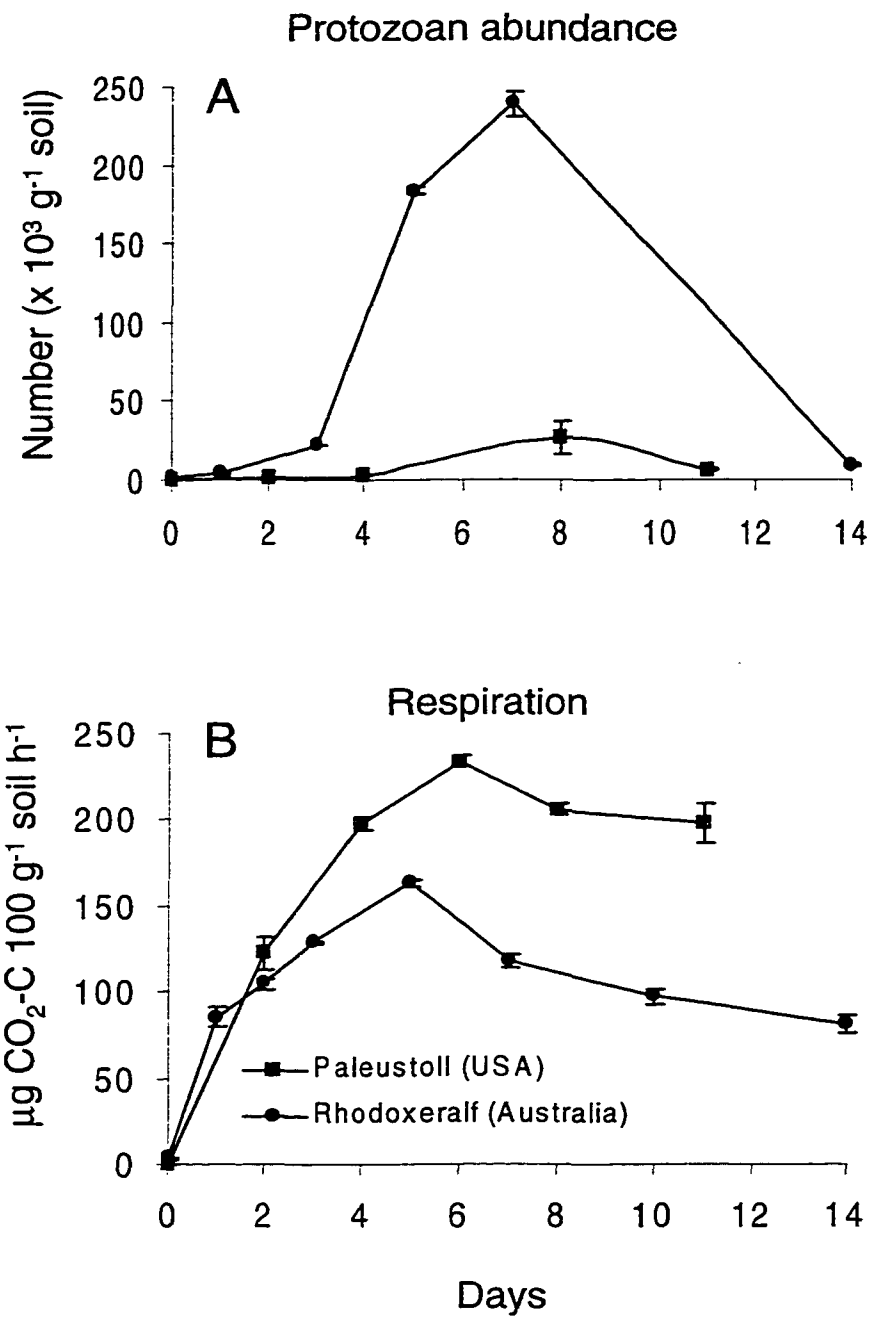


**Fig. 4.1.** Protozoan numbers (A) and respiration rate (B) for a Paleustoll amended with 1500 µg wheat straw and incubated at 25°C for 12 d and an unamended Rhodoxeralf incubated at 25 °C for 14 d. Values are means ± 1 standard error.

