environmental microbiology

Environmental Microbiology (2013) doi:10.1111/1462-2920.12081

An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition

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Summary

Arbuscular mycorrhizal fungi (AMF) perform an important ecosystem service by improving plant nutrient capture from soil, yet little is known about how AMF influence soil microbial communities during nutrient uptake. We tested whether an AMF modifies the soil microbial community and nitrogen cycling during litter decomposition. A two-chamber microcosm system was employed to create a root-free soil environment to control AMF access to ^{13}C- and ^{15}N-labelled root litter. Using a 16S rRNA gene microarray, we documented that approximately 10% of the bacterial community responded to the AMF, *Glomus hoi*. Taxa from the Firmicutes responded positively to AMF, while taxa from the Actinobacteria and Comamonadaceae responded negatively to AMF. Phylogenetic analyses indicate that AMF may influence bacterial community assembly processes. Using nanometre-scale secondary ion mass spectrometry (NanoSIMS) we visualized the location of AMF-transported ^{13}C and ^{15}N in plant roots. Bulk isotope ratio mass spectrometry revealed that the AMF exported 4.9% of the litter ^{15}N to the host plant (*Plantago lanceolata* L.), and litter-derived ^{15}N was preferentially exported relative to litter-derived ^{13}C. Our results suggest that the AMF primarily took up N in the inorganic form, and N export is one mechanism by which AMF could modify the soil microbial community and decomposition processes.

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Introduction

The arbuscular mycorrhizal association between the Glomeromycota fungi and land plants is widespread and ancient, and it is thought that this symbiosis enabled plants to colonize the land (Remy *et al*., 1994; Brundrett, 2002; Bonfante and Genre, 2008). Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that depend on their host plant for carbon nutrition (Parniske, 2008; Smith and Read, 2008; Leigh *et al*., 2011). However, AMF commonly proliferate in decomposing organic material (Nicolson, 1959; St. John *et al*., 1983). In one study, AMF preferentially colonized plant litter instead of an additional host plant, which represented a potential new carbon source (Hodge *et al*., 2001). In addition, AMF have been shown to stimulate the decomposition of plant material (Hodge *et al*., 2001; Atul-Nayyar *et al*., 2009; Cheng *et al*., 2012), although the mechanism of this effect is unknown. As soil microbial communities mediate many biogeochemical processes in soil, it is possible that AMF alter decomposition by influencing the saprotrophic microbial community. Changes in microbial community composition can alter the production of bioactive metabolites and decomposition processes, and ultimately affect long-term carbon stabilization (Schmidt *et al*., 2011). Since approximately 80% of all land plants form symbiotic associations with AMF (Smith and Smith, 2011), the effects of AMF on litter decomposition are likely widespread and are hypothesized to contribute more to terrestrial biogeochemical cycling than previously recognized (Hodge and Fitter, 2010).

An essential function of the AMF symbiosis is the bidirectional exchange of nutrients between the host plant and fungal symbiont. Arbuscular mycorrhizal fungi enhance nutrient acquisition for the plant by transferring phosphorus (P) captured from soil in exchange for carbon (C) derived from photosynthate (Smith and Read, 2008). Relatively recently, AMF have been found to transfer nitrogen (N) to the host plant from decomposing litter (Hodge *et al*., 2001; Hodge and Fitter, 2010). In one study, the AMF acquired approximately one-third of the N from decomposing litter and exported 3% of the litter N to the host plant (Hodge and Fitter, 2010); in another study, up to one-third of the litter N was exported to the host plant

(Leigh *et al*., 2009). Arbuscular mycorrhizal fungi are also large sinks for photosynthate (Johnson *et al*., 2002), and supply C beyond the rooting zone to decomposing litter (Herman *et al*., 2012). The composition of hyphal exudates contains low-molecular-weight sugars and organic acids such as glucose, formate and acetate, as well as unidentified high-molecular-weight polymeric compounds (Toljander *et al*., 2007). Arbuscular mycorrhizal fungi also contribute the glycoprotein glomalin to the soil carbon economy (Wright and Upadhyaya, 1996). These compounds are energy-rich, and can stimulate or otherwise affect the growth of soil bacteria (Toljander *et al*., 2007).

Despite the important functions of the arbuscular mycorrhizal symbiosis, we still know comparatively little about AMF and their interactions with other soil microorganisms (Hodge and Fitter, 2010). In the rhizosphere, which is the soil immediately adjacent to plant roots, plant growth-promoting rhizobacteria (PGPRs) have been studied extensively in the tritrophic plant–AMF–bacterial interaction (Artursson *et al*., 2006; Frey-Klett *et al*., 2007). The influence of AMF on soil microbial communities beyond the rhizosphere is largely unknown, even though extraradical hyphae are the main zone of interaction between AMF and the soil microbial community. As the soil surrounding hyphae is difficult to assay directly, *in vitro* studies using split plates and soil filtrates have been used to study bacterial attachment to surface of the hyphae (Toljander *et al*., 2006; Scheublin *et al*., 2010), as well as to examine the effect of AMF exudates on bacterial and fungal isolates (Filion *et al*., 1999). Some studies have found that AMF may repress members of the microbial community (Welc *et al*., 2010), or may be repressed themselves by the other microbes (Leigh *et al*., 2011). Currently, very little is known about the interaction between AMF and archaea.

Few studies have explicitly studied how AMF influence the soil bacterial and archaeal communities in decomposing litter. The microbial depolymerization of organic N is one of the rate-limiting steps during soil N cycling (Schimel and Bennett, 2004). Since AMF do not appear to acquire organic N directly (Parniske, 2008; Leigh *et al*., 2011; but see Whiteside *et al*., 2009), the surrounding microbial communities are likely responsible for mineralization of organic N and yielding the mineral N taken up by the AMF. Previous studies using phospholipid fatty acids (PLFA) found that AMF had no discernable effect on the composition of the microbial community present in litter-containing soil, but this technique only detects coarse changes in microbial community composition (Hodge *et al*., 2001; Herman *et al*., 2012). To our knowledge, no previous studies have investigated how AMF modify microbial communities using high-throughput molecular techniques with high phylogenetic resolution. Molecular analyses based on 16S rRNA have the potential to identify interactions between AMF and soil bacteria that would be undetectable by PLFA analyses (Herman *et al*., 2012) and culture-based techniques.

