Despite more than a century of study, fundamental questions remain about plant litter decomposition, a key control on carbon (C) sequestration. Changing soil C storage in response to nitrogen (N) deposition, elevated CO_2, plant community composition shifts, and climate change, is a topic of growing interest because of the potential CO_2 feedback the atmosphere. These disturbances influence soil C by altering the interactions between microbial activity and plant litter chemistry during decomposition. However, we are unable to predict the magnitude or sometimes even direction of microbial response in specific instances, as the mechanisms controlling changes in plant litter decomposition are not well understood. **We propose an integrated field, laboratory, and modeling study of the biochemical mechanisms driving interactions between organic matter stabilization, litter chemistry, microbial community composition and enzyme activities during decomposition.** Our central hypothesis is that during decomposition there is a predictable and quantifiable microbial succession tied to litter chemistry, and that changes in decomposition rates and litter chemistry in response to nutrient availability reflect changes in the composition and function of the microbial community.

Many models are available for predicting relative mass loss rates of different plant litters, but these models lack resolution with regard to the late stages of decomposition. Most predictive models of decomposition are driven by initial litter quality and environmental conditions rather than microbial dynamics, and fail to capture impacts arising from “bottom up” changes in microbial community composition or function. Moorhead and Sinsabaugh (2006) have developed a new model that incorporates microbial succession into the decomposition process. With this model, the effects of exogenous N on organic matter stabilization can be simulated. This model, the Guild Decomposition Model (GDM), has generated a series of specific hypotheses about the mechanisms controlling the interactions between plant litter chemistry and microbial community composition and activity during decomposition. The proposed research will evaluate these hypotheses experimentally by monitoring changes in litter chemical composition, microbial community composition, and the activities of the exoenzymes that degrade plant litter throughout the decomposition of contrasting litter types, with and without added N. We will also label leaf litter subsamples with different ^{13}C compounds representative of the primary leaf litter chemical constituents to determine the community composition of active microorganisms capable of metabolizing specific products of decomposition. GDM simulations will be run using these experimental data, and these experiments have been designed to test the structure of, and predictions from the GDM, which in turn explicitly defines functional links between activities of decomposer microorganisms, litter chemistry and labile N.

Intellectual Merit: Because soil organic matter represents a large pool of C relative to the atmosphere, there is great interest in understanding the conditions under which soils will behave as a source or sink for atmospheric C. Disturbances such as N deposition and CO_2 enrichment affect SOM dynamics but the magnitude and direction of responses vary among systems. The work we propose will provide mechanistic insight into this variation, particularly the extent to which litter decomposition can be modified by changes in litter chemistry and N availability through “bottom up” effects on the composition and substrate use of microbial communities. A key area of scientific significance is the explicit integration of microbial community and chemical interactions during the long-term degradation and stabilization of plant litter. There has not yet been a working synthesis of this knowledge expressed in a comprehensive quantitative framework.

Broader impacts: We are proposing an integrated education and outreach program to complement the proposed research. To make this effort as effective as possible we are collaborating with the University of Toledo’s Center for Creative Instruction (CCI). For this project, the CCI will set up an online **i**nteractive **m**odel **o**f **l**eaf **d**ecomposition (iMold) to provide educational outreach about decomposition for grades 5-12. The iMold virtual environment will include a web site which will consist of two distinct experiences: public and members-only community. The Public community will consist of interactive animations of decomposing leaf litter, GDM simulations, and learning components available both on the website and by a version designed for the SMART Board™. The interactive animations will include the ability to select from different leaf liter types of varying chemistry and then select the environmental conditions they will decompose within, including global change scenarios. Visitors will be able to visualize the progress of the decomposition of litter as a whole, or different individual litter chemical constituents, along a time line to see how time, litter type, and environment relate to how quickly or slowly something decomposes. The members-only community will give teachers and students the ability to network between researchers and classrooms at other schools, enabling them to share data, ask questions, and collaborate on experiments. A communication center will be created so that the researchers and classrooms can share ideas and results, as well as ask questions in a blog-like environment. Regularly scheduled video conferences will be given by project researchers directly to the schools. Students and teachers can ask questions during the presentation or experiment, and each presentation will be archived for review on the web site.

Results of Prior NSF Support

NSF Polar Programs to D. Moorhead. 1999-2006. $289,605 (subcontract). An Antarctic Dry Valley Long Term Ecological Research Site. NSF DPP-921173 (Lyons B., P. Doran, D. Freckman, A. Fountain, D. McKnight, D. Moorhead and J. Priscu). This project examined the lake, stream and soil ecosystems in the polar deserts of the McMurdo Dry Valleys, Antarctica. Moorhead's lab supported 2 PhD and 3 MS students, and generated 15 presentations, 2 manuscripts in preparation, and 14 publications.

NSF Polar Programs to J. Schimel ($514,926) and M. Weintraub ($30,979). 2007-2010. IPY: Microbial winter survival physiology: a driver on microbial community composition and carbon cycling. NSF ARC-0733074 (Schimel JP, Reardon KF, Wallenstein MD, and Weintraub MN). This project examines the effects of freezing on microbial community composition and C cycling in Arctic tundra soils in northern Alaska, USA, and Thule Greenland. We are now completing the initial phase of the research.

Response to reviews

This proposal is a resubmission. In the first version we proposed decomposition mesocosms in the lab rather than leaf litter bags in the field and did not include any microbial molecular genetic analyses. The major criticism of the proposal was that the laboratory mesocosms were too artificial, eliminating the contributions of broad groups, including mycorrhizal fungi. In response to reviews from the first submission, we revised the study to use *in situ* leaf litter bags in the field and expanded the scope of the research by adding molecular genetic analyses of microbial community composition and activity.

Some panelists felt our second submission was simply a case of "applying old ideas about community succession to a new group of organisms," and that our hypotheses were not novel. Our goal, however, is not the study of succession, per se, nor the simple correlative relationships between patterns in leaf litter chemistry and microbial communities that have already been determined. Instead, we propose to elucidate the mechanisms defining interactions between leaf litter chemistry, microbial community function and composition (which undergoes succession) during decomposition, quantifying for the first time how these interactions affect the trajectory of mass loss and organic matter stabilization during decomposition, and integrating this research with a conceptually new microbial guild-based model. We have tried to clarify this. While some hypotheses are grounded in ideas that go back to the pioneering work of Selman Waksman and others, only now does the technology exist to directly test these ideas.

Reviewers also noted that we did not consider the role of temperature and moisture as drivers of decomposition. Our goal, however, is not to determine the effects of these factors on decomposition, which many others have done, but rather to develop a mechanistic understanding of the interactions between leaf litter chemistry and microbial dynamics. However, there is a micro-meteorological station at our research site, and data on air and soil temperature, precipitation, and soil moisture are available to support our study. These data will enable us to include temperature and moisture drivers in our model and account for the environmental conditions our litter bags experience during decomposition.

The most recent panel was critical of our molecular methods for microbial community analysis. We had proposed using T-RFLP to determine changes in community composition, qPCR to determine relative abundances of bacteria and fungi, and ^{13}C stable isotope probing (SIP) to determine which taxa use different C substrates. The reviewers felt that T-RFLP was not desirable because it does not provide phylogenetic information, and that we were unlikely to be successful with either SIP or qPCR in these experiments. We have completely revamped our molecular approach and now propose using clone libraries to determine changes in bacterial and fungal community composition, and ^{13}C PLFA to determine relative abundances of bacteria and fungi and which bacterial groups use different substrates. Our use of ^{13}C vanillin as a lignin analog in the isotopic labeling experiments was also criticized. Fortunately, purified 97% ^{13}C labeled lignin is now commercially available, which we substitute for the vanillin.

In addition to the ^{13}C labeling experiments, previous versions of this proposal also included ^{15}N labeling to determine rates of N sequestered in reactions with lignin in the late stages of decomposition. This experiment was criticized as being ancillary to the objective and disconnected from our hypotheses and modeling. We agree, and have removed it. Additionally, we eliminated a C addition experiment, instead doubling the litter bag replicates from 4 to 8 to help overcome the variability inherent to litter bags.

Earlier panels also felt that a stronger broader impacts section was needed. We greatly expanded our education and outreach activities, and are now collaborating with the University of Toledo's Center for Creative Instruction (CCI) to create an online **i**nteractive **m**odel **o**f **l**eaf **d**ecomposition (iMold) to provide educational outreach about decomposition for grades 5-12. (see Broader Impacts).

