
UNIVERSITY OF TASMANIA

# The influence of plant species on soil processes in a Tasmanian grassland

by

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# Abstract

Many ecological processes are mediated by plant-soil interactions and feedbacks, thus the examination of interactions between the plant community and soil processes is crucial to further understanding how ecosystems function. Environmental change will influence how terrestrial ecosystem work but since plant communities are also likely to change in composition, it is possible that changes in plant community composition will have impacts on ecosystem processes larger than the impacts of the environmental changes themselves. Thus, this study investigated the effect of plant species on soil processes in order to understand the extent by which global change might affect soil processes via shifts in plant species composition. Using a native temperate grassland community co-dominated by a $C_4$ grass, *Themeda triandra,* and a $C_3$ grass, *Austrodanthonia caespitosa*, with a $C_3$ grass, *Austrostipa mollis*, as a sub-dominant species, this study examined the effect of plant species on soil microbial community composition, litter decomposition and nitrogen (N) transformation processes, as well as how plant species influences the impact of elevated carbon dioxide ($CO_2$) and warming on litter decomposition.

The co-occurring grass species differed in their associated microbial community composition examined by a molecular fingerprinting technique. The two dominant species, *Themeda triandra* and *Austrodanthonia caespitosa*, were more similar to each other in their bacterial and arbuscular mycorrhizal community composition than either was to the sub-dominant species, *Austrostipa mollis*, but not in their fungal community composition. Plant species not only affected microbial community composition but also microbial community function. Using a $^{15}N$ isotope tracing technique, coupled with quantitative molecular techniques and soil incubation assays, it was found that the co-occurring plant species differed substantially in N transformation rates as well as the abundance and activity of their associated microbial groups (ammonia-oxidising bacteria, ammonia-oxidising archaea and fungi) that are involved in N mineralisation and nitrification processes. Further examination also

revealed that autotrophic nitrification dominates nitrate production in this grassland, however, there was some indication that nitrification by fungi may also contribute substantially to nitrate production.

Litter decomposition was influenced by both physical and biochemical quality, as decomposition rates increased with decreasing litter particle sizes and were strongly correlated with litter quality measured by litter N content and C:N ratio. The effect of plant species on litter decomposition was therefore largely driven by differences in litter quality. One of the predicted impacts of global change is its effect on litter quality of individual species, with potentially significant ramifications for ecosystem nutrient cycling. Therefore, the effect of global change on litter decomposition was examined using a reciprocal incubation experiment in order to assess the relative importance of changes in litter quality and soil microbial community function in litter decomposition rates under simulated global change. The study utilised plant and soil materials from a long running free-air-$CO_2$-enrichment (FACE) facility established in the same grassland community, in which the community has been exposed to elevated $CO_2$ (550 ppm) and warming (2.0 °C) treatments since 2002. It was found that litter decomposition, assessed by C mineralization rates, was more strongly influenced by global change-induced alterations in soil community function than litter quality. Further, soil microbial communities exposed to both experimental warming and elevated $CO_2$ concentrations had a substantially increased ability to decompose added plant litter, regardless of that litter's source. Despite this, the consistent difference between $C_3$ and $C_4$ litter decomposition means that a shift in the relative abundance of $C_3$ and $C_4$ species is also likely to alter decomposition processes.

Therefore, these co-occurring grass species exert a very strong influence on both the soil microbial community and soil N and C cycling. Hence, any changes in the relative dominance of these species are likely to lead to relatively large and important alterations of nutrient cycling. Such fine-scale differences among largely similar and co-occurring species have not been demonstrated before. It is

therefore likely that such specific relationships also exist in many other systems, and that even slight changes in plant community composition, for whatever reasons, will lead to alterations of ecosystem function.

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# Chapter 1: Introduction

## INTRODUCTION

Constant interactions and feedbacks between plant and soil communities influence the rates and flows in which energy and nutrients move through an ecosystem thereby playing a crucial role in ecosystem function (Bardgett and Wardle 2010). The realisation that the global climate is changing has concentrated attention on improving understanding of ecosystem function, including plant-soil interactions. One of the key drivers of global change, the rising atmospheric $CO_2$ concentration, has been found to increase primary production (Ainsworth and Long 2005; Zak et al. 1993) and belowground carbon (C) input (Allard et al. 2006; Pendall et al. 2004b) while the resulting alteration to the organic input to the soil has been found to change the soil decomposer community (Drissner et al. 2007; Klironomos et al. 1997; Montealegre et al. 2002) thus affecting decomposition processes and nitrogen (N) mineralisation rates (de Graaff et al. 2006; Zak et al. 1993). Understanding the effects of these changes on ecosystem processes, particularly C and N dynamics, is imperative if we are to predict future ecosystem services (Reich et al. 2006; Zak et al. 1993). Nitrogen is required in relatively large amounts for plant growth, and thus N availability is particularly important in determining the response of plants to increased C availability in an atmosphere of elevated $CO_2$, as it potentially constrains the so-called "$CO_2$ fertilisation effect" on plant productivity (Luo et al. 2004; Reich et al. 2006). Therefore, it is undeniable that the understanding of ecosystem response to global change requires a thorough understanding of the interactions between plants and the soil community that regulate C and N dynamics within an ecosystem.

## ECOSYSTEM PROCESSES

*Carbon and nitrogen cycling*

The C and N cycles are tightly interconnected through plant-microbe interactions (Mary et al. 1996). Through photosynthesis, plants fix atmospheric $CO_2$ to produce organic molecules which serve as the energy base for most biological activity (Mikola et al. 2002). Organic molecules produced then enter the soil system directly through litter deposition both above- and belowground as well as root exudation or indirectly through animal excretion and death. The soil microbial community mediates the transformation of organic materials back into inorganic forms that are readily available for plant uptake. While it has been experimentally shown that plants can take up organic form of N (Jones et al. 2005; Nasholm et al. 2009; Paungfoo-Lonhienne et al. 2008), the availability of inorganic N is primarily dependent on the microbial mineralisation of organic N through ammonification and subsequent nitrification (Hamilton and Frank 2001).

It is generally accepted that increased C substrate availability increases microbial population size (Anderson and Domsch 1978) and thus activity, although substrate quantity alone does not necessarily correlate well with the capacity of the soil to supply nutrients through microbial mineralisation (Russell et al. 2004). The qualities of substrates, which reflects both biochemical composition and physical availability of the substrates, is also an important determinant of decomposition rates (Swift et al. 1979). The physical qualities of organic matter can affect mineralisation processes, as it determines the accessibility of the organic substrate to microbial degradation (Bremer et al. 1991). The biochemical qualities, such as C:N ratios and lignin contents, determines the availability of mineralised N through microbial immobilisation-mineralisation processes (Janssen 1996; Mary et al. 1996). Thus, C and N cycling is tightly interconnected through plant-microbe systems.

*Plant-microbe interactions*

The relationship between plants and the soil microbial community is highly complex. A plethora of studies exist in the literature demonstrating the effect of plant species on microbial community composition and structure (e.g. Grayston et al. 1998; Patra et al. 2006; Vandenkoornhuyse et al. 2003). The quality of litter inputs can directly translate into the quality of soil organic matter thereby affecting nutrient release (Enríquez et al. 1993; Hessen et al. 2004; Melillo et al. 1989) and population dynamics of the soil microbial community (Hu et al. 1999). Plant species can also affect the soil microbial community by secreting inhibitory or promotive compounds through root exudates (Blum et al. 2000; Fillery 2007; Hamilton and Frank 2001; Subbarao et al. 2007; Westover et al. 1997), thereby actively modifying the composition of soil microbial community in their rhizosphere. However, plant-microbe interactions are not only influenced by top-down processes. It is now well acknowledged that the soil microbial community can contribute to shaping the composition and structure of the plant community (Sanon et al. 2009; van der Heijden et al. 2003). Plant association with mycorrhizal fungi, for example, can enhance the dominance of a particular plant species (Hartnett and Wilson 1999) or facilitate plant species coexistence (Moora and Zobel 1996; van der Heijden et al. 2003), thus influencing the composition and diversity of the plant community. Thus, complex and often highly specific relationships exist between plants and soil microbes (Ayres et al. 2009), with their interactions and feedbacks ultimately shaping and determining the functioning of the whole ecosystem.

*Global change*

Superimposed on these complex interactions and feedbacks between plants and soil microbes are the effects of environmental influences that may strengthen or weaken the relationship between plants and the soil microbial community (Bezemer et al. 2006; Veresoglou et al. 2011), thereby altering ecosystem functioning. Global change is one such environmental influence that could

significantly alter both the plant community and the soil microbial community and therefore the interactions that determine the functioning of the ecosystem. Thus, the effect of global change drivers on various ecosystem processes has been extensively studied across a wide range of climatic regions, vegetation types and plant species (Blankinship et al. 2010; Hungate et al. 2009; Rustad et al. 2001; Wu et al. 2011). One global change driver, the increasing atmospheric $CO_2$ concentration, has been found to increase plant production (Ainsworth and Long 2005), photosynthetic rates (Zak et al. 1993), water use efficiency (Drake et al. 1997), rhizodeposition (Allard et al. 2006; Pendall et al. 2004b), and biomass C:N ratio (Cipollini et al. 1993), while concurrent increase in temperature often increases respiration, evapotranspiration, and the rates of biochemical reactions (Pendall et al. 2004a) as well as altering plant phenology (Badeck et al. 2004; Norby et al. 2003). Alterations to the input of organic matter to the soil caused by simulated global change affect soil community structure (Blankinship et al. 2010; Drissner et al. 2007; Klironomos et al. 1997; Montealegre et al. 2002) as well as C and N mineralisation rates (de Graaff et al. 2006; Dorrepaal et al. 2009; Zak et al. 1993), thereby altering decomposition processes and nutrient cycling of a community.

What is increasingly evident from many studies is that the responses of plants to elevated $CO_2$ and increased temperature are highly species-specific (Franck et al. 1997; Wand et al. 1999; West et al. 2005). Plant species can differ in the direction and in the degree of response (Kardol et al. 2010a; Moore et al. 1999), thus leading to the speculation that global change-induced shifts in plant species composition may have more pronounced effects than the changes themselves (Dukes and Field 2000). Indeed, changes in the quantity and quality of organic input to the soil can also occur through a shift in plant species composition (Chapin 2003), which is also one of the predicted consequences of global change (Williams et al. 2007). The importance of this indirect effect of global change was highlighted in a study by Langley and Megonigal (2010), who examined the effect of elevated $CO_2$ and N availability on a brackish wetland community. They found that the ecosystem response to global change was limited by global change-induced shifts in plant species composition. So far, few

studies have experimentally examined this indirect effect of global change on soil processes in order to quantify the relative importance of direct versus indirect effects of global change (e.g. Kardol et al. 2010b). However, it is clear that the concurrent changes in plant species composition need to be taken into account when examining global change impacts on ecosystem processes.

## RESEARCH OBJECTIVES

The aim of this study was to examine the effect of plant species composition on soil processes in a temperate grassland community dominated by native grass species. Temperate grasslands of south-eastern Australia have important economic and conservation values. While much of these "native pastures" are used for grazing animals, these grasslands also support rich and diverse native communities, containing large numbers of threatened genotypes, species and communities (Kirkpatrick et al. 1988). Thus, successful management of this vegetation types has an important national and global significance.

*Study site*

The study site is located in a species-rich temperate grassland in southeastern Tasmania (42° 42' S, 147° 16' E, 40 m asl) within the Australian Federal Department of Defense Pontville Small Arms Range Complex (Fig. 1.1). Vegetation is dominated by a perennial $C_4$ grass, *Themeda triandra* Forssk., which is the only widespread and abundant $C_4$ species in Tasmania and the sole $C_4$ species at the site, and the $C_3$ grass *Austrodanthonia caespitosa* (Gaudich.) H.P.Linder. Another $C_3$ grass *Austrostipa mollis* (R.Br.) S.W.L.Jacobs & J.Everett is present as a sub-dominant species (Fig. 1.2). Together these species average ~85% of aboveground production at the site. Introduced annual grasses and both native and introduced perennial herbaceous dicots are present in interspaces between grass tussocks. The list of vascular plant species composition at the site is found in Hovenden *et al.* (2006).

The soil is a clayey black vertisol formed on alluvium with mixed mineralogy, and is carbonate-free with neutral pH and low N content. The climate of the study area has warm, dry summers and cool, moist winters, with mean annual precipitation of 560 mm and mean annual temperature of 11.6°C. The study site has never been fertilized and has been grazed by stock only lightly.

**Fig. 1.1** A temperate grassland community dominated by *Themeda triandra* and *Austrodanthonia caespitosa* at Pontville, Tasmania

*Research questions*

Previous studies at this grassland community have found that simulated global change significantly affects C and N cycling (Hovenden et al. 2008a; Pendall et al. 2010). However, studies of plant population dynamics indicate that global change is also likely to shift plant species composition from

the currently $C_3/C_4$ co-dominated grassland towards a $C_4$ dominated grassland (Hovenden et al. 2008b; Williams et al. 2007). This raised the question of whether the observed global change effect on C and N cycling is a direct effect of climate change or an indirect effect through changes in plant species composition. Since the two dominant grass species differ in their photosynthetic pathway and biomass quality, both of which have been shown to respond differently to global change drivers and decomposition processes (Morgan et al. 2011; Ross et al. 2002; Wand et al. 1999), the changes in plant species composition may have a greater impact on nutrient cycling in this community than the changes in $CO_2$ and temperature themselves.

**Fig. 1.2** The two dominant species, $C_4$ grass *Themeda triandra* (left) and $C_3$ grass *Austrodanthonia caespitosa* (middle), and the sub-dominant $C_3$ grass *Austrostipa mollis* (right) at the study site

Therefore, this study focused on the effect of plant species on soil processes in order to investigate the extent of the indirect effect of global change on C and N cycling in this community. To achieve this, it is important to find out whether there are differences already existing between the co-occurring species in C and N cycling. Thus, this study examined the effect of plant species on microbial community composition, N transformations and litter decomposition. Specifically, the questions addressed in this thesis were:

- Are there any differences in the soil microbial community among the co-occurring grass species *Themeda*, *Austrodanthonia* and *Austrostipa*? (Chapter 2)
- Are there any differences in N transformation rates in soil associated with each of the co-occurring grass species *Themeda*, *Austrodanthonia* and *Austrostipa*? (Chapter 3)
- Are there any differences in the relative contribution of different microbial groups to nitrification among the co-occurring grass species *Themeda*, *Austrodanthonia* and *Austrostipa*? (Chapter 4)
- Do litter decomposition rates differ as a function of litter species, litter quality, litter types or litter particle size? (Chapter 5)
- What is the relative importance of global change-induced changes in litter quality and that of soil community function in litter decomposition under simulated global change? (Chapter 6)

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# Chapter 2

## Plant species effect on soil microbial community

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# Chapter 2: Plant species effect on soil microbial community

## INTRODUCTION

Plants have been known to modify their surrounding soil environment through root exudation or rhizodeposition which influence chemical and biological properties of the soil (Hartmann et al. 2009; Jones et al. 2009). Plant species may differ in the degree and manner in which they affect soil microbial community composition due to differences in the timing, quantity and quality of organic matter they provide to the belowground community (Smalla et al. 2001). Shifts in soil microbial community composition have been frequently documented following changes in plant community composition resulting from agricultural practices (Larkin 2003), land use (Carson et al. 2010; Hedlund 2002), invasive species (Batten et al. 2006; Wolfe and Klironomos 2005) and succession (Williamson et al. 2005), with its consequence often being linked to altered nutrient cycling. There is also strong evidence that the soil microbial community, most especially the mycorrhizal community, can influence the plant function and hence community dynamics (Hartnett and Wilson 1999; van der Heijden and Horton 2009). Specifically, plant-microbe feedbacks have been identified as one of key drivers of plant community composition (Bever 2003; Reynolds et al. 2003; Sanon et al. 2009; van der Heijden et al. 1998). Negative feedbacks (e.g. accumulation of harmful pathogens) between plant species and soil microbes can help maintain higher plant diversity or species richness, while positive feedbacks (e.g. accumulation of beneficial bacteria or symbionts) can lead to increased dominance of a particular species in a system (Bever 2003; Reynolds et al. 2003). The functional characteristics of dominant plant species can in turn exert strong selective pressures on the soil microbial community and hence soil nutrient cycling of a whole community (Bardgett et al. 1999). Therefore, understanding the relationship between plant species and the soil microbial community

is crucial in order to predict the consequences of alterations in plant community composition on nutrient cycling and ecosystem processes.

The composition of the soil microbial community differs between different vegetation types (Cookson et al. 2007; Zechmeister-Boltenstern et al. 2011) and the relationship between plant species and the soil microbial community is often found to be more specific in specific environments (Bezemer et al. 2006), especially those with low nutrient availability (Bardgett et al. 1999; Veresoglou et al. 2011). The influence of soil nutrient status on plant-microbe relations was highlighted in the study by Veresoglou *et al.* (2011) who found that the application of fertilisation masked the plant species effect on soil microbial community composition in a temperate upland grassland in northern Greece. The tight coupling observed between the dominant grass species and the soil community in intermediate and low-fertility temperate grasslands in North Wales (Bardgett et al. 1999) also provides evidence that the interaction between plant species and the soil microbial community can play a central role in nutrient cycling and ecosystem functioning of the whole community (Paterson 2003).

The current changes in global atmospheric carbon dioxide concentration and temperature has posed a threat to plant communities around the world with many studies suggesting shifts in plant species composition under global change (Harte and Shaw 1995; Kardol et al. 2010b; Shaver et al. 2000). While global change drivers have been demonstrated to alter key ecosystem processes such as nutrient cycling across various vegetation types (Rustad et al. 2001), some studies have indicated a greater effect on nutrient cycling as a result of shifts in plant species composition under global change (Dukes and Field 2000; Shaw and Harte 2001). Since changes in plant community composition, such as the invasion of exotic species, can alter the composition (Batten et al. 2006; Kourtev et al. 2002) and function (Ehrenfeld 2003) of the soil microbial community, it is also possible that changes in soil function could occur even due to alterations of dominance patterns of currently co-occurring plant species. Such a phenomenon has been demonstrated for a temperate native

grassland in southeastern Australia, in which the plant community is dominated by the warm season $C_4$ species *Themeda triandra* and the cool season $C_3$ species *Austrodanthonia caespitosa* with another $C_3$ species *Austrostipa mollis* as a common sub-dominant species. While these co-occurring species share the same ecological niche, they differed in their effects on soil processes such that there were substantial differences in nitrogen availability, autotrophic and heterotrophic nitrification rates and litter decomposition rates among the species (Osanai et al. 2012). It is unknown, however, whether the differences in soil processes were a result of differences in the microbial community composition among these co-occurring grass species. Thus, the aim of this study was to examine the effect of co-occurring grass species on the community composition of bacteria, fungi and arbuscular mycorrhizal (AM) fungi using a terminal restriction fragment length polymorphism (T-RFLP) analysis. T-RFLP analysis has high throughput capabilities allowing the extensive examination of large-scale community samples often required for ecological studies (Marsh 2005) and has been successfully used to describe the soil bacterial community (Fierer and Jackson 2006; Kuske et al. 2002; Osborne et al. 2011), fungal community (Okubo and Sugiyama 2009; Robinson et al. 2009) and AM fungal community (Hausmann and Hawkes 2009; Martinez-Garcia and Pugnaire 2011). Since different microbial groups have been shown to differ in the way they interact with plants (Costa et al. 2006; Mitchell et al. 2012), it is likely that there would be some differences in the strength of the plant species effect on their composition. This study also examined soil characteristics to see if any environmental variables correlated with the pattern observed in each of the three microbial community groups, as they also have been shown to respond differently to various environmental factors (Edel-Hermann et al. 2008).

## MATERIALS AND METHODS

*Sampling and chemical analysis of soil*

Soil samples were collected in the late spring of 2009 when the vegetative growth of both $C_3$ and $C_4$ species was apparent by taking 30 cores, each 15 mm in diameter, from the top 5 cm of the soil directly beneath each of the three grass species. These grass species have a caespitose habit with each plant forming a spatially distinct tussock clearly separated from all others, thus soil sampling was done with a maximum of one core being taken from any individual tussock, giving 90 core samples in total. Soil samples were transported to the laboratory on ice and stored at −20 °C until processed. Soil samples were homogenised and sub-samples were taken for determination of C and N concentration in a Perkin Elmer 2400 Series II Elemental Analyser (Perkin Elmer Australia). Soil ammonium and nitrate contents were determined using potassium chloride (KCl) extraction method. Briefly, sub-samples of 3 g fresh soil was mixed with 2 M KCl for an hour, followed by centrifugation at 13400 x g for 3 min. The concentrations of ammonium and nitrate of the extracts were determined colourimetrically using a SmartChem$^{TM}$ 200 Discrete Analyser (Westco). Soil pH measurements were taken by a pH meter (Eutech pH700, Thermo Fisher Scientific) after mixing 3 g of fresh soil with 15 ml of de-ionised water for a minute.

*Microbial DNA extraction and PCR*

DNA was extracted using a MoBio Powersoil kit (MoBio, CA, USA) according to the manufacturer's instructions with the modifications of 0.5 g of soil and 0.2 g of glass beads (100 mesh size) added to rupture microbial cells by bead beating for 3 min. DNA quantity was determined using a Nanodrop spectrophometer (Thermoscientific, DA, USA). PCRs were carried out to amplify the 16S rRNA region from bacteria as well as the ITS rRNA region from fungi and AM fungi from all soil samples. The group specific primers selected to amplify these regions included: 63f (AGGCCTAACACATGCAAGTC) (Marchesi et al. 1998) / 1087r (CTCGTTGCGGGACTTACCCC) (Hauben et al. 1997) for bacteria and

ITS1F (CTTGGTCATTTAGAGGAAG) / ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990) for fungi. As for AM fungi, a nested PCRs were carried out using primers AML1 (ATCAACTTTCGATGGTAGGATAGA) / AML2 (GAACCCAAACACTTTGGTTTCC) (Lee et al. 2008) on the amplified products of eukaryote specific primers NS1 (GTAGTCATATGCTTGTCTC) / NS4 (CTTCCGTCAATTCCTTTAAG) (White et al. 1990). The forward primer from each pair had a fluorescent label attached to the 5' end (6-FAM for ITS1F and 63f, and 5-HEX for AML1). Amplifications were carried out in 50 µl reaction volumes containing 30-50 ng of template DNA; 20 pmol of each of the primers; 1.5 mM $MgCl_2$; 200 µM of each of dATP, dCTP, dGTP and dTTP; 2.0 µl bovine serum albumin (BSA) (10 mg/ ml); 5.0 µl 10x reaction buffer; and 1.5 unit (1.5 µl) of Taq DNA polymerase (Bioline). All amplifications were performed on a PE Applied Biosystems GeneAmp® PCR System 9700 (Applied Biosystems) with an initial 5 min stage at 95$^{o}$C followed by 30 cycles of 95$^{o}$C for 30 s, 55$^{o}$C for 30 s and 72$^{o}$C for 1 min, followed by a final extension at 72$^{o}$C for 10 min for bacterial and fungal samples. For AM fungal samples, amplifications were first conducted with NS1 / NS4 primer set with an initial 3 min stage at 94$^{o}$C followed by 30 cycles of 94$^{o}$C for 30 s, 40$^{o}$C for one min and 72$^{o}$C for one min, followed by a final extension at 72$^{o}$C for 10 min. The amplification products were diluted to 1/100 (with MilliQ water) and used as a template for amplification with AML1 / AML2 primer set with an initial 3 min stage at 94$^{o}$C followed by 30 cycles of 94$^{o}$C for 1 min, 50$^{o}$C for 1 min and 72$^{o}$C for 1 min, followed by a final extension at 72$^{o}$C for 10 min. Amplification products were electrophoresed in 1.0 % (w/v) agarose gels, stained with SYBR Safe (Invitrogen) and visualised under UV light. The fluorescently labelled PCR products were purified using the QIAquick PCR Clean up Kit (QIAGEN) as per manufacturer's instructions.

*T-RFLP analyses for soil microbial communities*

The broad microbial groups of bacteria and fungi as well as a specific fungal group, the AM fungi were examined using a molecular fingerprinting technique. Microbial community fingerprint patterns based on 16S rRNA region for bacteria and the ITS rRNA region for fungi and AM fungi were

generated by T-RFLP analyses. Aliquots of purified DNA of each bacterial, fungal and mycorrhizal PCR products were digested using restriction enzyme *Msp*I. Each 20 µl reaction contained 0.5 µl restriction enzyme, 2.0 µl buffer, 0.2 µl BSA and ~150 ng of purified PCR product and was incubated for 4 h at 37°C. Digest products were desalted with ethanol prior to T-RFLP analysis. In 96-well plates, 2.0 µl of each of the desalted digest products were added to individual wells containing 0.05 µl of 500-LIZ size standard (Applied Biosystems) and 8.95 µl formamide (Applied Biosystems). Digested amplicons were separated on an ABI 3730 sequencer and terminal restriction fragment (T-RF) lengths were calculated with Genemapper® software (Applied Biosystems) where peak heights below 50 fluorescent units and peaks outside the size standard range were ignored. Based on the GeneMapper® output three binary tables (bacteria, fungi and AM fungi) were constructed to indicate only the presence or absence of T-RFs between 50 and 500 base pairs (rounded to the nearest base) and not their peak heights (fluorescence intensity) or peak areas. This binary information was used for the construction of bacterial, fungal and AM fungal community composition. It is recognised that plant mitochondria and plastids are descendants of bacterial endosymbionts, therefore it would be difficult to distinguish a plant derived peak from a bacterial peak based in T-RF length alone. However, a test of the specificity of 16s rRNA primers used for bacteria against the NCBI blastn suite revealed that they hit predominantly bacteria. Therefore, the majority of T-RFs will be of soil microbial origin because the primers are based on highly conserved regions of the bacterial small sub-unit, fungal ITS and AM fungal small sub-unit.

*Statistical analyses*

T-RFLP binary data was analysed by one-way analysis of variance using general linear model procedures in the SAS statistical software package (SAS Institute Inc. 2003) to determine if plant species affected the number of bacteria, fungal and AM fungal T-RFs and soil chemical properties. Ryan-Einot-Gabriel-Welsch post hoc multiple comparison test was used where ANOVA indicated significant differences among the species ($P<0.05$, Day and Quinn 1989). While the fungal

community composition also include some AM fungal community (Bellemain et al. 2010), the majority will be from Ascomycota and Basidomycota, therefore, it is unlikely that Glomeromycota data would skew the total fungal response studied with primers ITS1F and ITS4. Therefore, in this study, the fungal community composition and AM fungal community composition were treated as independent variables. The composition of each of the bacterial, fungal and AM fungal communities and the composition of the three groups combined were assessed using multivariate data analyses using Primer 6 (Clarke and Warwick 2001). Ordination plots based on the T-RFLP binary information from each of the three datasets plus the combined dataset and soil chemical information were constructed using non-parametric multidimensional scaling (nMDS) as an unconstrained ordination and canonical analysis of principal coordinates (CAP) discriminant analysis as a constrained ordination that maximises the differences among *a priori* groups (Anderson and Willis 2003). Unconstrained and constrained ordination methods were used to capture additional patterns in the multivariate data cloud that may not be detected in one method only (Anderson and Willis 2003; Ratkowsky 2007). Both analyses were performed with Bray-Curtis (Sørensen) dissimilarity measure. As the ordination plots for the AM fungal T-RFLP dataset showed clear differences in the spread of the community composition among the plant species, the AM fungal dataset was also analysed by PERMDISP, which is a distance-based test for homogeneity of multivariate dispersions among groups (Anderson 2004), to examine for the statistical significance. To assess the significance of differences in community composition among the plant species, T-RFLP binary data were compared using analysis of similarities (ANOSIM), which compares within group to between group variances using *R* statistics (Clarke and Warwick 2001; Rees et al. 2004). The *R* statistic is based on the ranks of dissimilarities and is calculated using a random permutation procedure, where the mean rank of between group dissimilarities is compared to the mean rank of within group dissimilarities. The value of R ranges from −1.0 to +1.0 with a value of 0 indicating no difference among groups. The significance is determined by comparing the observed *R* value to the distribution of *R* under the null hypothesis of no difference between groups ($\alpha$=0.05). Multiple comparisons were performed where

ANOSIM indicated the significance of the overall treatment effect (indicated as global *R*) on microbial community composition.

The relationships between the patterns observed in microbial community composition and those observed for soil chemical properties were assessed using BIOENV procedures in Primer 6, using the Spearman rank correlation coefficient (ρ). BIOENV allows the exploration of environmental variables that best correlate to the dissimilarity patterns observed in the biological community by calculating a rank correlation between the ranks of the elements of the Bray-Curtis matrix (biotic data) and the ranks of the elements of the Euclidean matrix (abiotic data) (Clarke and Ainsworth 1993). The statistical significance of the BIOENV results were tested using the RELATE procedure which compares the global ρ to the distribution of ρ under the null hypothesis generated by 999 random permutations.

**RESULTS**

Plant species had a significant impact on both T-RF numbers ($P>0.0001$) and composition ($P=0.0001$), indicating that the individual plant species had a significant effect on the soil microbial community composition. Overall microbial community composition was strongly affected by plant species (Fig. 2.1a, b). The ANOSIM procedure that allows the significance testing of treatment groups confirmed this strong species effect with a global *R* statistic of 0.165 ($P=0.0001$), with pairwise comparisons of the plant species demonstrating the greatest difference between *Austrostipa* and *Austrodanthonia* ($R=0.225$, $P=0.0001$), followed by *Austrostipa* and *Themeda* ($R=0.198$, $P=0.0001$) and *Themeda* and *Austrodanthonia* ($R=0.080$, $P=0.004$). While each of the three tested microbial groups contributed to the overall differences in the soil community among plant species, the degree of variation within each of the microbial groups differed.