We investigated how the AM fungus, *Glomus hoi*, altered the bacterial and archaeal communities and N cycling in soil containing decomposing litter. To accomplish this, we used a two-chamber system to allow or deny AMF hyphae access to a discrete patch of isotopically labelled litter in root-free soil (Fig. 1). A previous study of this plant–soil–mycorrhizal system indicated that after the AMF reached the decomposing litter, bacteria, rather than fungi, became the primary processors of litter-derived C. Fungi were primary decomposers before the arrival of the AMF (Herman *et al*., 2012). We hypothesized that: (1) the presence of AMF would modify the bacterial communities in decomposing litter and (2) the mycorrhizal fungus would alter the physicochemical environment for the microbial community by exporting N from the decomposing litter.

Results

Response of the bacterial and archaeal communities to AMF

We characterized the bacterial and archaeal communities in the soil containing decomposing root litter (litter soil) after the AMF had colonized the litter chamber (Fig. 1C). The AMF arrived after 21 days, and were clearly detectable in the AMF-permitted treatment by 42 days (1.02 ± 0.13 SE m hyphae g^{-1} dry weight soil, $n = 5$), while virtually no AMF hyphae were detected in the exclusion controls (AMF-excluded) at any time point (Fig. 1D). When the hyphae could access the litter chamber, by 70 days, the hyphae in the planted chamber colonized on average 2.1 times more root length (Bonferroni *post hoc* analysis: $P < 0.001$) and produced 4.0 times more arbuscules than the hyphae in the AMF-excluded treatment (Bonferroni *post hoc* analysis: $P < 0.001$) (Fig. S1).

We detected a total of 3007 taxa in the litter soil material using 16S rRNA gene-based microarrays (see *Experimental procedures* for presence–absence criteria) (Table S1). We detected 2751 ± 43 SE OTUs in the litter soil where AMF was permitted ($n = 5$), and 2831 ± 63 SE OTUs in the exclusion controls ($n = 5$). 9.5% (316) of the taxa significantly increased or decreased in response to AMF based on indicator species analysis (ISA: $n = 5$, $P < 0.05$). The presence of AMF did not significantly alter the total bacterial diversity of the soils; the average Shannon diversity was 8.1 ± 0.03 SE (two-tailed *t*-test: $n = 5$, $P = 0.5$).

The taxa that responded to AMF significantly clustered by treatment in NMDS ordination space (anosim: $R = 0.64$, $P = 0.008$) (Fig. 2). Litter colonized by the

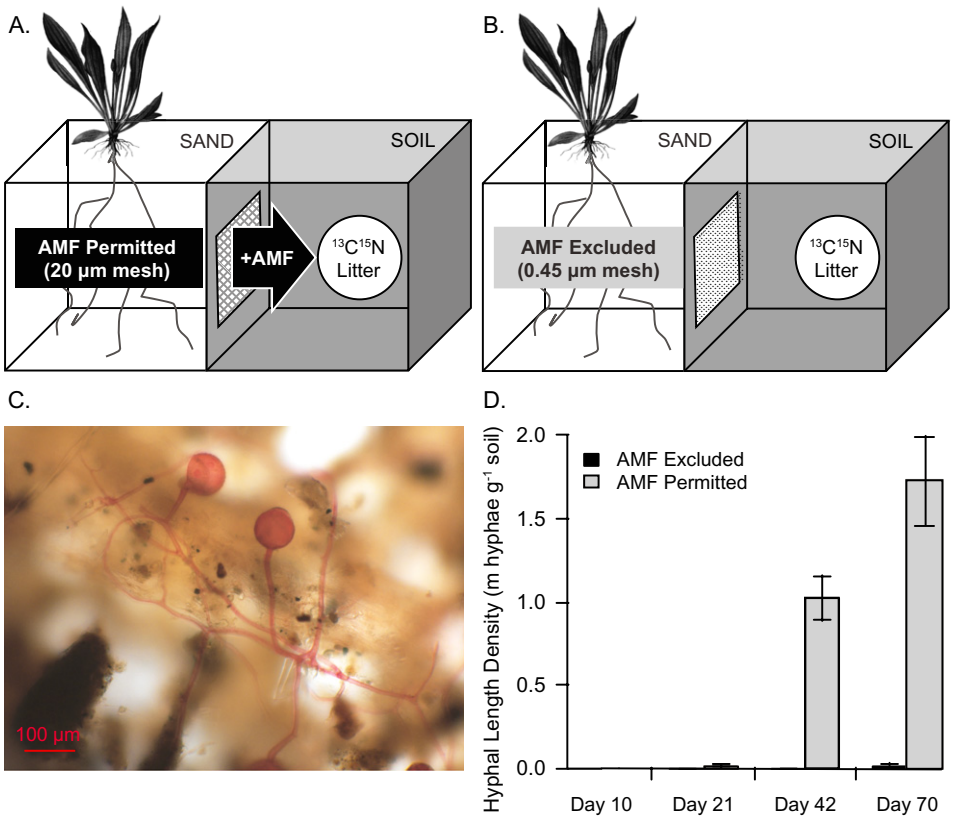

Fig. 1. Dual-chamber experimental design. Planted chambers contain *Plantago lanceolata* L and the AMF *Glomus hoi* in a sand media. Litter chambers contain field fresh soil and a region of ^{13}C- or $^{13}C^{15}N$-labelled root litter mixed with soil.
A. The AMF-permitted treatment has a 20 µm mesh barrier that allows AMF to access the litter.
B. The AMF-excluded treatment has a 0.45 µm mesh barrier that excludes AMF from the litter.
C. AMF hyphae with attached spores growing in decomposing litter imaged by light microscopy (42 days). Scale bar represents 100 µm.
D. Hyphal length densities in the litter chamber monitored over 70 days in soil where AMF was permitted or excluded from the litter (m hyphae g^{-1} dry soil ± standard error, $n = 5$).

AMF had a higher relative abundance of Firmicutes, Gemmatimonadetes, Deltaproteobacteria and Planctomycetes (Fig. 3). Within the Firmicutes, the taxa that increased in relative abundance were within the classes Bacilli and Clostridia (Table S2). We used net relatedness index (NRI) and nearest taxon index (NTI) to assess if the taxa that responded to AMF were more related (clustered) or unrelated (overdispersed) than expected by chance. Bacterial taxa that responded positively to the presence of AMF in the decomposing litter were significantly clustered within the deeper branches of the soil phylogenetic tree (NRI: 2.02 ± 0.09 SE, $n = 5$, $P = 0.03$), but were overdispersed at the terminal branches of the tree (NTI: –3.2 ± 0.04 SE, $n = 5$, $P = 0.001$). The bacteria that responded negatively to the presence of AMF showed significant phylogenetic clustering both basally and at the terminal branches of the phylogenetic tree (NRI: 1.6 ± 0.03 SE, $n = 5$, $P = 0.05$; NTI: 6.1 ± 0.09 SE, $n = 5$, $P < 0.001$).

