Title

Pastures to woodlands: changes in soil microbial communities and carbon following reforestation.

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Abstract

Reforestation of agricultural lands has the potential to sequester C, while providing other environmental benefits. It is well established that reforestation can have a profound impact on soil physicochemical properties but the associated changes to soil microbial communities are poorly understood. Therefore, the objective of this study was to quantify changes in soil physicochemical properties and microbial communities in soils collected from reforested pastures and compare then to remnant vegetation and un-reforested pastures. To address this aim, we collected soil from two locations (pasture and its adjacent reforested zone, or pasture and its adjacent remnant vegetation) on each of ten separate farms that covered the range of planting ages (0-30 years and remnant vegetation) in a temperate region of southeastern Australia. Soils were analysed for a range of physicochemical properties (including C and nutrients), and microbial biomass and community composition (PLFA profiles). Soil C:N ratios increased with age of tree planting, and soil C concentration was highest in the remnant woodlands. Reforestation had no clear impact on soil microbial biomass or fungal:bacterial ratios (based on PLFA's). Reforestation was associated with significant changes in the molecular composition of the soil microbial community at many farms but similar changes were found within a pasture. These results indicate that reforestation of pastures can result in changes in soil properties within a few decades, but that soil microbial community composition can vary as much spatially within pastures as it does after reforestation.

1. Introduction

Carbon sequestration in vegetation and soils has substantial potential to help mitigate further climate change (Lal, 2004; Swift, 2001). Reforestation of pastures is an important means of sequestering C in the soil (Hoogmoed et al., 2012; IPCC, 2013). Reforestation can provide other environmental benefits, such as the provision of habitat for native flora and fauna, increasing habitat connectivity, and reducing non-point source pollution from agriculture (Cunningham et al., 2015b). For this reason, reforestation of marginal agricultural land is seen as an important form of land-use change (Mackey et al., 2013).

In addition to increasing soil C levels, reforestation can change the chemical nature of C inputs into the soil (de Alcântara et al., 1996; Smith et al., 2012). Trees being long-lived perennial plants typically produce nutrient poor and resistant to decomposition tissues, whereas agricultural plants typically allocate most of their C to photosynthetically active, high nutrient and readily decomposed tissues (Aerts and Chapin, 2000). This can have important implications for soil C cycling, as the residence time of C in the soil is linked closely to its chemical nature and its accessibility to microbes (Conte et al., 2010; de Alcântara et al., 1996; Smernik and Oades, 2001). Additionally, the cycling of C in soil is determined to some degree by its C:N ratio and management (e.g. Giardina et al., 2000). For example, an increase in soil C:N ratio is often associated with the conversion from pasture to woodland, due to increased C:N ratio of the litter inputs, and reduced disturbance and fertiliser inputs (Hoogmoed et al., 2014; Hoogmoed et al., 2012; Ussiri et al., 2006).

Reforestation can change physicochemical properties of the soil (see Cunningham et al., 2015b, and references therein). For example, soil nutrient levels (especially N) often decrease following reforestation due to cessation of fertilizer addition, reduced levels of biological N fixation associated with leguminous species and increased nutrient immobilisation (Garten

69 and Ashwood, 2002; Hooker and Compton, 2003). However, increases in soil nutrients (both
70 N and P) have been reported following reforestation of highly-degraded soils (Jiao et al.,
71 2012) and centuries after reforestation (Wilson et al., 1997). Removal of livestock associated
72 with reforestation can change soil physicochemical properties due to reduced levels of
73 nutrient redistribution and grazing effects on plant-soil feedbacks (Holland and Detling, 1990;
74 Semmartin et al., 2008). These changes in soil properties may have significant effects on soil
75 biotic communities, including those that regulate the cycling of C and nutrients in soils
76 (Bardgett and Wardle, 2010; De Deyn et al., 2008; Ng et al., 2014b).

77 The biomass, activity and diversity of soil microbial communities is affected strongly by
78 changes in soil physicochemical properties (Bossio and Scow, 1998; Ng et al., 2014b), with
79 most of this information coming from agricultural systems. In contrast, few insights have been
80 gained about how soil microbial communities respond to reforestation. Soil microbial
81 communities can differ between forested (plantations and native woodlands) and agricultural
82 lands (Bossio et al., 2005; Singh et al., 2007), among different types of agriculture (Drenovsky
83 et al., 2010), and within a few years among different methods of revegetating agricultural
84 lands (Hedlund, 2002). However, how reforestation of pastures with mixed-species, affects
85 soil microbial communities remains largely unknown.

86 Despite the tremendous complexity of soil microbial communities, predictions can be
87 made about how different groups of soil microbes, such as fungi and bacteria, will respond to
88 revegetation. For example, following reforestation and afforestation (i.e. planting trees on
89 areas that were historically treeless) of agricultural lands, soil C:N ratios generally increase
90 (Berthrong et al., 2009; Cavagnaro, 2016), which is likely to cause a shift from bacterial to
91 fungal dominance in soil communities (Busse et al., 2009; Fierer et al., 2009; Högberg et al.,
92 2007). Given that soil communities play an important role in soil C and nutrient cycling

93 (Bardgett and Wardle, 2010; De Deyn et al., 2008; Ng et al., 2014b), it is valuable to determine
94 how reforestation alters the microbial composition of soils.

95 Here, we quantify changes in the microbial community and soil physicochemical
96 properties following the conversion of pastures to mixed-species plantings dominated by
97 species belonging to the genera *Eucalyptus* L'Hér. and *Acacia* Mill. We selected mixed-species
98 plantings because they are planted increasingly instead of single-species plantings, and their
99 higher above-ground biodiversity potential. We hypothesized that with time, the soil
100 physicochemical properties and microbial community composition of tree plantings would
101 become increasingly divergent from that of the adjacent pasture. To test this hypothesis, we
102 surveyed a replicated chronosquence of sites ranging from treeless pastures through to
103 remnant woodlands on ten farms in a temperate region of southeastern Australia. In order to
104 account for differences in soil properties among farms, at each farm we sampled soils from
105 both the reforested or remnant vegetation zones and an adjacent un-reforested pasture.
106

107 **2. Materials and Methods**

108 **2.1 Study area and design**

109 This study focused on tree plantings on formerly-grazed pastures in northern Victoria,
110 Australia (Table 1). Prior to European settlement in the 1840s, the region was dominated by
111 *Eucalyptus* woodlands (10-30 m tall, 10-30% projective foliage cover (i.e. percentage of the
112 sky blocked out by leaves and stems), Specht, 1981) with grassy understoreys. Since
113 European settlement the land has been cleared extensively and converted predominantly to
114 dryland cropping and pasture-based grazing systems. Consequently, this region offers
115 substantial opportunities for reforestation. The region has a temperate climate with seasonal

116 changes in mean monthly maximum temperature (12.8–31.0 °C) and minimum temperature
117 (3.2–14.9 °C), and a winter-dominant annual precipitation of 500-700 mm $year^{-1}$ (Table 1).

