1 Article Type: Article

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3 *Elevated N suppresses lignin decomposers*

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12 Anthropogenic N deposition increases soil C storage by reducing the relative abundance of
13 lignolytic fungi

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21 Manuscript submitted to: *Ecological Monographs*

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ecm.1288

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38

39 **ABSTRACT**

40 Atmospheric nitrogen (N) deposition has increased dramatically since preindustrial times and
41 continues to increase across many regions of the Earth. In temperate forests, this agent of global
42 change has increased soil carbon (C) storage, but the mechanisms underlying this response are
43 not understood. One long-standing hypothesis proposed to explain the accumulation of soil C
44 proposes that higher inorganic N availability may suppress both the activity and abundance of
45 fungi which decay lignin and other polyphenols in soil. In field studies, elevated rates of N
46 deposition have reduced the activity of enzymes mediating lignin decay, but a decline in the
47 abundance of lignolytic fungi has not been definitively documented to date. Here, we tested the
48 hypothesis that elevated rates of anthropogenic N deposition reduce the abundance of lignolytic
49 fungi. We conducted a field experiment in which we compared fungal communities colonizing
50 low-lignin, high-lignin, and wood substrates in a northern hardwood forest that is part of a long-
51 term N deposition experiment. We reasoned that, if lignolytic fungi decline under experimental
52 N deposition, this effect should be most evident among fungi colonizing high-lignin and wood
53 substrates. Using molecular approaches, we provide evidence that anthropogenic N deposition
54 reduces the relative abundance of lignolytic fungi on both wood and a high-lignin substrate.
55 Furthermore, experimental N deposition increased total fungal abundance on a low-lignin
56 substrate, reduced fungal abundance on wood, and had no significant effect on fungal abundance
57 on a high-lignin substrate. We simultaneously examined these responses in the surrounding soil
58 and forest floor, in which we did not observe significant reductions in the relative abundance of
59 lignolytic fungi or in the size of the fungal community; however, we did detect a change in

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60 community composition in the forest floor that appears to be driven by a shift away from
61 lignolytic fungi and towards cellulolytic fungi. Our results provide direct evidence that
62 reductions in the abundance of lignolytic fungi are part of the mechanism by which
63 anthropogenic N deposition increases soil C storage.

64 **Key words**: atmospheric N deposition, nitrogen, fungi, fungal communities, lignin, soil organic
65 matter, soil C, decomposition, forest, forest floor, Agaricomycetes

66 **INTRODUCTION**

67 Human activity has dramatically increased the production of reactive N (N_r) compounds,
68 with global N_r emissions projected to further double by mid-century (Galloway et al. 2004). As
69 a result, the annual amount of atmospheric N_r deposition in terrestrial and coastal ecosystems has
70 increased over historical levels and continues to rise in some parts of the Earth (Galloway et al.
71 2004). Rising rates of N_r deposition reduce soil respiration (Janssens et al. 2010) and increase
72 soil C content (Frey et al. 2014) in temperate forest soils. Therefore, atmospheric N deposition
73 has the potential to influence the storage of C in soils, which are a large and globally important C
74 pool (Prentice et al. 2001).

75 Field studies have suggested that elevated rates of N deposition may increase soil C
76 storage by depressing the rate of lignin decay in plant litter (Berg and Matzner 1997, Waldrop
77 and Zak 2006). Lignin is a protective compound found in plant secondary cell walls that limits
78 the rate of plant litter decay (Melillo et al. 1989). Fungi are the primary mediators of lignin
79 decay in terrestrial ecosystems (de Boer et al. 2005, Bugg et al. 2011b), with Agaricomycete
80 fungi being especially important for this process (Floudas et al. 2012). Changes in soil fungal
81 communities, mediated by greater rates of N deposition, represent a plausible mechanism by
82 which soil C storage has increased. For example, high inorganic N concentrations can suppress
83 fungal lignin decay in culture (Fenn and Kirk 1981, Leatham and Kirk 1983) as well as on
84 inoculated natural substrates (Osono et al. 2006); however this effect is not universal among
85 fungal species (Kaal et al. 1995). Similarly, increasing rates of N deposition reduce lignolytic
86 enzyme activity in forest floor, suggesting that high inorganic N availability also suppresses
87 fungal lignolytic enzyme activity under field conditions (Carreiro et al. 2000, DeForest et al.
88 2004a, Frey et al. 2004). Furthermore, it has been hypothesized that, by reducing the activity of
89 lignolytic fungi, anthropogenic N deposition will weaken the competitive advantage of these
90 organisms over other decomposers and thereby decrease their abundance within the soil

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microbial community (DeForest et al. 2004b). As such, the biological mechanism proposed to explain increased soil C storage under elevated N deposition posits that higher N availability reduces both the activity and abundance of fungi involved in lignin decay (DeForest et al. 2004b, a).

However, it has remained uncertain whether lignolytic fungi actually decline under elevated rates of N deposition. For example, elevated N deposition has been observed both to significantly alter (Eisenlord et al. 2013, Entwistle et al. 2013, Hesse et al. 2015, Morrison et al. 2016) and to have no effect (Freedman et al. 2015) on fungal community composition. Furthermore, even when elevated N deposition has been found to alter fungal community composition, it has not been clear whether such a change is driven by a decline in lignolytic fungi. This is, in part, because past studies have often examined fungal communities at taxonomic levels that were too coarse to obtain sufficient insight into the physiology and ecology of the affected taxa (Edwards et al. 2011, Entwistle et al. 2013). However, such limitations can now be overcome by taking advantage of recent improvements in next-generation sequencing, automated tools for fungal sequence classification (Liu et al. 2012, Schloss et al. 2016), and knowledge of the phylogenomic distribution of fungal genes involved in lignin decay (Floudas et al. 2012, Nagy et al. 2015).

A direct comparison of fungal communities colonizing low- and high-lignin substrates under ambient and elevated rates of N deposition should also improve our understanding of the role of lignin and lignolytic fungi in ecosystem responses to anthropogenic N. We hypothesized that anthropogenic N deposition slows decomposition by reducing the abundance of fungi that mediate the process of lignin decay, specifically the abundance of fungi with known lignolytic physiologies. If anthropogenic N deposition alters fungal community composition in a manner consistent with our proposed mechanism, we reasoned that these effects should be more evident in fungal communities colonizing lignin-rich substrates, relative to those colonizing low-lignin substrates. Furthermore, because cellulose and hemicellulose are decomposed more rapidly than lignin, making partially decayed materials relatively enriched in lignin compared to their initial condition, we similarly reasoned that the negative effects of anthropogenic N deposition on lignolytic fungi should become more apparent with time. Finally, if greater inorganic N availability suppresses lignin decay, undecayed lignin should protect larger proportions of cellulose and hemicellulose, making them inaccessible to the fungal community as an energy

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122 source. We hypothesized that, as a result, the overall size of the fungal community would be
123 smaller under experimental N deposition, particularly on decomposing plant litter and substrates
124 with intact lignocellulose (*i.e.*, wood and high-lignin substrates, in this study).

125 To test our hypotheses, we allowed low-lignin, high-lignin, and wood substrates to be
126 colonized by fungi in a northern hardwood forest that has received experimental N deposition
127 since 1994. We investigated whether experimental N deposition altered fungal community
128 composition on decomposing substrates that vary in their biochemical composition, and
129 subsequently examined which types of fungal physiologies primarily account for dissimilarities
130 in fungal community composition under experimental N deposition. Furthermore, we directly
131 tested whether fungal groups involved in lignin decay, specifically Agaricomycetes, as well as
132 highly lignolytic taxa within the Agaricomycetes, decline in response to experimental rates of N
133 deposition. Lastly, we assessed fungal abundance on each of our substrates over time using
134 quantitative PCR (qPCR). We also assessed fungal community composition and fungal
135 abundance in the mineral soil and the forest floor.

136 **METHODS**

137 **Site Description.** Our study site consists of a sugar maple (*Acer saccharum* Marsh.) dominated
138 northern hardwood forest in Lower Michigan, located at 43°40' N, 86°09' W. It is one of four
139 sites in the Michigan Gradient Experiment, a long-term elevated atmospheric N deposition
140 experiment in Michigan, USA. Soils are well-drained sandy typic Haplorthods of the Kalkaska
141 series. The forest floor (O_i) is dominated by sugar maple leaf litter.

142 The site contains six 30-m x 30-m plots. Three plots receive ambient N deposition,
143 whereas the other three have received ambient N deposition plus 3 g NO_3^- -N m^{-2} y^{-1} since 1994,
144 an amount consistent with future levels expected in some portions of northeastern North America
145 and Europe by 2050 (Galloway et al. 2004). Treatments are applied as $NaNO_3$ pellets in 6 equal
146 additions of 0.5 g N m^{-2} from April to September. Each treatment plot is surrounded by a 10-m
147 treated buffer to reduce edge effect, which also receives the N deposition treatment.
148 Experimental N addition has not altered soil pH, base saturation, matric potential, or forest floor
149 conductivity (Patterson et al. 2012).

150 **Substrate selection and deployment.** Different products of the forest industry vary in their
151 lignocellulose content and biochemistry; for example, newsprint is produced by mechanical

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pulping, with the resulting paper having a high lignin content (Ragnar et al. 2000). Kraft paper, by contrast, is subjected to chemical pulping which reduces lignin content by 50% (Kleppe 1970) as well as breaking the bonds between the remaining lignin, cellulose, and hemicellulose (Ragnar et al. 2000). Wood has not been subjected to physical or chemical alteration; therefore, it has both intact cell walls and intact lignocellulose. In our study, we use birch wood (*Betula* spp.; Woodsies, Loew Cornell, Cincinnati, OH), a high-lignin paper substrate (newsprint, white 3401, Pacon Corp., Appleton, WI), and a low-lignin paper substrate (kraft paper; #60, Caremail, ShurTech Brands, LLC Avon, OH) in a field decomposition experiment to make comparisons between fungal communities targeting weakly and highly lignified substrates. To characterize the initial biochemistry of these materials, we submitted dried, ground kraft paper, newsprint, and wood samples to the Soil & Forage Lab at the University of Wisconsin-Madison for analysis of lignin, cellulose, hemicellulose, and nitrogen content (Table 1).