Relative to the exclusion control, the AMF-permitted treatment had a lower relative abundance of Betaproteobacteria, Actinobacteria and Bacteroidetes, among others (Fig. 3). Within the Betaproteobacteria, the Comamonadaceae had the most taxa decline in relative abundance (25 taxa) (Table S3). The Actinobacterial taxa that decreased in relative abundance were from the families Thermomonosporaceae and Propionibacteriaceae (15 and 7 taxa respectively). Finally, within Bacteroidetes, the Flavobacteriaceae had the most taxa decrease in relative abundance (8 taxa). Only one archaeon (family Methanosarcinaceae) significantly responded to the presence of AMF, and it had a negative response.

Influence of AMF on litter N cycling

As mentioned previously, the AMF arrived after 21 days. The average C : N ratio of the host plants at 21 days was 37.1 ± 2.6 SE ($n = 10$), and was not significantly different between treatments (Bonferroni *post hoc* analysis: $P > 0.1$) (Fig. S2). By 42 days, the host plants with AMF access to the litter chamber had an average C : N ratio of 50.4 ± 2.2 SE ($n = 5$), which was approximately 30%

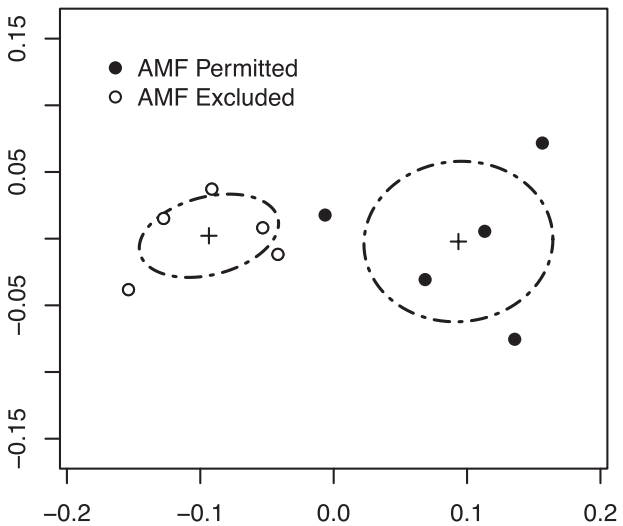

Fig. 2. The influence of AMF on the soil bacterial communities in decomposing litter after 42 days (NMDS ordination: stress = 3.23%). The filled circles represent units where AMF were permitted access to the litter chamber (AMF-permitted), and open symbols represent units where AMF were excluded from the litter chamber (AMF-excluded). Crosses indicate the mean centroid for each treatment, and the dashed ellipses represent the standard error of the mean at a 95% confidence level. The ordination was completed on the operational taxonomic units (OTUs) identified by microarray analysis that significantly responded to the presence of AMF. The bacterial communities in the two treatments are significantly different by anosim analysis (anosim: $R = 0.64$, $n = 5$, $P = 0.008$).

lower than the host plants whose fungal partners were denied access to the root material (72.36 ± 2.34 SE, $n = 5$) (Bonferroni *post hoc* analysis: $P < 0.001$). At 70 days, the host plant for the AMF-excluded treatment continued to show signs of N limitation with a C : N ratio of 70.9 ± 3.0 SE ($n = 5$), while the C : N ratio of the AMF-permitted host plant decreased from the previous time point to 45.5 ± 5.6 SE ($n = 5$) (Bonferroni *post hoc* analysis: $P < 0.001$). At day 70, the bulk soil surrounding the litter soil patch in both treatments contained 0.23% N ± 0.004 SE ($n = 10$) and had a C : N ratio of 14.4 ± 0.4 SE ($n = 10$).

By the end of the experiment, isotope ratio mass spectrometry (IRMS) analyses determined that AMF had exported 4.9% ± 0.3 SE ($n = 5$) of the litter ^{15}N to the host plant, whereas the plants whose AMF partner was excluded from the litter acquired only 0.05% ± 0.06 SE ($n = 5$) of the litter ^{15}N through mass flow (Table 1). The shoots acquired more of the litter ^{15}N than the roots, indicating that the enrichment in the plant was not simply due to the hyphae embedded in the plant roots (Table 1). The AMF did not export significant quantities of the litter ^{13}C; only 0.06% ± 0.005 SE ($n = 10$) of the litter ^{13}C was recovered in the plant shoots and roots, and there were no significant differences between the treatments (two tailed *t*-test: $n = 5$, $P > 0.1$). On average, the plant roots contained −18.5‰ ^{13}C ± 2.6 SE ($n = 10$), while the shoots contained −25.7‰ ^{13}C ± 0.6 SE ($n = 10$). For additional information on bulk ^{13}C results, see Herman and colleagues (2012).

Because of the intimate nature of AMF root colonization, it can be difficult to differentiate the ^{15}N enrichment of AMF hyphae from that of the associated root tissue using standard IRMS analysis. We used NanoSIMS to distinguish the relative enrichment of these two tissue types within a plant root. At the final harvest (70 days), NanoSIMS analyses of multiple AMF-colonized live roots showed that the hyphae associated with living roots in the planted chamber were significantly enriched in ^{15}N, demonstrating that the hyphae exported litter ^{15}N from the litter chamber to the plant roots (Fig. 4A). The average ^{15}N enrichment of the hyphae from the NanoSIMS analysis was 24000‰ ^{15}N ± 700 SE ($n = 28$), which was significantly higher than that of the associated roots (one-way ANOVA: $F_{1,75} = 259.77$, $P < 0.001$), which were enriched at 2800‰ ^{15}N ± 300 SE ($n = 53$). NanoSIMS analyses also showed a low but statistically significant ^{13}C enrichment in the individual hyphae (104‰ ^{13}C ± 12 SE, $n = 28$)

