**Working Title:** Intrinsic Traits of Ectomycorrhizal Fungal Necromass Predict Decomposition Across Different Vegetation Communities and Plant-Mycorrhizal Associations

**Authors**: Katilyn V. Beidler1\*, Erin Andrews2, Chris W. Fernandez2, Ryan M. Mushinski3, Richard P. Phillips1, Craig See2 and Peter G. Kennedy2.

**Affiliations:**

1. Biology Department, Indiana University, Bloomington IN USA
2. Department of Plant Biology, University Minnesota, Twin Cities, MN, USA
3. School of Public and Environmental Affairs, Indiana University, Bloomington, IN, USA

Introduction Sections

1. *Mycorrhizal decomposition is an important component of belowground carbon cycling.*

Mycorrhizal fungi form a continuum between soils and plants, connecting root carbon to soil nutrients via their extensive hyphal mycelium. Plants invest heavily in their mycorrhizal symbionts, allocating 20-70% of photosynthetically fixed carbon to mycorrhizal fungi (Hobbie 2006; Godbold *et al.* 2006). Which in turn contribute between 50-1000 kg of fungal biomass per hectare per year (Rilling 2004; Cairney 2012; Ekblad *et al.* 2013; Lenaers *et al.* 2018). Thus, the decomposition of mycorrhizal mycelia is an important pathway by which carbon and nutrients enter the soil food web. Fungal necromass (i.e. recently dead fungal biomass) serves as an attractive resource for microbial decomposers, as reflected in rapid rates of ectomycorrhizal (EcM) necromass decay (Koide and Malcolm 2009; Fernandez and Koide 2014; Brabcová *et al.* 2016, 2018; Fernandez and Kennedy 2017). Decay rates of EcM necromass range from 6.76 to 15.6 g g−1 yr−1 , which are considerably faster that rates of leaf litter decay ranging from 0.006 to 4.99 g g−1 yr−1 (Zhang *et al.* 2008; Brabcová *et al.* 2018). Similar to leaf litter decay, variation in decay rates is thought to be the result of interspecific differences in tissue quality, largely determined by the initial chemical composition of fungal tissues (Hurst and Wagner 1969; Fernandez *et al.* 2016).

1. *The rate at which EcM necromass decomposes is regulated by intrinsic properties of fungal biomass which in turn influences the composition of associated decomposer communities.*

Mycorrhizal fungal taxa differ in their intrinsic properties, namely the structure and biochemistry of their mycelia (Anderson and Cairney 2007; Siletti *et al.* 2017). Biochemical components of fungal cell walls known to influence rates of decomposition include but are not limited to: the structural polysaccharide chitin which has a high N content and the pigment melanin which is resistant to decay and protective in function (Malcom and Koide 2009; Fernandez and Koide 2012, 2014; Brabcová *et al.* 2018; Leaners *et al.* 2018). Initial fungal melanin: nitrogen ratios are analogous to plant lignin: nitrogen ratios, increased concentrations of melanin or lignin relative to N correspond with slower decay rates (Fernandez and Koide 2014). Nutrient rich tissues high in N are targeted by microbial decomposers and are considered “high-quality” tissues, whereas tissues that contain phenolic compounds resistant to decay are deemed “low-quality.” Because melanins are complex biopolymers consisting of phenolic compounds they require energetically expensive oxidative enzymes for degradation (Butler and Day 1998; Fernandez *et al.* 2016).

The unique chemistry of fungal tissues may require a specific guild of decomposer microbes with the necessary enzymatic machinery (Brabcová *et al.* 2018). both nitrogen and melanin content are known to affect the microbial decomposers that colonize fungal necromass throughout decomposition (Brabcová *et al.* 2018; Fernandez and Kennedy 2018). Specific communities of bacteria and fungi have been shown to colonize decomposing mycelium and these communities tend to differ between the initial and late phases of decomposition. During early stages of decay bacterial abundance has been shown to increase, especially for necromass rich in N, highlighting the important role bacteria are playing in the decomposition of fungal necromass (Brabcová *et al*. 2016, 2018; López Mondéjar 2018). Moulds and yeast are also early colonizers of necromass and can give way to EcM basidiomycetes in the later stages of decay and in response to increased melanin concentrations (Brabcová *et al.* 2018; Fernandez and Kennedy 2018).

