

1 RUNNING HEAD: NITROGEN ENRICHMENT AND FUNGAL BEHAVIOR

2 **Fungi exposed to chronic nitrogen enrichment are less able to decay leaf litter**

3 Linda T.A. van Diepen¹, Serita D. Frey^{1,3}, Elizabeth A. Landis¹,

4 Eric W. Morrison¹, and Anne Pringle²

5 ¹Natural Resources and the Environment, University of New Hampshire, Durham, NH, 03824 USA

6 ²Departments of Botany and Bacteriology, University of Wisconsin-Madison, Madison, WI, 53706 USA

7 ³Corresponding author E-mail: serita.frey@unh.edu; phone: 603-862-3880

8

9 *Abstract.* Saprotrophic fungi are the primary decomposers of plant litter in temperate forests, and
10 their activity is critical for carbon (C) and nitrogen (N) cycling. Simulated atmospheric N
11 deposition is associated with reduced fungal biomass, shifts in fungal community structure,
12 slowed litter decay, and soil C accumulation. Although rarely studied, N deposition may also
13 result in novel selective pressures on fungi, affecting evolutionary trajectories. To directly test if
14 long-term N enrichment reshapes fungal behaviors, we isolated decomposer fungi from a long-
15 term (28 year) N addition experiment and used a common garden approach to compare growth
16 rates and decay abilities of isolates from control and N amended plots. Both growth and decay
17 were significantly altered by long-term exposure to N enrichment. Changes in growth rates were
18 idiosyncratic, but litter decay by N isolates was generally lower compared to control isolates of
19 the same species, a response not readily reversed when N isolates were grown in control (low N)
20 environments. Changes in fungal behaviors accompany and perhaps drive previously observed
21 N-induced shifts in fungal diversity, community composition, and litter decay dynamics.

22 *Keywords:* *adaptation; common garden; evolution; fungi; global change; growth rate; litter*
23 *decay; Harvard Forest; nitrogen deposition; phenotypic plasticity.*

INTRODUCTION

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26 The fungi are a megadiverse group of microbial species that play critical roles in
27 biogeochemical cycles. Anthropogenically-mediated environmental changes are a serious threat
28 to biological diversity and may result in novel selective pressures (Sala et al. 2000), but whether
29 or how fungi are adapting to global change is largely unknown. Vulnerable fungi may move or
30 go extinct in response to environmental stresses. Shifts in fungal phenology in response to
31 climate change are documented (Boddy et al. 2014), as are fungal range expansions (Wolfe et al.
32 2012). However, whether fungi are prone to extinction remains an open question, largely because
33 conservation efforts rarely target fungi (Heilmann-Clausen et al. 2015). Species may also be
34 tolerant of changes, either because the stresses are irrelevant to the species in question, or
35 because the species can alter aspects of behavior or life history in response to changing
36 environments (West-Eberhard 1989).

37 Fungi are not static entities, and fungal lineages can evolve on very short time scales. For
38 example, individuals exposed to novel environments in the laboratory diverge and evolve over
39 weeks or months (Schoustra et al. 2005; Leu and Murray 2006; Dettman et al. 2007, 2008).
40 However, there is little research on the evolution of fungi in global change contexts, even though
41 evolutionary dynamics may accompany or drive shifts in fungal diversity that occur in response
42 to environmental change (Jaenike 1991; Avis et al. 2008; Bärlocher et al. 2008). As fungal
43 lineages evolve in response to global change, the physiology and function of these species may
44 change, and the evolutionary trajectories taken by individual lineages may aggregate to influence
45 ecosystem scale processes.