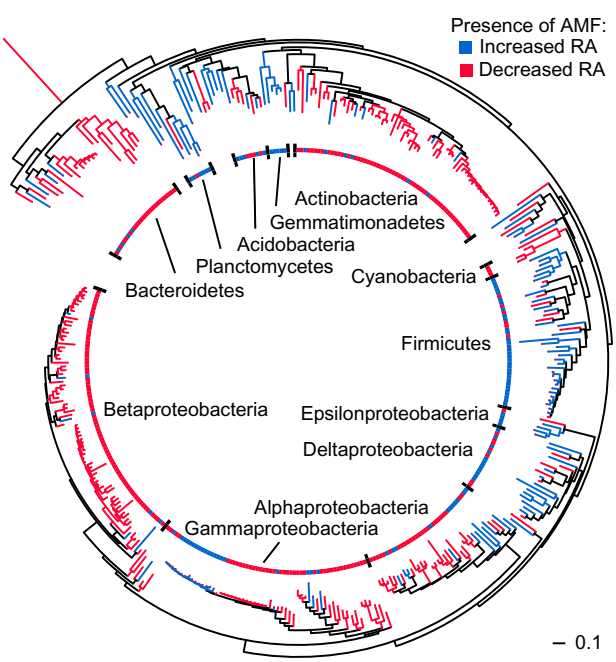

Fig. 3. Maximum likelihood tree displaying the subset of bacteria that significantly increased (blue) or decreased (red) in relative abundance (RA) in the presence of AMF (indicator species analysis: $n = 5$, $P < 0.05$). Labels indicate the phyla of the taxa, except for the Proteobacteria, which are labelled by class. The black tick marks on the centre ring surround the taxa within a given phylum. The outgroup is the single Archaeon that responded to AMF, which is from the phylum Euryarchaeota. The scale bar in the lower right corner indicates the number of nucleotide substitutions per site.

Table 1. Litter soil ^{13}C : ^{15}N ratio and percentage of litter ^{15}N that was recovered from the plant biomass and litter chamber at day 70 when AMF were present (AMF-permitted) or excluded (AMF-excluded) (± standard error, $n = 5$).

^{15}N location	AMF-permitted	AMF-excluded	*P*-value
Shoot	3.4% ± 0.2	0.01% ± 0.02	<0.001
Root	1.5% ± 0.5	0.04% ± 0.04	0.02
Plant total	**4.9% ± 0.3**	**0.05% ± 0.06**	**<0.001**
Microbial biomass	5.5% ± 0.3	5.8% ± 0.7	ns
Litter soil	36.8% ± 3.7	39.9% ± 3.0	ns
Bulk soil	22.9% ± 8.3	29.9% ± 13.6	ns
Litter chamber total	**65.2% ± 12.2**	**75.6% ± 17.3**	**ns**
^{13}C : ^{15}N in litter soil	28.6 ± 0.5	27.1 ± 0.4	0.05

Values in bold represent the totals for the plant biomass and the litter chamber. The microbial biomass only accounts for the litter soil patch. The litter soil values exclude the microbial biomass. The bulk soil is the soil surrounding the litter soil patch. Percentage litter ^{15}N was determined by dividing the mg of ^{15}N in each component at day 70 by the initial mg of litter ^{15}N added to the soil. Statistical significance was determined using two-tailed *t*-tests. The term 'ns' designates non-significance.

compared with adjacent plant roots (–14‰ ± 11 SE ^{13}C, $n = 53$) (one-way ANOVA: $F_{1,74} = 83.9$, $P = 0.001$) (Fig. 4B).

In the litter chamber, we determined that the presence of AMF slightly increased the ^{13}C : ^{15}N ratio of the litter and soil material ($P = 0.05$, Table 1). This indicates that proportionally more ^{15}N had been removed from the litter soil than ^{13}C. While there was a trend towards decreased ^{15}N in the litter chamber in the presence of AMF, the two treatments were not statistically distinguishable (Table 1). We also did not see a difference in C content or ^{13}C isotopic enrichment in the litter chamber between the two treatments (data not shown). Approximately 70% of the litter ^{15}N remained in the litter chamber at the end of the experiment (Table 1).

Discussion

Previous studies have shown that AMF alter the decomposition rate of organic material, but the mechanisms by which AMF modify decomposition are not understood (Hodge *et al.*, 2001; Hodge and Fitter, 2010). By microarray analysis, we demonstrated that the AMF *G. hoi* significantly altered the relative abundance of approximately 10% of the soil bacterial taxa inhabiting decomposing litter (Hypothesis 1). Previous work using a similar high-density microarray analysis shows that an actively growing root significantly alters about 7% of the total soil bacterial community (DeAngelis *et al.*, 2009). Thus, the impact of AMF hyphae in soil containing decomposing litter appears to be similar in magnitude to a plant root actively growing through soil. Interestingly, the taxa that responded to AMF exhibited significant patterns of phylogenetic clustering and overdispersion, which suggests that AMF may have influenced bacterial community assembly in decomposing litter. In addition, we showed that AMF altered the physicochemical environment of decomposing litter by exporting N liberated by the microbial community (Hypothesis 2). NanoSIMS provides the first isotopic images of AMF associated with root tissues by mapping the ^{15}N and ^{13}C that were exported from the decomposing litter to the host plant.

Arbuscular mycorrhizal fungi significantly modified the soil bacterial community in decomposing litter. We found that taxa within the phylum Firmicutes (Clostridia and Bacilli) increased in relative abundance in the presence of AMF, while taxa within the phyla Actinobacteria and Bacteroidetes decreased in the presence of AMF (Fig. 3). Previous stable isotope probing studies examining litter decomposition (in the absence of AMF) have identified taxa from the Actinobacteria, Firmicutes and Bacteroidetes as bacterial decomposers of plant material in soil (el Zahar Haichar *et al.*, 2007; Lee *et al.*, 2011). It is possible that the AMF shifted the relative abundances of the bacterial decomposer community from the phyla Actinobacteria and Bacteroidetes to the phylum Firmicutes. Many members of the bacterial class Clostridia are known to produce multienzyme cellulosome complexes, which are capable of catalysing the efficient degradation of cellulose (Bayer *et al.*, 1998). Previous work has shown that

