

1 Title: Shifts in rhizosphere fungal community during secondary succession following
2 abandonment from agriculture

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21 **Abstract**

22 Activities of rhizosphere microbes are key to the functioning of terrestrial ecosystems. It is commonly
23 believed that bacteria are the major consumers of root exudates and that the role of fungi is thought to
24 be limited to that of mycorrhizae and pathogens. In order to test the hypothesis that the role of
25 saprotrophic fungi in rhizosphere processes increases with increased time after abandonment from
26 agriculture, we determined the composition of fungi that are active in the rhizosphere along a
27 chronosequence of ex-arable fields in The Netherlands. Intact soil cores were collected from nine fields
28 that represent three stages of land abandonment and pulse labelled with $^{13}\text{CO}_2$. The fungal contribution
29 to metabolizing plant-derived carbon was evaluated using phospholipid analysis combined with stable
30 isotope probing (PLFA-SIP), whereas fungal diversity was analysed using DNA-SIP combined with 454-
31 sequencing. In long-term abandoned fields most of the root derived ^{13}C was found in fungal biomass while
32 in recently abandoned fields most of the root derived ^{13}C was taken up by bacteria. Furthermore, we
33 observed a shift along the chronosequence at the functional level from fast growing stress tolerant to
34 slower growing competitive fungi and from pathogenic to beneficial fungi and consequently a change in
35 network configuration.

36

37

38 **Introduction**

39 Studying chronosequences has increased our understanding of plant community succession, which is an
40 important theme in ecology (Tilman, 1988). Most chronosequence studies have focused on plants, but
41 they also offer great opportunities to study soil microbial community responses to primary or secondary
42 vegetation succession and coinciding changes in soil characteristics (Kuramae et al., 2010; Hell et al., 2013;
43 Dini-Andreote et al., 2014). One such a series of sites in the Netherlands represent a secondary succession
44 chronosequence of ex-agricultural fields abandoned at different times. All sites are located in the Veluwe,
45 a region in the centre of the Netherlands and have the same soil type. Since the Middle Ages species-rich,
46 semi-natural grasslands, heathlands and even drifts sands have developed as a consequence of agricultural
47 practices on formerly glacial sand deposits in several parts of the Netherlands. After the introduction of
48 artificial fertilizers in the beginning of the former century, and through intensification of agricultural
49 practice, land use change and increased levels of atmospheric nitrogen deposition, the distribution of
50 species-rich grasslands and heathlands declined dramatically. In order to restore the semi-natural
51 ecosystems, agricultural soils have been taken out of production at different times. This chronosequence
52 in this study (7-30 years of land abandonment) has served as a model system to study the relationship
53 between plant secondary succession and soil abiotic and biotic factors (Kardol et al., 2006; Van der Wal et
54 al., 2006; Holtkamp et al., 2008; van de Voorde et al., 2011).

55 It is acknowledged that soil microbes are strongly involved in plant community succession (Bardgett and
56 van der Putten, 2014). Microbes control soil carbon and nutrient cycling affecting plant community
57 indirectly (Wardle et al., 2004) but can also have direct effects on plants via mutualistic and pathogenic
58 interactions (Buee et al., 2010). Fungi are assumed to be more important in natural ecosystems than in
59 intensively managed systems that are largely dominated by bacteria (de Boer et al. 2005; de Vries et al.
60 2006). Thus, systems in transition such as this chronosequence of abandoned arable fields can give

61 valuable information on the shifts in microbial communities and consequently on their contribution to soil
62 ecosystem function.

63 It was initially assumed that saprotrophic fungal biomass would increase over time of secondary
64 succession due to changes in substrate quality, and at the same time associations between (arbuscular)
65 mycorrhizal fungi and plants would become more important due to depletion of easily available nutrients
66 (Holtkamp et al. 2008) contributing to the observed changes in plant species composition and cover
67 (Kardol et al., 2006). However, when the fungal biomass and fungal:bacterial biomass ratio were
68 investigated an initial increase during the first 2 years after abandonment was seen without any further
69 effect on fungal abundance or fungal:bacterial biomass ratio for the next 30 years (Van der Wal et al.,
70 2006). Yet, an increase in carbon and nitrogen mineralization rates was observed (Holtkamp et al., 2011;
71 Tardy et al. 2015) indicating an increase in activity of microbial decomposers which, however, did not
72 appear to have a positive effect on plant growth nor change the community (Holtkamp et al. 2011). When
73 the identities of the archaeal, bacterial and fungal communities along this chronosequence were assessed,
74 the microbial communities appeared to differ between soils abandoned for less than 10 years and for over
75 25 years (Thomson et al., 2015). However, this only partly explained changes in mineralization rates and
76 could not be related to the changes in plant community.