Whereas wood is a natural product, the paper (high-lignin and low-lignin) substrates do not have precise analogues in nature. Our primary motivation for using high-lignin, low-lignin, and wood substrates was that it specifically allowed us to examine decomposition of products which are different from one another in terms of plant biochemistry. Beyond allowing us to easily manipulate plant litter biochemistry in our decomposition experiment, using these commercially available forest-products as our substrates held several advantages over using plant litter and wood collected from our study site. The substrates are homogenous in biochemical composition within samples, unlike field-collected leaf litter or woody debris which contains tissues of varying biochemical composition within individual samples (*e.g.*, leaf petioles, bark). Additionally, factory-processing of forest products results in homogeneity in physical and chemical characteristics between samples, unlike field-collected materials which exhibit an unavoidable degree of biological variability. Furthermore, plant litter or woody debris from the field may have already experienced the very earliest phases of decomposition prior to collection; using forest industry products ensures we are starting with undecayed materials and removes another potential source of variation. Finally, by using materials which were not collected from our study sites, we avoid any potentially confounding effects of experimental N deposition on initial plant biochemistry (Zak et al. 2004, Xia et al. 2015).

In preparation for deploying substrates to the field, bags of nylon screen with a mesh size of 1.13 mm x 1.30 mm were filled with ~6 g of either kraft paper or newsprint, which consisted

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183 of four pieces of kraft paper or eight pieces of newsprint (10.8 cm x 13.97 cm), in order to give
184 the low- and high-lignin paper substrates similar initial mass and surface area. The wood
185 substrate (15 cm x 2 cm x 0.15 cm) was not placed in a litter bag. Processed forest industry
186 products are subjected to drying at elevated temperature for sterilizing purposes (Ross 2010);
187 because of this, we did not conduct an additional sterilization step prior to assembling litter bags
188 or deploying substrates to the field.

189 After recording the initial mass of each substrate sample, we tethered one substrate of
190 each type (high-lignin, low-lignin, wood) to each stake, using < 0.5 m of monofilament. In
191 November 2011, one stake for each collection date was placed at five points within each plot,
192 specifically 3 m from each corner of the rectangular plot and within 1 m of the plot center. This
193 design enabled us to collect decomposing substrates of each type over two dates from the same
194 locations within plots, thus minimizing spatial variability between samples. When placing
195 substrates in the field, we removed the O horizon, placed the substrates on top of the mineral
196 soil, and then covered the substrates by returning the O horizon.

197 **Field collection.** We collected substrates (30 of each type per collection date) from the field after
198 7 and 18 months of decay. During our first collection date, we also collected the forest floor (Oi,
199 Oe, Oa horizons) from a 100 cm^2 area and a 5 cm-deep mineral soil core from within 1 m of each
200 substrate location in order to assess the composition and abundance of the forest floor and soil
201 fungi. Samples were transported to the lab on ice, and stored at –80 °C prior to DNA extraction.

202 **Sample preparation and characterization.** Samples were weighed to determine mass loss.
203 Soil cores were manually homogenized inside a sterile plastic bag. We cut leaf litter and high
204 and low-lignin substrates into ~25-mm^2 pieces with sterilized scissors and manually
205 homogenized the material. Wood was first cut into ~8 mm^2 pieces with sterilized pruning
206 shears, which were then collected in a sterile plastic bag, shaken to mix, and pulverized with a
207 hammer. A subsample was dried for 24 h at 105 ° C to determine moisture content. Another
208 subsample was taken for DNA extraction.

209 **DNA extraction.** We extracted DNA from each sample in triplicate using a PowerLyzer®
210 PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). We added ~0.125 g
211 forest floor, ~0.18 g wood, or ~0.25 g soil, ~0.25 g high-lignin substrate, or ~0.25 g low-lignin
212 substrate to a Powerlyzer® Glass Bead Tube with 0.1 mm glass beads. We bead-beat the

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213 material for extraction on a PowerLyzer® 24 homogenizer for 45s at 2500 rpm for soil, 1 min at
214 3500 rpm for forest floor, 1 min at 4500 rpm for wood, or 45s at 4500 rpm for the low-lignin and
215 high-lignin substrates. We followed manufacturer's instructions with the several modifications.
216 Specifically, we increased 4 °C incubation times to 10 minutes and added two centrifugations
217 and a 10 minute ethanol evaporation step in order to remove residual wash buffer and ethanol
218 prior to DNA elution. In order to maximize DNA yield at the elution step, we incubated the
219 molecular biology grade water on the membrane for one minute before centrifuging; we then
220 repeated this process using the eluted DNA. The quality of the extracted DNA was measured on
221 a NanoDrop™ spectrophotometer (ThermoScientific, Waltham, MA, USA). Triplicate DNA
222 extractions were pooled prior to amplification.

223 **Fungal 28S rDNA amplification and sequencing.** We amplified the 28S rRNA gene using 10
224 barcoded pairs of fungal primers LR0R (5' ACCCGCTGAACTTAAGC 3') and LR3 (5'
225 CCGTGTTTCAAGACGGG 3') (http://sites.biology.duke.edu/fungi/mycolab/primers.htm).
226 Barcode pairs 1, 42, 3, 40, 7, 33, 11, 17, 20, and 24 were selected from among 96 paired
227 barcodes available for multiplexing on the Pacific BioSciences (PacBio) RS II sequencer
228 (https://github.com/PacificBiosciences/Bioinformatics-
229 Training/blob/master/barcoding/pacbio_barcodes_paired_nopadding.fasta). Reactions contained
230 2.5 µL dNTPs (2 mM each), 2.5 µL Expand™ High Fidelity 10x Buffer (Roche, Indianapolis,
231 IN) with 15 mM $MgCl_2$, 0.5 µL bovine serum albumin (20 mg mL^{-1}), 0.5 µL of barcoded LR0R
232 (20 µM), 0.5 µL barcoded LR3 (20 µM), 16 µL molecular biology-grade water, 0.5 µL
233 Expand™ High Fidelity DNA polymerase, and 2 µL of 10x diluted target DNA. For samples
234 that amplified weakly, PCR was repeated with template DNA using a lower dilution factor or no
235 dilution until products of satisfactory concentration were obtained. Thermocycling conditions
236 were: initial denaturation (5 min, 95˚C), followed by 25 cycles consisting of denaturat ion (30 s,
237 95 ˚C), primer annealing (30 s, 54 ˚C), extension (75 s, 72 ˚C), with cycling followed by a final
238 extension (7 min, 72˚C). Each sample was amplified in triplicate and these triplicate products
239 were pooled prior to purification.

240 We purified the PCR products using a MinElute PCR Purification Kit (Qiagen, Valencia,
241 CA). The quality of purified PCR products was measured on a NanoDrop™ spectrophotometer,
242 while the concentration of PCR products was measured fluorometrically using a Quant-IT™
243 PicoGreen® dsDNA kit (Life Technologies, Carlsbad, CA, USA) on a Synergy HT microplate

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244 reader (BioTek Instruments, Winooski, VT, USA). We combined 10 barcoded samples in
245 equimolar concentrations for multiplexing on a PacBio SMRT® chip. Samples were sequenced
246 at the University of Michigan sequencing core on a PacBio RS II sequencer with P6-C4
247 chemistry. We obtained circular consensus sequencing files with 5x coverage; these files have
248 been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read
249 Archive (SRA) as described in the Data Accessibility section of this manuscript.

250 **28S rDNA sequence data processing**. We processed the 28S rDNA sequences in mothur
251 v.1.31.2 (Schloss et al. 2009). We removed sequences that had quality scores lower than 25 over
252 windows of 50 bp, > 1 mismatch to the primer or barcode, > 1 ambiguous nucleotide,
253 homopolymers of > 8 nucleotides, or lengths that were outside of our expected amplicon size of
254 550-750 bp. We aligned sequences with the Ribosomal Database Project (RDP) version 7 fungal
255 28S rRNA (LSU) training set (Liu et al. 2012, Cole et al. 2014), which had been previously
256 obtained from http://mothur.org/wiki/RDP_reference_files and aligned (Freedman et al. 2015) in
257 Clustal Omega (Sievers et al. 2011) according to default parameters. Chimeric sequences were
258 identified with UCHIME (Edgar et al. 2011) and were removed. Sequences were classified
259 using the mothur-compatible taxonomy file that accompanied the RDP v.7 LSU fungal training
260 set (available at: http://mothur.org/wiki/RDP_reference_files) using a bootstrap cutoff of 51.
261 Non-fungal sequences and sequences that were not able to be classified beyond kingdom were
262 removed. We clustered operational taxonomic units (OTUs) at 97% similarity using the
263 cluster.split command with sequences split for clustering at the class level. The most abundant
264 sequence in each OTU was considered the representative sequence for that OTU and identified
265 using NCBI BLAST®. Our complete pipeline and all associated files used in sequence
266 processing are available with the archived data associated with this publication. To assist in
267 subsequent data interpretation of our fungal community composition analyses, we initially
268 explored the overlap of fungal OTUs occurring on substrates, soil, and the forest floor and
269 examined the effects of sample type, time, and experimental N deposition on fungal OTU
270 richness. For the examination of overlap of fungal OTUs among substrates, soil, and forest
271 floor, we present values averaged across substrates for simplicity, as the trends for each type of
272 substrate were similar in this regard.

273 **Fungal community composition and dissimilarity analyses.** To test the hypothesis that
274 experimental N deposition alters fungal community composition, we compared fungal

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275 communities from our ambient and experimental N deposition treatments with two-factor
276 permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) with 9999
277 permutations and the default options for sum of squares and permutation of residuals. Relative
278 OTU abundances, calculated after removing singleton OTUs, were used for analyses.
279 PERMANOVA analyses were performed in PRIMER-E (version 6) with the PERMANOVA+
280 add-on (Clarke and Gorley 2006). We restricted analysis to OTUs that comprised≥ 0.5% of at
281 least one sample. OTU abundances were square-root transformed, and pairwise distances among
282 samples were calculated as Bray-Curtis similarity.

283 For the high-lignin and wood substrates, we had data for samples collected at 7 and 18
284 months, allowing us to analyze communities on these substrates individually in response to N
285 deposition treatment and time as factors. However, we had only one time-point for forest floor
286 and soil, which was only collected once, and for the low-lignin substrate, which decayed too
287 rapidly in the field for a second collection date. We were unable to perform individual
288 PERMANOVA comparisons for samples obtained from only one time-point (*i.e.,* the low-lignin
289 substrate, soil, and forest floor) because the statistical power of our experiment ($n = 3$) is too low
290 for PERMANOVA to be able to detect differences between communities even where they are
291 present. Therefore, we analyzed all samples collected after 7 months of decay together with
292 sample type and N deposition treatment as factors; we followed PERMANOVA with pairwise
293 tests to further examine differences in fungal community composition between sample types.

