N-fixing trees in restoration plantings: Effects on nitrogen supply and soil microbial communities

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ABSTRACT

Mixed-species restoration tree plantings are being established increasingly, contributing to mitigate climate change and restore ecosystems. Including nitrogen (N)-fixing tree species may increase carbon (C) sequestration in mixed-species plantings, as these species may substantially increase soil C beneath them. We need to better understand the role of N-fixers in mixed-species plantings to potentially maximize soil C sequestration in these systems. Here, we present a field-based study that asked two specific questions related to the inclusion of N-fixing trees in a mixed-species planting: 1) Do non-N-fixing trees have access to N derived from fixation of atmospheric N₂ by neighbouring N-fixing trees? 2) Do soil microbial communities differ under N-fixing trees and non-N-fixing trees in a mixed-species restoration planting? We sampled leaves from the crowns, and litter and soils beneath the crowns of two N-fixing and two non-N-fixing tree species that dominated the planting. Using the ¹⁵N natural abundance method, we found indications that fixed atmospheric N was utilized by the non-N-fixing trees, most likely through tight root connections or organic forms of N from the litter layer, rather than through the decomposition of N-fixers litter. While the two N-fixing tree species that were studied appeared to fix atmospheric N, they were substantially different in terms of C and N addition to the soil, as well as microbial community composition beneath them. This shows that the effect of N-fixing tree species on soil carbon sequestration is species-specific, cannot be generalized and requires planting trails to determine if there will be benefits to carbon sequestration.

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1. Introduction

Afforestation of agricultural land may contribute to carbon sequestration, potentially mitigating climate change, and restoring of native ecosystems (Guo and Gifford, 2002; Hoogmoed et al., 2012; Paul et al., 2002). Single-species tree plantations for wood production are among the most common afforestation systems (Chazdon, 2008; Paul et al., 2002), although restoration plantings, which contain a mixture of native tree species that are not harvested, are becoming more widely planted (Cunningham et al., 2012). This is because in addition to their potential capacity to store carbon, both above- and below-ground, they provide a range of additional ecological benefits (Harrison et al., 2000), including increased habitat for native flora and fauna (Munro et al., 2009) and ecological stability (e.g. higher resilience to insect pests, Knoke et al., 2008), and nutrient interception when planted as buffer strips adjacent to waterways (Burger et al., 2010; Fennessy and Cronk, 1997).

A fundamental question in establishing mixed-species restoration plantings is which species to plant. One consideration in selecting tree species is whether individual species possess desirable traits. For example, nitrogen-fixing trees can directly fix atmospheric nitrogen (N) to support partly or totally their own growth, giving them an advantage over non-N-fixing tree species, especially in N limited systems (Gallanà et al., 1998). Consequently,
higher levels of soil C under N-fixing trees have been attributed to higher growth rates of N-fixing trees and subsequent higher C inputs into the soil via litter and root exudates (e.g. Resh et al., 2002; Wang et al., 2010). Including N-fixing tree species in mixed-species restoration plantations may increase and accelerate the carbon sequestration potential of the ecosystem (Kaye et al., 2000). In addition to increasing soil N (Kaye et al., 2000), heightened N levels may reduce lignin decomposition (e.g. Berg and Matzner, 1997; Carreiro et al., 2000), further slowing organic matter decomposition and increasing C sequestration (Prescott, 2010).

In mixed-species plantings, N-fixing trees can also facilitate the growth of non-N-fixers. The non-N-fixers may benefit from lowered competition for the available soil N, or they may be able to access the fixed atmospheric N pool (Forrester et al., 2006) after decomposition of the N-fixers litter (van Kessel et al., 1994), through root exudates, or via interconnected mycorrhizal networks between the trees (He et al., 2003). This facilitative effect of N-fixers on non-N-fixers is important for net primary production, as well as community development (Siddique et al., 2008) and successional processes (Chapin et al., 1994; Vitousek and Walker, 1989). Consequently, the inclusion of N-fixers in mixed species woody plantings may have an important impact upon N dynamics in these systems.

The stand-scale consequences of N2-fixation on soil C sequestration are ultimately driven by the effects of N on soil processes. This may include impacts on soil microbial communities, which play a key role in organic matter decomposition (Wardle, 2002). This process is governed by complex interactions among factors such as litter quantity and quality (nutrient content and chemical structure), soil microbial community composition and several biotic and abiotic factors (e.g. Prescott, 2010). Soil microbial communities are often found to differ among tree species (Priha et al., 2001), presumably, due to differences in litter quality and quantity (Bauhus et al., 1998; Hobbie, 1992; Schweiter et al., 2012). Higher amounts of N in litter and soil under N-fixing trees are likely to have a major effect on the soil microbial community beneath these trees (Allison et al., 2006). For example, higher available nitrogen or a lower C:N ratio under N-fixers may favour bacterial over fungal decomposers (Fierer et al., 2009; Harrison and Bardgett, 2010). Bacteria are generally less adapted to decompose recalcitrant litter as fungi (Henrikson and Breland, 1999; van der Heiden et al., 2008). Therefore, increased N levels under N-fixing trees may shift the microbial community towards bacterial dominance, slowing the rate of decomposition of organic matter and increasing the rate of soil C sequestration. In contrast, fungal biomass is more recalcitrant and fungi have a higher C assimilation efficiency compared with bacteria, therefore a shift towards more bacteria could also result in a reduction of soil C sequestration (Bailey et al., 2002b).