46 Fungi are the primary drivers of decomposition in temperate forest systems (Gadd 2006), and
47 altered rates of decomposition will directly influence future trajectories of climate change

48 (Kirschbaum 1995; Ise and Moorcroft 2006). Since saprotrophic fungi are the primary producers
49 of the extracellular enzymes involved in cellulose and lignin (lignocellulose) decay, it may be
50 especially critical to understand the evolution of saprotrophic fungi in global change contexts.
51 Global change experiments, in which the abiotic environment is manipulated to simulate future
52 scenarios of a particular (or multiple) global change driver(s) in the field, offer a unique
53 opportunity to explore changes in species' behaviors relevant to ecosystem-scale processes,
54 although these kinds of experiments are rarely used for this purpose (Bataillon et al. 2016). In
55 this study, we use a long-term (28 year) simulated nitrogen (N) deposition experiment—the
56 Chronic Nitrogen Amendment Study (CNAS) located at the Harvard Forest Long-Term
57 Ecological Research (LTER) site in Petersham, MA, USA—to explore fungal behaviors in
58 contexts of altered soil N availability. Although not originally designed as an artificial selection
59 experiment, a continuous and consistent exposure to elevated N within treatment plots has likely
60 acted as a novel selective pressure on fungi. Previous research from the CNAS shows that long-
61 term N enrichment reduces fungal biomass, causes changes in fungal diversity and community
62 composition (Frey et al. 2014; Morrison et al. 2016), slows plant litter decay (Magill and Aber
63 1998), and results in a significant accumulation of soil organic matter (Frey et al. 2014).
64 However, whether changes in fungal behaviors accompany or drive shifts in decomposition
65 dynamics remains untested.

66 Here we investigated whether the saprotrophic fungi that grow on plant litter of N-enriched
67 plots behave differently from the same species found in control plots. Have these isolates
68 evolved, compared to isolates of the same species from control plots, and does that evolution
69 impact decomposition? We first isolated fungi from decomposing plant litter in control and N-
70 enriched plots and next used a common garden design to study the behaviors of these individuals

71 in their respective home and away environments. To measure potential changes in fungal
72 behaviors, we first measured mycelial growth rate, often assumed to be an aspect of fitness
73 (Pringle and Taylor 2002) and next measured the ability of isolates to decay plant litter. We
74 compared isolates from the N treatments with isolates of the same species from control plots,
75 growing all isolates in identical laboratory environments (the same “gardens”) where N
76 availability was varied to reflect field conditions in the different N treatments at CNAS. Our
77 working assumption was that if fungal isolates from N-enriched plots grew similarly to those
78 from control plots when grown in a common environment, then isolates from treatment plots
79 have not evolved. By contrast, if we found differences—if isolates from the N treatment plots
80 showed consistent differences in growth and litter decay compared to control isolates—then data
81 would suggest that the lineages isolated from N treatment plots are distinct and that treatment
82 isolates have evolved to grow in the novel environments of the experimental plots.

83 METHODS

84 *Fungal cultures.* Fungi were isolated from decomposing leaf litter collected from each of
85 three N addition treatments within the CNAS which was established in 1988 to examine the
86 effects of simulated N deposition on ecosystem dynamics (Aber et al. 1993). The site is located
87 in a mixed hardwood forest dominated by black and red oak (*Quercus velutina* and *Q. rubra*).
88 The experiment consists of three 30 × 30 m plots which receive either ambient N deposition
89 (control, N0) or an additional 50 (N50) or 150 (N150) kg N ha⁻¹ yr⁻¹. The CNAS has resulted in a
90 wealth of data (Aber et al. 1993; Magill et al. 2004; Frey et al. 2004; Frey et al. 2014; Morrison
91 et al. 2016), and while it has been criticized for pseudo-replication, it remains one of the longest
92 running N addition experiments in existence. For this reason, it is a particularly appropriate
93 choice for experiments exploring fungal evolution where the continuous and consistent exposure

94 to elevated N has likely acted as a novel selective pressure on the fungi growing within the plots.
95 While soil fungi occasionally grow very large (Smith et al. 1992) and it is possible that a single
96 individual could grow between plots, the plots are large enough to encompass the entire canopies
97 and root structures of multiple individual trees (>100 in each plot; Fig. S1) and would, by
98 extension, house the fungal communities associated with these trees, especially saprotrophic taxa
99 housed within individual decomposing leaves and litter patches.

