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Nitrogen enrichment shifts functional genes related to nitrogen and carbon acquisition in the fungal community

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Abstract

To better understand mechanisms of carbon (C) and nitrogen (N) dynamics under anthropogenic N enrichment, we examined frequencies of C- and N-targeting genes in litter fungi. In particular, we tested the hypothesis that N enrichment selects for C-targeting genes but against N-targeting genes, if fungi preferentially invest resources in acquisition of growth-limiting nutrients. We conducted a fully-factorial litter and microbial transplant in a N fertilization experiment in Southern California grassland. The transplant design enabled us to contrast direct effects of N fertilization in the environment, indirect effects of N-induced shifts in the microbial community, and indirect effects of N-induced changes in plant litter chemistry. For each treatment, we assessed frequencies of select well-annotated fungal functional genes: cellulose-targeting AA9 genes (for C acquisition) versus ammonium transporter genes and amino acid permease genes (for N acquisition). We found that our hypothesis was upheld only with regard to shifts in the microbial community. Specifically, when grown in the same environment and litter, fungi from the N-fertilized plots displayed greater frequencies of cellulose-targeting AA9 genes from basidiomycetes, but smaller frequencies of ammonium transporter genes and amino acid permease genes, when compared to fungi from the control plots. In contrast, N fertilization in the plot environment was associated with higher frequencies of amino acid permease genes and ammonium transporter genes. Likewise, plant litter from the N-fertilized plots selected for higher frequencies of ammonium transporter genes. Altogether, we found fairly inconsistent effects of N enrichment on fungal functional genes related to C and N acquisition. Even if the genetic capacity of the fungal community to acquire C versus N changes owing to shifts in the microbial community, direct effects of N fertilization and indirect effects of litter chemistry may offset the response.

Keywords: *amino acid permease genes*, *ammonium transporter genes*, *fungal functional genes*, *lytic polysaccharide monooxygenase family AA9 genes*, *microbial community composition*, *nitrogen enrichment*

1. Introduction

Nitrogen (N) and carbon (C) are critical nutrients for fungi (Griffin, 1996). For example, fungi require C for energy and biomass production, and N for protein construction (Sinsabaugh et al., 2009). Fungi can acquire C by releasing extracellular enzymes into the environment to break down complex organic molecules such as crystalline cellulose (Lynd et al., 2002; Langston et al., 2011). They can also obtain N by incorporating transporter enzymes into their cell membranes to take up N-containing compounds like amino acids and ammonium (Grenson et al., 1970; Chalot and Brun, 1998; Mitsuzawa, 2006). In doing so, they contribute to C respiration, N mineralization, and microbial N immobilization within ecosystems (Dighton, 2016). A number of genes controlling these functions have been identified in fungi (Treseder and Lennon, 2015). Accordingly, we can assess the genetic potential of the fungal community to influence N and C cycling by examining the distribution of selected functional genes in fungi growing in the environment.

In fact, we can examine fungal functional genes in natural ecosystems to understand how these physiological capacities of fungi respond to N enrichment (Berlemont et al., 2014; Myrold and Nannipieri, 2014; Myrold et al., 2014). Nitrogen enrichment is an important element of global change, because human activity has about doubled the amount of biologically available N worldwide (Vitousek et al., 1997; Galloway et al., 2008). For example, in Southern California, anthropogenic N deposition adds more than 25 kg N ha^{-1} y^{-1}. It can be challenging to predict how soil C dynamics respond to N enrichment (Fog, 1988; Knorr et al., 2005; Hyvonen et al., 2007; Janssens et al., 2010). We may improve our

60understanding of links between N enrichment and C cycling by examining in detail the relevant processes
61governed by fungi.

62 For instance, since N is a macronutrient for fungi (Griffin, 1996), its enrichment could alter fungal
63investment in N versus C acquisition. Extracellular enzymes and transporters are proteins, which require
64N and C to construct (Elser et al., 2000). If a fungus allocates N and C to construction of an N-acquiring
65enzyme, those resources become unavailable for production of a C-acquiring enzyme. Thus, this
66allocation constraint creates a trade-off between the ability to acquire N versus C (Allison et al., 2010).
67Accordingly, we hypothesize that N enrichment will select for C acquisition genes, and against N
68acquisition genes, if fungi preferentially invest resources in enzymes that target growth-limiting
69nutrients. If such a trade-off exists, then we can consider it when predicting fungal contributions to C
70dynamics under N enrichment.

71 A trade-off between fungal genetic capacity for N versus C acquisition could manifest via several
72ecological pathways (Fig. 1a). First, N additions could *directly* increase the growth of fungi with greater
73genetic capacity for C acquisition, allowing them to outcompete individuals that invest instead in greater
74genetic capacity for N acquisition. Second, over a longer term, N enrichment could select for fungal
75species that favor C acquisition over N acquisition. In this way, N additions could *indirectly* influence the
76genetic capacity of the fungal community via shifts in species composition. Third, N enrichment could
77*indirectly* alter the genetic capacity of fungi via changes in plant litter chemistry. For instance, N
78concentrations in plant litter often—though not always—increase after N enrichment (Ostertag and
79DiManno, 2016). Thus, we extend our hypothesis to specify that N enrichment will change genetic
80capacities for N versus C acquisition via short term, direct effects of N on fungi; longer-term shifts in
81fungal community composition; or changes in plant litter chemistry.

To test our hypothesis, we used a reciprocal litter transplant to independently manipulate direct effects via N enrichment in the environment versus indirect effects via shifts in microbial community composition, or indirect effects via changes in litter chemistry (Figs. 1b & 2, Allison et al., 2013). This field experiment was located in Southern California grassland subjected to long-term N fertilization. Following decomposition, we shotgun-sequenced DNA in each litterbag (Martiny et al., 2017), and then measured the frequency of fungal genes involved in N acquisition (amino acid permeases and ammonium transporters) and C acquisition (enzymes that break down crystalline cellulose). Previous work in this experiment has established that N fertilization alters fungal community composition (Matulich et al., 2015; Amend et al., 2016; Martiny et al., 2017) and increases litter N concentration (Allison et al., 2013).

2. Material and Methods

2.1. Field site

We tested our hypothesis in an N-enrichment experiment established in a grassland in coastal Orange County, California USA (33° 44’ N, 117° 42’ W, 365 m elevation). The N-fertilization experiment is described in detail by Allison et al. (2013). Briefly, the grassland is dominated by *Nassella pulchra*, a native perennial grass, as well as exotic annual forbs and grasses (Potts et al., 2012). Mean annual precipitation is 325 mm y^{-1}, primarily as rain between October and April. Mean annual temperature is 17 °C. The Irvine Ranch Conservancy and Orange County Parks granted permission to use this field site.