INTRODUCTION

Somewhat surprisingly, fundamental questions remain about plant litter decomposition, one of the most basic terrestrial ecosystem processes and a key control on carbon (C) sequestration. While it has long been known that decomposition involves both chemical changes in leaf litter and a succession of microorganisms that consume the different chemical constituents (e.g. Tenney and Waksman 1929), until now we have lacked the techniques to elucidate the underlying biochemical interactions. **We propose an integrated field, laboratory, and modeling study of the biochemical mechanisms driving interactions between organic matter stabilization, leaf litter chemistry, microbial community composition and exoenzyme activities during decomposition.** Our central hypothesis, which dates back to the early research on decomposition by Waksman and others, (e.g. Waksman 1929; Waksman et al. 1928), and yet remains to be mechanistically tested, is that **during decomposition there is a predictable and quantifiable succession of microbial assemblages tied to leaf litter chemistry, and that changes in decomposition rates and litter chemistry in response to nutrient availability reflect changes in the composition and function of the microbial community.**

As leaf litter decomposition proceeds and the easiest to degrade chemical constituents are depleted by decomposers, mass loss rates decrease dramatically, from ~0.1% per day for fresh leaf litter to 0.00001% per day or lower for more decomposed litter (Berg 2000). Eventually mass loss rates become so slow that the leaf litter essentially stops decomposing and reaches the "limit of decomposition" (Berg 2000). This decrease in decomposability involves both chemical changes in the leaf litter and the succession of microorganisms that consume the different chemical constituents.

About 5% of the plant material that falls to the ground remains when the limit of decomposition is reached, entering the mineral soil as recalcitrant organic matter, and decomposing extremely slowly. It is this ~5% of leaf litter that becomes sequestered as part of the terrestrial C pool. However, the controls on the amount of C sequestered during decomposition are not well understood. In part, this is because the vast majority of decomposition studies have focused on the early stages of plant litter decay, seldom extending beyond the equivalent of 30-40% of initial mass remaining.

A critical feature of leaf litter decay is the interaction between cellulose and lignin, described by the lignocellulose index (LCI): LCI = lignin / [lignin + cellulose], which increases during decomposition. Aber et al. (1984) studied the chemistry of decomposing leaf litter from six tree species during a 2-year field study in Wisconsin and observed no net decrease in the absolute amount of lignin in decomposing leaf litter until LCI ~ 0.4, suggesting a consistent minimum value of LCI when lignin turnover can first be detected (Moorhead and Sinsabaugh 2006). In contrast to potentially rapid holocellulose decay at relatively low LCI values, there is little evidence for substantial lignin degradation when polysaccharides are abundant (Berg and McClaugherty 2008). A recent synthesis of data from detailed studies conducted in the USA and Europe (Berg et al. 1991, Berg and Johansson 1998) discovered that this pattern of lignin decay was conservative across sites and litter types, and was responsive to N fertilization (Herman et al. 2008). Finally, Melillo et al. (1982, 1989) found that LCI generally increases during decomposition to a constant value of 0.7, regardless of initial chemistry. This finding, however, is based on empirical observations with limited knowledge of the underlying mechanisms (Heal 1997, Berg and McClaugherty 2008). These studies leave questions unanswered that are central to understanding not only litter decay but also, more generally, the links between microbial communities and ecosystem function. For example, is the LCI threshold for lignin decomposition a function of microbial competition, facilitation or other processes related to litter chemistry-microbe interactions?

Atmospheric N deposition, elevated CO_2, climate shifts, and other environmental changes cause shifts in soil N availability and leaf litter quality and quantity that influence soil C storage by changing microbial community composition and activity during decomposition (Carreiro et al. 2000, Gallo et al. 2004, 2005, Finzi et al. 2006, Sinsabaugh et al. 2005). Several studies have shown that N availability can have a significant effect on C sequestration (e.g. Berg and Matzner 1997, Hobbie 2000, Berg and McClaugherty 2008, Waldrop et al. 2004, Pregitzer et al. 2004, Gallo et al. 2005). In general, higher N availability increases the decay rates of labile constituents, such as cellulose, and can accelerate the decomposition of leaf litter with low lignin content (Berg and Meentemeyer 2002). However, higher N also tends to retard lignin decomposition. These effects reflect interactions between N availability, microbial community composition, and exoenzyme activity (EEA). Activities of microbial exoenzymes associated with carbohydrate hydrolysis tend to increase in response to increased N availability, particularly for microbial communities associated with low lignin leaf litter. In contrast, activities of oxidative exoenzymes associated with the degradation of polyphenols such as lignin tend to decrease with added N (Carreiro et

al. 2000, Berg and Meentemeyer 2002, Saiya-Cork et al. 2002, Michel and Matzner 2003, DeForest et al. 2004, Gallo et al. 2004, Sinsabaugh et al. 2005); this effect is particularly pronounced for recalcitrant leaf litter. Some fungi are known to repress expression of lignolytic exoenzymes when labile N is present (Berg and Meentemeyer 2002). It is also likely that exogenous N availability alters selective pressures on community composition. A meta-analysis by Treseder (2004) suggests that high N availability reduces the diversity of both mycorrhizal and saprophytic fungi. High N availability may also promote C sequestration (Neff et al. 2002) through secondary reactions or greater incorporation of microbial biomass products into leaf litter residue (Berg and Meentemeyer 2002, Gallo et al. 2005). However, it is important to emphasize that these trends are generalities; detailed, mechanistic investigations of the links between leaf litter chemistry, N availability and the activity of the decomposer microbial community have yet to be conducted. While it is known that leaf litter chemistry, microbial community composition and exoenzyme activities change during decomposition, relationships among their dynamics are not understood. Further, while recent research has shown how long-term changes in enzyme activities associated with N enrichment can translate into changes in soil organic matter (SOM) chemistry (Grandy et al., 2008), a mechanistic explanation of these changes, in particular the role of microbial communities and their relationship to changing litter chemistry, remains elusive. We are currently unable to predict the magnitude or sometimes even the direction of microbial and SOM responses to N enrichment.

Most predictive models of decomposition are driven by initial litter chemistry and environmental conditions (Meentemeyer 1978, Heal et al. 1997, Gholz et al. 2000), rather than microbial dynamics, and fail to capture impacts arising from "bottom up" changes in microbial community composition or function (Carreiro et al. 2000, Gallo et al. 2004, 2005, Finzi et al. 2006, Sinsabaugh et al. 2005), that play a critical role in the stabilization of organic matter (Fog 1988, Berg and McClaugherty 2008). Building on efforts to refine decomposition models by including more ecological interactions between microorganisms and substrates (e.g. Sinsabaugh and Moorhead 1997, Jans-Hammermeister and McGill 1997, Schimel and Weintraub 2003), Moorhead and Sinsabaugh (2006) developed the Guild Decomposition Model (GDM), which resolves the decomposer community into three functional guilds, defined by different metabolic characteristics and exoenzyme activities (EEA), that are differentially associated with the decomposition of the three classes of leaf litter constituents (as defined by the most commonly used approach to litter chemical analysis): labile soluble compounds, holocellulose, and lignin. By combining observations about the physiology of key groups of decomposers and the biochemistry of different leaf litter constituents, this model explores the ramifications of microbial dynamics on decomposition. The GDM is the model that is most consistent with the generally accepted conceptual model of microbially mediated decomposition (e.g. Berg 2000). The GDM advances our ability to simulate the interaction between microbial behavior, leaf litter chemistry, and N availability as cellulose is depleted and decomposition transitions from being controlled by cellulose decay to lignin degradation, i.e., the threshold between leaf litter decay and stabilization. Many models include inhibitory effects of lignin on leaf litter decay (e.g., Melillo et al. 1982, Parton et al. 1987), but the GDM also describes how labile C and N availability can inhibit lignin degradation by influencing the expression of exoenzymes (e.g. Sinsabaugh et al. 2002, Berg and McClaugherty 2008). This interaction between leaf litter chemistry and microbial community behavior is sensitive to C and N availability because different groups of decomposers dominate different stages of leaf litter decay and respond differently to C and N availability (Fog 1988, Berg and McClaugherty 2008). GDM simulations have given rise to four inter-related, working hypotheses:

Hypothesis 1: Decomposition inextricably links microbial responses to- and effects on leaf litter chemistry, and so **the primary control on decomposition can shift between microbial activity and leaf litter chemistry.** Microbial activity should control decay rates when biomass is low and/or exoenzyme activity is low, such as early in the colonization of fresh leaf litter by microbes, when there is suppression in exoenzyme expression or activity, or with changes in community composition. In contrast, leaf litter chemistry should control decomposition when effective exoenzyme binding sites become limited or when inhibitory phenolic concentrations are high (see $\mathbf{H_2}$).

Hypothesis 2: While the lignin concentration of leaf litter influences the decomposition of more labile compounds, these same labile substrates in turn influence lignin decay. **Relative amounts of lignin and holocellulose define a threshold of chemical controls on decay.** High lignin concentrations directly inhibit holocellulose degradation by interfering with exoenzyme activity, largely by limiting the availability of effective exoenzyme binding sites. In contrast, high concentrations of holocellulose indirectly inhibit lignin degradation by influencing patterns of microbial substrate use and exoenzyme expression (see $\mathbf{H_1}$).