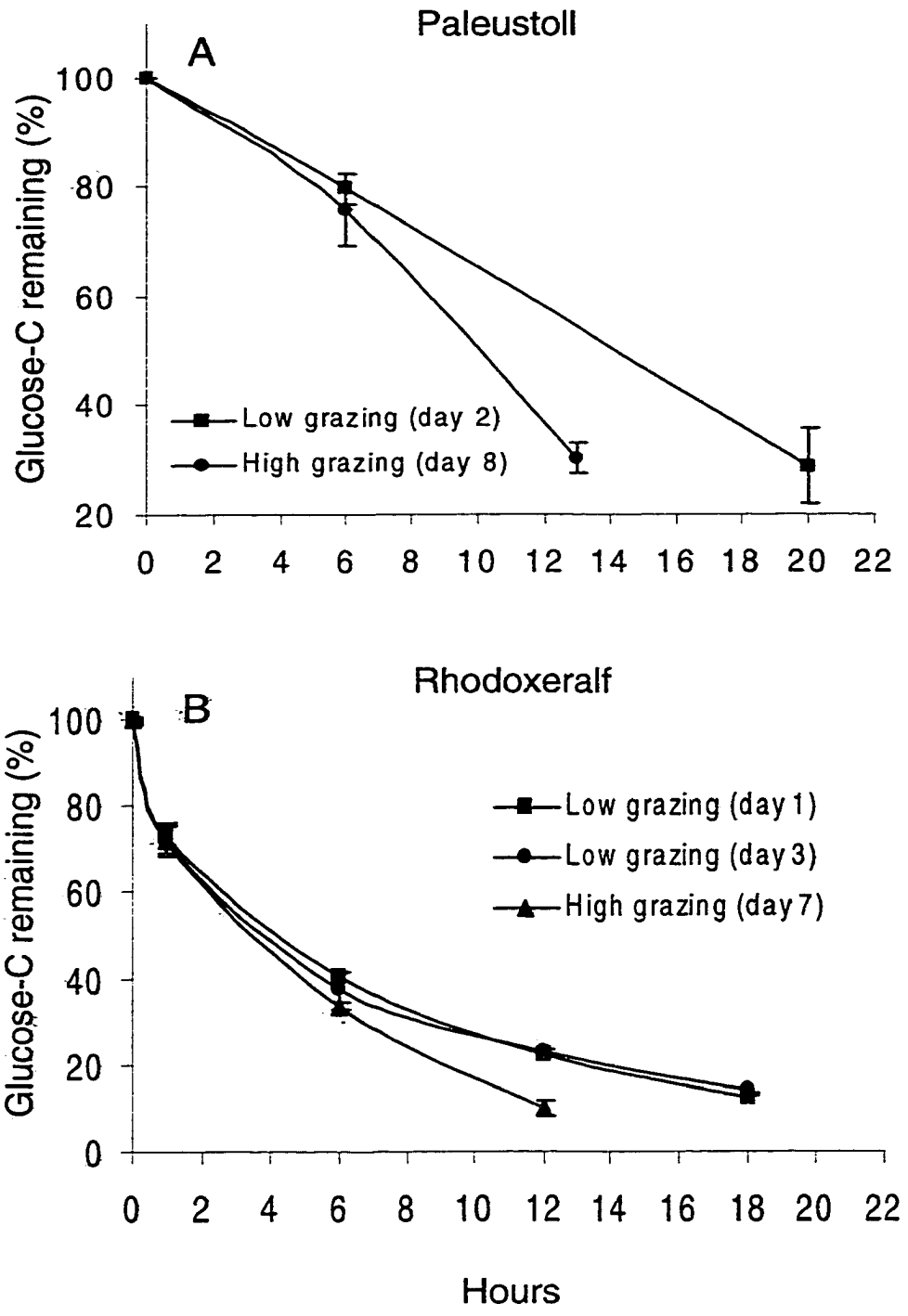


**Fig. 4.2.** Percentage glucose-C remaining for low vs high grazing treatments for a Paleustoll (A) and a Rhodoxeralf (B). Values are means ± 1 standard error.