294 We performed similarity percentages (SIMPER) analyses (Clarke 1993) in PRIMER-E
295 (Clarke and Gorley 2006) to determine whether the changes in fungal community composition
296 we detected in response to experimental N deposition were driven by shifts from strong lignin
297 decomposers to weaker decomposers of lignin. SIMPER analyses were one-factor, with each
298 sample type and collection date considered independently; all other conditions for SIMPER
299 analyses were the same as those previously used for PERMANOVA. From the results for each
300 of these comparisons, we report the ten OTUs accounting for the highest amount of dissimilarity
301 between fungal communities under ambient and experimental N deposition. We subsequently
302 identified the closest known species for each reported OTU using a megablast search through the
303 NCBI BLAST® portal. Based on our knowledge of the biology of these species, we
304 subsequently classified each OTU as either a white-rot and lignolytic litter decay fungus, a soft-
305 rot & cellulolytic/hemicelluloytic litter decay fungus, a brown-rot fungus, a weakly lignolytic

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306 fungus, or a mycorrhizal/biotrophic fungus, as summarized in Appendix S1: Table S1. Although
307 some ectomycorrhizal (ECM) fungi may be lignolytic (Shah et al. 2015), a paucity of data on the
308 extent and distribution of this ability among ECM fungi led us to classify them as
309 mycorrhizal/biotrophic fungi rather than include them in either the white-rot and lignolytic litter
310 decay fungi or weakly lignolytic fungi categories. In order to determine whether fungi from each
311 of these physiologies were responding positively or negatively to elevated rates of N deposition,
312 we calculated the mean abundance of each of these physiologies under ambient and experimental
313 N deposition, the 95% confidence intervals around those means, and the change in relative
314 abundance for these physiologies under experimental N deposition.

315 **Relative abundance of Agaricomycetes and highly lignolytic taxa.** We hypothesized that
316 experimental N deposition would reduce the relative abundance of lignolytic fungi, especially on
317 high-lignin and wood substrates. Therefore, we examined the relative abundances of two groups
318 of fungi colonizing our substrates as well as residing in soil and forest floor: (1) the class
319 Agaricomyetes, a class which contains many lignin-decomposing species, and (2) a selected
320 subset of Agaricomycete taxa which had been identified as having highly lignolytic physiologies.
321 We identified this suite of highly lignolytic Agaricomycete fungi *a priori* (Table 2 & Appendix
322 S2: Table S1) based on the criteria that they consistently demonstrated high lignin loss and/or
323 selectivity for lignin in published studies and were represented by > 20 sequences across the data
324 set, *i.e.*, > 0.006898% of all sequences in our data set. A list of taxa which we excluded from
325 consideration and our justifications for doing so are also provided in Appendix S3: Table S1.
326 These highly lignolytic taxa were treated as a group, rather than as individual taxa, for the
327 purposes of relative abundance comparisons. For each sample, we calculated the relative
328 abundance of Agaricomycetes as a proportion of total fungal sequences, and the relative
329 abundance of highly lignolytic taxa as a proportion of Agaricomycete sequences. Considering
330 Agaricomycetes as a class and "highly lignolytic taxa" as a group allowed us to make
331 comparisons across substrates, soil, and forest floor, which may have different fungal
332 communities from each other (Lindahl et al. 2007, Edwards and Zak 2010); for example, wood
333 and forest floor may harbor different lignolytic taxa from each other, as some species are
334 specialists for either leaf-litter or wood (Floudas et al. 2015, Nagy et al. 2015).

335 **Fungal ITS1 qPCR.** To test the hypothesis that the overall size of the fungal community would
336 be smaller on substrates with intact lignocellulose (*i.e.*, the wood and high-lignin substrate) as

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337 well as in in soil and forest floor under experimental N deposition, we conducted quantitative
338 PCR (qPCR) of the fungal ITS1 region to measure fungal abundance. We calculated ITS1 copy
339 number for each sample as both number of copies per µg of extracted DNA and number of
340 copies per g of soil, which enabled us to assess both the abundance of fungi as a proportion of
341 the total microbial community DNA and total fungal abundance in soil, forest floor, and
342 decomposing substrate biomass, respectively.

343 Quantification of fungi with qPCR can be influenced by other variables besides the
344 amount of fungi present including interspecific variation in either the genomic copy number or
345 length of the selected marker, as well as amplification biases from the selected primers. To
346 minimize these potential problems, we selected the fungal ITS1 region for fungal qPCR because
347 it is both short (~300 bp) and does not vary significantly in length among fungal phyla
348 (Bellemain et al. 2010, Toju et al. 2012). Furthermore, we selected fungal primers ITS1f (5'
349 CTTGGTCATTTAGAGGAAGTAA 3') and 5.8s (5' CGCTGCGTTCTTCATCG 3') to amplify
350 this region (Gardes and Bruns 1993, Vilgalys undated); prior to employing this primer set in
351 qPCR, we verified that it was both specific for fungi and had broad coverage across fungal taxa
352 (data not shown). Although the number of ITS copies within fungal genomes varies both within
353 and between species (Vilgalys and Gonzalez 1990), interspecific variation in genomic ITS copy
354 number did not prevent accurate assessment of fungal ITS copy number in qPCR of mixed
355 template samples in a previous study (Manter and Vivanco 2007); hence, we do not have any
356 reason to think variation in copy number between species in our template DNA is likely to be an
357 impediment to quantifying fungal ITS1 copy number in the soil, forest floor, and substrates here.

358 Standard DNA for qPCR was prepared by cloning the ITS1 region of *Agaricus bisporus*
359 with a TOPO® TA pCR 2.1 Cloning Kit (Life Technologies). The insert was subsequently
360 amplified with M13 primer to create a linear target appropriate for qPCR (Hou et al. 2010). The
361 DNA concentration of the qPCR standard was quantified fluorometrically, in the manner
362 previously described for 28S rDNA PCR products, and its copy number was calculated
363 according to manufacturer instructions (AppliedBiosystems 2003). Quantitative PCR was
364 calibrated with a standard curve ranging from 1.71×10^3 to 1.03×10^6 copies/µL. Prior to qPCR,
365 sample DNA was also quantified fluorometrically and diluted as needed in order to obtain an
366 appropriate target concentration.

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367 Each 25 µL qPCR consisted of 0.625 µL of each primer (20 µM), 0.375 µL ROX
368 reference dye (500x dilution), 0.5 µL bovine serum albumin (20 mg mL^{-1}), 9.375 µL molecular
369 biology-grade water, 12.5 µL Brilliant III Ultra-Fast SYBR® Green qPCR master mix (Agilent
370 Technologies, Santa Clara, CA, USA, cat. #600882), and 1 µL target DNA. Reactions for
371 samples contained 0.8 - 11 ng DNA. Quantitative PCR assays were performed on a Stratagene
372 Mx3000P qPCR System (Agilent Technologies) using the following thermocycling program:
373 initial denaturation (5 min, 95 ˚C), followed by 40 cycles of denaturation (45s, 95 ˚C), annealing
374 (30s, 53˚C), and extension (30s, 72 ˚C), and a final dissociation curve set at manufacturer default
375 settings. We ran all standards and no template controls in triplicate and all unknown samples in
376 duplicate, allowing us to run to run all samples from the same set (*i.e.,* same sample type &
377 collection date) in a single 96-well run. Each run was performed three times for quality control.
378 Efficiencies of all qPCR runs were ≥ 80 %. The R^2 values for each run ranged from of 0.991 -
379 0.999. More detailed quality control information and all qPCR data is available in the Dryad
380 data archive associated with this paper.

381 **Statistical analyses.** We performed statistical analyses for substrate mass loss, the relative
382 abundances of Agaricomycetes and highly lignolytic taxa, and qPCR data with IBM SPPS
383 Statistics v. 23 software. Fungal OTU richness analyses were performed in the R statistical
384 environment (R Core Team 2017) with the R commander interface (Fox 2005). Prior to analysis,
385 values for substrates, soil, and forest floor were averaged across each plot. No data
386 transformations were applied to mass loss, fungal richness, or relative abundance data, but qPCR
387 data were $\log_{10}$ transformed so that the data would meet the assumption of homoscedasticity
388 required for ANOVA. We conducted independent sample t-tests for comparisons with only one
389 factor (*i.e*., N deposition treatment) and two-way ANOVA for comparisons with two factors (*i.e*.,
390 N deposition treatment and either collection date or sample type). For comparisons in which
391 there was a significant interaction between N deposition and collection date, we also considered
392 collection dates separately with independent-sample t-tests.

393 **RESULTS**

394 **Field collection and mass loss.** High mass loss and evidence of faunal grazing (*e.g.,* distinct
395 holes in substrate) were visually apparent on low-lignin substrates after 7 months of decay. By
396 contrast, the high-lignin substrate was entirely intact after 7 months of decay, with visible
397 evidence of fungal colonization, but no apparent damage by soil fauna. Wood samples were

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398 intact at 7 months, with only some showing visible evidence of fungal colonization. We were
399 able to collect high-lignin and wood substrates after 18 months of decay, but the relatively rapid
400 decay of the low-lignin substrate prevented collection at this second time-point. At 18 months,
401 the high-lignin substrate was still intact, but visible evidence of fungal colonization was more
402 extensive. After 18 months of decay, many wood samples were structurally weak, suggesting
403 more extensive decay had occurred.

404 When mass loss was examined across both of the N deposition treatments, the percentage
405 of mass lost after 7 months of decay from the low-lignin (–61%), high-lignin (–26%), and wood
406 substrates (–10%) varied significantly among substrates of different types (two-way ANOVA; F
407 $= 218.947$, $P < 0.001$). This result confirmed that our selection of substrates, which varied in
408 their initial lignocellulose content, represented a range of decomposability under field conditions.
409 After 18 months of decay, the high-lignin substrate had lost 30% of its mass, an additional loss
410 of only 4% compared to that at 7 months, demonstrating that the high-lignin substrate was highly
411 resistant to decay.

412 After 7 months, substrates decomposed under experimental N deposition exhibited no
413 significant differences in the amount of mass lost compared to those decomposed under ambient
414 N deposition (Figure 1). Similarly, mass loss of the high-lignin substrate after 18 months of
415 decay under experimental N deposition (28%) was still not significantly different from the
416 amount of mass lost under ambient N deposition (32%) over that same time period. We were
417 unable to accurately assess mass loss of wood after 18 months of decay, because several wood
418 substrate samples were fragmented prior to or during collection of samples at this time-point.