77 In the current study, we want to relate the secondary succession to the active fungal community in the
78 rhizosphere using $^{13}\text{CO}_2$ pulse labeling of plants and subsequent isotope analysis of both fungal lipids and
79 DNA in intact soil cores collected along the Veluwe chronosequence. We focused on the active rhizosphere
80 fungi regulated mainly by the plant community (Strickland et al. 2016) and used the pulse labeling
81 approach to separate the fungi that are actively assimilating recently photosynthesized, plant derived
82 carbon from the inactive fungi or fungi fulfilling other ecological roles. Our main interest was in
83 saprotrophs using root exudates and thus priming SOM decomposition and affecting mineralization rates,

84 plant pathogens and AM fungi. The ITS-region of fungi was sequenced using 454-sequencing and the fungi
85 were divided into functional trait-based categories based on knowledge of the ecology of species
86 (Tedersoo et al., 2014, Nguyen et al., 2016).

87 The research questions of the current study were

- 88 (i) how does the diversity, functional composition and species interactions of the active
89 rhizosphere fungal community change during secondary succession
- 90 (ii) what are the main (biotic and abiotic) factors driving these changes.

Commented [HE1]: Consequences as well?

91

92 **Material and methods:**

93 *Experimental set-up and sampling*

94 In July 2012 intact soil cores were collected from 9 grassland sites that were taken out of agricultural
95 production 7-30 years prior to sampling. All fields are located in the south Veluwe region, in the centre of
96 the Netherlands (Suppl. Table 1). In three separate sampling locations per field 4 soil cores (12 cm
97 diameter, at least 20 cm deep) were collected with native vegetation growing on it. We selected sites that
98 were dominated by grasses, *Agrostis capillaris*, *Holcus lanatus* and *Plantago lanceolata* to decrease
99 variation due to differences in plant cover. More details on the sampling and sample handling are
100 presented in Morrien et al. (2017) and in supplementary material and methods.

101
102 Nine cores per field site were labeled with 99.99 atom-% $^{13}\text{CO}_2$ (Cambridge Isotope Laboratories, Andover,
103 MA, USA) in an artificially lit air-tight growth chamber for a total of 8 hours. Three other cores per field
104 site were placed in a similar chamber and kept under identical conditions but with a $^{12}\text{CO}_2$ atmosphere,
105 representing the control treatment. Samples were collected 24h, and 7 and 14 days after the start of
106 labeling. Samples from rhizosphere were collected from the upper, root filled part of the cores by brushing
107 the roots. More details on ^{13}C labeling and sampling are presented in supplementary material and
108 methods. Soil chemistry and analysis of soil biota was analyzed and data are given by Morrien et al. (2017).
109 Here these data are used for correlation analysis.

110

111 *PLFA and NLFA analysis*

112 PLFAs were extracted from freeze dried soil and concentrations and $\delta^{13}\text{C}$ values were measured on a
113 Finnigan Delta-S gas chromatograph - isotope ratio mass spectrometer (GC-IRMS) as described in
114 (Boschker, 2004). For more details see Suppl. Material and Methods.

115

116 *Molecular and bioinformatic analysis*

117 DNA was extracted from rhizosphere soil using MoBIO PowerSoil Kit according to manufacturer's
118 instructions. The quantity of DNA was inspected using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo
119 Life sciences) using λ -DNA as standard. ^{13}C -enriched ('heavy') DNA was separated from non-labeled ('light')
120 DNA by density-gradient centrifugation and analysed as described in Neufeld et al. (2007). Fungal PCR was
121 performed for combined 'heavy' and 'light' fractions using primers ITS4 and ITS9 (Ihrmark et al. 2012) and
122 subsequent 454-sequencing was performed. Resulted sequences were submitted to ENA under project
123 number PRJEB15250. For more details on molecular and bioinformatics analysis see Suppl. Material and
124 Methods.