118 This study involved a survey of ten grazing farms that were selected to cover a
119 representative range of time since reforestation (Table 1). At each of the 10 farms two sites
120 were established, one of which was a 'reference pasture site' and the other was a 'treatment
121 site' (Fig. 1). The two sites on each farm were located 50 m apart from one another, but were
122 in the same topographic position and on the same soil type (see below), and had the same
123 management prior to re-forestation. The treatment sites were of the following classes:
124 reforested patches, remnant woodland patches, or pastures. The reforested sites were planted
125 with trees 10, 18 or 30 years prior to sampling (i.e. there were two farms per age class) and
126 were included to provide an indication of changes in soil properties with time since tree
127 planting. The remnant sites were included to represent a potential trajectory for plantings at
128 maturity (two farms). The reference pasture –pasture comparison (two farms) was included
129 to provide a temporal reference without reforestation (0 years) for soil properties, and a
130 spatial reference for the variability of soil properties across a field. This paired design allowed
131 us to assess changes in soil properties under various stages of reforestation (i.e. treatment
132 sites) relative to a conventional pasture management scenario (i.e. reference pasture sites). It
133 also allowed us to partially account for differences among farms due to variation in land-use
134 history and local soil properties.

135 The treatment sites on each farm included the whole tree planting or patch of remnant
136 vegetation (approx. 2 ha), with an equivalent area sampled in the adjacent reference pastures.
137 The adjacent references pasture sites were located away from any remnant paddock trees to
138 remove the influence of trees. The soils at all sites were alfisols according to the FAO soil

139 classification system (IUSS Working Group WRB, 2014) and sodosols in the Australian soil
140 classification system (Isbell, 2002).

141 All tree plantings were planted with a mixture dominated by *Eucalyptus* L'Hér. and
142 *Acacia* Mill. species native to the region. All plantings include the regional dominants
143 *Eucalyptus macrocarpa* and *E. sideroxylon,* with seven to eleven woody species planted and
144 tree densities from 389-604 plant ha^{-1} when surveyed (see also Cunningham et al., 2015a).
145 The plantings were established by ripping the soil into furrows, fencing out livestock and
146 hand planting tubestock seedlings into the furrows. Following reforestation there was no
147 further active management intervention. The remnants patches were selected to represent
148 the target vegetation (plains woodland dominated by *Eucalyptus macrocarpa*) being restored
149 with these plantings and were among the most mature native woodlands in the region. While
150 the exact age of the remnants was unknown, it is likely that they post-date the widespread
151 clearance associated with the Gold Rush of the 1850s and 1860s in the region, and were not
152 actively replanted. At all sites the pastures, which were un-cultivated since establishment,
153 were dominated by perennial pasture species, typically including *Phalaris aquatica* L. and
154 *Lolium perenne* L.

155

156 **2.2 Sample collection**

157 Fieldwork was completed at the ten farms during the austral autumn from late-April to mid-
158 May, 2012. At each farm, a treatment site and an adjacent reference pasture site were
159 sampled (Fig. 1). Four 400-m^2 sampling plots were established randomly across each site.
160 These sampling plots were located in similar topographic positions so as to avoid potential
161 impacts of any underlying gradients within the sites. Within each of these sampling plots, soil
162 samples were sampled within five randomly-located quadrats. Soil was collected from the 0-

163 10 cm soil layer where microbial activity is highest in these soils (Cavagnaro, unpublished).
164 This sampling intensity within plot has been shown to provide a representative sample of soil
165 C in this region; that is, the probability of estimating within 10% of mean at this sampling
166 intensity is $\geq$ 0.8 (see Cunningham et al., 2012).

167 Prior to soil sampling, a 25 × 25 cm quadrat was placed at each soil sampling point.
168 Digital photographs were taken of the quadrat and of the canopy directly above the quadrat
169 for visual quantification of percentage cover of bare ground and canopy, respectively. Canopy
170 cover represents the projected cover of the canopy as a percentage of the sky blocked by
171 leaves and stems. Cover was estimated by placing a 25-cell grid laid over the image and
172 counting the number of cells dominated by canopy or bare ground (Cunningham et al. 2012).
173 All leaf litter and live plant biomass were collected from within the quadrats, and masses
174 weighed after being oven-dried at 50 ^{o}C for 48 h.

175 The five samples of soil from each 400-m^2 plot were bulked in the field and mixed
176 carefully to create one soil sample per plot. Consequently, there were four replicate soil
177 samples, from each site, which were composited from a total of 20 cores (Fig. 1). Each
178 composite sample was then stored at 4 ^{o}C (in a battery operated "car refrigerator") in the
179 field, and within 4 h it was divided into two sub-samples, the first of which was frozen (for
180 microbial analysis), and the second was stored at 4 ^{o}C for physicochemical analysis. These
181 samples were then returned to the laboratory for immediate analysis.

182

183 **2.4 Soil analysis**

184 Prior to physicochemical analysis, the soil samples stored at 4 ^{o}C (N = 4 per site) were sieved
185 to < 2 mm to remove large rocks, roots and macroinvertebrates. These samples were analysed
186 as follows. Gravimetric moisture was determined after drying 20 g of moist soil at 105 ^{o}C for

48 h. Duplicate soil samples (10 g moist soil) were extracted with 2M KCl, and inorganic N content determined colorimetrically using a modification (assays were downscaled for analysis on a 96 well plate reader) of the method for NO_3^--N (plus NO_2^--N) reported in Miranda et al. (2001) and the method for NH_4^+-N in Forster (1995). For each soil sample, potential mineralizable N (PMN) was determined by anaerobic incubation for 7 days (following Wong et al., 2015). Total soil C and N were determined in air-dried sub-samples by dry combustion, by the Environmental Analysis Laboratory, Southern Cross University (www.scu.edu.au/eal/; last accessed April, 2016).