419 **Fungal 28S sequences and OTUs.** Sequencing produced a total of 575,339 reads with 5x
420 circular consensus coverage. Forty-seven percent of sequences were removed during quality-
421 control steps, a percentage of sequence loss which is similar to those that have been previously
422 reported for targeted gene sequencing of soil microbial communities with the PacBio RS II
423 sequencing platform (Freedman and Zak 2014, Freedman and Zak 2015). Ninety-five percent of
424 the resulting sequences were fungal, yielding a total of 289,920 high quality fungal sequences; of
425 these, 126,371 sequences were unique.

426 These fungal sequences initially clustered to 9115 operational taxonomic units (OTUs) at
427 the 97% sequence similarity level; of these, 2589 OTUs remained after removal of singletons.
428 Most OTUs were of low abundance with only 33% of OTUs represented in the data set by more

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429 than 10 sequences and only 10% of OTUs represented in the data set by more than 100
430 sequences. Mean Good's coverage for 97% sequence similarity OTUs was 94% for substrates
431 and 88% for soil and forest floor samples.

432 Of the fungal OTUs occurring on each type of substrate after 7 months of decay, ~26% of
433 OTUs occurring on each substrate were also observed in both the mineral soil and the forest floor
434 fungal communities. Another ~25% of OTUs occurring on each substrate were detected in the
435 forest floor, but not in the soil, whereas ~8% of fungal OTUs occurring on each type of substrate
436 were also observed in the soil, but not in the forest floor, respectively. Thus, approximately one-
437 half of fungal OTUs colonizing substrates also colonized the forest floor, while approximately
438 one-third of OTUs occurring on each substrate were also detected in the mineral soil. The
439 remaining ~40% of OTUs on each type of substrate were not observed in either the forest floor
440 or soil fungal communities. Among fungi occurring on substrates which had decayed for 7
441 months in the field, 57% of observed OTUs were unique to only one type of substrate, 26% of
442 OTUs occurred on two different types of substrate, and 17% of fungal OTUs were found on all
443 three substrates.

444 Fungal OTU richness was not different among wood, high-lignin, or low-lignin substrates
445 after 7 months of decay (two-way ANOVA, $F = 1.73$, $P = 0.21$). However, when we compared
446 fungal OTU richness among substrates, forest floor, and soil collected during the first collection
447 date, observed fungal OTU richness was significantly different between sample types (one-way
448 ANOVA, $F = 14.3$, $P < 0.0001$); this response was driven by the significantly higher fungal
449 OTU richness of the forest floor in comparison to either mineral soil or substrate fungal
450 communities (Tukey tests, $P \leq 0.001$). Furthermore, time (*i.e.*, 7 or 18 months of decay) did not
451 alter fungal OTU richness on either high-lignin (two-way ANOVA, $F = 0.38$, $P= 0.55$) or wood
452 (two-way ANOVA, $F = 0.06$, $P = 0.81$) substrates. Experimental N deposition did not alter
453 fungal OTU richness either on substrates decayed for 7 months in the field (two-way ANOVA, F
454 $= 1.73$, $P = 0.21$), in the surrounding soil or forest floor collected on the same date (two-way
455 ANOVA, $F = 2.58$, $P = 0.15$), or on high-lignin substrate across 7 and 18 months of decay (two-
456 way ANOVA, $F = 0.21$, $P = 0.66$); however, experimental N deposition significantly increased
457 fungal OTU richness on wood substrates (+ 40%, two-way ANOVA, $F = 6.24$, $P = 0.04$) when
458 we considered substrates decayed at both 7 and 18 months.

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459 **Fungal community composition.** Experimental N deposition significantly altered fungal
460 community composition on both wood and the high-lignin substrate (Table 3). Whereas fungal
461 community composition significantly changed over time on the high-lignin substrate (Table 3),
462 time was not a significant factor for wood fungal community composition (P = 0.055, Table 3).
463 There was no significant interaction of N deposition treatment and time for fungal community
464 composition on either wood or the high-lignin substrate (Table 3).
465 When we compared community composition across all substrates collected after 7
466 months of decay, as well as in the soil and forest floor co-collected on the same date,
467 experimental N deposition significantly altered fungal community composition across sample
468 types (Table 3). Unsurprisingly, all substrates, as well as soil and forest floor, harbored distinct
469 fungal communities which varied significantly from each other (Table 3; all pairwise tests for
470 sample type, permutational $P < 0.01$); however, there were no significant interactions between N
471 deposition treatment and sample type (Table 3), indicating that a change in community
472 composition occurred in all substrates, as well as in the soil and forest floor under experimental
473 N deposition. We anticipated that change in community composition would be greatest on high-
474 lignin and wood substrates. Instead, it appears experimental N deposition altered fungal
475 community composition broadly across our samples, irrespective of the degree to which they are
476 protected by lignin.
477 **Fungal community dissimilarity.** We followed PERMANOVA with SIMPER analyses to
478 determine whether the changes in fungal community composition that occurred in response to
479 experimental N deposition were driven by a decline in lignolytic fungi (Appendix S4: Table S1).
480 Many of the OTUs on the high-lignin substrate, wood, and forest floor were white-rot and
481 lignolytic litter decay taxa (Table 4) and these fungi were less abundant under experimental N
482 deposition (Figure 2). This response was significant on wood after 7 months of decay, whereas a
483 negative but nonsignificant trend was observed for wood after 18 months of decay as well as for
484 the high-lignin substrate and forest floor (Figure 2). In contrast, no white-rot or lignolytic litter
485 decay fungi were among the top ten SIMPER results for the low-lignin substrate (Table 4, Figure
486 2). The only positive response of white-rot and lignolytic litter decay taxa occurred in mineral
487 soil, in which a single OTU (Table 4) slightly, but significantly, increased in response to
488 experimental N deposition (Figure 2).

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489 Experimental N deposition appears to stimulate fungi that decompose cellulose and
490 hemicellulose, but not lignin. For example, the relative abundances of OTUs associated with
491 soft-rot as well as cellulolytic and hemicelluloytic litter decay fungi significantly increased on
492 wood and in forest floor under experimental N deposition (Figure 2). Other comparisons
493 displayed apparent increases in brown-rot, soft rot and cellulolytic and hemicellulolytic, or
494 weakly lignolytic fungi; however, these were not significant (Figure 2).

495 In soil, most of the OTUs identified by SIMPER as the greatest contributors to
496 community dissimilarity were associated with biotrophic or mycorrhizal species (Table 4, Figure
497 2); overall, their abundance in soil declined under experimental N deposition, but not
498 significantly so (Figure 2).

499 **Relative abundance of Agaricomycetes.** Experimental N deposition reduced the relative
500 abundance of Agaricomycetes on wood across both collection dates (-46%, two-way ANOVA, F
501 = 18.368, P = 0.003, Figure 3a). However, experimental N deposition had no effect on the
502 relative abundance of Agaricomycetes on the high-lignin substrate (Figure 3c), a finding
503 inconsistent with our hypothesis and our results for wood. On the low-lignin substrate, the
504 relative abundance of Agaricomycetes was higher under experimental N deposition (Figure 3c),
505 although this increase was not statistically significant (independent-samples t-test, P = 0.057).
506 Because the low-lignin substrate had low amounts of intact lignocellulose, this result is more
507 likely to reflect an increase in cellulolytic Agaricomycetes than an increase in lignolytic taxa and
508 is, therefore, not necessarily inconsistent with our hypothesis. Finally, mean relative abundance
509 of Agaricomycetes was also lower under experimental N deposition in both soil (-15%) and
510 forest floor (-19%), but these differences also were not significant (Figure 4a,c).

511 We had further hypothesized that reductions in Agaricomycete abundance under
512 experimental N deposition would be enhanced with time. Instead, Agaricomycete relative
513 abundance was significantly higher (+37%) on wood at 7 months of decay relative to 18 months
514 of decay (two-way ANOVA, F = 10.757, P = 0.011). Agaricomycete abundance did not change
515 over time on the high-lignin substrate. There also was no interaction between N deposition and
516 time with a two-way ANOVA. Thus, the negative effect of experimental N deposition on
517 Agaricomycete abundance does not appear to be enhanced with time.

518 **Relative abundance of highly lignolytic taxa.** We hypothesized that experimental N deposition
519 would reduce the relative abundance of fungi with highly lignolytic physiologies, and that this

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520 effect would be most evident on substrates in which energy-rich organic compounds are
521 protected by lignin. Accordingly, experimental N deposition significantly reduced the
522 proportion of highly lignolytic fungi present on wood across both collection dates (Figure 3b,
523 two-way ANOVA, $F = 8.254$, $P = 0.021$), with no significant treatment by time interaction.
524 Experimental N deposition did not have a significant main effect on the abundance of highly
525 lignolytic taxa on the high-lignin substrate (Figure 3d), but there was a significant interaction
526 between treatment and collection date (two-way ANOVA, $F = 8.641$, $P = 0.019$). When we
527 analyzed collection dates separately, the relative abundance of highly lignolytic taxa on the high-
528 lignin substrate was significantly lower (–67%, independent samples t-test, $P = 0.015$) under
529 experimental N deposition at 7 months of decay, but not after 18 months of decomposition
530 (Figure 5). By contrast, highly lignolytic taxa were neither abundant (~9% of Agaricomycetes)
531 nor significantly affected by experimental N deposition on the low-lignin substrate (Figure 3f).
532 Consistent with our hypothesis, experimental N deposition reduced the abundance of highly
533 lignolytic taxa on both lignin-rich substrates, albeit in a time-dependent manner, while having no
534 effect on their abundance on the low-lignin substrates. Moreover, we found that the relative
535 abundance of highly lignolyic taxa in the forest floor declined (–20%) under experimental N
536 deposition, although not significantly (Figure 4b). Taxa which we had identified as highly
537 lignolytic were uncommon in mineral soil (~6% of Agaricomycetes, ~3% of all fungi), and their
538 relative abundance was not significantly altered by experimental N deposition (Figure 4d).
539 We had further hypothesized that experimental N deposition would most negatively
540 impact highly lignolytic taxa later in decay when substrates become enriched in lignin relative to
541 their initial state. However, our results did not support this hypothesis. Instead, experimental N
542 deposition reduced the abundance of highly lignolytic fungi colonizing the high-lignin substrate
543 early in decay (*i.e.*, after 7 months), but not after 18 months (Figure 5). Additionally, the
544 negative effects of experimental N deposition on the highly lignolytic taxa occurring on wood
545 were not greater after 18 months of decay than they were after 7 months of decay, as neither time
546 effects nor time-by-treatment interactions were statistically significant.
547 **Fungal abundance.** We reasoned that experimental N deposition should reduce energy flow to
548 the fungal community as a consequence of diminished fungal decay of lignin. Consistent with
549 this hypothesis, experimental N deposition reduced fungal ITS1 copy number per μg of extracted
550 DNA on wood (Figure 6a, two-way ANOVA, $F = 8.309$, $P = 0.020$). This trend was the same

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551 for wood when we calculated fungal ITS1 copy number per g of substrate, although no longer
552 statistically significant (Figure 6b). However, experimental N deposition did not alter fungal
553 ITS1 copy number on the high-lignin substrate (Figure 6c,d). On the low-lignin substrate,
554 experimental N deposition significantly increased fungal ITS1 copy number per g of substrate
555 (Figure 6f, independent samples t-test, $P = 0.003$), while having no significant effect on fungal
556 ITS1 copy number per µg of extracted DNA (Figure 6e). In contrast to our expectations,
557 experimental N deposition did not significantly reduce fungal ITS1 copy number in either forest
558 floor or soil (Figure 7), although there were nonsignificant trends toward lower ITS1 copy
559 numbers under experimental N deposition in soil (Figure 7 c, d) and forest floor (per g of
560 sample, Figure 7b).

