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High-throughput eDNA monitoring of fungi to track functional recovery in ecological restoration

Running headline: **Monitoring fungi for restoration**

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19 **Abstract**

20 Fungi are key functional components of ecosystems (e.g. decomposers, symbionts),
21 but are rarely included in restoration monitoring programs. Many fungi occur
22 belowground, making them difficult to observe directly, but are observable with
23 environmental DNA (eDNA) methods. Although eDNA approaches have been
24 proposed as ecological monitoring tools for microbial diversity, their application to
25 restoration projects is very limited. We used eDNA metabarcoding of fungal ITS
26 barcodes on soil collected across a 10-year restoration chronosequence to explore
27 fungal responses to restoration. We observed a dramatic shift in the fungal
28 community towards that of the natural fungal community after just 10 years of active
29 native plant revegetation. Agaricomycetes and other Basidiomycota – involved in
30 wood decay and ectomycorrhizal symbiosis – increased in rarefied sequence
31 abundance in older restored sites. Ascomycota dominated the fungal community, but
32 decreased in rarefied sequence abundance across the restoration chronosequence.
33 Our results highlight eDNA metabarcoding as a useful restoration monitoring tool
34 that allows quantification of changes in important fungal indicator groups linked with
35 functional recovery and, being underground, are normally omitted in restoration
36 monitoring.

37

38

39 **Keywords**: ecosystem function; eDNA; genomics; land degradation; microbiome;
40 restoration genomics

41

42 **1. Introduction**

43 Land clearing and unsustainable land use are driving a global land degradation crisis
44 (Gibbs and Salmon 2015; Nkonya et al. 2016). Ecological restoration is employed as
45 the primary intervention to repair degraded land, largely to re-instate functional
46 ecosystems and native biodiversity (Suding et al. 2015). Effective and targeted
47 restoration is required, in combination with accurate biological monitoring, to achieve
48 these restoration goals (Collen and Nicholson 2014).

49 Terrestrial ecosystems consist of aboveground and belowground
50 components that interact to shape ecological communities (Wardle et al. 2004).
51 Plants influence the composition of belowground biota, and in turn, belowground
52 biota feedback to influence plants. For example, fungi contribute major ecological
53 functions such as decomposition and nutrient cycling, especially carbon and soil
54 aggregation (Avis et al. 2017; Morriën et al. 2017). Mycorrhizal fungi can influence
55 the status of soil nutrients, and also the establishment, diversity and succession of
56 plants (Cavagnaro et al. 2005; Kulmatiski et al. 2008). Therefore, understanding the
57 dynamics of fungal communities is important to influencing ecosystem functions
58 (Gehring et al. 2014), and as such should be a key focus point of ecological
59 restoration.

60 Fungi are large components of the biodiversity in many soil ecosystems,
61 even in species-poor plant communities (Taylor et al. 2014). The diversity and
62 community dynamics of soil fungi are often linked to soil physical, chemical, and
63 biological properties (e.g. age, pH, nutrient levels) (Guo et al. 2016; Moon et al.
64 2016; Trivedi et al. 2016; Zechmeister-Boltenstern et al. 2011). These soil properties
65 are often influenced by vegetation cover, land-use, and revegetation practices. As
66 such, assessing changes in the fungal community during ecological restoration is an

67 important part of determining the return of functional ecosystems and native
68 biodiversity to restoration sites – key indicators of restoration success (Harris 2009).
69 Despite the potential for revegetation to influence fungal diversity, few restoration
70 projects have monitored changes in the fungal community, and used the fungal data
71 as part of the assessment of restoration progress and success (Harris 2003).

72 A primary reason why fungi often go unmonitored in restoration is that many
73 are belowground and microscopic, making them difficult to observe *in situ*. However,
74 with next generation sequencing approaches, researchers can now efficiently and
75 accurately assess such highly diverse and cryptic biological communities (Lindahl et
76 al. 2013). High-throughput amplicon sequencing of environmental DNA (eDNA) –
77 metabarcoding – can identify and quantify the biological sources of genetic material
78 (Barnes and Turner 2015; Corlett 2017; Ji et al. 2013). As such, metabarcoding has
79 been put forward as a cost-effective, efficient and easy-to-standardise approach that
80 can be used to survey and monitor even the most cryptic biodiversity. Metabarcoding
81 has already proven to be an effective and efficient method to survey soil bacterial
82 and fungal microbiomes (Rime et al. 2015; Taberlet et al. 2012). However, there are
83 few examples of using metabarcoding to explore changes in biodiversity in a
84 restoration context (Gellie et al. 2017b; Mills et al. 2017).

85 In this study, we tested the hypothesis that replanting the native plant
86 community into an ex-pasture will lead to restoration of the fungal community. To test
87 this hypothesis, we used metabarcoding to explore the soil fungal community across
88 a 10-year revegetation chronosequence, including samples from remnant sites (the
89 revegetation reference sites), and cleared sites. We analysed these samples to
90 address the following questions: (i) Does native overstory revegetation alter the soil
91 fungal community? (ii) Which functional groups of fungi are indicators of the different

92 stages of revegetation? (iii) How do soil physicochemical parameters respond to
93 revegetation, and do these changes associate with the fungal community?
94

95 **2. Material and Methods**

96 *2.1 Site description and sampling*

97 Our study system was an active restoration site at Mt Bold, a water catchment
98 reserve of the Mt Lofty Ranges in South Australia (35.07˚S, 138.42˚E), described in
99 detail in Gellie et al. (2017b). This catchment was dominated by open eucalypt
100 woodland, but has been cleared and grazed from early in the 20th century. Grazing
101 ceased in 2003, restoration began in 2005, and the restoration goal was to recreate
102 the local *Eucalyptus leucoxylon* grassy woodland community, as found in the
103 remnant, reference sites (Remnant A and B). Prior to 2005, Remnant A was
104 minimally cleared and had low-density grazing, and remnant B was protected from
105 clearing and had minimal human impact. Each reference site is in close proximity to
106 the restoration site (<1km).

107 Revegetation methods were consistent across the study system. This
108 included the use of the same site preparation method (i.e. shallow surface rip), plant
109 species mix (i.e. replanting the same subset of over-story and mid-story plant
110 species present in the local woodland community), timing (i.e. late winter planting),
111 and maintenance (i.e. fencing to exclude livestock, annual grass slashing, woody
112 weed removal). The sites restored between 6 and 10 years ago were revegetated
113 with the same local, native plant species, including the overstory South Australian
114 blue gum (*E. leucoxylon*) and manna gum (*E. viminalis*), and a shrub layer that
115 included golden wattle (*Acacia pycnantha*), sticky hop bush (*Dodonaea viscosa*) and

116 sweet bursaria (*Bursaria spinosa* ssp. *spinosa*). Remnant A had weed control, and
117 remnant B was managed for conservation.
118 In January 2015, we sampled soil from three randomly selected 25 x 25 m
119 quadrats at each of seven sites, including sites restored 6, 7, 8 and 10 years before
120 sampling, a cleared site, and the two remnant, reference sites (the restoration
121 reference sites; remnant A and B in Gellie et al.2017b), giving a total of 21 quadrats.
122 Soil was sampled from the 0-10 and 20-30 cm soil horizons at each quadrat. The
123 data used for this work was generated from the Biomes of Australian Soil
124 Environments (BASE) database workflow, and is downloadable as OTU abundance
125 tables from the BASE download portal (samples 102.100.100/19281 – 19322).
126 Below we briefly describe the BASE methods, which are described in detail in Bissett
127 et al. (2016). Sampling was conducted as part of the Biomes of Australian Soil
128 Environments (BASE) project according to the protocol described in Bissett et al.
129 (2016). Briefly, nine soil samples per quadrat were pooled into a sterile plastic bag,
130 homogenised using a sterilised trowel, and frozen on site in sterile 50 mL falcon
131 tubes – hereafter the replicates (n = 42). 300 g of homogenised soil was also
132 sampled for soil physicochemical analysis, quantifying soil moisture, ammonium,
133 nitrate, available phosphorus, sulphur, organic carbon, and soil pH (H_20).
134
135 2.2 *Genomic analyses*
136 DNA extraction and sequence analysis were conducted according to the methods
137 described in Bissett et al. (2016). Briefly, soil DNA was extracted in triplicate using
138 MoBio PowerSoil extraction kits according to manufacturer’s instructions, together
139 with extraction blank controls. We PCR-amplified the fungal internal transcribed
140 spacer (ITS) region for each replicate with negative controls using primers ITS1F

141 (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR products were
142 screened for negative control contamination with gel electrophoresis, purified using
143 the Agencourt AMPure XP bead PCR product purification kit as per manufacturer's
144 instructions, concentration normalised to 10 nM, and sized on an Agilent Bioanalyze.
145 Equal volumes of products were pooled, diluted to 4 nM and sequenced on the
146 Illumina MiSEQ platform with MiSeq Reagent Kit v3 600 cycle chemistry, to produce
147 300bp paired end reads.
148 Read analysis was also done as per Bissett et al. (2016) as part of the BASE
149 dataset analysis. Briefly, the ITS1 region was extracted from Illumina R1 reads using
150 ITSx (Bengtsson-Palme et al. 2013) and Operational Taxonomic Units (OTUs)
151 clustered at 97% sequence similarity between ITS1 reads using USEARCH
152 v8.0.1517 (Edgar 2010). OTUs were classified against the UNITE v7.0 fungal
153 database (Koljalg et al. 2013), using the Wang classifier (Wang et al. 2007) in
154 MOTHUR. We discarded OTUs not identified as belonging to fungi, unidentified at
155 the phylum level, or having <100 reads across the full BASE dataset (>900 samples)
156 as in Gellie et al. (2017b).
157
158 2.3 *Statistics*
159 We used R v 3.3.2 (R Core Team) for all statistical analyses. OTU abundance was
160 rarefied to the replicate with the lowest number of reads (49,724 reads for 0-10 and
161 51,138 reads for 20-30 cm soil samples, respectively) with the *rarefy* function in
162 *vegan* v 2.4-3 (Oksanen et al. 2017). OTU richness was measured using the Chao 1
163 nonparametric richness estimator. Diversity was estimated as the effective number
164 of species (Jost 2006) using the Shannon-Wiener index (H) and the Gini-Simpson
165 index (D), where the Shannon-Wiener index and Gini-Simpson index were

166 transformed by using the formula exp(H) and 1/(1-D), respectively, to evaluate the
167 true diversity of the fungal community.