Analysis of phopsholipid fatty acids (PLFA) allows the composition of the soil microbial community to be estimated, based on the profile of ester-linked fatty acids of phospholipids. This analysis provides information on the molecular composition of microbial communities, such as the relative biomass of bacteria and fungi or shifts in whole communities but cannot identify finer functional groupings (Bardgett and Wardle, 2010; Bossio and Scow, 1998). Presence of PLFA was estimated from the soil samples, frozen at -20^{o}C (in a battery operated "car freezer") in the field, following the methods of Bossio and Scow (1998), with slight modification (Mosse et al., 2012). Briefly, PLFAs were extracted from 4 g freeze-dried and finely ground soil samples, using a solvent containing citrate buffer (0.15 M, pH 4.0), chloroform and methanol, followed by transesterification of the polar lipid fraction containing the phospholipids. Individual PLFAs were separated using gas chromatography (30 m (5%-phenyl)-methylpolysiloxane column, Varian CP 3800). Peaks were identified and quantified by comparing with Supelco Bacterial Acid Methyl Ester (BAME) standard mix (product number 47080-U, Supelco, USA). Nomenclature of PLFAs followed that described by Frostegård and Bååth (1996). The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0cy, and 17:0 were chosen as bacterial biomarkers and linoleic acid (18:2ω6,9) was

chosen as the biomarker for decomposer fungi, based on Ng *et al*. (2014b). These PLFA's where then used to calculate Fungal:Bactirial PLFA ratios.

2.5 Data calculations and analysis

Data collected from the survey were analyzed, using the appropriate replicates. For the sites within each farm, we calculated the individual site means and standard errors (N = 4 plots per site; see Fig. 1). Differences in vegetation and soil properties between sites (e.g. reforestation versus pasture) within a given farm were identified using one-way ANOVA.

As we were also interested in assessing changes in selected soil and vegetation variables following reforestation, we calculated the change in properties between sites within each farm by subtracting the mean of adjacent reference pasture site from the mean of the treatment site. For the pasture-pasture (i.e. time zero) pairs, differences were calculated between the two pastures by subtracting the lower value from the higher value, to provide a measure of the average differences between two spatially related pastures (i.e. on the same farm; see above). As the age of the remnant woodlands were unknown, a categorical approach was taken instead of regressions against time. Significant changes ($P < 0.05$) following reforestation were then identified by comparing treatment classes (i.e. 0, 10, 18 and 30 years after reforestation, and remnant woodland) using one-way ANOVAs based on the mean difference between paired sites at each farm (N = 2 farms per class; see Fig. 1). All ANOVAs were performed using JMP statistical software (version 10.0.0).

Multivariate analyses were used to examine differences in the molecular composition (PLFAs) of microbial communities among farms and with between treatment and reference sites on each farm. We restricted these analyses to overall comparisons of molecular composition rather than a detailed analysis of individual PLFAs, as the specificity of such

biomarkers is the subject of growing debate (Frostegård et al., 2011). PLFA concentration values were ranged standardized (x - minimum / range) to avoid analyses being dominated by PLFAs with the highest values. Compositional differences among the samples were estimated using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957).

Analysis of Similarity (ANOSIM, Clarke and Green, 1988) was used to determine if microbial composition was significantly dissimilar ($P > 0.05$) among groups, using Primer 5 (www.primer-e.com; last accessed March, 2015). ANOSIM is analogous to a multivariate ANOVA. It tests the null hypothesis that the mean rank similarity *within* a group is the same as the mean rank similarity *among* groups. Tests are based on the rank similarities between samples and a test statistic R is calculated, which is close to zero when groups are similar. Comparison can be made across all groups (global R) and between specific groups (pairwise R). We used ANOSIM to determine if there were differences in the microbial composition (PLFA): a) of the pastures among the farms (N = 4 plots per site) and b) between the treatment site – reference pasture site at each farm (N = 4 plots per site). Given the low replication for treatment classes (N = 2 farms per class), there were not enough possible permutations to test for a significant difference ($P < 0.05$). Compositional differences among site means were visualized with non-metric multidimensional scaling (NMDS), which creates an ordination from the dissimilarity values among samples, using Systat 10. This provided a multivariate comparison of the treatment classes (pasture, 10-year-old reforestation, 18-year-old reforestation, 30-year-old reforestation and remnant woodland), with each class replicated by two farms.

3. Results

3.1 Ground layer

Reforestation and remnant woodland sites had more leaf litter mass than their adjacent pastures (Table 2). The difference in leaf litter biomass between the treatment and reference sites on each farm showed an increasing trend with time since planting (Fig. 2a), reaching a maximum in the 30-year-old plantings. However, the difference in leaf litter mass was only significantly higher (P = 0.04) in the 30-year-old plantings compared with the pasture-pasture (0-year-old) reference sites. The amount of bare ground was highly variable within and among farms with only three farms having significant differences, so significant changes following reforestation were not found (Table 2).

3.2 Soil properties

Several soil physicochemical properties showed significant differences between treatment and reference sites and with time since reforestation (Fig. 2, Tables 3 & 4). The difference in C concentration of soil between the treatment and reference sites was significantly higher (P = 0.004) at the remnant pairs than in all other categories (Fig. 2b). At individual farms, there were significant increases in soil C concentrations in the forested sites compared with their adjacent pasture sites in the two remnant woodlands, and one of the 18-year-old plantings (Table 4). The largest increase in total N was found between remnant woodlands and their adjacent pastures (P = 0.04, Fig. 2c). Total N concentration showed the same pattern as total C concentration within individual farms (Table 4). At all farms, soil C:N ratios were significantly higher in reforested sites than their adjacent pasture sites, except for one of the 10-year-old plantings (Table 4). The difference in soil C:N ratio between treatment and reference sites increased significantly after tree planting (P < 0.001; Fig. 2d). There were neither consistent nor significant (P > 0.05) changes in soil nitrate, ammonium, potentially mineralizable N (PMN), plant available (Colwell) P, pH or soil moisture content in response to reforestation

283 (change data not shown). When these soil physicochemical properties were compared within
284 individual farms, there were some differences among treatment classes but no consistent
285 patterns (Table 3).

286 There were no significant differences ($P > 0.05$) in the changes in total PLFA, fungal
287 biomass and F:B among the treatment classes (Fig. 2e, f, g). We do however, note the high
288 fungal biomass in the pasture of Site 8, the reason for which remains unknown. When these
289 microbial variables were compared at individual farms (Table 4), some differences were
290 found between land uses. Fungal biomass was higher ($P < 0.05$) 18 years following
291 reforestation, with the exception of one 30-year-old site where fungal biomass (and total
292 PLFA) was very high in the adjacent pasture. To further explore total PLFA, fungal biomass
293 and F:B ratio data, these data were correlated with the other soil physicochemical properties.
294 The only significant correlation was between total PLFA and soil moisture, and this
295 relationship was weak ($P < 0.01$, $R^2 = 0.37$).