Hypothesis 3: High N availability generally enhances cellulose degradation, particularly in low lignin leaf litters, but inhibits lignin breakdown. However, cellulose degradation may still be inhibited by high lignin concentrations (see $\mathbf{H_2}$). We hypothesize that **increased N availability will alter the thresholds of chemical controls on litter decay, and that these changes will be controlled by initial litter chemistry.** In low lignin litter cellulose will disappear faster with elevated N, but the inhibition of lignin degrading organisms both by competition with cellulose decomposers and by excess N will be exacerbated. In high lignin litter the stimulation of cellulose breakdown by increased N will be minimal, but the inhibition of lignin degradation is still likely to be significant. The GDM predicts that regardless of litter quality, elevated N availability should increase each guild's rate of substrate depletion, as well as increase the mass of litter remaining at the limit of decomposition due to decreased lignin degradation.

Hypothesis 4: During decomposition, changes in exoenzyme activities track changes in microbial community composition which in turn correlate with changes in leaf litter chemistry (see $\mathbf{H_1}$ and $\mathbf{H_2}$). These patterns result from differences between groups of decomposers in metabolic and life history characteristics, and exoenzyme production, and result in differential access to chemical constituents of leaf litter to different decomposers. **The activities of specific exoenzymes define potential decay rates for specific leaf litter constituents and reflect the composition of the microbial community.** Interactions between resource use efficiency and exoenzyme activities define community structure and function in response to leaf litter quality, resulting from competition between microbial groups.

We propose to test these hypotheses with measurements of microbial community composition, the activities of specific exoenzymes involved in microbial C and N acquisition, leaf litter chemistry, and decay rates for leaf litters of contrasting initial chemical composition coupled with a manipulation of N availability. We will also add pulses of different ^{13}C-labeled substrates to sub-samples at different stages of decomposition and follow the incorporation of ^{13}C into the microbial community. These experiments will elucidate microbial community composition and behavior in relation to decomposition rates, leaf litter chemistry, and N availability. The data we will generate will explicitly test the assumptions and predictions of the GDM, which quantitatively define functional links between decomposer microorganisms, leaf litter chemistry and labile N. To our knowledge, the GDM represents the most detailed, mechanistic description of these relationships to date. The work described in this proposal will generate data that will enable us to empirically evaluate the structure of the GDM and test its predictions, thus also testing the shared assumptions of the general conceptual model of microbially mediated decay.

MODELING RATIONALE

Decomposition models usually estimate the maximum rate of substrate decay (V_{max}) based on either substrate availability ($k \cdot C$) or microbial activity ($r \cdot B$), where r and k are rate coefficients, B is microbial biomass, and C is substrate, but in truth, decomposition is limited by both factors. Moorhead and Sinsabaugh (2006) calculated the realized rate of decomposition based on microbial activity in the substrate-limited case: $dC/dt = (k \cdot C) \cdot B/(K_b+B)$; and based on substrate availability in the microbial activity-limited case: $dC/dt = (r \cdot B) \cdot C/(K_c+C)$; where, K_c and K_b are half-saturation coefficients. These equations are equivalent when decay is optimal, suggesting that decay is limited by substrate availability if $C < r \cdot B/k$ or by microbial activity if $B < r/(k \cdot C)$. This conceptual approach can be applied to specific chemical constituents of leaf litter, which have different intrinsic decay rate coefficients, and guilds of decomposers, which have different metabolic and exoenzyme characteristics. A useful feature of this approach is that all model parameters and state variables can be determined either by direct, empirical measures or by calculations based on direct measures (Moorhead and Sinsabaugh 2006).

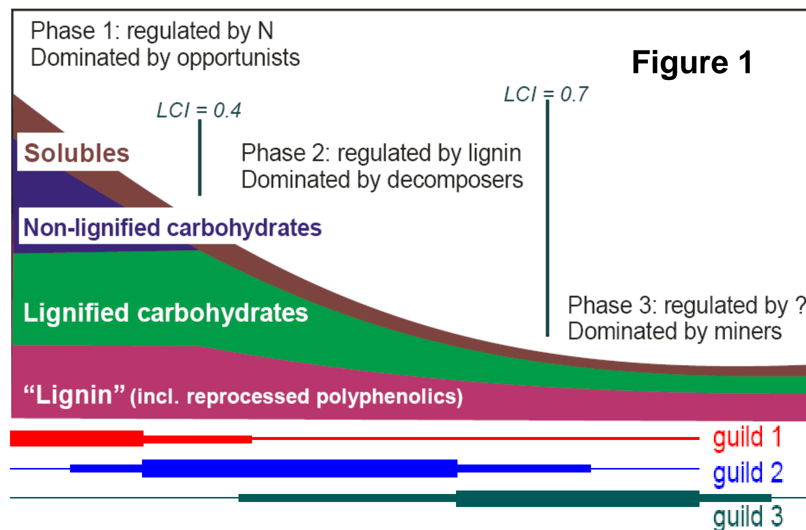
Moorhead and Sinsabaugh (2006) calculate the rate of cellulose decay with Michaelis-Menten reaction kinetics: $dC_2/dt = V_{max} \cdot C_2/(K_2+C_2)$ when LCI = 0 (i.e. no lignin); $dC_2/dt = 3/7 \cdot dC_3/dt$ when LCI = 0.7 and decomposition rates become extremely slow (see Melillo et al. 1982, 1989); and using an exponential function to scale K_2 (i.e., K_2') when $0<LCI\leq 0.7$, $K_2' = K_2 \cdot \exp[LCI \cdot (\ln(K_3+4/3 \cdot C_2)-\ln(K_2)]/0.7)$; where V_{max} and K_2 are maximum rate and half-saturation coefficients, respectively, C_2 is holocellulose and C_3 is lignin. The inhibition of lignin turnover by cellulose (Berg and McClaugherty 2008, Herman et al. 2008) is based on data from Aber et al. (1984) showing little evidence of lignin decay when LCI < 0.4: $dC_3/dt = -1 \cdot dC_2/dt \cdot (7.0E9 \cdot LCI-2.8E9)/(-5.6E9 + 7.0E9 \cdot LCI)$. These formulations are consistent with our current quantitative and qualitative knowledge of relationships between exoenzyme activities and substrate qualities, but have yet to be linked to specific, microbial activity.

Moorhead and Sinsabaugh (2006) define the decomposer community as three functional guilds that differ in their capacities to degrade the three most commonly defined classes of leaf litter constituents: soluble labile compounds, holocellulose and lignin. The presumption of three distinct guilds was based upon general patterns of microbial community succession in decomposing litter and differences in metabolic and enzymic capabilities of decomposer microorganisms, although available data are more suggestive than definitive. Nonetheless, this approach is consistent with the prevailing conceptual model of leaf litter decay as a collective process in which different groups of microorganisms degrade different constituents of leaf litter at progressive stages of decomposition (e.g. Neeley et al. 1991, Gessner and Chauvet 1994, Findlay et al. 2002, Sinsabaugh et al. 2002):

Guild 1: Opportunists rapidly colonize fresh leaf litter, consuming intermediate metabolites and soluble polymers. Organisms with fast growth rates and limited degradative capacities dominate early stages of decomposition, but slower-growing organisms with broader degradative capabilities displace them as labile substrates dwindle. These organisms are likely bacteria (perhaps dominantly β-proteobacteria and Bacteroidetes; Fierer et al. 2007) and fast growing fungi (such as the “sugar fungi” in the zygomycota).

Guild 2: Decomposers degrade cellulose and lignocellulose, which comprise the bulk of plant litter, by producing a variety of exoenzymes. They grow slower than Opportunists but access a broader range of substrates. Because plant cell walls contain little N and P, this guild is efficient at acquiring nutrients. This group likely includes actinomycetes in the bacteria and fungi spanning zygo-, asco-, and basidio-mycota.

Guild 3: Miners degrade recalcitrant organic matter with powerful oxidative exoenzymes that break the covalent bonds of aromatic rings and hydrocarbon chains, and thus gain access to protected glycosides, peptides and lipids. These exoenzymes offer a low yield of C and nutrients relative to their production costs, so these organisms grow very slowly. Their competitive advantages over other guilds are their capacities to metabolize reactive phenols, which inhibit many other organisms, and subsist on recalcitrant substrates. In litter decay, these organisms are thought to be dominantly fungi in the basidiomycota.