168 Differences in rarefied abundances of the sequence reads, OTU richness,
169 diversity indices, phyla, classes and soil characteristics across the restoration sites
170 (i.e. the restoration chronosequence), soil depths, and the interaction between
171 restoration site and soil depth were analysed using a multifactor permuted analysis
172 of variance (PERMANOVA) with the *aovp* function implemented in *lmPerm* 2.1.0
173 package with 5,000 permutations.

174 The effect of the restoration sites on fungal composition was visualised using
175 non-metric multidimensional scaling (NMDS) ordinations using Bray-Curtis (rarefied
176 abundance) and Jaccard (presence-absence) dissimilarity matrices, which were
177 generated with *vegan's vegdist, metaMDS, stressplot and ordiplot* functions
178 (Oksanen et al. 2017). Differences in fungal community composition across the
179 restoration chronosequence and soil depths were tested using ANOSIM analysis
180 (999 permutations) on Bray-Curtis dissimilarity matrices with the *anosim* function in
181 vegan, estimating R values, where R close to 1 indicates high separation between
182 groups (e.g. between restoration sites) and R close to 0 indicates little separation
183 between groups.

184 Distance-based redundancy analyses (db-RDA) were run to visualize the
185 relationships between soil physical and chemical variables and fungal community
186 composition based on Bray-Curtis and Jaccard distances. The *ordistep* function with
187 the forward procedure in the *vegan* package was used to select the soil physical and
188 chemical variables that best predicted the differences in fungal community structure.
189 The selected variables were then used to build a constrained ordination plot. This
190 procedure selects predictor variables that significantly improve model fit using a

191 permutation test with the *permutest* function, keeping the strongest variable in the
192 model, and repeats this process until no further predictor significantly improves the
193 model fit.

194 Indicator species analysis was run using the *multipatt* function implemented in
195 the *indicspecies* package with 99,999 permutations. *P* value correction for multiple
196 testing was run using the *fdrtool* function implemented in the *fdrtool* package
197 (Strimmer 2008) with a false discovery rate of 10% (q <0.10).

198 We analysed fungal trophic mode and guild with FUNGuild v1.0
199 (https://github.com/UMNFuN/FUNGuild) (Nguyen et al. 2016). FUNGuild v1.0 is a flat
200 database that contains a total of 9,476 entries, with 66% at the genus level and 34%
201 at the species level (Nguyen et al. 2016). Fungal OTU tables with OTUs in rows,
202 samples in columns, and a 'taxonomy' column were inputs (at
203 http://www.stbates.org/guilds/app.php). Outputs included the original OTU table,
204 sorted by sequence abundance, with trophic mode, guild, and confidence data.
205 Trophic modes for all rarefied OTUs were accepted if the match confidence was
206 'highly probable' or 'probable'. We then determined the fungal functional value of
207 each restoration site according to the methods described in Avis *et al.* (2017) and
208 Dighton (2003). Differences in rarefied abundance, richness of different trophic
209 modes, functional values and saprophyte:symbiotroph across the restoration sites
210 and soil depths were determined using multifactor PERMANOVAs with 5,000
211 permutations in *LmPerm* 2.1.0 package.

212

213 **3. Results**

214 *3.1 Fungal diversity and community composition*

215 We generated a total of 4,993,144 ITS fungal raw sequence reads (118,884 ±
216 42,210 SD per replicate) across the 42 replicates (Table 1). A total of 4,955,680
217 fungal sequences (117,430 ± 42,164 SD per replicate) remained for further analysis
218 after quality filtering. No significant differences in read abundance were observed
219 across the restoration chronosequence (Table 1). The number of fungal operational
220 taxonomic units (OTUs) was lower in the 20-30 cm than the 0-10 cm soil horizon
221 (observed and Chao 1; Table 1). Richness also varied significantly across the
222 restoration chronosequence, but did not correspond with time since revegetation
223 (Table 1). The effective species number based on Shannon's and Simpson's
224 diversity did not vary significantly across the restoration chronosequence or soil
225 depths (Table 1).

226 We observed clear directional changes in the fungal community across the
227 restoration chronosequence (Figs. 1, Fig. A1, Supplementary material). Recently
228 revegetated sites had fungal communities similar to cleared sites, and older
229 revegetated sites were similar to remnant sites. The ANOSIM showed that the fungal
230 community differed significantly across the restoration sites based on Bray-Curtis
231 dissimilarity ($R = 0.772$, $P < 0.001$) and Jaccard dissimilarity ($R = 0.650$, $P < 0.001$).

232 The fungal communities were dominated by four phyla at 0-10 cm and five
233 phyla at 20-30 cm (each with >1.0% of the total number of sequences), representing
234 98.9% and 99.9% of the sequence reads respectively (Fig. A2, Table A1). Of the
235 dominant phyla, Ascomycota was the most abundant, followed by Basidiomycota.
236 Across all sites, the total percentage of Ascomycota was 55.67% and 46.34%, and
237 Basidiomycota was 39.80% to 45.24%, at 0-10 and 20-30 cm respectively.

238 Ascomycota, Glomeromycota and Rozellomycota exhibited significant
239 decreases in rarefied sequence abundance across the restoration chronosequence,

240 and Basidiomycota showed a significant increase (Fig. A2, Table 2). Shannon's
241 diversity of the phyla Basidiomycota and Glomeromycota changed significantly, but
242 showed no directional changes with the restoration chronosequence and no
243 significant changes with soil depth, with only Chytridiomycota showing a significant
244 increase in diversity from 0-10 to 20-30 cm (Table 2).

245 A total of 6.05% and 8.81% of total sequence reads at 0-10 and 20-30 cm
246 depths, respectively, were unclassified at the class level. The classes
247 Eurotiomycetes and Sordariomycetes (both Ascomycota) were most abundant at
248 cleared sites, and both decreased in abundance significantly with time since
249 revegetation (Fig. A3, Table 2). Agaricomycetes (Basidiomycota) and Leotiomycetes
250 (Ascomycota) were the dominant classes at remnant and older restoration sites, and
251 both showed a pattern of increasing abundance with time since revegetation.
252

253 *3.2 Indicator and guild analysis*

254 Indicator species analysis revealed 26 and 42 fungal OTUs (each with >0.1% of the
255 total number of sequences in all samples) were associated with the restoration
256 chronosequence at 0-10 and 20-30 cm soil, respectively (q <0.10; Figs. 2, 3), and
257 largely formed distinct indicator species assemblages across the restoration sites.
258 Indicator genera, trophic modes and guilds for remnant and older revegetation sites
259 were similar, and were different from the younger restoration and cleared sites. For
260 example, OTUs in the genera *Chloridium*, *Paecilomyces* and *Ruhlandiella* (all
261 Ascomycota) were associated with cleared sites at both soil depths, while OTUs in
262 the genera *Clavulina*, *Tomentella* (both Basidiomycota) and *Archaeorhizomyces*
263 (Ascomycota) were characteristic of remnant and older restoration sites (Figs. 2, 3).
264 OTUs in the *Paecilomyces* and *Phialemonium* genera had high rarefied abundance

265 and indicator values in cleared sites at 0-10 and 20-30 cm soil depths, respectively,
266 and these genera are known saprotrophs. While OTUs in the *Tomentella* and
267 *Clavulina* genera had higher rarefied abundance in remnant and older restoration
268 sites, and these genera are known ectomycorrhizal symbiotrophs.

269 Trophic modes were successfully assigned to 69% and 62% of the fungal
270 OTUs at 0-10 and 20-30 cm soil depths, respectively. After trophic modes assigned
271 with 'possible' confidence were removed, we obtained four dominant trophic modes
272 (each mode >2% of total remaining OTUs), which included 62% and 58% of the
273 rarefied sequences at 0-10 and 20-30 cm soil depth across all restoration sites.
274 Rarefied abundance of all trophic modes, and richness of saprotrophs, pathotrophs
275 and pathotrophs-saprotrophs, varied significantly across the restoration
276 chronosequence, with only pathotroph richness showing a significant directional
277 trend in decreasing abundance across the restoration sites (Table A4, Fig. A4). The
278 ratio of saprotroph to symbiotroph OTUs in cleared sites was generally lower than
279 restored and remnant sites at the 0-10 cm soil depth, while the opposite trend
280 appeared at the 20-30 cm soil depth (Table A3, Fig. A4). The functional values were
281 significantly higher at the 0-10 cm than that at 20-30 cm soil depth, and varied
282 significantly across the restoration chronosequence but showed no pattern with time
283 since restoration (Table A3, Fig. A4).