296 We used ANOSIM to determine differences in microbial composition (PLFAs) among
297 farms and treatment classes. The microbial composition of the pasture sites were significantly
298 different among all farms (Global $R = 0.89$, $P < 0.01$; pairwise R, $P = 0.03$, $N = 4$ samples per
299 pasture). Within a farm, the paired land uses contained different microbial compositions
300 (Table 5). The 10-year-old reforestations tended ($P = 0.06$) to have different microbial
301 compositions to their adjacent pastures whereas the 18-year-old and 30-year-old
302 reforestations had significantly different ($P = 0.03$) microbial compositions to their adjacent
303 pastures. Both the pasture-pasture and the remnant woodland-pasture pairs did not show
304 consistent changes in microbial community composition, with one farm having a significant
305 change while the other farm did not.

306 The non-metric multidimensional scaling (NMDS) ordination provided a robust visual
307 representation (Stress = 0.10, variance explained = 96.7%) of the differences in microbial
308 composition (PLFAs) among the samples (Fig. 3). The ordination showed clearly that the
309 microbial composition of the pastures differed widely among the farms. Importantly, one of
310 the pasture-pasture pairs had as much difference in microbial composition as many of the
311 other pastures paired with a forested site. Therefore, there was not a trend of increasing
312 difference in composition between paired sites along the chronosequence. Together, these
313 results indicate that reforestation of pastures can result in significant changes in soil
314 properties within a few decades, but that soil microbial community composition can change
315 as much with local variation in pastures as it does with reforestation.

4. Discussion

Reforestation of pastures had a significant impact on litter mass and selected soil physicochemical properties (Fig. 2, Tables 2-4). There were no clear changes in microbial biomass (measured as total PLFA), or soil fungal:bacterial ratios with reforestation (Fig. 2, Table 4). This was unexpected given the general increase in soil C:N ratio with reforestation (Table 4) and the well established link between soil C:N and fungal:bacterial ratios (Busse et al., 2009; Fierer et al., 2009; Högberg et al., 2007). However, it is important to note that the range of C:N values in the present study (10.5-19.4) are much narrower than those in the earlier work by Fierer *et al*. (2009) (approx. 4-38) in which this correlation was observed. Nevertheless, there was a trend towards increased fungal biomass at most sites 18 years after reforestation (Fig. 2g). Further, whole soil microbial community profiles (based on PLFAs) differed with reforestation at some farms (Table 5, Fig. 3). We had anticipated a shift towards more distinct microbial communities with reforestation but it was not possible to attribute changes in whole soil microbial community profiles to specific soil physicochemical properties. Despite the fact that every effort was taken to ensure sites within farms were as similar as possible prior to reforestation (i.e. soil type, topography, prior management), at these farms it appears that spatial variation at the site level was the major determinant of microbial community composition. These differences may also be associated with the greater heterogeneity in the reforested sites (mixed species plantings) than in their adjacent pastures (pasture grasses), both in terms of litter composition and spatially.

There was a substantial and steady increase in leaf litter mass following reforestation of pastures, with the mass reaching that of the remnant woodlands within 30 years. This increase in litter mass, although less stable than soil, represents an important store of C in these low-rainfall ecosystems (Cunningham et al., 2015b). For example, working in the same

region we found a consistent increase in litter mass C over a 45 year period post-reforestation, where stocks equivalent to those in remnant woodlands were reached within ca. 25 years after reforestation (Cunningham et al., 2015a). This increase in leaf litter is presumably due to an increase in tree biomass at the site, which was, in part supported by a positive correlation between leaf litter and canopy cover ($P=0<0.0001$; $R^2 = 0.80$). Further work on the chemical nature of those inputs is needed. A shift from pasture to *Eucalyptus*-dominated woodland would be predicted to increase the relative amount of recalcitrant C containing compounds (e.g. lignin and cellulose) entering the soil compared with pasture (Smith et al., 2012), which may affect the residence time of C in the soil (Conte et al., 2010; Smernik and Oades, 2001), and its availability to soil microbes (Ng et al., 2014b). Further studies into the chemical nature of the C pools in these and other ecosystems are needed, if we are to develop a complete understanding of the residence time of C in these systems.

Reforestation of pastures affected significant change in some soil physicochemical properties (Fig. 2). There was a clear increase in the difference in soil C:N ratio between reference and treatment sites, between the different treatment classes (Fig. 2d). The larger difference in total soil C concentration between the pastures and the adjacent remnant vegetation plots compared with all other land-uses is consistent with earlier work indicating that an increase in the C concentration of soil is often found > 30 years after reforestation (Guo and Gifford, 2002; Paul et al., 2002; Post and Kwon, 2000). The increase in soil C:N ratio is likely due to larger C inputs (i.e. litter mass), and an increase in the C:N ratio of the litter produced by tree species compared with that of pasture species (Aerts and Chapin, 2000; De Deyn et al., 2008). This is further supported by a positive correlation between soil C:N leaf litter at the sites ($P<0.001$; $R^2 = 0.56$).

363 The impact of reforestation of pastures on soil microbial communities was considered
364 at the levels of the total microbial biomass (measured as PLFA), fungal:bacterial ratio, fungal
365 biomass (Fig. 2), and molecular composition (PLFA, Fig. 3). There was no consistent change in
366 the microbial community with reforestation at the level of total biomass or fungal:bacterial
367 ratio. There was no clear relationship between soil C:N ratio and fungal:bacterial ratio. Given
368 the wide range of C:N ratios (10-22) and fungal:bacterial ratios (0.04-0.47) in the soils studied
369 here, and earlier global studies showing a relatively strong relationship between these
370 ecosystem properties (Fierer et al., 2009; Waring et al., 2013), this was unexpected. The lack
371 of an observed relationship here may be associated with not only changes in soil C:N ratios,
372 but also the forms of C present in the soil. This further highlights the need to consider
373 composition of litter inputs as well as amounts of litter (Cunningham et al., 2015a; Giardina et
374 al., 2000; Hoogmoed et al., 2014).
375 While there were clear changes in microbial communities among the sites, the
376 underlying reasons for these changes remain elusive. When we compared (correlations) total
377 PLFA, F:B ratios and fungal biomass to all soil physicochemical and ground layer data, there
378 was only a weak relationship between total PLFA and soil moisture (P = 0.004, R^2 = 0.37).
379 Given that samples were collected at the same time of year, this response is not due to
380 seasonal differences, but variation in soil moisture among and within sites. The lack of a clear
381 response of the microbial community, to what was a major shift in above-ground community
382 composition, was unexpected given the clear links between above- and below-ground
383 communities (see Bardgett and Wardle, 2010, for detailed review). Soil microbial community
384 composition differed not only among farms, but also within farms (i.e. between the reference
385 and treatment sites, Fig. 3). These differences suggest a stronger response to local variation in

soils, as suggested by the significant differences in community composition between pasture-pasture pairs sampled on the same farm, than to reforestation.