1. *The influence of environmental factors on necromass decomposition are less well studied and may differ depending on the dominant mycorrhizal association of the plant community.*

Microbial colonization and corresponding rates of decomposition depend on both initial tissue chemistry of the litter and the nutrient status of the surrounding soil environment (Delgado-Baquerizo *et al.* 2015). Vegetation communities differ in the quality and type of plant litter inputs, which in turn shape microbial communities and soil nutrient conditions (Zhang *et al.* 2008; García-Palacios *et al.* 2013). Moreover, soil nutrient conditions predict dominant forms of plant-mycorrhizal symbioses; with arbuscular mycorrhizal (AM) and ectomycorrhizal (EcM) fungal associations being the most geographically and taxonomically pervasive (Read and Perez-Mereno 2003). Each mycorrhizal type is favored under a specific set of climatic and soil conditions (Read 1991; Soudzilvovskaia *et al.* 2015). Plants associated with EcM fungi dominate many nitrogen limited boreal and temperate forest ecosystems, whereas AM associated plants dominate many phosphorus limited tropical forests and grasslands (Read 1991; Dickie *et al.* 2014).

Differences between AM and EcM dominated systems are thought to be influenced by litter chemistry and the abundance of organic vs. inorganic nutrients (Read and Perez-Mereno 2003; Phillips *et al.* 2013; Dickie *et al.* 2014). Compared to EcM fungi, AM fungi have are limited in their capacity to utilize organic soil nutrients (Dickie *et al.* 2014: Hodge 2017). In mixed AM and EcM temperate forests, tree species that associate with AM fungi have been shown to generate fast decomposing leaf litter which increase the concentrations of inorganic nutrients in the soil. While, tree species that associate with EcM fungi tend to generate slower decomposing litters that release more organic forms of nutrients (Phillips *et al.* 2013; Midgley *et al.* 2015; Cheeke *et al.* 2016). It is unclear if rates of fungal necromass decomposition differ under AM and EcM dominated plant communities in either forested or non-forested ecosystems.

In situ mycorrhizal decomposition studies have been conducted in ectomycorrhizal dominated temperate oak and pine forests exclusively. It is also important to note that decomposition rates have only been reported for EcM fungal species, given the difficulties associated with producing enough intact AM mycelium from pure cultures to measure AM fungal mass loss or C mineralization over time. While it is known that rates of fungal necromass are partially determined by interspecific differences in biochemical traits, less in known about how EcM necromass decomposition varies under vegetation communities that differ with respect to soil conditions and dominant mycorrhizal type. In this study we wanted to investigate the impact of different mycorrhizal plant groups (AM-associated trees, EcM-associated trees, AM-associated grasses and EcM associated forbs) on fungal necromass decomposition, for two EcM fungal species known to differ in their melanin and N content (i.e., representing a high and low quality litter).

1. *Study Approach and Predictions*

This study combines results from experiments in which fungal necromass was deployed under three different vegetation communities and in different EcM/AM settings. Necromass was decomposed in (1) prairie fields containing AM-associated grasses and patches of the EcM-associated forb, *Helianthemum bicknellii* (2) along an oak savannah forest to grassland gradient and (3) in AM and EcM associated temperate forest stands. To test the potential interactive effects of litter quality and decay environment on decomposition we utilized two types of fungal necromass that vary with respect to litter quality (1), *Mortirella elongata*,an EcM fungal species that produces necromass high in N and low in melanin and (2) *Meliniomyces bicolor* an EcM fungal species that produces melanized necromass with a lower N content than *M. elongata*. We also assessed how litter quality and decay environment may influence decomposer community composition for the temperate forest and oak savanna vegetation sites. We predicted that *M. elongata* would decompose more quickly than *M. bicolor* and that decomposition dynamics would differ between mycorrhizal types. With faster decay of *M.bicolor* occurring in EcM settings thought to contain