If the potential for N-fixers to increase soil C sequestration in mixed-species afforestation plantings is to be maximized, we need to better understand the role of N-fixers in these plantings. An extensive literature exists on interactions between N-fixing and non-N-fixing trees (e.g. Bouillet et al., 2013; Forrester, 2014), albeit predominantly in relation to tree growth and wood production (e.g. Binkley et al., 2003; Parrotta, 1999) but also soil C sequestration (e.g. Kaye et al., 2000) or nutrient cycling (e.g. Khanne, 1997). However, there is a lack of consensus about how N-fixers and non-N-fixers interact and what drives differences among studies. Further, little is known about the impact of N-fixers on soil microbial communities in mixed-species plantings. Here, we present the results of a field-based study in which we investigated two important aspects of restoration plantings including both N-fixing and non-N-fixing tree species: 1) the pathways that fixed atmospheric N takes within the stand and 2) the effect of N-fixers on the soil microbial community. We asked two specific questions:

1. Do non-N-fixing trees have access to N derived from the fixation of atmospheric N2 by neighbouring N-fixing trees, in the early development of a tree planting?
2. Do changes in the N dynamics associated with N-fixing trees, result in changes in soil microbial communities in a mixed-species restoration planting?

To address these questions, we focused on a young (14 yr) mixed-species planting in southeastern Australia.

2. Materials and methods

2.1. Site description

A field study was conducted in November 2011, in a mixed-species restoration planting along Castle Creek near Euroa (36°86′S, 145°58′E) in northern Victoria, south-eastern Australia. The region has a temperate climate with an annual rainfall of 650 mm, ranging from 30 to 80 mm month−1, monthly maximum temperatures between 12.3 and 29.7°C and monthly minimum temperatures between 4.1 and 15.3°C (1981–2010, Australian Bureau of Meteorology, 2011). The site was previously a pasture that was replanted in 1997 with a mixture of tubestock seedlings of N-fixing and non-N-fixing trees. The N-fixers were Acacia dealbata Link., Acacia implexa Bentham, Acacia melanoxylon R. Br., and the non-N-fixers were Eucalyptus camaldulensis Dehnh., Eucalyptus polyanthemos Schauer, Eucalyptus macrorrhyncha F. Muell, Eucalyptus macrocarpa Maiden and various shrubs. Tree density was ca 700 trees ha−1 and basal area was 13.9 m2 ha−1 at the time of sampling. Soil was a Chromosol loam, classified as Pb1 according to the Australian Soil Classification System (ABARES, 2004), with a mean pH of 5.1.

2.2. Sampling

The two dominant N-fixing tree species, A. dealbata and A. implexa, and the two dominant non-fixing tree species E. camaldulensis and E. polyanthemos, were selected to study N cycling and soil microbial communities in the restoration planting. Ten trees of each species were randomly selected within a 1 ha plot, and sampled for soil, litter and fresh leaves. The selected trees covered the range of DBH (diameter at breast height) of each species within the planting: A. dealbata (14–23 cm), A. implexa (7–20 cm) E. camaldulensis (9–25 cm) and E. polyanthemos (15–35 cm). Soil was sampled from two depth layers (0–10 and 10–20 cm) under the crown of each of the selected trees, on average 50 cm, and never more than 1 m away from the base of the stem. In the 0–10 cm layer, four subsamples (ca 100 g) were collected around the stem in different directions and then bulked to make one composite sample. In the 10–20 cm layer, two samples (ca 200 g) were collected to make one composite sample. Given limited differences δ15N among the tree types (see results), we collected additional soil samples from a large patch of non-N-fixing trees to provide a reference value for δ15N in soil with negligible influence of N-fixing trees. In June 2013, five soil samples were collected in the patch from the 0–10 cm layer, which was ca 10 m away from the nearest N-fixing tree. All soil, from both sampling campaigns was stored immediately at 4°C for 2 days until further processing in the laboratory. Soil bulk density samples were taken at both depth layers, under six of the N-fixers and six of the non-fixers, making sure that trees were spread across the whole sampling area, following Minoshima et al. (2007).