100 Litter samples for culturing fungi were obtained from a litterbag experiment described in
101 van Diepen et al. (2015). The culture media used to isolate fungi and the protocol used to
102 identify them are detailed in the Supplementary Information. Briefly, we targeted those fungal
103 species that were isolated from both the control and N treatment plots. To minimize the
104 probability of using the same individual twice, we never used more than one isolate of a species
105 from the same litterbag and made sure that isolates of the same species came from different
106 litterbags collected at least 5 m apart. Approximately 1500 isolates were cultured, and in this
107 isolate collection, more than 100 different morphologies were distinguishable. A subset of 60
108 morphologies was found in both the control and at least one N treatment, and following
109 identification using the internal transcribed spacer (ITS) barcode, the different morphologies
110 grouped into a collection of 41 different species. The majority of species were from the phylum
111 Ascomycota or Zygomycota, and 21 species (51%) matched an operational taxonomic unit
112 (OTU) found in a high-throughput sequencing dataset (Morrison et al. in prep) obtained from the
113 same litterbags. Nineteen of these 21 species had either representative isolates from all three
114 field treatments or representatives from the control and one of the N treatments. Subsequent
115 experiments targeted ten ascomycetes (Supplementary Table S1). All were 1) identified and
116 grouped using the ITS barcode, 2) found in the high-throughput sequencing dataset (providing a

117 context for future work) and 3) found in the control and at least one N treatment plot. In the
118 sequencing dataset, the relative abundances of the ten taxa ranged from 0.01 to 1.2% (Table S1),
119 typical for saprotrophic taxa at our site (Morrison et al. 2016). Collectively, the relative
120 abundance of these taxa was 1.4% in the control treatment, increasing to 1.9 and 3.9% in the N50
121 and N150 treatments, respectively. We also included one basidiomycete in this study, although
122 basidiomycetes were harder to culture as they were mostly outgrown by ascomycetes on all
123 culture media. To facilitate communication, we assigned a provisional name to each group of
124 isolates based on the best match to a voucher species within NCBI, with a minimum requirement
125 of 97% sequence similarity and a bit score of 800 (Table S1). From this point forward, we refer
126 to each of our species using its provisional generic epithet. Seven of the 11 selected taxa were
127 available as multiple biological replicates (more than one isolate was found in each plot). Eight
128 of the species were cultured from all three N treatments, but two (*Discosia* and *Phacidium*) were
129 only isolated from the control and N50 treatments and one (*Irpex*) was only isolated from the
130 control and N150 treatments.

131 *Fungal growth and litter decay.* To measure potential changes in the physiology of isolates,
132 we compared the behaviors of isolates from the two N treatments (N isolates) with isolates of the
133 same species collected from the control treatment (control isolates). We use the term “isolate
134 origin” to indicate the field treatment (N0, N50, or N150) from which each culture was isolated
135 and “laboratory environment” to indicate the N treatment simulated by our common garden
136 experiment (described below). Isolates from the control treatment were grown in their home
137 environment, as well as N50 and N150 (away) environments, and isolates from the N treatments
138 were grown in the control (away) environment and their home (N50 or N150) environment.

139 Mycelial growth rates are often used as a measure of fungal fitness (Pringle and Taylor
140 2002). Here we compared mycelial growth rates among isolates of the same species by growing
141 them at 25°C in darkness in ‘race tubes’ (sensu White and Woodward 1995) created by filling
142 serological pipettes with a horizontal strip of culture medium: 1.5% potato dextrose agar
143 dissolved in half-strength Hoagland nutrient solution supplemented with inorganic N at levels
144 representative of the available N ($\text{NH}_4 + \text{NO}_3$) in the organic soil horizon of each respective field
145 treatment (Supplementary Fig. S2). Growth was measured daily until each isolate reached the
146 end of its race tube when growth rate was calculated in mm day^{-1} . We ran three laboratory
147 replicates per biological isolate in each environment, and whenever possible, growth rates were
148 calculated for the multiple biological replicates per species isolated from each field treatment.