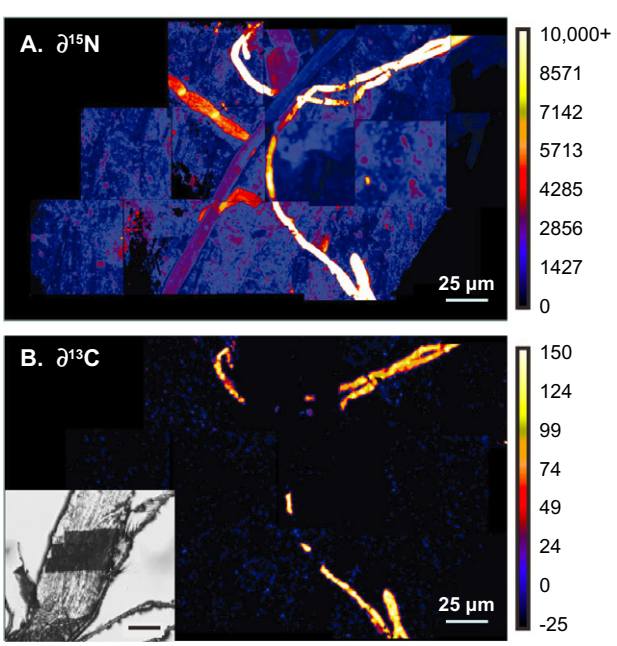

Fig. 4. Composite of 14 NanoSIMS images of a *P. lanceolata* root colonized by *G. hoi* hyphae. The colour scale bar represents (A) $\delta^{15}N$ enrichment and (B) $\delta^{13}C$ enrichment ranging from natural abundance (black) to 10 000+‰ ^{15}N or 150‰ ^{13}C. Black and white inset image in B is a secondary electron micrograph taken post analysis that indicates the location of the NanoSIMS sputtering path as it transected the width of the colonized root. Black scale bar in inset represents 0.1 mm.

Bacilli associate with AMF (Andrade *et al*., 1997), and in particular with decomposing hyphae (Artursson and Jansson, 2003; Toljander *et al*., 2006). As extraradical hyphae have a fast C turnover (Staddon *et al*., 2003), AMF hyphae could be an important form of substrate that stimulates Bacilli in soil. While some strains of Actinobacteria have been shown to have a beneficial impact on AMF hyphal growth and root colonization (Franco-Correa *et al*., 2010), our study suggests that AMF have a negative effect on many Actinobacteria in soil during litter decomposition. Actinobacteria are renowned for producing a wide variety of secondary metabolites and antibiotics (Bérdy, 2005), and altering the composition of Actinobacteria could provide either an advantageous or an inhospitable environment to neighbouring fungi or bacteria. However, we did not observe an overall change in bacterial diversity in the presence of AMF.

A notable result from this study was the widespread decrease of Proteobacteria in response to AMF, particularly within the betaproteobacterial family Comamonadaceae. The Comamonadaceae are a physiologically heterogeneous group of bacteria; they are known to consume a broad spectrum of organic carbon compounds that range from simple sugars to complex aromatic compounds, as well as assimilate inorganic carbon autotrophically (Kersters *et al*., 2006). In the *Medicago truncatula* rhizosphere, the presence of AMF increased the relative abundance of Comamonadaceae taxa (Offre *et al*., 2007). However, further study found that Comamonadaceae strains isolated from the *Medicago* rhizosphere had no effect on or decreased root colonization, and in one case depressed AMF spore germination and hyphal proliferation (Pivato *et al*., 2009). We found that 25 taxa from the Comamonadaceae decreased in relative abundance in decomposing litter in the presence of AMF, which suggests that members of the Comamonadaceae may be repressed by the presence of AMF in decomposing litter. While AMF are not known to produce antibiotics, the presence of AMF has been shown to repress some members of the microbial community (Welc *et al*., 2010), including fungal pathogens (Filion *et al*., 1999). The mechanisms for these interactions are unknown, and may result from the direct or indirect manipulation of the community through hyphal exudates (Toljander *et al*., 2007), or the AMF may occupy the same niche as these microbes and compete with them during nutrient acquisition (Veresoglou *et al*., 2011).

It is well known that AMF can transport P to the host plant, which may induce competition for P uptake between the soil microbial community and the extraradical hyphae when P is a limiting nutrient (Leigh *et al*., 2011; Smith and Smith, 2011). As our work and the recent literature show, it is becoming more apparent that AMF may perform a similar process with N (Hodge and Fitter, 2010). Arbuscular mycorrhizal fungal competition for N with some soil microbes could have a significant effect on the microbial community composition. In one study, the presence of AMF appeared to reduce potential nitrification rates, suggesting that AMF may be capable of outcompeting nitrifiers for ammonium in an N-limited Mediterranean soil (Veresoglou *et al*., 2011). In our work, the AMF exported 4.9% of the litter ^{15}N to the host plant. Our detection of this effect in live soil confirms that this AMF is capable of drawing significant amounts of N away from decomposing litter, even when the AMF is competing for N acquisition with a diverse and populous soil microbial community. In addition, it appears that the AMF took up N primarily in the inorganic form. While recent work has suggested that AMF can uptake N as simple amino acids (Hawkins *et al*., 2000; Rains and Bledsoe, 2007; Whiteside *et al*., 2009), or even as the complex N macromolecule chitosan (Whiteside *et al*., 2009), we did not see evidence for robust organic N uptake in our study. By IRMS, we were able to quantify significant transfer of litter ^{15}N to the host plant; however, we were not able to detect significant ^{13}C enrichment in the hyphae or roots (Herman *et al*., 2012). By NanoSIMS analysis, we were only able to detect a slight enrichment of ^{13}C in the hyphae relative to the plant root (Fig. 4B). Together, these results suggest that the AMF primarily relied on the surrounding microbial community to depolymerize macromolecular compounds and make inorganic N and small-molecular-weight organic compounds available to the AMF. The trace levels of ^{13}C enrichment we detected in the hyphae by NanoSIMS analysis were likely due to a small amount of C fixation by the fungus through gluconeogenesis (Pfeffer *et al*., 2004), although it is possible that amino acids were taken up in very small amounts.

We also observed that the presence of AMF slightly increased the ^{13}C : ^{15}N ratio in the litter soil material, which indicates that litter-derived ^{15}N was preferentially removed relative to litter-derived ^{13}C. During decomposition, the C : N ratio of litter usually declines due to the loss of litter C as CO_2. In numerous studies, the rate of litter decomposition has been shown to be a function of N availability, where lower soil N concentrations can stimulate decomposition (Knorr *et al*., 2005; Bird *et al*., 2011). Although our experimental design did not allow us to directly quantify the rate of litter decomposition, accelerated litter decomposition in the presence of AMF has been reported in similar systems (Hodge *et al*., 2001; Atul-Nayyar *et al*., 2009; Larsen *et al*., 2009; Cheng *et al*., 2012). Arbuscular mycorrhizal fungi have also been shown to reduce extractable inorganic N from soil (Tu *et al*., 2006). It has been suggested that AMF reduce the available NH_4^+ pool, which could stimulate accelerated rates of decomposition (Cheng *et al*., 2012). We hypothesize that the preferential

export of N is one mechanism by which AMF alter the microbial community in decomposing litter.