To assess the presence of fixed atmospheric N in litter and fresh leaves, standing litter was collected from within a randomly placed 20 cm × 20 cm quadrat underneath the crown of each tree, within
1 m of the base of the stem. Representative samples of fully-expanded leaves were collected from each tree from four locations in the crown: at two randomly selected sides of the tree and at two heights (from the lowest branches and up to 10 m) using pruning shears on an extension pole.

2.3. Sample processing

Soil samples were passed through a 2 mm sieve. A subsample was frozen immediately at −20 °C for phospholipid fatty acid (PLFA) analysis (see below). Soil moisture was determined by drying a subsample of ca 10 g moist soil samples at 105 °C for 48 h.

All remaining soil was air-dried, and a subsample was ground to a fine powder using a mill and analysed for total C and total N, and the δ15N value and pH. Values of δ15N are defined as the ratio between 15N and 14N isotopes in the sample, and are used to trace the fate of N in ecosystems (Robinson, 2001). Elemental and isotope analysis was done using dry combustion in an ANCA GSL 2 elemental analyzer (Sercon Ltd., UK), coupled to a 20–22 isotope ratio mass-spectrometer (Sercon Ltd., UK). The precision for δ15N is 0.1‰. Please note: total C and total N means per tree type (i.e. the species grouped as N-fixing and non-N-fixing) were published previously in Hoogmoed et al. (2014). The pH of the air dried soil was measured in a 1:5 soil water slurry using a TPS WP-81 pH, TDS, Temperature & Conductivity Meter (EnviroEquip Biolab, Australia).

Bulk density samples were dried at 105 °C for 48 h. Stones were retained to estimate stone volume in each sample using displacement of water in a measuring cylinder. Bulk density was calculated by dividing the oven-dried soil mass by the steel cylinder volume less the stone volume.

To compare soil microbial communities among the tree species, phospholipid fatty acid (PLFA) analysis following the procedures of Bossio et al. (1998) with slight modifications (Mosse et al., 2012). PLFA analysis was performed on the 0–10 cm soil layer only, as we assume that microbial activity is most predominant in this soil layer (e.g. Fierer et al., 2003; Hossain et al., 1995).

Briefly, PLFAs were extracted from 4 g freeze-dried, 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. Separation of PLFAs was done 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 of Frostegård and Bååth (1996).

Litter and fresh leaves were oven dried at 60 °C for 48 h. Identifiable leaves of the tree species under which the sample was taken were removed from the bulk sample for analysis of the species-specific leaf litter. The remaining litter and the species-specific leaf-litter were then ground to powder with a biomass grinder (IKA, Malaysia). Small subsamples (ca 5 mg) of the species-specific leaf-litter were used for total C, total N and δ15N analysis. The remaining species-specific leaf-litter was returned to the main litter sample and analysed to obtain total C, total N and δ15N for the whole litter sample. Fresh leaves were also ground and analysed for C, N and δ15N.

2.4. Statistical analysis

All statistical analyses were performed using the statistical software R (version 3.0.0., R Core Team, 2013). To trace the fate of atmospheric N (δ15N), total C, total N and C:N ratio in the restoration planting, the effects of tree type (A. dealbata and A. implexa pooled as ‘N-fixers’ and E. camaldulensis and E. polyanthemos pooled as ‘non-N-fixers’) was analysed by a nested-analysis of variance (ANOVA), with tree species nested in tree type, for each sample type separately. Paired t-tests were performed to test differences among species. Differences in δ15N, total C, total N and C:N ratio among sample types within a tree species were analysed by one-way-ANOVA. For the one-way-ANOVA of total C and total N content of the sample types, the analysis only included leaves, species-specific litter and litter, because of different measurement units used for the soil C and N stocks (t ha−1) compared with the leaves and litter (%). For δ15N and C:N ratio, all sample types were compared. Results for total C and total N content in the 0–10 cm and 10–20 cm soil layer have been reported previously in Hoogmoed et al. (2014) but are included in the results section for completeness.

Non-metric multidimensional scaling (NMDS) was performed using the metaMDS function within the vegan package (Oksanen et al., 2013) to explore dissimilarities in PLFA communities among tree types and tree species. PLFAs with a concentration of less than 0.1 mg g−1 were considered absent. Only PLFAs detected in more than 4% of the samples were included in the analysis and some of the PLFAs were excluded when also found in the blank samples. In total, 16 PLFAs were used in analysis. The PLFA data were first normalised by sample mass and then range-standardised to scaling values between 0 and 1. The dissimilarities in PLFA communities among the samples was estimated using the Bray–Curtis metric (Bray and Curtis, 1957). Analysis of dissimilarity was performed using the adonis function within the vegan package, to test whether PLFAs were significantly dissimilar between N-fixers and non-N-fixers, and among the individual tree species. To determine which environmental variables explained most of the variation in microbial community composition beneath trees, we used the envfit function in the vegan package. Vectors of variables that were significantly correlated (P < 0.05) and explained more than 50% of the variation (R2 > 0.50) in the microbial communities were plotted on the NMDS ordination (Fig. 1). The following environmental variables were included in the analysis from the 0–10 cm soil layer: PMN, NH4-N and total mineral N (NH4-N + NO3-N), as reported in Hoogmoed et al. (2014), Table S2, site R1), soil moisture, total N, total C, C:N ratio, pH and total amount of PLFA.