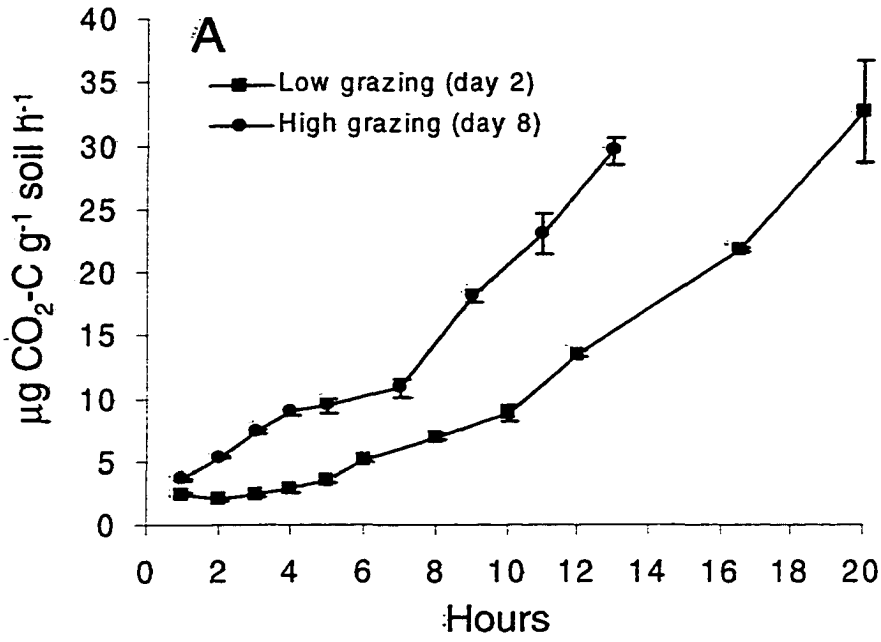


**Fig. 4.3.** Rates of $CO_2$-C evolution for low vs high grazing treatments for a Paleustoll (A) and a Rhodoxeralf (B). Values are means ± 1 standard error.

# CHAPTER 5

# APPLICATION OF THE HEXOKINASE/GLUCOSE-6-PHOSPHATE DEHYDROGENASE ENZYMATIC ASSAY FOR MEASUREMENT OF GLUCOSE IN AMENDED SOIL

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Soils are often amended with glucose to examine patterns of carbon utilization and stabilization by the soil microbial community. In such studies it is often desirable to quantify glucose loss and microbial growth and respiration over a short incubation period. This information can then be used to determine the amount of glucose used for microbial growth versus that used for energy acquisition. Soil glucose concentrations can be determined chemically or enzymatically, although chemical analyses are most commonly used. Typically, the anthrone (Brink *et al.*, 1960; Oades, 1967) or phenol-sulfuric acid method (Dubois *et al.*, 1956) is used. These methods are time-consuming, require the use of concentrated sulfuric acid, and are nonspecific since the chemical reagents used react with carbohydrates other than glucose. The enzymatic approach has been applied to soil

(Anderson and Domsch, 1985; Parsons and Smith, 1989); however, little detailed information is available on appropriate methodological protocol and the efficiency with which glucose can be extracted from soil using this method. We describe a simple procedure for the enzymatic measurement of glucose in short-term soil amendment experiments and evaluate the extraction efficiency of this method over a range of glucose concentrations.

The hexokinase/glucose-6-phosphate dehydrogenase (HK) enzymatic assay is used routinely in clinical laboratories for the determination of glucose concentrations in blood serum and plasma, cerebral spinal fluid, and urine (Stein, 1963). A glucose diagnostic kit containing the necessary reagents is available (Procedure No. 16-UV, Sigma Chemical Co., St. Louis, MO). Two coupled enzymatic reactions are involved in the assay:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \qquad (1)$$

$$\text{glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH} \qquad (2)$$

In reaction (1) hexokinase catalyzes the phosphorylation of glucose. In reaction (2) the glucose-6-phosphate formed in the first reation is oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalyzed by glucose-6-phosphate dehydrogenase (G-6-PDH) and is accompanied by an increase in absorbance at 340 nm as an equimolar amount of NAD is reduced to NADH. The increase in absorbance is directly proportional to the solution glucose concentration

(Procedure No. 16-UV, Sigma Chemical Co., St. Louis, MO).

Glucose was extracted from soil samples by shaking 10 g moist soil in 20 ml cold (4°C) deionized water for 15 min. at 200 rpm on a reciprocal shaker. Cold water was used to minimize further glucose utilization by the microbial community. The soil suspension was centrifuged at 8,000 g for 15 min. at 4°C and 10 ml of the supernatant was then filtered through a 0.45 $\mu$m MF-Millipore membrane filter to remove particulate organic matter that did not settle out during centrifugation. Preliminary tests indicated that these filters did not bind glucose. Nine ml of filtered soil extract was preserved with 1 ml 20% $H_3PO_4$ and the samples were stored at 4°C for up to two weeks. A 0.5 M $K_2SO_4$ solution was initially examined as a possible soil extractant since it is commonly used to extract soluble carbon from soil following chloroform fumigation (Vance *et al.*, 1987). However, water proved to be a superior extractant since a precipitate, presumably $CaSO_4$, formed in many of the $K_2SO_4$ extracts, interfering with the glucose determination.