*Bacterial community composition*

The total number of bacterial T-RFs was the highest in *Themeda* (120 T-RFs) followed by *Austrodanthonia* (105 T-RFs) and *Austrostipa* (94 T-RFs). The percentage of T-RFs unique to a single plant species was also highest in *Themeda* which had 17.0% unique T-RFs, followed by *Austrodanthonia* with 13.1% and *Austrostipa*, 5.9%. Thus, approximately a third of all T-RFs were unique to a single plant species, whereas, 44.4% of T-RFs were ubiquitous (i.e. found in all three species). The average number of bacterial T-RFs differed significantly among plant species ($F_{2,87}$=4.17, $P$=0.02) with *Austrostipa* (mean=30.1±1.0) having fewer T-RFs than either *Austrodanthonia* (mean=34.6±0.9) or *Themeda* (mean=34.2±1.1). The plant species identity also had a significant effect on bacterial community composition (Fig. 2.2a, b). In the unconstrained ordination plot (nMDS), the bacterial communities associated with *Austrodanthonia* and *Austrostipa* tended to cluster according to their associated grass species, while the bacterial community associated with *Themeda* showed a substantial overlap with the communities associated with the other two species (Fig. 2.2a). The effect of plant species on the bacterial community composition was even more pronounced in the constrained ordination plot (CAP), where *Austrostipa* clearly separated from the two dominant species along the first axis (Fig. 2.2b). The ANOSIM results corresponded to this significant effect of plant species on clustering of bacterial communities (global $R$=0.102, $P$=0.0001) indicating that the bacterial communities differed by approximately 10% over all samples. Pairwise comparisons of the plant species demonstrated that the greatest difference in the bacterial community occurred between the two $C_3$-species, *Austrostipa* and *Austrodanthonia* ($R$=0.136, $P$=0.0001). The bacterial community associated with *Austrostipa* was also significantly different to that associated with the $C_4$ grass *Themeda* ($R$=0.121, $P$=0.0002), however, the difference between the bacterial communities associated with *Themeda* and *Austrodanthonia* was less pronounced, despite being statistically separated ($R$=0.051, $P$=0.01). The bacterial community associated with *Themeda* showed much wider spread on the ordination plots and therefore lower specificity of community, despite the high number of unique T-RFs. The bacterial communities

associated with *Austrodanthonia* and *Austrostipa* on the other hand showed much smaller spread, thus more specific in their community composition.

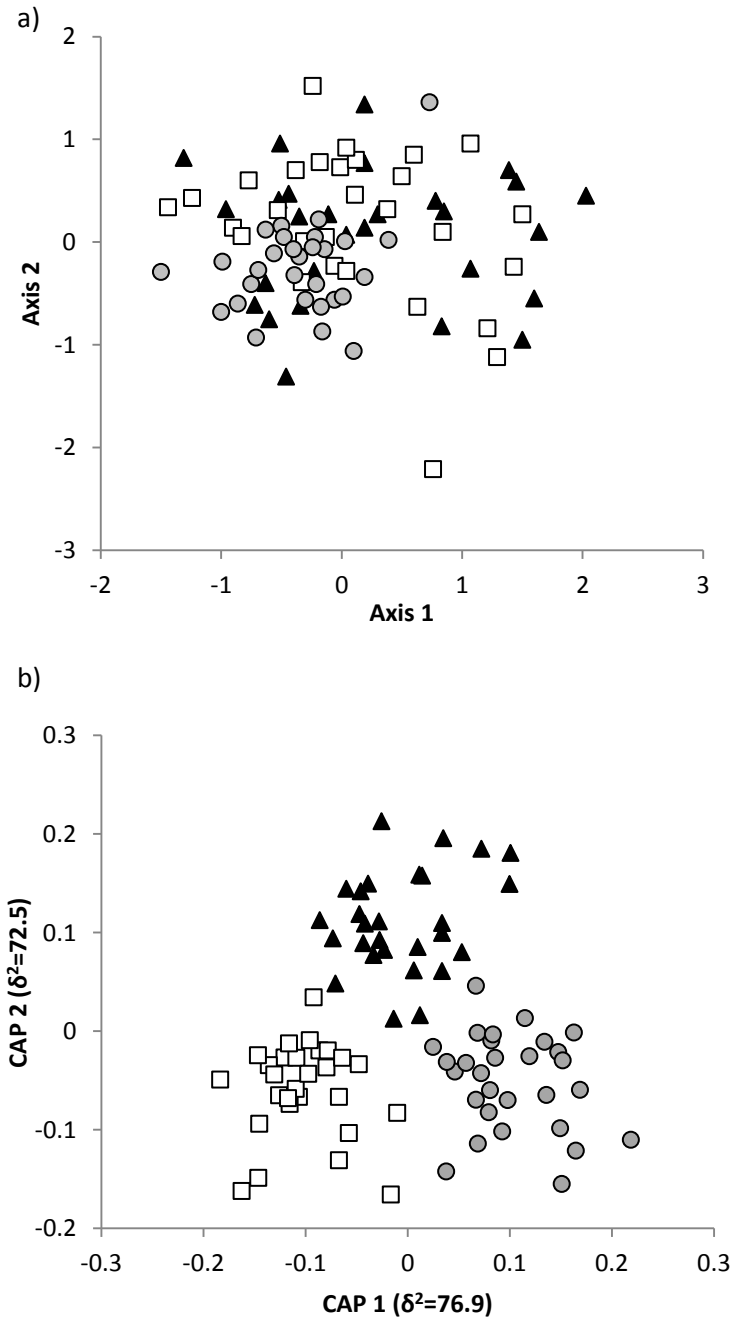


**Fig. 2.1** Ordination plots from non-parametric multidimensional scaling analysis (a) and canonical discriminant analysis (b) of overall soil microbial community composition using the combined bacterial, fungal and AM fungal T-RFLP data from soil samples associated with *Themeda* (▲), *Austrodanthonia* (□) and *Austrostipa* (●). For the ordination from canonical discriminant analysis, values on axes 1 and 2 are squared eigenvalues ($\delta^2$).

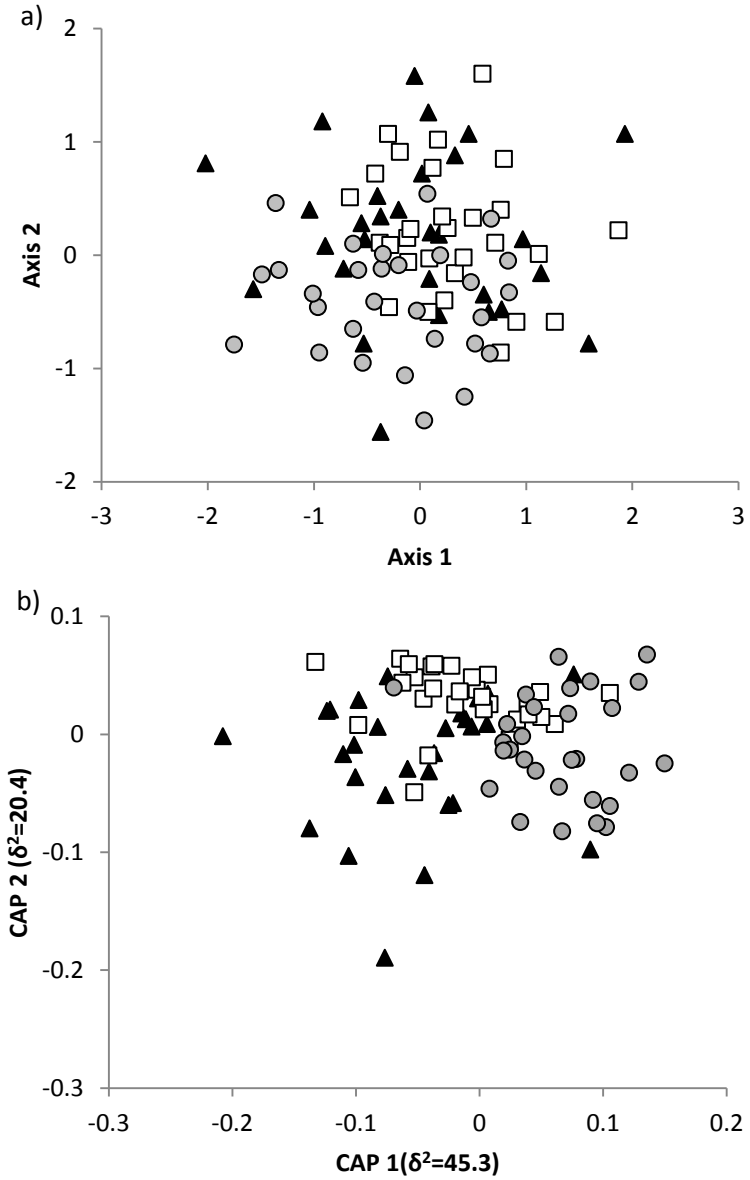


**Fig. 2.2** Ordination plots from non-parametric multidimensional scaling analysis (a) and canonical discriminant analysis (b) of soil bacterial community composition using bacterial T-RFLP data from soil samples associated with *Themeda* (▲), *Austrodanthonia* (□) and *Austrostipa* (●). For the ordination from canonical discriminant analysis, values on axes 1 and 2 are squared eigenvalues ($\delta^2$).

*Fungal community composition*

The impact of plant species on fungal T-RFs was less pronounced than for bacterial communities in the unconstrained ordination plot (Fig. 2.3a) but more pronounced in the constrained ordination plot (Fig. 2.3b) . Fungal T-RF numbers were much higher than those of bacteria with a mean fungal

T-RF number of 61.3±2.0 as compared to a mean bacterial T-RF number of 33.2±0.6. Soil associated with *Austrodanthonia* yielded 390 fungal T-RFs in total with a mean of 65.9±3.8, while soil from *Themeda* yielded 360 with a mean of 58.3±4.0 and *Austrostipa* 358 with 59.6±2.5 on average, although these means did not differ significantly ($F_{2,86}$=1.37, $P$=0.3). The percentage of unique T-RFs as a function of total T-RFs was much lower than with bacterial T-RFs, with the highest in *Austrodanthonia* (6.8%) followed by *Austrostipa* (4.2%) and *Themeda* (4.0%). The percentage of ubiquitous T-RFs on the other hand was much higher than with bacterial T-RFs with 58.2% of T-RFs occurring in all plant species. Due to this high abundance of ubiquitous T-RFs, fungal community composition appeared less strongly affected by plant species than was bacterial community composition, as indicated by a general lack of structure in the nMDS plot (Fig. 2.3a). However, the constrained ordination plot showed clear separations among all the species (Fig. 2.3b). The ANOSIM results mirrored the results of the CAP analysis showing a statistically significant plant species effect (global $R$=0.114, $P$=0.0001), with the greatest difference in fungal community composition observed between *Austrostipa* and *Themeda* ($R$=0.121, $P$=0.0002), followed by *Austrostipa* and *Austrodanthonia* ($R$=0.120, $P$=0.0001) and *Themeda* and *Austrodanthonia* ($R$=0.103, $P$=0.001). Therefore, the lack of plant species effect on nMDS plot is likely to be caused by the high abundance of ubiquitous T-RFs combined with the low abundance of unique T-RFs which overshadowed the effect of plant species on fungal community composition.

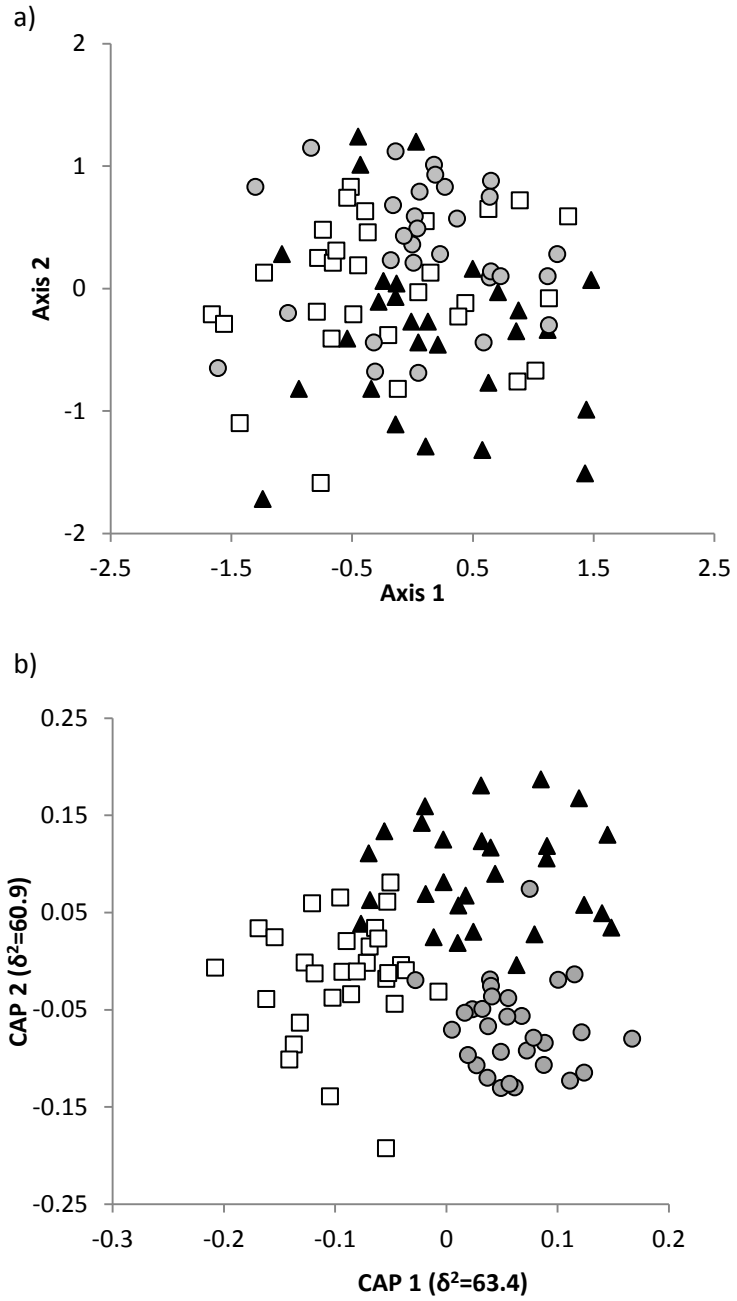


**Fig. 2.3** Ordination plots from non-parametric multidimensional scaling analysis (a) and canonical discriminant analysis (b) of soil fungal community composition using fungal T-RFLP data from soil samples associated with *Themeda* (▲), *Austrodanthonia* (□) and *Austrostipa* (●). For the ordination from canonical discriminant analysis, values on axes 1 and 2 are squared eigenvalues ($\delta^2$).

*AM fungal community composition*

The manner in which plant species affected AM fungal community composition was quite different to that with the other two microbial groups. The total number of AM fungal T-RFs was the highest in *Austrostipa*, followed by *Themeda* and *Austrodanthonia* (150, 143 and 135, respectively). The percentage of unique T-RFs did not follow the same pattern, with the highest in *Austrostipa* (13.2%) followed by *Austrodanthonia* (9.6%) and *Themeda* (8.1%), with 48.2% of the total T-RFs being ubiquitous. On average, *Austrostipa* had a higher AM fungal T-RFs (mean=42.8±2.1, $F_{2,87}$=5.36, *P*=0.01) than both *Themeda* (mean=34.4±2.4) and *Austrodanthonia* (mean=32.6±2.5). The ordination plots for AM fungal community showed a different pattern to those of bacterial and fungal communities (Fig. 2.4a, b). The AM fungal communities associated with the grass species showed a clear effect of plant species with the community associated with *Austrostipa* showing a much tighter clustering compared to those associated with *Austrodanthonia* and *Themeda* in both ordination plots. This plant species effect on AM fungal community was also evident in the ANOSIM results (global *R*=0.100, *P*=0.0001), where the community associated with *Austrostipa* was significantly different from that of *Themeda* (*R*=0.157, *P*=0.0001) and that of *Austrodanthonia* (*R*=0.145, *P*=0.0001), while no difference was observed between the communities associated with *Themeda* and *Austrodanthonia* (*R*=0.010, *P*=0.2). Due to the obvious differences in the spread of AM fungal community composition amongst the plant species, the difference in the dispersion was tested using a PERMDISP and found that *Austrostipa* indeed had a statistically tighter community composition than the other two species (*P*<0.0001). Thus, the AM fungal community composition was highly uniform across *Austrostipa* samples, despite having the highest number of total, average and unique T-RFs. *Austrodanthonia*-associated soils, on the other hand, did not differ from *Themeda*-associated soils in their AM fungal community composition, despite having a relatively high proportion of unique T-RFs. Therefore, it is likely that the occurrence of these unique T-RFs within *Austrodanthonia*-soils was rather rare, thus contributing to the high variability of *Austrodanthonia* soil samples in the ordination plot.

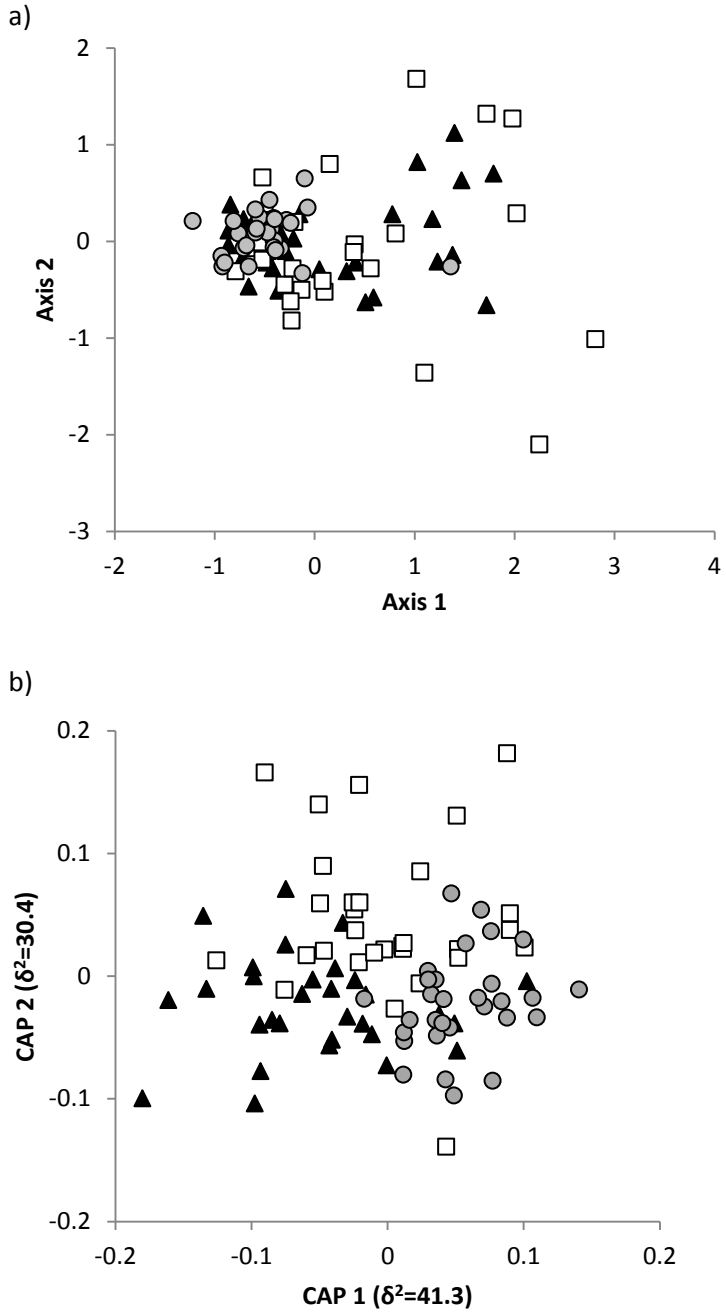


**Fig. 2.4** Ordination plots from non-parametric multidimensional scaling analysis (a) and canonical discriminant analysis (b) of soil AM fungal community composition using mycorrhizal T-RFLP data from soil samples associated with *Themeda* (▲), *Austrodanthonia* (□) and *Austrostipa* (●). For the ordination from canonical discriminant analysis, values on axes 1 and 2 are squared eigenvalues ($\delta^2$).

*Soil chemical properties*

The plant species identity had a significant influence on their surrounding soil chemical properties with six out of eight variables tested differing significantly among the species, despite growing within a close proximity to one another (Table 2.1). While the dominant species (i.e. *Themeda* and *Austrodanthonia*) differed significantly in the amount of soil nitrate, C and N contents, they did not differ in soil pH and C:N ratio. *Austrostipa* did not differ in soil C and N contents from either of the species, and the amount of nitrate differed only from that of *Themeda*. *Austrostipa* however did have a lower soil pH and C:N ratio than the dominant species. The ordinations of soil chemical properties associated with the plant species showed a relatively smaller distribution amongst *Themeda* soils compared to those of *Austrodanthonia* and *Austrostipa* (Fig. 2.5a, b). This was reflected in the ANOSIM results that indicated a significant difference in the soil chemistry amongst the plant species (global $R$=0.06, $P$=0.001) with *Themeda* soils showing greater differences from both *Austrostipa* ($R$=0.08, $P$=0.01) and *Austrodanthonia* ($R$=0.06, $P$=0.02) compared to the difference between *Austrodanthonia* and *Austrostipa* ($R$=0.05, $P$=0.03). The pattern observed in the soil chemical characteristics was compared against the patterns observed in the microbial community composition using the BIOENV procedures. The ordination plots of the microbial community composition showed a relatively strong separation of the communities associated with *Austrostipa* from that of *Austrodanthonia* in particular, and the environmental differences between those species were observed in soil pH and C:N ratios. Soil pH and C:N patterns however did not correlate with the patterns observed in microbial community composition, assessed by the BIOENV procedures, nor did other soil characteristics ($\rho$=0.14, $P$=0.12). Thus, it appears that the apparent influence of plant species on the soil microbial community composition was not related to differences in soil chemistry but rather to a direct influence of the plants themselves.

**Table 2.1** Chemical properties of soils associated with the co-occurring grass species analysed with analysis of variance (ANOVA). F-ratios and probabilities (F>P) with significant effects are shown in bold (P<0.05). The unit for the amount of ammonium, nitrate and mineral nitrogen is mg N/g soil.

| | *Themeda* | *Austrodanthonia* | *Austrostipa* | $F_{2,89}$ | *P* |
|---|---|---|---|---|---|
| Soil pH | 5.90 ±0.03 a | 5.86 ±0.04 a | 5.73 ±0.03 b | 4.92 | **0.01** |
| Total carbon (%) | 2.96 ±0.12 b | 3.41 ±0.12 a | 3.19 ±1.13 ab | 3.32 | **0.04** |
| Total nitrogen (%) | 0.21 ±0.01 b | 0.24 ±0.01 a | 0.24 ±0.01 ab | 3.8 | **0.03** |
| Carbon:nitrogen | 13.9 ±0.11 a | 13.8 ±0.11 a | 13.5 ±0.10 b | 4.15 | **0.02** |
| Ammonium | 0.024 ±0.007 | 0.041 ±0.008 | 0.027 ±0.004 | 2.39 | 0.1 |
| Nitrate | 0.009 ±0.001 b | 0.013 ±0.001 a | 0.016 ±0.001 a | 8.2 | **0.001** |
| Mineral nitrogen | 0.034 ±0.007 | 0.053 ±0.007 | 0.042 ±0.005 | 2.95 | 0.06 |
| Ammonium:nitrate | 3.92 ±1.24 | 4.07 ±1.03 | 2.09 ±0.32 | 3.03 | **0.05** |

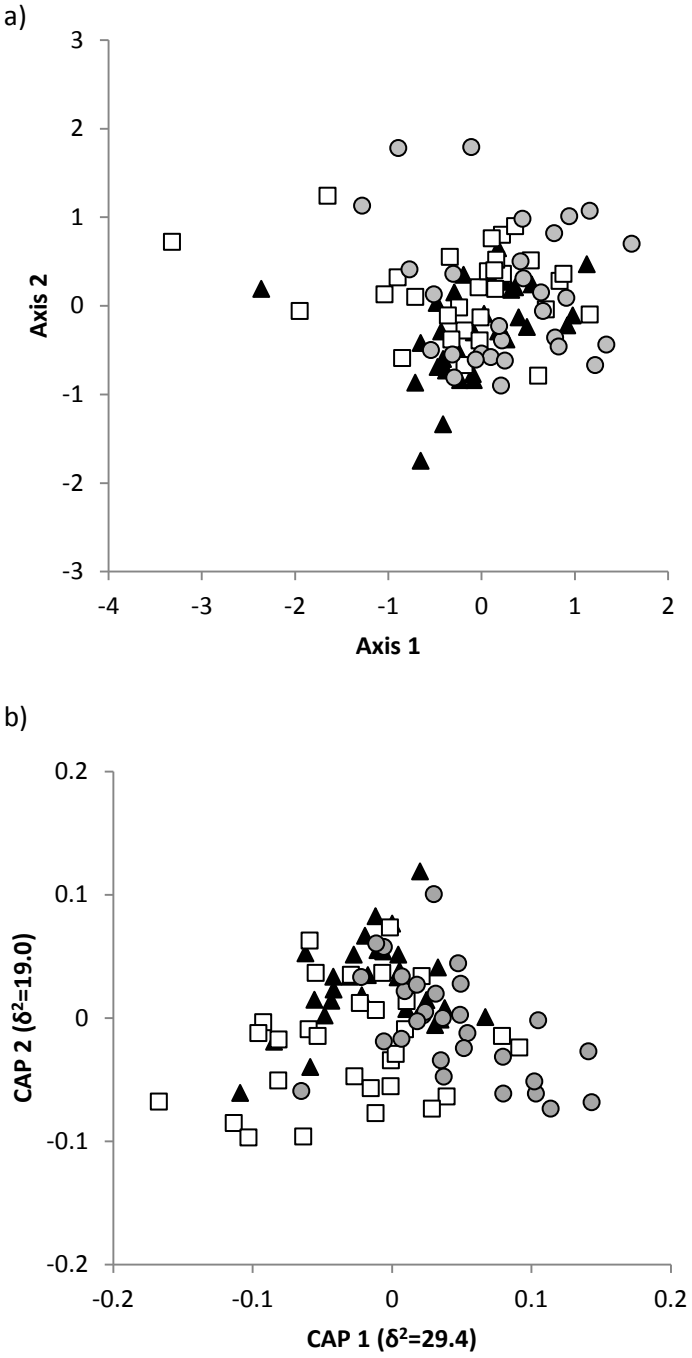


**Fig. 2.5** Ordination plots from non-parametric multidimensional scaling analysis (a) and canonical discriminant analysis (b) of soil chemical composition from soil samples associated with *Themeda* (▲), *Austrodanthonia* (□) and *Austrostipa* (●). For the ordination from canonical discriminant analysis, values on axes 1 and 2 are squared eigenvalues ($\delta^2$).

## DISCUSSION

Using a T-RFLP analysis, this study examined three different microbial groups in order to investigate the extent to which microbial community composition is influenced by plant species that co-occur in a southern temperate grassland. The three co-occurring grass species examined do have statistically different associated bacterial, fungal and AM fungal communities, but there was no clear evidence that those differences were linked to soil chemical properties. Interestingly, the two dominant species, *Themeda* and *Austrodanthonia*, were more similar to each other in their bacterial and AM fungal community composition than either was to the sub-dominant species, *Austrostipa*. This is despite the fact that *Themeda* is a warm-season $C_4$ grass while *Austrodanthonia* is a cool-season $C_3$ species. Despite this, only fungal community composition differed substantially between *Themeda* and *Austrodanthonia*. While the results demonstrate that co-occurring plant species from a temperate grassland do differ in their associated soil microbial communities, these differences were generally smaller than is the case for species that generally do not co-occur naturally, such as those from agricultural crop systems (Costa et al. 2006) and improved grassland systems (Patra et al. 2006) or species that are grown in pots or monoculture (Bezemer et al. 2006; Grayston et al. 1998; Haichar et al. 2008; Söderberg et al. 2004). The findings from those studies that have examined the effect of plant species identity on microbial community in natural systems (Khidir et al. 2010; Kuske et al. 2002; Vandenkoornhuyse et al. 2003; Veresoglou et al. 2011) are often variable. For instance, Nunan *et al.* (2005) found little or no effect of plant species on the composition of the root-colonising bacterial community when comparing five co-occurring grass species from an upland grazed grassland in Scotland. In contrast, Vandenkoornhuyse *et al.* (2002) found a large difference in the AM fungal community among the roots of three co-occurring grass species from a semi-natural grassland in Scotland. However, most of the studies published focus on a single microbial group and comparisons among studies are made difficult because of differences in collection, processing and experimental techniques of samples. By comparing plant-microbe relationships on three microbial

groups using the same samples, this study provides strong evidence for the high degree of specificity of each of the bacterial, fungal and AM fungal communities to their plant species on the smallest scale with ecologically similar species.

While the results show that each of the three tested microbial groups demonstrated statistically significant specificity to plant species, there were differences in the strength of the plant species effect for the different microbial groups, as has been reported in other studies (Bardgett et al. 1999; Costa et al. 2006; Hossain and Sugiyama 2011). The popular notion that the plant species effect on microbial community composition is due to plant species-specific patterns of root exudation (Grayston et al. 2001; Grayston et al. 1998) has led many studies to focus on rhizosphere soils, often collected away from the soil surface. However, for fungi which are generally saprophytic in nature and have the ability to utilise the more complex organic carbon sources often found in plant litter (Meidute et al. 2008; Sagova-Mareckova et al. 2011), the differences in plant litter quality may have exerted a stronger effect on fungal community composition in surface soil. The greater responsiveness of the fungal community to plant litter has been demonstrated by litter amendment studies in which litter identity and mixing influenced the fungal community only and not the bacterial or AM fungal communities (Hossain and Sugiyama 2011). Thus, the greater sensitivity of the fungal community to aboveground litter chemistry might explain the difference observed in fungal community composition between the two dominant species that differ in their litter chemistry (Osanai et al. 2012).

The AM fungal community composition, on the other hand, showed more pronounced differences between the sub-dominant *Austrostipa* and the two dominant species, implying a role for mycorrhizal association in determining plant community structure and dynamics. It has been suggested that mycorrhizal associations can either reduce plant diversity by increasing dominance of a particular species (Hartnett and Wilson 1999) or promote plant coexistence by increasing the ability of less competitive species to access nutrients (Moora and Zobel 1996; Opik et al. 2006; Sanon

et al. 2009; van der Heijden et al. 1998; Wagg et al. 2011). In this study, the latter was the likely case as the sub-dominant species, *Austrostipa*, had a higher number of total and average AM fungal T-RFs than the dominant species. Furthermore, soils associated with *Austrostipa* also contained the highest proportion of unique AM fungal T-RFs. This high specificity of the AM fungal association in *Austrostipa* is also in line with the suggestion that the AM fungal association becomes more specific when plants are under environmental stress (Martinez-Garcia and Pugnaire 2011), which in this case could be attributed to interspecific competition for resources. This high specificity of AM fungal association in *Austrostipa* could also explain the sub-dominance of this plant species in this grassland community, as AM fungal diversity has been found to correlate positively with phosphate availability (Alguacil et al. 2010), thus affecting the ability of plant species to access phosphate via AM fungal association. While phosphate availability was not measured in this study, phosphate availability in this grassland is generally low (Hovenden pers. comm.). Still, it is possible that the difference in AM fungal specificity observed reflect the difference in the accessibility to phosphate among the plant species and therefore explains their success in this grassland community.