The N-fertilization experiment began in February 2007. Plots were set up in eight blocks. In each block, one plot remained unmanipulated as a control, and the other was N-fertilized. Each plot covered 13.3 x 9.3 m. Each year, we N-fertilized the plots with soluble calcium nitrate (20 kg N ha^{-1}) before the growing season, plus 100-day release calcium nitrate (40 kg N ha^{-1}) within the growing season.

2.2. Microbial cages

First, we collected plant litter to serve as a decomposition substrate. We collected litter from four 0.07 m^2 quadrats haphazardly located in each control plot and N plot on 29 June, 2 July, and 14 September 2010. (We used multiple collection dates, because deep-rooted annual forbs senesced later in the season than did the other plants.) We pooled and hand-homogenized litter from all plots within each treatment.

Second, we assembled "microbial cages" within which we decomposed the plant litter (sensu Reed and Martiny, 2007). Microbial cages are litterbags made from nylon membrane with 0.45 µm pores. Fungi and most larger bacteria cannot pass through these pores, but water, nutrients, and unusually small bacteria can. We added 2 g air-dried litter to each cage, and then sterilized the completed cages with at least 22 kGy of gamma irradiation. We verified sterility by placing subsamples of irradiated litter in 50 mL sterile tubes with either potato dextrose broth (for fungi growth; Becton, Dickinson and Company; Franklin Lakes, NJ) or lysogeny broth (for bacterial growth, Fisher Scientific, Pittsburgh PA), shaking at 37 ℃ (three days for fungi and 12 hours for bacteria). Then, we plated out 100 µl of the media on potato dextrose plates or lysogeny broth. We included a positive control (i.e., non-irradiated litter) to confirm that this procedure yielded colony growth.

Third, microbial inoculum was collected from control and N-fertilized plots to add to the microbial cages. On 30 November 2010, we hand-collected three haphazardly located litter samples (~5 g each) from each of the eight control and eight N plots. We generated two batches of microbial inoculum: "control microbes" and "N microbes", by combining litter samples within each treatment. The inoculum was air dried, ground in a Wiley mill to 1 mm mesh, and then added as 50 mg aliquots to microbial cages containing sterilized litter.

2.3. Reciprocally transplanted litter

Fungi can acquire N from two major sources in the environment: plant litter and soil. Nitrogen fertilization in this field site increases N availability in both sources (Allison et al., 2013). Prior to decomposition, litter from the N-fertilized plots had significantly higher concentrations of total N (i.e., %N), cellulose, and hemicellulose compared to litter from control plots (Allison et al., 2013). In contrast, C:N ratios, lignin concentrations, and sugar concentrations were significantly lower in litter from the N-fertilized plots than the litter from the control plots (Allison et al., 2013). Concentrations of total C (i.e, %C), protein, starch, and fat did not differ significantly by litter origin (Allison et al., 2013). Litter chemistry was originally analyzed by Allison et al. (2013) from the same litter used in the current study. They determined total C, total N, and C:N by elemental analysis, and the other fractions by near infrared spectroscopy.

For the current study, we isolated the effects of these changes in litter chemistry by decomposing control ("control litter") and N-fertilized plant litter ("N litter") in the control and N plots (Figs. 1b & 2). Accordingly, we placed four microbial cages (one of each microbe x litter combination) in each of eight control plots and eight N plots. The cages were placed within standing grass litter. One corner of each microbial cage was tethered to the soil surface, and the remainder rested on the standing grass litter. This way, the orientation of the microbial cage matched that of the standing litter. Each cage contained either control or N litter inoculated with control or N microbes in a factorial design. Thus, there were 64 microbial cages total. Microbial cages were incubated in the plots for three months, from 15 December 2010 to 3 March 2011.

2.4. Survey of non-transplanted litter

We also examined components of fungal functional genes in naturally occurring, non-transplanted plant litter in the N experiment. Our objective here was to determine overall effects of N enrichment on functional gene frequencies (Fig. 1b). In this design, N fertilized samples represented microbes from the

149N fertilized plots decomposing N fertilized plant litter in the N fertilized plot environment. Likewise,
150control samples were control microbes decomposing control litter in control plots.

151 We gathered samples of plant litter from haphazardly located 0.07-m2 quadrats in each plot (N-
152fertilized and control) once per season for 15 months (December 2010, February 2011, June 2011,
153September 2011, December 2011 and March 2012). Altogether, we collected eight control samples and
154eight N-fertilized samples in each of six dates, totaling 96 samples.

155 2.5. DNA sequencing and annotation

156 To balance cost limitations of sequencing with replication, we pooled eight plots from each treatment
157(each plot sampled and extracted separately) into two duplicate samples for sequencing. This approach
158allowed us to capture spatial variation across our study site, while still maintaining moderate statistical
159power.

160 For sequencing and annotation, we followed procedures previously described in Berlemont et al
161(2014). We ground about 20 g of each litter sample in a mixer, subjected it to direct DNA isolation as
162described before (DeAngelis et al., 2010), and then normalized for the amount of leaf litter material used
163in the extraction. (We note that Smets et al. (2016) suggest including an internal DNA standard, which
164can be used to adjust gene copy number. We support this approach for future studies.) We used Covaris
165to fragment DNA to 300 bp. We then pooled equal amounts of DNA extracts into two replicates for
166sequencing. From the litter manipulation, 64 microbial cages were processed, yielding 16 metagenomic
167libraries. From the survey of unmanipulated litter, a total of 96 litter samples were processed to create
16826 metagenomic libraries [2 treatments × 6 dates × 2 replicates = 24 samples, plus two sequencing
169controls]. We prepared metagenomic libraries by using a Truseq library kit (Illumina, San Diego, CA, USA),
170and then sequenced them via Illumina HiSeq2000 (100 bp-paired ends). We treated sequences as single
171reads for subsequent analysis. We uploaded sequences onto the MG-RAST server (Glass and Meyer,

1722011) to make them publically accessible (Berlemont et al., 2014; Martiny et al., 2017). Altogether, we 173obtained 107.4 Gbp after quality control.