For glucose determination, the HK enzymatic reagent was reconstituted according to package instructions (Procedure No. 16-UV, Sigma Chemical Co., St. Louis, MO) and reagent and samples were warmed to room temperature. Two ml of HK reagent and 20 $\mu$l of sample were pipetted into a test tube cuvette and vortexed briefly to mix. This mixture was incubated in a water bath set at 37°C for 15 min before reading the absorbance at 340 nm on a spectrophotometer (Spectronic 301, Milton Roy). Two analytical replicates of each sample were analyzed and the average value was used in the glucose determination. The coefficient of variation for analytical replicates was routinely < 2%. Glucose concentrations were calculated using absorbance values obtained from

known glucose standards. Blanks were also routinely analyzed and consisted of 20 $\mu$l $H_3PO_4$-preserved water in 2.0 ml of reagent.

To examine the efficiency with which glucose could be extracted from soil using the above procedure, glucose was added to sterile soil in a range of concentrations. Soil sterilization was necessary since glucose is rapidly utilized by the microbial community under non-sterile conditions. A silty clay loam (7% sand, 61% silt, 32% clay) was collected from the surface 0-20 cm of an agricultural plot at a long-term tillage comparison experiment at the University of Kentucky, Lexington, KY. This soil is classified as a fine, mixed, mesic Typic Paleudalf. The soil was sterilized by autoclaving for 1 h at 121 °C and 18 psi on three consecutive days.

Glucose was added to soil in sufficient deionized water to bring the soil moisture content to field capacity and the glucose concentration to the target level. Target glucose levels were 0, 150, 250, 500, 1000, 2000 $\mu$g glucose $g^{-1}$ soil, equivalent to 0-800 $\mu$g glucose-C $g^{-1}$ soil. This range of glucose concentrations is representative of glucose levels typically used in soil amendment studies (Elliott *et al.*, 1983; Parsons and Smith, 1989; Ladd *et al.*, 1992; Bremer and Kuikman, 1994). After amendment, glucose was immediately extracted as described previously.

In addition to evaluating extraction efficiency over a range of glucose concentrations, the method was evaluated for extraction efficiency over a 48 hr period since glucose may, given enough time, diffuse to and become bound by clay and iron or aluminum oxide sorption sites. Sterile soil was asceptically amended with 2000 $\mu$g glucose $g^{-1}$ soil and extracted at 0, 24 and 48 h after amendment.

Glucose recovery from sterile soil ranged from 97% ± 1.0 to 102% ± 1.6 for a concentration range of 150-2000 $\mu$g glucose $g^{-1}$ soil (Table 5.1). Overall recovery was 100% ± 2.7. The glucose concentration in unamended samples was low (6.1 ± 8.6) but highly variable, suggesting that this method is not generally suitable for accurate measurement of background glucose levels in unamended soil. Efficient recovery was not affected when this soil was exposed to glucose for a 24-48 hr period (Table 5.2), indicating that the soil we used does not bind glucose over short periods of time. In fact, glucose concentrations increased slightly during this incubation period, due possibly to enzyme activity that was not completely inactivated by our sterilization protocol. Analytical precision was determined to be 1.00 ± 0.01 $\mu$g glucose $ml^{-1}$ for a known standard (n = 5) and 0.80 ± 0.02 $\mu$g glucose $ml^{-1}$ for a soil extract (n = 10). Glucose concentrations of preserved samples stored at 4°C were monitored over a two week period and found to be stable.

To determine if glucose loss could be reliably monitored over a short incubation period using soil with an actively growing microbial community, we collected fresh samples from the surface 0-5 cm of three no-tillage plots at the Lexington, KY site. Soil characteristics are as described previously. The soil was sieved (2 mm) and 25 g subsamples were amended with 2000 $\mu$g glucose $g^{-1}$ soil and incubated at 25°C for 40 hr. Soil glucose concentrations and $CO_2$-C evolution rates were determined at 0, 14, 25 and 40 hr following amendment. Maximum glucose utilization coincided with maximum respiratory response at 14 hr (Fig. 5.1). Seventy-three percent of the added glucose was utilized within this period of time. For the remainder of the incubation, respiration rates declined as glucose levels dropped to near background levels by 40 hr.