Competition for resources has been regarded as one of the major forces shaping plant community structure and, according to classical theory, species coexistence is possible due to niche separation in resource use (Gotzenberger et al. 2012). In this study, a greater difference in microbial community composition was observed between the two $C_3$ species, *Austrostipa* and *Austrodanthonia*, than between the two dominant species *Themeda* ($C_4$) and *Austrodanthonia* ($C_3$), suggesting that niche separation in phenology (i.e. growing season) may have alleviated the competition between the two dominant species. The two $C_3$ species on the other hand showed a greater difference in their associated soil microbial communities, particularly in bacteria. The bacterial community has been found to be more responsive to root exudates than other microbial groups (Costa et al. 2006). As *Austrodanthonia* and *Austrostipa* have a similar phenology, selective pressure from those species on bacterial community are likely to be stronger when they are actively

growing in order to compete each other for growth-limiting resources. Meanwhile, the selective pressure from *Themeda* is likely to be less pronounced during cooler months, which are the growing season for $C_3$ species. This role of plant species in modifying their associated microbial community is further supported by the lack of correlation between soil chemical properties and microbial community composition, indicating the importance of plant species identity on microbial community composition.

Soil pH is often found to influence bacterial community composition (Osborne et al. 2011) and even the changes in the order of 0.1 in pH have been reported to significantly influence the composition of bacterial community (Sagova-Mareckova et al. 2011). There was no evidence of such an effect in this study, despite the differences in soil pH among the plant species. It is possible that this lack of soil pH effect on the bacterial community composition may be due to the limitation of T-RFLP analysis to detect differences in such a small spatial scale study. However, it is interesting to note that soil chemistry differed more between the two dominant species than between the dominant species and the sub-dominant species. Thus, it is likely that a difference in microbial community composition does not directly translate into a difference in microbial activity and function, or that soil chemistry is strongly influenced by litter quality and nutrient acquisition by plant species (Chapman et al. 2006). As the effect of plant species on soil microbial community can vary throughout over time (Bremer et al. 2007), in future studies it would be beneficial to take repeated samples to examine the generality of responses.

*Conclusions*

The results from this study demonstrate that co-occurring plant species have distinct associated microbial communities but there was no clear evidence that those differences were linked to differences in soil chemical properties and thus the differences in microbial composition must be due to inherent differences between the plant species themselves. The lack of correlation between

soil chemical properties and the microbial community composition may be due to the limit of T-RFLP analysis to deal with a small spatial scale study, with such a small magnitude of difference in soil chemical properties. However, the technique was still able to detect the effect of plant species on microbial community composition as well as provide an insight into the differences in response of bacterial, fungal and AM fungal communities to their associated plant species. Further investigation is required to examine the functional role of the microbial community associated with these grass species in order to understand the importance of plant species identity in affecting microbially-mediated soil processes, and therefore predict the potential consequences of shifts in plant species composition on ecosystem processes and functioning.

# Chapter 3: Plant species effect on nitrogen transformations

## INTRODUCTION

Nitrogen (N) mineralisation can vary across climatic regions, vegetation types and soil types (Reich et al. 1997; Rustad et al. 2001), however, unlike the case with decomposition in which the global pattern of carbon (C) mineralisation is strongly influenced by climate (Raich and Schlesinger 1992), the release of mineralised N from soil plant litter is primarily controlled by the initial chemical composition of the litter itself, most importantly the carbon to nitrogen (C:N) ratio and lignin:N ratio (Manzoni et al. 2008; Parton et al. 2007). Plant species can influence N cycling through differences in the provision of organic inputs to the soil as well as through their interaction with the soil microbial community that catalyses the mineralisation processes (Fillery 2007; Knops et al. 2002). While litter input influences the population size and activity of the soil microbial community (Dornbush 2007; Jin et al. 2010), it also influences the balance between mineralisation and immobilisation (Knops et al. 2002; Murphy et al. 2003; Swift et al. 1979) thereby affecting the availability of plant available N in the soil. Plant available N is commonly measured by soil incubation assays, both *in situ* and *ex situ*, by examining the changes in the size of inorganic N pools such as ammonium ($NH_4^+$) and nitrate ($NO_3^-$) during the incubation period. Final calculations of rates of conversion using this method, however, are based on net changes and therefore do not provide the information required to develop a mechanistic understanding of the processes involved (Davidson et al. 1992; Davidson et al. 1991; Hart et al. 1994a). For example, Davidson *et al.* (1992) found that low net nitrification rates observed in a mature conifer forest, compared to a young conifer forest, did not necessarily indicate that the turnover rates of $NO_3^-$ were lower in the mature forest, as gross nitrification rates were actually higher in the mature forest than the young forest. This apparent disagreement was caused by high $NO_3^-$ consumption rates rather than low $NO_3^-$

production rates, emphasising the importance of microbial immobilisation of $NO_3^-$ in controlling N availability in this forest system. This, therefore, demonstrates the importance of measuring both gross and net N transformation rates in obtaining mechanistic insights into the control of N cycling.

While the quality of plant litter has been shown to influence nitrification rates (Laughlin 2011; Orwin et al. 2010), it is also recognised that plants can actively influence nitrification processes through the secretion of organic compounds from roots that inhibit the activity of the nitrifying community (Subbarao et al. 2009; Subbarao et al. 2007). The first step of nitrification is mediated by ammonia-oxidising bacteria (AOB) and archaea (AOA) that convert $NH_4^+$ into $NO_3^-$. This is often regarded as the rate-limiting step of the nitrification processes (De Boer and Kowalchuk 2001), and the abundance of AOA and AOB has been shown to correlate with nitrification rates (Di et al. 2010; Gubry-Rangin et al. 2010). While autotrophic nitrification has been reported to predominate in various systems (Bollmann and Conrad 1997; Herrmann et al. 2007), even in those environments that were thought to inhibit the growth and activity of autotrophic nitrifiers (Hayatsu and Kosuge 1993; Pennington and Ellis 1993), a substantial contribution of heterotrophic nitrification has been demonstrated in some soils (e.g. Killham 1990; Pedersen et al. 1999) including grassland soils (e.g. Cookson et al. 2006; Laughlin et al. 2008). For instance, Cookson *et al.* (2006) found that up to 50% of nitrification could be attributed to heterotrophic rather than autotrophic nitrification in a semi-arid grassland in Western Australia. Heterotrophic nitrification can be performed by two microbial groups, heterotrophic bacteria and fungi (De Boer and Kowalchuk 2001). While the importance of fungal nitrification is well-documented in acidic forest systems (Killham 1990; Schimel et al. 1984), fungal biomass can contribute substantially to the total microbial biomass in grassland systems (Bittman et al. 2005; Laughlin and Stevens 2002). Therefore, fungi could make a considerable contribution to heterotrophic nitrification in grassland systems.

A study conducted in a native temperate grassland community has demonstrated that N cycling differed amongst co-occurring grass species, with a substantial contribution of heterotrophic

nitrification observed under some species (Osanai et al. 2012). Therefore, to obtain a better understanding of factors driving these differences in N cycling, N transformations among the co-occurring grass species from the temperate grassland community were examined by comparing gross N transformation rates, the abundance of autotrophic ammonia-oxidisers, and the relative contributions of autotrophic and heterotrophic nitrification as well as the contribution of fungal nitrification to the overall nitrification processes in soils associated with the co-occurring grass species. Specifically, the study aimed to answer the following three questions:

- Do co-occurring species differ in gross ammonification, gross $NH_4^+$ immobilisation, gross nitrification and gross $NO_3^-$ immobilisation?
- Are there any differences in the abundance of autotrophic ammonia-oxidisers, AOA and AOB, among the co-occurring species?
- Do co-occurring species differ in the relative contributions of autotrophic and heterotrophic nitrification as well as the contribution of fungal nitrification to the overall nitrification processes?

## MATERIALS AND METHODS

*Nitrogen transformation rates*

Gross rates of ammonification, nitrification and microbial immobilisation were measured using the $^{15}N$ pool dilution technique (Hart et al. 1994b) to examine the effect of plant species on N transformation rates. Soil samples were collected in the autumn of 2010 in three separate, randomly chosen sites within the grassland. At each site, soil samples were collected by taking twelve cores, each 35 mm in diameter, from the top 5 cm of the soil directly beneath each of the three grass species. These grass species have a caespitose habit with each plant forming a spatially

distinct tussock clearly separated from all others, thus soil sampling was done with a maximum of two cores being taken from any individual tussock. These twelve cores per species per site were composited at the site, giving approximately 600 g fresh soil for each of three species in each of three sites, giving nine soil samples in total. Soil samples were homogenised by passing through a 2 mm-sieve which also removed large roots and litter. Sub-samples were taken for determination of relative water contents by drying in an oven at 105°C for 24 h. Total C and N concentration of soils were also determined using a Perkin Elmer 2400 Series II Elemental Analyser (Perkin Elmer Australia).

For each soil sample, 145 g of fresh soil was spread as a thin layer on plastic trays and sprayed with 4.4 ml of either $(^{15}NH_4)_2SO_4$ or $K^{15}NO_3$ solutions to achieve N addition of 2 mg N/kg soil with an excess of 6 atom% $^{15}N$. From this labelled sample, four sub-samples of 30 g soils (t1 and t2 measurements x 2 replicates) were placed in 250 ml bottles and pre-incubated for 18 h in dark at room temperature (~15 °C). Soil samples were extracted for the determination of mineral N ($NH_4^+$ and $NO_3^-$), before (t0) and after 24 h incubation period (t1). Mineral N was extracted after shaking 30 g of soil with 60 ml of 1 M KCl for 1 h. The extracts were analysed for $NH_4^+$ and $NO_3^-$ concentration using a FIAstar 5000 flow injection analyser (Foss Tecator AB, Hoeganaes, Sweden). The $^{15}N$ enrichment of $NH_4^+$ in the extracts was determined by generating $NH_3$ by the addition of MgO. The $NH_3$ generated was absorbed by a $H_2SO_4$-soaked filter, which was later dried and analysed for isotope ratio determination using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The $^{15}N$ enrichment of $NO_3^-$ in the extracts was determined by generating $NH_3$ by the addition of MgO first to diffuse out the existing $NH_4^+$ from the extracts, followed by the addition of Devarda's alloy to reduce $NO_3^-$ to $NH_4^+$. The $NH_4^+$ produced from $NO_3^-$ was then collected following the same method as above.

Rates of gross ammonification, $NH_4^+$ immobilisation, gross nitrification and $NO_3^-$ immobilisation were calculated using the equations of Kirkham and Bartholomew (1954).

$$m = \frac{M_o - M_1}{t} \times \frac{\log\ H_o \times M_1\ /\ H_1\ \times M_o}{\log\ M_o\ /\ M_1} \qquad \text{when } i \neq m$$

$$i = \frac{M_o - M_1}{t} \times \frac{\log\ H_o\ /\ H_1}{\log\ M_o\ /\ M_1} \qquad \text{when } i \neq m$$

where *m* is the mineralisation rate per unit mass of soil per time (µg N g soil$^{-1}$ d$^{-1}$); *i* is the immobilisation rate; *t* is time; $M_0$ is the initial $^{14+15}$N pool; $M_1$ is the post-incubation $^{14+15}$N pool at time *t*; $H_0$ is the initial $^{15}$N pool; $H_1$ is the post-incubation $^{15}$N pool at time *t* (Kirkham and Bartholomew 1954).

*Quantification of bacterial and archaeal amoA genes*

The abundance of ammonia-oxidising bacteria (AOB) and archaea (AOA) in the soils associated with the three grass species was examined by quantifying the functional gene marker ammonia monooxygenase (*amoA*) using realtime PCR. Soil samples were collected in the late spring of 2009 when the vegetative growth of both $C_3$ and $C_4$ species was apparent by taking 30 cores, each 15 mm in diameter, from the top 5 cm of the soil directly beneath each of the three grass species with a maximum of one core being taken from any individual tussock, giving 90 core samples in total. Soil samples were transported to the laboratory on ice and stored at −20 °C until processed. DNA was extracted using a MoBioPowersoil kit (MoBio, CA, USA) according to the manufacturer's instructions with the modifications of 0.5 g of soil and 0.2 g of glass beads (100 mesh size) added to rupture microbial cells by bead beating for 3 min. DNA quantity was determined using a Nanodropspectrophometer (Thermoscientific, DA, USA). Realtime PCR was performed to quantify copy numbers of bacterial and archaeal *amoA* genes. For the bacterial *amoA* quantification, the amplification was carried out in 10 µl reaction volumes containing 40-60 ng of template DNA; 5 µl 2x BioRadiTaq SYBR Mix with ROX; 0.4 µM of the forward primer *amoA*-1F (5'-GGGGTTTCTACTGGTGGT) and 0.6 µM of reverse primer *amoA*-2R (5'-CCCCTCKGSAAAGCCTTCTTC) (Hayden et al. 2010; Rotthauwe et al. 1997). For the archaeal *amoA* quantification, the amplification was carried out in

25 µl reaction volumes containing 40-60 ng of template DNA; 12.5 µl 2x BioRadiTaq SYBR Mix with ROX and 0.4 µM of the forward primer Arch-amoAF (5'-STAATGGTCTGGCTTAGACG) and reverse primer Arch-amoA R (5'-GCGGCCATCCATCTGTATGT) (Francis et al. 2005). Standard curves where developed as described in Hayden *et al.* (2010). Representative genes were amplified from a type strain with specific primers for bacterial *amoA* and from environmental DNA from a peat bog for archaeal *amoA* and the resultant PCR product isolated from an agarose gel, purified and then cloned into a TOPO-TA plasmid vector (Invitrogen Corporation,USA) in *E. coli.* Plasmid DNA preparations were obtained from clones by using a QiagenMiniprep kit (Qiagen, Germantown, MD, USA). The number of copies of the target gene in a nanogram of plasmid DNA was determined, and then a serial dilution was prepared from 107 to 101 copies to use as an external standard curve ($R^2 > 0.9$), allowing determination of the number of copies of the gene in each sample of soil DNA.

All amplifications were carried out in duplicate on a Mx3005P QPCR system (Stratagene) with an initial denaturation stage of 3 min at 95$^{o}$C, 40 cycles of 95$^{o}$C for 15 sec and 60$^{o}$C for 45 sec, followed by a melt curve cycle of 95$^{o}$C for 1 min, 60°C for 30 sec then temperature increments of 0.5$^{o}$C each 10 sec until 95$^{o}$C for both the bacterial and archaeal *amoA* assays. The copy number of *amoA* genes per nanogram of DNA was determined for each sample of soil DNA by comparison with a standard curve of101 to 107 gene copies in the assay. Efficiencies of 78 to 81% were obtained for bacterial *amoA* amplification, with the $R^2$ values ranging from 0.830 to 0.991, and efficiencies of 76 to 84% were obtained for archaeal *amoA* amplification with the $R^2$ values ranging from 0.994 to 0.999. Alongside the duplicate reactions for copy number determination a third representative of each sample was included in the assay which had been "spiked" with 0.5 ml of DNA from a type strain containing high copy numbers of the gene of interest. Due to the generally low numbers of AOB in the soil DNA preparations, some reactions failed to generate a threshold cycle, but the possibility of reaction inhibition was ruled out by the positive response of spiked samples. The detection limits of the realtime-PCR reactions were 10 copies of bacterial and archaeal *amoA*.

*Nitrification inhibition*

To examine the relative contributions of autotrophic and heterotrophic nitrification as well as fungal nitrification, a 28-day soil incubation study was conducted using acetylene which inhibits autotrophic nitrification (Hyman and Wood 1985) and cycloheximide which inhibits fungal nitrification (Castaldi and Smith 1998; Landi et al. 1993). Soil samples were collected in the spring of 2009 in five separate, randomly chosen sites within the grassland. At each site, soil samples were collected by taking 20 cores, each 15 mm in diameter, from the top 5 cm of the soil directly beneath each of the three grass species. Soil sampling was done with a maximum of one core being taken from any individual tussock. These 20 cores per species per site were composited at the site, giving 15 soil samples in total. Soil samples were homogenised using a 4 mm-sieve and large roots, litter and gravels were removed. Sub-samples were taken to measure relative water contents and field capacity at 105°C for 24 h.

For each soil sample, 10 g of fresh soil was processed immediately to determine $NH_4^+$ and $NO_3^-$ concentrations using the following method: 10 g of fresh soil was added to 100 ml 2 M KCl and mixed on an orbital shaker for one hour, left to settle for a further hour and then filtered through Whatman No. 42 filter paper. Extraction blanks were processed similarly with each sample run. The soil extracts were analysed for $NH_4^+$ and $NO_3^-$ using an FIAstar 5000 flow injection analyser (Foss Tecator AB, Hoeganaes, Sweden). For the remaining soil samples, each soil sample was divided into four sub-samples (10 g fresh weight) for the four treatments (untreated control, acetylene, cycloheximide and combined acetylene and cycloheximide) and was placed in airtight jars. De-ionised water was added until each sample was at 60% field capacity.

Acetylene and combined acetylene and cycloheximide samples were exposed to acetylene (1% v/v) for 24 h to inhibit autotrophic nitrification using the method adapted from Herrmann *et al.* (2007). Cycloheximide and combined acetylene and cycloheximide samples were treated with cycloheximide

(2 mg/g soil) mixed with talc powder (1:2) to achieve even distribution of the inhibitor treatment. Talc powder was also applied to all other samples in order to prevent a confounding influence of the talc in the cycloheximide-treated samples. All jars were incubated in dark at 25°C for 28 days. Since microbial decomposition of inhibitors have also been shown to occur in soils (Badalucco et al. 1994; De Boer et al. 1993), inhibitor treatments were reapplied every five days, as initial investigations indicated that this was sufficient to completely prevent nitrification. After the 28 day incubation period all soil samples were extracted with 2 M KCl and analysed as above. Net N mineralisation and nitrification were calculated as the difference in $NH_4^+$ and $NO_3^-$ concentration between incubated and initial samples.

*Statistical analyses*

N transformation data were analysed by one-way analysis of variance (ANOVA) using general linear model procedures in the SAS statistical software package (SAS Institute Inc. 2003) to determine if plant species affected the gross and net rates of mineralisation, nitrification and immobilisation and the soil C and N contents. Ryan-Einot-Gabriel-Welsch post hoc multiple comparison test was used where ANOVA indicated significant differences among the species (Day and Quinn 1989). Nitrification inhibition data were analysed by two-way ANOVA with plant species and inhibitor treatments as factors, followed by post hoc multiple comparison test as above. The realtime-PCR data were analysed by non-parametric significance testing, Kruskal-Wallis test, due to the non-normal distribution of the data (Ruxton and Beauchamp 2008).

## RESULTS

*Soil chemistry and inorganic N availability*

The soil C content did not differ among the plant species (Table 3.1), however, significant differences were found in soil N ($P$=0.05) and consequently C:N ratio ($P$=0.05; Table 3.1). Soil N content was higher in *Austrodanthonia* soil than *Themeda* soil, with *Austrostipa* soil not differing from either of them. Soil C:N ratio on the other hand was higher in *Themeda* soil than *Austrostipa* soil, and *Austrodanthonia* soil did not differ from either of them in soil C:N ratio. The amount of extractable $NH_4^+$ and $NO_3^-$ differed significantly amongst the plant species. Soils associated with *Austrodanthonia* had a higher $NH_4^+$ than *Themeda* and *Austrostipa* (Table 3.2). The availability of $NO_3^-$ showed a different pattern with the highest availability found in soils associated with *Austrostipa*, followed by *Austrodanthonia* and *Themeda*. $NH_4^+$ was the predominant form of inorganic N in all soils (Fig. 3.1), and the plant species differed significantly in their relative proportion of $NH_4^+$ to $NO_3^-$ ($F_{2,9}$=97.75, $P$<0.0001). *Themeda* soil had the highest proportion of $NH_4^+$ (85.1%), followed by *Austrodanthonia* soil (76.8%) and *Austrostipa* soil with the lowest proportion of $NH_4^+$ (67.4%).

**Table 3.1** Soil total carbon (C) and nitrogen (N) contents and C:N ratios of soil samples collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa*. Different letters indicate statistical differences between the species (P<0.05).

| | C (%) | N (%) | C:N |
|---|---|---|---|
| *Themeda* | 2.84 ±0.09 | 0.21 ±0.01 b | 13.80 ±0.10 a |
| *Austrodanthonia* | 3.11 ±0.06 | 0.23 ±0.01 a | 13.61 ±0.08 ab |
| *Austrostipa* | 2.94 ±0.13 | 0.22 ±0.01 ab | 13.38 ±0.15 b |
| $F_{2,24}$ | 2.11 | 3.44 | 3.54 |
| $P$ | 0.14 | **0.048** | **0.045** |

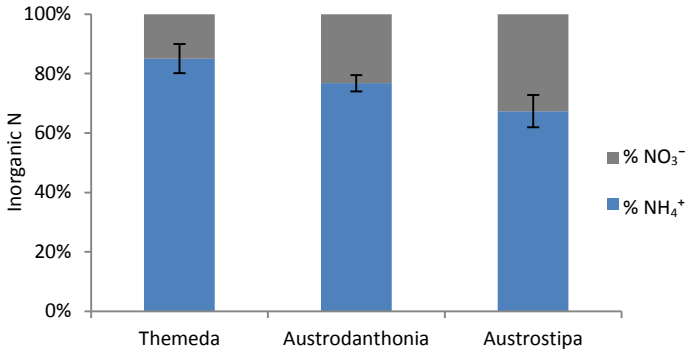


**Fig. 3.1** The relative proportion of soil inorganic N measured in soil samples collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa.* Vertical lines are ±SE.

*N transformations*

Gross rates of ammonification, nitrification, $NH_4^+$ immobilisation and $NO_3^-$ immobilisation were examined using a $^{15}N$ pool dilution technique. Gross ammonification rate differed significantly among the plant species ($P$=0.01, Table 3.2) with *Austrodanthonia* soil having a rate 44.6% and 35.0% lower than *Austrostipa* and *Themeda* soils, respectively. $NH_4^+$ immobilisation rates followed the same pattern as gross ammonification rates in the plant species effect ($P$=0.04) with *Austrodanthonia* soil having 33.6% and 25.3% lower rate than *Austrostipa* and *Themeda* soils, respectively. Net ammonification rates calculated from the difference between gross ammonification and $NH_4^+$ immobilisation were negative in all species, indicating that net $NH_4^+$ immobilisation occurred since $NH_4^+$ consumption exceeded production in all species. Despite the significant plant species effect on those gross rates, the differences among the plant species were such that there were no differences in net ammonification rates (Table 3.2). Gross nitrification rates also differed among the plant species ($P$<0.05) with *Austrostipa* soil having a significantly higher rate than *Austrodanthonia* soil, with *Themeda* soil not significantly differing from either of them. Plant species also differed in $NO_3^-$ immobilisation rates ($P$=0.003) with *Themeda* and *Austrodanthonia* soils producing negative values while *Austrostipa* soil had a positive $NO_3^-$ immobilisation rate. Significant

differences among the plant species were also observed in net nitrification rates (*P*=0.03) calculated from gross nitrification and $NO_3^-$ immobilisation. Despite the low gross nitrification rate, *Austrodanthonia* soil had a higher net nitrification rate than that of *Austrostipa* soil, while *Themeda* soil did not differ from either of them (Table 3.2).

**Table 3.2** Initial soil inorganic N contents (µg N/g soil dwt) and gross N transformation rates (µg N/g soil dwt/d) of soils collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa*. Different letters indicate statistical differences between the species (P<0.05).

| | *Themeda* | *Austrodanthonia* | *Austrostipa* | $F_{2,9}$ | *P* |
|---|---|---|---|---|---|
| Initial $NH_4^+$ | 6.95 ±0.09 b | 7.36 ±0.33 a | 6.90 ±0.14 b | 12.98 | **0.002** |
| Initial $NO_3^-$ | 1.34 ±0.46 c | 2.35 ±0.43 b | 3.74 ±0.98 a | 128.27 | **0.0001** |
| Gross ammonification | 7.38 ±1.86 a | 5.47 ±0.62 b | 7.91 ±0.41 a | 8.19 | **0.01** |
| $NH_4^+$ immobilisation | 8.25 ±2.16 b | 6.69 ±0.56 b | 8.59 ±0.41 a | 4.56 | **0.04** |
| Net ammonification | -0.99 ±0.23 | -1.22 ±0.05 | -1.02 ±0.12 | 1.65 | 0.25 |
| Gross nitrification | 0.12 ±0.07 ab | -0.01 ±0.07 b | 0.33 ±0.15 a | 4.43 | **0.046** |
| $NO_3^-$ immobilisation | -0.06 ±0.36 b | -0.54 ±0.21 b | 0.88 ±0.41 a | 11.99 | **0.003** |
| Net nitrification | 0.18 ±0.31 ab | 0.53 ±0.25 a | -0.55 ±0.36 b | 5.12 | **0.03** |

The relationship between gross ammonification and $NH_4^+$ immobilisation and that of gross nitrification and $NO_3^-$ immobilisation showed different patterns to each other. There was a very strong positive correlation between gross ammonification and $NH_4^+$ immobilisation ($r^2$=0.97, Fig. 3.2a), which explains the lack of difference in net ammonification rates among the plant species despite the differences in gross ammonification rates. On the contrary, no correlation was observed between gross nitrification and $NO_3^-$ immobilisation ($r^2$=0.35, Fig. 3.2b).

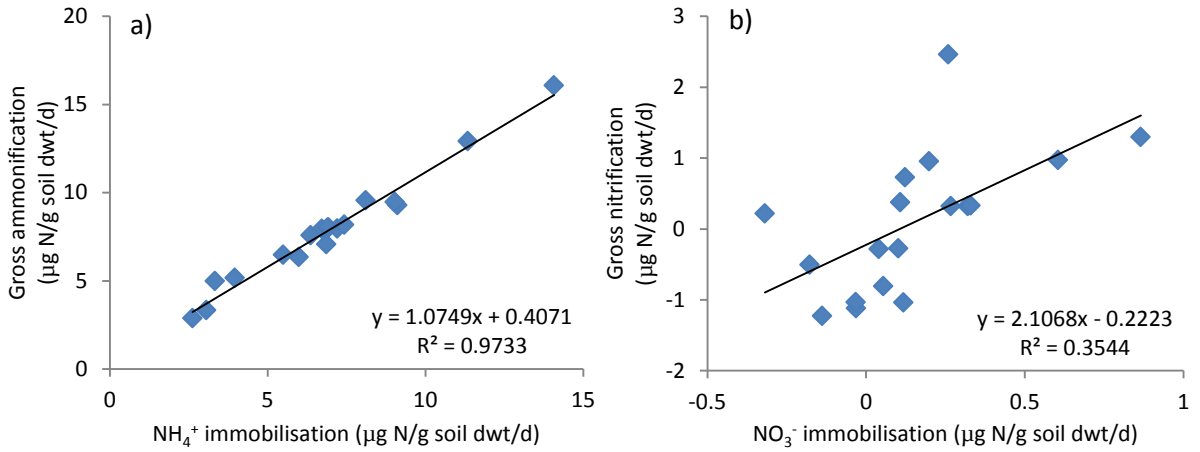


**Fig. 3.2** Relationships between gross ammonification and $NH_4$+ immobilization a), and between gross nitrification and $NO_3$- immobilisation b).

*Ammonia-oxidising bacteria (AOB) and archaea (AOA)*

The bacterial *amoA* gene copy number was quantified by realtime PCR to examine the population of ammonia-oxidising bacteria (AOB) in soils associated with *Themeda*, *Austrodanthonia* and *Austrostipa*. The total bacterial *amoA* gene copy number was the highest in *Themeda*, followed by *Austrostipa* and *Austrodanthonia*. However, the detection of bacterial *amoA* gene was very low in all species, with only one out of 30 samples tested for *Austrodanthonia* soil having a detectable *amoA* gene copy number (Table 3.3). Statistically, *amoA* gene copy number was significantly lower in *Austrodanthonia* soil than *Themeda* soil ($P$=0.03) with *Austrostipa* soil being intermediate and not significantly different from either of the other species (Table 3.3). The archaeal *amoA* gene copy number was also quantified to examine the ammonia-oxidising archaea (AOA) population. The archaeal *amoA* gene copy number was much greater than that of the bacterial *amoA* gene in soil from all plant species (Table 3.3), with over 86% detection rates among soil samples tested. The average archaeal *amoA* gene copy number was the highest in *Austrostipa* soil, followed by *Austrodanthonia* and *Themeda* soils. The number of archaeal *amoA* gene copy was significantly greater in *Austrostipa* soil than *Themeda* soil ($P$=0.04) and *Austrodanthonia* soil ($P$=0.02), while no

statistical difference was found between *Themeda* and *Austrodanthonia* soils in the *amoA* gene copy number. These results indicate that the two dominant species, *Themeda* and *Austrodanthonia*, differed significantly in AOB population size, which was very small in all cases, yet did not differ in AOA population size, which was approximately 10 times more abundant than the AOB population (Table 3.3). Interestingly, the sub-dominant species *Austrostipa* soil had the highest apparent population size of both AOB and AOA.

**Table 3.3** The number of bacterial and archaeal *amoA* gene copy (*amoA* copies/ng DNA) quantified by realtime-PCR on DNA extracted from soil samples collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa*. The number of samples that contained detectable amount of *amoA* gene copy out of 30 samples examined is also indicated in the table due to the low detection rate of bacterial *amoA*.

| | Total number of *amoA* copy detected | Number samples with *amoA* detected | Mean of the detected samples |
|---|---|---|---|
| **Bacterial *amoA*** | | | |
| *Themeda* | 195 | 6 | 27.9 ±5.4 |
| *Austrodanthonia* | 25 | 1 | 25.2 |
| *Austrostipa* | 128 | 4 | 30.4 ±13.6 |
| **Archaeal *amoA*** | | | |
| *Themeda* | 6707 | 27 | 248.4 ±93.6 |
| *Austrodanthonia* | 7114 | 26 | 273.6 ±148.1 |
| *Austrostipa* | 12294 | 28 | 439.1 ±89.1 |

Note: A detectable amount of bacterial *amoA* was found in only one soil sample out of 30 samples tested in *Austradanthonia* soil.

*Nitrification inhibition*

Net nitrification rate was examined using the selective inhibitors, acetylene and cycloheximide singly and in combination, to separate the contribution of autotrophic nitrifiers and fungi to the overall nitrification rates. All inhibitor treatments resulted in a significant reduction in net nitrification rates compared to the control ($F$=9.24, $P$<0.0001, Table 3.4), with the acetylene treatment having the strongest inhibitory effect overall (94% reduction), followed by the combined acetylene +

cycloheximide treatment (84%) and the cycloheximide treatment (44%). While statistically non-significant, a slightly higher nitrification rate in the combined acetylene + cycloheximide treatment than the acetylene only treatment was unexpected, however, the examination of net ammonification revealed that there was an order of magnitude increase in extractable $NH_4^+$ in soils treated with cycloheximide (Appendix 1). Since the rate of nitrification is strongly influenced by the supply rate of $NH_4^+$, this increase is the likely cause of the higher nitrification rates in the combined treatment (Table 3.4). Plant species did not differ in net nitrification rates with or without the inhibitors, and the degrees of inhibitory effect of the treatments were similar, except for the cycloheximide-only treatment which had different impacts on the soil from the different plant species. Cycloheximide resulted in 63% reduction in nitrification rate in *Themeda* while it only reduced the rate by 30% and 36% in *Austrostipa* and *Austrodanthonia*, respectively (Table 3.4).