149 To test the ability of the fungal isolates to decompose plant litter, we used a common garden
150 approach where fungi isolated from both control and N enriched field plots were grown in the
151 laboratory in their respective home and away environments. Petri dishes were filled with 30 ml
152 of culture medium (1.5% agar dissolved in half-strength Hoagland nutrient solution) which was
153 then covered with ~0.75 g of sterile oak litter (Fig. S3a). The underlying medium mimicked the
154 organic (O_e/O_a) horizon in the field plots, with N levels representative of available N ($\text{NH}_4 +$
155 NO_3) within each of the three field treatments (N0, N50, or N150), while the oak litter mimicked
156 the O_i (litter) layer. Fresh oak litter was collected from each treatment plot using litter traps.
157 Collected oak litter was air dried at room temperature and the leaves cut into ~2 x 2 cm pieces
158 after stem removal. Before use, litter was oven-dried (60°C for 48 hours) and subsequently
159 sterilized (three rounds of autoclaving at 121°C). Because these experiments were extraordinarily
160 time intensive, we made the deliberate choice to include more species with a single biological
161 replicate each, rather than fewer species with more biological replicates. We manipulated one

162 biological replicate per species per treatment and ran three lab replicates for each isolate in each
163 of its test environments, with the exception of *Trichoderma koningii* for which we used three
164 independent biological isolates per treatment for an initial proof-of-concept study. Each Petri
165 dish was inoculated with one plug of an isolate's stock culture placed on top of the litter at the
166 center of each plate (Fig. S3a). Petri dishes were incubated for seven weeks at 25°C in darkness
167 (Fig. S3b). At harvest, all litter was removed from each Petri dish by hand using forceps,
168 weighed, and subsequently oven dried at 60°C for 48 hr to determine moisture content. The
169 ability of each isolate to decay litter in each environment was calculated as percent litter mass
170 loss (dry wt basis).

171 *Statistical analyses.* Two-way analysis of variance (ANOVA) was used to test whether long-
172 term exposure to N enrichment significantly affected the growth rate or litter mass loss
173 associated with each species, with isolate origin and laboratory environment as independent
174 variables (R version 3.0.1, R Core Team, 2013). We compared the responses of N50 or N150
175 isolates with control isolates of the same species. When interactions between isolate origin and
176 laboratory environment were significant, we analyzed the data of each environment separately to
177 test if isolates of different origin behaved differently in a common environment. When
178 necessary, variables were transformed to ensure a normal distribution and homogeneity of
179 variance. Using the same approach, we also tested for a general fungal response, by pooling the
180 data across all species.

181 RESULTS AND DISCUSSION

182 Global change experiments offer a unique opportunity to investigate and record evolutionary
183 responses to rapid environmental change in the field, but their potential remains largely
184 unexploited (Weese et al. 2015; Bataillon et al. 2016). Microbial evolution is likely to impact

185 critical ecosystem processes, including organic matter decomposition. We tested whether chronic
186 soil N enrichment has affected the ability of diverse fungi to decompose plant material. Using a
187 common garden approach, we grew control and N-exposed fungi isolated from a 28 year
188 simulated N deposition experiment in identical laboratory environments to test whether fungi
189 exposed to chronic N enrichment consistently grow differently, or decompose litter differently,
190 compared to control fungi. Experiments tested whether environment is the main driver of
191 behaviors by comparing the same individuals across different laboratory environments, while
192 simultaneously testing whether the identity of the individuals themselves is instead the main
193 driver of behaviors by comparing different individuals isolated from different treatment plots
194 (from control plots or N exposed plots) in the same laboratory environments (the same
195 “gardens”). To our knowledge, this study is the first to test if and how fungal behaviors relevant
196 to a critical ecosystem process evolve in response to long-term environmental change.