284

285 *3.3 Soil physicochemical effects*

286 Notable changes in soil physical and chemical characteristics were observed across
287 the restoration chronosequence (Tables A5, A6). Soil nitrate and phosphorous
288 significantly decreased with time since restoration, and organic carbon and sulphur
289 significantly increased. Phosphorous, organic carbon, ammonium and sulphur

290 significantly decreased with depth. The fungal community strongly associated with
291 soil physical and chemical variables (Figs A5, A6). Seven soil variables explained
292 63.70% of the variance in fungal community ($F_{7,34}$ = 8.524, P < 0.001). Of these
293 seven variables, nitrate, soil moisture, phosphorous and organic carbon were the
294 variables that best explained variance in the fungal community (Fig. A5), explaining
295 46.87% of the variance ($F_{4,37}$ = 8.159, P < 0.001).
296

297 **4. Discussion**

298 We used eDNA metabarcoding to demonstrate a significant shift towards a restored
299 state in the soil fungal community after just 10 years of active restoration of a retired
300 pasture. Our study is consistent with previous work on fungal communities, that has
301 shown them to change with ecological processes such as soil development with the
302 retreat of glaciers (Rime et al. 2015) and the emergence of islands (Clemmensen et
303 al. 2013). However, the dramatic shift in the natural fungal community over the
304 decade we observed in our study is in contrast with these previous studies where
305 changes occurred over decades to millennia. Here we also show that active
306 replanting of native vegetation can clearly lead to the return of important functional
307 groups of fungi, including lower rarefied abundance of pathotrophs and saprotrophs
308 (especially those in phylum Ascomycota) and increases in symbiotrophs (particularly
309 ectomycorrhizas in phylum Basidiomycota). Fungal OTU richness did not correspond
310 with time since revegetation, indicating that fungal community composition rather
311 than the number of fungal taxa responded to restoration. Our study indicates that
312 quantifying changes in the fungal community has great potential to be a robust
313 diagnostic tool in demonstrating the success trajectory of restoration practices, as
314 has been shown in allied areas of ecology (Thomsen and Willerslev 2015; Valentini

315 et al. 2016). Our results strongly support the case for using eDNA metabarcoding as
316 a functionally relevant monitoring tool of restoration projects.
317 Previous work has shown that fungal communities often show a
318 corresponding shift with changing vegetation communities, with most studies
319 focussing on natural ecological changes (e.g. succession) (Clemmensen et al. 2013;
320 Li et al. 2013; Rime et al. 2015). It is rare for changes in fungal communities to be
321 monitored or manipulated as part of the restoration process (Avis et al. 2017; Prober
322 et al. 2015). Recent work has shown that inoculating soils as part of the restoration
323 process can have strong impacts on the plant community (Delgado-Baquerizo et al.
324 2016; Soliveres et al. 2016; Wubs et al. 2016), carbon uptake by fungi increases
325 independently of fungal biomass and bacterial-to-fungal ratios (Morriën et al. 2017),
326 grass cover of sand dunes associates with fungal diversity (Zuo et al. 2016), and
327 replanting riparian zones can restore bacterial-to-fungal activity ratios (Mackay et al.
328 2016). However, few studies have characterised the return of fungal microbial
329 communities or key functional groups with restoration (Avis *et al.* 2016), and we
330 suggest that the lack of studies is due to the difficulty in studying changes in fungal
331 communities without eDNA approaches, such as the metabarcoding method we
332 employed.
333 Ascomycota and Basidiomycota were the two most abundant phyla in our
334 study, and both showed clear changes in rarefied abundance across the restoration
335 chronosequence, particularly in the 0-10 cm soil horizon. Basidiomycota, particularly
336 symbiotrophs in the class Agaricomycetes, increased in rarefied abundance across
337 the restoration chronosequence. In contrast, OTUs assigned as saprotrophs within
338 Ascomycota showed a pattern of decreasing rarefied abundance. Such trends are
339 consistent with studies on degraded land, including low nutrient content soil and

340 managed lands such as rice paddies (Burton et al. 2016; Corneo et al. 2014), alpine
341 grasslands (Pellissier et al. 2014) and oak forest soils (Varela et al. 2015).
342 Supporting our results, Gourmelon et al. (2016) showed that a larger representation
343 of Ascomycota in the fungal community can be an indicator of ecosystem
344 degradation, however more work is clearly needed to explore changes in this phylum
345 in more detail. The class Agaricomycetes (phylum Basidiomycota) is widespread in
346 many terrestrial ecosystems, and is involved in the decay of wood and is a common
347 ectomycorrhizal symbiont of forest trees (Bonfante and Genre 2010). In our study,
348 symbiotrophs in Agaricomycetes were characteristic at remnant and older restoration
349 sites and showed a significant increase in rarefied abundance across the restoration
350 chronosequence. These results suggest that Agaricomycetes may also be a good
351 indicator of restoration success in woodland and forest systems.
352 Indicator species and trophic mode analysis identified several OTUs that were
353 characteristic with time since restoration, although they generally clustered within
354 study sites. For example, an OTU in the genus *Chloridium* (OTU141; phylum
355 Ascomycota, class Sordariomycetes, trophic mode symbiotroph, guild
356 ectomycorrhiza) was associated with the cleared sites at both soil depths,
357 particularly in the 20-30 cm soil horizon (up to 4% of total fungal rarefied
358 abundance). An OTU in the genus *Clavulina* (OTU93; phylum Basidiomycota, class
359 Agaricomycetes, trophic mode symbiotroph, guild ectomycorrhiza) was associated
360 with remnant vegetation at both soil depths. Identifying ectomycorrhizas at higher
361 rarefied abundance in remnant and older restoration sites is supported by their
362 known association with forest and woodland tree species (Brundrett 2009). However,
363 with limited taxonomic and functional knowledge, the roles of the indicator fungi we

364 identified in the restoration of the woodland ecosystem require further exploration,
365 but our approach holds promise as a diagnostic tool.

366 Using field observations of macrofungi, Avis *et al.* (2017) derived functional
367 values and saprophyte to symbiotroph ratios as indicators of restoration success.
368 These approaches did not follow clear patterns across our restoration
369 chronosequence. We observed higher functional values and saprotroph to
370 symbiotroph ratios at the 0-10 cm than at the 20-30 cm soil depth, indicating that
371 revegetation may be having a stronger influence on shallow soil depths than deeper
372 soil depth. Further, and in contrast to our community and indicator taxon analyses,
373 the functional values and saprophyte to symbiotroph ratios did shift significantly, but
374 did not show any clear trends across our restoration chronosequence. We suggest
375 that future work should attempt to better integrate these field macrofungi approaches
376 with eDNA metabarcoding, as both approaches have benefits and problems. For
377 example, the field identification method suffers from ascertainment biases as it is
378 restricted to only sampling present macrofungi. Assigning function to fungi identified
379 with eDNA metabarcoding is problematic as it relies on external database curation.

380 We observed soil nitrate and phosphorous concentrations to significantly
381 decrease, and organic carbon concentration to significantly increase, across the
382 revegetation chronosequence. These edaphic changes are consistent with
383 expectations of the rehabilitation of pastoral lands (Cramer et al. 2008; Cunningham
384 et al. 2015). These soil characteristics were also strong predictors of changes in the
385 fungal community, which supports the general expectation that these soil
386 physicochemical properties strongly shape changes in the soil fungal community
387 (Zumsteg et al. 2011; Zuo et al. 2016). Such abiotic soil responses are expected to
388 associate with restoration, but importantly are also expected to be strong drivers of

389 fungal community structuring in soils (Tedersoo et al. 2014), as supported by our
390 results. For example, changes in vegetation should effectively modify the resource
391 availability and microclimate in soils, in which heterotrophic microbial communities
392 (e.g. fungi) will respond (McGuire et al. 2012; Zak et al. 2003).
393 The development of environmental DNA sequencing methods offers
394 extraordinary scientific and practical opportunities for better understanding soil fungal
395 dynamics, changes in functional diversity, and biodiversity diagnosis. However,
396 additional work is required to address some technical limitations of this approach.
397 For example, overcoming methodological biases, standardisation of methods, and
398 further methodological development for additional taxonomic groups require careful
399 consideration. Indeed, read abundance is commonly interpreted as biological
400 abundance, but read abundance is only an approximate quantification of biological
401 abundance and should be interpreted with caution (Amend et al. 2010). Better
402 understanding of functional diversity at lower taxonomic levels (e.g. genus) is
403 important to determine symbioses and trophic interactions (e.g. changes in the
404 rhizosphere during the restoration process) (Requena et al. 2001). Extending soil
405 assessments to include the study of metaproteogenomics has potential to yield high-
406 resolution functional data about these changing communities that cannot be derived
407 by eDNA metabarcoding (Seifert et al. 2013; Wilmes et al. 2015). Such an approach
408 can provide information on the biological activities of species within the community
409 such as carbon conversion, metal contamination metabolism, and niche partitioning,
410 by linking genomic sequences with functional proteins (Bastida et al. 2016; Gillan et
411 al. 2015; Knief et al. 2012).
412

413 **5. Conclusions**

414 The evidence we report here suggests that, at least in our study system, replanting
415 native vegetation can bring about a dramatic shift in the fungal community towards
416 that of the natural fungal community. Further, we demonstrate these soil microbiome
417 changes with high-throughput amplicon sequencing, which holds great promise to be
418 an efficient and standardisable tool to monitor and predict functional restoration
419 processes. Many questions do remain, such as how plant functional diversity
420 influences the fungal community? Which abiotic factors play the principal roles in
421 driving fungal dynamics? How the fungal community changes through time (e.g.
422 diurnal, seasonal)? From a monitoring perspective, when would interventions be
423 indicated based on such monitoring data? Answering such questions requires an
424 improved understanding of the link between abiotic factors and fungal community
425 dynamics with restoration practice. With modest investments into the knowledge
426 gaps, restoration science could embrace such novel technology and become a more
427 efficient and targeted practice.
428

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442
443 **Data accessibility**

444 All sequence data are available from the Biomes of Australian Soil Environments
445 (BASE) database (https://data.bioplatforms.com/bpa/base/amplicon/amplicons/ITS)
446 under sample numbers 102.100.100/19281 to 102.100.100/19322 and have been
447 deposited in the National Center for Biotechnology Information Sequence Read
448 Archive, under bioproject ID PRJNA317932. All OTU pipelines can be found at
449 (http://www.bioplatforms.com/soil-biodiversity/) under "BASE Protocols and
450 Procedures". OTU and soil chemistry data available from AEKOS Digital repository.
451 OTU raw data matrix (Gellie 2017) doi: 10.4227/05/58ca32e5ef782 and soil
452 chemistry (Gellie et al. 2017a) doi:10.4227/05/587d63e2dd056.
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687 **Table 1.** Richness and diversity indices of rarefied fungal abundance data across the restoration chronosequence assessed by

688 permuted analysis of variance (PERMANOVA), with *P* values < 0.05 in bold.