**Table 3.4** The effect of acetylene (Acet) and cycloheximide (Cyclo) on net nitrification rates (mg N/g soil dwt/28 d) of soil samples collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa* during 28-d laboratory incubation. Different letters indicate statistical differences between the treatments ($P<0.05$).

| | Control | Acetylene | Cycloheximide | Acet + Cyclo |
|---|---|---|---|---|
| *Themeda* | 0.018 ±0.007 a | 0.000 ±0.002 b | 0.007 ±0.001 ab | 0.002 ±0.001 b |
| *Austrodanthonia* | 0.015 ±0.010 | 0.002 ±0.001 | 0.009 ±0.001 | 0.003 ±0.001 |
| *Austrostipa* | 0.016 ±0.005 a | 0.001 ±0.001 c | 0.012 ±0.002 ab | 0.002 ±0.001 bc |

## DISCUSSION

The effect of plant species on N transformations in a temperate grassland was examined in order to identify the underlying drivers of the differences in N cycling observed amongst the co-occurring grass species. Using the $^{15}N$ pool dilution technique, it was found that gross N transformation rates

differed substantially among the co-occurring grassland species, whereas net rates did not necessarily show differences among the species. Thus, substantial differences among the species in the gross rates of N transformation are not apparent in measurements of net rates, most particularly with rates of ammonification. The impact of plant species on the autotrophic nitrifier community was also examined by quantifying the functional gene marker *amoA*. This approach demonstrated that the abundance of autotrophic ammonia-oxidising bacteria and archaea differed markedly among the plant species and that archaeal nitrifiers far outnumbered bacterial nitrifiers in this grassland. As nitrification can occur through autotrophic and heterotrophic pathways, the relative contributions of autotrophic and heterotrophic nitrification were also examined using selective inhibitors. While the predominance of autotrophic nitrification over heterotrophic nitrification was observed in all species, apparent fungal nitrification rates differed among the co-occurring grass species, therefore the relative contribution of fungi in N cycling in this grassland may also depend on plant species identity. Overall, the differences observed in N transformation rates as well as the abundance and contribution of the nitrifying communities among the plant species suggest the importance of plant species identity on N cycling in this grassland community.

Native grassland systems such as these tend to have very low rates of net ammonification (Cookson et al. 2006; Osanai et al. 2012). Similarly, extremely low levels of net ammonification and consequently no real differences among the plant species were observed in this study. However, these soils sustained substantial rates of both gross ammonification and $NH_4^+$ immobilisation which showed a clear difference in N cycling under these co-occurring plant species. Thus the high gross ammonification rates in *Themeda* and *Austrostipa* soils were accompanied by high $NH_4^+$ immobilisation rates, whereas the low ammonification rate in *Austrodanthonia* soil was accompanied by a low rate of $NH_4^+$ immobilisation. Indeed, there was a very strong positive correlation between gross ammonification and $NH_4^+$ immobilisation, while no correlation was observed between gross nitrification and $NO_3^-$ immobilisation (Fig. 3.2). This indicates that the

microbial community in these soils might be strongly $NH_4^+$-limited. The results from this study agree with other studies that have obtained similarly strong correlations between gross mineralisation and $NH_4^+$ immobilisation yet weak relationships gross nitrification and $NO_3^-$ immobilisation (Booth et al. 2005; Chen and Stark 2000; Verchot et al. 2001). The gross mineralisation assay often stimulates $NH_4^+$ consumption or immobilisation rates due to the addition of $^{15}NH_4^+$ (Booth et al. 2005). This is often attributed to the preferential consumption of applied $NH_4^+$ by soil microbes during the assay, as the aqueous $^{15}NH_4^+$ is likely to be more accessible to soil microbes than indigenous soil $NH_4^+$ that may exist within soil aggregates or microsites (Watson et al. 2000). The preferential consumption of applied $^{15}NO_3^-$ in the other hand is less likely to occur, as $NO_3^-$ is already relatively mobile in soil solution. Furthermore, microbial demand for $NH_4^+$ is thought to be greater than that of $NO_3^-$, as $NH_4^+$ is consumed by both heterotrophic microorganisms and nitrifiers (Murphy et al. 2003). Therefore, the results from this study indicate the strong microbial control over $NH_4^+$ and that N cycling in this grassland is likely to be driven by the rate of ammonification and the microbial demand for $NH_4^+$, as observed in other grassland systems (Booth et al. 2005).

Both net and gross nitrification rates were also influenced by plant species, and the high gross nitrification and $NO_3^-$ immobilisation rates in *Austrostipa* soil combined with the high initial $NO_3^-$ availability suggest that microbial activities that are linked to N transformations might be more active under *Austrostipa* than the two other species. The use of net rates in this case is not a good measure of microbial nitrifying activity, as the low net nitrification rate under *Austrostipa* was caused by rapid $NO_3^-$ immobilisation rather than slow nitrification, as gross nitrification was in fact the highest under *Austrostipa*. While the study by Davidson *et al.* (1992) also found the importance of gross rates in characterising N cycling under different vegetation, Verchot *et al.* (2001) found that net rates were good indicators of differences in N cycling between forest types in eastern US, as gross rates did not show any differences between stands where they found differences in net rates. Nevertheless, the results from this study highlight that the balance between mineralisation and

immobilisation is strongly dependent on subtle differences in these two opposing processes, and that it is likely to change in time and space depending on various environmental factors that affect these processes.

The differences among the plant species in N transformation rates might be driven by differences in organic matter quality. Booth *et al.* (2005) reviewed 100 isotope pool dilution studies across various vegetation types and found that both soil organic matter quantity and quality were important drivers of N transformation rates. While the comparisons were based on vegetation types rather than plant species, they found that gross mineralisation is higher in low C:N soils than high C:N soils. Thus, it is possible that the greater N transformation rates observed in the soil from under *Austrostipa* than from under the other two plant species might be induced by low C:N ratios of *Austrostipa* soil. The differences observed in this study in gross nitrification may also have reflected differences among the plant species in the abundance of the nitrifier community. The abundance of autotrophic nitrifiers is commonly measured by the quantification of the functional gene marker, *amoA*, which is found in AOA and AOB (e.g. Adair and Schwartz 2008; Leininger et al. 2006). In this study, higher archaeal *amoA* abundance was observed in *Austrostipa* soil than *Themeda* and *Austrodanthonia* soils. While this does not necessarily relate causally to the higher gross nitrification and $NO_3^-$ availability measured in soils under *Austrostipa*, these results provide further support for the role of AOA as an important driver of N cycling in grassland systems (O'Sullivan et al. 2011). Furthermore, the copy number of the archaeal *amoA* gene outnumbered that of the bacterial *amoA* gene in all plant species examined in this study, to a factor of approximately 10. This predominance of archaeal *amoA* over bacterial *amoA* is in agreement with many other studies including those from Australian soils (Adair and Schwartz 2008; O'Sullivan et al. 2011). One common explanation for this pattern is that AOB and AOA differ in their $NH_4^+$ requirements, thus AOA are more abundant in low $NH_4^+$ environments while AOB predominate in high $NH_4^+$ environments such as agricultural soils that receive regular fertilisation (Di et al. 2010; Shen et al. 2011). Thus, it is not surprising that soils from

this natural temperate grassland community contain more AOA than AOB. The link between the abundance and the functional activity of AOA and AOB is, on the other hand, found to be more variable among studies. Some studies have found a correlation between AOA abundance and nitrification rates (Gubry-Rangin et al. 2010; Wessen et al. 2010; Yao et al. 2011), while others have found that nitrification rates are linked to the abundance of AOB and not that of AOA (Di et al. 2010; Ying et al. 2010). It is also possible that the abundance of AOA and AOB may not necessarily translate into nitrification rates, due to the high spatial variability of soil environments or high functional redundancy between AOA and AOB (Bernhard et al. 2010). However, the dominance of AOA over AOB and the occurrence of higher gross nitrification rates in soils with higher AOA abundance suggest that AOA may also dominate nitrification processes in this grassland community.

It is also possible that the discrepancies between the effect of AOB and AOA abundance on nitrification rates might be a result of heterotrophic nitrifier activity which is often not examined in many studies, due to the predominance of autotrophic nitrification frequently observed in many systems, including those systems that were previously thought to be dominated by fungi (Barraclough and Puri 1995). Similar to many other studies (Bollmann and Conrad 1997; Pennington and Ellis 1993), autotrophic nitrification was the predominant process in soil from all species examined, as 94% of nitrification was inhibited by acetylene, which inactivates ammonia-monooxygenase and thus suppresses autotrophic nitrifier activity (Hyman and Wood 1985). This predominance of autotrophic nitrification was also evident from the effect of the fungal inhibitor cycloheximide, which showed less inhibitory effect on nitrification than that of acetylene. However, the effect of cycloheximide was much greater than what would be expected from the effect of acetylene. The specificity of acetylene on autotrophic nitrifiers was questioned by Castaldi and Smith (1998) who suggested that fungi may oxidise $NH_4^+$ via an enzyme similar to ammonia-monooxygenase. Furthermore, it is also suggested that heterotrophic bacteria may also produce ammonia-monooxygenase-like enzymes (Daum et al. 1998; Lin et al. 2010; Moir et al. 1996).

Therefore, a possible non-target effect of acetylene on heterotrophic organisms could result in an overestimation of autotrophic nitrification. Furthermore, the use of cycloheximide can also be problematic, as $NH_4^+$ availability has been shown to increase following the addition of cycloheximide (Badalucco et al. 1994; Boyle et al. 2008; Castaldi and Smith 1998; Landi et al. 1993). While Boyle *et al.* (2008) attributed this increase in $NH_4^+$ to the combined effect of $NH_4^+$ assimilation inhibition by cycloheximide and the continued activity of extra- and intracellular enzymes that produce $NH_4^+$, it is also possible that increased $NH_4^+$ was caused by microbial utilisation of cycloheximide as a C substrate during the incubation, thereby stimulating decomposition activity (Badalucco et al. 1994) or the release of $NH_4^+$ from lysed microbial cells (Landi et al. 1993). While it is not possible to determine the cause of this increase in $NH_4^+$ availability, the effect of cycloheximide may explain the higher nitrification rates observed in the combined acetylene and cycloheximide treatment than the acetylene only treatment in this study. Therefore, a possible non-target effect on acetylene and the effect of cycloheximide on $NH_4^+$ availability could explain the disagreement between autotrophic and heterotrophic contribution to overall nitrification measured by acetylene and cycloheximide in this study.

Some studies from other grassland systems showed a much greater contribution of heterotrophic nitrification to total nitrification than found in this grassland (Cookson et al. 2006; Laughlin et al. 2009; Laughlin et al. 2008). Cookson *et al.* (2006) used a conceptual model to examine N transformations in a semiarid grassland in Western Australia and found that heterotrophic nitrification via organic N accounted for more than 50% of total $NO_3^-$ production. Using the $^{15}N$ dilution technique with selective bacterial and fungal inhibitors (streptomycin and cycloheximide, respectively), Laughlin *et al.* (2009; 2008) also found that N cycling in temperate grasslands in Northern Ireland and UK were dominated by fungi, demonstrating that fungi dominated $NH_4^+$ consumption in these grasslands. While the exact mechanism by which fungi oxidise $NH_4^+$ to $NO_3^-$ is still largely unknown, if the oxidation of $NH_4^+$ by fungi involves an ammonia-monooxygenase-like

enzyme, then that may also explain the discrepancy observed between studies that used biocide inhibitors and those that have used acetylene. Therefore, it is possible that autotrophic nitrification in this study may have been overestimated at the expense of fungal nitrification, which accounted for up to 60% of nitrification in *Themeda* soil when calculated from the inhibitory effect of cycloheximide. This is comparable to the findings of Cookson *et al.* (Cookson et al. 2006) who also examined the grassland community dominated by *Themeda*, thus further supporting the possibility of a non- target effect by acetylene on fungal nitrification.

The results from this study also showed that plant species differed in the inhibitory effect of cycloheximide, indicating that fungal nitrification rates may differ among the plant species. To my knowledge, there is only one study that examined fungal nitrification under different plant species using cycloheximide (Boyle et al. 2008). Boyle *et al.* (2008) found higher gross and net nitrification rates in red alder stands than Douglas fir stands owing to the high N inputs from red alder. These differences however were mediated by prokaryotes, not fungi. They also found that the effect of cycloheximide on $NH_4^+$ availability differed between the tree species examined, thus making it difficult to compare the effect of cycloheximide on subsequent nitrification, as it would have been affected by the differences in $NH_4^+$ availability. On the contrary, no differences in the effect of cyclohemixide on $NH_4^+$ availability were found among the plant species in this study, thus the differences observed in fungal nitrification were most likely due to the differences in the abundance and/or activity of fungi. Fungal biomass typically has a higher C:N ratio than bacterial biomass (Six et al. 2006; Swift et al. 1979), thus the higher soil C:N ratio observed under *Themeda* may reflect the higher abundance of fungal biomass under *Themeda* soil. Therefore, this study also highlights the importance of the fungal-to-bacterial biomass ratio in determining the relative contribution of fungal nitrification to overall nitrification in the grassland community.

*Conclusions*

The co-occurring plant species from a temperate grassland differed in N transformation rates as well as the abundance and activity of microbial groups that are involved in N mineralisation and nitrification processes. Plant species with greater abundance of autotrophic nitrifiers also had higher gross N transformation rates than the species with lower abundance of nitrifiers, thus highlighting the importance of autotrophic nitrifiers, particularly of AOA, in N cycling in this grassland community. While autotrophic nitrification was found to be the dominant nitrification pathway, a considerable contribution of fungal nitrification was also observed under $C_4$ species, *Themeda*, thus further strengthening the importance of the association between plant species and microbial community in determining N cycling and the relative contribution of different microbial groups to overall nitrification processes in this grassland.

# Chapter 4: Plant species effect on nitrification

## INTRODUCTION

Classically, nitrification was thought to be performed only by a specific group of bacteria, autotrophic nitrifying bacteria, involving two separate processes performed by two different bacterial groups. Firstly, ammonium ($NH_4^+$) is converted to nitrite ($NO_2^-$) by ammonia-oxidising bacteria (AOB) that use $NH_4^+$ to generate metabolic energy (Prosser 1989). The resultant $NO_2^-$ is released to the environment and rapidly utilised by nitrite-oxidising bacteria (NOB) which use $NO_2^-$ to generate energy, thus $NO_2^-$ rarely accumulates in the environment (Norton and Stark 2011; Prosser 1989). NOB are thought to be far more abundant in most environments, as they can also utilise organic matter and thus grow by heterotrophy (Bock 1976; Degrange et al. 1997) or grow by dissimilatory nitrate ($NO_3^-$) reduction where $NO_3^-$ produced is used by NOB as an electron acceptor in the presence of an organic electron donor and converted back to $NO_2^-$ (Freitag et al. 1987). The oxidation of $NH_4^+$ is therefore regarded as the rate-limiting step of the nitrification processes (Norton and Stark 2011).

The oxidation of $NH_4^+$ is catalysed by an enzyme called ammonia-monooxygenase (AMO) found in AOB (Hyman and Arp 1992). The discovery of the functional gene marker *amoA* that encodes for this enzyme allowed the problems associated with culture-dependent assays to be overcome, thus enabling finer-scale investigation and the improved quantification of AOB community size and activity (Kowalchuk and Stephen 2001; Rotthauwe et al. 1997; Stephen et al. 1999). The understanding of the genes involved in $NH_4^+$ oxidation has also led to the discovery of ammonia-oxidising archaea (AOA) that can also perform $NH_4^+$ oxidation (Konneke et al. 2005; Schleper et al. 2005). Extensive metagenomic studies on archaea have found putative AMO genes in

Crenarchaeota (Schleper et al. 2005; Treusch et al. 2005; Venter et al. 2004), suggesting the genetic capability of $NH_4^+$ oxidation by certain archaea. This possible archaeal $NH_4^+$ oxidation was confirmed by the cultivation of $NH_3$-oxidising crenarchaeon *Nitrosopumilus maritimus* from a seawater aquarium, which grew chemoautotrophically (Konneke et al. 2005). Since then, the abundance of archaeal *amoA* genes has been reported from various environments (Adair and Schwartz 2008; Francis et al. 2005; Leininger et al. 2006; O'Sullivan et al. 2011), prompting a question as to how important AOA are in mediating N cycling. The function of AOA and AOB is still not well understood, as they seem to behave differently in many aspects, such as their response to $NH_4^+$ availability (Di et al. 2009; 2010) and sensitivity to nitrification inhibitors (Hatzenpichler et al. 2008). Therefore, the functional importance of AOA in nitrification is still unclear (Bernhard et al. 2010; Gubry-Rangin et al. 2010; Wessen et al. 2010; Ying et al. 2010). However, this previously uncharacterised group has been showed to be numerically more abundant than AOB in many environments (Adair and Schwartz 2008; Bernhard et al. 2010; He et al. 2007; Leininger et al. 2006; O'Sullivan et al. 2011), suggesting that AOA could play an important role in the nitrification process.

Nitrification is not only performed by autotrophic nitrifiers (i.e. AOB and AOA) but also by heterotrophic organisms (De Boer and Kowalchuk 2001). While autotrophic nitrification has frequently been found to be dominant in many environments (Bollmann and Conrad 1997; Pennington and Ellis 1993), some studies have discovered a substantial contribution of heterotrophic nitrification (Cookson et al. 2006; Killham 1990) with some reporting that nearly all $NO_3^-$ production could be attributed to heterotrophic nitrification (Pedersen et al. 1999). One pathway by which heterotrophic nitrification occurs is when heterotrophic bacteria perform $NH_4^+$ oxidation using an enzyme that shares structural similarities to AMO (Daum et al. 1998; Hooper et al. 1997; Moir et al. 1996). However, unlike the case with AOB, the oxidation of $NH_4^+$ by heterotrophs is not linked to cellular growth (Bedard and Knowles 1989). The most well-known group of organisms that has the ability to oxidise $NH_4^+$ is methanotrophs, with all of the 104 isolates examined by Whittenbury *et al.*

(1970) producing $NO_2^-$ from $NH_4^+$. Such an ability has also been demonstrated for other heterotrophic bacteria, such as *Thiosphaera pantotropha* (Robertson et al. 1988) and *Pseudomonas putida* (Daum et al. 1998).

Another heterotrophic pathway in which $NO_3^-$ is produced is through fungal nitrification (Laughlin et al. 2008). Most research efforts have been directed at fungal nitrification in forest systems, due to the high fungal biomass in those systems (Fierer et al. 2009) and low pH which inhibits the growth and activity of autotrophic nitrifiers (Allison and Prosser 1993; De Boer and Laanbroek 1989). Therefore, the dominance of fungal nitrification is frequently reported in acidic forest systems (Killham 1990; Schimel et al. 1984). Recent studies, however, have shown that an important contribution of fungal nitrification is not limited to acidic forest systems (Killham 1990; Schimel et al. 1984) but is also found in other vegetation types including grasslands (Cookson et al. 2006; Laughlin et al. 2008). Thus, fungal nitrification should not be overlooked when examining key microbial processes that are responsible for $NO_3^-$ production, particularly in a natural system where environmental conditions (e.g. high litter C:N and low soil moisture) tend to favour fungal dominance over bacteria (Cornejo et al. 1994; Fierer et al. 2009).

Therefore, this study examined the contributions of AOA, AOB and fungi in $NO_3^-$ production in a native temperate grassland system dominated by a $C_4$ grass species *Themeda triandra* and a $C_3$ species *Austrodanthonia caespitosa*. As some N transformation processes have been shown to differ under different plant species (Osanai et al. 2012), the effect of plant species on the relative contributions of AOA, AOB and fungi to $NO_3^-$ production was also examined among the co-occurring grass species. The relative contributions of AOA and AOB to nitrification were assessed by examining the differences in sensitivity to temperature (Avrahami and Bohannan 2007; Oishi et al. 2012) and to a nitrification inhibitor allyl thiourea (ATU) between AOA and AOB (Hatzenpichler et al. 2008). This study also examined the recovery of nitrification after a short exposure to acetylene to quantify autotrophic nitrification and heterotrophic nitrification by inactivating AMO possessed by

autotrophic nitrifiers (Hyman and Wood 1985; Offre et al. 2009). By combining acetylene exposure with the application of bacterial and fungal protein synthesis inhibitors, the contribution of bacterial and fungal nitrification in the recovery was also examined. Specifically, the following questions were asked:

- Is there any evidence that the relative contributions of AOA and AOB differ in soils associated with different co-occurring grass species?
- Do the relative contributions of autotrophic and heterotrophic nitrification differ in soils associated with these grass species?
- Does the relative contribution of fungal nitrification differ in soils associated with these grass species?

## MATERIALS AND METHODS

*Soil sampling and processing*

Soil samples were collected in the spring of 2011 when both $C_3$ and $C_4$ species were actively growing. Samples were collected in three separate, randomly chosen sites within the grassland. At each site, soil samples were collected by taking four cores, each 35 mm in diameter, from the top 5 cm of the soil directly beneath each of the three grass species. These four cores per species per site were composited at the site, giving nine soil samples in total. Soil samples were transported to the laboratory on ice where they were homogenised by passing through a 4 mm-sieve which also removed large roots and litter. Sub-samples were taken for determination of relative water contents by drying in an oven at 105°C for 24 h. Total C and N concentration of soils were also determined using a Perkin Elmer 2400 Series II Elemental Analyser (Perkin Elmer Australia).

*Nitrification potential assay*

Nitrification potential was measured using a shaken slurry method with some modifications (Hart et al. 1994b). For each soil sample, 5 g of soil (fresh weight) was added to 50 ml of reaction buffer (pH 7.2) containing 0.3 mM potassium monobasic phosphate, 0.7 mM potassium dibasic phosphate and 0.75 mM ammonium sulphate in a 250 ml bottle. Supplemental 1 mM $NH_4^+$ was added to assure a maximum $NO_3^-$ production rate by reducing the possibility of $NH_4^+$ limitation. Soil slurries were shaken at 200 rpm on an orbital shaker for 48 h in a temperature-controlled growth cabinet to examine the effect of incubation temperature (18°C, 25°C and 35°C) on nitrification potential to assess the contribution of AOA and AOB in $NO_3^-$ production. An aliquot of soil slurry (1.8 ml) was taken periodically from the bottles (at 0, 6, 12, 18, 24, 36 and 48 h) and centrifuged for 3 min at 134000 x g to collect the supernatant for the determination of $NO_3^-$ concentrations using a SmartChem 200 discrete analyser (Westco, USA). To assess the contribution of AOA and AOB on nitrification potential, the effect of allyl thiourea (100 µM) was also examined with each of the three incubation temperatures. The effects of bacterial protein synthesis inhibitors (streptomycin and kanamycin), a fungal protein synthesis inhibitor (cycloheximide) and a fungicide (nystatin) were also assessed to examine their effect on nitrification potential rates at 25°C. The treatments consisted of four groups; bacterial inhibitors (+BI), fungal inhibitor plus fungicide (+FI+FC), bacterial plus fungal inhibitor (+BI+FI), all combined (+All) and no inhibitors (Control). All inhibitors of protein synthesis were applied to the soil slurries at the rate of 800 µl/ml and the fungicide nystatin was applied at the rate of 24000 unit/ml (Taylor et al. 2010). Nitrification potential was measured as above.

*Nitrification potential recovery assay*

The recovery of nitrification activity was examined by the method of Taylor *et al.* (2010) following the inactivation of AMO by a short exposure to acetylene gas. As acetylene works on this key enzyme that is produced by AOA and AOB, the change in $NO_3^-$ production following acetylene

removal should reflect the recovery of autotrophic nitrifier activity and thus indicate the activity of the initial autotrophic nitrifier population. The use of specific inhibitors should also allow the quantification of different microbial groups to the recovery, as demonstrated by Taylor *et al.* (2010). Nitrification potential was measured by a shaken slurry method with some modifications as above. Acetylene (0.025%) was applied to the headspace of 250 ml bottles containing slurries for the initial 6 h. At 6 h, applied acetylene was removed from the bottles by ventilating the bottles for 10 min in the open air, and the recovery of $NO_3^-$ production were measured for the following 42 h in the presence or absence of bacterial and fungal inhibitors as above to quantify the contribution of bacterial and fungal nitrification to the recovery of nitrification potential. To test for the efficiency of acetylene treatment and to evaluate heterotrophic nitrification, the assay also included acetylene-control samples where acetylene was applied for the entire incubation period.

*Statistical analyses*

Nitrification potential and nitrification potential recovery data were analysed by two-way analysis of variance (ANOVA) using general linear model procedures in the SAS statistical software package (SAS Institute Inc. 2003) to determine if plant species affected nitrification potential rates and the recovery rates in the presence or absence of specific inhibitors. Ryan-Einot-Gabriel-Welsch post hoc multiple comparison test was used where ANOVA indicated significant differences among the species (Day and Quinn 1989). Soil carbon and nitrogen data were analysed by one-way ANOVA to examine the plant species effect, followed by post hoc multiple comparison test as above.

## RESULTS

*Soil chemistry*

The soil C and N content did not differ among the plant species (Table 4.1), however, significant differences were found in soil C:N (*P*=0.02, Table 4.1). Soil C:N ratio was higher in *Themeda* soil than both *Austrostipa* and *Austrodanthonia* soils.

**Table 4.1** Soil total carbon (C) and nitrogen (N) contents and C:N ratios of soils collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa*. Different letters indicate statistical differences between the plant species (P<0.05).

| | C (%) | N (%) | C:N |
|---|---|---|---|
| *Themeda* | 2.83 ±0.38 | 0.20 ±0.03 | 13.90 ±0.17 a |
| *Austrodanthonia* | 3.24 ±0.26 | 0.25 ±0.02 | 13.11 ±0.06 b |
| *Austrostipa* | 3.30 ±0.48 | 0.25 ±0.04 | 13.16 ±0.21 b |
| $F_{2,9}$ | 0.44 | 0.88 | 7.89 |
| *P* | 0.66 | 0.46 | **0.02** |

*Nitrification potential*

The effect of ATU on nitrification potentials at three different temperatures was examined in order to quantify the contribution of AOA and AOB to nitrification potential, as ATU sensitivity and temperature optimum differ between these two autotrophic nitrifiers (Avrahami and Bohannan 2007; Hatzenpichler et al. 2008; Oishi et al. 2012). Plant species differed in nitrification potentials with *Themeda* soil having a lower nitrification potential than the soils from the other two species at all temperatures (Fig. 4.1, *P*=0.01). While the incubation temperature had a negligible effect on nitrification potentials overall, small differences in the response to the temperature treatment among the plant species were such that a substantial difference was observed between *Themeda*

soil and *Austrodanthonia* soil at 35°C where *Themeda* only produced 60% of the $NO_3^-$ produced by *Austrodanthonia* soil (Fig. 4.1). There were no differences in nitrification potentials between *Austrodanthonia* and *Austrostipa* soils at any temperature (Fig. 4.1). Allyl thiourea, which has been shown to completely suppress AOB activity (Hatzenpichler et al. 2008), only suppressed nitrification potentials by 24%, 19% and 17% on average at 18°C, 25°C and 35°C, respectively, indicating that the contribution of AOB to nitrification potentials was rather small in these soils. A substantial inhibition by ATU was only observed in *Austrodanthonia* soil, in which nitrification potential was reduced by 26% ($P$=0.06), and thus AOB would seem to make a greater contribution to nitrification in *Austrodanthonia* soil than in that of the other species. While there were no clear differences in nitrification potentials in the presence of ATU among the plant species at 18°C and 25°C, the nitrification potential of *Austrodanthonia* soil was slightly higher than that of *Themeda* soil at 25°C. While there were no interaction effects of temperature and ATU treatments among the plant species, plant species showed different responses to ATU at different temperatures (Fig. 4.1). In *Themeda* soil, the effects of ATU were similar at all temperatures, being ~28% inhibition on average. On the other hand, ATU had no inhibitory effect on nitrification potential at 35°C in *Austrostipa* soil while it reduced the rate by 35% at 35°C in *Austrodanthonia* soil, suggesting that temperature might affect the nitrification activity of AOA and AOB populations differently in the different plant species.

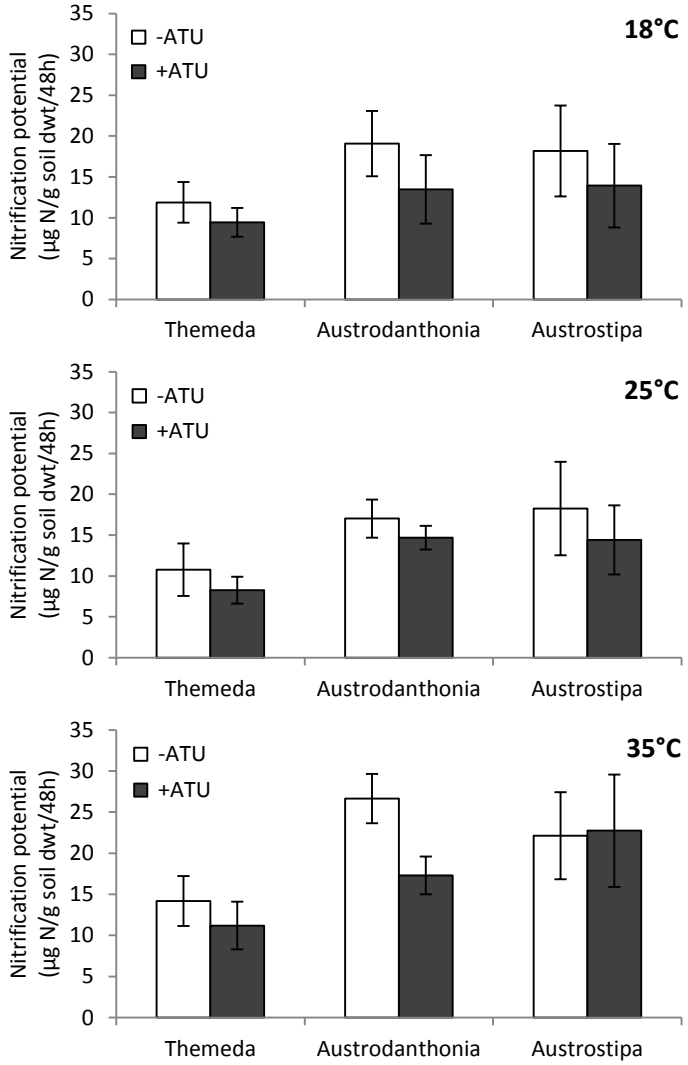


**Fig. 4.1** The effect of incubation temperature and nitrification inhibitor allyl thiourea (ATU) on nitrification potential of soils collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa* during 48h laboratory incubation. Data are means±SEM

Nitrification potentials were also examined in the presence of bacterial and fungal inhibitors, and the application of the inhibitors mostly resulted in reduction in nitrification potentials (Table 4.2). However, due to the high variability within each plant species, statistical significance of the treatment effects was only observed in *Austrodanthonia* soil. In *Austrodanthonia* soil, the presence of the fungal inhibitor cycloheximide significantly and substantially (54%) reduced $NO_3^-$ production from 17.0 µg g soil$^{-1}$ dwt at 48 h in Control to 7.8 µg g soil$^{-1}$ dwt on average in treatments that

included cycloheximide (*P*=0.03), while the treatment without cycloheximide (i.e. bacterial inhibitor only) did not differ in the amount of $NO_3^-$ produced from the Control. This lack of reduction in nitrification potentials in the presence of bacterial inhibitors was especially pronounced in *Austrodanthonia* soil, as nitrification potential was reduced by only 15% in *Austrodanthonia* soil compared to the reduction of 39% and 31% in *Themeda* and *Austrostipa* soils respectively. Therefore, these results may indicate the greater contribution of fungal nitrification in soils under *Austrodanthonia* than the other two species. When all the inhibitors were applied, the greatest suppression of nitrification potential was only observed in *Themeda* soil (Table 4.2).