197 *Growth rates.* All species grew with a single growth front and in a consistent linear fashion
198 along race tubes, except *Cladosporium* whose growth was patchily distributed throughout the
199 race tubes. This growth pattern made it impossible to calculate a growth rate and so this species
200 was excluded from subsequent growth rate analyses. The remaining species grew at very
201 different rates; the rapidly growing *Trichoderma* raced at $\sim 22 \text{ mm dy}^{-1}$ while growth rates of the
202 other species ranged from 1.0 to 10 mm dy^{-1} .

203 While biological replicates of a species from a specific treatment plot behaved similarly to
204 each other, different species showed idiosyncratic growth responses, with one species
205 (*Paraconiothyrium*) exhibiting faster growth of N isolates compared to control isolates and two
206 species (*Cylindrium*, *Trichoderma*) showing slower growth (Fig. 1). Two species (*Alternaria*,
207 *Discosia*) showed no response of long-term N enrichment on growth rate, while four species

208 (*Epicoccum* I and II, *Pestalotiopsis*, *Phacidium*) showed mixed results. Thus the majority of
209 ascomycete species proved sensitive to chronic N additions, with isolates from N amended plots
210 growing differently (faster or slower) from control isolates, even when N isolates were grown in
211 the away (control, low N) environment. However, the direction of change was not predictable,
212 with the average response being no effect. The basidiomycete *Irpex* showed significantly higher
213 growth rates for the N isolates (9.8-10 mm dy⁻¹) in both their home (N150) and away (N0,
214 control) environments compared to the control isolates (7.6-7.8 mm dy⁻¹; origin: $P < 0.0001$,
215 environment: $P = 0.1976$; Fig. 2A).

216 *Litter decay.* While growth responses were inconsistent, the capacity to decay plant litter,
217 potentially a more relevant measure of ecosystem function, was significantly and consistently
218 less for fungi isolated from N enriched plots compared to the same species isolated from the
219 control treatment. Mass loss of litter averaged 19.6-34.9% for the white-rot fungus *Irpex*,
220 compared to 7.0-12.6% for the ascomycete species (Figs. 2B and 3), which is not surprising
221 since white-rot fungi can typically fully decompose lignocellulose, while most ascomycetes
222 specialize in cellulose breakdown and only partially decompose lignin. Mass loss for litter
223 decomposed by N isolates of *Irpex* was significantly lower compared to control isolates and this
224 result was independent of the laboratory environment in which the isolates were grown (origin: P
225 < 0.0001 ; environment: $P = 0.3803$; Fig. 2B). Mass loss for litter decomposed by ascomycete
226 species was on average 17.9 and 26.2% lower for N50 and N150 isolates, respectively, compared
227 to control isolates (Fig. 3). While isolate origin was significant ($P < 0.01$), there was no
228 significant origin \times environment interaction, meaning that N50 and N150 isolates decomposed
229 litter more slowly than control (N0) isolates, irrespective of the laboratory environment in which
230 they were grown (Fig. 3 inset). In terms of species-level responses, *Cylindrium* proved to be an

231 exception and the only species able to decay litter more effectively after exposure to chronic N
232 enrichment and this was only for isolates collected from the highest N addition (N150) treatment.
233 There was no relationship between litter mass loss and growth rates ($r^2 = 0.04$; $P = 0.1063$; Fig.
234 S4), suggesting that growth rate, as measured here, is not a useful predictor of decay dynamics.