Microbial control of decomposition within the GDM emerges from combinations of values for V_{max} and K assigned to different guilds for the degradation of different leaf litter constituents. General knowledge of the physiology, growth, and enzymology of different groups of microorganisms associated with different stages of leaf litter decay define roughly different characteristics of the guilds used in GDM, i.e., V_{max} is a function of growth rate and thus is greatest for Opportunists and lowest for Miners. The half-saturation coefficients for substrate degradation are specific to both substrate and guild, i.e., all guilds have high affinity (= low K_s values) for labile substrates, only Decomposers and Miners have relatively high affinity for cellulose, and only Miners have relatively high affinity for lignin. Thus all guilds have access to all chemical constituents of litter and generally coexist throughout much or all of the decomposition process. However, differences in substrate affinities and growth rates favor different guilds at different stages of litter decay. Competition between guilds results from the relative activity of each guild, scaled to the degradative potential of each guild for each substrate and drives changes in the relative contribution of each guild to the total community biomass (i.e., community structure). The GDM makes no assumptions about decomposer taxonomy and assumes that all guilds have access to all substrates (i.e., they are functionally similar), but differ with respect to the rates at which they may utilize (hence degrade) different substrates. If we include contributions from microbial turnover (which the GDM can either account for or ignore), then all three guilds are likely to persist throughout any decomposition simulation.

The stimulatory effect of N on early stages of decay is determined by comparing C and N availability to the C:N ratio of microbes, defining a stoichiometric relationship between litter decay and microbial growth (Moorhead and Sinsabaugh 2000). Conversely, N inhibition is simulated in late stages of decay by retarding lignin degradation proportional to the amount of N exceeding microbial demand.

This simple model generates behavior consistent with observed patterns of community succession, changes in leaf litter chemistry, and exoenzyme activity. It allows us to view the microbial community in guilds that are defined by a variety of coupled growth-strategy characteristics (growth rate, substrate use, etc.), rather than by the presence of individual genes. Opportunists first dominate the community, followed by Decomposers and then Miners. Substrate turnover rates peak first for labile compounds, next for cellulose, and finally for lignin. Added N stimulates turnover of soluble and cellulose fractions of leaf litter but retards lignin decay. However, the timing and amount of available N has differential effects on organic matter stabilization due to the interactions between lignin and cellulose and how they regulate the three microbial guilds contributions to overall decay. As far as we know, no data set exists to systematically test these predictions. This project will generate the data to empirically evaluate the structure of the GDM and test its predictions, enabling us to comprehensively test the assumptions of the general conceptual model of microbially mediated decay. It will also provide detailed information on the coupled dynamics of litter chemistry and microbial community dynamics that will allow us to develop the next generation of both intellectual and mathematical models of litter decay that go beyond the simplistic formulation in GDM to understand litter decay at a more refined chemical and organismal level.

OBJECTIVES

We propose an integrated experimental and modeling study to examine specific interactions between changes in leaf litter chemistry, exoenzyme activities and community dynamics of decomposers in the absence and presence of exogenous N inputs. We will test the above hypotheses by meeting the following objectives:

1. Determine the interactions between leaf litter chemistry, N content, microbial community composition and exoenzyme activities, and mass loss of contrasting leaf litter types during decomposition; do metabolically-defined guilds correspond with distinct communities and chemistry, and if so, do guilds overlap taxonomically and/or temporally?
2. Determine how N availability affects these interactions during leaf litter decay.
3. Determine how the availability of N and different C forms affect interactions between microbial community composition and litter chemistry using microbial community analysis and ^{13}C tracers.
4. Empirically evaluate the structure of the GDM, testing its predictions and assumptions to advance our conceptual model of microbially mediated decay, and making it more mechanistic and quantitative.

Hypothesis 1 states that when microbial EEA is low, it is the rate-limiting factor in decomposition; otherwise leaf litter chemistry controls the decomposition rate. We will determine how microbial responses to leaf litter chemistry control the trajectory of leaf litter decay by simultaneously tracking changes in litter chemistry, microbial community composition, and the activities of exoenzymes involved in degrading specific compounds throughout the decomposition of contrasting leaf litter types. Comparing activities of microbial guilds with decay rates and pool sizes of leaf litter constituents, across leaf litter types, will determine the controlling factors at different stages of decomposition. While it is known that litter chemistry, EEA, and microbial community composition change through the course of decomposition, the relationships between their dynamics are not understood nor have they been rigorously quantified.

Hypothesis 2 states that relative concentrations of holocellulose and lignin define thresholds of alternative chemical controls on leaf litter decay. We will test this by determining leaf litter cellulose and lignin contents, the activities of cellulose- and lignin-degrading exoenzymes, and the composition of the microbial community at regular intervals during decomposition of a variety of leaf litter types with different chemistries. We will also conduct detailed analyses of leaf litter chemistry to gain compound specific insights into chemistry and its relationship to microbial exoenzymes and communities at different stages of decomposition. Hypothesis 3 states that the thresholds of chemical controls on litter decay are altered by increased N availability due to differential responses of microbial cellulose and lignin breakdown (as well as possible changes in the decomposability of lignin) to N. In a subset of samples we will increase N availability during decomposition to determine how changes in N availability affect microbial community composition and exoenzyme activity, and thereby leaf litter mass loss, LCI, N content, and stabilization.

According to Hypothesis 4, as leaf litter chemistry changes during decomposition there is a succession of microbial guilds based on their relative growth rates on different substrates; changes in microbial community composition and substrate consumption ultimately determine the proportion of leaf litter that will be stabilized. We will test this by monitoring changes in leaf litter chemistry, microbial community composition, and exoenzyme activity throughout the course of decomposition. We will use relatively simple, robust, and targeted molecular microbial analyses to determine the extent to which guilds overlap taxonomically and/or temporally, and which groups of organisms are associated with each guild. We will also label leaf litter subsamples with different ^{13}C compounds representative of the primary leaf litter chemical constituents to determine the community composition of active microorganisms capable of metabolizing specific products of decomposition. These amendments will be added at key points during decomposition, as defined by changes in exoenzyme activity (e.g., oxidative:hydrolytic ratios) and leaf litter chemistry (e.g., LCI), when different microbial guilds should dominate the community.

RESEARCH METHODS

Our field site is the Oak Openings community, an oak (*Quercus spp.*) dominated forest in NW Ohio (N 41°33', W 83°50'), consisting of a mosaic of oak savanna, oak woodland, and wet prairie occurring on a series of post-glacial beach ridges and swales (Moseley 1928, Brewer and Vankat 2004). We will work within the 15 km^2 Oak Openings Preserve managed by the Toledo Area Metroparks, where we are already conducting research. The forest at Oak Openings is dominated by *Q. rubra* (red oak; 28%), *Q. alba* (white oak; 26%), *Q. velutina* (black oak; 10%) with an *Acer rubra* (red maple; 18%) component in the low lying mesic areas. The soils are sandy, and are usually mixed, mesic Typic Udipsamments, with sandy, mixed mesic Typic Endoaquolls occurring in wetter areas. Since these soils occur over fine-textured glacial till, a perched water table (~2 m) is prevalent in the area and the soil is typically mesic, even when precipitation is low. Mean annual precipitation is 970 mm and mean annual temperature is 10.4°C. The growing season lasts an average of 167 days from mid May - September.

<u>Objective 1:</u> Determine the interactions between chemistry, N content, microbial community composition and exoenzyme activity during the decomposition of contrasting leaf litter types. Initial LCI and N content determine the trajectory of leaf litter decomposition and microbial exoenzyme production through their effects on substrate availability to the decomposer community. As relative lignin concentrations increase during decomposition, lignin begins to have a retarding effect on leaf litter breakdown, and overall mass loss rates eventually become tied to lignin degradation rates (Berg 2000). Available N can also interact with lignin in the later stages of decomposition to slow mass loss rates. Initially, growth of the microorganisms that degrade cellulose and other relatively labile C rich compounds in leaf litter is limited by a lack of available N. As decomposition proceeds, leaf litter C:N ratio declines, relative N availability increases and begins to interfere with lignin degradation by suppressing the microbial production of lignolytic exoenzymes, altering community composition, and reacting with the lignin breakdown products to form recalcitrant complexes (Berg 2000). **We propose to resolve the interactions between leaf litter chemistry and the composition and function of the microbial community over the course of decomposition, and determine how these interactions affect organic matter stabilization.** This will be accomplished by monitoring changes in leaf litter chemical composition, N content, microbial community composition, and the activities of the exoenzymes that degrade both cellulose and lignin throughout the decomposition of contrasting leaf litter types, and determining how mass loss rates respond to these patterns.

We will set up a field decomposition leaf litter bag experiment with 4 types of litter (dogwood, sugar maple, white oak, and red pine) with varying initial lignin, cellulose, and N contents (Sinsabaugh et al. 2002b). These leaf litter types have been widely used in decomposition studies, allowing us to place our measurements in a broader context. We will run the study until the residual chemistry has stabilized (empirically determined to be LCI ~ 0.7), or by the middle of the 3rd year of the experiment, whichever is first. Even if the LCI doesn't reach 0.7 before the final harvest, we will still be able to observe the shift in decomposition pattern at LCI = 0.4, and the more gradual shift as litter begins to stabilize as LCI approaches 0.6 (Moorhead and Sinsabaugh 2006).