174 2.6. Fungal functional genes

175 We counted frequencies of fungal functional genes within each metagenomics library by comparing 176sequences to described genes in the InterPro database (https://www.ebi.ac.uk/interpro/). Nitrogen-177targeting genes included those that facilitated N uptake from the environment: amino acid permease 178genes (e.g., *AAP1* and *GAP1*, InterPro domain: IPR004762) and ammonium transporter genes (e.g., 179*AMT2*, IPR001905)(Nehls et al., 1999; Mitsuzawa, 2006; Gresham et al., 2010). For C-targeting genes, we 180focused on lytic polysaccharide monooxygenase family AA9 (IPR005103)(Levasseur et al., 2013). 181Extracellular enzymes of this gene family contribute to the breakdown of relatively recalcitrant forms of 182cellulose, including highly crystalline cellulose as well as cellulose molecules that are cross-linked with 183lignin (Harris et al., 2010; Langston et al., 2011; Villares et al., 2017). Since the copy number of a given 184functional gene can vary among individuals as well as taxa (Stajich, 2017), changes in gene frequencies 185could result from shifts in community composition, selection within populations, or a combination of the 186two.

187 We used annotations of all the reads in order to evaluate and compare samples without potential 188assembly biases based on composition or coverage. For cellulose-targeting AA9 genes, sequences were 189assigned to the phylum Ascomycota or Basidiomycota. We examined genes from these two phyla 190separately, because ascomycetes and basidiomycetes can differ in traits related to N responses or 191ecosystem dynamics (Treseder and Lennon, 2015). For ammonium transporter genes, we restricted our 192analyses to genes characterized in the phylum Ascomycota, as this gene family has not yet been well 193annotated in the Basidiomycota. For amino acid permease genes, kingdom-level annotations were not 194available. Instead, we focused on amino acid permease genes that were assigned to the Eukaryota 195domain (excluding bacteria). We used the M5NR database for annotation (Wilke et al., 2012). Positive

matches were considered for sequences with e-value ≤10^{-5}. Using this cut-off, 0.15% of the microbial cage sequences and 0.43% of the survey sequences were identified as fungal functional genes of interest.

2.7. Statistics

To test our hypothesis on reciprocally transplanted litter from the microbial cages, we conducted a series of fully-factorial analyses of variance (ANOVAs). In each case, plot environment, microbe origin, and litter origin were the independent variables. The dependent variable was frequency of the functional gene of interest (e.g., amino acid permease, cellulose-targeting AA9 basidiomycete genes, etc). Where appropriate, we used Tukey post hoc comparisons to check for pairwise differences among treatments. Significant increases in frequencies of C acquisition genes (or decreases in N acquisition genes) via plot environment, microbe origin, or litter origin would support our hypothesis.

For the non-transplanted litter, we used repeated measures ANOVAs to test our hypothesis. The independent variable was N treatment (control versus N-fertilized plots), and the six collection times were the repeated measure. The dependent variable was the frequency of the functional gene of interest. Significant differences between the control and N-fertilized plots—with higher gene frequencies for C acquisition or lower frequencies for N acquisition—would support our hypothesis.

In all cases, data were ranked because they did not meet assumptions of ANOVA regarding normality and homogeneity of variances. Gene frequencies of zero were uncommon in the samples. Differences between treatments were considered statistically significant when $P < 0.05$, and marginally significant at $P < 0.10$. We used Systat 13 to run each of the ANOVAs (SPSS, 2009).

3. Results

3.1. Reciprocally transplanted litter

Microbe origin. Our hypothesis was supported only with respect to indirect effects via long-term alteration of the microbial community (Table 1, Figs. 1c & 3). Specifically, the microbial community that originated from the N fertilized plots displayed significantly lower frequencies of amino acid permease genes (Fig. 3a, $P = 0.004$) and ammonium transporter genes (Fig. 3b, $P = 0.044$) than did microbes from the control plots, even when grown under common conditions. The frequency of cellulose-targeting AA9 genes from basidiomycetes increased (Fig. 3d, $P = 0.022$), which also supported our hypothesis. On the other hand, cellulose-targeting AA9 gene frequency from ascomycetes decreased marginally significantly in the N fertilized microbes (Fig. 3c, $P = 0.073$). This latter result was not consistent with our hypothesis.

Plot environment. Direct, short term effects of N enrichment primarily contradicted our predictions (Table 1, Figs. 1c & 3). Although we had expected decreases in frequencies of N acquisition genes with N fertilization, we observed the opposite: the frequency of amino acid permease genes (Fig. 3a, $P = 0.021$) and ammonium transporter genes (Fig. 3b, $P < 0.001$) each increased significantly in N-fertilized plot environments compared to control plot environments. In slight support of our hypothesis, cellulose-targeting gene frequency in ascomycetes rose marginally significantly in the N-fertilized plot environment (Fig. 3c, $P = 0.073$). The remaining gene families did not change significantly in response to plot environment ($P > 0.10$).

Litter origin. For the most part, litter origin did not influence fungal gene frequency as we had hypothesized (Table 1, Figs. 1c & 3). The only exception—which was minor—was a marginally significant increase in the frequency of cellulose-targeting AA9 ascomycete genes in N-fertilized litter compared to control litter (Fig. 3c, $P = 0.062$). In contrast to our predictions, frequencies of ammonium transporter genes and amino acid permease genes rose significantly (Fig. 3b, $P = 0.013$) and marginally significantly

239(Fig. 3a, P = 0.090), respectively, on litter from the N-fertilized plots versus the control plots. None of the
240other gene families changed significantly with litter origin (P > 0.10).

241 Interactions. Significant interactions between microbe origin, plot environment, or litter origin were
242uncommon (Table 1, Fig. 3). Ammonium transporter gene frequencies responded significantly to
243interactions between plot environment and litter origin (Fig. 3b, P = 0.024), and between plot
244environment and microbe origin (P = 0.032). All other interactions for each gene family were either non-
245significant or marginally significant (P > 0.05).

246 3.2. Survey of non-transplanted litter

247 In the survey of non-transplanted litter, the only significant N fertilization effect was a decrease in
248cellulose-targeting AA9 gene frequency for basidiomycetes, compared to controls (Table 2, Figs. 1c, 4).
249This response was opposite to our prediction. In addition, the remaining functional genes did not change
250significantly with N fertilization. Thus, the survey results did not support our hypothesis.