561 Fungal ITS1 copy number (per µg of extracted DNA) on wood was greater at 7 months
562 than after 18 months of decomposition in the field (two-way ANOVA, $F = 16.263$, $P = 0.004$).
563 Time did not significantly affect fungal ITS1 copy number on the high-lignin substrate, nor was
564 the interaction of N deposition treatment and time a factor significantly affecting ITS1 copy
565 number on either wood or the high-lignin substrate.
566

567 **DISCUSSION**

568 We have found evidence that greater soil C storage under chronic N deposition arises
569 from a decline in the abundance of soil fungi with lignolytic physiologies. In this study,
570 experimental N deposition, at rates predicted for midcentury (Galloway et al. 2004), reduced the
571 relative abundance of fungi with lignolytic capacity on both wood and a high-lignin substrate.
572 Furthermore, experimental N deposition induced overall changes in fungal community
573 composition, including a shift toward cellulolytic fungi on decomposing wood and in the forest
574 floor. Finally, we found that experimental rates of N deposition decreased fungal abundance on
575 wood, but increased fungal abundance on a low-lignin, cellulose-rich substrate. Thus, our
576 observations are consistent with those of previous studies, wherein elevated rates of N deposition
577 suppressed lignin decay, but stimulated cellulose decay (Berg and Matzner 1997, Carreiro et al.
578 2000, Talbot and Treseder 2012). Although a decline in the relative abundance of lignolytic
579 fungi has long been postulated as a response to elevated N deposition (Fog 1988, DeForest et al.
580 2004b), to our knowledge, this is the first study to document shifts in fungal community

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581 composition which are physiologically consistent with observations of reduced lignin decay
582 under experimental N deposition.

583 Previous work in both our long-term field study and elsewhere has provided evidence
584 that experimental N deposition increases soil C storage by altering microbial decomposition of
585 lignin, not by changing plant litter production or biochemistry. For example, experimental N
586 deposition has reduced decay of leaf and root-litter (Zak et al. 2008, Xia et al. 2017a) and
587 increased retention of lignin in decaying leaf and root-litter (Xia et al. 2017b), while increasing
588 neither the production of plant litter nor the lignin content of plant tissues (Burton et al. 2004,
589 Frey et al. 2014, Xia et al. 2015). Furthermore, experimental N deposition has decreased soil
590 respiration without altering root respiration (Burton et al. 2004, Burton et al. 2012), suggesting
591 that reduced soil microbial respiration underlies this response. Moreover, stable isotope tracing
592 with ^{13}C-labelled plant litter has revealed that experimental N deposition diminishes the amount
593 of C transferred from plant litter into the soil microbial community (Gan et al. 2013). Finally,
594 long-term experimental N deposition has negatively affected fungal lignocellulolytic potential
595 (Eisenlord et al. 2013), fungal lignocellulolytic gene expression (Edwards et al. 2011, Hesse et
596 al. 2015), and fungal lignolytic enzyme activity (DeForest et al. 2004b, a, Sinsabaugh 2010,
597 Freedman and Zak 2014), as well as fungal capacity for litter decomposition (van Diepen et al.
598 2017). Our results thus add to a growing body of evidence that experimental N deposition
599 negatively affects lignolytic soil fungi, organisms that mediate the rate-limiting step of organic
600 matter decay.

601 However, lignin-decay is not strictly limited to fungi, because some bacterial
602 decomposers also possess lignolytic activity (McCarthy et al. 1986, Bugg et al. 2011a, Bugg et
603 al. 2011b) In our study system, experimental N deposition has had an overall positive effect on
604 the abundance of bacterial genes mediating plant cell-wall decay (Freedman et al. 2016).
605 Furthermore, experimental N deposition has increased the abundance of bacterial laccase-like
606 multicopper oxidase (LMCO) genes (Freedman and Zak 2014), which may play similar roles to
607 fungal laccases in lignin decay (Ridge et al. 2007, Reiss et al. 2013, Lu et al. 2014). However,
608 this expanded bacterial role in lignin-decay has apparently not offset reductions in fungal lignin
609 decay, given the reductions in lignin decay occurring under experimental N deposition in our
610 field experiment. This may be because, while both bacteria and fungi share laccase or laccase-
611 like enzymes that act upon polyphenolics (Baldrian 2006, Ausec et al. 2011, Lu et al. 2014),

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612 fungi additionally possess other, more powerful lignolytic enzymes (*e.g.,* fungal class II
613 peroxidases) which bacteria do not, giving fungi an overall greater oxidative capacity to decay
614 lignin than that of bacteria (Kirk and Farrell 1987, Floudas et al. 2012).

615 Our working model for conceptualizing these responses predicts that higher N
616 availability, resulting from increased rates of inorganic N deposition, leads to lower rates of
617 fungal lignin decay (DeForest et al. 2004b). This idea originates from the observation that the
618 suppression of lignolytic activity appears to be a physiological response to high inorganic N
619 concentrations for some species of lignolytic fungi (Leatham and Kirk 1983, Commanday and
620 Macy 1985), with the highly lignolytic wood decay fungus *Phanerochaete chrysosoporium* being
621 the most extensively studied species in this regard. For example, lignolytic activity in *P.*
622 *chrysosoporium* cultures is suppressed by the additions of high amounts of inorganic N (Jeffries
623 et al. 1981) and stimulated by conditions of N starvation (Keyser et al. 1978). Interestingly, we
624 observed the strongest and most consistent negative effects of experimental N deposition on
625 lignolytic fungi on the wood substrate. It is plausible that high N availability may have similar
626 negative effects on the activity of the lignolytic wood-decomposing species colonizing our wood
627 substrates, as it does on *P. chrysosporium*.

628 Although it is not clear why saprotrophic lignolytic fungi would reduce lignin decay in
629 response to the presence of high inorganic N, several physiological explanations for this
630 phenomenon have been proposed. For example, low N conditions in culture may stimulate
631 lignin and polyphenol decay because those conditions are similar to those of its natural
632 environment, *i.e.* wood (Keyser et al. 1978), making it possible that inorganic N concentration
633 serve as a signal for environmental conditions appropriate for lignin and polyphenol decay. In
634 culture, lignin decay occurs as part of fungal secondary metabolism; Keyser and colleagues
635 (1978) suggest that the sensitivity of fungal lignolytic activity to N availability may be related
636 more broadly to the role of N in secondary metabolism, as N limitation has been observed to
637 induce expression of some other fungal secondary metabolites (Gold et al. 1973, Bu'Lock et al.
638 1974). Additionally, Fog (1988) suggested that nitrogenous compounds may react with the
639 intermediate products of lignin decay, thereby preventing effective fungal decay of lignin under
640 high N conditions and making low N conditions ideal for this activity. While N suppression of
641 fungal lignolytic activity is consistent with our results and those of previous studies in our long-
642 term field experiment, not all fungal species appear to reduce lignin decay in response to

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643 increased N availability (Boyle et al. 1992, Kaal et al. 1995) and many of the lignolytic taxa
644 (Tables 2, S2, and S4) in our study system have not yet been studied in this regard. Direct
645 observation of the taxa in question under laboratory culture could provide further insight as to
646 whether this mechanism underlies the responses of lignolytic fungi to experimental N deposition
647 that we have observed in the field.

648 Because anthropogenic N deposition is also known source of acid-deposition (Liechty et
649 al. 1993), that, on poorly-buffered soils inherently low in base cations, reduces soil pH and
650 thereby increases leaching of cationic nutrients (Long et al. 1997, Bowman et al. 2008, Lieb et
651 al. 2011), the question often arises as to whether soil microbial responses to anthropogenic N
652 deposition result from the effects of increased N availability or, instead, reflect the effects of
653 reduced soil pH or cationic nutrient availability. For example, reduced soil pH could reduce the
654 activity of lignolytic N enzymes (Sinsabaugh et al. 2008) or induce the loss of cations (*e.g.*,
655 Mn^{2+}) required by fungal lignolytic enzymes (*e.g.*, manganese peroxidases) (Hofrichter 2002, De
656 Santo et al. 2009). However, our study sites are located on well-buffered calcareous sands (Zak
657 et al. 2008). Our long-term experimental N deposition treatment has not altered soil pH, base
658 saturation of the cation exchange complex, nor the concentrations of exchangeable Ca^{2+} in soil
659 (Zak et al. 2008, Patterson et al. 2012). By extension, it also is unlikely that our N deposition
660 treatment has differentially affected the loss of Mn^{2+} from soil in our experiment. Therefore,
661 cation loss is unlikely to explain the declines in lignolytic fungi we have documented in our
662 study.

663 While the mechanisms we have discussed thus far focus on the physiological effects of
664 anthropogenic N deposition on lignolytic fungal physiology, ecological explanations have also
665 been proposed to explain reduced fungal lignolytic activity under experimental N deposition.
666 For example, fungi can use the same oxidative enzymes involved in lignin decay to "mine" N
667 which is either bound in soil organic matter (SOM-N) or protected by lignin in plant litter. It has
668 been thought that fungi may reduce their production of lignolytic enzymes under experimental N
669 deposition because mining for N in SOM becomes less necessary when inorganic N availability
670 is high (Craine et al. 2007). However, reduced fungal assimilation of N which is protected by
671 lignin is unlikely to explain the reduced abundance of lignolytic fungi on high-lignin and wood
672 substrates observed in our study, as these substrates are energy-rich, but N-poor (Table 1).
673 Similarly, a reduction in fungal metabolism of SOM-N also seems insufficient to explain the

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674 negative effects of experimental N deposition on lignolytic saprotrophs in our study, as SOM-N
675 mining is an activity typically attributed to some ectomycorrhizal fungi in the mineral soil
676 (Lindahl et al. 2007, Talbot et al. 2013); by contrast, we observed a decline in lignolytic
677 saprotrophic decomposer fungi in the forest floor and on decomposing high-lignin and wood
678 substrates. In a decomposition experiment conducted in a boreal forest, experimental N
679 deposition reduced lignin decay but not N loss in plant litter, suggesting that reduced fungal
680 foraging for plant litter N did not underlie this observation. Our conclusions here are consistent
681 with those of Talbot and Treseder (2012).