125

126 The fungi were divided into functional groups when possible. This approach is similar to the one taken
127 earlier by Tedersoo et al. (2015) and refined by Nguyen et al. (2016). The functional groups used in this
128 study were: AMF, ericoid mycorrhizae, coprotrophic fungi, endophytes and potential plant pathogens,
129 molds, nematophagous fungi, saprotrophic fungi, wood parasites and - decomposers, yeasts,
130 entomopathogens, animal pathogens, lichens, ectomycorrhizae and mycoparasites. The groups not
131 expected to have functions in rhizosphere that also had little reads/OTUs assigned to them (incl.
132 entomopathogens, animal pathogens, lichens, ectomycorrhizae and mycoparasites) were later grouped
133 into a group 'other'. The types that could not be identified into functional groups were categorized as
134 unclassified Chytridiomycota, unclassified Mucoromycota, unclassified Ascomycota, unclassified
135 Basidiomycota and unclassified fungi. Species could belong to multiple functional groups, especially yeasts
136 and molds (lifestyles) were also members of other groups (i.e. saprotrophs and pathogens). Both data on

137 OTUs presence-absence and OTUs percentage of total reads were used in the analysis. For each analysis,
138 the data used is highlighted.

139 The relative labeling of each OTU was determined as the ratio of the abundance of that OTU in the heavy
140 ¹³C fraction to that of the abundance in the light ¹²C fraction

141 *Statistical analysis*

142 The difference in the amount of reads per sample was standardized by using % of total OTUs in a sample
143 as a measure for the relative abundance of each OTU (McMurdie & Holmes 2014). Absolute values were
144 used for diversity and rarefaction calculations. The effect of treatment (recent, mid- and long-term
145 abandonment) on fungal community composition at the level of phylum, class, order and OTU was
146 estimated using ANOVA combined with Tukey's pairwise comparisons as the post-hoc test. When the
147 distribution of data was not in accordance with assumptions of ANOVA (i.e. due to complete absence of a
148 group in one or more treatments) Kruskal-Wallis test was used in combination with Mann-Whitney as a
149 post-hoc test. The effect of abandonment time was evaluated using Pearson's correlation coefficient.

150 The OTUs explaining most differences between treatments were analyzed using SIMPER in program PAST
151 (Hammer et al., 2001) and their statistical significance confirmed with ANOVA. Cluster analysis (using
152 WARD linkages) was performed to analyze the similarity between 'heavy' and 'light' fractions from the
153 same sample. NMDS using both presence-absence data and the % data was applied to visualize the effects
154 of time since labeling, the fraction (i.e. presence in 'heavy' or 'light' fraction) and the agricultural
155 abandonment time on community composition. Differences in beta-diversity between abandonment
156 categories were evaluated using ANOSIM with Jaccard as a distance measure in PAST.

157 In order to assess α -diversity both Simpson and Shannon-H indexes were calculated using the non-
158 transformed raw data and resulting values were subjected to ANOVA to compare treatment effects.

159 Rarefaction curves of the observed richness were calculated in PAST using 1000-fold resampling without
160 replacement.

161 Variance partitioning was performed in CANOCO (ter Braak and Smilauer, 1998) using both forward
162 selection of the variables and canonical correspondence analysis (CCA) with data derived from Morrien et
163 al. (2016).

164 In order to explore which species are most connected to others in different abandonment categories, co-
165 occurrence networks were used (Barberan et al. 2012). Data on only labeled (i.e. 'heavy' fractions) OTUs
166 were used and time points were combined to get a better view on the system making the total number of
167 samples used for correlations 3 (replicates) x 3 (time points sampled) x 3 (soils in the land abandonment
168 category) = 27 . Only species present in more than 3 samples per abandonment category and with relative
169 abundance of over >1 % were used in analysis. Significant correlations (Pearson's rho >0.5, p<0.05,
170 adjusted using the false discovery rate method in R) were used to plot the networks in Cytoscape (Shannon
171 et al., 2003).

172 Data from labeling status of fungal feeding soil biota was taken from another data set (Morrien et al. 2016)
173 and correlated with the fungal data in R using Pearson's correlation coefficient.