**Table 4.2** Nitrification potential (µg N/g soil dwt/48h) of soil collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa* during 48 h laboratory incubation at 25°C. BI = bacterial inhibitors, FI = fungal inhibitors, FC = fungicide. Different letters indicate statistical differences between the treatments (P<0.05).

| | *Themeda* | *Austrodanthonia* | *Austrostipa* |
|---|---|---|---|
| Control | 10.77 ±3.21 | 17.03 ±2.34 a | 18.25 ±5.70 |
| + BI | 6.58 ±2.40 | 14.45 ±3.98 ab | 12.61 ±4.49 |
| + FI + FC | 6.79 ±2.44 | 7.40 ±0.80 b | 9.41 ±3.48 |
| + BI + FI | 5.66 ±1.66 | 7.60 ±1.27 b | 7.95 ±3.18 |
| + All | 5.16 ±1.69 | 8.46 ±1.11 b | 8.49 ±3.18 |

*Nitrification potential recovery*

The recovery of nitrification potential was measured by applying acetylene for the first 6 h of the incubation to suppress autotrophic nitrification activity and examining the recovery following the removal of acetylene. At the end of the acetylene treatment phase (i.e. 0 h to 6 h), $NO_3^-$ production was reduced by 50%, from 1.67±0.4 µg g $soil^{-1}$ dwt in Control (i.e. no acetylene treatment) to 0.8±0.4 µg g $soil^{-1}$ dwt in samples subjected to acetylene. The efficiency of acetylene was also tested by the

inclusion of samples with continuous acetylene application over 48 h, which suppressed nitrification potential by 97% in *Austrodanthonia* soil and by 91% in *Themeda* and *Austrostipa* soils (Fig. 4.2). Nearly 80% of the total $NO_3^-$ accumulated during this continuous acetylene treatment was produced in the first 6 h, with $NO_3^-$ production being very slight from 6 h onwards (Fig. 4.2). Thus, these results also indicate that only a small proportion of $NO_3^-$ (less than 10%) was produced heterotrophically. For the rest of the samples, the recovery of nitrification potential was measured from 6 h onwards. As acetylene irreversibly inactivates AMO, the enzyme responsible for the oxidation of $NH_4^+$, the recovery of $NH_4^+$ oxidation activity requires *de novo* protein synthesis (Hyman and Arp 1992). Therefore, by using inhibitors of bacterial and fungal protein synthesis, the contribution of bacteria and fungi in the recovery of nitrification potential was examined. There was a slight lag in the onset of the recovery, as noticeable increases in the nitrification potential were not observed until 18 h after the removal of acetylene (Fig. 4.2). The recovery in the absence of inhibitors was similar among the plant species, with $NO_3^-$ production recovered to 5.0 to 6.7 µg g $soil^{-1}$ dwt (~38% of $NO_3^-$ produced in the untreated control) at the end of 48 h. The proportion of nitrification potential recovered was the highest in *Themeda* soil with 46%, followed by *Austrostipa* soil with 37% and *Austrodanthonia* soil with 34%.

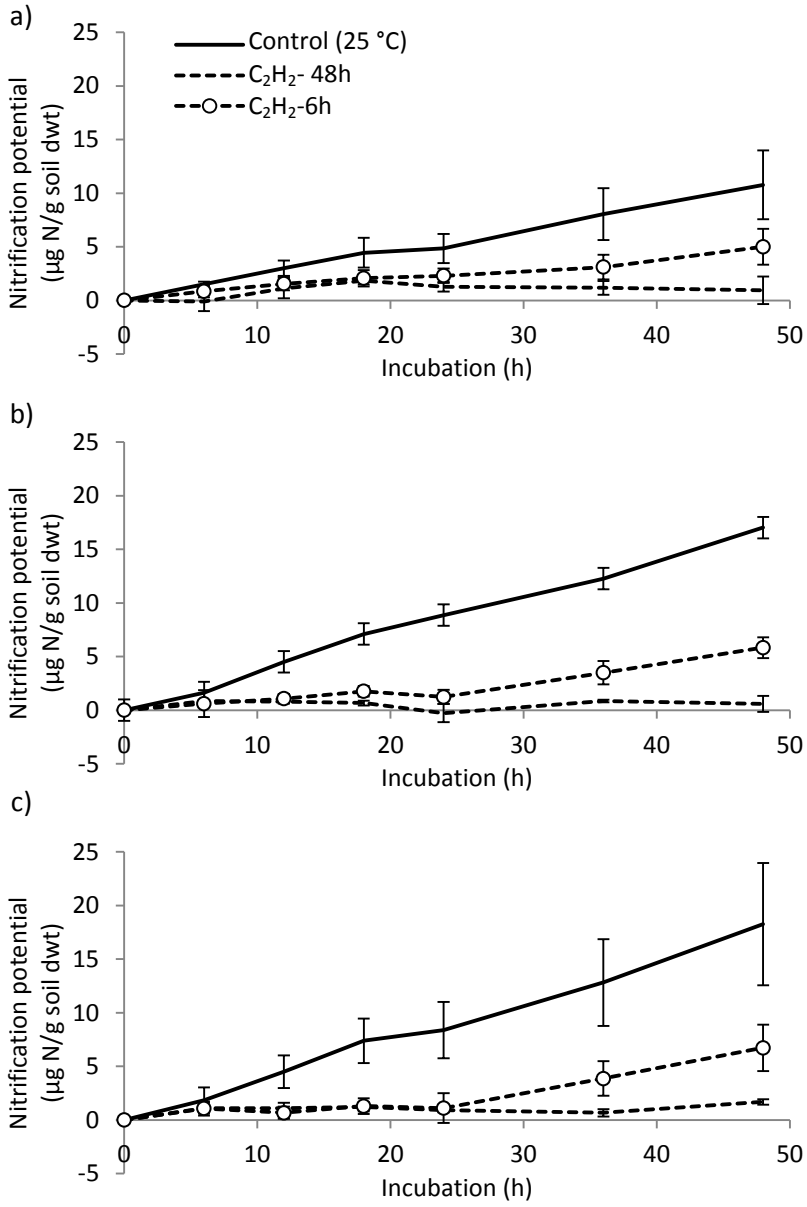


**Fig. 4.2** The effect of acetylene ($C_2H_2$) applied for 6 h (dashed line open symbols) and 48 h (dashed line no symbols) compared to controls (solid line no symbols) on nitrification potential of soils collected from underneath *Themeda* a), *Austrodanthonia* b) and *Austrostipa* c) during 48 h laboratory incubation at 25°C. Data are means±SEM

The addition of bacterial inhibitors reduced the recovery of nitrification potential by 10% in *Themeda* soil compared to the recovery without the inhibitors (Table 4.3), while it caused an increase of 25% in *Austrodanthonia* soil and of 22% in *Austrostipa* soil, thus suggesting a relatively small contribution of bacterial activity in the recovery of nitrification potential. These results however could also

indicate the possibility of microbial utilisation of bacterial inhibitors which enhanced $NO_3^-$ production in *Austrodanthonia* and *Austrostipa* soils. The addition of a fungal inhibitor combined with a fungicide on the other hand showed a significant suppression of the recovery in all species ($P$<0.0001), with only 1.8 to 3.5 µg g soil$^{-1}$ dwt of $NO_3^-$ produced (~18% of $NO_3^-$ produced in the untreated control) at the end of 48 h. The proportion of reduction was the greatest in *Themeda* soil with 65%, followed by *Austrodanthonia* and *Austrostipa* soils both with 48%, indicating that almost half of the nitrification recovered after acetylene treatment was performed by fungal activity.

**Table 4.3** The recovery of nitrification potential rates (µg N/g soil dwt) after the removal of acetylene ($C_2H_2$) at 6 h and of the continuous acetylene treatment ($C_2H_2$ 48h) of soil collected from *Themeda*, *Austrodanthonia* and *Austrostipa* during 48 h laboratory incubation at 25°C. BI = bacterial inhibitors, FI = fungal inhibitors, FC = fungicide. Different letters indicate statistical differences between the treatments (P<0.05).

| | *Themeda* | *Austrodanthonia* | *Austrostipa* |
|---|---|---|---|
| $C_2H_2$ 6h | 5.00 ±1.68 | 5.82 ±0.97 ab | 6.72 ±2.16 |
| $C_2H_2$ 6h + BI | 4.49 ±1.08 | 7.80 ±1.26 a | 8.61 ±2.72 |
| $C_2H_2$ 6h + FI + FC | 1.76 ±0.63 | 3.01 ±0.61 b | 3.47 ±1.58 |
| $C_2H_2$ 6h + BI + FI | 2.21 ±1.57 | 3.91 ±0.57 b | 4.09 ±0.82 |
| $C_2H_2$ 6h + All | 2.89 ±0.84 | 3.29 ±0.87 b | 3.34 ±1.32 |
| $C_2H_2$ 48h | 0.94 ±1.29 | 0.59 ±0.75 | 1.67 ±0.26 |

The nitrifying activity of the acetylene-insensitive microbial community was also assessed by applying both bacterial and fungal protein synthesis inhibitors to examine the production of $NO_3^-$. The effect of these combined inhibitors were slightly less than that of combined fungal inhibitor and fungicide, suppressing the recovery of nitrification potential by 56% in *Themeda* soil, followed by *Austrostipa* soil with 39% and *Austrodanthonia* soil with 33%. Thus, these results suggest that the application of the fungal protein inhibitor cycloheximide had the greatest inhibitory effect on $NO_3^-$ production and that fungal activity contributes substantially to the production of $NO_3^-$ in these soils. Furthermore, the effect of combined bacterial and fungal protein synthesis inhibitors was lower in *Austrostipa* and *Austrodanthonia* soils than that of *Themeda* soil, further suggesting that the

bacterial inhibitors may have been used as organic substrates by microbes and thus stimulated $NO_3^-$ production in these soils.

## DISCUSSION

This study examined the effect of plant species on nitrification potentials using various inhibitors in order to investigate the contributions of archaea, bacteria and fungi to $NO_3^-$ production. Due to the large variability within each grass species, the soils associated with different grass species did not differ greatly in the contribution of different microbial groups to $NO_3^-$ production. The responses to acetylene, which inactivates AMO possessed by autotrophic nitrifiers, suggest that $NO_3^-$ production was dominated by the activity of autotrophic nitrifiers, AOA and AOB, and that heterotrophic nitrifiers contributed minimally in this grassland soil. However, $NO_3^-$ production was substantially reduced by the fungal inhibitor cycloheximide, contradicting the results obtained from the acetylene treatment. Thus, this study demonstrates the problems associated with the selectivity and efficiency of these inhibitors, as different conclusions may be reached as to the relative importance of these microbial groups to $NO_3^-$ production depending on the choice of inhibitors used to suppress certain groups of soil microbes.

The suppression of autotrophic nitrification using biochemical inhibitors often results in substantial reduction in nitrification rates in various systems due to the predominance of autotrophic nitrification over heterotrophic nitrification (Bollmann and Conrad 1997; Pennington and Ellis 1993). This study also found this predominance of autotrophic nitrification in temperate grassland soils, as over 90% of nitrification was inhibited by acetylene which inactivates AMO that catalyses the first step of nitrification processes performed by AOB and AOA (Gubry-Rangin et al. 2010; Hyman and Wood 1985; Offre et al. 2009). This contrasts with some of the findings from other grassland

systems (Cookson et al. 2006; Laughlin et al. 2008) as well as the findings from the earlier study conducted at the same grassland where heterotrophic nitrification accounted for over 70% of the total nitrification under *Austrodanthonia* (Osanai et al. 2012). While the differences in experimental setups such as types and amounts of inhibitors used, incubation temperature and duration, and soil processing could affect the outcomes of such experiments, it is also possible that nitrification processes are highly dynamic and that the importance of different microbial groups in $NO_3^-$ production may change substantially through time.

Nitrification inhibitors that target AMO have been frequently used to examine the contribution of autotrophic nitrifiers and heterotrophic nitrifiers to $NO_3^-$ production (Osanai et al. 2012; Pedersen et al. 1999; Pennington and Ellis 1993). AMO has a broad substrate range for catalytic oxidation, with over 40 compounds that can competitively inhibit $NH_4^+$ oxidation having been identified as substrates of this enzyme (McCarty 1999). Acetylene is one of the most potent inhibitors AMO activity and irreversibly inactivates the enzyme (Hyman and Wood 1985), thus the recovery of nitrifying activity requires *de novo* protein synthesis (Hyman and Arp 1992). Studies that use acetylene as the inhibitor of autotrophic nitrification often find the majority of nitrification to be autotrophic (Bollmann and Conrad 1997; Herrmann et al. 2007; Taylor et al. 2010), even in environments that are thought to inhibit autotrophic nitrifier activity (Hayatsu and Kosuge 1993; Pennington and Ellis 1993). The selectivity of acetylene for autotrophic nitrifiers, however, has been questioned in some studies due to its inhibitory effect on methane-monooxygenase of methanotrophs that can also oxidise $NH_4^+$ (Roy and Knowles 1995) and thus the possible involvement of AMO-like enzymes of heterotrophic nitrifying bacteria (Hooper et al. 1997; Moir et al. 1996). While fungal nitrification has been thought to occur through an organic pathway (Islam et al. 2007; Pedersen et al. 1999), there is evidence that fungi can oxidise $NH_4^+$ (Laughlin et al. 2008), thus the effect of acetylene on fungal nitrification is also unclear (Castaldi and Smith 1998). Therefore, the possible inhibitory effect of acetylene on heterotrophic nitrifiers may have caused

overestimation of autotrophic nitrification in those studies, and thus may also explain the discrepancy between the inhibitory effects of acetylene and the other inhibitors on nitrification rates in this study.

The contribution of autotrophic nitrification and heterotrophic nitrification is also studied using selective inhibitors that target either bacterial or fungal growth and activity (Boyle et al. 2008; Castaldi 2005; Castaldi and Smith 1998; Landi et al. 1993). Most inhibitors work by interfering with protein synthesis of either prokaryotic or eukaryotic cells, thereby leading to a reduced cellular growth or even death of affected cells (Badalucco et al. 1994). Bacterial inhibitors such as those derived from *Streptomyces* are commonly used in laboratory incubation studies to separate bacterial activity from fungal activity (Alphei et al. 1995; Crenshaw et al. 2008; Landi et al. 1993; Susyan et al. 2005; Zeglin et al. 2011). While some studies have claimed to successfully demonstrate the validity of these inhibitors (Alphei et al. 1995; Velvis 1997), others have questioned their ability to suppress target organisms selectively and effectively (Bailey et al. 2003; Nakamoto and Wakahara 2004; Rousk et al. 2009). This study found a much weaker effect of bacterial inhibitors on nitrification compared to that of acetylene, which could imply either incomplete suppression of bacterial activity or the suppression of heterotrophic nitrification by acetylene. While it is possible that this weaker response to bacterial inhibitors may have been due to the dominance of AOA in autotrophic $NO_3^-$ production, the substantial contribution of fungi in $NO_3^-$ production was also evident from the effect of fungal inhibitors cycloheximide and nystatin, which also does not agree with the results from acetylene treatment. While the possible effect of acetylene on fungal $NH_4^+$ oxidation is largely unknown (Castaldi and Smith 1998), non-target effects of cycloheximide on bacteria and archaea have been reported in the literature (Taylor et al. 2010; Velvis 1997), thus questioning the selectivity of cycloheximide on fungal community. Furthermore, when these inhibitors were applied in combination in this study, it did not always result in the greatest inhibition on nitrification. Therefore, the discrepancy in the estimates of autotrophic and heterotrophic nitrification could also

be attributed to the differences in the efficiency of these inhibitors to suppress target organisms selectively and effectively.

Another problem concerning the use of biochemical inhibitors is that these inhibitors themselves can become substrates for microbial activity, thereby influencing nitrification rates (Badalucco et al. 1994; Landi et al. 1993). In this study, the addition of bacterial inhibitors did not suppress the recovery of nitrification but instead increased it in two $C_3$ species. Landi *et al.* (1993) also reported that the addition of streptomycin increased $NO_3^-$ production substantially in the first 24 h in unamended soils, while a complete lack of inhibitory effect of bacterial inhibitors for pasture soils was observed by Taylor *et al.* (2010), leading to a conclusion that nitrification in these pasture soils were not mediated by AOB. While it is possible that the lack of inhibitory effect was due to the low abundance of AOB in these soils examined, it is interesting to note that the stimulation of $NO_3^-$ production in the presence of the bacterial inhibitors occurred in the soils of both $C_3$ species but not of $C_4$ species. As the abundance and activity of heterotrophic microbial community is strongly influenced by plant growth and activity that affect organic substrate supply to the soil (Hooker and Stark 2008; Lu et al. 2002), it is reasonable to suggest that the microbial community may have been less abundant or active under warm-season $C_4$ species than cool-season $C_3$ species when the study was conducted in spring. If so, the difference in response to the bacterial inhibitors between $C_3$ and $C_4$ soils could be explained by the difference in the abundance or composition of the heterotrophic community that can utilise the inhibitors as organic substrates in these soils. Furthermore, it is also possible that this increase in $NO_3^-$ production may have been caused by a shift in the microbial utilisation of soil organic matter from assimilation to mineralisation. As bacterial inhibitors interfere with *de novo* protein synthesis, it is possible that soil organic matter was used to maintain cellular activity rather than cellular growth thereby causing net mineralisation to prevail. Such increase in net mineralisation is likely to be more pronounced when the heterotrophic microbial population is abundant. Therefore, this could indicate that greater N mineralisation may have occurred in soils of

$C_3$ species than $C_4$ species in the presence of bacterial inhibitors. It is therefore likely that $C_3$ and $C_4$ species differed in their associated microbial community at the time of sampling and thus repeated sampling throughout a year is necessary to thoroughly understand the effect of plant species on nitrification processes.

The effect of plant species on $NO_3^-$ production in their soils was clearly evident, as nitrification potential was substantially lower in soils collected under the $C_4$ species *Themeda* than in soils from under the $C_3$ species *Austrodanthonia* and *Austrostipa*. It is thought that the difference in nitrification potential most likely reflects the relative differences in the abundance of ammonia-oxidisers among the plant species, due to the slow growth rate of ammonia oxidisers (Paul 2007). Some plant species have been shown to affect nitrifying activity by secreting organic compounds from roots in order to actively inhibit nitrification (Fillery 2007; Subbarao et al. 2009; Subbarao et al. 2007). These compounds inhibit autotrophic nitrification by blocking both AMO and hydroxylamine-oxidoreductase pathways (Subbarao et al. 2009). Subbarao *et al.* (2007) examined 18 plant species including pastures, cereals and legumes and showed that greater nitrification inhibition was observed in tropical $C_4$ grasses (e.g. *Brachiaria* sp.) than temperate $C_3$ grasses (e.g. *Lolium* sp.). While this study did not characterise organic compounds exuded by the co-occurring grass species examined, it is possible that *Themeda*, a $C_4$ species, may produce such compounds, thereby suppressing autotrophic nitrification as observed in other $C_4$ species (Subbarao et al. 2007). It is however also possible that the observed difference in nitrification rates between $C_3$ and $C_4$ species might be attributed to the difference in growing season between $C_3$ and $C_4$ species, thereby affecting the way in which plant species interact with their associated microbial community. Therefore, it is reasonable to suggest that the microbial community may have been more active under cool season $C_3$ species than warm season $C_4$ species when the sampling was done in spring, and therefore repeated sampling would be necessary to examine the generality of plant species effects on nitrification.

Until the recent discovery of AOA, studies on plant effects on nitrification have mostly focused on autotrophic nitrification by AOB community (e.g. Hawkes et al. 2005; Nugroho et al. 2006; Patra et al. 2006). Since the discovery of AOA in 2005, many studies have found that AOA are abundant in many environments (Bates et al. 2011; Francis et al. 2005; Gubry-Rangin et al. 2010; Long et al. 2012; O'Sullivan et al. 2011), prompting a question as to the role of AOA in N cycling (Francis et al. 2007; Hayatsu et al. 2008; Nicol and Schleper 2006). Despite the definitive evidence of $NH_4^+$ oxidation by AOA (Konneke et al. 2005), the functional role of AOA in N cycling has not fully been understood. Most studies have examined the correlation between bacterial/archaeal *amoA* gene copy number and nitrification rates to assess the relative importance/contribution of AOA and AOB, and results have been highly variable. Some studies found correlation between the abundance of AOB and nitrification (Di et al. 2010; Ying et al. 2010), while other found correlation between AOA and nitrification (Gubry-Rangin et al. 2010; Wessen et al. 2010; Yao et al. 2011). A different approach was taken by Taylor *et al.* (2010) who examined the relative contribution of AOA and AOB by utilising differences between AOA and AOB in temperature optimum (Avrahami and Bohannan 2007; Oishi et al. 2012) and sensitivity to nitrification inhibitor ATU (Hatzenpichler et al. 2008). They examined a range of soil types and concluded that AOA were more important than AOB or fungi in nitrification processes in pasture soils when compared to forest, cropped and fallowed soils based on the occurrence of nitrification at high temperature and insensitivity to nitrification inhibitor ATU and fungal inhibitors (Taylor et al. 2010). This study followed the same approach to examine the relative contribution of AOA in $NO_3^-$ production in a native grassland soil and found some differences in the pattern of ATU sensitivity at different temperatures among the plant species, thus suggesting the possible differences in the composition of autotrophic nitrifiers among the plant species. However, the magnitude of temperature and ATU effects was relatively small, and the effect of fungal inhibitors on $NO_3^-$ production was substantial. Therefore, further investigation is required to better quantify the contribution AOA in $NO_3^-$ production in this grassland.

*Conclusions*

The contribution of different microbial groups to $NO_3^-$ production under different co-occurring grass species showed slight but noticeable differences. The differences in $NO_3^-$ production under the grass species were likely to be influenced by temporal differences in plant activity and therefore the interactions between plants and soil microbial community, thus further study is required to understand the mechanisms by which plant species may affect the relative importance of different microbial groups in nitrification processes. Autotrophic nitrification seems to be the dominant pathway in this grassland soil, however, the heterotrophic nitrification by fungi can be substantial depending on the biochemical inhibitors used to measure their activity. Therefore, the specificity and efficiency of inhibitors as well as the modes of inhibition need to be thoroughly understood before they can be used for the quantification of nitrifying activity by different microbial groups. As $NO_3^-$ production by fungi occurs through various enzymatic processes and pathways, further research into fungal nitrification is required to fully understand the role of fungi which may play an important role in N cycling in this temperate grassland.

# Chapter 5: Litter decomposition

## INTRODUCTION

At a global scale, the decomposition rate is primarily influenced by climatic factors such as temperature and precipitation (Aerts 1997; Couteaux et al. 1995). At a more local scale, the effect of litter quality becomes a stronger determinant of carbon mineralisation (Aerts 1997; Cornwell et al. 2008), with evidence that decomposition is faster for plant species growing in fertile environments and that produce litter richer in nitrogen (N) than those that grow in infertile environments (Hobbie 1992; Hossain et al. 2010; Wardle et al. 2004). Many examinations of decomposition have been based on mass loss measurements using litter bags buried or placed in the field for extended periods (Parton et al. 2007). However, it can be hard to examine the effect of litter quality on decomposition using such a method, as environmental variables such as microclimates (Wang et al. 2010), faunal activity (Tian et al. 1992) and nutrient leaching (Handayanto et al. 1994) can interfere with the effect of litter quality. In recent years, laboratory incubation has become commonly used to study the influence of litter quality on decomposition-based net fluxes of carbon (C) and/or N (e.g. Jensen et al. 2005; Trinsoutrot et al. 2000). This method, in which net mineralisation of C and N is measured, not only enables the optimisation of the incubation environment to reveal the maximum effect of litter quality but also enables relatively rapid estimates of decomposition rates as compared to the litter bag method (Vanlauwe et al. 1997). Such laboratory studies have demonstrated relationships between N mineralisation and litter N content (Abiven et al. 2005; Constantinides and Fownes 1994; Jensen et al. 2005; Vanlauwe et al. 1996), litter C:N ratio (Jensen et al. 2005; Vanlauwe et al. 1996) and litter lignin content (Scott and Binkley 1997) as well as between C mineralisation with those quality parameters (Johnson et al. 2007; Osanai et al. 2012; Vanlauwe et al. 1996). Laboratory incubation studies are also useful in determining the proportion of soil C in various decomposition

pools as well as their turnover rates (de Graaff et al. 2010; Liu et al. 2009; Pendall et al. 2010), thus making a major contribution to our understanding of the global C cycle and global climate models.

Studies based on laboratory incubations, however, often show differences in the litter chemical characteristics that correlated best with mineralisation of C and N. Differences in methodological approach may explain such inconsistency in the effect of litter quality on C and N mineralisation rates. In a natural environment, litter decomposition involves the fragmentation of litter by physical weathering and faunal activity, both of which condition the litter for decomposition by the soil microbial community (Petersen and Luxton 1982; Seastedt 1984; Swift et al. 1979). Laboratory incubations often fragment added litter mechanically to achieve even mixing of litter with soil, thereby eliminating the confounding effect of leaf morphology on the litter chemistry effect and effectively circumventing the physical disruption of litter normally achieved by macroinvertebrates and physical processes. Differences in litter particle size used for incubation, however, can affect decomposition rates (Ambus and Jensen 1997; Angers and Recous 1997; Bremer et al. 1991; Swift et al. 1979). Decomposition rate generally increases with decreasing litter particle size especially in the early stages of decomposition (Ambus and Jensen 1997; Angers and Recous 1997), due to the increased surface to volume ratio, allowing the microbial community greater access to the organic material. This is particularly important in examining the relationship between litter chemistry and N mineralisation rates, as mineralisation-immobilisation turnover of N is both substrate- and time-dependent (Constantinides and Fownes 1994; Swift et al. 1979). Thus, any variation in litter particle size might distort the effect of litter chemistry on N mineralisation at a given time. Furthermore, fragmentation of plant tissue by grinding not only increases the contact surface but also breaks up structural bonds, thereby increasing microbial access to material otherwise chemically protected (Jensen 1994). Such an effect of litter size may also contribute to the differences in the effect of certain chemical properties such as lignin on decomposition rates (Jensen et al. 2005; Johnson et al. 2007; Vanlauwe et al. 1996).

The inconsistency amongst studies on litter chemistry effects on decomposition may also arise from the use of different parts of a plant. The majority of decomposition studies examine aboveground leaf materials and relatively fewer studies have focused on decomposition of both aboveground and belowground materials and their relationship with litter quality (Abiven et al. 2005; Johnson et al. 2007; Moretto et al. 2001; Osanai et al. 2012; Vanlauwe et al. 1996). While some studies have demonstrated a significant relationship between litter decomposition rates and litter chemistry using a wide range of plant materials that include various plant parts (e.g. Jensen et al. 2005; Trinsoutrot et al. 2000), other studies have found root materials to show no or reduced correlations between litter chemistry and mineralisation of C or N compared to the aboveground counterpart (Abiven et al. 2005; Moretto et al. 2001). Similarly, several studies have indicated the differential effects of litter chemistry on decomposition between green leaves and senesced leaves (Fonte and Schowalter 2004; Sanaullah et al. 2010). Thus, not only the particle size of litter but also the use of different plant parts could contribute to the inconsistencies observed in the effect of litter chemistry on decomposition.

Therefore, this study examines the effect of litter particle size and chemistry on C and N mineralisation in laboratory incubations using two perennial grass species important in Australian native grasslands and native pasture, namely *Themeda triandra* and *Austrodanthonia caespitosa*. Specifically, the study aimed to answer the following three questions:

- Does litter decomposition rate correlate with litter quality regardless of tissue type or particle size?
- Does decomposition rate differ intrinsically between tissue types independent of litter quality effects?
- Does litter size influence litter decomposition-quality relationships?

## MATERIALS AND METHODS

*Plant biomass harvests and soil sampling*

Plant and soil samples were collected on September 3rd 2009 from a native grassland community dominated by *Themeda triandra* and *Austrodanthonia caespitosa* at Pontville in southeastern Tasmania. Several whole tussocks of both *Themeda triandra* and *Austrodanthonia caespitosa* were collected with roots and soil intact, returned to the laboratory and processed immediately. Roots were separated from the aboveground biomass and then washed free of soil with deionised water in a sonic bath for 3 min. Shoots were separated into green and senesced leaf materials. All samples were dried at 60°C for 24 h. Soil was collected from beneath the sampled tussocks of *Themeda* and *Austrodanthonia*, sieved (4 mm) and combined into a single, homogenised soil sample. Visible plant materials (e.g. litter, roots) and gravel were removed by hand.

*Reciprocal soil incubation*

All plant materials were prepared in an identical fashion. Materials were either cut coarsely to 5 mm, ground in a coffee grinder to approximately 1 mm or ground to powder in a ball mill (MM200 Mixer Mill, Retsch GmbH, Haan, Germany). To eliminate the effect of existing soil organic matter interfering with the effect of litter amendment on the incubations, all incubations were done using 10 g of pasteurised, washed river sand to which 1 ml of a soil slurry was added. Soil slurries were prepared by mixing 50 g (fresh weight) of soil with 100 ml of distilled, deionised water. Thus, the soil microbial community was added to the incubation containers with a minimum of additional soil organic matter. Litter was added at the rate of 15 mg litter per gram of sand. Control incubations consisting of sand and the soil slurry but no added litter were also prepared. Three replicates were prepared for each incubation. Each incubation sub-sample was placed in a 60 ml plastic container in a 500 ml glass jar sealed with a lid equipped with a septum to allow headspace gas sampling. The sealed jars were incubated in the dark at 25°C. Gas samples (10 ml) were periodically obtained from

the headspace with a syringe and directly injected into an infrared gas analyser (LiCor LI-6262, John Morris Scientific, Melbourne, Australia) to estimate headspace $CO_2$ concentration. Gas samples were collected after 1, 2, 4, 6, 9, 12, 16, 21 and 28 days of incubation. After each measurement, lids were removed and headspace gas was allowed to equilibrate with the atmosphere, sealed and then re-sampled to gain initial headspace $CO_2$ concentration. C mineralisation was calculated as the difference in $CO_2$ evolution rate between the samples and the unamended control incubations.