The fungal-to-bacterial ratio (F:B ratio) was calculated using the PLFA marker 18:2ω6c, as an indicator of fungal biomass, and the sum of PLFA markers i15:0, a15:0, i16:0, i17:0, 17:0cy, 17:0 and 19:0cy as an indicators of total bacterial biomass (Frostegård and Bååth, 1996). Differences in F:B ratio, total PLFA and individual PLFAs were tested using the same nested-ANOVA design as described above. Pearson correlation analysis was used to test relationships between F:B ratio and C:N ratio, total C and total N, and between total fungal PLFA and C:N ratio, total N and total C, in the 0–10 cm soil layer.

3. Results

3.1. Nitrogen cycling

There were no significant differences (P < 0.05) in the δ15N values between tree types (N-fixers and non-N-fixers) for any of the sample types (leaves, species-specific litter, litter, 0–10 cm soil layer and 10–20 cm soil layer, Table 1). However, there were significant differences in δ15N value of the soil (both 0–10 and 10–20 cm soil layers) among tree species within tree type (P < 0.01, Table 1). Soil underneath A. dealbata had a significantly higher δ15N value compared with the other tree species in the 0–10 cm soil layer (P ≤ 0.03). In the 10–20 cm soil layer, δ15N under A. implexa was significantly higher compared with A. dealbata and E. camaldulensis (P ≤ 0.02).
E. polyanthemos represents the relative magnitude of explained variation and the direction indicates that of a positive increase. Table 2

Mean ± standard errors of δ15N (%e) in the leaves, species-specific litter, litter, 0–10 cm and 10–20 cm soil layer of the individual tree species. Different letters indicate a significant difference (P < 0.05) among tree species (N = 10, compare letters horizontally).

Table 1

Nested- and one-way-ANOVA results, comparing δ15N (%e) value. Nested ANOVAs were performed on tree types and tree species nested within tree type, separate for each sample type: leaves, species-specific litter, litter, soil 0–10 cm and soil 10–20 cm. One-way-ANOVA were performed among sample types, separate for each tree species: Acacia dealbata, A. implexa, Eucalyptus camaldulensis and E. polyanthemos. A significant (P < 0.05) difference is indicated by an asterisk (*).

3.2. Carbon

There were no differences in total C content between N-fixers and non-N-fixers of any of the sample types (Table 3). However, species within tree-type effects were found for species-specific litter, and both soil layers. Carbon content was higher in samples of A. dealbata compared with A. implexa. However post-hoc testing revealed that for species-specific litter, this difference was only marginal compared with A. implexa (P = 0.051). There was no difference in C content among samples of the non-N-fixing species.

There were no significant differences in C content between leaves and species-specific litter, for any of the tree species, but trees that were grown closer together. As these samples were collected at different times, the difference between the values should be treated as an indication of relative difference rather than an absolute difference.

Total N content was higher in leaves and litter of the N-fixing trees compared with the non-N-fixing trees. However, a species within tree-type effect was also found for leaves, litter and both soil layers (Table 3). In leaves and species-specific litter, total N content was significantly higher in E. camaldulensis compared with E. polyanthemos (P < 0.01). In the soil layers, total N content was significantly higher under A. dealbata compared with A. implexa (P < 0.01).

Comparing total N concentration among leaves, species-specific litter and litter (soil was not compared as the units differed, see Materials and methods) for each tree species, showed significant differences among sample types for all tree species (P < 0.01). Leave N content was significantly higher compared with litter N content, for all species (P ≤ 0.03). Species-specific litter N content was significantly lower compared with leaves for A. dealbata, A. implexa and E. polyanthemos (P ≤ 0.01), and significantly lower than litter underneath E. polyanthemos (P < 0.01).

The δ15N values of the different sample types were significantly different in all tree species (P < 0.01, Table 1). A. dealbata, A. implexa and E. camaldulensis showed no significant differences among leaves, species-specific litter and litter, but these were significantly lower compared with the δ15N value of the soil, in both depths (P < 0.01). E. polyanthemos showed a significantly lower δ15N value in the species-specific litter compared with the leaves and litter (P < 0.01).