251 4. Discussion

252 We independently manipulated microbial communities, litter chemistry, and plot environment to
253examine consequences of N enrichment on the genetic capacity of fungi to contribute to N and C
254dynamics in decomposing litter. We tested the hypothesis that N enrichment should augment the
255frequency of fungal genes related to C-acquisition, but reduce the frequency of those related to N-
256acquisition, based on economic principles of resource allocation (Fig. 1a, sensu Allison et al., 2010). We
257further predicted that N effects would manifest directly via immediate effects of N availability in the
258environment; indirectly via longer-term changes in the microbial community; indirectly via alterations in
259plant litter chemistry; or some combination of the three. Our predictions were partially upheld, but only
260with respect to changes in microbial community composition (Figs. 1c, 3). Moreover, the shift in
261functional gene capacity in the microbial community was not matched in the unmanipulated, standing

litter in the control and N fertilized plots (Fig. 4). There, C-acquisition genes were less frequent in some fungi, while N-acquisition genes did not change significantly. Contrasting direct environmental effects and indirect litter effects may have offset the shifts in functional gene frequency in the fungal community (Fig. 1c). We discuss below each direct and indirect effect of N enrichment in turn.

Microbe origin. When incubated under common conditions, fungi from N-fertilized plots displayed higher gene frequencies related to C acquisition, and lower gene frequencies related to N acquisition, compared to fungi from control plots ("Microbial community change" in Fig. 1c). Specifically, frequencies of amino acid permease genes (Fig. 3a) and ammonium transporter ascomycete genes (Fig. 3b) declined significantly in N-fertilized microbes, while the frequency of cellulose-targeting AA9 genes in basidiomycetes increased significantly (nitrogen microbes versus control microbes, Fig. 3d). These responses are consistent with expectations that fungi will invest resources in acquisition of limiting resources (Read, 1991; Allison et al., 2010). Following N-fertilization, C may have become more limiting. Over time, basidiomycetes with greater ability to break down crystalline cellulose may have been selected over other fungi with lesser ability. At the same time, those with higher capacity to take up amino acids and ammonium may have been at a disadvantage, perhaps owing to trade-offs associated with maintaining this capacity (Treseder et al., 2011). Nevertheless, we note that the cellulose-targeting AA9 gene frequency of ascomycetes decreased marginally significantly in N microbes compared to control microbes (Fig. 3c), which did not support our hypothesis.

The taxonomic composition of the fungal community also differs between N-fertilized microbes and control microbes in this field experiment (Matulich and Martiny, 2014). For example, taxa within the Davidiellaceae (Ascomycota) and the Sirobasidiaceae (Basidiomycota) tend to increase in abundance with N fertilization, whereas taxa in the *Cryptococcus* (Basidiomycota) and Hypocreales (Ascomycota) tend to decline (Matulich and Martiny, 2014; Amend et al., 2016). These changes can persist for years after transplanting the N-fertilized microbes to control plots, which suggests a long-term legacy of N

enrichment in the fungal community (Martiny et al., 2017). We note that fungi from the subphylum Agaricomycotina tend to possess a relatively high number of cellulose-targeting AA9 genes within their genomes (Treseder and Lennon, 2015). Since the Sirobasidiaceae belong to this subphylum, increases in this group could have contributed to the higher frequency of basidiomycete-associated cellulose-targeting AA9 genes in N-fertilized microbes compared to control microbes (Fig. 3d).

Notably, although cellulose-targeting AA9 genes in basidiomycetes became more common with N enrichment, those of ascomycetes did not. We cannot simply attribute this to shifts between basidiomycetes and ascomycetes themselves; the relative abundance of these two phyla did not change significantly with N fertilization (Martiny et al., 2017). Perhaps other traits associated with the basidiomycetes allowed them to take advantage of crystalline cellulose when N was more available. For example, if basidiomycetes possessed additional gene families that could target the downstream products of cellulose-targeting AA9, then they could have more easily obtained energy from the molecule (Treseder and Lennon, 2015).

In this field manipulation, microbial activities and related biogeochemical processes do not necessarily match the functional gene profile we characterized (Table 3). For example, empirically-measured activities of the extracellular enzymes β-glucosidase and cellobiohydrolase each decline in the N-fertilized microbial community compared to the control microbial community when both experience a common environment (Alster et al., 2013). These enzymes help break down cellulose. Activities of the cellulose-targeting AA9 enzyme family have not yet been measured in this experiment, so we cannot make a direct comparison between its actual activity and the fungal community's genetic potential. Even so, Allison et al. (2013) reported that microbial origin did not significantly affect loss rates of cellulose, N, or protein from litter during decomposition in this field site. In addition, uptake rates of the amino acid glycine are higher in N-fertilized fungi than control fungi (Hynson et al., 2015), even though we found that the frequency of amino acid permease genes declined (Fig. 3a). Of course, just because the fungal

community possesses particular functional genes does not mean that they express them. For one thing, shifts in fungal taxa may have led to changes in the prevalence of other traits—such as dormancy—that interacted with gene expression (Krause et al., 2014). Environmental conditions can mediate gene expression as well.

Plot environment. We examined direct environmental effects of N enrichment by reciprocally transplanting microbes and litter to N fertilized versus control plots. We found that all else being equal, fungi growing in the N fertilized plots tended to possess greater frequencies of N acquisition genes (i.e., amino acid permeases and ammonium transporters) than those growing in control plots (Fig. 3a,b). This response is the opposite of our prediction. It is also opposite the microbial origin effect, in which these gene frequencies declined in response to N. It is possible that, over the three months that the microbial cages were in the field, excess N in the environment improved the growth of fungi that possessed the genetic capacity to take up that N. This response may have been ephemeral, though. Over the longer term, trade-offs associated with the ability to acquire N might have selected against the fungi that possess that trait. These trade-offs may include the cost of maintaining or expressing those N acquisition genes (Treseder and Lennon, 2015). Another possibility is that N enrichment may shift the type of N that fungi target—fungi may reduce investment in acquisition of more complex N (e.g., chitin and chitosan) in favor of simpler forms that are easier or less expensive to acquire (e.g., ammonium and amino acids) (Hynson et al., 2015).

The observed shift in functional gene composition with plot environment was not matched by changes in fungal taxonomic composition or litter decomposition rate (Allison et al., 2013; Martiny et al., 2017). In fact, the latter two parameters are not significantly different between the N and control plot environments (Allison et al., 2013; Martiny et al., 2017).