**Fig. 5.1** Microcosm setup (left photo) and comparison of three different litter particle sizes (right photo), coarsely-cut (top), medium (middle) and finely ground (bottom) of fresh *Themeda* leaf litter.

Another set of litter-amended sand mixture was prepared as above and was immediately extracted with 2 M KCl for pre-incubation analysis for $NH_4^+$ and $NO_3^-$ concentrations. Briefly, each sample (10 g) was mixed with 100 ml 2 M KCl solution and shaken for an hour and then left to sit overnight. The mixtures were filtered using Whatman No. 42 filter paper and the extracts analysed for $NH_4^+$and $NO_3^-$ concentrations on an FIAstar 5000 flow-injection analyser (Foss Tecator AB, Hoeganaes, Sweden). After 28 days, the incubated samples were extracted with 2 M KCl for post-incubation analysis for $NH_4^+$and $NO_3^-$ concentrations, exactly as described above. Net ammonification was

calculated as the difference in $NH_4^+$ concentrations at the beginning and end of the incubation periods, while net nitrification was calculated as the difference in $NO_3^-$ concentrations. Potential N mineralisation was calculated as the change in mineral N ($NH_4^+ + NO_3^-$) over the incubation period.

*Decomposition rate calculations*

A two-parameter one-pool exponential decay model (Eqn. 1) was fitted to the daily respiration rates from each replicate sample to obtain the parameters $C_l$ and $k$:

$$dC/dt = C_l.e^{-kt} + r \quad (1)$$

where dC/dt is the microbial respiration rate at time $t$, $C_l$ is the labile C pool size, $k$ is the apparent decay constant of the labile pool and $r$ is the decomposition rate of the resistant C pool, which is considered to be constant (e.g. Dijkstra et al. 2006; Pendall et al. 2010). Data were fitted to Eqn. 1 using the non-linear curve fitting routine in SAS v. 9.2 (PROC NLIN), with the default Gauss-Newton algorithm (SAS Institute Inc. 2003).

*Statistical analyses*

All data were analysed by analysis of variance (ANOVA) using general linear model procedures in the SAS statistical software package, ver. 9.2 (SAS Institute Inc. 2003). A three-way ANOVA was used to test the differences amongst species, litter tissue types and litter particle sizes plus all interactions between them. All data were checked for normality and heteroscedasticity and were log-transformed where required. The Ryan-Einot-Gabriel-Welsch post hoc comparison was used to compare means where ANOVA indicated significant effects (Day and Quinn 1989). Correlation analyses were performed using SAS to estimate Pearson's correlation coefficient amongst initial chemical properties of litter, litter particle sizes and decomposition rate parameters.

## RESULTS

*Litter quality*

Tissue type had an overwhelming effect on litter quality (Table 5.1), as defined by C:N ratio ($P$<0.0001) but there was also a strong effect of species ($P$<0.0001) as well as a species × tissue type interaction ($P$<0.002). Thus, the C:N of senesced leaves of *Themeda* was 63.1±0.83 compared to 25.7±1.10 for green leaves and 35.5±0.85 for roots. For *Austrodanthonia*, however, the pattern was different with roots having the highest C:N of 76.3±1.61 compared to 26.4±0.46 for green leaves and 40.0±1.72 for senesced leaves. These differences in C:N were almost entirely driven by differences in N content, as tissue C content varied only slightly across tissue type and species. Litter size had a negligible influence on C, N and C:N.

**Table 5.1** Carbon and nitrogen contents and C:N ratio of green leaf, senesced leaf and root of *Themeda* and *Austrodanthonia* for each litter particle size (coarse, medium and fine).

| Species | Tissue type | Litter size | Carbon (%) | Nitrogen (%) | C:N |
|---|---|---|---|---|---|
| *Themeda* | Green leaf | Coarse | 41.88 ±0.46 | 1.53 ±0.09 | 27.46 ±1.25 |
| | | Medium | 42.82 ±0.21 | 1.98 ±0.04 | 21.65 ±0.53 |
| | | Fine | 41.76 ±1.42 | 1.49 ±0.04 | 28.07 ±0.32 |
| | Senesced leaf | Coarse | 40.77 ±0.42 | 0.63 ±0.02 | 64.85 ±2.13 |
| | | Medium | 39.55 ±0.07 | 0.63 ±0.01 | 62.81 ±0.91 |
| | | Fine | 40.42 ±1.63 | 0.66 ±0.03 | 61.56 ±0.41 |
| | Root | Coarse | 43.99 ±0.45 | 1.33 ±0.02 | 33.18 ±0.78 |
| | | Medium | 43.63 ±0.23 | 1.25 ±0.01 | 34.82 ±0.56 |
| | | Fine | 43.75 ±0.08 | 1.13 ±0.00 | 38.60 ±0.12 |
| *Austrodanthonia* | Green leaf | Coarse | 40.05 ±0.05 | 1.58 ±0.07 | 25.49 ±1.08 |
| | | Medium | 40.33 ±0.06 | 1.47 ±0.01 | 27.51 ±0.30 |
| | | Fine | 38.55 ±1.40 | 1.48 ±0.03 | 26.09 ±0.45 |
| | Senesced leaf | Coarse | 40.75 ±1.82 | 0.90 ±0.06 | 45.59 ±1.10 |
| | | Medium | 37.15 ±0.05 | 0.92 ±0.01 | 40.39 ±0.42 |
| | | Fine | 36.57 ±0.15 | 1.08 ±0.00 | 33.96 ±0.24 |
| | Root | Coarse | 43.57 ±0.25 | 0.55 ±0.01 | 79.24 ±1.23 |
| | | Medium | 43.48 ±0.28 | 0.55 ±0.01 | 79.13 ±1.94 |
| | | Fine | 42.46 ±0.48 | 0.60 ±0.01 | 70.39 ±0.44 |

*C mineralisation*

Litter particle size exerted a strong and significant influence on the total amount of C mineralised, the apparent labile C pool size and the inherent decomposition rate of the labile pool (Fig. 5.2). The total amount of C mineralised in the incubations over 28 d was strongly affected by litter size ($P$<0.0001), litter type ($P$<0.0001) and, to a lesser degree, plant species ($P$<0.02). However, these three terms interacted strongly ($P$<0.0001) indicating that the impact of litter size on total C mineralisation was dependent upon both plant species and tissue type (Fig. 5.2a). The litter effect was most evident in green and senesced leaf material where the amount of C mineralised declined as litter particle size decreased, with an exception of finely ground green *Austrodanthonia* leaf litter which had a higher C mineralisation than that of medium ground litter (Fig. 5.2a). The effect of litter size on total C mineralisation was more pronounced in *Themeda* litter, in which C mineralisation declined by 40% as litter size decreased from coarsely cut to finely ground for green leaf litter and by 59% in senesced leaf litter. In *Austrodanthonia* litter, total C mineralisation of medium ground litter was 16% and 21% lower than that of coarsely cut litter in green and senesced leaf litter respectively with finely ground litter intermediate (Fig. 5.2a).

The calculated labile C pool size ($C_l$) was much less affected by litter particle size, although there were some differences, most notably with root material (Fig. 5.2b). While there was again a significant species × litter type × litter size interaction ($P$<0.001), this was mostly driven by the influence of litter size on $C_l$ of *Themeda* root material, which was 37% higher when derived from medium and fine litter sizes than for the coarse litter size (Fig. 5.2b). There was no significant effect of litter particle size on labile pool size for the other tissue types of *Themeda* and no difference for any tissue type of *Austrodanthonia* ($P$>0.05). Plant species also had a very strong impact on labile C pool sizes estimated from root litter, as $C_l$ in *Themeda* root litter was 175% greater than that of *Austrodanthonia* root litter (Fig. 5.2b). The labile C pool size from *Themeda* root litter was also 224%

greater than in *Themeda* senesced leaf litter (Fig. 5.2b). *Austrodanthonia,* on the other hand, had similar labile C pool sizes in root and senesced leaf litter (Fig. 5.2b).

Litter size had a substantial impact on the estimation of the apparent decomposition rate of the labile C pool (*k*) in both species (Fig. 5.2c), although this effect was again dependent upon both species and litter type (species × litter size × litter type $P<0.01$). In general, the estimate of *k* tended to increase consistently with decreasing litter particle size (Fig. 5.2c), largely irrespective of litter source. Thus, when averaged across all tissue types and both species, estimates of *k* were nearly 60% higher for medium than for coarse litter particle sizes and almost double for fine than for coarse particle sizes. However, with senesced leaf material, the effect was even more pronounced with *k* increasing by 46% and over 180% with litter particle size declining from coarse, through medium to fine, respectively, for *Austrodanthonia* and increasing by 360% and over 850%, respectively for *Themeda* (Fig. 5.2c). This variation in the impact of litter particle size on estimates of *k* greatly affected comparisons between species (Fig. 5.2c), especially with senesced leaf and root materials. Thus, *k* of *Themeda* root litter was almost double that of *Austrodanthonia* for coarse and fine particle sizes, but only 50% greater for the medium particle size (Fig. 5.2c).

*Nitrogen mineralisation*

Just as the case with C mineralisation, potential net N mineralisation was affected by a 3-way interaction between plant species, tissue type and litter particle size ($P<0.0001$; Fig. 5.3a). The impact of particle size on net N mineralisation was more pronounced with *Themeda* litter than with *Austrodanthonia* litter and more pronounced with green leaves and roots than with senesced leaves (Fig. 5.3a). Incubation of coarse green leaf material of *Themeda* resulted in an increase in soil mineral N of 35.8 mg N $g^{-1}$ added N as compared to a decline in mineral N of 33.6 mg N $g^{-1}$ added N with finely ground material (Fig. 5.3a). Similarly, incubation of coarse root material of *Themeda* resulted in an increase in mineral N of 66.7 mg N $g^{-1}$ added N but no significant change in soil mineral

N when finely ground (Fig. 5.3a). These patterns of N mineralisation were dominated by changes in soil $NH_4^+$ concentration (Fig. 5.3b) with nitrification generally being only a minor contributor to net N mineralisation in these incubations (Fig. 5.3c). The exception occurred with coarsely cut root material of *Austrodanthonia* in which there was substantial net nitrification (Fig. 5.3c), however, changes in soil inorganic N were relatively small in *Austrodanthonia* litter than *Themeda* litter (Fig. 5.3). The strong effect of particle size on N mineralisation in *Themeda* resulted in both net mineralisation in some incubations, mostly those with large particle sizes, and net immobilisation or no net change in those with fine particles (Fig. 5.3).

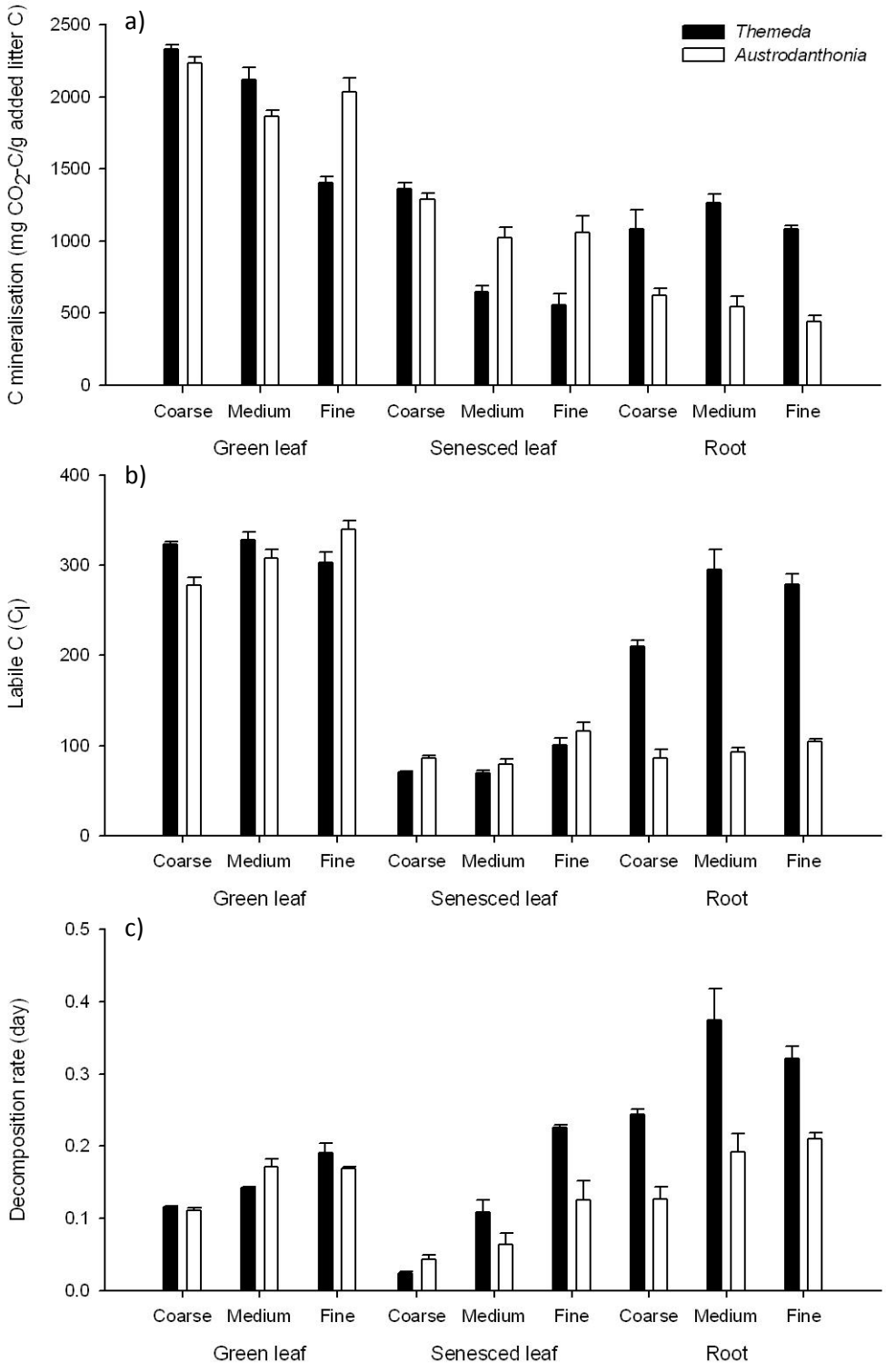


**Fig. 5.2** The cumulative C mineralisation a), labile C pool size b) and decomposition rate c) of green leaf, senesced leaf and root litter of *Themeda* and *Austrodanthonia* for each litter particle size (coarse, medium and fine), calculated from incubation curve fits from a 28d reciprocal transplant incubation. Data are means±SEM.

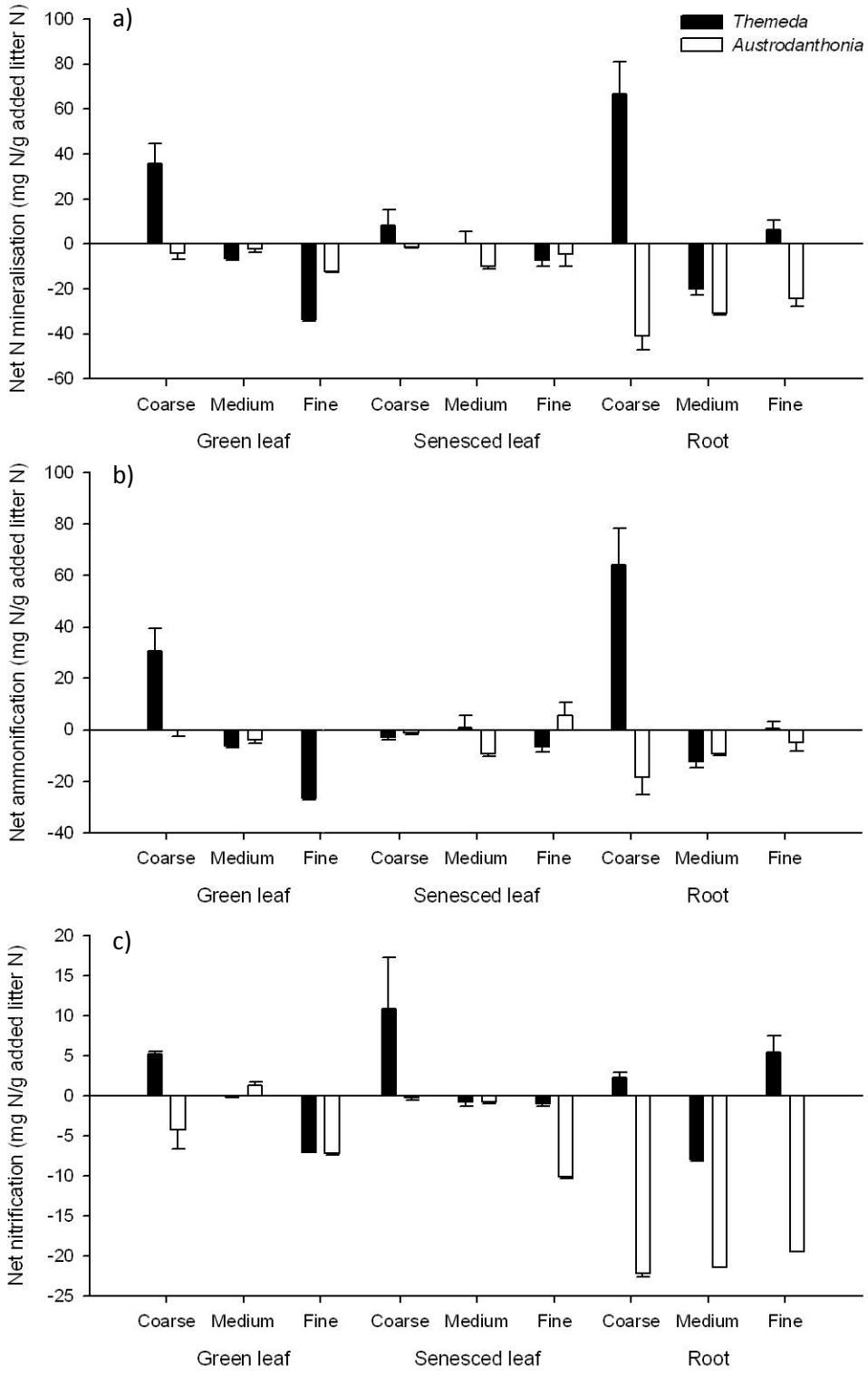


**Fig. 5.3** Net N mineralisation a), net ammonification b) and net nitrification c) of green leaf, senesced leaf and root litter of *Themeda* and *Austrodanthonia* for each litter particle size (coarse, medium and fine), from a 28d reciprocal transplant incubation. Data are means±SEM.

*Correlations between litter quality and C and N mineralisation*

Despite the overall correlation between the total C mineralisation and the initial litter N content ($r$=0.85, $P$<0.0001, Fig. 5.4a) and litter C:N ratio ($r$=-0.8, $P$<0.0001, Fig. 5.4b), the relationship between the total C mineralisation and litter N contents and C:N ratios showed a marked reduction in the correlation coefficients with coarse litter ($r$=0.79 and $r$=-0.77 for N contents and C:N ratio respectively) compared to those of medium-sized litter ($r$=0.97 and $r$=-0.91, respectively) and fine litter ($r$=0.91 and $r$=-0.88, respectively). Potential N mineralisation, ammonification and nitrification rates, on the other hand, did not correlate with any of the litter chemistry parameters, regardless of litter sizes.

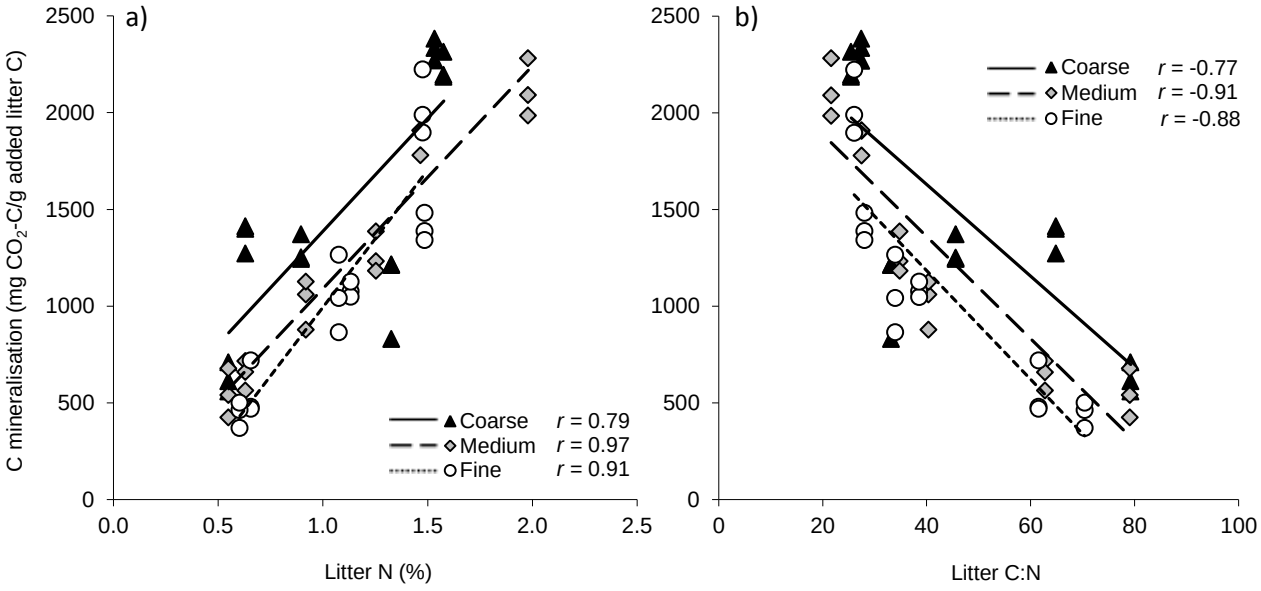


**Fig. 5.4** The relationship between cumulative C mineralisation rates and litter N contents a) and cumulative C mineralisation and litter C:N ratio b) by litter sizes (coarse, medium and fine) for all litter types (green leaf, senesced leaf and root) and litter species (*Themeda* and *Austrodanthonia*).

## DISCUSSION

Laboratory incubations are an important tool for those studying both organic matter decomposition and nutrient mineralisation and, as such, are widely used to examine the influence of various factors on these processes (e.g. Cochran et al. 2007; Feng and Simpson 2009; Trinsoutrot et al. 2000). Understanding such biogeochemical processes is vital for economic crop production (Abiven et al. 2005; Angers and Recous 1997; Angus et al. 2006; Collins et al. 1992) as well as understanding and predicting function of ecosystems (De Deyn et al. 2008; Kardol et al. 2010b). In particular, studying the mineralisation of organic C is a key component of efforts to disentangle various processes in the land surface-atmosphere relationship necessary for predicting future climates (Davidson and Janssens 2006; Luo et al. 2011; Luo and Weng 2011). Hence, it is important to know whether variation in the methods used to determine C and N mineralisation rates influences the results to a substantial degree, and most particularly whether such influences alter observed, presumed "fundamental" relationships involved in the underlying processes. This study aimed to determine whether litter quality correlated with litter decomposition regardless of tissue type or litter particle size, whether the intrinsic difference between tissue types influenced decomposition independent of any litter quality effect, and whether the litter decomposition-quality relationship was affected by litter particle size.

While the strength of correlation between litter quality and C mineralisation differed among litter sizes, this study found that C mineralisation generally correlated with litter quality, adding further support for the role of litter quality as a key determinant of litter decomposition (Ayres et al. 2009; Cornwell et al. 2008; Fornara et al. 2009; Melillo et al. 1982). Such a relationship was not observed, however, with N mineralisation, as N mineralisation was related to neither litter N content nor C:N ratio. Nitrogen limitation of the microbial community may explain the lack of correlation between the initial N contents and C mineralisation rates in this study. Nitrogen limitation of the soil microbial community has been suggested to cause N immobilisation rates to dominate over N

mineralisation rates (Trinsoutrot et al. 2000), thereby confounding the effect of litter quality on N mineralisation. Likewise, the opposite results were obtained from studies which were conducted under non-limiting soil N conditions (Abiven et al. 2005; Jensen et al. 2005; Trinsoutrot et al. 2000). In those cases, sufficiently high availability of soil N allowed net N mineralisation to take place as soil microbes decomposed litter for C uptake, causing net N mineralisation to correlate with the initial N contents of litter materials. Carbon mineralisation rates on the other hand did not correlate with the initial N contents in these studies (Abiven et al. 2005; Jensen et al. 2005; Trinsoutrot et al. 2000), as N was no longer the limiting factor for microbial activity (Sall et al. 2007). Therefore, the inconsistency in the effect of litter quality on decomposition in the literature could also be attributed to the differences in soil nutrient status affecting the relationship between litter quality and mineralisation of C and N differently as observed in this study.

Immobilisation of N is often observed in laboratory incubations where a rapid expansion of the microbial community occurs in response to an increase in substrate C availability due to litter amendments (Sall et al. 2007; Trinsoutrot et al. 2000). Thus, it is thought that the amount of mineralisable substrate and decomposition rate are interrelated and that the product of these two parameters, termed initial potential rate of mineralisation, can be a more precise estimate than only one parameter (Campbell et al. 1991; Saviozzi et al. 1993; Vahdat et al. 2010). Therefore, the product of decomposition rate and labile C pool ($C_l \cdot k$) was examined to see if there was any relationship with net N mineralisation rate. There was a positive relationship between $C_l \cdot k$ and net N mineralisation but only when litter was coarsely cut ($r$=0.69, $P$=0.001, Fig. 5.5). No such relationship was observed in ground litter ($r$=0.24 for finely ground litter, $r$=-0.22 for medium-sized litter). C and N dynamics are strongly linked during litter decomposition due to the simultaneous assimilation of C and N by the microbial community (Mary et al. 1996). While gross immobilisation and mineralisation have been found to correlate well with C mineralisation, as net N mineralisation is a product of these two opposing processes, it does not necessarily correlate with C mineralisation

(Luxhoi et al. 2006). Mineral N availability can strongly affect the mineralisation-immobilisation turnover of N, thereby affecting both C and N mineralisation rates and their relationship (Sall et al. 2007). Hence, the difference observed in the relationship between C and N mineralisation amongst litter sizes in this study may be due to the effect of grinding which induced N limitation of the microbial community by increasing C availability. Unground, coarsely cut litter, on the other hand, restricted C availability thereby causing N mineralisation to prevail over N immobilisation as decomposition proceeded (Jensen 1994). This study, therefore, highlighted the importance of litter size on the correlation between litter quality and mineralisation of C and N through its effect on substrate availability.

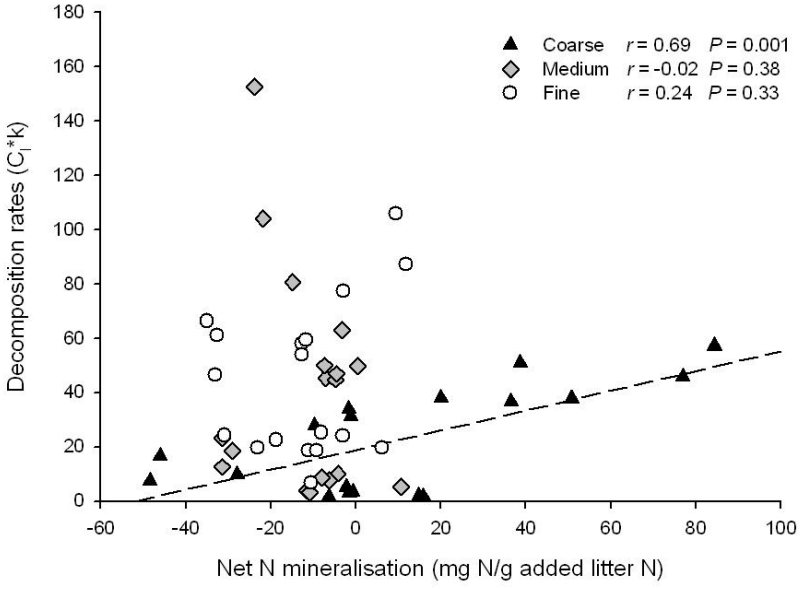


**Fig. 5.5** The correlation between decomposition rate and net N mineralisation as a function of litter particle size (coarse, medium and fine). Dash line indicates a significant correlation found between decomposition rates and net N mineralization rates in coarsely cut litter.

The effect of litter size on decomposition was also evident in the discrepancy between decomposition rates and the total amount of C mineralised during the 28-day incubation. In general,

the decomposition rate increases as litter particle size decreases (Ambus and Jensen 1997; Sims and Frederic 1970), but this was only evident for coarsely-cut litter in this study. Despite this, significantly more C was mineralised from coarse litter than from finely ground litter. Since there were no substantial differences in litter quality between the coarse and finely-ground material, this suggests that there might have been a shift in microbial community composition during the incubation. Fungi are more efficient in decomposing materials with high lignin contents (Romani et al. 2006) and their metabolism is characterised as having higher C use efficiency than that of bacteria (Bailey et al. 2002; Keiblinger et al. 2010). Grinding not only breaks up structural components that are inaccessible to certain microbial groups but also provides larger contact surface for microbial attack. Thus, ground litter may have provided a more favourable environment for heterotrophic bacterial growth (Holland and Coleman 1987), leading to a rapid release of $CO_2$ and exhaustion of C substrate. Thus, ground litter may only be able to sustain a large heterotrophic microbial population for a short period of time whereas coarsely-cut litter may be able to sustain a small population of certain microbial groups for a longer period of time. Therefore, differences in physical quality of litter could influence C mineralisation rates via shifts in microbial community composition.

Shifts in microbial community composition occur in the early stages of decomposition (Marschner et al. 2011), and thus the relationship between litter quality and decomposition could also be affected by the duration of the laboratory incubations (Jensen et al. 2005; Trinsoutrot et al. 2000). This study showed that a higher correlation was observed in the initial C mineralisation measured at day 1 with litter N ($r$=0.95, Appendix 2a) and with litter C:N ($r$=-0.85, Appendix 2b) than that of the cumulative C mineralisation at day 28. This is in agreement with the findings of Trinsoutrot *et al.* (2000) who found that the litter quality effect tends to be strongest in the early stages of decomposition, when labile organic matter from added litter is predominantly utilised by soil microbes. Such a change in the relationship between litter quality and litter decomposition rate was predominantly caused by the decline in the correlation coefficient of coarsely cut material from 0.96 at day 1 to 0.79 at day 28

for litter N and -0.87 at day 1 to -0.77 at day 28 for litter C:N (Appendix 2a, b). Thus, the effect of litter quality on C mineralisation rate is not only influenced by the microbial community composition but also by their utilisation patterns, which change during incubation.