Fig. 1. Non-metric multidimensional scaling (NMDS) ordination of range standardized PLFAs from surface (0–10 cm) soil samples under different tree species. ■: A. dealbata, ●: A. implexa, ▽: E. camaldulensis, ◀: E. polyanthemos. Stress value was 0.07. Figure contains only significant vectors that explain more than 50% of the variation. Vector length represents the relative magnitude of explained variation and the direction indicates that of a positive increase.
litter had a significantly lower C content compared with leaves for all species \((P < 0.05)\) except for *E. polyanthemos* \((P = 0.17)\).

### 3.3. C:N ratio

The C:N ratio was significantly lower in litter under N-fixing trees compared with the non-N-fixing trees \((P < 0.02, \text{Table 3})\). A species within tree type effect was found for all sample types except litter \((P = 0.73, \text{Table 3})\). The C:N ratio was significantly higher in *E. polyanthemos* compared with *E. camaldulensis* in all sample types \((P < 0.04)\) expect litter \((P = 0.64)\). Between the N-fixing trees, the C:N ratio was only significantly higher in the 0–10 cm soil layer under *A. implexa* compared with *A. dealbata* \((P < 0.01, \text{Table 4})\).

There were several differences the C:N ratio among sample types within each tree species. Leaves, species-specific litter, litter and soil had significantly different C:N ratios for *A. dealbata* and *A. implexa*. Soil under both non-N-fixing species had a significantly lower C:N ratio compared with the rest of the sample types \((P < 0.01)\). The C:N ratio in litter of *E. camaldulensis* was significantly higher compared with leaves \((P < 0.01)\), but litter and leaves did not differ significantly from species-specific litter \((P = 0.96)\). For *E. polyanthemos*, the C:N ratio of leaves and litter did not differ, but it was significantly higher in species-specific litter.

### 3.4. Soil microbial community

Soil microbial community composition, measured using PLFAs, did not differ significantly under N-fixing and non-N-fixing trees \((P = 0.08, \text{Fig. 1})\). Among all soil samples irrespective of tree type, total PLFA concentration explained most of the variation in microbial communities \((\text{R}^2 = 0.84, P < 0.01)\) followed by total C and total N content of the soil \((P < 0.01, \text{R}^2 = 0.54, \text{and} P < 0.01, \text{R}^2 = 0.51\) respectively). Comparing individual tree species, soil microbial

<table>
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<th>Sample type</th>
<th>Test</th>
<th>Total N</th>
<th>Total C</th>
<th>C:N ratio</th>
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<td></td>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
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<td>Leaves (%)</td>
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### Table 3

Nested-and one-way-ANOVA results, comparing total N (%), total C (%) and C:N ratio. One-way-ANOVA was performed on tree types and tree species nested within tree type, separate for each sample type: leaves, species-specific litter, litter, soil 0–10 cm and soil 10–20 cm. One-way-ANOVA was performed on sample types, separate for each tree species: *Acacia dealbata*, *A. implexa*, *Eucalyptus camaldulensis* and *E. polyanthemos*. A significant \((P < 0.05)\) difference is indicated by an asterisk (*). N.B. One way-analysis for sample type comparing Total N and Total C does not include contents in the 0–10 and 10–20 cm soil layers, as these could not be compared due to different measurement units.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Test</th>
<th>Total N</th>
<th>Total C</th>
<th>C:N ratio</th>
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<tr>
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<td>P</td>
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<td>Leaves (%)</td>
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<td>2.06</td>
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<tr>
<td>Soil 10–20 cm (t ha(^{-1}))</td>
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<td>2.06</td>
<td>0.18*</td>
<td>0.18</td>
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### Table 4

Means and standard errors of total N and total C and C:N ratio in the leaves, species-specific litter, litter, 0–10 cm and 10–20 cm soil layer of the individual tree species. Different letters indicate a significant difference \((P < 0.05)\) among tree species \((N = 10, \text{compare letters horizontally})\).

### Table 5

Nested-ANOVA results, comparing individual PLFAs, total PLFA and Fungal-bacterial ratio, between tree types and species nested within tree type. A significant \((P < 0.05)\) difference is indicated by an asterisk (*).
4. Discussion

There were indications that the N fixed by the N-fixing trees was redistributed and utilized by the non-N-fixing trees. Overall, there was a strong species effect within the N-fixing tree types, whereas the non-N-fixing species were more similar to each other. Characterization of the soil microbial community showed no differences among the N-fixers and the non-N-fixers, but some differences in communities under different tree species.

4.1. Nitrogen cycling

The total amount of N in the leaves and total litter of N-fixing trees was significantly higher than that of non-N-fixing trees (Table 3). In addition, total N content of the soil was significantly higher under A. implexa, compared with the other tree species (Table 4). Similarly, δ15N value was significantly higher in both soil layers under A. implexa compared with the other tree species (Table 2). Values of δ15N in soil and leaves found here were higher compared with other studies in southeastern Australia (2–3‰, Forrester et al., 2007; May and Attiwill, 2003) but are within the range of values reported globally (−7‰–15‰, Pörtle et al., 2007; Roggy et al., 1999; Shearer and Kohl, 1986). The other studies in Australia measured rotational plantations, whereas our site had been pasture for many decades prior to planting. Use of fertilizer and/or livestock manure in the previous pasture may have increased the initial soils’ δ15N values (Watzka et al., 2006).