Finally, we observed that the AMF-responsive taxa exhibited significant patterns of phylogenetic clustering using the metrics NRI and NTI. The NRI provides an index of basal clustering of taxa on a phylogenetic tree, while NTI assesses local clustering at the terminal branches of the phylogenetic tree, independent of deeper clustering (Webb *et al*., 2002). Significant patterns of clustering and overdispersion can both indicate phenotypic attraction among taxa, such as when traits have been conserved among closely related taxa (clustered), or when distantly related taxa have converged upon similar niche use (overdispersed) (Webb *et al*., 2002). Phenotypic attraction was indicated for the taxa that responded to AMF; the taxa that responded positively were clustered basally and were overdispersed at the terminal branches of the tree, while the taxa that responded negatively clustered basally and strongly clustered at the terminal branches of the tree. Since bacteria are highly diverse, interact at microscopic spatial scales and have undergone billions of years of evolution, it is difficult to invoke a single mechanism of community assembly to explain phenotypic attraction in bacterial communities (Vamosi *et al*., 2009). We posit that one way AMF drive phenotypic attraction is by acting as a habitat filter. As shown in this study, AMF can modify the physicochemical environment of the soil by exporting litter N. In addition, AMF export P to the plant (Smith and Read, 2008), and import photosynthate C from distant roots (Herman *et al*., 2012). The removal of the P and inorganic N by AMF could deplete microsites of readily accessible forms of nutrients, and instigate inter-domain competition between the bacterial community and AMF (Schimel and Bennett, 2004; Leigh *et al*., 2011; Smith and Smith, 2011; Veresoglou *et al*., 2011). Additional mechanisms that may drive phenotypic attraction are mutualisms that have developed between the AMF and the microbial community, such as endosymbioses (Naumann *et al*., 2010), or the facilitation of bacterial populations by imported C (Toljander *et al*., 2007).

We demonstrated that the AMF *G. hoi* significantly modified the bacterial community in decomposing litter. The responding taxa exhibited significant patterns of phylogenetic clustering and overdispersion, which suggests that AMF may have a role in bacterial community assembly. We also showed that the AMF modified the physicochemical environment in the decomposing litter by preferentially exporting N, and the AMF appeared to uptake N primarily in the inorganic form. We propose that the export of N from litter is one mechanism by which AMF alter the composition of the bacterial community and alter decomposition processes in soil. As the AMF–plant symbiosis is ubiquitous in terrestrial ecosystems, the influence of AMF on decomposition is broadly relevant across terrestrial ecosystems for the utilization and ultimate stabilization of plant carbon.

Experimental procedures

Experimental overview

Plants were grown in microcosm units constructed by connecting two plastic boxes ($13.5 \times 14.0 \times 14.0$ cm) via a double-mesh barrier (Fig. 1). To establish an AMF monoculture, the planted chamber contained, the common plantain *Plantago lanceolata* L. and the AMF *G. hoi* (University of York isolate #110) in a sterile sand and Terra-Green® mixture (a calcined attapulgite clay soil conditioner; Oil-Dri, Cambridgeshire, UK). To simulate decomposition in a live soil, the litter chamber contained freshly harvested loam soil and a discrete patch of dried ^{13}C-only or ^{13}C and ^{15}N dual-labelled *P. lanceolata* root litter. The 'AMF-permitted' treatments had a 20 μm mesh barrier (John Stanier, Whitefield, Manchester, UK) that allowed hyphae to pass into the litter chamber, but excluded plant roots (Fig. 1A). The 'AMF-excluded' treatments had a 0.45 μm mesh barrier (Anachem, Bedfordshire, UK) that excluded hyphae from the litter chamber but permitted solute diffusion via mass flow between the chambers (Fig. 1B). In total, 50 dual-chamber units were established: two treatments with five replicates each receiving ^{13}C-only labelled litter for harvest after 10, 21, 42 and 70 days, and two treatments with five replicates each receiving ^{13}C^{15}N-labelled litter for harvest after 70 days.

Experimental setup

To establish AMF colonization, 2 weeks prior to the start of the experiment, the planted chamber received 120 g of fresh weight inoculum comprised of *G. hoi* colonized roots (*P. lanceolata*) in a sand and Terra-Green® growth medium The inoculum was mixed thoroughly with 1.85 l of a 50:50 mix of sand : Terra-Green® and 0.3 g l^{-1} sterilized bone meal (a complex P and N source to encourage mycorrhizal development). The *P. lanceolata* seeds (Emorsgate Seeds, Norfolk, UK) were planted in this plant compartment (one seed per unit).

The litter chamber contained freshly harvested loam soil (2 mm sieved, pH 6.8 in 0.01 M $CaCl_2$) collected from an experimental garden at the University of York, UK. The soil was thoroughly homogenized after sieving to minimize differences in the starting microbial communities. No fertilizer had been added to this soil for at least the previous 10 years, and none was added during the experiment. A PVC pipe was used to precisely add the isotopically labelled root litter 2 weeks after setup, while ensuring minimal disturbance to the system (internal diameter 6.5 cm, depth 8 cm). The litter was added as 2 g of dried root material (*P. lanceolata*, C : N = 26.7) either labelled with ^{13}C only or dual-labelled with ^{13}C and ^{15}N. Root litter was mixed with 60 g of loam soil prior to burial at 5 cm depth. All root litter was enriched to 40 atom% ^{13}C ± 0.3 SE ($n = 6$). Dual-labelled root litter was enriched to 40 ± 0.3 SE atom% ^{13}C and 27 ± 0.6 atom% ^{15}N ($n = 3$). Litter soil zones were *c.* 1 cm in depth, 6.5 cm in

diameter, had a bulk density of 1.9 mg m^{-3} and were placed at a distance of 3 cm from the mesh to the litter soil perimeter. Production and characteristics of the litter material are described in Herman and colleagues (2012).