The differences in decomposition traits amongst different parts of a plant have been attributed to the differences in chemical and physical makeup of tissue (Abiven et al. 2005; Hobbie et al. 2010). Root decomposition especially has been shown to be slower than leaf and/or stem decomposition, as the presence of the suberin-lignin complex in roots can both chemically and physically protect roots from microbial degradation (Abiven et al. 2005). The results showed that C mineralisation was much slower in roots than green leaves, but this difference was largely explained by the difference in litter quality. The comparative study between leaf and root decomposition by Hobbie *et al.* (2010) found that root and leaf decomposition were influenced by different tissue traits, and that the trait which correlates well with leaf decomposition rates may have no or reduced influence over root decomposition rates. In this study, senesced leaves decomposed more slowly than root material of a similar C:N in the early stage of incubation. Thus, these tissues may have differed in chemical composition in a way not reflected in litter C and N content or ratio. Senescence involves substantial changes in tissue chemistry (Aerts 1996; Sanaullah et al. 2010), so the senesced leaves used in this study might have been depleted in soluble C and N but still contained large amounts of structural C such as lignin and polyphenol (Fonte and Schowalter 2004), all of which have been found important in determining litter decomposition rates (Abiven et al. 2005; Constantinides and Fownes 1994; Vanlauwe et al. 1996). Thus, the differences amongst tissue types that were unaccounted for by the litter N contents or C:N ratios in this study are likely to be explained by the differences in those unmeasured chemical traits.

*Conclusions*

This study has demonstrated that both litter chemical quality and physical quality are important in determining litter decomposition rates. While C mineralisation rates were predominantly influenced by differences in litter chemical quality, N mineralisation showed a complex interplay of litter particle size and decomposition progress in affecting microbial N mineralisation-immobilisation balance. The differences in C mineralisation rates between the tissue types and litter species were largely driven by the differences in litter N contents and C:N ratios. Unlike C mineralisation, neither litter N contents nor C:N ratio correlated with net N mineralisation, most likely due to N limitation of the soil microbial community developed during the laboratory incubation. This N limitation of the microbial community was most pronounced in ground litter, which provided a greater C accessibility than unground litter, resulting in a poor correlation between C and N mineralisation rates. As for coarsely cut unground litter, there was a significant net N mineralisation as decomposition proceeded, indicating that litter particle size has an important influence on the balance between the mineralisation and immobilisation of N and thus its relation with C mineralisation through microbial substrate availability. Therefore, the results from this study suggest that litter size needs to be taken into a consideration when investigating C and N dynamics in laboratory litter decomposition studies in order to improve our understanding of the underlying processes and environmental control of organic matter mineralisation.

# Chapter 6: Litter decomposition under simulated global change

## INTRODUCTION

The net annual exchange of carbon (C) between the atmosphere and terrestrial ecosystems is of prime importance in determining the concentration of carbon dioxide ($CO_2$) in the atmosphere and consequently future climate (Heimann and Reichstein 2008). C storage in a terrestrial ecosystem depends upon the balance of C inputs from photosynthesis and C losses (Chapin et al. 2009). The losses largely arise as an efflux of $CO_2$ from respiration by autotrophs (plants and some bacteria) and heterotrophs (fungi, animals and some bacteria) with some other non-$CO_2$ losses such as methane gas and leached dissolved organic C (Chapin et al. 2009; Schulze et al. 2009).

Heterotrophic respiration is often the largest source of ecosystem C loss, frequently accounting for over 50% of the $CO_2$ efflux in grassland (Schulze et al. 2009), and occurs when microbes break down organic matter, mostly plant litter. Climate and soil type are important determinants of decomposition rate (Aerts 1997) but the rate can be controlled by the chemical composition of the litter (Cornwell et al. 2008) and/or the activity of the microbes (Ayres et al. 2009; Strickland et al. 2009). Both litter chemistry and microbial activity are known to be influenced by atmospheric $CO_2$ concentration and temperature (Blankinship et al. 2010; Cotrufo et al. 1998; Knops et al. 2007) which are currently changing and are projected to change further. Most studies with elevated $CO_2$ have shown increased soil respiration (Ross et al. 2002), which is frequently ascribed to increased plant and microbial activity (Adair et al. 2011) arising either from increased soil moisture content or greater C inputs. Similarly, warming consistently stimulates respiration (Dorrepaal et al. 2009; Rustad et al. 2001). However, if these results actually reflect a fundamental change in the function of the soil microbial community and an enhanced ability to mineralise organic matter, rather than

changes in C inputs, then future C storage in the soil may be much less than currently believed. Determining whether the function of the soil community is altered in response to environmental manipulations requires the separation of environmental controls and organic matter supply from intrinsic soil community influences on C mineralisation.

To test the actual C mineralisation ability of soil microbial communities developed in response to warming and elevated $CO_2$ manipulations, plant and soil samples from a long-running free air $CO_2$ enrichment (FACE) experiment on Australian native grassland were used to construct different combinations of litter and microbial communities in a reciprocal transplant experiment. Carbon mineralisation was measured in incubated artificial soil communities created by inoculating sterilised river sand with soil communities from experimental plots that had been exposed to warming, elevated $CO_2$ and their combination and controls. To each artificial community, root or shoot material from either $C_3$ or $C_4$ grasses from one treatment combination was added so that the study had a full reciprocal transplant arrangement. By controlling the origin of the soil community, the origin of the plant litter and incubating under standard conditions, it was possible to separate the influence of the soil community from the influence of the organic matter on C mineralisation. Specifically, this study aimed to answer the following questions:

- What is the relative importance of changes in litter quality and changes in the soil microbial function induced by simulated global change in influencing litter decomposition rates?
- Are there any differences in the influence of such changes on litter decomposition between $C_3$ and $C_4$ litters and for shoot and root materials?

## MATERIALS AND METHODS

*Study site*

The TasFACE climate change impacts experiment was established in a species-rich temperate grassland in southeastern Tasmania (42° 42' S, 147° 16' E, 40 m asl) in February, 2002 and has run continuously since then (Hovenden et al. 2006). The TasFACE experiment is located in the same native grassland as used in the previous five chapters of this thesis. Free-air $CO_2$ enrichment (550 µmol $mol^{-1}$ year-round) using pure $CO_2$ injection and warming (2°C) by ceramic infrared heat lamps (Salamanda ESE250 240 V/250 W Emerson Solid Ceramic Infrared Emitter) were applied in a full factorial design on 12, 1.5-m diameter plots (3 replicates, Fig. 6.1). Atmospheric [$CO_2$] was controlled to 549 ± 0.1 µmol $mol^{-1}$ in FACE plots, compared to 372 ± 0.3 µmol $mol^{-1}$ in control plots. Soil temperature at 5-mm depth (measured with Type-T thermocouples with a 1-min sample interval) in warmed plots was 1.8 ± 0.1°C higher than unwarmed plots at night time, and 0.14 ± 0.01°C higher during the day. Bulk density of the soil (1.8 ± 0.32 g $cm^3$, n = 42) did not vary among treatments in October 2009 (P>0.05). The climate of the study area has warm, dry summers and cool, moist winters, with mean annual precipitation since 2002 being 402 ± 34 mm and mean annual temperature of 11.6°C.

*Reciprocal transplant incubation*

A reciprocal transplant incubation was conducted using plant and soil materials collected from each of the 12 experimental plots consisting of unwarmed control (Control), warmed control (Warming), unwarmed FACE (FACE) and warmed FACE (F+W), which were replicated in three. Two soil cores were collected to 5 cm depth in two random locations in each plot. Soil was hand-picked to remove course organic matter and roots. A 50:50 soil-water slurry was prepared using 40 g soil and 40 ml distilled water for each experimental plot, thus 12 soil slurries were prepared. Standing shoot material was collected from both $C_3$-and $C_4$-dominated patches in each experimental plot by cutting

1 cm above ground level. Belowground plant material was collected from $C_3$- and $C_4$-dominated patches by coring to 5 cm. Root material was sorted by hand, washed to remove soil contaminations with associated microbial communities and air dried. Plant samples were pooled according to plant functional type and treatment. Thus, a single pooled sample was prepared for unwarmed control, warmed control, unwarmed FACE and warmed FACE for leaf and root matter for each of $C_3$ and $C_4$. Plant material was ground to powder in a Retsch MM200 ball mill. A sub-sample was removed for determination of C and N concentration in a Perkin Elmer 2400 Series II Elemental Analyser (Perkin Elmer Australia). The soil samples were also analysed for C and N concentrations and did not differ between the treatments.

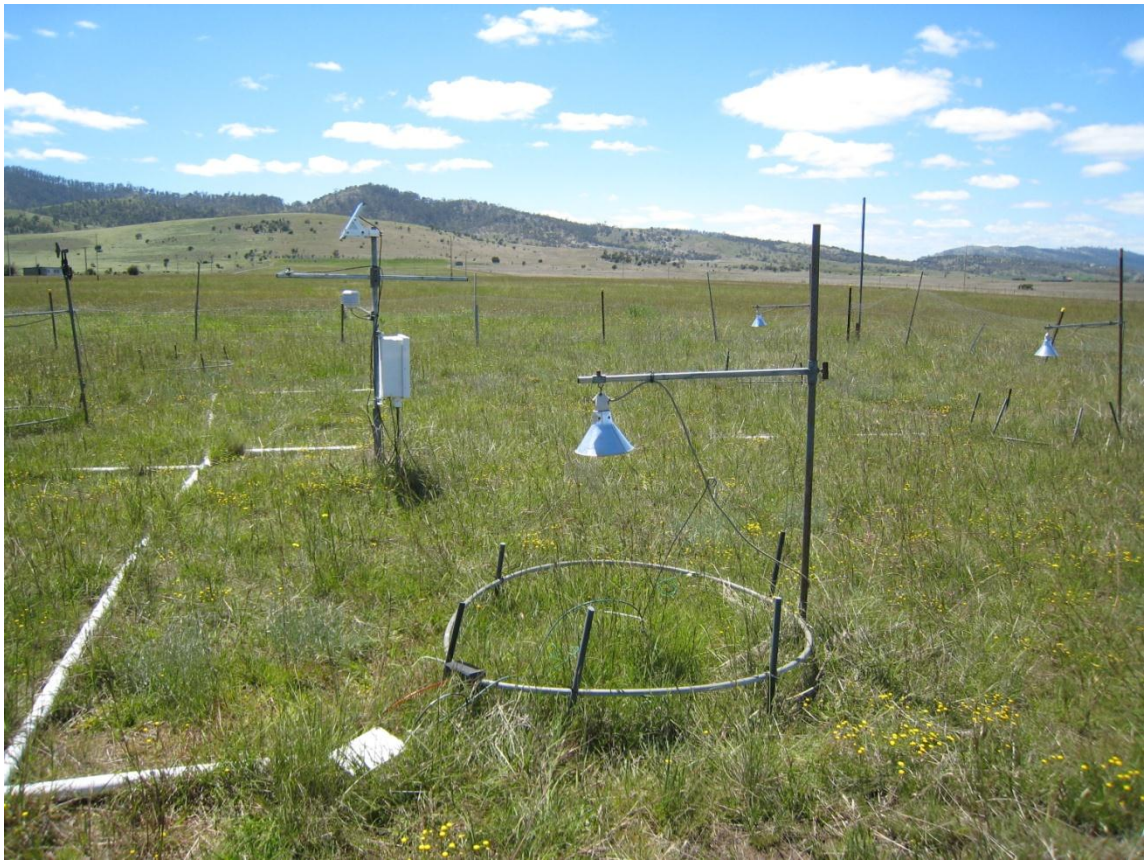

**Fig. 6.1** TasFACE climate change impacts experiment at Pontville, Tasmania

Artificial soil communities were constructed using 10 g pasteurised, washed river sand in 50 ml plastic cups. Twelve cups were each amended with 0.1 g of ground leaf or root material from either

$C_3$- or $C_4$-dominated vegetation from each of the four TasFACE treatments and mixed thoroughly. Soil communities were completed by adding 1 ml of soil slurry solution, described above, to each cup such that each of the 12 replicate soil-litter mixtures received one of the 12 soil slurries. Therefore, each different litter source (plant functional type, leaf/root, TasFACE treatment) was added to a soil inoculum from each TasFACE plot and conversely, each soil microbial community sample was added to every litter source, creating a full reciprocal transplant experiment. A soil slurry-blank was created for each TasFACE plot by adding soil slurry to 10 g of sand to which no litter was added. All microcosms were brought to 60% of water holding capacity with deionised water and incubated at 25°C in 500 ml jars with $CO_2$ efflux measured as previously described (in Chapter 5). Total $CO_2$ evolution was summed over the 28 d duration of the incubation. Daily soil $CO_2$ evolution rates were fit with a two-pool, three parameter exponential decay model to determine the apparent labile C pool size, $C_l$, as well as the apparent decay constant of the labile pool, $k$, and the intrinsic decay rate of the resistant C pool, $r$. Data were fit to the model using non-linear curve fitting procedures in the SAS statistical software package as described (in Chapter 5).

*Statistical analyses*

All data were analysed by analysis of variance (ANOVA) using general linear model procedures in the SAS statistical software package (SAS Institute Inc. 2003). Data from the reciprocal transplant experiment were analysed by 5-factor ANOVA with a model that included soil warming, soil elevated $CO_2$, litter warming, litter elevated $CO_2$ and plant functional type plus all interactions for each litter type (root or shoot). Litter chemical quality was analysed by a 3-factor model with litter warming, litter elevated $CO_2$ and plant functional type for each tissue type. All data were checked for normality and heteroscedasticity and log-transformed where necessary. Where ANOVA indicated there were significant effects, means were compared using the Ryan-Einot-Gabriel-Welsch *post hoc* comparison (Day and Quinn 1989).

## RESULTS

*Soil community effect on C mineralisation*

The source of the soil community had the greatest impact on C mineralisation in this experiment both for $C_3$ and $C_4$ litter. In other words, the exposure of the grassland ecosystem to experimental warming and the elevation of $CO_2$ concentration altered the function of the soil microbial community in terms of its ability to decompose plant litters, irrespective of the source of that litter. The microbial community from soil exposed to warming was more able to decompose both shoot and root litter in both functional types (Figs 6.2, 6.3), although the exact extent of the increase in decomposition ability depended strongly upon $CO_2$ concentration and whether the litter was from shoots (Fig. 6.2) or roots (Fig. 6.3; soil $CO_2$ x soil Temp interaction, $F_{1,64}$=9.91, $P$=0.003 in shoots, $F_{1,64}$=67.61, $P$<0.0001 in roots). In shoots, warming increased C mineralisation of $C_3$ litter by 17% at elevated $CO_2$ concentration, whereas no warming effect was apparent at ambient $CO_2$ (Figs 6.2, 6.4). The situation was similar but more pronounced (+29%) with $C_4$ litter (Figs 6.2, 6.4). This positive effect of warming was more pronounced with root litter, in which warming increased C mineralisation of elevated $CO_2$–treated samples by 72% in $C_3$ litter and 65% in $C_4$ litter (Fig. 6.3a, b).

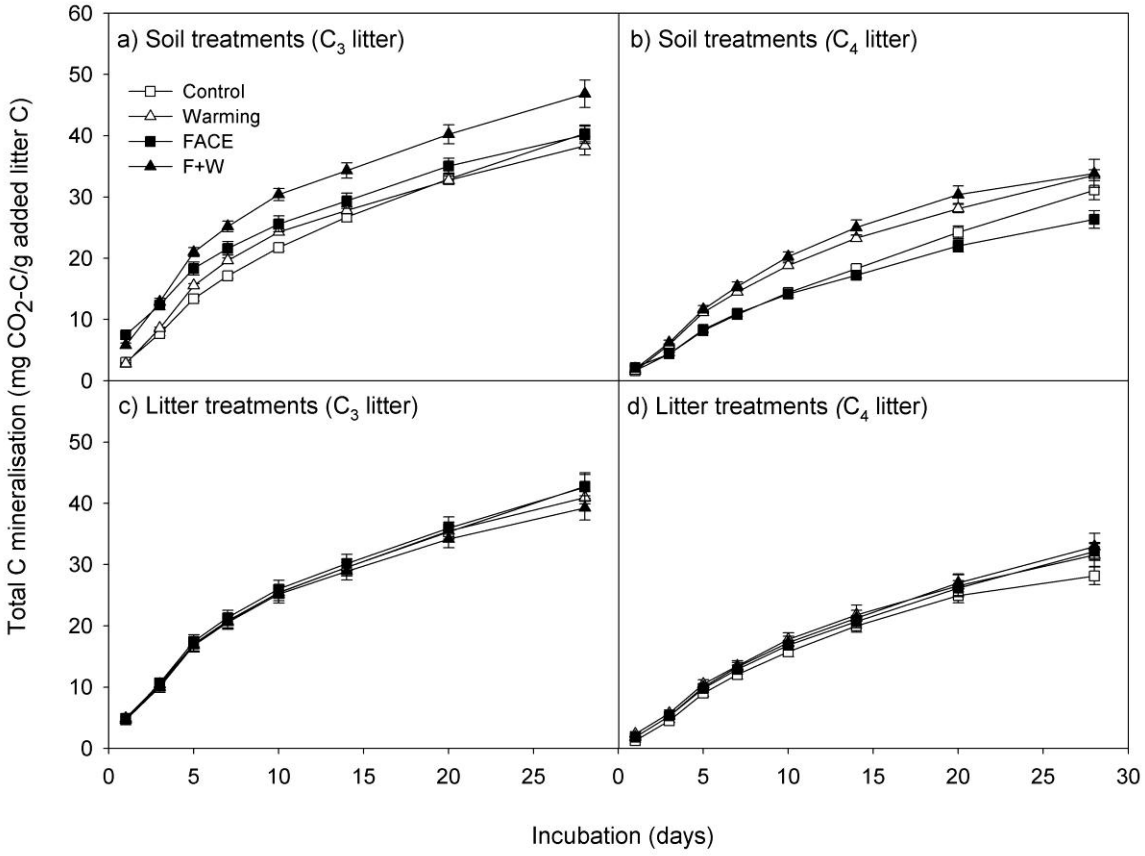


**Fig. 6.2** Cumulative total C mineralisation of shoot litter showing the impact of microbial community source (soil treatments-upper panels) or litter source (litter treatments-lower panels) during a 28-day incubation for $C_3$-derived litter (left panels) and $C_4$-derived litter (right panels). Data are means±SEM.

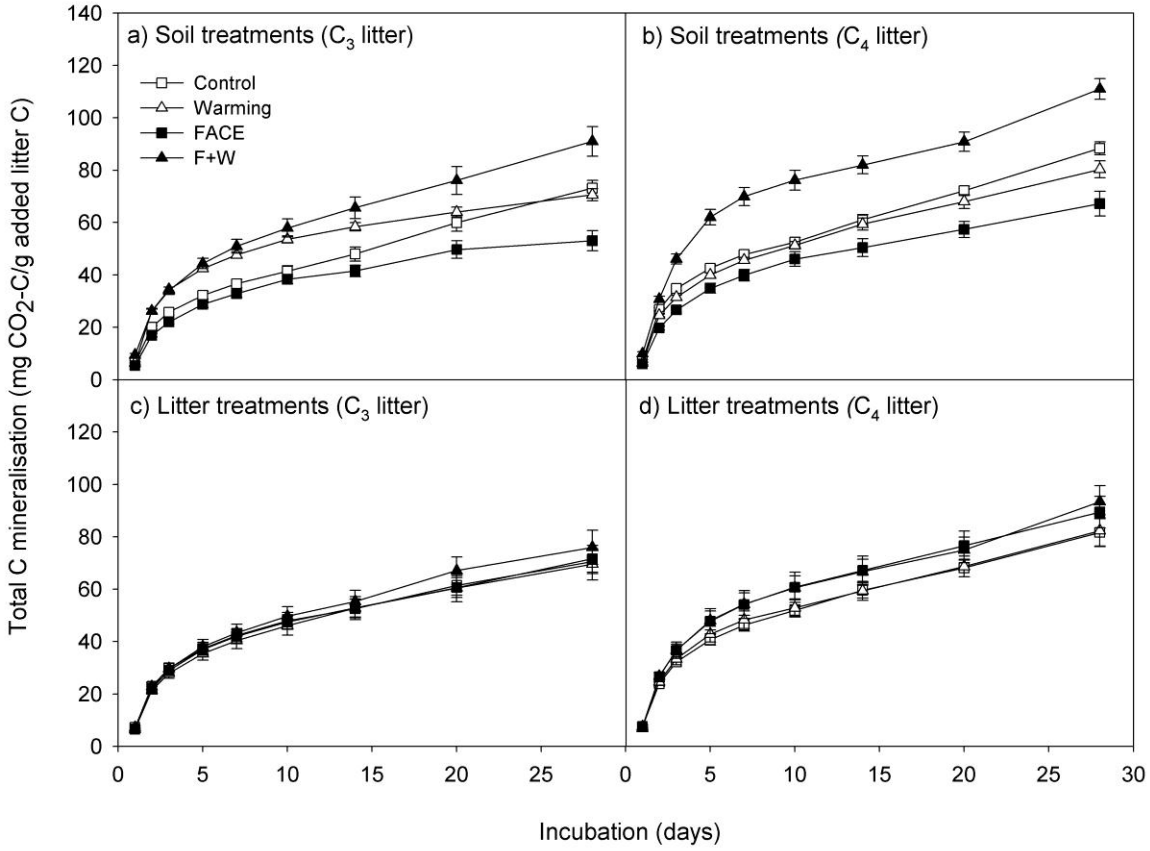


**Fig. 6.3** Cumulative total C mineralisation of root litter showing the impact of microbial community source (soil treatments-upper panels) or litter source (litter treatments-lower panels) during a 28-day incubation for $C_3$-derived litter (left panels) and $C_4$-derived litter (right panels). Data are means±SEM.

*Litter quality effect on C mineralisation*

Treatment effects on litter quality had little influence on C mineralisation (Figs 6.2, 6.3) compared to soil community effects. However, some significant effects of litter source were observed. In shoots, litter temperature treatment interacted with litter functional type and soil warming ($F_{1,64}$=7.88, $P$=0.01), increasing C mineralisation of warming-treated $C_4$ litter by 20% (Fig. 6.2d). In roots, elevated $CO_2$-treated litter produced 9% higher C mineralisation than ambient $CO_2$ samples but only when $C_3$ and $C_4$ are combined ($F_{1,64}$=5.49, $P$=0.02). Decomposition rates are strongly dependent upon litter quality, of which one of the best descriptors is C:N ratio (Aerts 1997). Global change

manipulations had statistically significant but slight effects on litter C:N ratio relative to that of litter functional type (Table 6.1), thus having little effect on C mineralisation. In contrast, plant functional type had a substantial impact on C:N ratio (35% lower in $C_3$ than in $C_4$ shoot litter, $F_{1,32}$=9542.5, $P$<0.0001), which was reflected in C mineralisation rates (33% higher in $C_3$ shoot litter, $F_{1,64}$=86.77, $P$<0.0001, Fig. 6.4a, Table 6.1). This strong functional type effect was also evident in roots, however, C mineralisation was 20% higher in $C_4$ litter (Fig. 6.4b), despite having 12% higher C:N ratio than that of $C_3$ root litter (Table 6.1).

**Table 6.1** Carbon and nitrogen content and C:N ratios of plant material used in incubations. Material was sourced from the TasFACE experiment. Data shown are means with SEM in parentheses. Results of ANOVA are shown. n.s. = non-significant (P>0.05).

| | | Shoot | | | Root | | |
|---|---|---|---|---|---|---|---|
| | | C (%) | N (%) | C:N | C (%) | N (%) | C:N |
| *$C_4$ litter* | | | | | | | |
| Control | Unwarmed | 41.06 (1.55) | 0.45 (0.02) | 91.95 (0.37) | 36.12 (0.60) | 0.78 (0.02) | 46.31 (0.18) |
| | Warmed | 41.44 (0.53) | 0.52 (0.01) | 79.69 (0.50) | 37.03 (0.96) | 0.83 (0.02) | 44.61 (0.36) |
| FACE | Unwarmed | 41.10 (0.29) | 0.39 (0.00) | 105.38 (0.73) | 35.96 (1.14) | 0.70 (0.02) | 51.60 (0.28) |
| | Warmed | 40.31 (0.70) | 0.50 (0.01) | 80.12 (0.53) | 33.99 (1.17) | 0.67 (0.02) | 50.73 (0.34) |
| *$C_3$ litter* | | | | | | | |
| Control | Unwarmed | 41.73 (0.25) | 0.63 (0.01) | 66.24 (0.27) | 33.85 (0.41) | 0.83 (0.01) | 40.62 (0.31) |
| | Warmed | 40.14 (0.81) | 0.70 (0.01) | 57.33 (0.23) | 36.66 (0.94) | 0.87 (0.02) | 42.29 (0.33) |
| FACE | Unwarmed | 38.13 (1.66) | 0.74 (0.03) | 51.50 (0.42) | 34.75 (1.60) | 0.69 (0.02) | 50.07 (0.95) |
| | Warmed | 42.60 (0.10) | 0.74 (0.01) | 57.57 (0.35) | 34.08 (0.31) | 0.91 (0.00) | 37.31 (0.33) |
| *ANOVA (P-values)* | | | | | | | |
| Functional type (FT) | | n.s. | <.0001 | <.0001 | n.s. | <.0001 | <.0001 |
| $CO_2$ | | n.s. | 0.06 | n.s. | n.s. | <.0001 | <.0001 |
| Temp | | n.s. | <.0001 | <.0001 | n.s. | <.0001 | <.0001 |
| FT x $CO_2$ | | n.s. | <.0001 | <.0001 | n.s. | 0.01 | <.0001 |
| FT x Temp | | n.s. | 0.01 | <.0001 | n.s. | 0.0002 | <.0001 |
| $CO_2$ x Temp | | 0.08 | n.s. | n.s. | 0.04 | 0.04 | <.0001 |
| FT x $CO_2$ x Temp | | 0.01 | 0.01 | <.0001 | n.s. | <.0001 | <.0001 |

*Decomposition parameters*

Changes in the rate of C mineralisation could be caused by a simple acceleration or deceleration of labile organic matter decomposition, thereby shortening or lengthening mean residence time (MRT). For shoot litter, warming-induced increases in C mineralisation did appear to be associated with reductions in MRT (Fig. 6.4c), most especially for $C_4$ litter. However, the increased C mineralisation of root litter by the microbial community exposed to both elevated $CO_2$ and warming was not associated with a reduction in MRT, rather the reverse (Fig. 6.4d). This means that the increase in C mineralisation of root litter was not simply due to an acceleration of decomposition of the labile C pool. In fact, it appears that elevated $CO_2$ changed the soil microbial community such that the labile pool MRT increased in both shoot ($F_{1,64}$=7.59, $P$=0.01, Fig. 6.4c) and root litter ($F_{1,64}$=81.63, $P$<0.0001, Fig. 6.4d) with the effect more pronounced with $C_4$ than $C_3$ litter ($CO_2$ x functional type interaction, $F_{1,64}$=11.52, $P$=0.001 in shoots, $F_{1,64}$=16.94, $P$<0.0001 in roots).

It is possible that a change in the C mineralisation rates could be due to a change in the ability of the soil microbes to access the various organic C pools. If microbial community function was altered by exposure to the experimental treatments such that the microbes could more readily access resistant pools, this would be indicated as an increase in the "apparent" labile C pool size. Where shoot litter was concerned, warming did increase the labile C pool ($C_l$) size as a proportion of total C ($F_{1,64}$=57.00, $P$<0.0001, Fig. 6.4e), especially when combined with elevated $CO_2$ (soil $CO_2$ $\times$ soil temperature, $F_{1,64}$=5.47, $P$<0.02). The $C_l$ of shoot litter was greater in $C_3$ litter than $C_4$ litter ($F_{1,64}$=166.61, P<0.0001, Fig. 6.4e) and the species showed a different response to soil $CO_2$ treatment ($F_{1,64}$=18.39, P<0.0001). Consistent with the C mineralisation results, elevated $CO_2$ decreased $C_l$ by 25.8% in $C_4$ litter whereas it increased $C_l$ by 35.8% in $C_3$ litter. This increase in $C_3$ litter, however, was largely driven by a substantial increase in $C_l$ with elevated $CO_2$ in the presence of warming, hence causing a significant soil $CO_2$ x temperature interaction. Rather surprisingly, where root litter was concerned, the relative size of $C_l$ was decreased by elevated $CO_2$ in both $C_4$ and $C_3$ litter ($F_{1,64}$=79.58, $P$=0.0002, Fig. 6.4f),

with a much stronger effect observed on $C_4$ root litter than $C_3$ root litter (Soil $CO_2$ x Litter species interaction, $F_{1,64}$=7.96, $P$=0.007, Fig. 6.4b). Elevated $CO_2$ decreased $C_l$ by 72.7% in $C_4$ root litter while it only reduced $C_l$ by 40.4% in $C_3$ root litter. This resulted in $C_4$ root litter less labile C than $C_3$ root litter under elevated $CO_2$ treatment regardless of the warming treatment.

For shoot litter, the decomposition rate of the resistant C pool ($r$) differed between the litter species ($F_{1,64}$=11.58, $P$=0.001) and soil $CO_2$ treatment ($F_{1,64}$=8.16, $P$=0.006, Fig. 6.4g). The effect of elevated $CO_2$-treated soil on $C_4$ litter $r$ was also consistent with that of C mineralisation rates, showing a reduction in $r$ of 28.7% with elevated $CO_2$ treatment. The soil $CO_2$ treatment also interacted with soil temperature ($F_{1,64}$=8.00, $P$=0.006), with warming treatment counterbalancing the effect of elevated $CO_2$ on $r$. Litter species also interacted with litter $CO_2$ treatment ($F_{1,64}$=5.05, $P$=0.03) where elevated $CO_2$ reduced $r$ in $C_4$ litter but not in $C_3$ litter. The effect of soil $CO_2$ and warming on root $r$ showed a different pattern between the litter species ($F_{1,64}$=9.08, $P$=0.004, Fig. 6.4h). In $C_3$ root litter, both elevated $CO_2$ and warming reduced $r$ by 32.5% and 19.4% respectively, but these reductions only occurred in isolation, as $r$ under elevated $CO_2$ treatment in the presence of warming did not differ from the control. In $C_4$ root litter, on the other hand, elevated $CO_2$ treatments reduced $r$ by 30.5%, regardless of warming treatment.