Litterbags will be created using window screen with ~2 mm mesh, and will be made to contain a relatively large amount of leaf litter (30-40 cm), yet lay flat on the ground. We acknowledge the potential for particle loss with this experimental design, but it is unlikely to alter remaining leaf litter chemistry or microbial community composition. We considered using a finer mesh to prevent loss; however this would

create water-retention problems and make the results less comparable to other studies, which generally use ~2 mm mesh. We will use a high number of replicates (8) to reduce error and improve statistical power. We will also subject the mass loss data to statistical outlier analysis, and will remove outliers.

Freshly senesced leaf litter will be collected in the fall of 2009 from the Oak Openings area near Toledo, Ohio, where all four species grow. The leaf litter will be sealed into nylon mesh bags and placed on the ground in eight replicate subplots the forest where it was collected. Samples will be harvested monthly, and we anticipate the incubations will continue through the second year after litter bags are deployed. The ground is rarely covered with snow in winter, allowing year-round access to the forest floor to maintain the experiments. We will monitor mass loss, N content, chemical composition, microbial community composition, and the activities of microbial exoenzymes associated with organic matter degradation over the course of decomposition.

Cellulose and lignin content in the leaf litter sub-samples will be determined by a series of extractions following the methodology of Melillo et al. (1989). Methylene chloride is first used to extract the non-polar soluble compounds from leaf litter, which is then extracted with hot water to remove the polar extractables. The water insoluble residue will then be digested with H_2SO_4 (Effland 1977) and the resulting solution will be analyzed for acid soluble carbohydrates using the phenol sulfuric acid assay for D-glucose (Dubois et al. 1956). The acid soluble fraction is considered to be holocellulose. The acid insoluble fraction is assumed to be lignin. Leaf litter C and N content will be conducted by the Schimel Lab using their Fisons CN analyzer (Fisons Instruments, Milan Italy). We will measure microbial biomass C and N using the chloroform fumigation-extraction technique (Horwath and Paul 1994). DOC and DON in these extracts will be analyzed using the Shimadzu TOC-V total organic C and total N analyzer (Shimadzu Scientific Instruments, Inc., Columbia MD) in Weintraub's lab at UT.

We will examine the chemical structure of litter using pyrolysis-gas chromatography/mass spectroscopy (Py-GC/MS) using previously described methods (Grandy et al., 2007; Grandy et al., 2008). We will analyze litter chemistry at 3 sampling dates (initial and at LCI ~ 0.3 and LCI > 0.4) to gain compound specific insights into chemistry and its relationship to microbial exoenzymes and communities at different stages of decomposition. Recent studies indicate that pyrolysis products can be linked to enzymes and soil communities (Table 1; Grandy et al., 2007; 2008), suggesting that combining Py-GC/MS with traditional chemical methods will provide new analytical insights into decomposition models. These analyses will also enable us to test the assumption that the acid insoluble litter fraction is purely lignin; as decomposition proceeds, this fraction may also contain an increasing proportion of microbial products, which would not be revealed by the traditional litter chemical analyses described above.

Soil C Compound	class	lap	bg	ph	per	f/b
Acetovanillone	lignin	–	ns	ns	ns	+
Phenol, 2-methoxy- (guaiacol)	lignin	–	–	ns	ns	+
2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	lignin	ns	ns	ns	ns	+
Pyridine	N	ns	+	+	ns	–
Pyrrole	N	ns	+	+	+	–

Table 1. Relationship between soil organic matter components determined using Py-GC/MS, EEA, and fungal/bacterial ratios in the Calhoun Experimental Forest. Sign indicates significant positive correlation (+), significant negative correlation (-), or no correlation (ns). Clear, predictable relationships are shown between lignin derivates, N compounds and biological components in the soil. Enzymes: lap (L-leucine aminopeptidase), bg (b-1,4-glucosidase), ph (phosphatase), per (peroxidase); f/b (fungal/baterial ratios) Grandy et al., in review

Samples will be pulse-pyrolyzed (CDS Analytical Pyroprobe Model 5150, CDS Analytical, Oxford, PA) with pyrolysis products transferred online to a gas chromatograph (ThermoQuest Trace GC, Thermo Finnigan, San Jose, CA) and mass spectrometer (Polaris Q, Thermo Finnigan, San Jose, CA). In addition to standard sample analysis we will also consider thermochemolysis in the presence of TMAH (tetramethylammonium hydroxide), which can help resolve lignin derivatives and lipids (e.g. Saiz-Jimenez et al., 1994; Filley et al., 2002). Peaks corresponding to pyrolysis products will be compared to reference spectra after deconvolution and extraction using AMDIS v 2.64 and National Institute of Standards and Technology mass spectral libraries and the literature. The Grandy lab has a custom soil compounds library, initially developed in J.C. Neff's lab at the University of Colorado, that contains >700 pyrolysis GC/MS products present in litter and soils. This library is coupled with a new Matlab program that uses a series of selection criteria to find the compound that best matches each peak. Data are stored in MS Access. These steps allow us to process samples efficiently.

We anticipate analyzing 140 Py-GC/MS samples, total. At each of the 3 sampling points we will run 5 replicates of each litter type from the ambient and elevated N treatments (=120 samples). We will run an additional 20 samples throughout the experiment to monitor litter decay to help us determine the optimum times to measure microbial communities and litter chemistry in all of the samples.

We will profile exoenzyme activities in decaying leaf litter using high-throughput protocols that we have developed (e.g. Saiya-Cork et al. 2002, Gallo et al. 2004, Sinsabaugh et al. 2003, Weintraub et al. 2007). These allow us to rapidly measure many samples for the potential activities of the major classes of exoenzymes that degrade organic matter. Preliminary evidence suggests that exoenzyme activities determined using these methods correlate strongly with chemical compounds in litter determined using Py-GC/MS (Table 1; Grandy et al., 2007; Grandy et al., 2008). We will assay for phenol oxidase, peroxidase, cellobiohydrolase, β-glucosidase, β-xylosidase, β-N-acetylglucosaminidase, leucine aminopeptidase, β-glucosidase, and phosphatase, using fluorigenic methylumbelliferyl-linked substrates for the hydrolytic exoenzymes and colorigenic L-3,4-dihdroxyphenylalanine (L-DOPA) for the oxidative exoenzymes. We will also estimate rates of exoenzyme turnover, which are implicit in hypothesis 4, in each stage of decomposition by assaying EEA over a time course (to be determined experimentally) on litter continuously fumigated with chloroform to inhibit microbial growth and enzyme production (Asao and Schimel, *in prep.*). The decline in EEA will provide an estimate of enzyme turnover times.

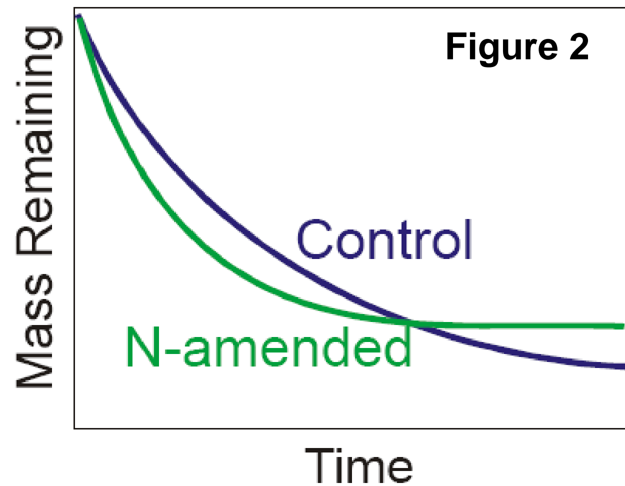
Microbial community composition will be assayed using an intensive cloning and sequencing approach. Sub-samples of leaf litter from each harvest date will be subjected to particle filtration to remove dormant fungal spores and other loosely-adhering organisms (Schmit and Lodge 2005). Samples from all litter harvests will be stored at -80°C for analysis. Litter chemistry and EEA will be used to retrospectively choose three sampling times which we expect to be dominated, on average, by the different microbial guilds. Analyzing four litter types will also provide substantial additional variability in litter chemistry and exoenzyme activity, allowing us to conduct a rigorous test of the hypothesis that there are strong correlations among microbial community composition, EEA, and litter biochemistry.