682 While all of these aforementioned mechanisms focus on the direct effect of high N
683 availability on lignolytic fungi, an alternative mechanism proposes that experimental rates of N
684 deposition negatively affects lignolytic fungi indirectly, through increased competition with fast-
685 growing fungal decomposers of energy-rich organic substrates (Fontaine et al. 2003, Talbot and
686 Treseder 2012). According to this mechanism, experimental N deposition removes N-limitation
687 of cellulose decay thereby stimulating the growth of fungi that efficiently decompose cellulose
688 but inefficiently decompose lignin. Indeed, elevated rates of N deposition have been
689 documented to stimulate cellulose decay, while slowing lignin decay (Berg and Matzner 1997,
690 Carreiro et al. 2000, Talbot and Treseder 2012). Furthermore, experimental N deposition in a
691 boreal forest slowed lignin decomposition, while simultaneously increasing both cellulase
692 activity and fungal antagonism, leading the authors to conclude that increased competition
693 between cellulolytic and lignolytic fungi was responsible for slowed lignin decay (Talbot and
694 Treseder 2012).

695 In our study, experimental N deposition increased fungal biomass on a cellulose-rich,
696 low-lignin substrate (Figure 6a), suggesting that a similar stimulation of cellulolytic fungi may
697 have occurred here. At the same time, we also observed increases in cellulolytic fungi in wood
698 and in forest floor under experimental N deposition (Figure 2), a result which we attributed to
699 decreased competition from the suppressed lignolytic fungi. Nevertheless, we cannot rule out
700 the alternate possibility that higher N availability instead directly stimulated growth of
701 cellulolytic fungi, thus increasing competition for energy-yielding substrates between cellulolytic
702 and lignolytic fungi, and that increased completion from cellulolytic fungi led to the decline in
703 lignolytic fungal relative abundance that we observed. However, if experimental N deposition
704 directly enhanced cellulose decay, this should also increase mass loss of the high-cellulose, low-

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705 lignin substrate, an effect which we did not observe (Figure 1). Furthemore, a recent study (Xia
706 et al. 2017a) revealed that experimental N deposition did not change the amount of plant cell-
707 wall carbohydrate fraction which was lost during decay (Xia et al. 2017b). Nevertheless, future
708 studies should additionally assess both fungal competition and the rate of cellulose loss in order
709 to bring further clarity to our understanding of the mechanism through which experimental N
710 deposition alters fungal community composition and lignin decay.

711 We had additionally hypothesized that we would observe the largest reductions in
712 lignolytic fungi later in decay when substrates are more enriched in lignin relative to their initial
713 state, a time when lignin-decomposing fungi should be more abundant. Surprisingly, we
714 observed the greatest suppression of lignolytic fungi under experimental N deposition at our first
715 time point, not later in the decay process as we had hypothesized. Our results suggest
716 experimental N deposition suppresses lignolytic fungi early in decay when the labile C
717 compounds that may "prime" lignin decay are most abundant (Fontaine et al. 2003), while
718 having a neutral or even positive effect on cellulolytic and hemicellulolytic fungi (Figure 2).
719 Therefore, plant litter may be entering later stages of decay with higher lignin-to-cellulose ratios
720 under experimental N deposition than under ambient N deposition. Because plant litter decay
721 plateaus as the ratio of lignin-to-cellulose increases (Melillo et al. 1989), it is possible that
722 reduced abundance of lignolytic fungi early in decomposition process ultimately exerts a large
723 influence on both the lignin content of decaying litter and the rate and extent of plant litter decay.
724 We note, however, it is also possible that we were able to most strongly detect declines in
725 lignolytic fungi early in decay, because our selection of lignolytic taxa is biased towards early-
726 colonizing species. Most published studies of fungal decomposition are of relatively short
727 duration and involve inoculating a single species on fresh litter or wood, which may have led us
728 to underrepresent late-stage decomposers which may grow slowly or prefer to colonize substrata
729 that are already partially decomposed.

730 Interestingly, a recent experiment (Xia et al. 2017a, b) in our study sites found that
731 experimental N deposition retarded lignin decomposition in sugar-maple leaf litter after 3 months
732 of decomposition, but not at later stages of decomposition; by contrast, lignin decay of sugar-
733 maple root litter was retarded after 3 years of decomposition under experimental N deposition,
734 but not at earlier time-points examined (Xia et al. 2017a). At the stage in decomposition at
735 which experimental N deposition increased lignin retention in sugar-maple plant litter (*i.e.*, 3

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736 months for leaf litter, 3 years for root litter), 35 – 49% of plant litter mass had been lost,
737 respectively (Xia et al. 2017a), an extent of decay that exceeds those of our high-lignin and wood
738 substrates here. This suggests it is plausible that the biological response to experimental N
739 deposition (*i.e.,* decline in lignolytic fungi) might precede the chemical response (*i.e.,* reduced
740 lignin decay), although we presently lack data on lignin-loss in our substrates, data from later
741 points in decomposition of our substrates, or microbial data from the root and leaf-litter in the
742 experiment conducted by Xia and colleagues (2017 a,b) which would allow us to confirm
743 whether this is true. Moreover, in contrast to our expectations, we did not observe significant
744 declines in high-lignin or wood substrate mass loss under experimental N deposition, which we
745 would anticipate given the reduced forest floor mass loss documented in our study system (Zak
746 et al. 2008); we cannot rule out that this may not be strongly evident until later in decomposition,
747 as has been observed for root-litter (Xia et al. 2017b).

748 In this experiment, the most consistent and significant negative responses to experimental
749 N deposition were observed among fungi colonizing wood. Many of the most powerful
750 lignolytic fungi are wood-decomposers, making wood an excellent substrate on which to observe
751 the effects of N deposition on lignin decomposers. Because experimental N deposition
752 simultaneously reduced the relative abundance of lignolytic fungi (Figure 3) and the size of the
753 fungal community (Figure 6) on wood, elevated rates of N deposition led to a reduction in the
754 absolute abundance of lignolytic fungi on wood. Our results are consistent with recent
755 observations that experimental N deposition has slowed the decay of coarse woody debris in our
756 long-term experiment (B. Lyons, unpublished data). Although the dominant overstory tree in our
757 system is sugar maple (*Acer saccharum*), the results we obtained with birch (*Betula spp.*) substrates are
758 likely broadly reflective of responses of wood-decay fungi to experimental N deposition at our field sites,
759 as birch and maple are both hardwoods with similar lignin and cellulose contents (Pettersen 1984).
760 Furthermore, experimental N deposition has also increased woody biomass production (Pregitzer
761 et al. 2008, Ibáñez et al. 2016). If higher rates of N deposition both increase the production and
762 slow the decay of wood in temperate forests, this could dramatically increase ecosystem C
763 storage.

764 ***Conclusions***

765 Increased soil C storage may be a widespread response to anthropogenic N deposition in
766 temperate forests (Nave et al. 2009, Janssens et al. 2010, Frey et al. 2014). Negative effects on

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767 lignin-decomposing fungi, like those documented here, may explain soil C accumulation under
768 elevated rates of N deposition. It is possible that increases in anthropogenic N deposition which
769 have occurred over the past century and a half may have already had widespread effects on
770 fungal biodiversity, as well as elicited increases in soil C in temperate forests. Our results
771 indicate that further increases in anthropogenic N deposition, which have been predicted for
772 some parts of Earth, could both increase soil C storage and have consequences for fungal
773 biodiversity in temperate forests.

774 **ACKNOWLEDGEMENTS**

775 This work was supported by funding from the United States Department of Energy Biological
776 and Environmental Research program. Maintenance of our long-term N deposition project is
777 supported by the National Science Foundation Long-term Research in Environmental Biology
778 program. Donald Zak, along with collaborators, established the Michigan Gradient Project and
779 has overseen the continuation of this long-term research project since 1994. Elizabeth Entwistle
780 and Donald Zak co-designed the field experiment presented in this manuscript. As principal
781 investigator on this project, Donald Zak conceived the larger project funding this particular piece
782 of research, provided intellectual input throughout this project, and edited drafts of this
783 manuscript. Elizabeth Entwistle created the protocols for DNA extraction and sequencing,
784 performed the bioinformatic and statistical analyses, and wrote the manuscript. William Argiroff
785 created standards, optimized, performed, and assembled the data for quantitative PCR. He also
786 was heavily involved in fungal community PCR and sequencing efforts and designed our
787 protocol for cleaning up PCR products. The authors would like to acknowledge the invaluable
788 assistance of several people. Rima Upchurch assisted with litter bag assembly and deployment.
789 Seongjun Kim assisted with mass loss measurements and sample processing. Karl Romanowicz
790 and Andrew Warner extracted DNA from our samples. We thank them for their important roles
791 in this project. We also thank Drs. Lauren Cline, Gregory Dick, Timothy James, and Inés Ibánez
792 for their feedback on earlier versions of this manuscript.

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1165 **DATA AVAILABILITY**

1166 Circular consensus sequencing files for this manuscript have been deposited in the National
1167 Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession
1168 numbers SAMN06475267–SAMN06475287 in BioProject PRJNA222775. All other underlying
1169 data files are available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.5bv7p.

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1171 **TABLES**

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Table 1. Summary of results of biochemical analysis of low-lignin, high-lignin, and wood substrates. Measurements were performed by the Soil & Forage Lab, UW-Madison. Results are reported as a percentage of dry weight.

Substrate	Material	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Nitrogen (%)
Low-lignin	Kraft paper	14.1[†]	64.1	14.1	0.14
High-lignin	Newsprint	27.6	46.7	23.1	0.09
Wood	Birch wood	11.8	52.4	28.9	0.13

[†]Because chemical pulping breaks bonds between lignins, cellulose, and hemicelluloses (Ragnar et al. 2000), the low-lignin substrate is more labile than its lignin content alone suggests.