235 *Synthesis.* In the aggregate, our data suggest that fungi growing in N enriched plots have
236 evolved and are less able to decompose plant litter than the same species growing in control
237 plots. Individual fungal isolates behaved consistently across home and away environments; an
238 isolate less able to decompose in one environment was less able to decompose in all
239 environments. Isolates from N treatment plots were generally less able to decay litter than
240 isolates of the same species from control plots, and the behaviors of these N isolates did not
241 revert or resemble control isolate behaviors even when the N isolates were grown in control
242 environments. An especially striking difference was recorded among isolates of the
243 basidiomycete, lignin-degrading species *Irpex*; litter mass loss was 30-44% lower for the N
244 isolates compared to the control isolates in both control and N enriched laboratory environments.

245 Whether or how these altered behaviors will benefit a fungus remains untested and would
246 require the tracking of entire life cycles in nature. The mechanism causing behavioral changes
247 also remains unknown. Changes may be mediated by altered patterns of gene expression,
248 including, for example, gene expression alterations caused by novel patterns of DNA
249 methylation; changes in allele frequencies between the populations of control and N treatment
250 plots so that rare alleles become dominant in treatment plots; novel DNA sequence evolution
251 with downstream effects on enzyme functions; or some combination of these or additional
252 forces. Regardless of mechanism, it appears that long-term exposure to chronic N additions may
253 have acted as a novel selective pressure on the behaviors of these saprotrophic fungi. And while

254 there may be gene flow across field treatment plots, the fact that we found consistent changes in
255 species behaviors suggests that whatever dispersal there is among plots is not enough to counter
256 natural selection. Our data connect to a classic literature on plant adaptation, including
257 experiments documenting rapid adaptation to pollution and across sharp clines or adjacent
258 treatment plots (e.g., Snaydon and Davies 1982). Early research on the heavy metal tolerance of
259 plants growing on toxic mine spoils (Antonovics et al. 1971) served to both document plant
260 adaptation to environmental change and provide evidence for genetic differences among closely
261 spaced populations. The fungal behaviors described here are clearly an analogous result.

262 Previous research at the CNAS has demonstrated that N enrichment increases the species
263 richness and diversity of ascomycetes generally (cellulose degraders), with an especially
264 pronounced effect on the relative abundance of several ascomycete genera (*Trichophyton*,
265 *Phialophora*, *Hypocrea/Trichoderma*, and *Rhizoscyphus* spp.) (Morrison et al. 2016). Others
266 have also observed increased ascomycete richness and abundance with soil N enrichment
267 (Allison et al. 2007; Weber et al. 2013). A shift in the fungal community toward taxa whose
268 primary niche appears to be cellulose (rather than lignin) decay coupled with the fundamental
269 changes in fungal behaviors we observed here (among ascomycetes and a basidiomycete) may
270 accompany or perhaps even drive previously observed N-induced declines in fungal biomass,
271 ligninolytic enzyme activity, and plant litter decay (Magill and Aber, 1998; Frey et al. 2004,
272 2014). Because observed changes in decay abilities were not readily reversed when N isolates
273 were grown in control environments, whether or how quickly a fungal community will recover
274 following the cessation of N enrichment emerges as another critical, unanswered question.
275 Meanwhile, and for as long as N pollution continues to be a feature of our rapidly changing

276 world, the evolution of fungal behaviors in these novel selective contexts will likely shape
277 ecosystem function.

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361 SUPPORTING INFORMATION

362 Additional supporting information may be found in the online version of this article at
363 <http://onlinelibrary.wiley.com/>.