Microbial DNA will be extracted from three replicate litterbags of each litter type and N treatment using a commercial kit designed for soil. We recently completed an optimization study of this kit in which we showed that DNA yield is maximized by repeated extractions involving freezing, heating, and bead-beating. Following this procedure, fungal and bacterial community profiles are not affected by extraction bias (Feinstein and Blackwood, in review). Standard PCR primers will be used to amplify a phylogenetically-informative portion of the 16S ribosomal gene for bacteria (primers Eub338F and 1392R; e.g. Blackwood and Buyer 2007) and the internal transcribed spacer (ITS) region for fungi (primers NSI1F and NLB4R; Martin and Rygiewicz 2005). *E. coli* clone libraries will be generated with standard methods. We plan to isolate approximately 384 clones per litterbag sample, each of which will be sequenced and archived as a glycerol stock at -80°C. An ARB database (Ludwig et al. 2004) will be constructed for alignment and pairwise distance calculation of fungal genes. Cluster analysis will be performed to define operational taxonomic units (OTUs) approximately equivalent to genus. OTUs will be identified using BLAST searches in GenBank and phylogenetic analysis with reference sequences (e.g., following O'Brien et al. 2005; Lynch and Thorn 2006; Waldrop et al. 2006). Bacterial 16S ribosomal sequences will be analyzed (checked for chimeras, aligned, pairwise distance calculations performed, and classified) using the tools at the RDP and GreenGenes websites (DeSantis et al. 2006, Cole et al. 2007).

Using the approach described above, sequences will be classified into both OTUs and higher-level taxonomic groups (e.g. bacterial phyla and fungal orders; Garrity et al. 2007, Hibbett et al. 2007), including groups characterized only by environmental sequences (e.g., Porter et al. 2008). Sequences can be aggregated to different levels of phylogenetic resolution by adjusting the sequence similarity cutoff used to define OTUs, or by use of different levels in the taxonomic hierarchy after classification. This will allow us to identify dominant members of the community and test our hypotheses using complementary datasets at both fine and broad levels of taxonomic resolution. Making use of clone libraries from replicate field samples, we will use redundancy analysis (RDA), a multivariate variance partitioning technique, to determine the amount of variability in community composition that can be attributed to substrate type and sampling time (Legendre and Anderson 1999, Peres-Neto et al. 2006). Monte Carlo randomization procedures are used in conjunction with RDA to assess significance of these treatments. The correlation between community composition, exoenzyme activity, and litter chemistry will be determined by RDA and Mantel tests. Rarefaction curves will be constructed to assess the completeness of sequence-based surveys and compare diversity of communities (Hughes and Bohannon 2004).

In summary, these analyses of leaf litter chemistry, exoenzyme activities and microbial community composition will provide the simultaneous evaluations of the functionally linked elements of decomposition necessary to test the prevailing conceptual model of microbially mediated leaf litter decay.

Objective 2: Determine how N availability affects interactions between mass loss, LCI, N content, organic matter stabilization, and microbial community composition and exoenzyme activity. N availability, including the effects of added labile N (e.g., N deposition), can have a significant but variable impact on C sequestration during decomposition. Higher N availability can accelerate the decay of cellulose (Berg and Meentemeyer 2002), but retard the decomposition of lignin (Berg and McClaugherty 2008). These effects result from differing responses to increased N by the different groups of microorganisms that degrade these different compounds (Fog 1988). Thus, it is difficult to predict if the decomposition of a particular type of leaf litter will increase or decrease with increased N availability (Waldrop et al. 2004). Previous work suggests the pattern shown in Fig. 2—increasing N should increase initial mass loss, increasing LCI more quickly, but retarding lignin decay in the later phases and increasing the amount of material stabilized. However, this remains a poorly tested hypothesis and the mechanisms remain surprisingly unclear. Finally, does initial leaf litter N content still play an important role in determining leaf litter mass loss rates when exogenous N availability is high? **We will address these questions by duplicating the experiments in Objective 1 with supplemental additions of NH_4NO_3.**



This experiment will run identically to the experiment outlined in Objective 1, but with added N. We will repeat mass loss, litter chemistry, exoenzyme activity, and community sequence analyses on N-amended samples. N application rates will be set at 3 times the ambient N deposition rate for the study region (~9 kg-N ha^{-1} y^{-1}; Waldrop et al. 2004). To ensure even distribution, we will apply the NH_4NO_3 solution to eight replicate 10x10 m subplots monthly using a backpack sprayer. To account for associated changes in soil moisture, we will apply an equivalent amount of N free water to the subplots not receiving N additions.

In brief, these experiments will enable us to test the sensitivity of microbial community structure and function (exoenzyme profiles), and leaf litter decay (changing chemistry) to changes in N availability.

Objective 3: Determine how the availability of C from different substrates affects microbial community composition, uptake rates, and assimilation efficiencies at different stages of decomposition using microbial community analysis and ^{13}C tracers. We propose to add a ^{13}C labeling component to the above experiments to determine the community composition of *active* microorganisms capable of metabolizing specific leaf litter substrates. Our model has three guilds (Opportunists, Decomposers, and Miners) that do not necessarily match specific phylogenetic groups that can be identified a priori. In addition to the sequencing approach to identifying microbial guilds described above, **we will use ^{13}C-phospholipid fatty acid (PLFA) analysis to monitor the activities of particular functional groups.** This will be done by amending samples with ^{13}C substrates characteristic of the substrates each guild is defined by, and monitoring their uptake into different PLFAs. We will add phylogenetic resolution to the PLFA work by coupling it with our clone library analyses described above.

The approaches that might first come to mind to address this challenge are stable isotope probing (SIP; adding ^{13}C substrates and isolating the "heavy" DNA or RNA from organisms that assimilated the label), or analyzing functional genes. However, neither of these is suitable for this project. SIP requires substantial DNA enrichment (> 10%), which means adding a lot of labeled substrate and having a lot of microbial growth on it. That is not possible with complex natural substrates such as cellulose and lignin and so is inapplicable in this context. Functional genes can be analyzed using specific primers for known genes, but connecting those to the biomass/population size of the organisms that produced them requires a functional metagenomics approach requiring a massive effort and so is equally unsuitable.

Our approaches for evaluating membership in functional guilds will be twofold—for bacteria we will use ^{13}C-phospholipid fatty acid (PLFA) analysis and for fungi, we will use a correlative approach, evaluating changes in community composition relative to changes in litter chemistry and processes.

For overall microbial use, and for identifying active groups of bacteria, ^{13}C-PLFA is powerful and directly targeted at the appropriate level of resolution for this project. It allows us to add tracer levels of ecologically relevant substrates and measure the uptake and enrichment of specific PLFAs that are biomarkers for particular groups of bacteria — e.g., gram negatives, gram positives, actinomycetes (as a subset of gram positive). These groups behave differently and appear to act as "functional groups" at a large scale; e.g. in Arctic shrub tundra gram positive bacteria are protein specialists (McMahon et al., in prep.). Thus, for bacteria, this technique directly gives activity information at a reasonable scale to match to the model. We will analyze ^{13}C PLFAs according to procedures in use in the Schimel lab—this a modified Bligh-Dyer method (White and Ringelberg, 1998) and is described in (McMahon et al., 2005).

^{13}C-PLFAs do not adequately identify active groups of fungi, however. While PLFAs can provide a breakdown between bacteria and fungi, they do not distinguish among the fungi, and it is likely that members of zygomycota, ascomycota, and basidiomycota fall into different guilds. There is no available tracer-based method that will allow us to identify which fungal taxa are using which substrates. Therefore we will use a correlative approach using the data from the fungal clone-libraries. Sequence information can be aggregated to the level of genera and above to provide quantitative information at the group level, which is appropriate for identifying the phylogenetic composition of functional guilds (Wallenstein et al. 2007). By analyzing communities at time points where there are clear changes in system behavior, we can evaluate quantitatively how the different groups change with changing chemistry and process.

Thus, by combining ^{13}C tracer approaches and quantitative clone library analysis, we will be able to identify the organisms that comprise the specific crude guilds of our existing model, and we lay the groundwork to refine the model in the future, by narrowing down and refining our understanding of the organisms that are present and active at different stages in decomposition.

For the labeling we will separately add ^{13}C to subsets of the control and +N treatments in short-term (~1 week; exact length to be determined in preliminary tests) lab incubations of leaf litter in different stages of decomposition harvested from litter bags in the field (at field temperature and moisture). For the ^{13}C substrates we will use >97% enriched glucose/acetate, cellulose, and lignin (^{13}C cellulose and lignin are available from Isolife Inc.), substrates characteristic of Opportunists, Decomposers, and Miners, respectively. These ^{13}C substrates will be added in separate treatments at each of 3 timepoints. We will first label shortly into the experiments, when Opportunists are most active, next during the mid-point of decomposition (LCI < 0.4), when Decomposers predominate, and again late in decomposition (as LCI approaches 0.7), when Miners are most active. We will use data from the exoenzyme assays and chemical analyses to help determine the optimum timing of the last two label additions for the four litter types. Labeling at the final time-point will help to elucidate C turnover in the late stages of decomposition. Labeling at the earlier timepoints will provide much needed data on the microbial assimilation efficiencies of different substrates. The GDM assumes that assimilation efficiency is substrate-specific and that uptake rate is guild-specific, and has values for all 3 guilds and substrates in each stage of litter decomposition. This experiment will enable us to test these assumptions and more accurately parameterize the model. We will follow the fates of the different ^{13}C substrates into respired CO_2 and microbial biomass to determine how the composition and C assimilation efficiency of the active microbial community changes as decomposition proceeds and in response to increases in N. We will also analyze the whole leaf litter, and the acid insoluble and extracted leaf litter fractions for ^{13}C to determine if any label is incorporated into these pools. We will conduct preliminary labeling experiments to optimize the tracer additions and label recovery in order to balance the tradeoffs between adding C in ecologically meaningful quantities and adding enough label to trace into the various pools. This will also enable us to optimize the incubation times with the labels to prevent the uniform distribution of added ^{13}C among the common PLFAs as a result of cross-feeding.