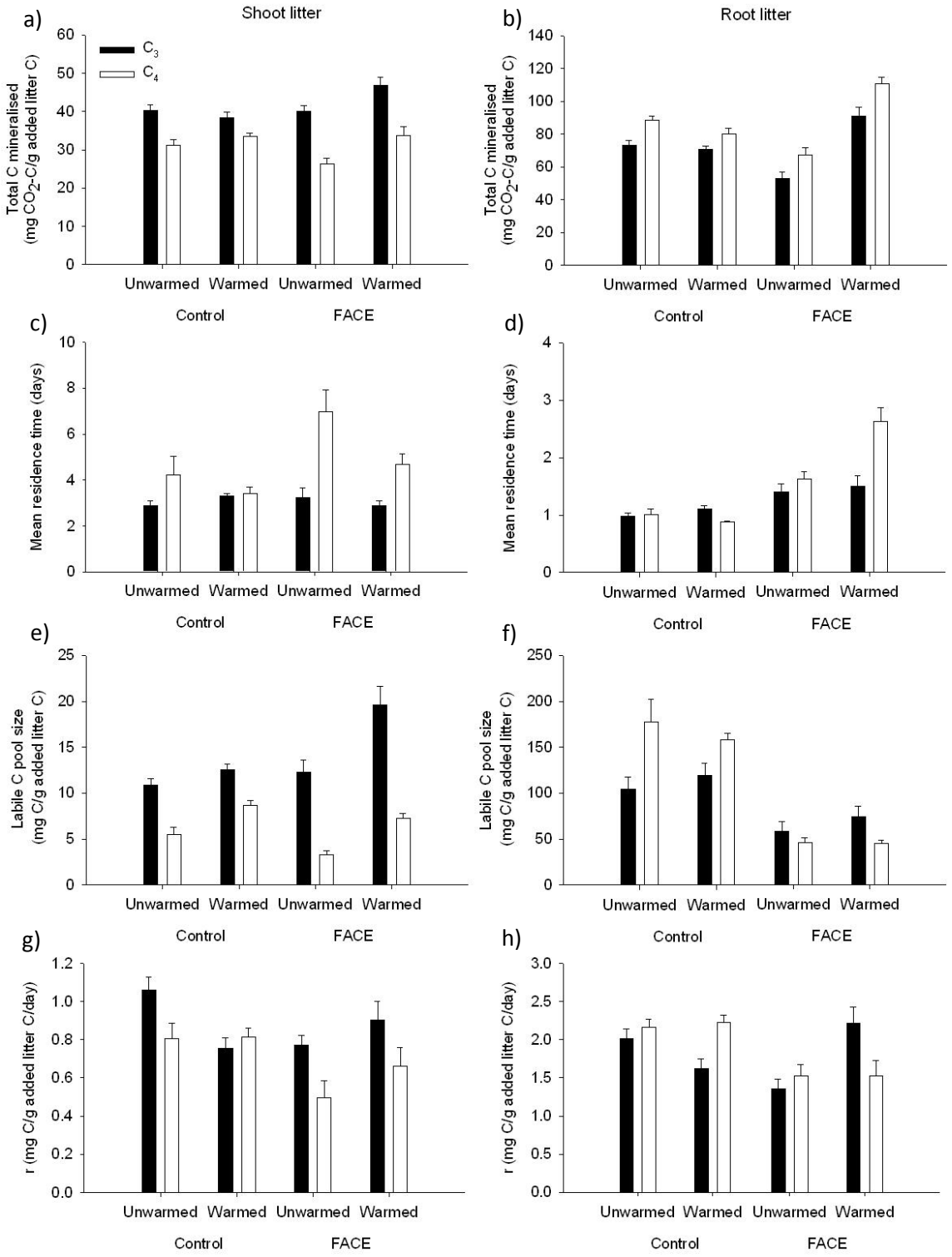


**Fig. 6.4** The impact of elevated $CO_2$ and warming on C mineralisation capability of soil microbial communities from the TasFACE experiment, showing treatment and litter source ($C_3$- versus $C_4$-derived litter) on total C mineralisation (a, b), mean residence time of the labile C pool (c, d), labile C pool size (e, f) and decomposition rate of resistant pool, *r*, (g, h) of shoot (left panels) and root litter (right panels), calculated from incubation curve fits from a 28 d reciprocal transplant incubation. Data are means±SEM.

## DISCUSSION

Global change can influence decomposition through changes in litter chemistry and/or changes in the function of soil microbial community. A growing line of evidence supports such changes and their potential consequences to the terrestrial ecosystem through the alteration of decomposition and nutrient cycling, yet the mechanism whereby global change affects decomposition is not well understood. Therefore, a reciprocal transplant incubation was conducted to examine the relative importance of global change-induced changes in litter chemistry and the soil community on litter decomposition. The study found that global change manipulation altered litter chemistry and the magnitude of the changes differed between the species (or functional types). Despite this, however, the changes in litter chemistry had a negligible effect on C mineralisation when compared with that of global change-induced changes in the soil community. Soil treatments dominated the effect of global change on litter decomposition, showing a substantial reduction in the ability of the soil community to decompose litter when subjected to elevated $CO_2$ treatment. This negative effect of FACE was however reduced or even reversed when elevated $CO_2$ was combined with warming. While such effects were observed in both shoots and roots, the way in which global change influenced the functional activity of soil community differed between shoots and roots as well as between the litter species, indicating the intricate interaction between litter chemistry and the soil community in determining the effect of global change manipulations on litter decomposition.

The effect of global change on litter chemistry has been extensively studied due to its implications for decomposition and thus ecosystem-level responses and feedbacks to global change (Cotrufo et al. 1998). Particularly, the effect of elevated $CO_2$ on litter C:N ratio, a common indicator of litter quality, is well studied across a wide range of ecosystems and vegetation types (e.g. Franck et al. 1997; Weatherly et al. 2003). The increase in litter C:N ratio under elevated $CO_2$ observed in this study is in agreement with the pattern frequently reported from other grassland systems (Gorissen and Cotrufo 2000; King et al. 2004). However, much less known is the effect of warming on litter chemistry and

its potential interaction with elevated $CO_2$. Pendall *et al.* (2010) found that the effect of warming and $CO_2$ were simply additive, while Kandeler *et al.* (1998) observed an interactive effects of elevated $CO_2$ and warming on litter chemistry, suggesting that various interactions can occur between elevated $CO_2$ and warming.

The difference in the responsiveness to global change manipulations can also occur due to the traits associated with photosynthetic pathway. Evidence suggests that $C_3$ species are often more responsive to global change manipulations than $C_4$ species in terms of not only biomass production (An et al. 2005; Sage and Kubien 2003) but also tissue chemistry (Ball and Drake 1997). In the comparison between $C_3$ and $C_4$, Ball and Drake (1997) found that only $C_3$ species showed an increase in litter C:N in response to elevated $CO_2$. The responsiveness of $C_4$ litter to global change manipulations observed in this study may be due to the lower N content of $C_4$ species compared to that of $C_3$ species, as the $C_4$ species examined by Ball and Drake had a higher N content than that of $C_3$ species. Nevertheless, such a difference in response remains important especially when global change is likely to favour $C_4$ species over $C_3$ species in this grassland (Pendall et al. 2010; Williams et al. 2007). Duke and Field (2000) suggested that changes in plant species composition may have more pronounced effect on the quality of litter input to the ecosystem than the effect of elevated $CO_2$ on the litter quality of individual species. Indeed, a study by Dijkstra *et al.* (2006) demonstrated that interspecific variation in litter inputs and chemistry caused much greater effect on decomposition than the variation induced by the global change manipulation. The results from this study are in agreement with their findings, as a strong effect of litter species (or functional group) on shoot and root decomposition was persistent while global change manipulations on litter had a minimal effect.

The dominant effect of global change manipulations on litter decomposition, however, came from their effect through soil microbial community function, which masked the effect of the altered litter chemistry on litter decomposition. The effect of global change on soil microbial community has

been extensively studied, yet the published reports have been highly variable in their responses (Blankinship et al. 2010; Brosi et al. 2010; Cornejo et al. 1994; Denef et al. 2001; Klironomos et al. 1996; Treseder 2004) with interacting effects of elevated $CO_2$ and warming also being observed (Castro et al. 2010). A previous study conducted in this grassland found that global change manipulations influenced both bacterial and fungal community composition with evidence of interaction between elevated $CO_2$ and warming treatments (Hayden et al. 2012). Although the link between the microbial community composition and functional activity is yet to be fully understood, it is plausible that the soil community was changed in a way to decrease decomposition under elevated $CO_2$ but to increase decomposition when elevated $CO_2$ was combined with warming in this study.

Soil community can affect decomposition in various ways. Changes in decomposition rate are often suggested under soil warming where increased microbial activity and/or biomass increases the rate in which substrate C is mineralised, thereby shortening the mean residence time (MRT) of decomposing materials. Alternatively, it is also possible that changes in microbial community composition leads to an increase in accessibility of C substrates which are otherwise inaccessible to microbial degradation without any changes in decomposition rates (Zogg et al. 1997).

Soil organic matter is usually considered to exist in various “pools”, which have different inherent decomposition rates. Organic matter that is readily decomposed is usually described as labile, while more resistant organic matter is considered to exist in slow or recalcitrant pools. The results from this study only apply to the labile C pool, as the study used short-term incubations, however it is this fraction of soil C that is most pertinent to changes in soil C storage, since these pools have the most rapid turnover and are more responsive to elevated $CO_2$ and warming (Schlesinger and Andrews 2000). Plants increase the release of root exudates rich in readily available C when grown at elevated $CO_2$ (Pendall et al. 2004b) and it is possible that this increase in the availability of labile C alters microbial community composition such that more recalcitrant organic matter, such as added

litter, is less readily decomposed (Cheng et al. 2007). This could explain the reduction in decomposition of added litter by microbial communities from unwarmed elevated $CO_2$ plots, but the combination of elevated $CO_2$ and warming actually increased litter decomposition. This increase in the rate of C mineralisation could be caused by a simple acceleration of labile organic matter decomposition, shortening mean residence time (MRT), but could also be caused by a change in the ability of the soil microbes to access C substrates. For shoot litter, warming-induced increases in C mineralisation did appear to be associated with reductions in MRT, most especially for $C_4$ litter. However, the increased C mineralisation of root litter by the microbial community exposed to both elevated $CO_2$ and warming was not associated with a reduction in MRT, rather the reverse. This means that the increase C mineralisation of root litter was not due to simply an acceleration of decomposition of the labile C pool. In fact, it appears that elevated $CO_2$ changed the soil microbial community such that the labile pool MRT increased in both shoot and root litter with the effect more pronounced with $C_4$ than $C_3$ litter. This means that elevated $CO_2$ decreased the actual decomposition rate of the labile C pool, which has also been demonstrated elsewhere (Cheng et al. 2007).

It is possible that a change in the C mineralisation rates could be due to a change in the ability of the soil microbes to access the various organic C pools. If microbial community function was altered by exposure to the experimental treatments such that the microbes could more-readily access resistant pools, this would be indicated as an increase in the "apparent" labile C pool size. Where shoot litter was concerned, warming did increase the labile C pool size as a proportion of total C, especially when combined with elevated $CO_2$. Work in other grasslands has also shown increases in the labile C pool size under elevated $CO_2$ (Pendall and King 2007). The results from this study indicate that the soil microbes exposed to warming and the combination of warming and elevated $CO_2$ had an increased ability to access the added litter, meaning that a greater proportion of total soil C was perceived as being labile in these incubations, but only where shoot litter was concerned. Rather

surprisingly, where root litter was concerned the relative size of the labile C pool was decreased by elevated $CO_2$ in both $C_4$ and $C_3$ litter.

This study showed that simulated global changes affected shoot decomposition consistently. The reduced C mineralisation of $C_4$ litter in the soil community exposed to elevated $CO_2$ corresponded with longer MRT, reduced labile C pool, and decreased decomposition rate of resistant C pool. The effect on $C_3$ litter was less pronounced, however, the increase in the amount of C mineralised by the soil community exposed to both elevated $CO_2$ and warming can be explained by the corresponding increase in the labile C pool size. The changes in the rate of microbial activity and microbial community composition were found responsible for the loss of soil C under elevated $CO_2$ in a scrub-oak community in Florida (Carney et al. 2007). This loss of C was found to be a result of increased priming of soil organic matter following litter addition under elevated $CO_2$ and that the microbial community utilised more C from soil organic matter than those from the ambient soils. It is therefore possible that elevated $CO_2$ changes the soil microbial community to preferably decompose soil organic matter, thereby negatively affecting their efficiency to decompose litter. This is consistent with the previous study conducted in this grassland community where elevated $CO_2$ increased the decomposition of resistant soil C pool while reducing the decomposition of labile C, which is largely composed of freshly added C inputs (Pendall et al. 2010).

Root decomposition, on the other hand, showed an inconsistent pattern where the increased C mineralisation did not necessarily correspond with the changes in the decomposition parameters and was strongly affected by the interaction between litter species and global change manipulations. While the increased decomposition rate of the resistant C pool seems to be responsible for the increased C mineralisation of $C_3$ litter by the soil community under elevated $CO_2$ and warming, no apparent causes were found for the increase in $C_4$ litter by the same community.

The difference in chemical composition of aboveground and belowground tissues may also explain the difference observed in the effect of global change on decomposition dynamics. Due to the presence of the suberin-lignin complex, root tissues are thought to be more recalcitrant than their aboveground counterparts (Abiven et al. 2005). The recalcitrance of a tissue however may change following a shift in the functional activity of a soil community. Zogg *et al.* (1997) observed an increase in the cumulative C mineralisation in response to soil warming with a corresponding increase in the amount of labile C. Without a corresponding increase in decomposition rate constant, they concluded that the increase in C mineralisation was caused by a temperature-induced shift in microbial community composition. This shift resulted in the enhanced ability of the soil community to metabolise substrates that were inaccessible to the community favoured under lower temperature environments. The higher recalcitrance of root tissues than shoots means that changes in the accessibility of C substrates under global change are likely to be more pronounced in root decomposition than shoot decomposition. Therefore, such a change in microbial accessibility may explain the differential effect of soil community on decomposition between roots and shoots.

*Conclusions*

This study demonstrates that elevated $CO_2$ and warming is likely to affect the ability of the microbial community to decompose litter and that the change in composition caused by combined warming and elevated $CO_2$ increases access to more resistant C pools. This could well explain the warming-induced increase in turnover of non-labile soil C (Knorr et al. 2005). Further, the changes in soil microbial function are likely to have a more pronounced and stronger effect on decomposition than the changes in litter chemistry induced by global change. Yet, the differences in the effect of soil microbial function and litter chemistry between $C_3$ and $C_4$ plant species suggest that the projected shift towards $C_4$ vegetation under global change (Morgan et al. 2011; Williams et al. 2007) is likely to affect decomposition to an even greater extent. What compositional changes occur in the soil microbial community to produce this marked change in C mineralisation ability needs to be

determined to complete the understanding of how soil function will respond to a changing world, but it is clear that the inherent functional ability of soil microbes is responsive to predicted changes to the atmosphere and the climate.

# Chapter 7: General discussion

Plant-soil interactions and feedbacks mediate many ecological processes, thus a thorough understanding of plant-soil interactions is a crucial part of understanding how an ecosystem responds to environmental changes. Environmental changes can be large in scale such as land use conversions where wholesale changes in plant species identity and traits, such as occurs in the conversion of forest to pasture or crops, substantially alter soil processes and nutrient cycling of the whole community (e.g. Cochran et al. 2007; Guo et al. 2008). Environmental changes can also induce small-scale changes such as shifts in the relative abundance of already co-existing species. The impact of such small-scale changes on soil processes is not well studied, despite the growing number of studies demonstrating the specificity of the association between plant species and the soil microbial community that mediates important soil processes (Bardgett et al. 1999; Chen and Stark 2000; Kourtev et al. 2002; Osanai et al. 2012). Therefore, this study aimed to investigate the influence of co-occurring grass species on key soil processes to test whether even a shift in the relative abundance of co-occurring species could lead to a large impact on carbon (C) and nitrogen (N) dynamics at a community-level, through changes in the associated soil microbial community composition (Chapter 2) and their functional activities that are related to N cycling (Chapters 3 and 4) and litter decomposition (Chapters 5 and 6).

The examination of soil microbial community composition associated with the three grass species (Chapter 2) demonstrated that plant species differed in their associated bacterial, fungal and arbuscular mycorrhizal community composition, and that each microbial group seemed to be influenced by different mechanisms. The composition of the bacterial community seemed to reflect their responsiveness to root exudates while the fungal composition seemed to reflect their saprophytic nature and thus were influenced by litter quality. The community composition of

arbuscular mycorrhizal fungi on the other hand exhibited patterns that provide support for the role of the mycorrhizal association in species coexistence and niche separation (Moora and Zobel 1996; van der Heijden et al. 1998). The patterns observed in the soil microbial community had no clear correlation with the soil chemical characteristics measured, further suggesting the influence of plant species identity on microbial community composition. It is, however, interesting to note that soil chemical characteristics differed among the plant species on average, indicating that the differences in soil microbial community composition may translate into differences in soil microbial activity that affect soil chemical characteristics. In summary, the terminal restriction fragment length polymorphism analysis indicated that functionally similar plant species occurring within centimetres of each other had distinct microbial communities associated with them and the nature of the influence differed among different microbial groups.

Chapter 3 focused on soil microbial activities that are related to N cycling and demonstrated that the co-occurring grass species differed in both soil N transformation rates and the abundance of the microbes responsible for those transformations. The sub-dominant grass species, *Austrostipa*, had higher N transformation rates than either of the dominant species and also had higher abundance of ammonia-oxidising archaea (AOA) than the other species. Therefore, this indicates that the autotrophic nitrifying community, especially AOA, is important in controlling N transformation rates in this grassland and supports the results of Chapter 2 since co-occurring species differed in microbial function as well as microbial community composition. The abundance of the nitrifying community is also influenced by the abundance and activity of the heterotrophic microbial community whose population expands rapidly with the supply of C substrates (Anderson and Domsch 1978). It is possible that relatively low C availability in *Austrostipa* soil restricted the growth of heterotrophic microbes and allowed net N mineralisation to prevail and the nitrifying community to compete effectively for the available ammonium ($NH_4^+$). *Austrostipa* litter however generally has a high C:N ratio, and since soil organic contents are largely influenced by litter quality (Vesterdal et al.

2008), seasonal variations in litter quality or root exudation may play an important role in determining N transformation rates. Furthermore, the examination of autotrophic nitrification and heterotrophic nitrification showed that soil associated with *Themeda*, a $C_4$ species, had a higher contribution of heterotrophic nitrification than that of the $C_3$ species, which could be driven by the differences in phenology and concomitant microbial community dynamics, especially in regards to bacterial to fungal ratios (Lipson et al. 2002). It is possible that subtle changes in the balance between gross mineralisation and immobilisation controlled the N status and differences among the plant species. These differences could easily be influenced by differences in the quantity, quality and timing of plant nutrient uptake and organic supply to the soil. Thus, this study highlights the complexity of interactions and feedbacks between plant and soil communities in influencing N cycling and that the influence of plant species on N cycling is highly dynamic through time and space.

The effect of plant species on N cycling was further examined in Chapter 4 in which the relative contribution of different microbial groups to nitrate ($NO_3^-$) production was assessed. Consistent with the finding from the previous chapter, the production of $NO_3^-$ was dominated by autotrophic nitrifiers in this grassland. Despite this, there were also some indications that fungi may contribute substantially to $NO_3^-$ production, and that the relative contribution of fungi differed between *Themeda* and the $C_3$ species, further supporting the findings of the previous chapter. The study on the relative contribution of different microbial groups to $NO_3^-$ production is challenging, due to the difficulty in quantifying the activity of one microbial group while effectively and selectively suppressing the others (Castaldi and Smith 1998; Rousk et al. 2009). Differences in sensitivity to a particular inhibitor within the target microbial community also add to the difficulty in quantifying the functional activity of a particular microbial community (Taylor et al. 2010). Therefore, the specificity and efficiency of inhibitors as well as the modes of inhibition need to be thoroughly understood before they can be used for the quantification of nitrifying activity by different microbial groups.

Chapter 5 focused on another important microbially-mediated process, litter decomposition. Litter decomposition is a crucial part of nutrient cycling, and the quality of litter influences the rate of decomposition greatly (Aerts 1997; Cornwell et al. 2008; Jensen et al. 2005). The results demonstrated that the chemical quality of litter is indeed an important determinant of decomposition rates, with N contents and C:N ratios correlating significantly with C mineralisation rates. Consequently, plant species that differed in litter chemistry also differed in C mineralisation rates. Such a relationship between litter chemistry and N mineralisation rates was not observed due to the effect of physical quality in the form of litter particle size in affecting the balance between mineralisation and immobilisation of N and possible C limitation in the experimental system. Thus, this study highlights that both chemical and physical quality of litter influence litter decomposition and that standardisation of litter particle size is also required in order to assess the effect of litter chemistry decomposition rates.

Litter chemical quality especially in terms of C:N ratio is predicted to change under global change, thereby potentially altering decomposition processes significantly. Litter decomposition is however mediated by the soil microbial community which could also affect the rate of decomposition (Ayres et al. 2009; Osanai et al. 2012). Chapter 6 therefore examined the relative importance of changes in litter quality and changes in the soil microbial community in affecting decomposition processes under simulated global change. The results showed the changes in soil microbial community had much greater impact on decomposition than that of litter quality, and that the microbial community exposed to experimental warming and elevated $CO_2$ concentrations had a substantially increased ability to decompose added plant litter, regardless of that litter's source (i.e. $C_3$, $C_4$, shoots or roots). Thus, the study demonstrates that environmental changes will stimulate the microbial decomposition of organic matter, reducing soil carbon storage and increasing soil carbon emissions. However, the results also showed the consistent differences between $C_3$ and $C_4$ litter in decomposition rates, therefore, predicted shifts in the relative abundance of $C_3$ and $C_4$ species

(Williams et al. 2007) would also have a significant impact on decomposition processes in this grassland. This study therefore further highlights the importance of soil processes and functions in regulating ecosystem nutrient cycling and that soil processes play a crucial role in determining ecosystem responses to global changes

## IMPLICATIONS OF THIS STUDY

The importance of a plant species effect on soil processes and functions is clearly evident throughout the study. Even a shift in the relative abundance of already co-occurring species could lead to substantial changes in soil processes and functions, with a potentially large impact on ecosystem nutrient cycling of the whole ecosystem through plant-soil feedbacks. This study provides further support to the growing body of evidence for the specificity of plant-microbe interactions and demonstrates that even functionally similar, co-occurring species can differ in their association with the soil microbial community at a very small spatial scale. The generality of such plant species effects on the soil microbial community, however, needs to be assessed through repeated sampling throughout a year, as the phenology of each plant species is likely to affect the way in which plant species influence microbial community composition and community dynamics in the soil. Such seasonal or temporal variation in the effect of plant species on microbial community composition may also contribute to the difficulty in linking microbial community composition to microbial community function, such as soil N transformations and litter decomposition. N transformations differed among the co-occurring species, however, they seemed to be controlled by various processes and factors that affect the fine balance between mineralisation and immobilisation. Such factors include substrate availability for both autotrophic and heterotrophic microorganisms, litter quality, microbial nutrient requirements, soil nutrient status and plant organic supply and nutrient uptake (Bardgett et al. 2005), all of which interact and influence one another. Thus, a subtle change in any of these factors can result in substantial changes in soil N dynamics, and

therefore the strength of the plant species effect on soil N dynamics is also likely to vary through time. While sampling was carefully timed so that both $C_3$ and $C_4$ plant species were present, fresh samples were collected from the field prior to each experiment in order to meet the specific soil sampling requirements for each study. This has made the interpretation of the findings across the chapters challenging, given the intricate interactions between plants and soil affecting the strength of plant species effect on these soil processes examined in this study. Thus, a large-scale study that allows the various aspects of plant species effect on soil processes to be examined simultaneously would be necessary to assess the generality and dynamics of plant species effect on these soil processes examined in this study.

The high specificity of plant species association with the soil microbial community observed also furthers our understanding of the ecological role of the soil microbial community in plant coexistence. While the temporal difference in phenology between the two dominant species, warm-season $C_4$ species *Themeda* and cool-season $C_3$ *Austrodanthonia*, may explain their coexistence and similarities between these species in their associated soil microbial community and soil processes, the sub-dominant $C_3$ species, *Austrostipa*, showed much clearer differences in the associated microbial community composition and soil nutrient relations, especially when compared with the dominant $C_3$ species. It is unknown whether the difference observed in the soil microbial community composition is directly linked to the differences observed in N transformations between the dominant species and the sub-dominant species, however, specialisation of soil microbial activity to their associated plant species has been reported in the literature (Ayres et al. 2009; Vivanco and Austin 2008). Thus, such feedbacks between plant species and their associated soil microbial community could determine the success of plant species in a given environment, promoting dominance or coexistence of plant species and shaping plant community structure (Bever 2003). Thus, this study also highlights the importance of feedbacks between plant species and the soil

microbial community in order to fully understand the plant species effect on soil processes and functions.

Identifying key processes or mechanisms is crucial in understanding the potential consequences of certain impacts on ecosystem processes and functions. The reciprocal transplant study demonstrated that changes in soil microbial function are likely to exert a stronger influence over litter decomposition than changes in litter quality under global change, further highlighting the importance of soil processes in ecosystem nutrient cycling. Despite this, changes in litter quality as a result of shifts in plant species composition between $C_3$ and $C_4$ vegetation would also likely to affect litter decomposition rates. This has particularly important ramifications for this $C_3$/$C_4$-dominated grassland where the dominance of $C_4$ grass *Themeda* is predicted to increase at the expense of $C_3$ grass *Austrodanthonia* under global change (Williams et al. 2007). These two species differ in litter quality, and thus the shift in the relative abundance of these two species would have a strong influence on the fungal community that is largely influenced by litter quality. However, this study also demonstrates that such a change in community composition is also likely to be accompanied by changes in microbial community composition and function for both C and N dynamics. While fungal nitrification was relatively small compared to autotrophic nitrification in this grassland community, the difference observed in the relative contribution of fungal nitrification between these two dominant species means that the shift in the dominance could have a large impact on N as well as C dynamics of this grassland. In contrast, the two dominant species did not differ greatly in soil bacterial composition and the abundance of autotrophic nitrifying bacteria. It is therefore extremely important to understand the key drivers of soil processes in order to identify the underlying mechanisms by which environmental changes alter ecosystem processes and functions. In particular, the role of heterotrophic microorganisms including fungi in nitrification processes in grassland community has only begun to attract attention in recent years, and the recent studies tend to suggest the greater contribution of heterotrophic nitrification to overall nitrification than previously

thought (Cookson et al. 2006; Laughlin et al. 2008). Thus, further research is needed to develop a reliable assay to efficiently separate the activity of different microbial groups in order to understand the relative importance of different microbial groups to key soil processes.

This study has examined the effect of plant species on soil processes in a native Tasmanian grassland community. These functionally similar co-occurring grass species still had a strong impact on the soil microbial community composition and on important soil functions. Changes in microbial community function can be great even among co-occurring species at a very small spatial scale. Both the composition and function of the soil microbial community are very responsive to differences in plant species and therefore must be taken into account for a complete understanding of ecosystem function. Future research should therefore focus on linking changes in microbial community composition to microbial community function. Advances in molecular technology have increased our understanding of the soil microbial community greatly in the last decade or two, however, it is also important to develop reliable and easily repeatable alternative techniques for examining microbial community functions in order to fully understand the dynamics of plant-microbe interactions and feedbacks that regulate ecosystem processes and functioning.

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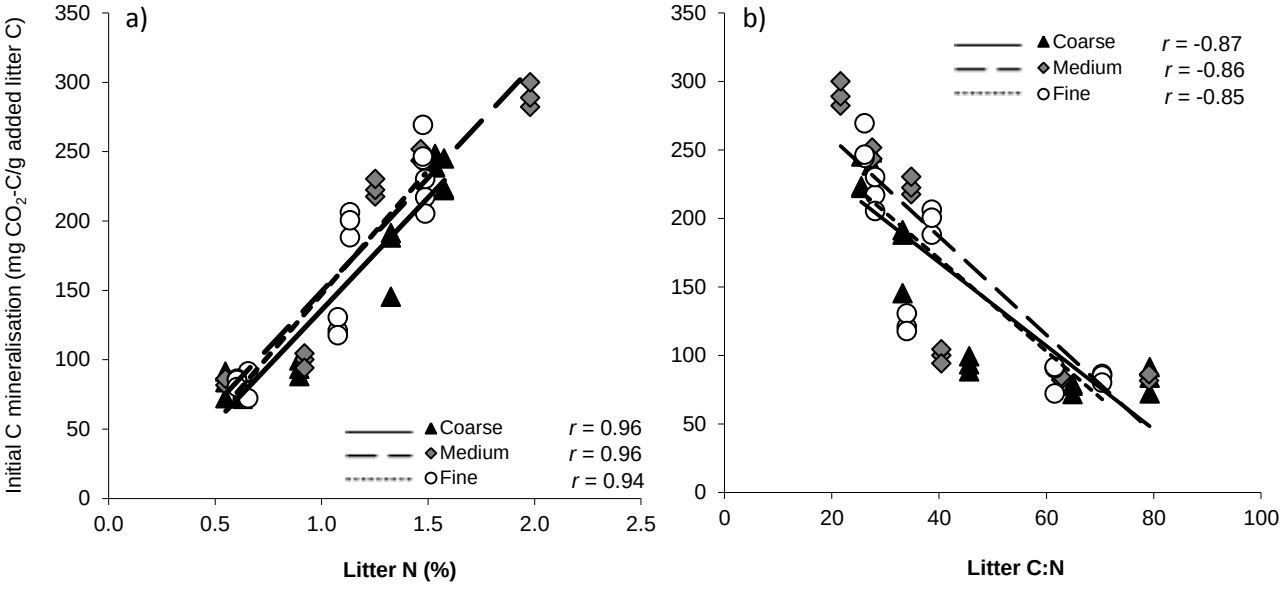
# Appendix

**Appendix 1** The effect of acetylene (Acet) and cycloheximide (Cyclo) on net ammonification rates (mg N/g soil dwt/28 d) of soil samples collected from underneath *Themeda*, *Austrodanthonia* and *Austrostipa* during 28-d laboratory incubation. Different letters indicate statistical differences between the treatments (P<0.05).

| | Control | Acetylene | Cycloheximide | Acet + Cyclo |
|---|---|---|---|---|
| *Themeda* | -0.002 ±0.001 b | 0.014 ±0.009 b | 0.118 ±0.004 a | 0.136 ±0.019 a |
| *Austrodanthonia* | -0.004 ±0.001 b | 0.001 ±0.001 b | 0.132 ±0.005 a | 0.141 ±0.011 a |
| *Austrostipa* | -0.001 ±0.000 b | 0.010 ±0.003 b | 0.123 ±0.010 a | 0.128 ±0.004 a |




**Appendix 2** The relationship between initial C mineralisation rates and litter N contents a) and initial C mineralisation and litter C:N ratio b) by litter sizes (coarse, medium and fine) for all litter types (green leaf, senesced leaf and root) and litter species (*Themeda* and *Austrodanthonia*).