Atmospheric N has a δ15N value of 0‰, whereas N pools in the soil have a higher δ15N value (between 5 and 6‰ at this site). Therefore, we expected to find a lower (diluted with atmospheric N) δ15N value in leaves and species-specific litter of the N-fixing species (and the soils below them) compared with the non-N-fixing species. However, we found no significant difference in δ15N values between the N-fixing and non-N-fixing trees (Table 1). Several possible mechanisms could explain this finding. As available N content was low in the soil at this site (Hoogmoed et al., 2014), the non-N-fixing tree species may be able to take up organic forms (‘unavailable’) of N from the litter layer (Averill and Finzi, 2011; Schimel and Bennett, 2004), which has a significantly lower δ15N value compared with the soil (Table 2). Another explanation could be that the N-fixing trees species are not, or only at low rates, fixing atmospheric N due to low available phosphorus (e.g., Battan et al., 2013).

The N-fixing and non-N-fixing trees would then share the same primary N source (e.g. bulk soil or organic N from the litter layer) and this could explain the similar values of δ15N in leaves and species-specific litter. However, it could also be that the non-N-fixing trees have access to the N that was fixed from the atmosphere by the N-fixing trees. To explore if the δ15N signature measured under both tree types was a result of fixed atmospheric N, we collected soil from a large patch of non-N-fixing trees assuming there was negligible influence of N-fixing trees. The δ15N value of this reference soil, was indeed higher (6.1‰) compared with when both tree types were growing close together. While these samples were collected at a later time, the results suggest that the lower value of δ15N in soil under both tree types is associated with similar access to fixed atmospheric N from the N-fixing trees.

Fixed atmospheric N can be redistributed in ecosystems and acquired by non-N-fixers through a number of possible pathways (Fig. 2). The most commonly suggested pathway (Fig. 2, pathway 1–2–3) is via the bulk soil pool that contains atmospheric N released from decomposed N-fixing litter and dead roots (e.g. Forrester et al., 2006; May and Attiwill, 2003; van Kessel et al. 1994). If the non-N-fixing trees at the site took up N primarily from the bulk soil, the δ15N value of their biomass would be more similar to the δ15N value of the soil, whereas the δ15N value of the N-fixers biomass would be more similar to that of the atmosphere (0‰, Shearer and Kohl, 1986). However, as the δ15N value in leaves of both N-fixers and non-N-fixers were similar and significantly lower than that of the soil, this suggests the non-N-fixing trees were not taking N solely from the bulk soil. We propose the non-N-fixing trees may have been able to access the fixed atmospheric N pool directly after it has been fixed by the N-fixers and before it is cycled through the tree biomass and to the soil through decomposing litter (Fig. 2, pathway 1–4). The roots of the N-fixers and non-N-fixers may be closely connected, physically or possibly by mycorrhizal fungi (He et al., 2003), so that N-fixers roots slough cells that are mineralized, the non-N-fixers rapid takes up the newly fixed mineral N. With such tight N cycling, these small-scale rhizosphere processes may not reduce the δ15N of the bulk soil. Similarly, in another study, in the first three years after planting, a significantly higher concentration of N was found in fine roots of Eucalyptus when grown together with Acacia than when grown in communities under A. dealbata were significantly different compared with the other three tree species (P ≤ 0.02).

Among the individual PLFAs, no significant differences were found between the N-fixing and non-N-fixing species. Several PLFAs showed a species within tree type effect. Generally, we found that most of the PLFAs were more abundant under A. dealbata than A. implexa and E. camaldulensis, whereas the amount of PLFA under E. polyanthemos was intermediate between them (Table 6). This trend was also reflected in the total amount of PLFA (Table 6). The fungal-to-bacterial (F:B) ratio did not differ between N-fixers and non-N-fixers (P = 0.64, Table 5), but some difference among the species was found. The F:B ratio was significantly lower under A. dealbata compared with A. implexa (P = 0.03), but neither differed significantly from the non-N-fixers. Ignoring tree type and species, there was a significant but very weak positive correlation (R² = 0.16, P = 0.01) between C:N ratio and F:B ratio of all samples, whereas no correlation was found between F:B ratio and total N (R² = −0.02, P = 0.57), or total C (R² = −0.03, P = 0.93). Total fungal PLFA was not correlated with C:N ratio (R² = −0.02, P = 0.68) but weakly positively correlated to total N (R² = 0.29, P < 0.01) and total C (R² = 0.34, P < 0.01).
monoculture, suggesting that N transfer was occurring belowground before litter was input to the soil (Khanna, 1997).