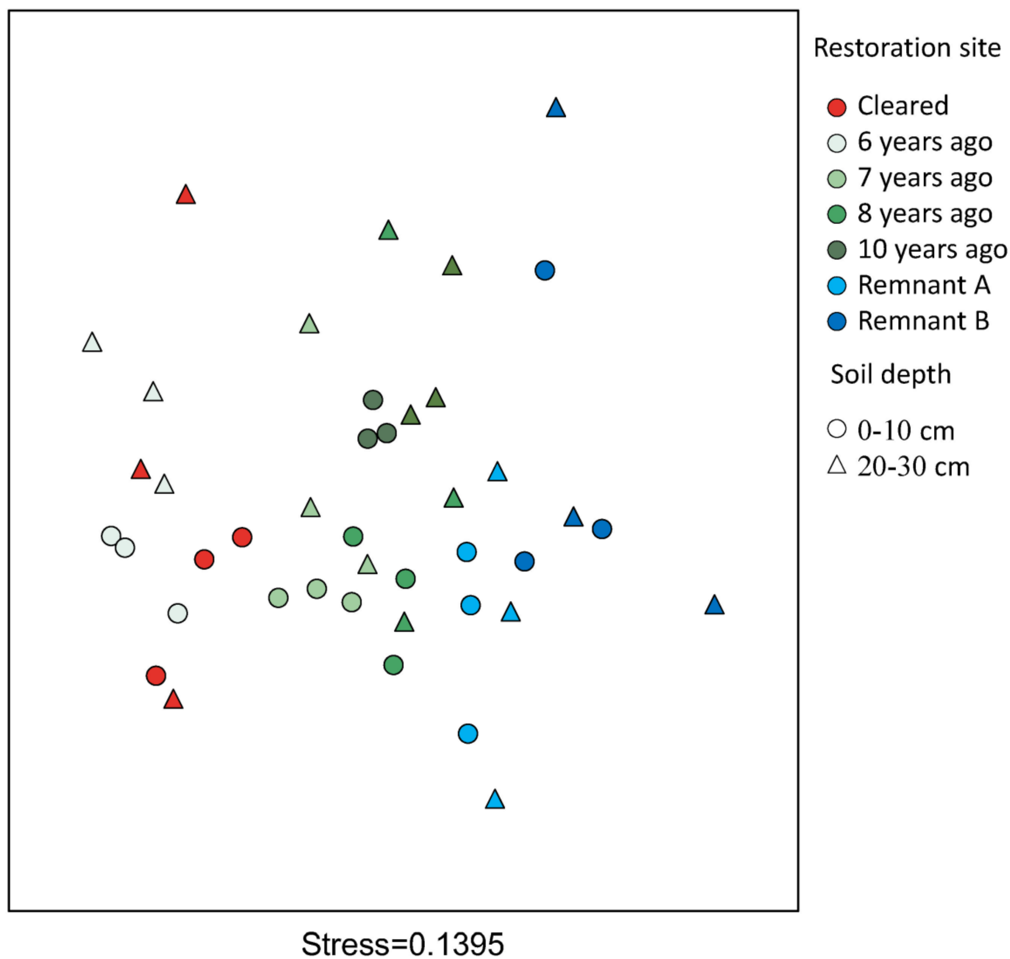
Site	Depth (cm)	OTUs[a] (±SD)		Diversity[b] (±SD)	
		Observed	*Chao* 1	*Shannon*	*Simpson*
Cleared	0-10	413 ± 26	477 ± 36	51.67 ± 12.09	17.70 ± 7.01
6 year	0-10	283 ± 58	304 ± 64	36.29 ± 33.00	15.44 ± 16.39
7 year	0-10	400 ± 114	432 ± 121	43.51 ± 14.37	17.89 ± 5.79
8 year	0-10	437 ± 136	484 ± 144	50.85 ± 21.50	16.84 ± 12.27
10 year	0-10	331 ± 11	370 ± 39	46.36 ± 13.58	17.14 ± 4.65
Remnant A	0-10	406 ± 49	446 ± 63	46.66 ± 21.69	17.31 ± 9.81
Remnant B	0-10	270 ± 81	295 ± 80	41.90 ± 11.89	16.68 ± 6.11
Cleared	20-30	303 ± 74	361 ± 54	48.39 ± 11.09	20.78 ± 3.02
6 year	20-30	231 ± 47	273 ± 47	32.24 ± 22.14	13.26 ± 12.57
7 year	20-30	309 ± 78	332 ± 81	48.73 ± 16.22	21.90 ± 4.74
8 year	20-30	277 ± 87	329 ± 103	36.62 ± 20.46	13.15 ± 11.72
10 year	20-30	335 ± 59	368 ± 72	65.12 ± 11.03	28.13 ± 6.26
Remnant A	20-30	332 ± 55	359 ± 60	54.87 ± 28.61	20.83 ± 18.35
Remnant B	20-30	218 ± 57	258 ± 43	31.50 ± 1.28	14.99 ± 0.21
PERMANOVA *P* values	Site	**0.017**	**0.006**	0.355	0.817
	Depth	**<0.001**	**0.001**	1.000	0.581
	Site x Depth	0.654	0.718	0.783	0.926

689 [a] The richness is calculated using the OTU number and Chao's species richness estimator (*Chao* 1).
690 [b] The diversity is effective number of species based on Shannon-Wiener H (*Shannon*) and Gini-Simpson's D indices (*Simpson*).
691
692

693 **Table 2.** Effects of restoration site and soil depth on rarefied abundance and diversity of fungal phyla and dominant classes

694 assessed by permuted analysis of variance (PERMANOVA), with *P* values < 0.05 in bold.

Taxon	Rarefied abundance					Shannon's diversity				
	Site	Direction of effect	Depth	Direction of effect	Site x Depth	Site	Direction of effect	Depth	Direction of effect	Site x Depth
Ascomycota	**<0.001**	Decreasing	0.069		0.234	0.055		0.843		0.841
Archaeorhizomycetes	**0.015**	Variable	0.060		0.632	0.215		0.067		0.556
Dothideomycetes	**0.013**	Decreasing	0.114		0.815	0.636		**0.027**	Decreasing	0.628
Eurotiomycetes	**<0.001**	Decreasing	**<0.001**	Decreasing	0.093	**<0.001**	Increasing	0.980		0.216
Leotiomycetes	**<0.001**	Increasing	1.000		0.109	**0.004**	Variable	**0.040**	Decreasing	0.307
Pezizomycetes	**<0.001**	Variable	0.521		1.000	**0.007**	Variable	0.129		0.335
Sordariomycetes	**<0.001**	Decreasing	**<0.001**	Decreasing	0.379	0.331		0.961		0.780
Incertae_sedis	**0.029**	Variable	**<0.001**	Increasing	0.438	0.099		**0.039**	Decreasing	0.491
Basidiomycota	**<0.001**	Increasing	0.062		0.301	0.127		0.451		0.118
Agaricomycetes	**<0.001**	Increasing	0.152		0.739	0.059		0.843		0.436
Tremellomycetes	0.623		1.000		0.256	**0.024**	Decreasing	0.076		0.541
Glomeromycota	**<0.001**	Decreasing	**0.026**	Increasing	0.452	**0.010**	Variable	0.556		0.383
Rozellomycota	**<0.001**	Decreasing	0.053		0.144	0.224		0.706		0.143
Zygomycota	0.090		**0.001**	Increasing	0.816	0.174		0.136		0.693
Chytridiomycota	**0.013**	Variable	0.143		0.524	0.170		**<0.001**	Increasing	0.480



696

697 **Fig. 1 Non-metric multidimensional scaling (NMDS) plot of the fungal**

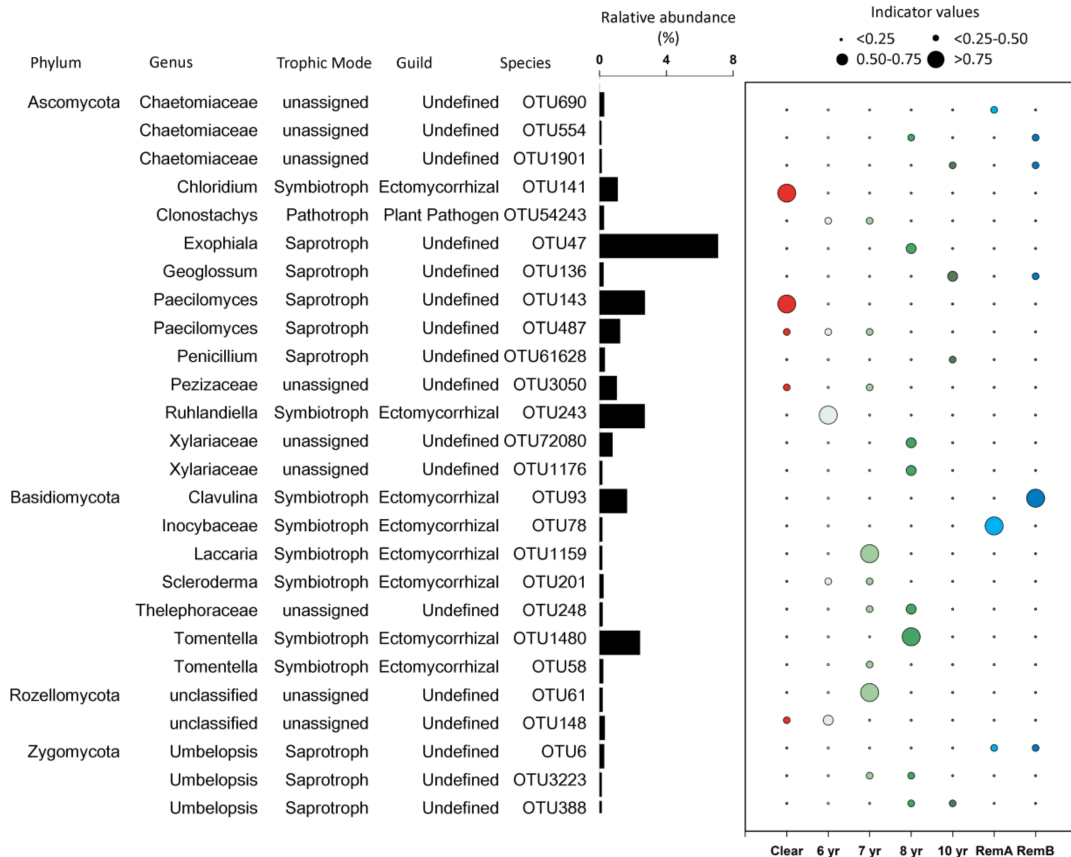
698 **community.** NMDS of dissimilarity of the restoration chronosequence sites based on

699 a Bray-Curtis distance matrix of rarefied fungal OTU abundances.