Methods

*Study Locations and Sites Within Locations*

This research was conducted at the Cedar Creek Ecosystem Science Reserve (CCESR) in central Minnesota and Moores Creek Research Preserve (MCRP) in south-central Indiana. Cedar Creek is a 5,600 acre ecological field station that borders prairie and forest ecoregions with a mean annual temperature of 6.7°C and mean annual precipitation of 801 mm. Moores Creek is comprised of 261 acres of mixed deciduous hardwood forest (~ 80 years in age) with a mean annual temperature of 11.6°C and a mean annual precipitation of 1200 mm. Sites were selected within CCESR and MCRP to study the effects of vegetation type on fungal decomposition. At CCESR decomposition experiments were conducted within prairie and oak savanna sites and at MCRP litter was incubated in temperate forest stands. Within sites, vegetation communities differed in their AM and ECM associated plant species (see Table 1). Plot or transect locations within the different vegetation communities were chosen to test the effects of dominant plant mycorrhizal type on fungal decomposition.

Plots were chosen within 3 prairie fields at CCESR. In prairie plots fungal litter was decomposed beneath patches of the ECM associated forb, *Helianthemum bicknellii* (hoary frostweed) and nearby patches of AM associated grass species (see Table 1). *H. bicknellii* a herbaceous perennial which grows in prairies and oak savannas in the Eastern and Midwestern United States; It is known to maintain patches of elevated ectomycorrhizal fungal abundance while growing in a matrix of AM-associated vegetation (Dickie et al. 2004a).

Within oak-savanna plots, a mycorrhizal gradient that was established along a 30 meter transect. Transects started in the interior of an ECM associated oak stand (10 meters into the forest) and then continued into the interior of an AM associated grassland (20 meters into the grassland). Previous studies conducted at this site report a gradient in EcM abundance, in which EcM abundance is uniformly low 20 meters from the forest edge (Dickie *et al.* 2004b, 2005).

Temperate forest plots were established according to known mycorrhizal associations of dominant tree species. In all plots, trees from the dominant mycorrhizal type (AM or ECM) represented >85% of the basal area of the plot. AM and EcM dominated forest plots were paired according to geographic proximity. For more information on temperate forest plot design at MCRP refer to Midgley *et al.* (2016).

*Fungal Necromass Generation and Decomposition*

We selected two ectomycorrhizal fungal species, *Meliniomyces bicolor* and *Mortierella elongata*, known to differ in their C:N values and melanin concentrations. *Meliniomyces bicolor* and *Mortierella elongata* represent a low and high quality fungal litter, respectively (see Table 2). For each species, fungal biomass was produced in liquid cultures by inoculating 50 mL of half-strength potato dextrose broth with 3mm diameter mycelial plugs. Following inoculation, cultures were transferred to an orbital shaker and left to shake at 80 rpm for at least 30 days or until growth stopped (growing times differed among fungal species as melanized species tended to grow more slowly)

To produce the fungal necromass, cultures were rinsed with distilled water and dried at 26°C for 24 hours. Dried fungal cultures (~25mg) were then placed into nylon mesh litter bags constructed from 53 micron mesh (Elko, Minneapolis, MN, USA). Separate litter bags were constructed for each fungal species. During deployment, litter bags were buried at the interface between organic and mineral soil. Upon collection the necromass was removed from the litterbag and dried to a constant mass to determine mass remaining. Following mass measurements, a subset of remaining necromass was stored at -20oC for DNA analyses. While the preparation and processing of necromass was standardized across the three sites, study durations and incubation times differed.

Two successive studies were conducted at the prairie site; in the first study *Mortierella elongata* necromass was incubated for 7, 14, 21, 30 and 65 day periods beginning in July of 2017 in one of the fields (n = 4 litter bags of each fungal species for each mycorrhizal association treatment for each sampling date). The second prairie study was meant to replicate the results of the first study, but with higher spatial replication (i.e. was conducted in two additional fields). In this second study, *Meliniomyces bicolor* and *Mortierella elongata* necromass was incubated for 11 and 34 days starting in late August of 2017 (n = 6 litter bags of each fungal species for each mycorrhizal association treatment for each sampling date). *Mortierella elongata* and *Meliniomyces bicolor* necromass was incubated in the oak-savanna site for 14, 28, 42 and 56 day increments starting in July of 2017 (n = 3 litter bags of each fungal species for each mycorrhizal association treatment for each sampling date.) *Mortierella elongata* and *Meliniomyces bicolor* were decomposed at the forest site over 14 day, 31 and 92 days beginning in late July of 2017 (n = 7 litter bags of each fungal species for each mycorrhizal association treatment for each sampling date).