174

175 **Results:**

176 The total number of good quality reads was $1.8 \cdot 10^6$ which were divided into 3562 OTUs. The average
177 number of reads per sample was 10 123 and the average number of OTUs per sample was 412. There were
178 no significant differences in total number of reads or numbers of identified OTUs between heavy and light
179 fractions, time points (incubation) or time of abandonment. The rarefaction curves of all samples are
180 presented in Supplementary Figure 1.

181 Of the total raw reads, 80.5 % was certainly identified using ITSx (Bengtsson-Palme et al., 2013) to be ITS
182 sequences from Fungi. The remaining reads were categorized mainly as plant derived (14 %) and *Alveolates*
183 (superphylum of protists) (4 %). These sequences were removed from the dataset and are not presented
184 nor discussed here. Approximately 10 % of the reads (and 20 % of the OTUs) assigned as fungi could not
185 be assigned to finer taxonomic level than 'unknown fungi'. The fungal communities across the samples in
186 these abandoned ex-arable ecosystems consisted predominantly of Sordariomycetes (making up 25.1 %
187 of all reads and 19.5 % of all OTUs) and of Dothideomycetes (21.7 % and 12.0 %, respectively). According
188 to the functional classification, the majority of reads (70.1 %) and OTUs (52.7 %) were classified as
189 'saprotrophic'.

190 There were 18 OTUs with average abundance of >1 % across the samples and fractions, and 141 OTUs with
191 an average abundance of >0.1%. These 141 OTUs are referred to as the dominant OTUs in the dataset. The
192 most commonly found OTU with an average abundance of 7.12 % was a *Pleosporales* sp. (OTU 5302),
193 which was also present in all samples.

194

195 *Fungal contribution to the carbon flow*

196 Neither the amount of fungi as based on the PLFA signature of 18:2 ω 6c nor the fungi to bacteria ratio (F:B
197 ratio) changed during the secondary succession following land abandonment ($F_{2,24}=1.19$, $p=0.32$).
198 However, when the excess ^{13}C in fungal and bacterial PLFAs (calculated compared to PLFAs in ^{12}C labeled
199 control samples) was used to calculate the 'active F:B ratio', we observed that fungi were receiving
200 relatively more carbon from the plants in the long-term abandoned fields than in recent and mid-term
201 abandoned sites (Fig. 1). Furthermore, the 'active F:B ratio' was positively correlated with time since
202 abandonment from agriculture ($R^2=0.48$, $p<0.05$).

203

204 *Active vs. total fungi in the rhizosphere*

205 Heavy and light fractions (i.e. fungal communities enriched in ^{13}C and ^{12}C) were similar to each other in the
206 general composition of the fungi when all samples were included in the analysis (data not shown). ANOSIM
207 analysis confirmed that the fraction 'heavy' or 'light' was not a significant factor affecting the fungal
208 community structure ($R < 0.20$, $p > 0.20$). Furthermore, the Ward linkage analysis revealed that in 10 of the
209 18 soil samples tested at the first time point, heavy and light fractions from the same sample clustered
210 together. However, the light fractions were significantly more diverse (measured by Shannon-H index)
211 than the heavy fractions when all the samples were combined ($t = 2.6$, $p < 0.05$), which was mainly explained
212 by observed differences between the fractions at the first sampling point 24h after labeling (Suppl. Fig. 2).

213

214 *Active rhizosphere communities in soils and between treatments*

215 Land abandonment time had no significant effect on the α -diversities of the fungi in the heavy fraction at
216 any time point after labeling (ANOVA: $F = 0.74$, $p = 0.48$) (Suppl. Fig. 3). A significant effect of the
217 abandonment time on fungal community structure in the 'heavy' fraction was found in samples that were
218 taken 1 day after labeling (ANOSIM: $R = 0.42$, $p = 0.024$, Suppl. Fig. 4). No effect of time since abandonment
219 was detected for the two other sampling time points or in the 'light' fractions. For this reason, a more
220 detailed analysis was made for fungi assimilating recently photosynthesized carbon (i.e. in the ^{13}C labeled
221 'heavy' fraction in the first time point).