Here, there was a clear difference in the C:N ratio of the reforestation soils compared with their adjacent pastures with increasing time since reforestation. The results also indicate that it will take > 30 years for total soil C concentrations to reach levels similar to those in remnant forests in the region. Changes in soil microbes with reforestation were less clear. With the importance of above-ground communities well recognised as drivers of below-ground communities (and vice versa) (Bardgett and Wardle, 2010), we conclude that the apparent lack of differences in microbial community composition is due more to high spatial variation within sites, than land-use having little impact on microbial community composition. This conclusion is supported by the fact that the remnant sites did not have a distinct microbial community compared to the other land-uses. This further highlights the need to study changes in soil physicochemical properties and microbial communities at sites that have been reforested for a longer time and with higher replication ($N > 20$). Finding older sites (> 30 yr) was not possible in this system, and may not be possible in many systems due to the recent development of such land practices (e.g. mixed-species plantings). There is also need for further studies that investigate changes in the nature of C containing compounds in soils, and link them to microbial community composition and activity (Ng et al., 2014a; Ng et al., 2014b). Understanding the forms of C stored in the soil following reforestation will tell us about the potential cycling of that carbon, and will be invaluable to including microbial responses in predictive models for C and nutrient cycling.

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Table 1: Environmental characteristics of the survey sites from the ten farms.

Farm	Land Use	Age	Latitude	Longitude	Rainfall	Max temp.	Elevation	Landform	Soil Texture	Basal area	Tree density
		(yr)†	(°S)	(°E)	(mm yr^{-1})	(°C)	(m)			(m^2 ha^{-1})	(trees ha^{-1})
1	Pasture	0	36.65	145.58	581	21.3	150	plain	sandy loam	0	0
2	Pasture	0	36.39	145.95	563	22.0	225	gentle slope	sandy loam	0	0
3	Planting	9	36.46	145.77	556	21.8	145	plain	sandy loam	3.6	456
4	Planting	10	36.50	146.13	629	21.6	180	gentle slope	sandy loam	7.4	474
5	Planting	17	36.00	145.91	487	22.5	120	plain	clay loam	39.3	604
6	Planting	18	36.58	146.11	684	20.6	240	gentle slope	sandy loam	9.9	493
7	Planting	30	36.53	145.75	581	21.6	175	gentle slope	sandy loam	9.7	389
8	Planting	31	36.17	146.95	510	22.2	190	plain	sandy loam	39.1	581
9	Remnant	na	36.58	145.62	580	21.3	160	gentle slope	sandy loam	13.5	342
10	Remnant	na	36.68	145.03	566	20.9	140	gentle slope	loam	10.2	263

†Age = years since planting. Age for the pastures was zero as they were not reforested and was unknown (na) for the remnant woodlands (see text).

Table 2. Key structural properties of the ground layer in the treatment site – reference pasture site pairs at each farm. Values are means ± SE (*N* = 4 plots for each site at a farm – see Figure 1). Results of one-way ANOVAs comparing the treatment sites (TREAT) and the reference pastures (PAST) sites within each farm are provided, with significant differences ($P < 0.05$) between sites are indicated by asterisks.

Age†	Farm	Bare ground		Litter mass	
		TREAT	PAST	TREAT	PAST
		--------(%)--------		----------(g m^{-2})----------	
0	1	6 ± 3*	28 ± 5*	10±4	24±15
	2	0 ± 0	0 ± 0	26±21	12±7
10	3	9 ± 6	9 ± 5	528±76*	24±11*
	4	30 ± 7*	0 ± 0*	574±114*	233±39*
18	5	3 ± 2	0 ± 0	1906±186*	291±35*
	6	4 ± 3	1 ± 1	711±64*	9±5*
30	7	8 ± 7*	0 ± 0*	1435±211*	214±21*
	8	2 ± 1	1 ± 1	1977±192*	28±7*
Remnant	9	3 ± 3	55 ± 6	1425±328*	81±20*
	10	10 ± 4	18 ± 6	1169±101*	26±9*

†N.B. Age = 0 are an unplanted pastures (see text).

Table 3. Key soil physicochemical properties of the treatment site – reference pasture site pair sites at each farm. Values are means ± SE (*N* = 4 plots for each site at a farm – see Figure 1). Results of one-way ANOVAs comparing the treatment sites (TREAT) and the reference pastures (PAST) sites within each farm are provided, with significant differences ($P < 0.05$) between sites are indicated by asterisks.

		Nitrate		Ammonium		PMN‡		Colwell P		pH		Moisture content	
Age§	Farm	TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST
		----------(μg g^{-1})----------		--------(μg g^{-1})--------		----------(μg g^{-1})----------		----------(μg g^{-1})----------				----------(%)----------	
0	1	18.4±2.4	20.2±3.2	5.2±2.6	4.0±2.0	37.7±4.2	30.2±4.8	52.2±3.8	58.5±6.8	5.3±0.1	5.4±0.0	12.2±1.5*	18.0±0.5*
	2	6.8±1.5	3.3±0.5	5.9±2.2	5.5±2.5	45.6±5.8	57.5±6.2	57.6±4.8	55.4±5.0	6.2±0.0	6.2±0.1	7.1±0.4	7.0±0.3
10	3	32.8±3.51	2.2±0.4	1.6±0.3	2.6±0.4	35.9±1.3*	52.1±5.4*	26.2±1.6	39.0±8.8	5.2±0.1*	5.5±0.1*	9.0±0.7	8.9±0.5
	4	23.3±3.3	14.8±4.0	0.4±0.2*	8.1±1.6*	38.4±5.6	44.9±2.9	12.7±1.1	14.4±0.6	5.0±0.0*	5.5±0.1*	7.1±1.0*	15.8±2.8*
18	5	13.4±4.4	11.0±1.1	0.9±0.1*	2.0±0.3*	50.9±7.9	30.3±10.0	36.2±4.4*	20.6±1.9*	6.2±0.1*	5.6±0.0*	6.3±0.9	7.7±1.0
	6	2.7±0.5	13.6±5.6	1.0±0.3*	7.9±0.7*	39.8±2.8	66.3±17.5	10.9±1.2*	62.8±4.3*	5.0±0.1*	6.3±0.3*	14.3±0.3*	18.5±0.9*
30	7	6.2±4.6	4.5±1.6	1.7±0.3*	5.2±0.6*	26.8±2.2	23.7±2.5	8.3±1.0	7.6±0.8	5.1±0.1*	5.4±0.0*	10.9±0.8	12.7±1.6
	8	11.8±2.3*	2.3±0.6*	6.0±4.2	5.4±0.9	55.1±10.9	29.7±6.4	9.9±3.4	10.4±0.7	5.4±0.1	5.2±0.1	18.0±7.5	22.6±1.3
REM†	9	11.6±4.2*	72.4±21.1*	0.9±0.2*	0.1±0.1*	50.7±15.4	28.6±9.5	18.0±4.6*	287.8±10.2*	6.1±0.2*	6.7±0.0*	9.9±1.0	13.1±1.4
	10	1.5±0.4	3.8±2.4	0.6±0.2	0.5±0.1	27.9±6.9	24.2±4.0	16.4±8.0	5.3±0.5	5.4±0.2	5.5±0.1	9.6±0.8	9.7±0.4