*Soil Factors*

Soil moisture was collected at the same time litter bags were harvested and one time pH measurements were made…[Still need to get this info from Erin and Craig]

*Identification of Bacterial and Fungal Decomposer Communities*

For a comparison of microbial decomposer communities among sites we performed molecular analyses for the black morph of *Meliniomyces bicolor* and *Mortierella elongata* at the oak savanna and temperate forest sites. Microbial genomic DNA was isolated from soil and necromass samples using DNeasy PowerSoil Extraction Kits (QIAGEN, Germantown, MD, USA). DNA extractions were done according to the manufacturer’s instructions with the addition of a bead-beating step prior to the start of the extraction to better homogenize necromass samples (Fernandez and Kennedy 2018). Positive and negative controls were included for both bacteria and fungi. Positive controls included mock communities from the Human Microbiome Project for bacteria and for fungi, the “SynMock” synthetic mock community developed by Palmer *et al.* (2017). We also extracted DNA from necromass that had not been incubated in litterbags. Negative controls included lysis tubes lacking substrate and PCR reactions with no DNA template added

Bacteria and fungi colonizing necromass samples and nearby soil were identified using high-throughput sequencing. For fungal identification we used the ITS2 primer pair designed by Taylor *et al.* (2016). For bacteria, we used 515F-806R primers to target the V4 region of the 16S rRNA gene. PCR reaction reagents and cycling conditions followed the Illumina MiSeq two-step PCR amplicon sequencing protocol. If initial PCRs were not successful, we performed dilutions or increased cycle numbers. Following amplification PCR products were visualized by gel electrophoresis and cleaned using the Charm Just-a-Plate Purifiation and Normalization Kit (Charm Biotech, San Diego, CA, USA). Successful bacterial and fungal amplicons for each sample were pooled at equimolar concentration and sequenced together on a full MiSeq lane (2 x 300 bp V3 Illumina chemistry) at the University of Minnesota Genomic Center.

To process bacterial and fungal HTS amplicon sequences we used the AMPtk pipeline (amplicon toolkit; Palmer *et al.* 2017) v1.1 primarily utilizing the default settings (<https://amptk.readthedocs.io/en/latest/index.html>). In summary, paired end reads were merged using VSEARCH (check this) and then subjected to quality trimming. Following pre-processing the UNOISE3 OTUs were clustered using the denoising algorithm run in USEARCH10. We applied a 0.0005 abundance cut-off to the bacterial data to eliminate very low abundance OTUs that were thought to be spurious. For the fungal data, SynMock abundances were used to determine the filtering threshold. For Fungi, functional assignments (i.e., saprotrophic, symbiotrophic, and pathotrophic trophic guilds) were made with FUNGuild (Nguyen *et al*. 2016).

*Statistical Analyses*

Statistical analyses were carried out in R version 3.5.1 (R Core Team, 2018). Linear mixed effect models were used to analyze fungal necromass mass remaining for each site. Fixed factors included mycorrhizal association (AM or EcM associated vegetation), necromass species (*M. bicolor* or *M. elongata*), incubation period (see discussion of differing incubation periods above). Replicate sampling locations (either plots or plot pairs) were designated as a random factor. To compare across sites, decay constants were calculated using exponential decay models and models were compared with ANOVA and AIC.