The experiment was set up in a randomized design in a glasshouse at the University of York, UK. The daily mean temperature during the experiment was 19.4°C ± 0.07 SE ($n = 126$). Photosynthetically active radiation flux was recorded weekly at noon and averaged 190.5 ± 37.2 SE μmol m^{-2} s^{-1} ($n = 12$) at plant level. All compartments were watered daily with deionized water. The planted compartment was irrigated twice weekly with 50 ml of nutrient solution (Leigh *et al.*, 2009). The litter compartment received no additional nutrients.

Mycorrhizal analysis

At 10, 21, 42 and 70 days the microcosm units were destructively harvested. A subsample of fresh root material from the planted compartment of each microcosm unit was taken and assessed individually for mycorrhizal colonization. This subsample was washed, cleared, acidified and stained with acid fuchsin for mycorrhizal assessment (as Hodge, 2003). Mycorrhizal colonization was examined with a Nikon Optiphot-2 microscope using bright-field and epifluorescence settings at × 200 magnification and numbers of arbuscules, vesicles and total root length colonized (RLC; the percentage of total intercepts where hyphae or other AMF structures were present) quantified (Hodge, 2003). A minimum of 100 intersections were checked for each subsample of root material.

To assess the mycorrhizal hyphal development in the litter soil material, AMF extraradical mycelium were extracted using a modified membrane filter technique, stained with acid fuchsin and counted with at least 50 fields of view at × 125 magnification using the gridline intercept method (Leigh *et al.*, 2009). Hyphal lengths were then converted to hyphal length densities (m hyphae g^{-1} soil dry weight).

Isotope ratio mass spectrometry analysis

Carbon and nitrogen content and isotope ratios of plant roots, shoots, litter soil material, litter soil microbial biomass and the bulk soil (soil surrounding the litter soil patch) were analysed at 70 days using a Roboprep automated nitrogen–carbon analyser coupled to a model 20–20 isotope ratio mass spectrometer (IRMS) (Sercon, Crewe, Cheshire, UK) (Herman *et al.*, 2012). At the earlier time points, only plant material was analysed for carbon and nitrogen content (10, 21 and 42 days). Prior to analysis, the soil and plant material was pulverized and homogenized to a fine powder and measured into tin capsules. The soil microbial biomass in the litter soil material was analysed by chloroform fumigation–extraction (CFE) of 4 g of samples (Brookes *et al.*, 1985). Subsequently, about 18 ml of the extract was lyophilized, and the dried crystals were analysed for C and N content. To determine the percentage litter ^{15}N that was in the plant shoots, roots, litter soil microbial biomass, or remained in the litter soil material or surrounding bulk soil, the atom percentage excess ^{15}N in each component at day 70 was divided by the atom percentage excess ^{15}N in the litter initially added to the soil: $\%^{15}N_{\text{Derived from litter}} = \frac{^{15}N_{\text{Component}_i}(\text{mg})}{^{15}N_{\text{Litter}_i}(\text{mg})}$. The same calculation was used to determine the percentage litter ^{13}C found in the bulk plant material (shoots + roots) by the end of the experiment.

NanoSIMS analysis

NanoSIMS analyses were performed to localize and quantify $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios in *P. lanceolata* roots and associated AMF hyphae using the Lawrence Livermore National Laboratory, USA (LLNL) NanoSIMS 50 (Cameca, Gennevilliers, France). Roots from three of the five 70 day dual-label microcosms were prepared for NanoSIMS analysis. Filtered colonized fine roots and AM extraradical mycelium (from the slides prepared for AM hyphal length and root length colonization assessment) were transferred to the surface of a 7 × 7 mm silica wafer, and then coated with 10 nm Au to ensure conductivity and prevent charging. Samples were mapped with a reflected light microscope at 20 × and 40 × magnification to select locations of infection and aid in sample orientation within the NanoSIMS. Then these areas were analysed in the NanoSIMS by stepping over target areas with a series of 5–15 contiguous 30–50 μm^2 ion image analyses. Samples were simultaneously imaged by secondary electrons to guide root and hyphae identification. Details regarding NanoSIMS run conditions can be found in the supplemental methods. After NanoSIMS imaging, the sputtering path was mapped using scanning electron microscopy (SEM) on a 7401 SEM (JEOL, Tokyo, Japan) at LLNL with an accelerating voltage of 1 kV.

Data were processed to generate quantitative isotopic ratio images from ion images ($^{15}N/^{14}N$ and $^{13}C/^{12}C$) using custom software (LIMAGE, L. Nittler, Carnegie Institution of Washington) and were corrected for effects of detector dead time and image shift from layer to layer. Details regarding standardization of the NanoSIMS data can be found in the supplemental methods. In total, 28 isotope ratio images were created. Sections of individual hyphae and colonized roots were separately defined (using reflected light micrographs and secondary electron images as guidance) as regions of interest (ROI) by encircling pixels with $^{12}C^{14}N^-$ counts > 30% of the maximum counts in the image. The isotopic composition in each ROI was calculated by averaging over all replicate layers where both C and N isotopes were at sputtering equilibrium. We defined 28 total ROI for the hyphae, and 53 total ROI for the root material.

Soil DNA extraction

Soil taken from the litter soil material was frozen at –80°C within 1 h of harvest. The 42 day harvest was selected for molecular characterization because the AMF were clearly present in the root litter soil at this time point (Fig. 1C and D). DNA was extracted in triplicate from 0.25 g aliquots of freeze-dried soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, USA), where samples were bead-beaten for 30 s at 5.5 m s^{-1} with a FastPrep Instrument (Qbiogene, Irvine, USA).

PCR amplification of target DNA

Bacterial and archaeal 16S rRNA genes were PCR amplified using the primers 1492rpl (universal reverse), 8F* (bacterial) and 4Fa (archaeal) (Brodie *et al*., 2007). PCR amplifications were performed as eight replicates of 25 μl final volumes of 1× Takara buffer, 0.3 μM primers, 1.5 U Takara ExTaq, 0.8 mM dNTP and 20 μg of BSA (Takara Mirus Bio, Madison, USA). 0.25 μl volume of DNA extract was added to each replicate as undiluted archaeal template or 1:10 diluted bacterial template. Templates were amplified in a Bio-Rad myCycler (Bio-Rad, Hercules, USA) using the following conditions: 95°C (3 min), 25 cycles at 95°C (30 s), 48–58°C gradient (25 s) and 72°C (2 min), followed by 72°C (10 min). Bacterial PCR products were purified using the UltraClean PCR Clean-Up Kit (MoBio Laboratories, Solana Beach, USA). Archaeal PCR products were gel purified using the QiaQuick Gel Extraction Kit (Qiagen Sciences, Valencia, USA). PCR products were electrophoresed on a 2% E-Gel (Invitrogen, Carlsbad, USA) for 18 min and quantified using Quantity One 1-D analysis software (Bio-Rad, Hercules, USA). Purified PCR products were concentrated using microcon-30 centrifugal filters (Millipore, Billerica, USA).