364

FIGURE LEGENDS

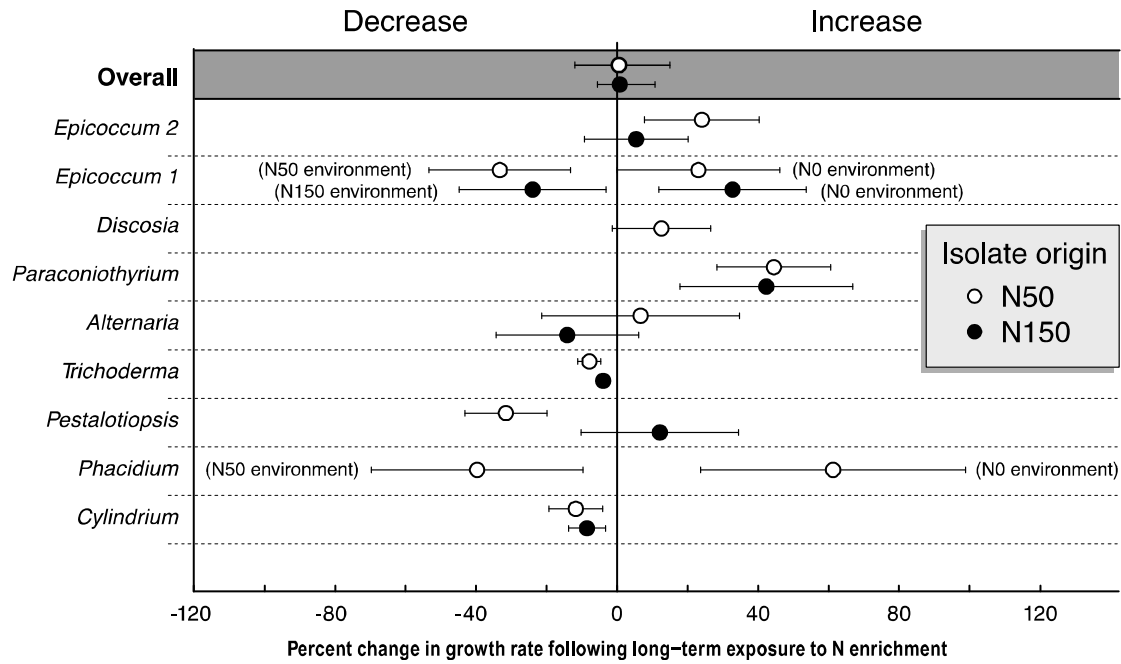
365 **FIG. 1.** Percent change in growth rates of cellulolytic ascomycetes following long-term exposure
366 to soil N enrichment. Open and filled circles represent N50 or N150 isolates, respectively,
367 compared to control (N0) isolates. Note *Discosia* and *Phacidium* were only isolated from control
368 plots and one of the two N treatment plots. Error bars are 95% confidence intervals. When error
369 bars do not overlap with zero, the change in growth rate is significant at $\alpha < 0.05$. Because there
370 was a significant statistical interaction between isolate origin and laboratory (common garden)
371 environment for *Epicoccum 1* and *Phacidium*, the growth rates of these species in each of the lab
372 environments are shown separately.

373 **FIG. 2.** Mycelial growth rate (A) and litter mass loss (B) (mean and SE) for the white rot
374 basidiomycete *Irpex* grown in home and away environments. Isolates of *Irpex* were not cultured
375 from the N50 treatment.

376 **FIG. 3.** Percent change in litter decay capacities of cellulolytic ascomycetes following long-term
377 exposure to soil N enrichment. Open and filled circles represent N50 or N150 isolates,
378 respectively, compared to control (N0) isolates. Note *Discosia* and *Phacidium* were only isolated
379 from control plots and one of the two N treatment plots. Error bars are 95% confidence intervals.
380 When error bars do not overlap with zero, the change in litter mass loss is significant at $\alpha < 0.05$.
381 There were no significant interactions between isolate origin and laboratory environment. Inset
382 graph on right shows litter mass loss averaged across all ascomycete species grown in their home
383 versus away environment. Error bars indicate one standard error of the mean.