222 At the level of classes, using the proportional data, some significant directional changes in the composition
223 of fungi using recently photosynthesized carbon were detected despite the large variation between soils
224 (Fig. 2). The classes most affected by the time since land abandonment were: dothideomycetes ($F_{2,27} 13.2$,

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225 $p < 0.01$, $R^2 = 0.46$, $p < 0.05$) and agaricomycetes ($F_{2,27} = 4.5$, $p = 0.06$, $R^2 = 0.40$, $p = 0.07$) which decreased in
226 abundance with increasing abandonment time, leotiomycetes ($F_{2,27} = 5.4$, $p < 0.05$) which showed the
227 highest abundance in mid-term abandoned fields and lecaronomycetes ($H = 7.6$, $p = 0.06$) which were
228 completely absent in mid- and long-term abandoned fields (Suppl. Fig. 5). When looking at a more detailed
229 level, the orders affected by the treatment were Capnodiales ($F_{2,27} = 12.5$, $p < 0.01$), Pleosporales ($F_{2,27} = 12.0$,
230 $p < 0.01$) and unclassified Dothideomycetes ($F_{2,27} = 5.5$, $p < 0.05$).

231 When OTUs were divided into functional categories, significant effects of the time since land abandonment
232 were detected: 'potential plant pathogens and endophytes' and 'yeasts' were proportionally more
233 abundant in recently and mid-term abandoned fields than in long-term abandoned fields ($F_{2,18} = 5.5$, $p < 0.05$
234 and $F_{2,18} = 11.88$, $p < 0.05$, respectively) and also decreased in proportion as a function of time since
235 abandonment ($R^2 = 0.52$ and $R^2 = 0.48$, for both $p < 0.05$) (Fig. 3). Saprotrophic fungi were most abundant
236 in the mid-term abandoned fields ($F_{2,18} = 11.60$, $p < 0.01$) and the proportion of 'molds' was highest in long-
237 term abandoned fields and increased with time since abandonment ($F_{2,18} = 5.68$, $p < 0.05$, $R^2 = 0.62$, $p < 0.05$)
238 (Fig. 3). Furthermore, there was a trend that the proportion of AMF of total fungi receiving carbon from
239 the plants increased with time since abandonment ($F_{2,18} = 2.64$, $p = 0.15$). When a ratio between AMF to
240 potential pathogens was calculated, a significant effect of the treatment ($F_{2,18} = 9.97$, $p = 0.01$) and time since
241 abandonment ($R^2 = 0.51$, $p < 0.05$) was found (Fig. 4).

242 Furthermore, some of the soils had a significantly larger proportion of certain functional groups: for
243 example long-term abandoned field BB had significantly more OTUs categorized as wood decomposers or
244 - parasites and nematophagous fungi than other fields (Fig. 3). These differences were not consistent over
245 abandonment time but rather related to unique attributes to the field probably related to the abiotic or
246 biotic factors in that specific field. Similarly, each soil had several OTUs that were unique: they were
247 present in at least two soil samples from that site but absent in other sites. One of the sites abandoned

248 for 30 years (BB) had the most unique OTUs (64) while a recently abandoned site REY had no unique OTUs
249 at all (Fig. 5).

250 There were 816 OTUs present in at least two sites for each abandonment category. 58 OTUs were found
251 in at least two long-term abandoned fields but were not found in earlier abandoned fields. Of these, only
252 2 OTUs were consistently found in all long-term abandoned fields but not in any other field (Suppl. Table
253 4). The recently abandoned fields had 33 OTUs that were not present in older abandoned sites and only 4
254 of them were present in all three recently abandoned soils (Suppl. Table 4). When the number of shared
255 OTUs between fields was related to the time difference since abandonment, a significant negative
256 correlation was found ($R^2 = 0.25$, $p < 0.01$) indicating that there is a directional succession in the fungal
257 community composition.

258 The contribution of individual OTUs to the observed differences between the abandonment times was
259 evaluated using SIMPER and the significance in presence of OTUs between treatments was confirmed
260 using ANOVA. The top 20 most abundant OTUs explaining 32.4 % of the observed differences between
261 abandonment time categories are shown in Table 1. Due to large variation between soils, only 4 out of the
262 20 OTUs selected by SIMPER were significantly different between abandonment categories when
263 evaluated with ANOVA (Table 1). These OTUs were mostly categorized as ascomycetes with unknown
264 function.