$^{15}$N natural abundance studies are complicated due to isotopic fractionation processes that occur during the (biochemical) cycling of N in the system (Robinson, 2001), which can mask differences in N sources among the tree species. Our experimental design allowed us to examine the pathways of fixed N but not to quantify the amount of fixed N in a mixed-species planting. Isotopic fractionation has been observed during biochemical N cycling processes in the soil (e.g. Templier et al., 2007), as well as during uptake by plants and allocation to different plant tissues (e.g. Gathumbi et al., 2002). However, it is often hypothesized that in soils with low available N, such as our planting (8.6 kg ha$^{-1}$ in the 0–20 cm soil layer, Hoogmoed et al., 2014), little to no fractionation during N uptake by plants takes place, as the plants will utilize all available N sources, irrespective of their isotopic composition (Högberg et al., 1996). Furthermore, we assume that possible differences in fractionation during translocation within the tree, among different tree species will be negligible.

The mycorrhizal status of a plant can also cause fractionation and affect the $\delta^{15}$N of its biomass (Högberg et al., 1996; Zeller et al., 2007). Acacia and Eucalyptus species form symbiotic relationships with both arbuscular (Adjoud-Sadadou and Halli-Hargas, 2000; Birhane et al., 2013; Chilvers et al., 1987) and ectomycorrhizal fungi (Chilvers et al., 1987; Diagne et al., 2013; Jumpponen et al., 2004). Many mycorrhizal fungi are not host-specific, and different tree species within a forest often have associations with the same species of mycorrhizal fungi (He et al., 2003). To our knowledge, the exact species of mycorrhizal fungi that may colonize the study tree species are unknown or whether they are host specific. The possibility of different rates of isotopic fractionation due to association with different mycorrhizal fungi species is possible.

Resorption of N from leaves during senescence was not significant for N-fixers and non-N-fixers based on comparison of concentration of total N in leaves and species-specific litter (Table 3). However, N concentration was significantly lower in species-specific litter than leaves in A. implexa only, indicating significant resorption of N. Values of $\delta^{15}$N were slightly lower in the species-specific litter compared with leaves for both N-fixing and non-N-fixing tree types suggesting some fractionation of $\delta^{15}$N during senescence. In contrast, in 10-year-old mixed-species plantation, a significantly lower $\delta^{15}$N in litter compared with leaves was found in Acacia mearnsii but not the co-occurring Eucalyptus globulus (Forrester et al., 2007). These species-specific responses underline the importance of measuring $\delta^{15}$N in leaves, litter and soil, when studying the cycling of fixed N in forest systems. Here, the $\delta^{15}$N value of the total litter, which contained litter inputs from various surrounding tree species, was not significantly different among the tree types or tree species, so the soil under each tree species received litter with the same $\delta^{15}$N value. The higher $\delta^{15}$N value of the total litter pool compared with the species-specific litter, although only significant for E. polyanthemos, may be because in addition to the leaves, the total litter pool includes twigs, bark and pods, which can have a higher $\delta^{15}$N value (Stähli et al., 2005; Templier et al., 2007) and different decomposition rates.

While not statistically significant, the $\delta^{15}$N value in the leaves among some of the tree species differed. The $\delta^{15}$N value of E. camaldulensis was higher (but not significantly, $P = 0.11$) than that of A. dealbata. This could indicate that the N pool accessed by E. camaldulensis contains less atmospherically-fixed nitrogen compared with A. dealbata. Interestingly, soils beneath A. dealbata had a significantly higher $\delta^{15}$N value compared with the other tree species, despite no differences in litter $\delta^{15}$N inputs. This could point to lower litter inputs into the soil under A. dealbata, which would cause less dilution of the soils $\delta^{15}$N value (i.e. a higher $\delta^{15}$N value compared with the other tree species). However, we found that soil underneath A. dealbata contained almost double the amount of total C and total N, compared with the other tree species, which indicates either higher litter and/or root inputs or lower uptake of these nutrients, and would cause a lower $\delta^{15}$N value under A. dealbata. One potential explanation is rapid nitrogen cycling under A. dealbata, which was found at this site (Hoogmoed et al., 2014) and is common under N-fixing trees (e.g. Boyle et al., 2008; Kaye et al., 2000). Many N-cycling processes discriminate against
the heavier $^{15}$N isotope and use the lighter $^{14}$N. This results in higher levels of $^{15}$N in the soil, as the $^{14}$N-enriched end products are more prone to leave the soil via plant uptake, leaching or volatilization (Pörtle et al., 2007; Templner et al., 2007).