<u>**Modeling Objectives:**</u> **Testing the assumptions and predictions of the guild decomposition model (GDM) against experimental results:** The experiments outlined above will enable us to explicitly test the assumptions and predictions of the GDM, which in turn explicitly defines functional links between decomposer microorganisms, leaf litter chemistry and labile N. The GDM represents a "working hypothesis" of shifting controls on the decomposition of leaf litter, based on our current state of knowledge regarding these relationships. To our knowledge, the GDM represents a novel, mechanistic

link between biological, chemical and environmental controls on decomposition, not previously examined in such an integrated fashion. While soil animals such as earthworms (which are excluded from the litterbags) may also impact leaf litter decomposition, the focus of the GDM and of the proposed research is to elucidate the interactions between litter chemistry and microbial community function and composition during decomposition; determining the effects of soil animals on decomposition is outside of our scope.

Objective 1: The GDM predicts that initial leaf litter quality determines mass loss rates and changes in leaf litter chemistry, comparable to many decomposition models (e.g. Melillo et al. 1982, Parton et al. 1987, Moorhead et al. 1999). Decomposition rate is higher for litters with more N and less lignin, and LCI increases during decay to a stable value of ~0.7. The GDM differs from most other models for the interaction between lignin and holocellulose; in the GDM, lignin decay is inhibited when LCI < 0.4. The GDM also predicts that leaf litter quality determines the functional behavior of the microbial community, resulting from- and further affecting litter chemistry. This includes the timing, magnitude, and duration of activities associated with early, middle and late-succession communities. Experiments in Objective 1 will provide the concurrent measures of leaf litter chemistry, N content, microbial community composition and exoenzyme activity, necessary to test the functional links between litter chemistry and microbial activity proposed by GDM. For example, simulated rates of turnover in specific litter pools can be interpreted as indices of specific extracellular enzyme activities (Moorhead and Sinsabaugh 2000).

The GDM resolves microbes into three guilds defined by metabolic characteristics and exoenzyme activities. As leaf litter chemistry changes during decomposition the model hypothesizes a succession of dominant decomposer guilds based on their growth rates on different substrates. Determining this pattern of microbial succession is critical to predicting the generality of responses to changes in the availabilities of C and N, and to estimate the degree of cellular turnover during transitions between guilds. The scenario that we have incorporated into the GDM is a predictable succession of co-adapted assemblages of taxa, with the biomass of one guild largely replacing the previous one according to a trajectory that mirrors the succession in exoenzyme activity and leaf litter chemistry.

Data do not exist that would allow us to evaluate how closely this “deterministic hypothesis” of succession of microbial assemblages resembles reality. We will use multivariate canonical ordination techniques to quantify the effects of time and leaf litter chemistry on microbial community and EEA profiles (Legendre and Anderson 1999). Quantitative comparison of the ordinations and group dispersions for metabolic (EEA), taxonomic (sequence and PLFA), and chemical (Py-GC/MS) assays will allow us to test the above hypotheses concerning patterns of succession and chemistry (Fukami et al. 2005, Anderson et al. 2006). This research will determine the links between the composition and function of the microbial community and leaf litter chemistry at different stages of decomposition, enabling us to critically evaluate the three guild structure of microbial succession in the GDM and to empirically test its predictions. While the three guild model is a simplistic representation of reality, our goal is to refine our understanding of the hypothesized three guild idea so we can start to expand it, moving toward more realistic structures, which requires more sophisticated information about chemistry and microbial dynamics that we can start to tie together with existing data.

Objective 2: The GDM predicts that exogenous N will accelerate the decomposition of soluble and cellulose fractions of leaf litter and the growth of decomposers, and alter patterns of exoenzyme activities, i.e., enhance cellulose but decrease lignin degrading exoenzyme activity in the early stages of decomposition. However, these effects change when LCI exceeds 0.4, the point up to which the model predicts that holocellulose degradation retards the establishment of Miners. When LCI > 0.4 excess N reduces oxidative exoenzyme activity, retards lignin degradation and thus indirectly retards the lignin-shielded portion of cellulose decay, reduces late-stage leaf litter mass loss rates, and increases values of LCI at equivalent mass loss, compared to controls. The experiments in Objective 2 will determine if the GDM accurately predicts these shifting effects of increased N on early versus late stages of decomposition (i.e., LCI < 0.4 vs. LCI > 0.4). Indeed, Herman et al. (2008) recently discovered that N availability had a significant effect on this LCI threshold in previously reported field studies of litter decomposition, increasing it with increasing N.

Temporal patterns in the responses to the N additions should provide insight to the sensitivity of decomposition and the composition of the decomposer community to the timing of the addition. For example, N additions should accelerate early stages of decay when LCI < 0.4 and promote the growth of Decomposers, but have an inhibitory effect on Miners (when LCI ≥ 0.4). N addition should also increase activities of exoenzymes associated with carbohydrate degradation, but decrease oxidative exoenzyme activity. However, few data are available to identify the timing, magnitude and variation in these shifts, or

if they are tightly coupled. This work will provide the observations of N impacts on these aspects of decomposition necessary to link specific microbial activities to decomposition, verifying or refuting relationships proposed in the GDM, and thus likely providing insights to mechanisms underlying the conflicting reports of the impacts of N additions on litter decay.

Objective 3: These experiments will enable us to quantify the uptake rates and assimilation efficiencies of C substrates associated with different stages of decomposition for specific groups of microorganisms, and determine how much biomass each group contributes to the whole. By partitioning the active microbial community among functionally defined guilds we can then estimate C uptake rates, C assimilation efficiencies, and biomass of the different guilds. Assimilation can be estimated from the fraction of total label incorporated by microbial biomass; uptake (i.e., direct measures of *r*) can be derived from the rate of label incorporation into microbial biomass over time. Once we quantify how much label goes into each guild, and how much biomass is contributed by each guild, we can then calculate uptake rates by different guilds. Determining the fates of C sources of different qualities will test model parameters for uptake (*r*, assumed to be guild specific) and assimilation coefficients (ε; assumed to be substrate specific); at present, the GDM uses "generic" values for *r* derived from ranges reported in the literature, and arbitrarily "slides" them up or down in a relative sense for the different guilds. Similarly, the GDM uses generic literature values for maximum assimilation efficiency for specific substrates. Even small variations in ε can have large cumulative impacts on the amounts of stabilized organic matter.

The GDM predicts that added N increases leaf litter stabilization rates by increasing the active period for Opportunistic microorganisms, retarding the activity of slower growing guilds and repressing lignolytic exoenzyme activity, resulting in decreased overall lignin decomposition. Following ^{13}C tracers added at the beginning, intermediate, and later stages of decomposition in the leaf litter and leaf litter +N decomposition experiments, will enable us to test GDM predictions regarding the fate of C and N during leaf litter decay. Isotopic analysis of residual leaf litter chemistry after labeling with different C forms will provide estimates of the timing and quantities of microbial inputs to the recalcitrant C pool and will enable us to determine how this is affected by elevated N availability. The ^{13}C labeling experiments will also enable us to test the GDM's prediction that N addition will increase the growth of Opportunists, by determining the responses of the active microbial community in early stages of decay to added N.

As Moorhead and Sinsabaugh (2006) note, the most important challenge to interpreting the GDM results is the lack of clear quantitative relationships between decay rate coefficients normally reported for leaf litter decomposition (k) and the equivalent uptake rate coefficients (r) describing microbial activity. Although the relative patterns of leaf litter decay and concomitant microbial activities suggested by GDM simulations are consistent with the many reported studies of decomposition, it remains to establish these quantitative links. The proposed research program described herein is designed to do so.