265 To reveal the connectedness of the species and possibly find more indicator species of land abandonment
266 time, co-occurrence networks were constructed for the different abandonment stages (Fig. 6). Network
267 topology revealed that the highest density of networks was at mid-term abandoned fields (0.13) and
268 lowest at long-term abandoned fields (0.05). Species in the networks from mid-term fields had also highest
269 number of direct neighbors (average of 14 per OTU). Interestingly, we found a split of network into two

270 subclusters ('hubs') starting in mid-term abandoned fields and continuing to long-term fields (Fig. 6) where
271 the two 'hubs' were completely separated.

272 We looked in more detail to the OTUs with most neighbors in each of the three land abandonment
273 categories in order to identify potentially important species in the systems. In recent fields the most
274 connected OTUs were two unclassified Ascomycota, OTU4843 and OTU5273, both with 24 neighbors. In
275 mid-term abandoned fields the most connected OTU was identified as *Coniochaetales* sp. with 36
276 neighbors. In long-term fields the OTU with most neighbors was *Lecythophora* sp. with 23 neighbors
277 (Suppl. Table 5). Furthermore, some OTUs identified as indicators by SIMPER analysis were among the top
278 connected OTUs in respective fields (table 1). Notably, OTU5177, unclassified ascomycete, was more
279 abundant and also among the top connected species in the long-term fields making it a good candidate
280 for further analyses as a keystone OTU.

281 *Abiotic factors affecting rhizosphere fungi*

282 Variance partitioning was performed in order to find out which abiotic and biotic factors affect the fungi
283 in the 'heavy' fraction the most. Factors tested were: soil pH, N-mineralization, soil C, total N, total P and
284 C:N ratio. Biotic factors included were: $\delta^{13}\text{C}$ in root, fungal PLFA and fungal feeding groups, and dominant
285 plant species in the core. Abiotic variables explained 23.7 % of the total variation in CCA (F=1.2, p=0.012)
286 but the measured biotic variables failed to explain significant variation alone or in combination. Of the
287 abiotic variables, pH and organic carbon in the soils were factors significantly explaining most of the active
288 rhizosphere fungal composition (Table 2). Time since abandonment in years (explaining 3.6 % of the
289 variation, F=1.3, p=0.049), time category (4.0 % of the variation, F=1.4, p=0.016) and soil (4.3 % of the
290 variation, F=1.5, p=0.012) were found to effect OTU composition when constrained and unconstrained
291 CCAs were compared (Table 2). The sampling time (1 day, 1 week or 2 weeks) did not significantly affect
292 the overall OTU composition in the heavy fraction.

293 *Fungal feeding organisms*

294 Data on the labeling status of morphologically identified soil animals collected one week after labeling was
295 correlated with fungal orders and functional groups. This revealed that relative abundance in ¹³C fraction
296 of groups such as ericoid mycorrhizae, molds, wood decomposers and parasites and nematophagous fungi
297 was related to ¹³C carbon detected in fungal feeding cryptostigmatic mites one week later (Suppl. Table
298 7). Labeling of fungivorous nematodes with ¹³C was correlated with labeling of coprophilic,
299 entomopathogenic and potential plant pathogenic fungi. Labeling of fungivorous prostigmatic mites was
300 related to general saprotrophic fungi (Suppl. Table 7) and amount of ¹³C in protist PLFA was positively
301 correlated with % of Saccharomycetes (yeasts) and Chytridiomycetes in the heavy fractions of the samples
302 (Suppl. Table 6).

303

304 **Discussion:**

305 Fungal α - and β -diversity was determined for a total of 9 ex-arable fields that were abandoned 7-30 years
306 prior to sampling. As expected, we observed that a large proportion of the variation in the diversity of
307 fungal communities could be explained by abiotic factors and a lesser part by abandonment time. High
308 explanatory values based on abiotic variables is more rule than exception for soil fungal community
309 composition (Tedersoo et al. 2015; Thomson et al. 2015). With the current set-up, we could, however,
310 separate the effects of abandonment time from the abiotic effects and found a shift in the active
311 community of fungi consuming recently fixed (¹³C labeled) carbon in relation to abandonment time. We
312 showed that fungi become a more important sink of recently photosynthesized carbon during secondary
313 succession (Fig. 1), although the fungal:bacterial ratio or fungal biomass did not increase (van der Wal et
314 al. 2007; Morrien et al. 2017). Earlier it has been shown that shifts in flow of carbon and relative
315 contribution of fungi compared to bacteria has direct consequences for the food-web architecture and