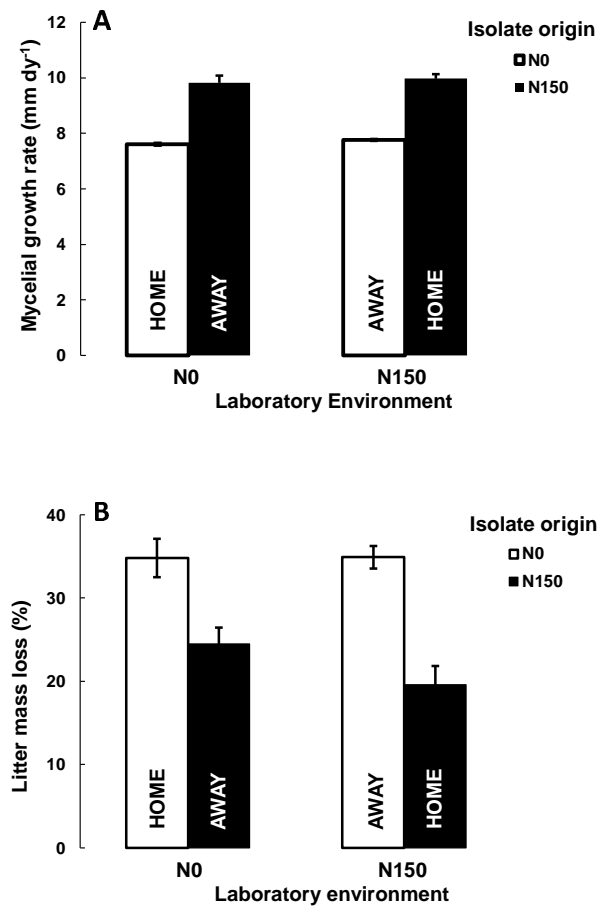
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387 FIG. 1.

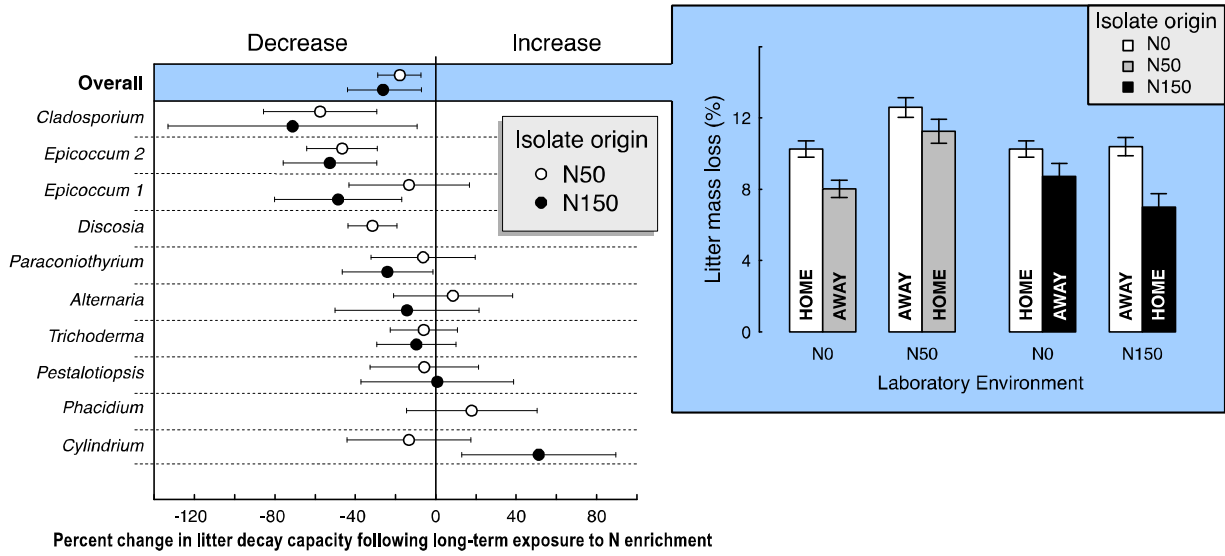


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389

390 FIG. 2.

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393 FIG. 3.

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