700

701 *Single column figure*

702



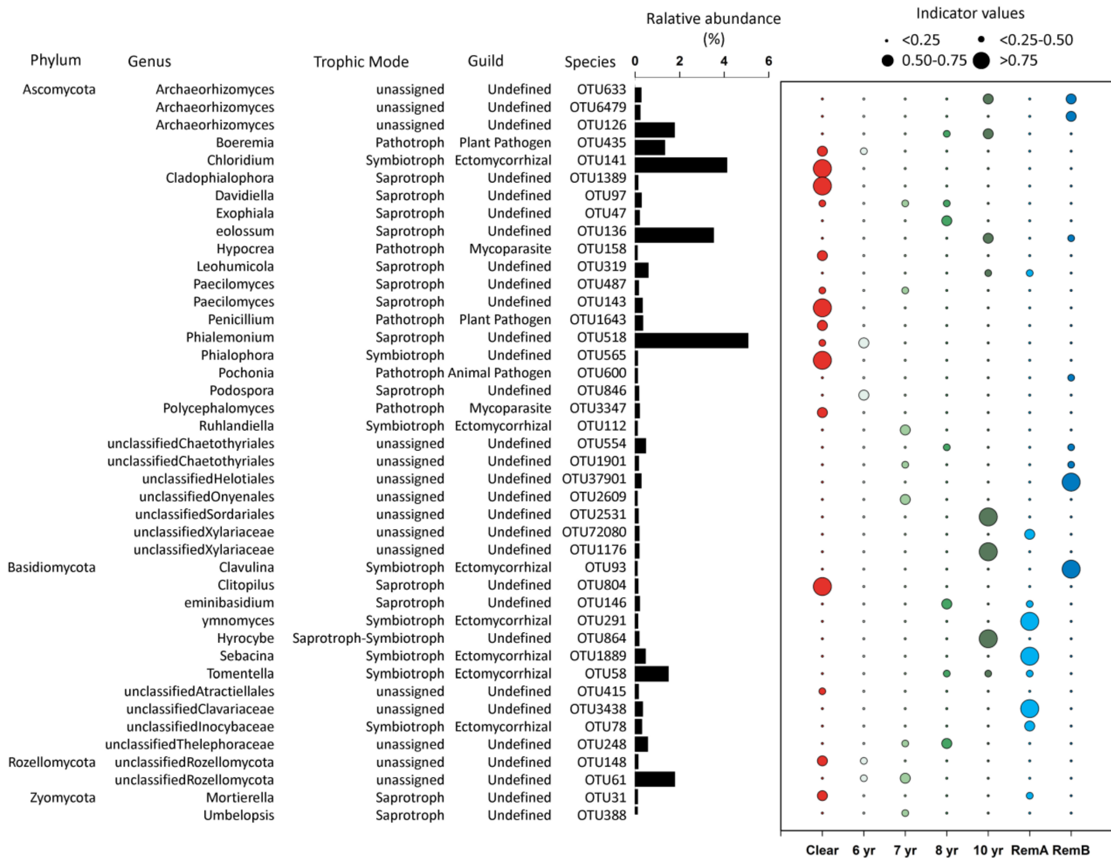
703

704 **Fig. 2 Fungal indicator OTU plot across the restoration chronosequence in the**
705 **0-10 cm soil horizon.** Indicator species analysis plots showing taxa, trophic modes
706 and guilds associated with the restoration chronosequence sites at $q < 0.10$. The
707 bars represent the relative abundance of each indicator OTU. The size of each circle
708 represents the association strength (i.e. indicator values), where 0-0.25 = no
709 association; 0.25-0.50 = weak association ; 0.50-0.75 = association ; 0.75-1.00 =
710 strong association.

711

712 *1.5 column figure*

713



714

715 **Fig. 3 Fungal indicator OTU plot across the restoration chronosequence in the**

716 **20-30 cm soil horizon.** Indicator species analysis plots showing taxa, trophic modes

717 and guilds associated with the restoration chronosequence sites at $q < 0.10$. The

718 bars represent the relative abundance of each indicator OTU. The size of each circle

719 represents the association strength (i.e. indicator values), where 0-0.25 = no

720 association; 0.25-0.50 = weak association ; 0.50-0.75 = association ; 0.75-1.00 =

721 strong association.

722

723 *2 column figure*

724

1 **SUPPLEMENTARY MATERIAL**

2 **Table A1** Rarefied abundances of the dominant fungal phyla at 0-10 and 20-30 cm depths, respectively.

Phylum	Depth (cm)	Cleared	6 years ago	7 years ago	8 years ago	10 years ago	Remnant A	Remnant B
Ascomycota	0-10	81.45%	66.21%	47.99%	51.01%	55.67%	34.51%	52.88%
Basidiomycota	0-10	11.03%	26.75%	47.28%	45.78%	39.81%	63.00%	44.93%
Rozellomycota	0-10	2.69%	4.51%	2.19%	0.34%	0.74%	0.23%	0.24%
Zygomycota	0-10	2.84%	1.11%	1.54%	2.09%	3.09%	1.56%	1.11%
Rare	0-10	1.99%	1.42%	1.01%	0.77%	0.69%	0.69%	0.85%
Ascomycota	20-30	56.77%	54.76%	43.23%	43.27%	57.11%	41.34%	28.57%
Basidiomycota	20-30	28.78%	32.62%	44.32%	52.69%	37.02%	54.39%	66.85%
Glomeromycota	20-30	3.24%	1.32%	0.97%	1.02%	1.50%	1.05%	0.73%
Rozellomycota	20-30	4.82%	8.20%	9.08%	0.21%	0.27%	0.15%	0.15%
Zygomycota	20-30	6.25%	3.06%	2.33%	2.61%	3.98%	2.88%	3.68%
Rare	20-30	0.13%	0.04%	0.07%	0.20%	0.12%	0.19%	0.02%

3

4 **Table A2** Rarefied abundances of the dominant fungal classes at 0-10 and 20-30 cm depths, respectively.

Class	Depth (cm)	Cleared	6 years ago	7 years ago	8 years ago	10 years ago	Remnant A	Remnant B
Agaricomycetes	0-10	6.65%	19.03%	44.54%	42.84%	33.88%	55.78%	40.74%
Archaeorhizomycetes	0-10	0.18%	0.00%	0.20%	1.40%	2.54%	4.11%	3.96%
Dothideomycetes	0-10	8.17%	8.01%	3.05%	3.49%	5.37%	2.06%	3.88%
Eurotiomycetes	0-10	30.32%	11.51%	10.27%	12.06%	12.35%	10.87%	7.78%
Leotiomycetes	0-10	2.03%	1.79%	0.82%	2.58%	15.08%	2.43%	15.39%
Pezizomycetes	0-10	1.27%	21.06%	21.04%	4.84%	2.75%	4.19%	4.14%
Sordariomycetes	0-10	34.73%	18.79%	9.27%	17.30%	12.61%	6.53%	10.24%
Tremellomycetes	0-10	2.67%	5.80%	2.13%	2.02%	5.30%	3.26%	3.38%
Incertae sedis	0-10	3.52%	1.36%	1.72%	2.41%	3.54%	1.72%	1.20%
rare	0-10	2.84%	3.38%	1.55%	1.40%	1.41%	1.16%	1.07%
unclassified	0-10	7.60%	9.25%	5.41%	9.69%	5.17%	7.89%	8.22%
Agaricomycetes	20-30	9.96%	27.87%	39.65%	48.26%	33.76%	51.14%	59.23%
Archaeorhizomycetes	20-30	0.04%	0.01%	1.62%	4.14%	8.72%	7.14%	2.20%
Dothideomycetes	20-30	8.38%	4.34%	3.37%	2.56%	1.39%	1.76%	1.65%
Eurotiomycetes	20-30	16.85%	6.20%	9.83%	8.17%	11.39%	7.09%	4.22%
Leotiomycetes	20-30	1.02%	1.00%	1.30%	6.72%	13.03%	7.26%	7.07%
Pezizomycetes	20-30	0.57%	23.73%	14.90%	0.91%	3.23%	3.64%	0.58%
Sordariomycetes	20-30	18.97%	11.39%	6.97%	9.91%	6.47%	6.45%	6.79%
Tremellomycetes	20-30	10.19%	3.90%	2.75%	2.38%	1.37%	1.41%	2.17%
Incertae sedis	20-30	12.77%	3.35%	2.50%	2.98%	4.59%	2.97%	4.49%
rare	20-30	2.12%	0.73%	1.87%	2.18%	0.89%	1.17%	0.43%
unclassified	20-30	19.13%	17.49%	15.25%	11.78%	15.15%	9.96%	11.18%

5

6 **Table A3** Raw sequences, quality-filtered sequences, saprotroph to symbiotroph ratio and functional value across the restoration

7 chronosequence assessed by permuted analysis of variance (PERMANOVA), with *P* values < 0.05 in bold.