†REM = Remnant. ‡PMN = potentially mineralizable N. §N.B. Age = 0 are unplanted pastures (see text).

Table 4. Total soil carbon and nitrogen (concentrations), and microbial biomass (measured as total PLFA – see text), fungal:bacterial (F:B) ratio and fungal biomass (measured using PLFA – see text), (0-10 cm soil layer). Values are means ± SE (*N* = 4 plots for each site at a farm – see Figure 1). Results of one-way ANOVAs comparing the treatment sites (TREAT) and the reference pastures (PAST) sites within each farm are provided, with significant differences ($P < 0.05$) between sites are indicated by asterisks.

Age‡	Farm	Total C		Total N		C:N		Total PLFA		F:B ratio		Fungal biomass	
		TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST	TREAT	PAST
		----------(%)---------		----------(%)----------				----------(nmol g^{-1})----------				---------(nmol g^{-1})--------	
0	1	3.1±0.2	3.0±0.3	0.29±0.02	0.28±0.02	10.4±0.0	10.5±0.2	175.7±29.1*	261.1±5.0*	0.09±0.02	0.08±0.02	12.8± 1.2	19.5± 4.6
	2	2.5±0.2	2.6±0.1	0.23±0.01	0.23±0.01	11.1±0.2	11.1±0.1	145.5±28.2	119.4±7.6	0.23±0.01	0.24±0.01	27.6± 6.2	23.2± 1.8
10	3	3.4±0.1	3.1±0.3	0.31±0.01	0.26±0.02	11.0±0.2*	11.7±0.2*	94.5±8.0	95.7±16.8	0.15±0.01*	0.20±0.02*	11.8± 0.5	15.6± 2.1
	4	2.7±0.1	2.6±0.3	0.23±0.00	0.24±0.03	11.6±0.4	11.1±0.2	65.1±4.7	79.0±10.3	0.22±0.03	0.15±0.02	11.6± 1.0	10.9± 2.8
18	5	2.6±0.2	2.4±0.3	0.19±0.02	0.21±0.03	13.7±0.4*	11.3±0.3*	139.7±21.8	103.2±5.6	0.15±0.02	0.10±0.01	17.0± 0.8*	9.5± 1.2*
	6	5.0±0.3*	6.1±0.3*	0.35±0.02*	0.48±0.03*	14.3±0.1*	12.7±0.5*	160.6±11.4	197.2±11.2	0.15±0.01	0.11±0.02	21.1± 1.1	19.3± 3.4
30	7	5.8±0.7	4.0±0.4	0.30±0.04	0.27±0.03	19.4±1.0*	14.8±0.6*	134.9±14.5*	80.7±16.6*	0.20±0.03	0.21±0.09	22.2± 3.4*	11.0± 1.2*
	8	3.8±0.3	3.0±0.1	0.23±0.02	0.26±0.01	16.3±0.1*	11.5±0.1*	89.5±19.6*	502.4±38.9*	0.36±0.04*	0.17±0.02*	22.9± 4.4*	73.9± 10.1*
REM†	9	6.2±0.6*	2.6±0.1*	0.34±0.03*	0.22±0.00*	17.9±1.0*	11.9±0.4*	185.4±19.8*	112.9±7.6*	0.18±0.02	0.18±0.02	26.9± 2.5*	17.3± 2.2*
	10	6.2±0.4*	2.0±0.1*	0.32±0.03*	0.17±0.01*	19.3±0.6*	12.3±0.3*	184.1±33.4	103.3±16.3	0.18±0.02*	0.33±0.05*	28.1± 6.1*	23.9± 1.5*

†REM = Remnant. ‡N.B. Age = 0 are unplanted pastures (see text).

Table 5. Results of ANOSIM comparing the molecular composition (PLFAs) between each treatment site – reference pasture site pair within a farm (N = 4 plots for each site at a farm – see Fig. 1).

Age	Farm	*R*	*P*
0	**1**	0.656	0.03
	2	0.479	0.09
10	**3**	0.510	0.06
	4	0.552	0.06
18	**5**	0.708	0.03
	6	0.719	0.03
30	**7**	0.438	0.03
	8	0.865	0.03
Remnant	**9**	0.771	0.03
	10	0.573	0.06

Fig. Captions

Fig. 1. Schematic diagram of the sampling hierarchy used in the field survey: farm > paired reference pasture - treatment sites > plot > quadrats. Each treatment class (e.g. 18-year-old planting) was replicated at two farms, giving a total of 10 farms (boxes with dashed lines). Within a farm, a treatment class was represented by a treatment site (e.g. remnant vegetation) and an adjacent reference pasture site, with the average distance between paired sites (within farms) also indicated. Four plots (dimension shown) were established randomly within each site and five quadrats were established within each plot. Note the Fig. is not drawn to scale and farms were not uniformly distributed across the landscape.