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Table 2. Agaricomycete taxa selected as highly lignolytic. All taxa are represented by >20 sequences in our data set. More details on taxa selected below are in Appendix S2: Table S1; excluded taxa are described in Appendix S3: Table S1.

Order	Family or genera included from this order	Citations documenting lignolytic activity
Agaricales		
	Marasmius[1]	(Osono and Takeda 2002, Steffen et al. 2007)
	Gymnopus[2]	(Osono et al. 2003, Osono and Takeda 2006, Valaskova et al. 2007, Šnajdr et al. 2010, Cline and Zak 2015)
	Mycena[3]	(Worrall et al. 1997, Osono and Takeda 2002, Steffen et al. 2007, Liers et al. 2011, Cline and Zak 2015)
	Clitocybe[4]	(Osono et al. 2003, Osono et al. 2011)
	Crepidotaceae	(Gutiérrez et al. 1999, Del Rıo et al. 2001, Martínez Ferrer et al. 2005)
Polyporales[5]		
	Ganodermataceae	(Blanchette 1984)
	Antrodiella	(Patel and Rao 1993)
	Phanerochaete[6]	(Kirk and Farrell 1987, Hatakka 1994, Del Rıo et al. 2002)
	Scopuloides[6]	(Kuuskeri et al. 2015)
Russulales[7,8]		
	Lachnocladiaceae	(Cline and Zak 2015)

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	Auriscalpiaceae	(Miller and Stewart 1971, Miller and Methven 2000)

1. Also included sequences that were assigned to the closely related genus *Amyloflagellula* (Douanla-Meli and Langer 2008).
2. Some *Gymnopus* species were formerly *Collybia*.
3. Also included sequences assigned to closely related genus *Poromycena* (Moncalvo et al. 2002).
4. Also included sequences classified to closely related genus *Lepista* (Matheny et al. 2006).
5. White-rot Polyporales are broadly highly lignolytic (Campbell 1932, Ander and Eriksson 1977, Ruiz-Duenas et al. 2013). The selected Polyporales taxa listed above are the white-rot Polyporales present in our data set (Binder et al. 2013).
6. Sequences assigned to the "Aphyllophorales" by the RDP v7 classifier represent these genera in our data set (see Appendix S2: Table S1 for more details). "Aphyllophorales" is no longer a recognized taxonomic group (Hibbett and Donoghue 1995, Binder and Hibbett 2002).
7. These families are now recognized as saptroptrophic Russulales (Hibbett and Donoghue 1995, Larsson and Larsson 2003, Miller et al. 2006), but are placed in Polyporales by RDP v7 fungal LSU classifier.
8. Saprotrophic Russulales are white-rot fungi which have broadly been observed to be highly lignolytic (Blanchette 1984, Otjen and Blanchette 1984, Del Rıo et al. 2002, Speranza et al. 2009, Floudas et al. 2012, Nagy et al. 2015). The selected Russulales taxa listed above are the saprotrophic Russulales present in our data set.

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Table 3. PERMANOVA results for comparisons of fungal communities under ambient and experimental rates of N deposition. Comparisons for wood and the high-lignin substrate are across both time-points. Comparisons for all substrates plus soil and forest floor are across sample types for substrates collected after 7 months of decomposition and soil and forest floor samples co-collected on the same date. Columns represent PERMANOVA values for the degrees of freedom (df), sum of squares (SS), mean square (MS), pseudo-F statistic and permutational *P* value (P(perm)).

Samples included	Factors	df	SS	MS	Pseudo-F	*P*(perm)
Wood	**N deposition treatment**	1	2686.7	2686.7	1.8946	**0.0476**
	Time	1	2443.4	2443.4	1.723	0.0553
	N deposition treatment x Time	1	994.72	994.72	0.70145	0.7487
High-lignin	**N deposition treatment**	1	2600.9	2600.9	1.7597	**0.0266**

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substrate	**Time**	1	4405.4	4405.4	2.9806	**0.0023**
	N deposition treatment x Time	1	1362	1362	0.92149	0.6118
Low-lignin, high-lignin, & wood substrates; soil, forest floor	**N deposition treatment**	1	2856.8	2856.8	2.5307	**0.0001**
	Sample type	4	39395	9848.7	8.7244	**0.0001**
	N deposition treatment x Sample type	4	4807.8	1202	1.0647	0.2909

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Table 4. SIMPER results for the ten OTUs with the highest average dissimilarity between fungal communities under ambient and experimental N deposition, including their cumulative contribution to total dissimilarity and the fungal physiologies they represent. Detailed results for individual OTUs can be found in Appendix S4: Table S1.

	Months of decomposition	Sample type	Cumulative contribution to dissimilarity (%)	Number of OTUs assigned to each fungal physiology				
				White-rot & lignolytic litter decay	Soft-rot & cellulolytic/ hemicellulolytic litter decay	Brown-rot	Weakly lignolytic	Mycorrhizal/ biotrophic
Substrates	7	low-lignin	24.39	0	6	1	1	2
		high-lignin	30.25	4	3	2	0	1
		wood	44.74	5	2	0	2	1
	18	high-lignin	33.18	7	1	1	1	0
		wood	47.19	3	3	0	4	0
Environmental samples	NA	forest floor	33.11	7	3	0	0	0
		soil	18.92	1	1	0	3	5

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NA, not applicable

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FIGURE LEGENDS

Figure 1. Mass loss of wood, high-lignin, and low-lignin substrates under ambient (open bars) and experimental N deposition (filled bars) after 7 months of decomposition in the field. Error bars represent 2 SE. Experimental N deposition did not significantly alter mass loss any substrate (independent sample t-tests).

Figure 2. The response (% change in relative abundance) of fungi of different physiologies to experimental N deposition among the ten OTUs with the largest dissimilarity scores according to SIMPER analysis. Significant comparisons (marked with an asterisk) are those for which the 95% confidence intervals for relative abundance under ambient and experimental N deposition did not overlap.

Figure 3. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) in fungal communities on a wood (top), high-lignin (middle), and low-lignin (bottom) substrates after 7 and 18 months of decomposition in the field. Relative abundance of Agaricomycetes was calculated as a proportion of fungi. Relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars represent 2 SE. Fungal communities on the low-lignin substrate paper, for which we have only one time point, were analyzed with independent sample t-tests. High-lignin and wood were analyzed across collection dates with a two-way ANOVA. Significance is indicated by * $P < 0.05$, ** $P < 0.01$.

Figure 4. Relative abundance of Agaricomycetes (left) and highly lignolytic taxa (right) of fungal communities in forest floor (top) and soil (bottom). Relative abundance of Agariomycetes was calculated as a proportion of fungi, while relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars indicate 2 SE.

Figure 5. Relative abundance of highly lignolytic taxa on the high-lignin substrate collected after 7 and 18 months of decomposition under ambient (open bars) and experimental (solid bars) rates of N deposition. Relative abundance of highly lignolytic taxa was calculated as a proportion of Agaricomycetes. Error bars represent 2 SE. Each collection date was analyzed with independent samples t-tests, for which significance is indicated with * $P < 0.05$.

Figure 6. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies μg^{-1} DNA (left) and ITS1 copies g^{-1} sample mass (right) on decomposing substrates of varying

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recalcitrance (wood, high-lignin, low-lignin) collected after 7 and 18 months of decomposition in the field. We compared ITS1 copy number on the wood (top) and high-lignin (middle) substrates between time points and treatments with two-way ANOVAs; we present data across time-points because there was no significant time-by-N deposition interaction. Differences in ITS1 copy number under experimental N deposition for the low-lignin substrate (bottom), for which we have only one time-point, were tested with an independent samples t-test. Error bars represent 2 SE. Significant comparisons are indicated with * $P < 0.05$.

Figure 7. Fungal abundance as measured by quantitative PCR and presented as ITS1 copies μg^{-1} DNA (left) and ITS1 copies g^{-1} sample mass (right) in forest floor (top) and soil (bottom). Error bars indicate 2 SE. ITS1 copy numbers under ambient and experimental N deposition were compared with independent samples t-tests. No comparisons were significant.

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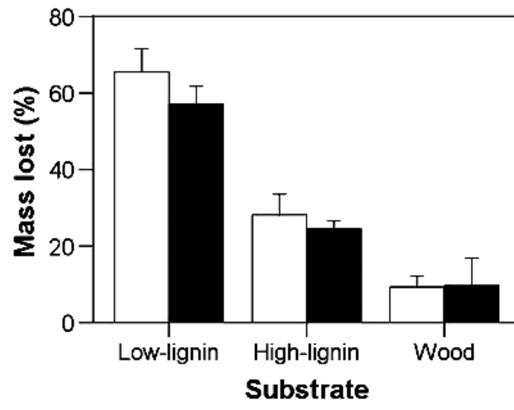

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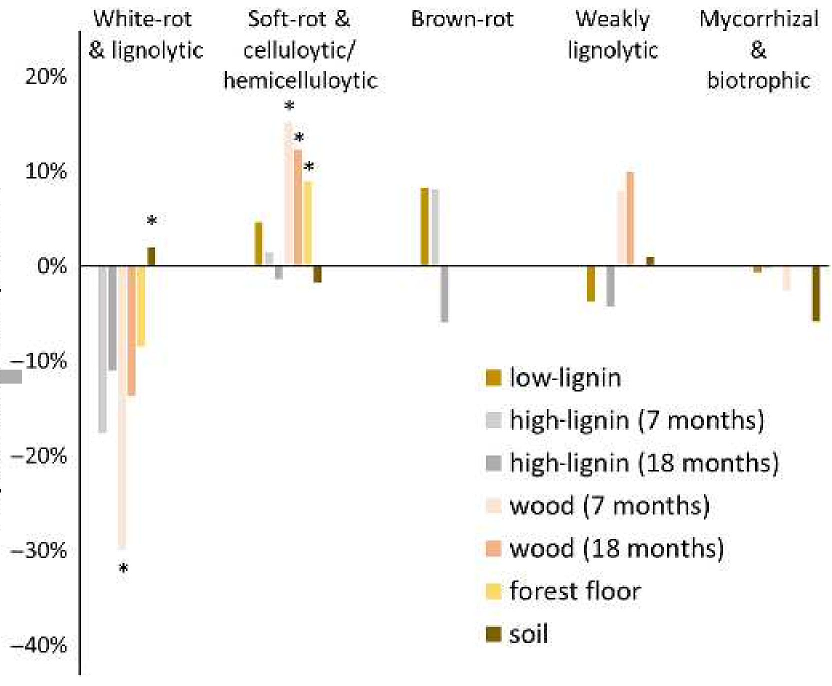