Site	**Depth (cm)**	**Raw sequences (±SD)**	**Quality-filtered sequences (±SD)**	**Saprotroph:Symbiotroph (± SD)**	**Functional value (± SD)**
Cleared	0-10	166,722 ± 84,085	165,053 ± 84,070	2.12 ± 0.07	34.64 ± 0.02
6 years ago	0-10	153,554 ± 13,957	151,747 ± 13,901	2.45 ± 0.09	31.25 ± 1.45
7 years ago	0-10	104,829 ± 14,952	103,436 ± 14,639	2.42 ± 0.03	34.01 ± 2.15
8 years ago	0-10	126,580 ± 49,086	124,515 ± 48,603	2.84 ± 0.05	34.17 ± 3.16
10 years ago	0-10	89,381 ± 41,232	88,053 ± 41,364	3.55 ± 0.01	31.72 ± 1.11
Remnant A	0-10	123,173 ± 44,255	121,163 ± 44,655	2.35 ± 0.02	33.94 ± 1.03
Remnant B	0-10	109,122 ± 4,868	107,154 ± 5,394	2.45 ± 0.02	30.23 ± 2.57
Cleared	20-30	148,605 ± 23,156	147,674 ± 23,370	2.23 ± 0.02	31.10 ± 2.19
6 years ago	20-30	132,175 ± 6,225	130,987 ± 6,168	1.75 ± 0.04	29.62 ± 1.21
7 years ago	20-30	83,263 ± 3,646	82,164 ± 3,972	1.81 ± 0.02	31.08 ± 1.53
8 years ago	20-30	97,826 ± 38,196	96,867 ± 38,279	2.15 ± 0.04	30.68 ± 2.99
10 years ago	20-30	122,997 ± 48,767	121,414 ± 48,833	1.80 ± 0.02	31.73 ± 1.73
Remnant A	20-30	80,713 ± 25,282	79,354 ± 24,953	2.19 ± 0.02	32.21 ± 1.04
Remnant B	20-30	125,440 ± 65,941	124,447 ± 66,100	1.55 ± 0.05	28.22 ± 2.68
PERMANOVA *P* values	Site	0.091	0.088	0.497	**0.016**
	Depth	0.194	0.270	**<0.001**	**0.001**
	Site x Depth	0.598	0.704	0.412	0.084

8

9 **Table A4** Effect of restoration chronosequence sites and soil depths on trophic modes assessed by permuted analysis of variance

10 (PERMANOVA), with *P* values < 0.05 in bold.

Trophic modes	**Rarefied abundance**				**Richness**				
	Site	**Direction of effect**	**Depth**	**Site x Depth**	**Site**	**Direction of effect**	**Depth**	**Direction of effect**	**Site x Depth**
Symbiotroph	**<0.001**	Variable	0.368	0.825	**0.028**	Variable	0.187		0.788
Saprotroph	**<0.001**	Variable	0.581	0.357	**0.004**	Variable	**<0.001**	Decreasing	0.609
Pathotroph-Saprotroph	**<0.001**	Variable	0.077	0.544	**0.004**	Variable	**<0.001**	Decreasing	1.000
Pathotroph	**<0.001**	Decreasing	0.221	0.354	**0.008**	Variable	**<0.001**	Decreasing	0.125

11

12

13 **Table A5** Soil characteristic values across restoration chronosequence sites. Mean values and standard deviations are provided (n

14 = 3 for each site).

Site	Depth (cm)	Nitrate (mg/Kg)	Phosphorus (mg/Kg)	Ammonium (mg/Kg)	Sulphur (mg/Kg)	Organic carbon (%)	pH (H_2O)	Soil moisture (%)
Cleared	0-10	16.00 ± 2.00	20.67 ± 4.41	6.67 ± 0.88	5.23 ± 0.13	3.15 ± 0.23	5.60 ± 0.06	2.49 ± 0.11
6 years ago	0-10	7.67 ± 0.88	11.33 ± 0.67	10.33 ± 1.20	4.90 ± 0.35	2.98 ± 0.16	5.63 ± 0.03	2.33 ± 0.23
7 years ago	0-10	7.33 ± 3.28	16.00 ± 1.15	7.00 ± 1.00	7.03 ± 0.68	4.87 ± 0.09	5.67 ± 0.07	3.96 ± 0.34
8 years ago	0-10	0.83 ± 0.17	8.33 ± 1.86	12.67 ± 1.86	4.27 ± 0.46	3.03 ± 0.62	5.80 ± 0.12	2.63 ± 0.33
10 years ago	0-10	3.00 ± 0.58	11.00 ± 1.00	7.00 ± 2.08	5.57 ± 0.52	4.15 ± 0.19	5.30 ± 0.06	2.64 ± 0.22
Remnant A	0-10	1.17 ± 0.44	11.00 ± 2.08	12.33 ± 2.73	5.53 ± 0.87	4.27 ± 0.23	6.10 ± 0.32	4.26 ± 1.04
Remnant B	0-10	3.00 ± 0.58	8.00 ± 0.00	6.33 ± 1.20	6.87 ± 1.11	3.74 ± 0.18	6.17 ± 0.24	3.34 ± 0.35
Cleared	20-30	12.67 ± 3.79	17.00 ± 6.56	<1	2.40 ± 0.36	1.46 ± 0.28	5.70 ± 0.26	4.66 ± 1.56
6 years ago	20-30	4.00 ± 1.73	7.33 ± 0.58	5.67 ± 3.51	2.50 ± 0.70	1.38 ± 0.14	6.10 ± 0.17	3.55 ± 0.69
7 years ago	20-30	6.67 ± 3.06	6.67 ± 0.58	2.67 ± 0.58	3.73 ± 1.29	2.03 ± 0.52	6.10 ± 0.10	2.41 ± 0.89
8 years ago	20-30	3.33 ± 1.53	3.33 ± 0.58	8.33 ± 3.21	2.43 ± 0.32	1.18 ± 0.02	5.63 ± 0.06	1.78 ± 0.30
10 years ago	20-30	1.67 ± 0.58	4.00 ± 0.00	3.00 ± 1.00	2.47 ± 0.06	1.97 ± 0.25	5.87 ± 0.15	2.59 ± 0.26
Remnant A	20-30	4.00 ± 2.83	4.33 ± 0.58	4.33 ± 0.58	3.50 ± 0.66	2.09 ± 0.56	5.73 ± 0.15	3.82 ± 0.81
Remnant B	20-30	2.67 ± 0.58	4.33 ± 0.58	5.33 ± 4.93	3.83 ± 1.36	2.00 ± 0.46	5.73 ± 0.12	4.16 ± 0.45

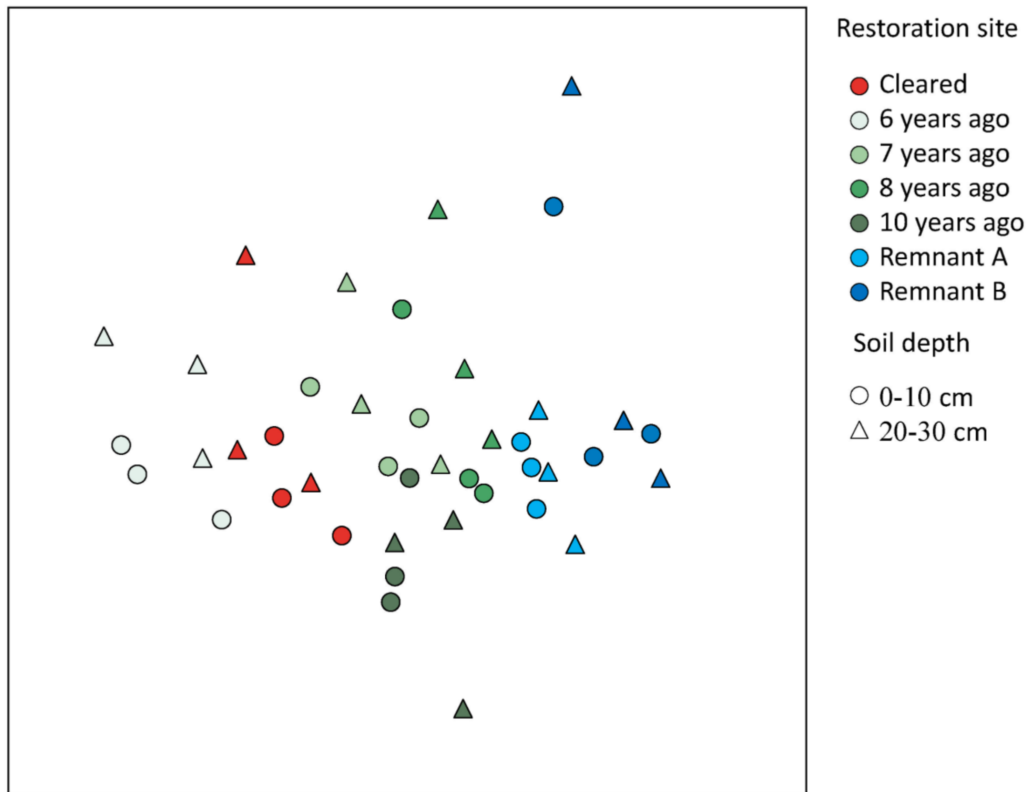
15
16
17

18 **Table A6** Effect of restoration chronosequence sites and soil depths on soil characteristics assessed by permuted analysis of

19 variance (PERMANOVA), with P values < 0.05 in bold.