Fig. 2. Difference (Diff.) (treatment site – reference pasture) in (a) leaf litter mass, (b) soil C concentration, (c) soil N concentration, (d) soil C:N, (e) total PLFA, (f) fungal:bacterial (F:B ratio) and (g) fungal biomass following reforestation. Values are means (±SE, N = 2 farms, Fig. 1) of the difference in mass between treatment sites and the adjacent reference pasture sites. For the pasture-pasture pairs, one of each pair was treated as a treatment site and the other as the reference pasture in this calculation (see Methods). Means followed by the same letter are not significantly different ($P > 0.05$, see text for further details).

Fig. 3. NMDS ordination of sites based on their soil microbial composition (PLFAs). Symbols are the mean PLFA composition from a site with land uses denoted as follows: pasture (P), 10-year-old reforestation (10), 18-year-old reforestation (18), 30-year-old reforestation (30) and remnant woodland (R). Treatment and reference pasture sites from the same farm are linked by dashed lines.

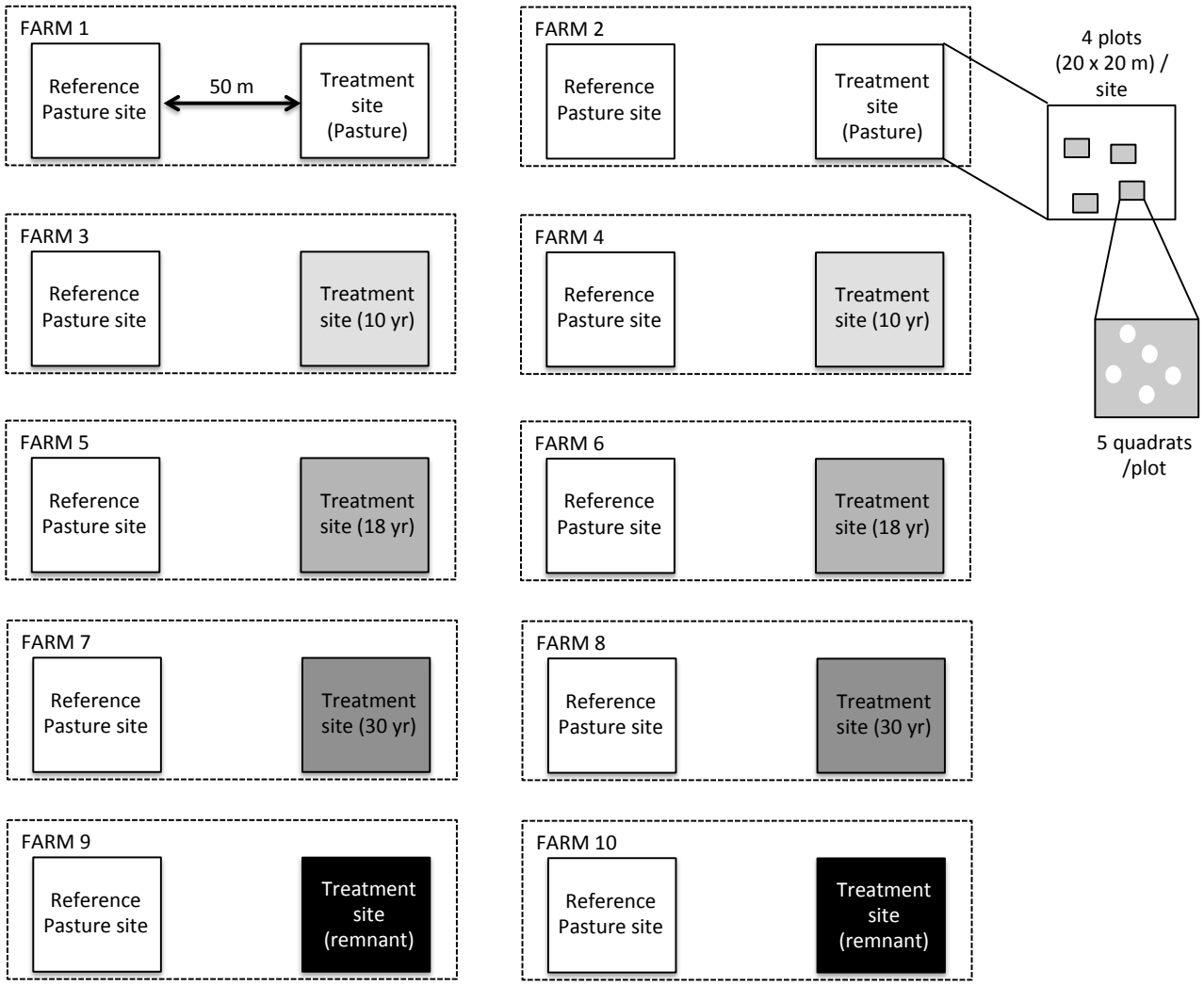
FARM 1
Reference Pasture site
50 m
Treatment site (Pasture)
FARM 2
Reference Pasture site
Treatment site (Pasture)
4 plots (20 x 20 m) / site
5 quadrats /plot
FARM 3
Reference Pasture site
Treatment site (10 yr)
FARM 4
Reference Pasture site
Treatment site (10 yr)
FARM 5
Reference Pasture site
Treatment site (18 yr)
FARM 6
Reference Pasture site
Treatment site (18 yr)
FARM 7
Reference Pasture site
Treatment site (30 yr)
FARM 8
Reference Pasture site
Treatment site (30 yr)
FARM 9
Reference Pasture site
Treatment site (remnant)
FARM 10
Reference Pasture site
Treatment site (remnant)

Figure 1.