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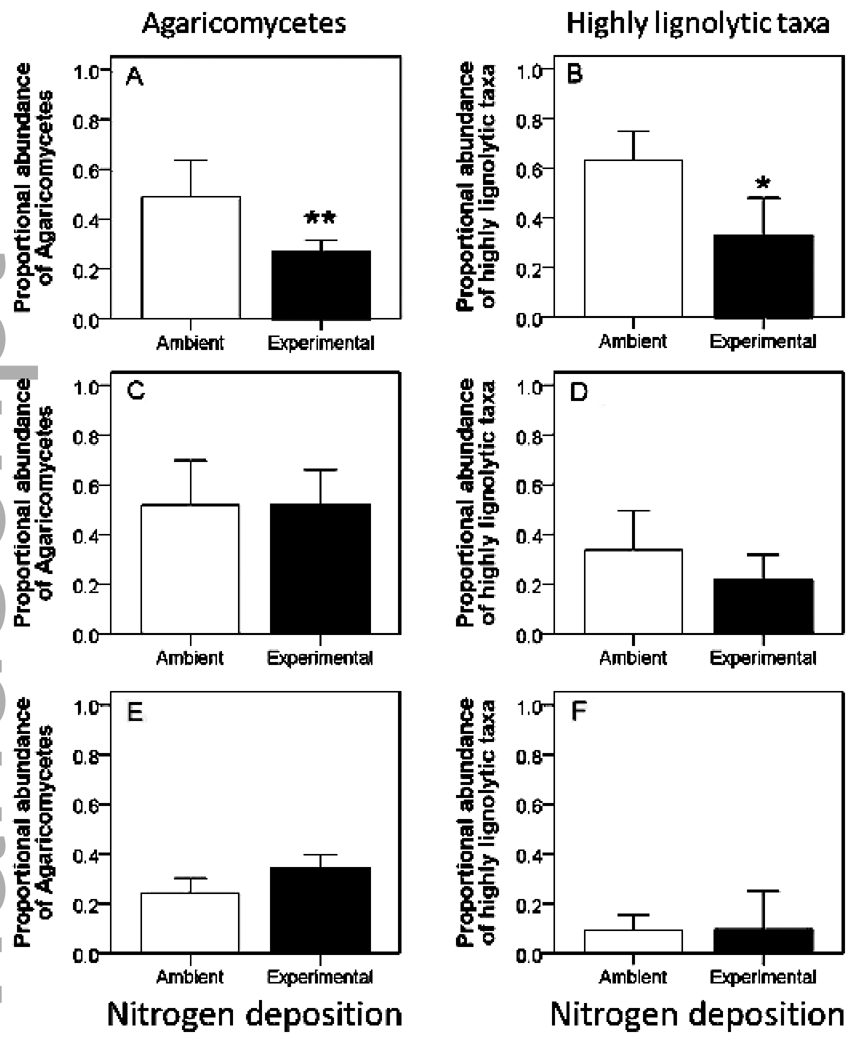

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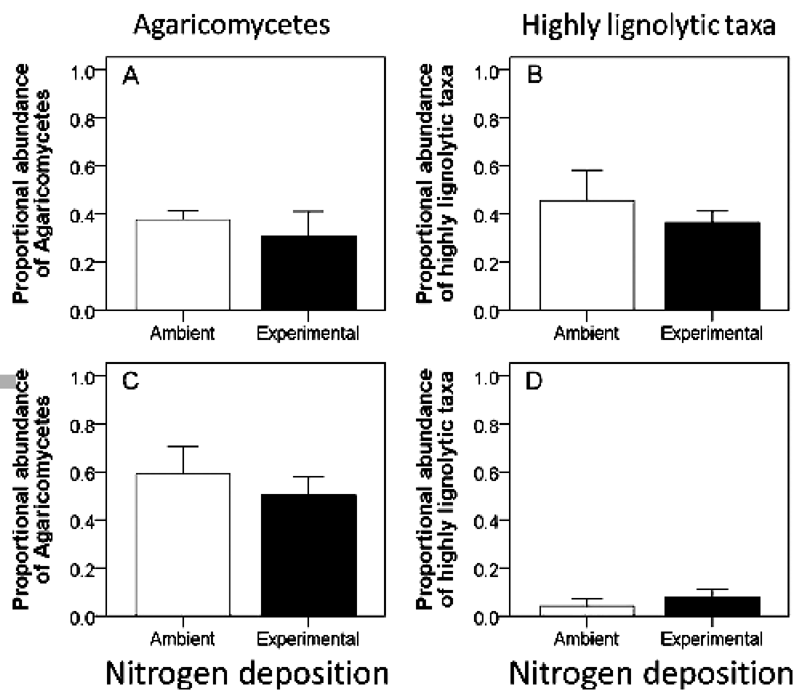

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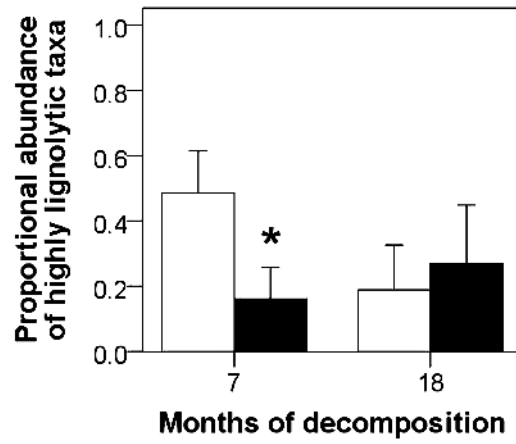

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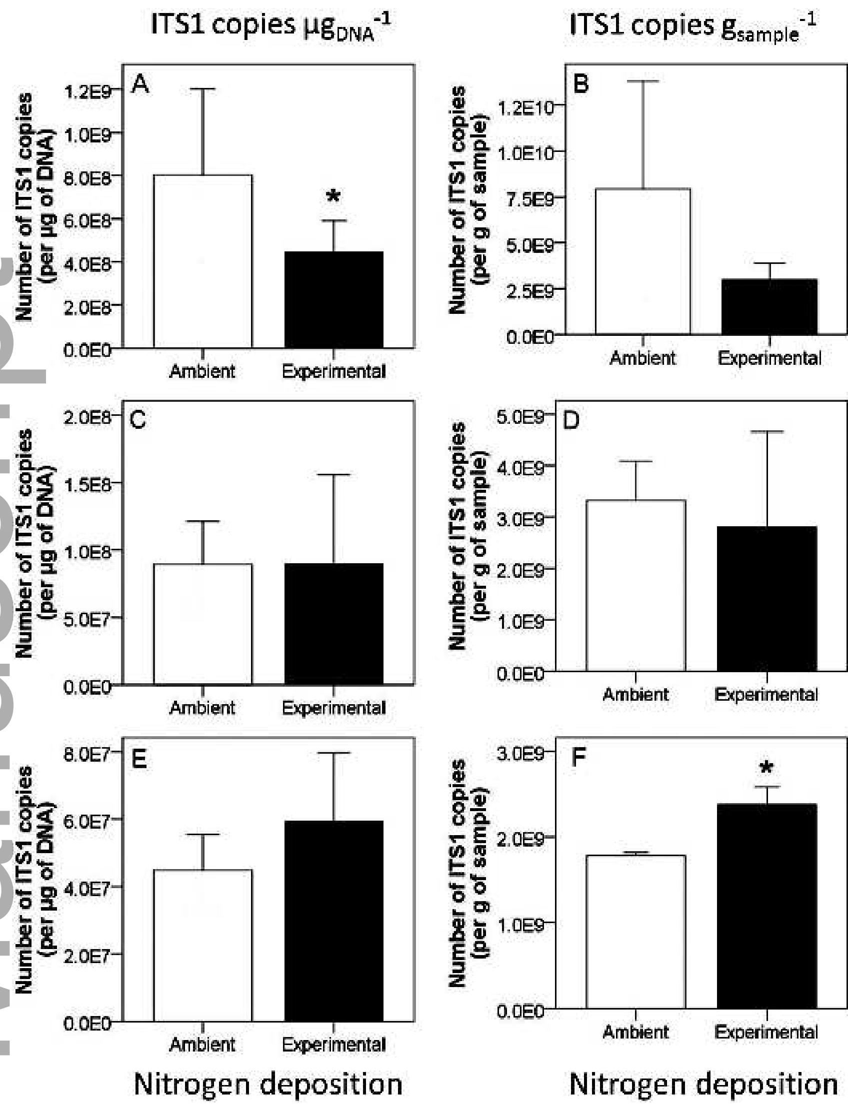

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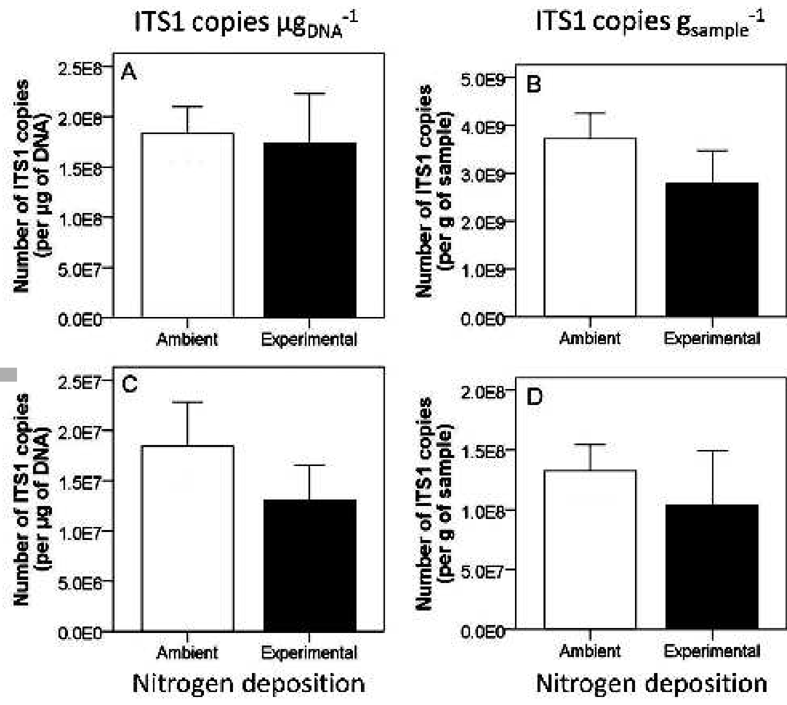

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