Variable	Site	Direction of effect	Depth	Direction of effect	Site x Depth
Nitrate	**<0.001**	Decreasing	0.380		0.339
Phosphorous	**<0.001**	Decreasing	**<0.001**	Decreasing	0.686
Organic Carbon	**<0.001**	Increasing	**<0.001**	Decreasing	0.223
pH (H_2O)	**<0.001**	Variable	0.619		0.880
Soil moisture	**0.027**	Variable	0.980		**0.010**
Ammonium	**0.006**	Variable	**<0.001**	Decreasing	0.577
Sulphur	**<0.001**	Increasing	**<0.001**	Decreasing	0.894

20



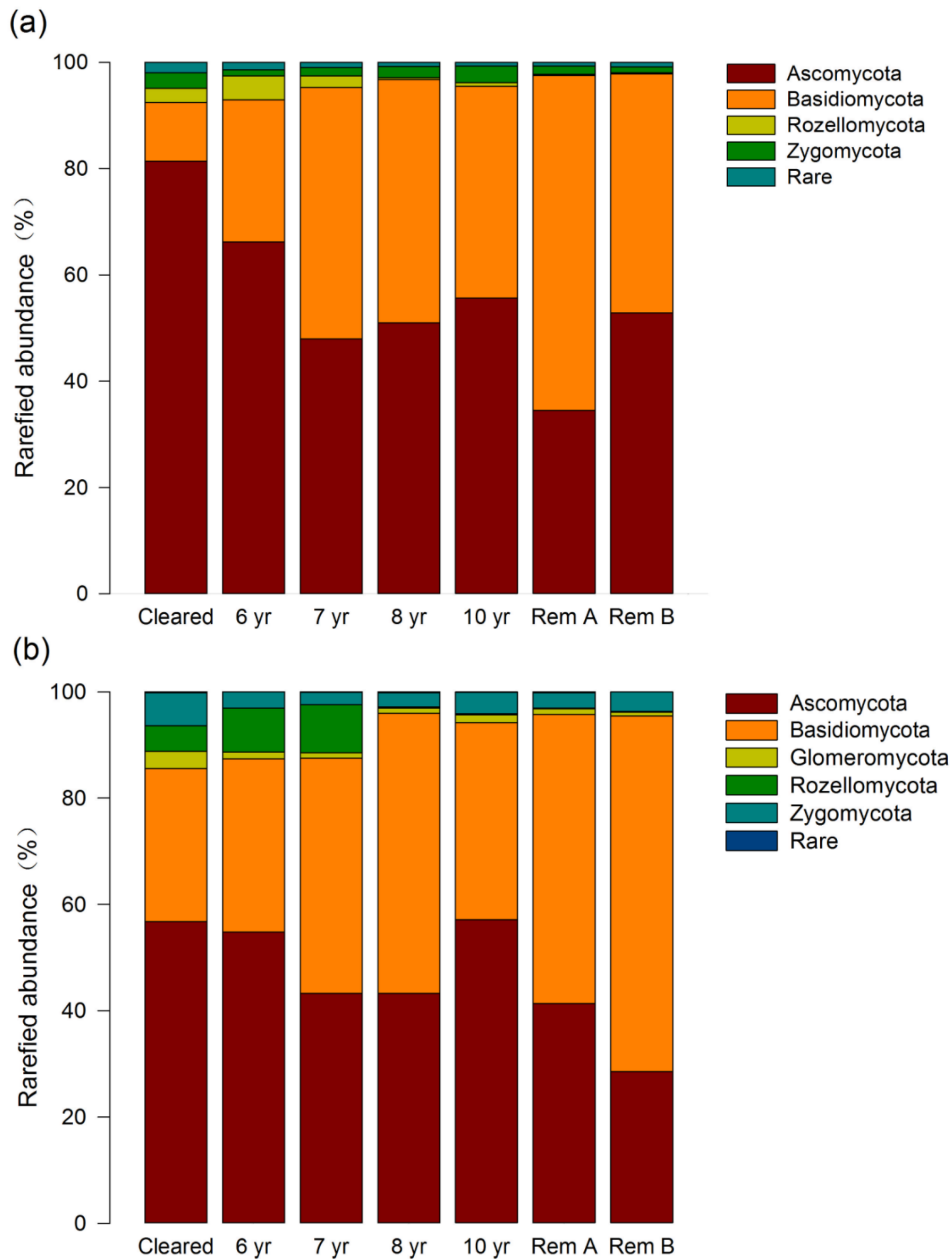
21

22 **Figure A1** Non-metric multidimensional scaling plots of dissimilarity of the

23 restoration chronosequence sites based on Jaccard distance matrix of rarefied

24 fungal ITS OTU abundances.

25



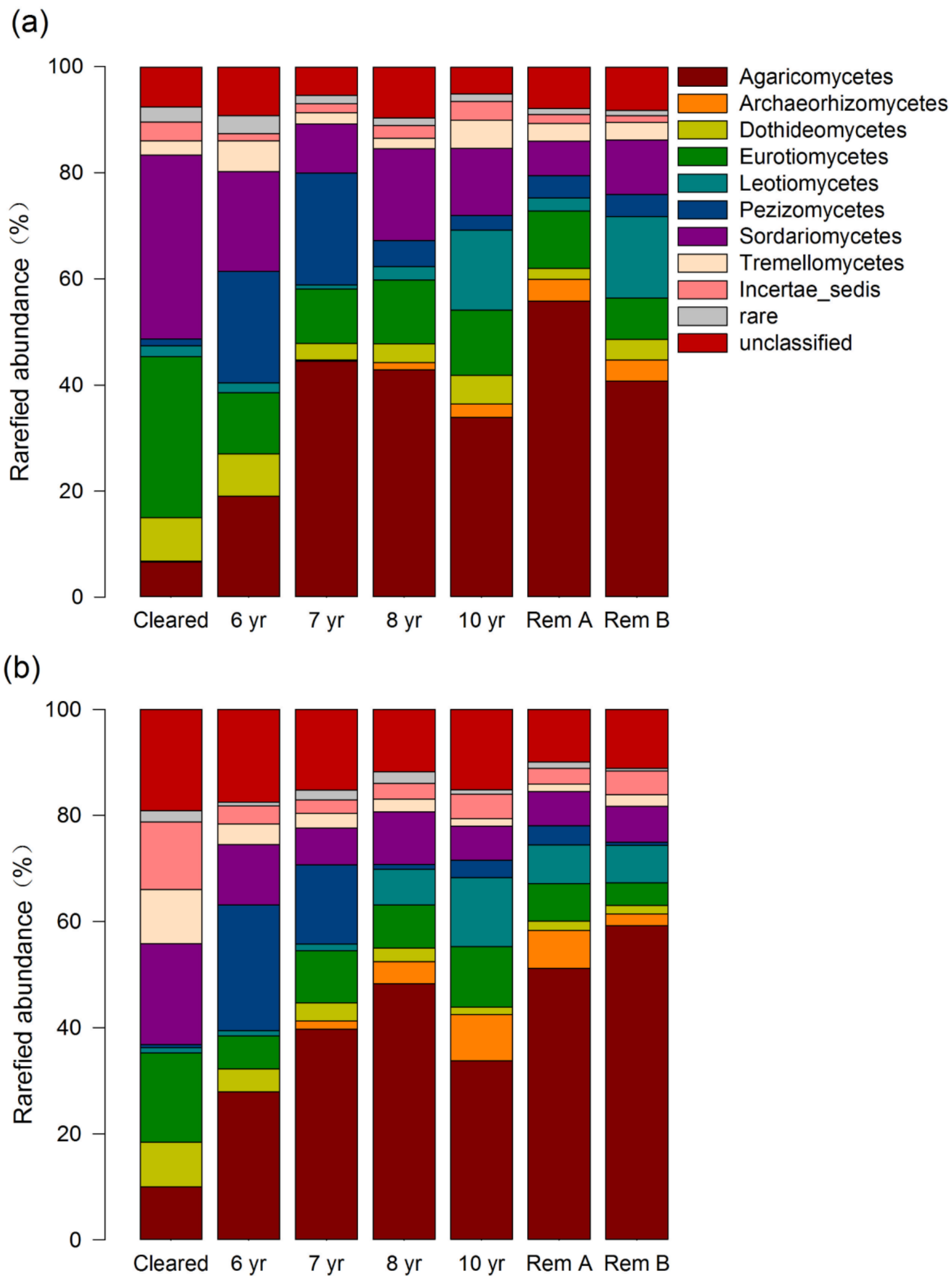
26

27 **Figure A2 Stackplot showing changes in rarefied abundance of fungal phyla.**

28 Rarefied abundances of the dominant fungal phyla and rare fungi at (a) 0-10 cm and

29 (b) 20-30 cm depths across the restoration chronosequence.

30

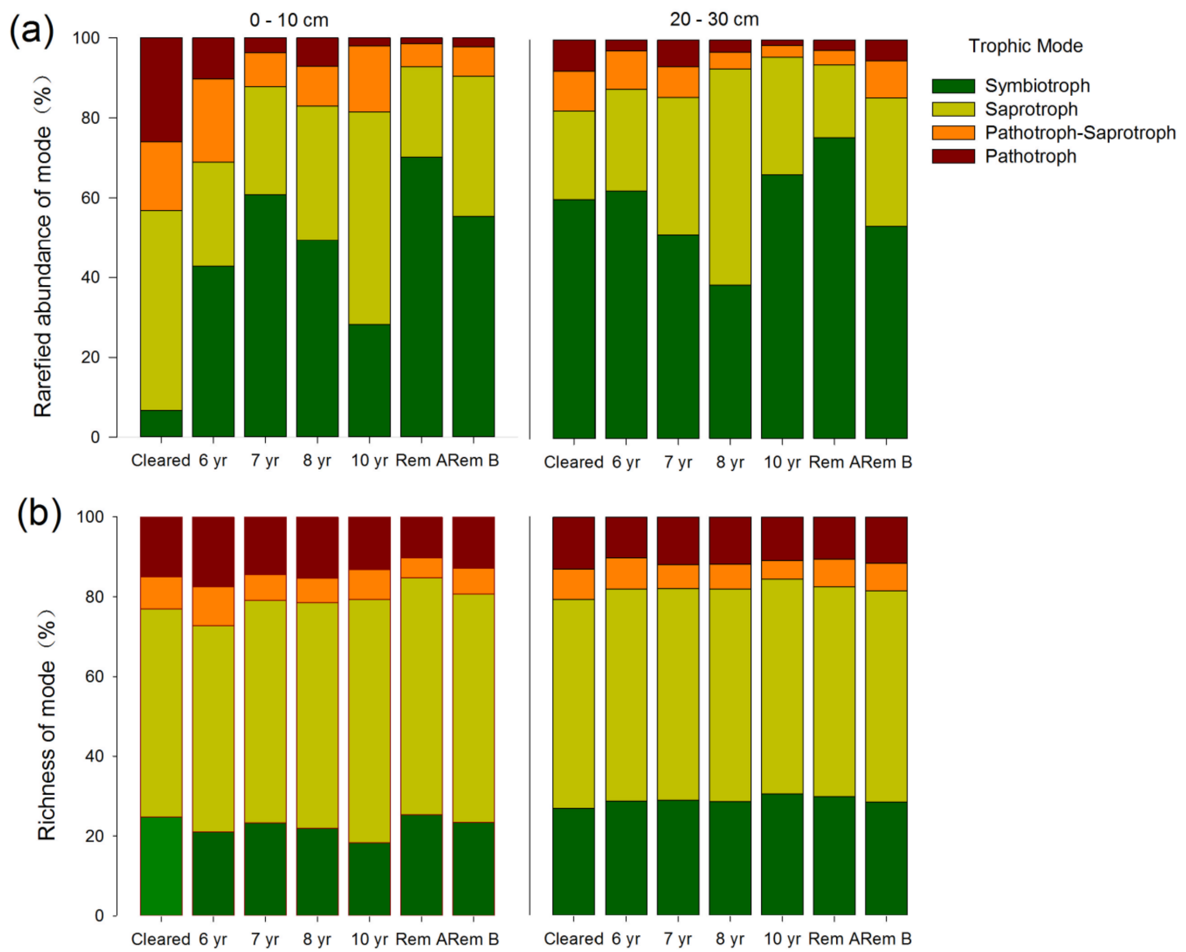


31

32 **Figure A3 Stackplot showing changes in rarefied abundance of fungal classes.**

33 Rarefied abundances of the dominant fungal classes and rare fungi at (a) 0-10 cm

34 and (b) 20-30 cm depths across the restoration chronosequence.