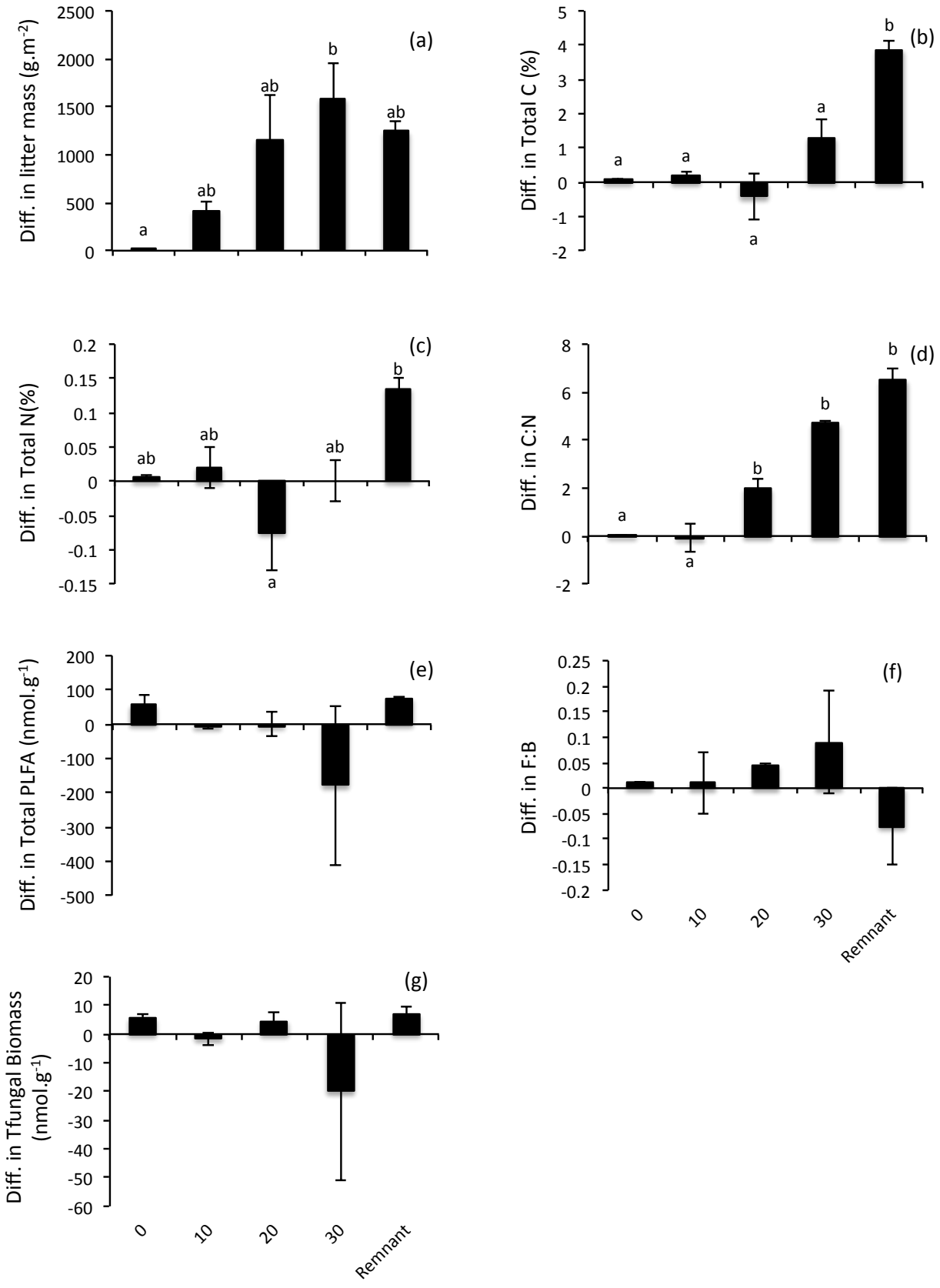
(a)
Diff. in litter mass (g.m-2)
2500
2000
1500
1000
500
0
a
ab
ab
b
ab
(b)
Diff. in Total C (%)
5
4
3
2
1
0
-1
-2
a
a
a
a
b
(c)
Diff. in Total N(%)
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
ab
ab
a
ab
b
(d)
Diff. in C:N
8
6
4
2
0
-2
a
a
b
b
b
(e)
Diff. in Total PLFA (nmol.g-1)
200
100
0
-100
-200
-300
-400
-500
(f)
Diff. in F:B
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
(g)
Diff. in Tfungal Biomass (nmol.g-1)
20
10
0
-10
-20
-30
-40
-50
-60
0
10
20
30
Remnant

Figure 2.

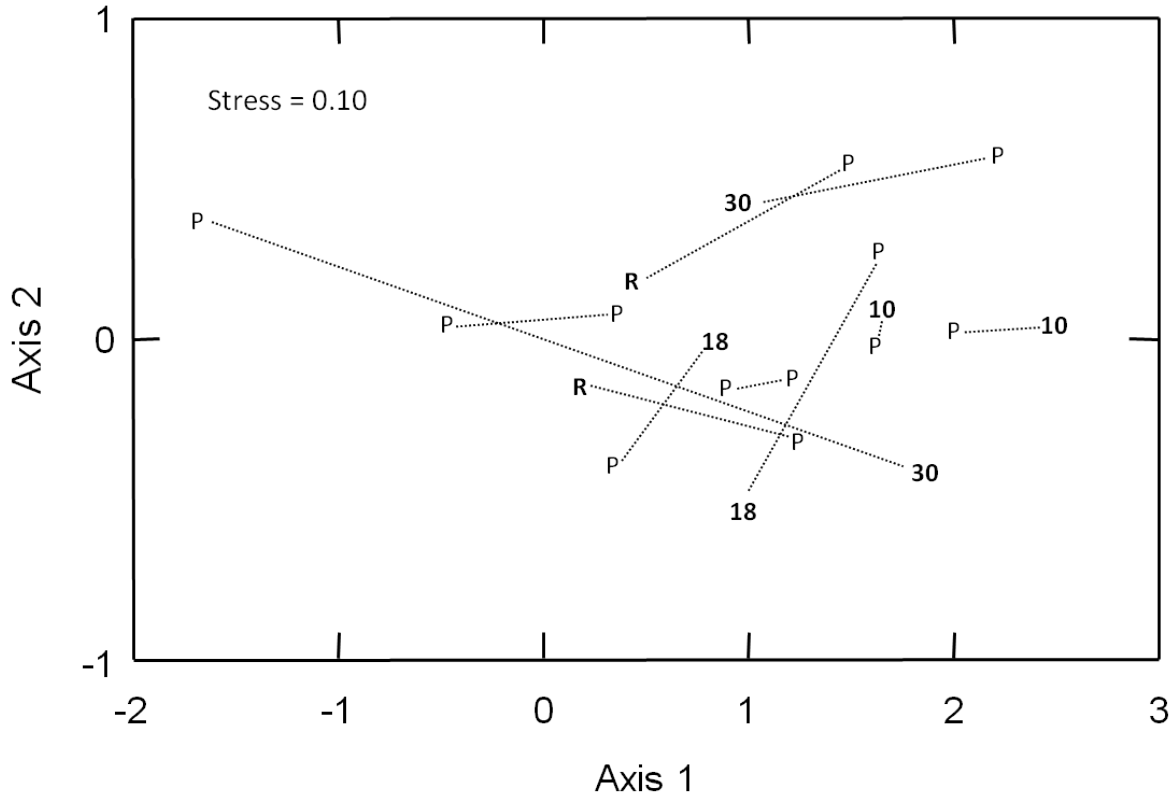

Figure 3.