Litter origin. The frequency of ammonium transporter ascomycete genes was higher in litter from the N fertilized plots than in litter from the control plots, when plot environment and microbial origin were held constant. Amino acid permease genes increased as well, albeit only marginally significantly. As with the plot environment effect, these responses are contrary to our hypothesis. Plant litter from the N fertilized plots contains higher initial N concentrations than does litter from the control plots (Allison et al., 2013). Thus, N availability to fungi was likely higher in the N litter. Potentially, this N enrichment may have, in the short term, encouraged the growth of fungi with better capacity to acquire N. In this respect, the mechanism underlying the litter origin effect may have been similar to that of the plot environment effect. Despite the increase in frequency of N acquisition genes, fungal taxonomic composition in this study is unaltered by litter origin (Martiny et al., 2017). Previously, Hobbie (2005) noted that litter N enrichment can differ from "external" N fertilization (akin to plot environment) in effects on decomposition in eight Minnesotan forests and grasslands. Moreover, in contrast with our findings, litter N enrichment was associated with shifts in microbial community composition in the Minnesota ecosystems (Hobbie et al., 2012).

Survey of non-transplanted litter. To determine broad effects of N enrichment, we assessed fungal functional genes in naturally occurring plant litter from control and N-fertilized plots. With this design, we compared N microbes on N litter in N plots with control microbes on control litter in control plots. Aside from applying N fertilizer, we did not manipulate the litter before collecting it. Thus, the samples represented a relatively naturally occurring collection of senesced plant litter at varying stages of decomposition. Under these conditions, the only significant change under N fertilization was a decline in cellulose-targeting AA9 gene frequency in basidiomycetes (Fig. 4). Once again, these results did not align with our hypothesis that fungal functional gene frequency should shift from N acquisition to C acquisition with N enrichment. To be sure, we focused on one cellulose-targeting gene family (AA9) in fungi. Even so, in a broad survey of known gene families for cellulolytic enzymes in bacteria + fungi,

Berlemont et al. (2014) reported no significant shift with N fertilization in these same samples. Activities of β-glucosidase and cellobiohydrolase do not change, either (Matulich and Martiny, 2014).

In other field studies, N fertilization can alter metagenomic profiles of bacteria (Fierer et al., 2012; Leff et al., 2015; Freedman et al., 2016). In some cases, the frequencies of carbohydrate metabolism genes increase (Leff et al., 2015). Few studies have explicitly examined shifts in metagenomic profiles of fungi, although Freedman et al (2016) noted no clear change with N fertilization in fungal genes associated with plant litter degradation. As the annotation of functional genes of fungi expands, so will our ability to identify their genetic profiles in environmental samples (Grigoriev et al., 2014), and to compare responses among N enrichment studies.

Overview. Taken together, our results suggest a rather subtle effect of N addition on fungal functional gene composition. In contrast to temperate forest soils which can exhibit symptoms of N saturation after long-term N deposition (e.g., Aber et al., 1989; Aber et al., 1998), our fertilization treatment did not consistently alter the genetic capacity for microbes to obtain N from grassland litter. Despite sustained N inputs, litter fungi at our site may still experience some degree of N limitation. Litter C:N ratios are relatively wide, and slight increases N in litter may not be sufficient to alleviate N limitation and reduce abundance of N-acquisition genes. On the other hand, the microbial inoculum in our experiment may have experienced higher N availability prior to sampling, potentially explaining why the microbial origin effect was consistent with our hypothesis but the litter effect was not. These seemingly contradictory results could be explained if our litter system fluctuates between N limitation and N excess over time or space.

We recognize a number of caveats in interpreting our results. For example, annotating functional genes of fungi is an active area of research, specifically with respect to taxonomic variation (Grigoriev et al., 2006). At the time of writing, fewer than 1000 whole genomes of fungi have been published, and

379they primarily represent the Ascomycota and Basidiomycota (Grigoriev et al., 2014). Many functional
380genes have been experimentally characterized in only a subset of fungal taxa (Grigoriev et al., 2006),
381limiting our ability to account for gene sequence variation across the fungal phylogeny. Accordingly,
382sequence matches from InterPro may be biased toward certain taxa, depending on the gene (McDowall
383and Hunter, 2011). This limits our ability to assign particular functional genes to even broad taxonomic
384groups. For instance, we did not have access to enough sequences of ammonium transporters of
385basidiomycetes to assign them with confidence to this phylum. Thus, we do not report ammonium
386transporter basidiomycete genes here. In addition, because we focused on a selection of functional
387genes that are well-annotated, we omitted other traits that could also be relevant. These include the
388extracellular cellulases β-glucosidase and cellobiohydrolase, which are difficult to distinguish between
389fungi and bacteria with current annotations (Berlemont and Martiny, 2013). Certainly, this will change as
390fungal gene annotation progresses, particularly owing to the 1000 Fungal Genome Project (Grigoriev et
391al., 2014). Of course, we again emphasize that the possession of a particular gene does not mean that
392the gene is expressed by the fungus.

393 Conclusion. Even though indirect N effects via microbial community changes largely followed our
394hypothesis, these effects were not realized in the standing litter in the experimental plots. Instead, the
395frequency of basidiomycete genes that break down crystalline cellulose displayed the *opposite* response,
396and none of the other gene families changed. It is possible that short-term increases in growth of fungi
397with more N acquisition genes were favored directly by N enrichment in the plot environment, and
398indirectly by higher N concentrations in N-fertilized plant litter. The plot environment and litter origin
399effects may have offset the microbe origin effects, leading to no significant change in genetic capacity for
400N acquisition overall. Even so, it is unclear why cellulose-targeting AA9 genes in basidiomycetes
401increased in the microbial community, but declined in the standing litter. Perhaps our system is close to
402the balance between N limitation and N excess, leading to conflicting results based on temporal or

spatial variation. Our results illustrate that even if the fungal community responds to N enrichment in accordance with allocation constraints, we may not necessarily expect corresponding changes to N or C dynamics.

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555Table 1. Statistical results for gene frequencies on reciprocally transplanted litter, including summary
556statistics.