316 influence through microbial community function ecosystem functions such as carbon and nitrogen
317 mineralization (Holtkamp et al. 2011; Malik et al. 2016; Morrien et al. 2016). Recently, it has been
318 proposed that fungi in many systems might be more important consumers of labile organic compounds,
319 such as root exudates, than thought (Hannula et al. 2012; Morrien 2016; de Vries & Caruso 2016).
320 However, the identity and functionality of these rhizosphere fungi was unknown. We identified these fungi
321 and could show that active fungal community using these label compounds is very diverse and largely
322 influenced by soil and plant.

323 The α -diversity of fungi actively assimilating recently photosynthesized carbon was not significantly
324 affected by abandonment time. Lowest diversity of active fungi was found in mid-term abandoned fields,
325 which is contrary to findings of studies done on same soils on total fungal community in bulk soil where
326 'hump-shaped' diversity patterns with highest diversity in mid-term abandoned fields were observed
327 (Tardy et al. 2015; Morrien et al. 2016). This discrepancy in the results can only be explained by the
328 presence of a high number of OTUs not actively using recently photosynthesized carbon present in mid-
329 term abandoned fields.

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330 The division of fungi into functional guilds based on phylogeny and literature is becoming a common
331 practice in fungal ecology (Nguyen et al. 2016). We acknowledge that such division into functional guilds
332 is rather artificial as in different circumstances fungi can have different roles, for example along the
333 continuum pathogen-endophyte-saprotroph (Arnold 2007; Aguilar-Trigueros et al. 2015). However, when
334 done conservatively and based on available literature, it will give a more complete picture on the function
335 soil fungal communities. Following this approach, it appeared that abandonment time had the strongest
336 effect on communities of potential endophytes and - plant pathogens and yeasts (decrease with
337 abandonment time), AMF and molds (increase with abandonment time) and saprotrophic fungi (hump-
338 shaped pattern) (Fig 3). Furthermore, there was an increase in the AMF:pathogen ratio with abandonment

339 time (Fig 4). Our study is the first to demonstrate pathogen enrichment in early and AMF enrichment in
340 late-secondary succession stages. The confinement of pathogens to recent abandonment stages fits well
341 with observations that plant species in recently abandoned soils have negative feedback interactions
342 through soil (Kardol et al. 2006). In that study, long-term abandoned field soil provided more positive
343 feedback effects to the plant species that are typical for that succession stage (Kardol et al. 2006), which
344 corresponds well with our finding that AMF are more prominent in long-term abandoned soil.
345 Furthermore, we found a significant negative relationship between agricultural abandonment time and
346 abundance of yeasts indicating that there is a shift from fast growing, single cell fungi to more resilient,
347 hyphal growing fungal community using root exudates. Yeasts are more common in more disturbed
348 systems growing on labile carbon sources such as root exudates efficiently contributing probably very little
349 to the maintenance of the soil structure and nutrient cycling as compared to hyphal growing fungi that are
350 usually better at producing extracellular enzymes (Mestre et al. 2011; Hannula et al. 2012; Treseder and
351 Lennon 2015).

352 Besides observed changes in the proportion of functional groups and classes with abandonment time,
353 changes at Order and Taxa level were detected and even individual OTUs were significantly affected by
354 treatment. Furthermore, highly connected species, specific to certain abandonment stages (so called
355 keystone OTUs; for synthesis see van der Heijden & Hartmann 2016) could be identified and may be of
356 interest for speeding up nature restoration (Wubs et al. 2016). A hypothesis presented recently is that
357 plant performance in a given ecosystem depends on the presence of keystone species that make the
358 system more stable by protecting against invasion (Banerjee et al. 2015; Agler et al. 2016; van der Heijden
359 & Hartmann 2016; Wubs et al. 2016). Here, the fungal taxa we found to be differentially present and highly
360 connected during secondary succession belong mainly to Ascomycota and more specifically to orders
361 *Pleosporales* and *Eurotiales*. Also a species of yeast from basidiomycete class *Tremellomycetes* was
362 indicated as a significant keystone species by network analysis.