Microarray analysis

For high-throughput identification of bacteria and archaea, 500 ng of bacterial amplicons, 100 ng of archaeal amplicons and a known concentration of synthetic control DNA were fragmented, biotin-labelled and hybridized to the Phylochip G2 microarray (Brodie *et al*., 2007). Microarrays were washed, scanned and normalized using the same criteria as Brodie *et al*. 2007. An additional normalization was used to account for differences in chip brightness, where the intensity units for individual taxa were divided by the average probe brightness for all detected taxa. Taxa were identified using the 2010 Phylochip probe set analysis and classified using the 2011 *Greengenes* taxonomy (McDonald *et al*., 2012). To qualify as present in the data set, taxa were required to pass a probe quartile cut-off (Hazen *et al*., 2010), have a probe fraction of 0.9 or higher and be detected in a minimum of three microarray replicates for at least one treatment.

Microarray statistical analysis

Community structure was ordinated by non-metric multidimensional scaling (NMDS) using the *vegan* R package (distance measure: Bray-Curtis) (Oksanen *et al*., 2010). Analysis of similarities (anosim) was used to evaluate the null hypothesis of no difference between groups (distance measure: Bray-Curtis). Operational taxonomic units (OTUs) that significantly differed between the treatments were determined using ISA on relative abundance data (Dufrene and Legendre, 1997). Indicator species analysis determines which taxa are uniquely present for each treatment, and accounts for both the relative abundance and frequency of an OTU (Dufrene and Legendre, 1997). Mean differences in relative abundance (RA) between the two treatments were used to confirm whether taxa increased or decreased in relative abundance in the presence of AMF ($\Delta\overline{RA} = \overline{RA}_{Permitted} - \overline{RA}_{Excluded}$, $n = 5$).

Phylogenetic analysis

Net relatedness index (NRI) and nearest taxon index (NTI) analyses were used to determine if the taxa that responded to AMF were phylogenetically clustered or overdispersed (Webb *et al*., 2002). Phylogenetic clustering was evaluated separately for the positive and negative AMF responders. The NRI and NTI values were calculated as $NRI = SES_{MPD} \times -1$ and $NTI = SES_{MNTD} \times -1$ and weighted by relative abundance in the *picante* R package (Kembel *et al*., 2010). Significance was assigned to any NRI or NTI value falling in the top or bottom 2.5% of randomized communities created using the independent swap null model (10 000 randomizations, each with 100 000 swaps). The independent swap algorithm maintains species occurrence frequency and species richness, and this null model performs well when detecting niche-based assembly processes compared with other null models (Kembel, 2009). The NRI values significantly > 0 indicate clustering within deeper branches of the tree, while NTI values significantly > 0 indicate clustering at the terminal branches of the tree. The NRI and NTI values significantly < 0 indicate that the traits are evenly dispersed on the tree (overdispersed), while values that are indistinguishable from 0 indicate random dispersal on the tree. The phylogenetic tree used for NRI and NTI analyses was constructed from all bacterial taxa detected in this study. The sequences corresponding to the probe sets were compiled and aligned using *Greengenes* (DeSantis *et al*., 2006). The maximum likelihood tree was created in *FastTree* using the generalized time reversible model and the gamma setting to optimize branch lengths (Price *et al*., 2010). A smaller maximum likelihood tree was generated using the same conditions to display the subset of the bacteria that responded to AMF.

Acknowledgements

This work was funded by an Underwood Fellowship (BBSRC) and California Experiment Station project 6117-H to M. K. F., a BBSRC research grant BB/E016359/1 to A. H., a DOE Genomic Science Program grant (FOA DE-PS02-09ER09-25 award #0016377) to M. K. F. and J. P. R., LLNL LDRD 10-ERD-021 to J. P. R., and a UC Laboratory Research Fees grant 116577 to M. K. F. and J. P. R. E. E. N. was funded by a UC TSR&TP fellowship. We thank Alastair Fitter for comments on this manuscript, Eoin Brodie and Todd DeSantis for helpful conversations regarding microarray analyses, Yvette Piceno for microarray technical assistance, Rebecca A. Daly and David Armitage for advice regarding phylogenetic analyses, and Jeremy Bougoure for assistance with SEM imaging. NanoSIMS analyses were performed under the auspices of the US DOE at LLNL (DE-AC52-07NA27344). The authors have no conflicts of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Supplemental methods.
Fig. S1. (A) Percent root length colonized and (B) percent arbuscules detected on *P. lanceolata* roots in the planted chamber for the AMF Permitted (closed circles) and AMF Excluded treatments (open circles). Error bars represent standard error (± SE, $n = 5$). Letters indicate significant differences between the time points and treatments by Bonferroni *post-hoc* analysis.
Fig. S2. Carbon to nitrogen (C : N) ratio of host plant material over the course of the experiment where the root-associated AMF was either permitted (filled squares, solid lines) or denied access (open squares, dashed lines) to the soil chamber containing decomposing litter. Error bars represent standard error (± SE, $n = 5$). Letters indicate significant

differences between the time points and treatments by Bonferroni *post-hoc* analysis.

Table S1. Richness of all taxa detected by 16S microarray analysis in this study grouped by phyla. See *Experimental procedures* for presence–absence criteria.

Table S2. Richness of the bacteria and archaea that significantly increased or decreased in relative abundance (RA) in response to AMF (increased RA = + RA; decreased RA = – RA). Taxa are grouped by taxonomic class. The single archaeal taxon that responded to AMF is denoted by (A). Increases and decreases in relative abundance were calculated in the following manner: Avg. $RA_{AMF\text{-}permitted}$ – Avg. $RA_{AMF\text{-}excluded}$. The statistical significance of these changes was determined by indicator species analysis (ISA).

Table S3. Richness of the taxa that significantly increased or decreased in relative abundance (RA) in response to AMF. Taxa are grouped by taxonomic family. Taxa are bacteria except when denoted by (A) for archaea.