Factor	F-ratio	P-value	N-fertilized (# per 100,000 reads) mean ±SE (n)	Control (# per 100,000 reads) mean ±SE (n)
Amino acid permease genes				
Plot environment	$F_{1,8}$ = 8.186	P = 0.021*	11.20 ±0.45 (8)	10.10 ±0.42 (8)
Litter origin	$F_{1,8}$ = 3.718	P = 0.090†	11.10 ±0.52 (8)	10.20 ±0.38 (8)
Microbe origin	$F_{1,8}$ = 16.34	P = 0.004**	9.93 ±0.43 (8)	11.38 ±0.37 (8)
Plot * litter	$F_{1,8}$ = 4.739	P = 0.061†		
Plot * microbe	$F_{1,8}$ = 0.870	P = 0.378		
Litter * microbe	$F_{1,8}$ = 1.253	P = 0.295		
Plot * litter * microbe	$F_{1,8}$ = 0.387	P = 0.551		
Ammonium transporter ascomycete genes				
Plot environment	$F_{1,8}$ = 43.68	P < 0.001***	5.81 ±0.13 (8)	5.00 ±0.19 (8)
Litter origin	$F_{1,8}$ = 9.991	P = 0.013*	5.60 ±0.23 (8)	5.21 ±0.19 (8)
Microbe origin	$F_{1,8}$ = 5.734	P = 0.044*	5.20 ±0.28 (8)	5.61 ±0.11 (8)
Plot * litter	$F_{1,8}$ = 7.716	P = 0.024*		
Plot * microbe	$F_{1,8}$ = 6.688	P = 0.032*		
Litter * microbe	$F_{1,8}$ = 0.083	P = 0.781		
Plot * litter * microbe	$F_{1,8}$ = 0.083	P = 0.781		
Cellulose-targeting AA9 ascomycete genes				
Plot environment	$F_{1,8}$ = 4.261	P = 0.073†	11.54 ±0.65 (8)	9.95 ±0.48 (8)
Litter origin	$F_{1,8}$ = 4.709	P = 0.062†	11.52 ±0.72 (8)	9.96 ±0.38 (8)
Microbe origin	$F_{1,8}$ = 4.261	P = 0.073†	10.18 ±0.72 (8)	11.30 ±0.48 (8)
Plot * litter	$F_{1,8}$ = 2.042	P = 0.191		
Plot * microbe	$F_{1,8}$ = 2.042	P = 0.191		
Litter * microbe	$F_{1,8}$ = 0.070	P = 0.798		
Plot * litter * microbe	$F_{1,8}$ = 0.473	P = 0.511		
Cellulose-targeting AA9 basidiomycete genes				
Plot environment	$F_{1,8}$ = 0.314	P = 0.590	1.08 ±0.07 (8)	1.00 ±0.08 (8)
Litter origin	$F_{1,8}$ = 0.089	P = 0.773	1.05 ±0.08 (8)	1.03 ±0.08 (8)
Microbe origin	$F_{1,8}$ = 8.067	P = 0.022*	1.18 ±0.06 (8)	0.89 ±0.06 (8)
Plot * litter	$F_{1,8}$ = 0.804	P = 0.396		
Plot * microbe	$F_{1,8}$ = 0.274	P = 0.615		
Litter * microbe	$F_{1,8}$ = 0.068	P = 0.800		
Plot * litter * microbe	$F_{1,8}$ = 0.873	P = 0.377		

557 †P < 0.10, *P < 0.05, **P < 0.01, ***P < 0.001

558

559Table 2. Statistical results for gene frequency on non-transplanted litter.

<u>Amino acid permease genes</u>		
N fertilization	$F_{1,2} = 0.013$	P = 0.921
Date	$F_{5,10} = 8.861$	P = 0.002**
N fertilization * date	$F_{5,10} = 1.305$	P = 0.336
<u>Ammonium transporter ascomycete genes</u>		
N fertilization	$F_{1,2} = 0.371$	P = 0.604
Date	$F_{5,10} = 4.863$	P = 0.016*
N fertilization * date	$F_{5,10} = 1.375$	P = 0.312
<u>Cellulose-targeting AA9 ascomycete genes</u>		
N fertilization	$F_{1,2} = 0.652$	P = 0.504
Date	$F_{5,10} = 8.465$	P = 0.002**
N fertilization * date	$F_{5,10} = 2.096$	P = 0.149
<u>Cellulose-targeting AA9 basidiomycete genes</u>		
N fertilization	$F_{1,2} = 186.8$	P = 0.005**
Date	$F_{5,10} = 43.44$	P < 0.001***
N fertilization * date	$F_{5,10} = 1.835$	P = 0.194

560*P < 0.05, **P < 0.01, ***P < 0.001

561Table 3. Synthesis of differences between microbial communities originating from the control plots
562versus the N-fertilized plots.

Greater in N microbes than control microbes	No significant difference	Lower in N microbes than control microbes
Fungal community (Allison et al., 2013; Matulich et al., 2015; Martiny et al., 2017)		
Relative abundance of e.g., Davidiellaceae & Sirobasidiaceae taxa	Fungal biomass	Relative abundance of e.g., *Cryptococcus* & Hypocreales taxa
Fungal functional genes frequency (this study)		
Cellulose-targeting AA9 basidiomycete genes	Cellulose-targeting AA9 ascomycete genes	Ammonium transporter ascomycete genes
		Amino acid permease eukaryote genes
Extracellular enzyme activity (Alster et al., 2013)		
	α-glucosidase (starch breakdown)	β-xylosidase (hemicellulose breakdown)
	Leucine aminopeptidase (peptide breakdown)	β-glucosidase (cellulose breakdown)
	Peroxidase (catalyzes oxidation reactions)	Cellobiohydrolase (cellulose breakdown)
	Acid phosphatase (mineralizes organic P)	N-acetyl-β-D-glucosaminidase (chitin breakdown)
		Polyphenol oxidase (lignin breakdown)
Biogeochemical processes (Allison et al., 2013; Hynson et al., 2015)		
Glycine uptake rate into fungi	Chitin uptake rate into fungi	
Litter mass loss (in N plots only)	Loss of C, N, protein, cellulose, lignin, and sugar from litter during decomposition	Loss of hemicellulose during decomposition