363 We explored the co-occurrence networks (Barberan et al. 2012) in order to assess how the connectedness
364 of fungi is influenced by time since abandonment. These analyses revealed that the highest network
365 densities of fungi using plant-derived carbon in terms of species connectedness were not found in later
366 successional stages but in the mid-term abandoned fields. In long-term abandoned fields, the network
367 configuration was different than in recently abandoned fields: there was a clear split of the network into
368 two 'hubs' instead of one tightly clustered hub. This increase in modularity and in number of 'hubs' has
369 been recently proposed to result in increased benefits to the plant and soil health through recruitment of
370 species and prevention of pathogen invasion through highly connected hubs (Agler et al. 2016; van der
371 Heijden & Hartmann 2016). If modularity increases over time, this is indicative that community
372 development follows a non-random succession pattern (autogenic succession) which is driven by biotic
373 interactions (Frankland 1998). In our system the second 'hub' started to form in the mid-aged fields
374 indicating that it took some time after agriculture for the system to gain its modularity by recruiting new
375 taxa and creating new connections. This coincided with increase in AMF receiving carbon from the plant
376 and decrease in potential pathogenic fungi.

377 Last we explored the effects of changes in fungal community on fungal feeding organisms. We could show,
378 based on correlations of the labeling data in time, that in recently abandoned fields, protists and
379 fungivorous nematodes both seem to be using yeasts, usually fast growing and stress tolerant fungi
380 (Treseder and Lennon, 2015) as a primary source of carbon. This can be due to the shorter generation
381 times and relatively small body size of the animals and fungi compared to, for example, mites (Yeates et
382 al. 1993) and filamentous fungi (Treseder and Lennon, 2015). In later successional fields the fungal feeders
383 receiving most of the carbon were cryptostigmatic mites. In microcosms, it has been shown that fungal
384 feeders do have preferences on the fungal species and groups they feed on (Crowther et al. 2015). Here,
385 we provide preliminary evidence that preference of fungal feeders to certain fungal taxa also affects the
386 communities in soils.

387 In conclusion, we show that thirty years of land abandonment result in shifts of active plant associated
388 communities from bacterial dominated to fungal dominated communities. The composition of active
389 functional fungal community changed from one composed of fast growing and pathogenic species to one
390 consisting of beneficial and slower growing fungal types affecting the carbon flow through the soil food
391 web and consequently the nutrient cycling and the plant succession (model fig).

392

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396

397 **Conflict of interest statement**

398 Authors declare no conflict of interest

399 **Figure legends**

400 **Figure 1.** The share of functional groups of fungi incorporating recently photosynthesized carbon by
401 vegetation on soil cores of 3 land abandonment categories. The total amount of carbon from plants to
402 fungi is calculated as an average of 3 soils for each of the 3 time categories using the excess ¹³C labeling
403 information of the PLFA marker 18:2ω6c and set to 1 for the long-term soils. For assignment of ITS
404 sequences to functional groups, see text.

405 **Figure 2.** Effect of the time since abandonment from agriculture on fungal classes in the samples
406 calculated from the % data. For significant differences between treatments see figure S5.

407 **Figure 3.** Fungal functional groups using recently photosynthesized carbon from plants affected by the
408 time since agricultural abandonment. The recently abandoned fields are represented by red bars, mid-
409 term abandoned fields by blue and long-term abandoned fields by green. The bars represent averages
410 and error bars represent standard error. The significant trends in time are shown in the figure, for
411 significance between treatments, see text. The groups are ordered based on their abundances in
412 samples starting from the most abundant group.

413 **Figure 4.** The AMF/pathogen ratio changing in time since agricultural abandonment. The recently
414 abandoned fields are marked as red bars, mid-term abandoned fields as blue and long-term abandoned
415 fields with green. The bars represent averages and error bars represent standard error.

416 **Figure 5.** Number of unique OTUs present in the heavy fractions and the OTUs shared between
417 treatments and soils. See text for definition of OTU present in a soil.

418 **Figure 6.** Development of network topology abandonment time. Only significant (p<0.05) positive
419 correlations are visualized. The node size is proportional to the % abundance of the organism and edge
420 darkness is scaled to the strength of the correlation (Pearsons rho). OTUs presented in the figure are
421 grouped to functional groups (see text) and colored based on function. Network parameters such as
422 clustering coefficient, number of nodes, density and number of neighbors are presented in the figure.

423

424

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