In summary, the hexokinase/glucose-6-phosphate dehydrogenase enzymatic assay is a simple, rapid and accurate method for measuring glucose in amended soil. The method, unlike chemical approaches, is specific for glucose and results in complete recovery over a range of glucose concentrations. We obtained 97-102% recovery of glucose from an agricultural, silty clay loam soil amended with 150-2000 $\mu$g glucose $g^{-1}$ soil. We have subsequently applied this method to quantitatively monitor glucose loss from other soil types and for soils differing in the initial amount of microbial biomass and activity. However, the efficiency of glucose extraction using this approach should be determined for each soil on which glucose determinations are to be made.

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**Table 5.1.** Recovery of glucose from sterile, amended soil. Values are means of three replicates. Values in parentheses are standard deviations.

| Glucose added (ug $g^{-1}$ soil) | Glucose recovered ($\mu g$ $g^{-1}$ soil) | Recovery of added glucose (%) |
|---|---|---|
| 0 | 6.1 (8.6) | N/A[§] |
| 150 | 148 (4.2) | 98 (2.3) |
| 250 | 242 (3.2) | 97 (1.0) |
| 500 | 512 (3.7) | 102 (0.6) |
| 1000 | 1016 (19.3) | 102 (1.6) |
| 2000 | 2027 (19.4) | 101 (0.7) |
| | Overall recovery: | 100 (2.7) |

[§]Not applicable since no glucose added.

**Table 5.2.** Glucose recovery over a 48 hr period for sterile soil amended with 2000 $\mu$g glucose $g^{-1}$ soil. Values are means of three replicates. Values in parentheses are standard deviations.

| Extraction time (hr) | Glucose recovered ($\mu g\ g^{-1}$ soil) | Recovery (%) |
|---|---|---|
| 0 | 2027 (19.4) | 101 (0.7) |
| 24 | 2041 (19.4) | 102 (0.7) |
| 48 | 2091 (7.9) | 104 (0.3) |

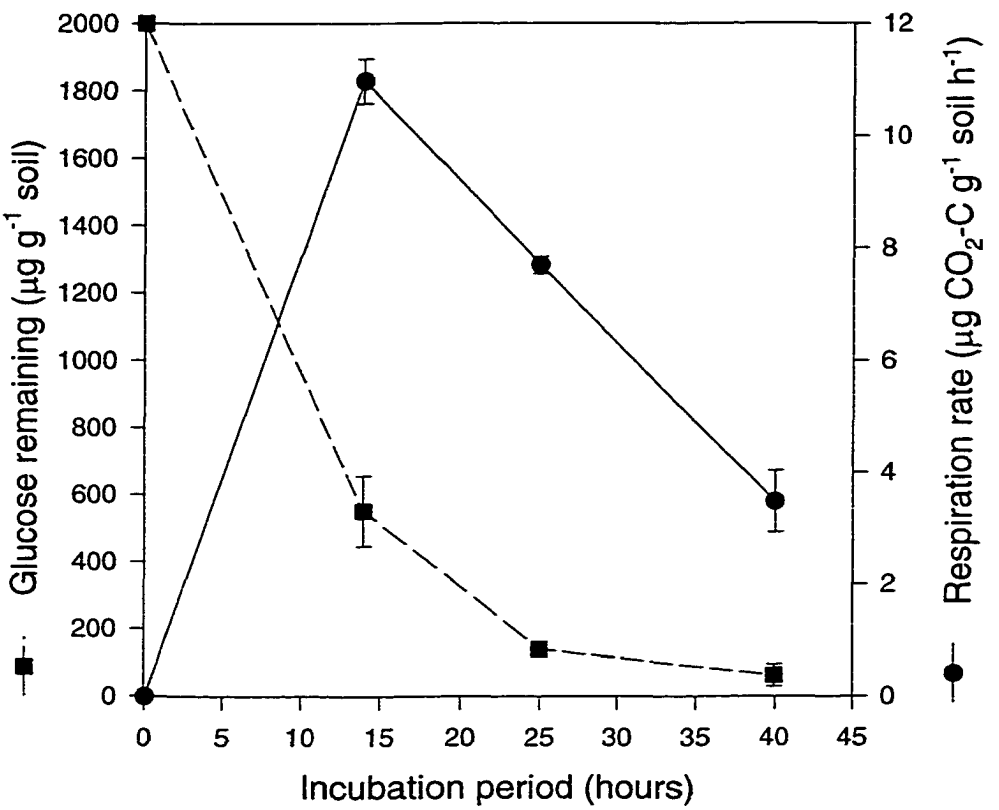


**Figure 5.1.** Glucose loss and $CO_2$-C evolution rate from non-sterile soil amended with 2000 µg glucose $g^{-1}$ soil and incubated at 25°C for 40 h. Error bars represent one standard deviation.