563

564

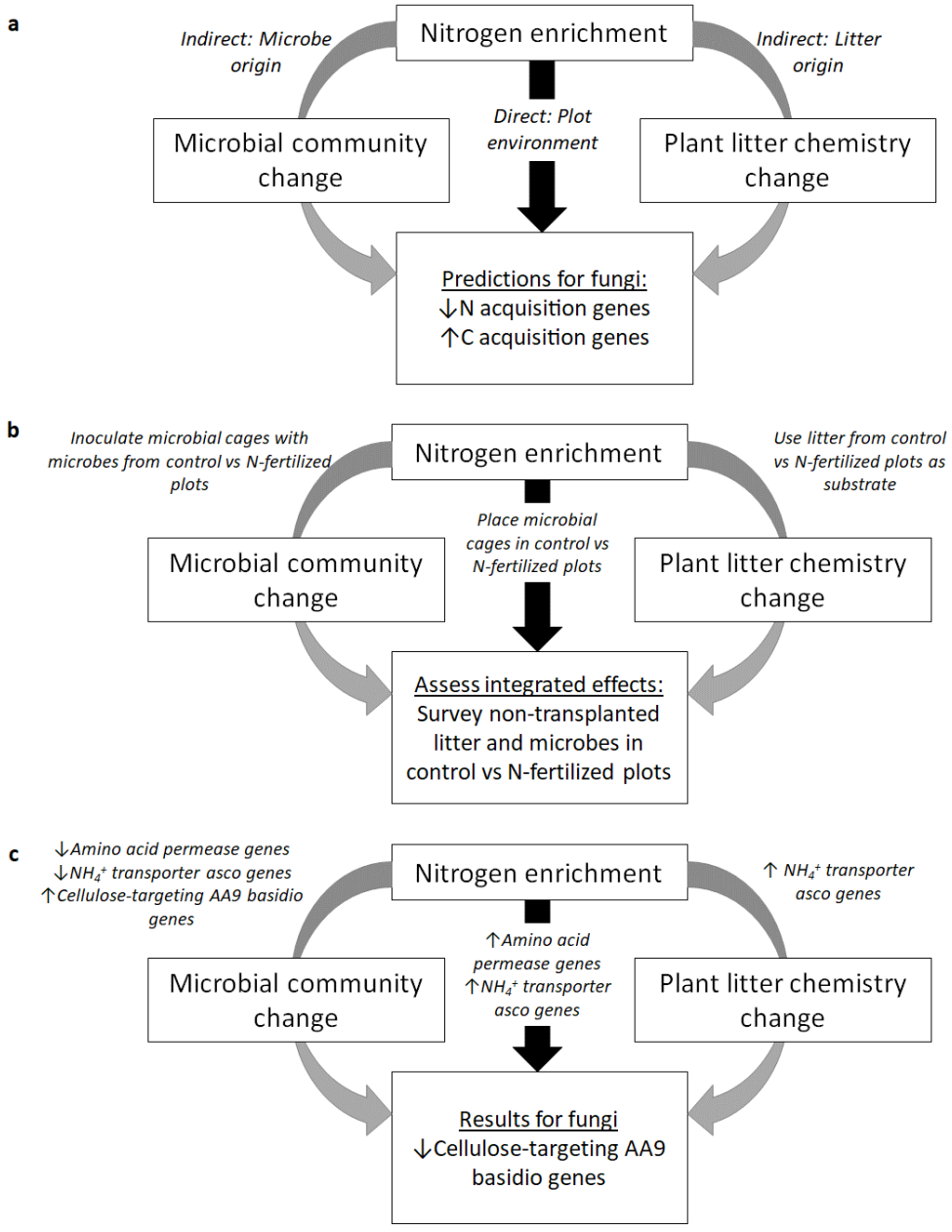
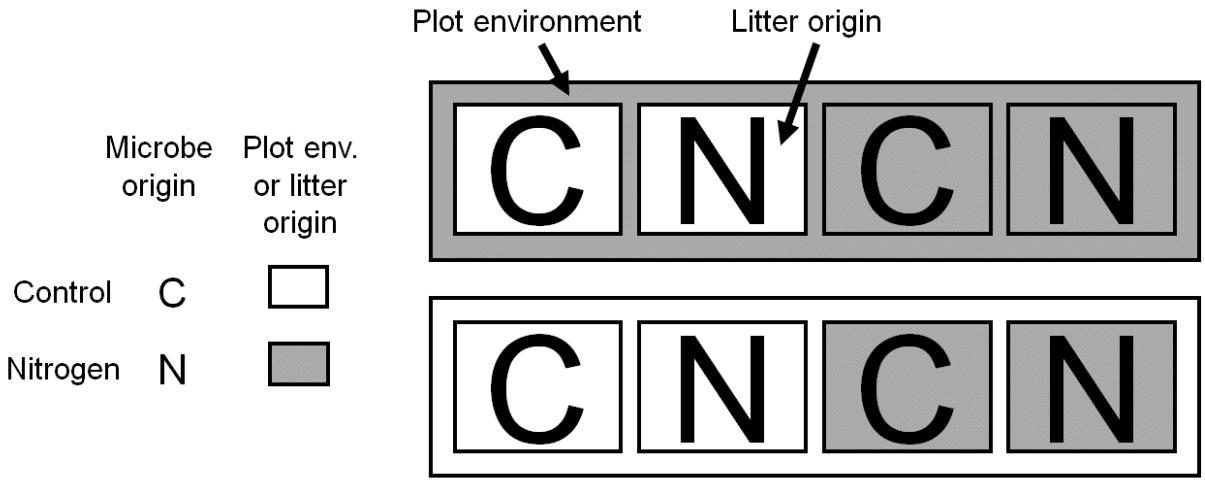


Figure 1. Hypothesized effects of N enrichment (a), approaches for testing effects (b), and observed effects (c) on the frequency of fungal functional genes related to N acquisition and C acquisition. For observed effects, only significant responses are shown. The decrease in cellulose-targeting AA9 basidiomycete genes in "Results for fungi" refers to results from a s urvey of unmanipulated litter. All other results are from a reciprocal transplant experiment.