Initial Simulations

We used the GDM to explore possible outcomes of the proposed experiments. The amount of initial leaf litter for simulations was 500 gC/m^2, at the upper end of autumn leaf litter-fall for forest stands of the Oak Openings field site (personal observation), with published data on leaf litter chemistry for the selected species (Sinsabaugh et al. 2002). The modeled N regime simulated regional annual atmospheric deposition (National Atmospheric Deposition Network for 1999-2003; ca. 9 kg N ha^{-1} y^{-1}). Preliminary results revealed unrealistic accumulations of mineral N during decomposition and concomitant inhibition in lignin decay, unless leaching losses $\geq$ 1% of the mineral N pool per day (Moorhead and Sinsabaugh 2006), similar to leaching of labile materials in other models of decomposition (e.g., Currie and Aber 1997) and suggesting that the current N deposition rate may already inhibit late stages of leaf litter (lignin) decay. Otherwise, altering relative leaf litter C and mineral N regimes had different impacts on the rates of leaf litter decay, depending on initial leaf litter chemical characteristics, affecting the amount and quality of leaf litter residues and relative flow of carbon through each guild of microorganisms. These results were comparable to simulation results of Moorhead and Sinsabaugh (2006) for field studies on Blackhawk Island, Wisconsin (Aber et al. 1984) and Harvard Forest (Magill and Aber 1998) but remain to be tested by experimental data on our site. Thus the GDM predicts patterns of leaf litter decay for the Oak Openings field site that are consistent with expectations, suggesting the utility of coupling this model and proposed experiments to elucidate finer scale mechanisms underlying decomposition than have not been previously explored in such a comprehensive, integrated study.

Timetable and Responsibilities

We envisage this as a 3-yr project beginning in August 2009. Once the litter bags are deployed in fall 2009, we will harvest replicates monthly during the first year and bi-monthly after that. We anticipate that the incubations will continue through year 2 and into year 3 before residual leaf litter LCI values approach 0.7, at which point we will stop the experiments. We will conduct labeling experiments, molecular analyses, and detailed Py-GC/MS chemical analyses at 3 times when processes (EEA) and preliminary chemical analyses (LCI and Py-GC/MS) suggest that different guilds dominate:

Time 1) early in decay, before the most labile C substrates are depleted (guild 1) – Late Fall 2009

Time 2) when LCI is ~0.3 (guild 2) before lignin should start to decay – Fall 2010 – Winter 2010-2011

Time 3) after LCI exceeds 0.4, as guild 3 is increasingly active – Fall 2011 – Winter 2011-2012

Initial modeling will involve predicting decay dynamics based on initial litter chemistry and the N additions, and translating the model into a form that can drive the interactive model of leaf decomposition (iMold) that will be used for education and outreach activities (see Broader Impacts). As the experiments proceed, modeling efforts will test the preliminary GDM predictions of early decomposition with data from the first year of incubations, and use these results to update the iMold for outreach activities. Most modeling will occur in year 3, once all data will be available to test model predictions and assumptions.

Activity	Responsibility
Fall 2009 - litter collection, litterbag creation/deployment	Weintraub/Moorhead Labs
Litterbag harvests	Weintraub/Moorhead Labs
Litter EEA assays	Weintraub Lab
Litter chemical fractionations	Weintraub Lab with Sinsabaugh consulting
Litter CN analyses	Schimel Lab
^{13}C labeling experiments	Weintraub Lab (^{13}C CO_2 and solid samples to be analyzed by a commercial lab)
^{13}C PLFA on samples from ^{13}C labeling experiments	Schimel Lab
Clone libraries	Blackwood Lab
Py-GC/MS	Grandy Lab
Modeling	Moorhead and Sinsabaugh

Scientific Merit/Synthesis

The last phase of leaf litter decomposition, when mass loss rates decline dramatically, is the least studied stage of decomposition. The transition occurs as LCI approaches a value of 0.7. Most models of decomposition predict relative mass loss rates, but do not focus on the endpoint. The rate of increase in LCI with mass loss determines what fraction of initial leaf litter mass remains as the limit of decomposition is reached. A deviation in the trajectory of decomposition that increases the abundance of residual organic matter from 5% to 7% of initial leaf litter mass makes little difference in the evaluation of decomposition models, but represents a large increase in the rate of C input to the slow-turnover SOM pool. Because SOM represents a large C pool relative to the atmosphere, there is great interest in understanding the conditions under which soils will behave as a source or sink for atmospheric C. Disturbances like N deposition and CO_2 enrichment affect SOM dynamics but the magnitude and direction of responses vary among systems. This work will provide mechanistic insight into this variation, particularly the extent to which the trajectory of decomposition can be modified by C and N availability through "bottom up" effects on the composition and substrate use of microbial communities.

The GDM currently represents the most comprehensive working synthesis of the underlying biological and chemical processes and relationships expressed in a mathematical model. To date, patterns of leaf litter decay have largely been approximated by empirical relationships that do not include driving mechanisms and thus lack the generality to adequately address perturbations such as N deposition. We propose to generate the comprehensive suite of data necessary to test the general behavior and predictions of the GDM and its underlying hypotheses.

Broader Impacts

We are proposing an integrated education and outreach program to complement this research and achieve the broadest possible dissemination of the knowledge gained from this work. To make this

effort as effective as possible we are collaborating with the University of Toledo's Center for Creative Instruction (CCI). The CCI supports and enhances the academic mission of the university through the development and dissemination of educational information technology. The CCI collaborates with many UT faculty and departments on web based educational initiatives, training applications, research studies, and the distribution of content and data. Examples of the CCI's work include the award winning Anatomy & Physiology Revealed software http://www.aprevealed.com; the Paired Kidney Donation website http://paireddonation.org/; and the NW Ohio Heals site http://hsc.utoledo.edu/org/nwoheals.

For this project, CCI personnel will set up an online **i**nteractive **m**odel **o**f **l**eaf **d**ecomposition (iMold) to provide educational outreach about decomposition for grades 5-12. The iMold virtual environment will include a web site which will consist of two distinct experiences: public and members-only community. The Public community will consist of interactive animations of decomposing leaf litter, GDM simulations, and learning components available both on the website and by a version designed for the SMART Board™. The interactive animations will include the ability to select from a handful of different leaf litter types of varying chemistry and then select the environmental conditions it will decompose within (including global change scenarios). Users will be able to visualize the progress of the decomposition of leaf litter as a whole, or different individual chemical constituents, at different places in a time line to see how time, leaf litter type, and environment relate to how quickly or slowly something decomposes.

The SMART Board™ is an interactive, electronic whiteboard which can enhance instruction and learning, and is now commonly used in K-12 classrooms. The SMART Notebook software makes it possible to create content rich, dynamic lessons. For example, a simulation lesson could illustrate a decomposition model where the time-lapsed to complete decomposition can be manipulated by the student to better visualize decomposition. This learning component can be downloaded to a SMART Board in a classroom. More and more classrooms are equipped with this technology every year, and it is relatively easy to make SMART Board based lessons available to teachers using this technology.

The members-only community will provide classrooms (teachers and students) with the ability to network between the researchers and each other (classrooms at different schools) to share data, ask questions, and collaborate on experiments. Each participating classroom will register online. Members will be able to upload images to demonstrate their own decomposition experiments. A communication center will be created so that the researchers and classrooms can share ideas and results, as well as, ask questions in a blog-like environment. Regularly scheduled video conferences will be given by project researchers directly to the schools. Students and teachers can ask questions during the presentation or experiment. Each presentation will be archived for review at any time on the web site.

We have discussed these education and outreach activities with several science teachers in the Toledo Public Schools who participate in the UT Dept. of Environmental Sciences NSF sponsored GK-12 program, and they agree that these activities would benefit the ecology and ecosystem units in their biology courses, and their environmental science classes. A teacher at Start High School commented that iMold could be "an engagement tool that would intrigue my students and motivate them to learn more."

To maximize participation in the members only community we will raise teacher awareness about the iMold by recruiting the active participation of existing education and outreach programs in our region, such as the SATELLITES (Students And Teachers Exploring Local Landscapes to Interpret The Earth from Space) program run by UT's Dept. of Geography and Planning, and TECHS (The Early College High School) program, with which co-PI Moorhead currently interacts. PIs Moorhead and Weintraub are also participants in a Dept. of Education Teachers for a Competitive Tomorrow (TCT) grant to UT that will pay for science teachers in the Toledo Public School system to receive their M.S. in Biology in the Dept. of Environmental Sciences beginning in summer 2009. Both PIs will be serving as M.S. advisors and course instructors to these teachers, and we will recruit their participation in the iMold outreach activities. We will also disseminate this program through SMART Board™ user groups and forums (teachers equipped with this technology are always looking for "out of the box" activities that will work with it), and to schools affiliated with UT's college of education. We budgeted for CCI personnel to develop the iMold and SMART Board™ activities. Moorhead and Weintraub will coordinate the outreach with teachers, with all PIs participating in the activities involving researchers.

The project will also provide training opportunities for 3 graduate students (1 for Weintraub, 1 for Blackwood, and 1 for Moorhead) and several undergraduates. The University of Toledo and Kent State University serve significant African-American populations. Individuals from groups under-represented in science will be recruited to this project. Weintraub has a history of hiring and training minority students. Selecting graduate students including members of underrepresented groups is a priority.