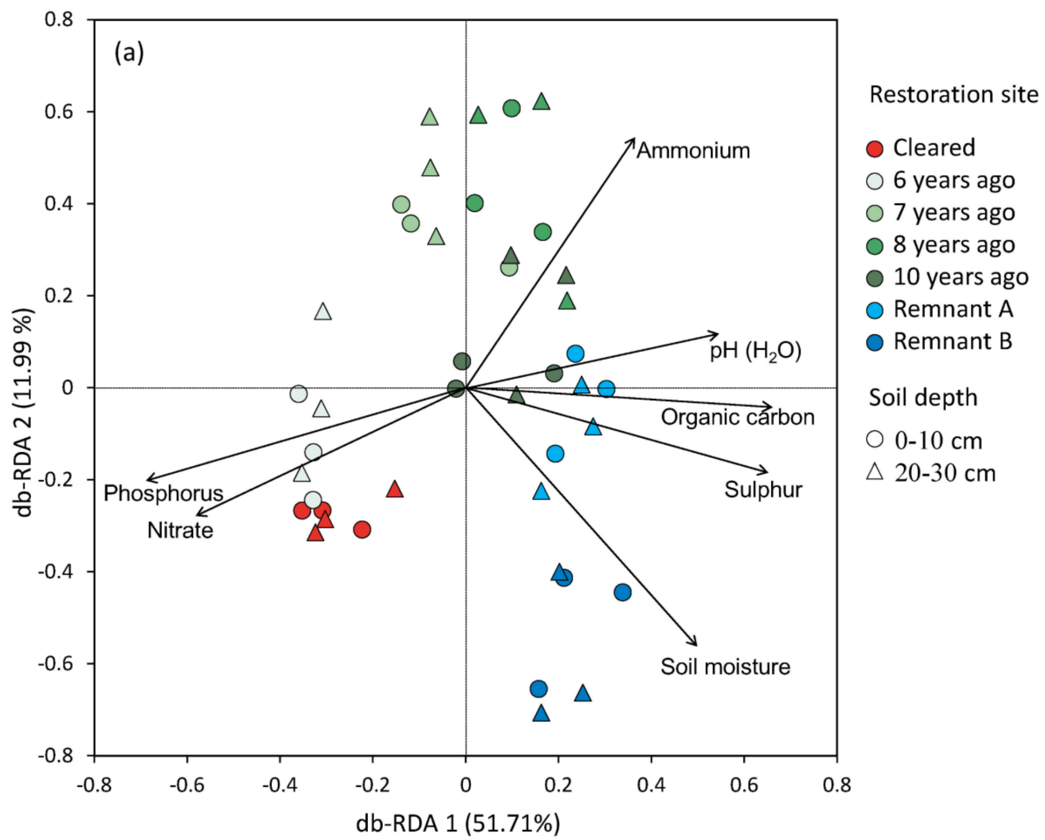
35

36 **Figure A4 Stackplot showing changes in trophic modes across the restoration**

37 **chronosequence.** Rarefied abundances (a) and richness (b) of the dominant trophic

38 modes across the restoration chronosequence.

39



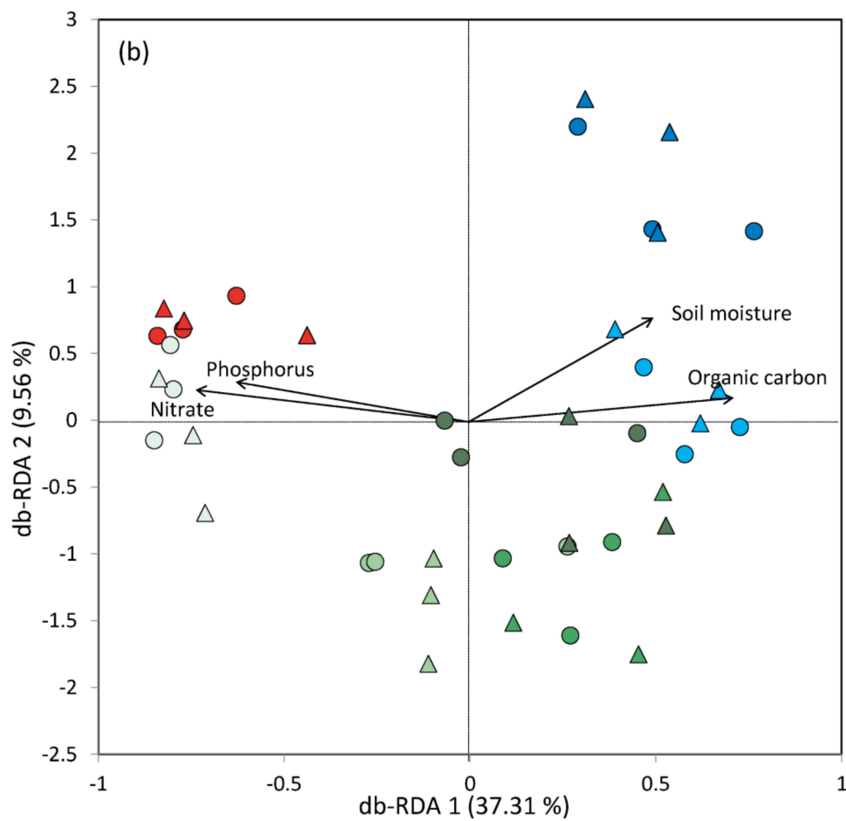
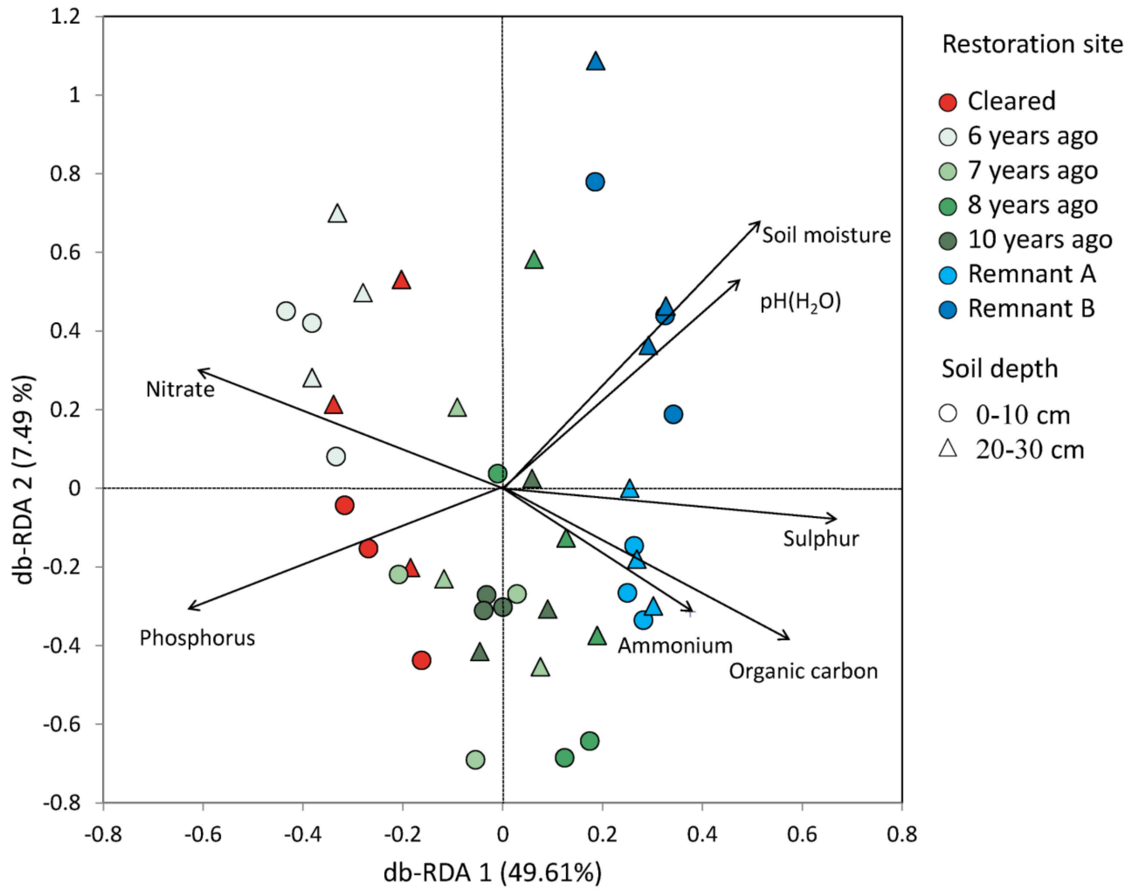


Figure A5 Distance based redundancy analysis (db-RDA) of the soil physicochemical variables and fungal community based on Bray-Curtis distance

43 matrix of fungal ITS OTUs. The db-RDA ordination is shown before (a) and after (b)

44 variable selection tests.



45

46 **Figure A6** Distance based redundancy analysis (db-RDA) of soil physicochemical

47 variables to explain variation in the fungal community based on Jaccard distance

48 matrix of fungal ITS OTUs.

49