Taken together, our results suggest that there was facilitation by N-fixers by supplying N to non-N-fixers in this relatively young tree planting. Likely pathways by which the non-N-fixing trees acquired this newly fixed N include through root interactions between the tree types or via utilization of organic forms of nitrogen from the litter layer, instead of indirectly from decomposed litter inputs, or decreased competition for soil available N. This means that even in a dry climate where litter decomposition is a slow process (as was the case at our study site, ca. 600 mm yr$^{-1}$), the inclusion of N-fixing trees in a mixed species forest may provide fast, short-term benefits in terms of N supply to non-N-fixing trees.

4.2. Microbial communities

Overall, the soil microbial community composition, as measured by PLFAs, was not significantly different under N-fixing and non-N-fixing trees ($P = 0.07$, Fig. 1). To our knowledge, few studies have compared microbial communities under N-fixing and non-N-fixing trees (Bini et al., 2013; Boyle et al., 2008) but some insights have been gained. Similarly, there was no difference in microbial community composition between the non-N-fixing *Pseudotsuga menziesii* (Douglas fir) and N-fixing *Alnus rubra* (red alder) trees in forest of north-western North America (Boyle et al. (2008) or in microbial biomass C or N under *Acacia mangium* compared with *Eucalyptus grandis* in a 20-month-old mixed-species planting in Brazil (Bini et al., 2013). However, there was significantly more dehydrogenase enzyme activity under *A. mangium* than *E. grandis*, suggesting some differences in the microbial community composition or activity underneath these N-fixing and non-N-fixing tree species. Differences in microbial community have been in ecosystems invaded by exotic N-fixers, which may have a larger effect on the microbial community than native species (e.g. Allison et al., 2006; Lorenzo et al., 2010; Remigi et al., 2008).

Regardless of tree species, any difference in microbial community among the soil samples from our site was most strongly correlated ($R^2 > 0.5$) with total amount of PLFA, followed by total C and total N in the 0–10 cm soil layer (Fig. 1). Increasing amounts of total C and N indicate increasing amounts of organic substrate for microbial growth (i.e., total amount of PLFA), which has been found in previous studies to be correlated with microbial biomass C (Bailey et al., 2002a; Potthoff et al., 2006).

Although no differences in microbial communities were found under N-fixer and non-N-fixers, some differences were found at the species level. The soil microbial community in soil under *A. dealbata* trees was significantly different to that of the other tree species ($P = 0.02$, Fig. 1). Furthermore, the amount of several specific PLFAs were significantly higher under *A. dealbata* (Table 6), contributing to the significantly higher total amount of PLFA underneath *A. dealbata* compared with the other tree species. The higher amounts of PLFA under *A. dealbata* further support the mechanism of higher nutrient cycling rates (i.e., higher microbial activity due to larger microbial population) which may accelerate $^{15}$N fractionation processes and explain the high $^{15}$N value of the soil under *A. dealbata*.

We hypothesized a decrease in the F:B ratio under N-fixing trees due to increased levels of soil N (Rachid et al., 2013), as N content of the soil is often negatively correlated with F:B ratio in forest ecosystems (Högberg et al., 2007). However, there was no difference in F:B ratio between N-fixing and non-N-fixing trees, or among individual tree species at the site. Regardless of tree type or species, no correlation was found between F:B ratio and total N ($R^2 < 0.01$), total C ($R^2 < 0.01$) or C:N ratio ($R^2 = 0.16$). A meta-analysis found a positive correlation between F:B and C:N, but only when the C:N ratio was higher than 18.4 (Waring et al., 2013). The C:N ratio in our soils was lower and the small range of C:N ratios (11.0–15.5) may not have provided sufficient spread in the data to detect a relationship between these factors. Total fungal PLFA was slightly better correlated to total C and total N ($P < 0.01$, $R^2 = 0.34$ and 0.29 respectively). While the amount of PLFA is a proxy for microbial biomass, and should only cautiously be considered for microbial activity, our results may correspond to Bailey et al. (2002b), who found that fungal activity rather than F:B ratio was positively correlated with soil C.

5. Conclusion

The results presented here suggest that even in a young planting in a dry environment (<800 mm yr$^{-1}$) where litter decomposition is slow, N-fixers may play an important role in facilitation of non-N-fixing trees. Possible pathways by which non-N-fixing trees could take up newly fixed N include direct below-ground exchange of fixed atmospheric N from N-fixing trees to the non-N-fixing trees, or via the uptake of organic forms of N from the litter layer, instead of via the slower process of decomposition of litter from N-fixers. While both N-fixing tree species appeared to fix atmospheric N, they were substantially different in terms of C and N addition to the soil, as well as microbial community composition. *A. dealbata* had significantly higher levels of soil C and N and a larger microbial mass compared with the other N fixer *A. implexa*. This shows that the effect of N-fixing tree species on soil carbon sequestration is species-specific, cannot be generalized and requires planting trials to determine if there will be benefits to carbon sequestration.

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