Results

*Soil Factors*

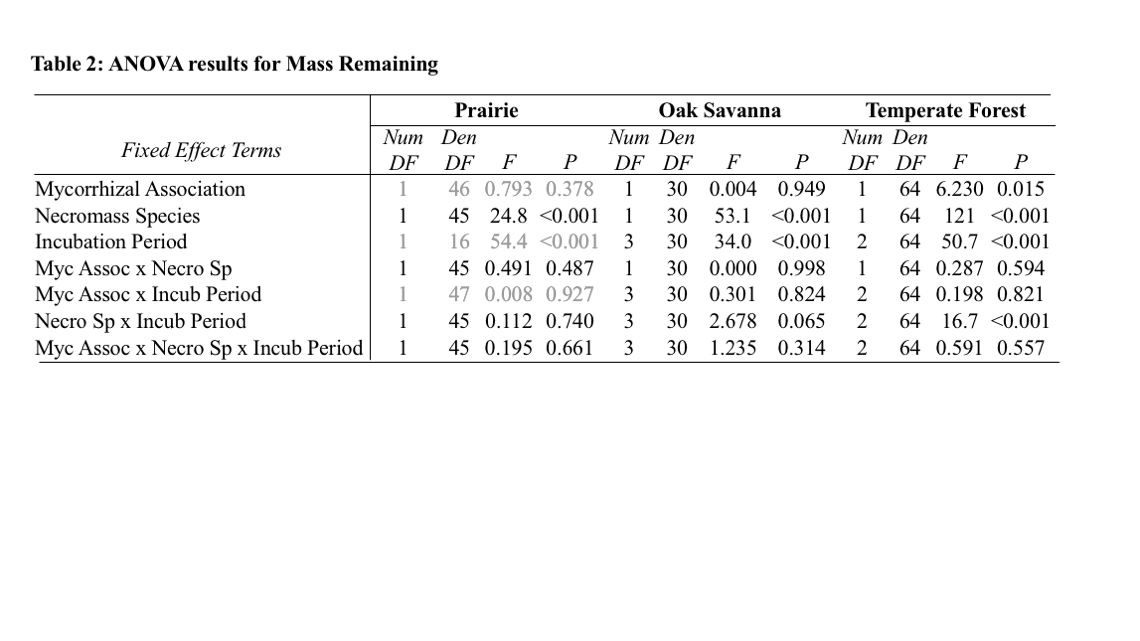
Sampling date did not have a significant effect on soil moisture or pH measurements across sites and differences among vegetation types depended on plant mycorrhizal association (interaction term F=; P<.01). Soils under EcM associated vegetation tended to have lower pHs compared to soils under AM associated vegetation, but only in the forest and oak savanna sites (See Table 1). Temperate forest soils dominated by EcM associated trees had the lowest pH values and oak savanna soils dominated by AM associated grasses had the highest pH values. Forest AM, prairie AM and prairie EcM soils did not differ from one another with respect to pH. Soil moisture only differed between vegetation mycorrhizal associations at the temperate forest site. Average soil moisture was lowest for the forest soils dominated by EcM associated vegetation and highest for prairie soils. Soil moisture values were similar between temperate forest and oak savanna AM soils, as well as, oak savanna and prairie EcM soils.

*Fungal Necromass Remaining*

Mycorrhizal association only had an effect on mass remaining of fungal necromass at the temperate forest site (See Table 2). Mass remaining was ~17% higher in EcM dominated forest plots on average (24.1 ± 2.1% AM ; 28.2 ± 2.2% EcM, mean ± 1SE ). Necromass species decomposed at a similar rate under AM and EcM associated vegetation across sites (See Figure 1). Mass remaining differed among incubation periods and fungal necromass species (See Figure 1 and Table 3). On average *M. bicolor* decomposed more quickly than *M. elongata* at each of the sites (see Table 3). However, the effect of necromass species was mediated by a significant necromass species x incubation period interaction at the temperate forest site and a moderately significant interaction at the oak savanna site. The greatest differences in mass remaining between *M. bicolor* and *M. elongata* occurred early on in decomposition (11-14 days). During this time period ~80%, 60% and 20% more *M. bicolor* mass remained compared to *M. elongata* mass at the temperate forest, oak savanna and prairie sites respectively.



\*



*Decay Rates*

I will present these results in lab meeting…



*Decomposer Communities*

Still waiting on this…