572



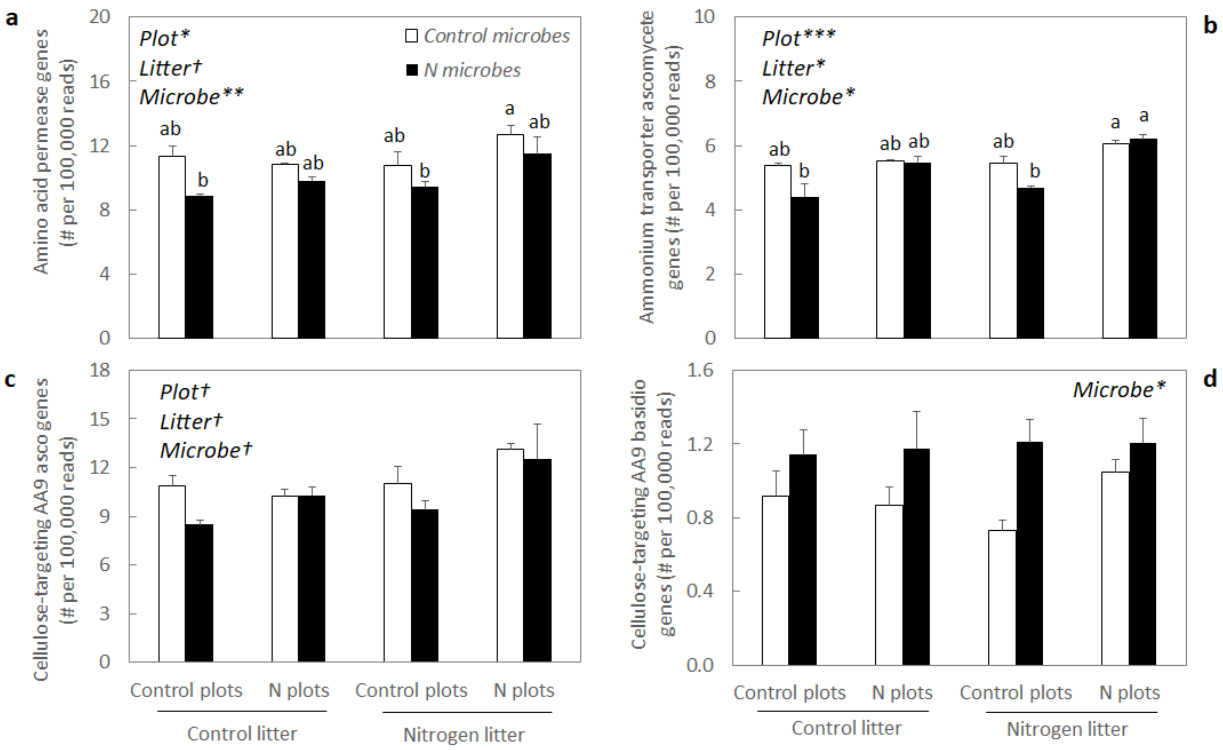
573

574**Figure 2.** Experimental design for one block in the litter manipulation. Plot environment, litter origin,

575and microbe origin were crossed in a fully factorial design. There were two treatments for each factor:

576control and N-fertilized. Control plots were unmanipulated, whereas N-fertilized plots received 60 kg N

577ha^{-1} y^{-1}.



578

579**Figure 3.** Frequencies of fungal functional genes in a reciprocal litter transplant using microbial cages.

580Microbial inocula collected from control versus N-fertilized plots were added to plant litter harvested

581from control versus N fertilized plots, and each combination was decomposed in control versus N-

582fertilized plots for three months. Microbial cages were collected March 2011. Cellulose-targeting AA9 is

583an enzyme that targets crystalline cellulose. Significant and marginally-significant effects are listed for

584each gene family. Treatment combinations associated with different letters were significantly different

585from one another. Bars are means +1SE of 2 replicates. †P < 0.10, *P < 0.05, **P < 0.01, ***P < 0.001

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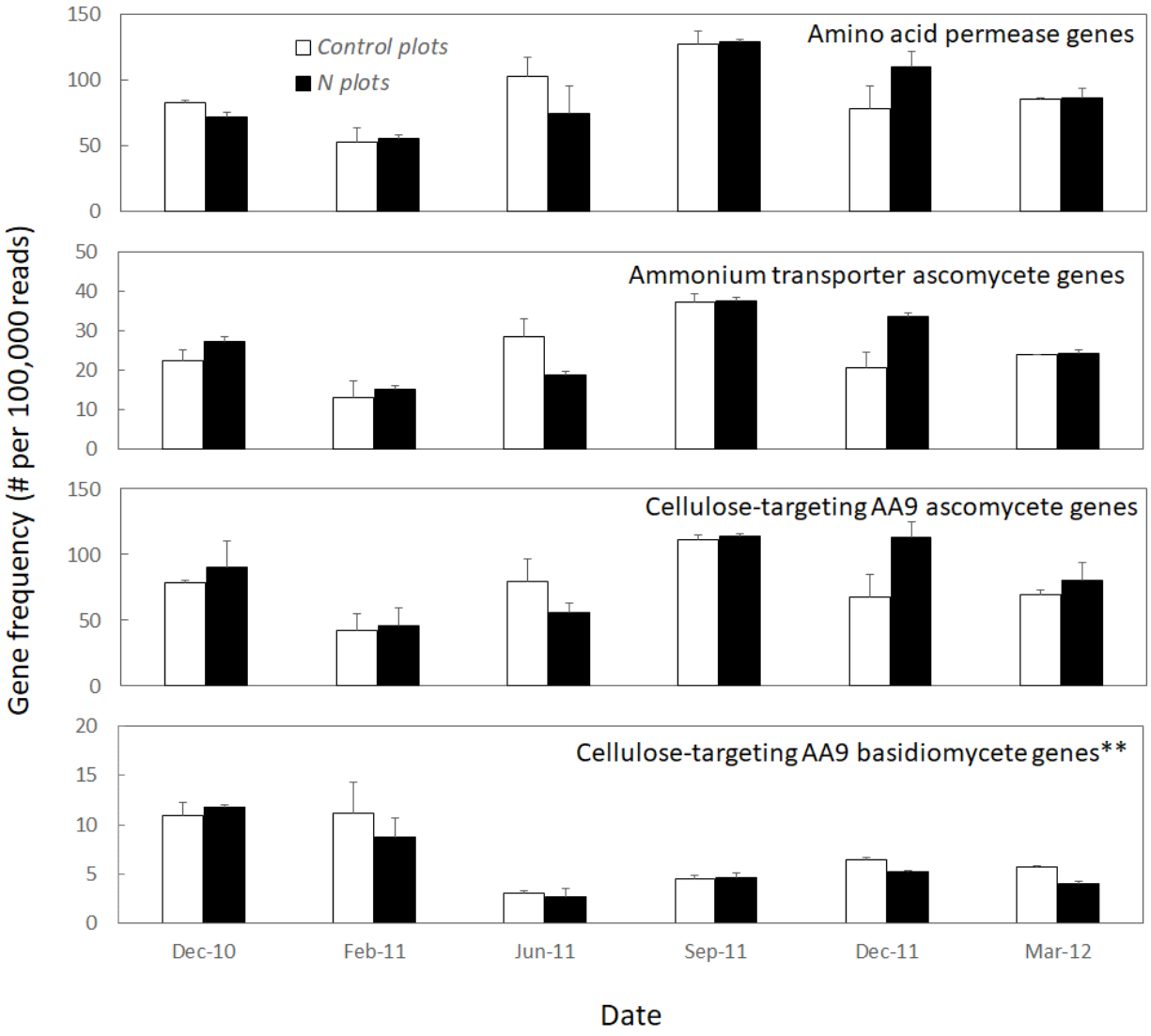

586

587**Figure 4.** Frequencies of fungal functional genes in a survey of non-transplanted, standing litter. The N

588effect was only significant for cellulose-targeting AA9 basidiomycete genes. Bars are means +1SE of 2

589replicates. **P < 0.01