Interactions between native and exotic plants in the context of grassland restoration and the importance of below-ground processes

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Thesis abstract

The importance of native ecosystems is being ever more realised as human-induced environmental change leads to ecosystem degradation. This is spurring increased efforts to restore ecosystems. In previously cultivated landscapes (old-fields) the legacy of farming practices can persist for decades and present many challenges for restoration. This thesis is focussed on identifying and overcoming some of these challenges that limit restoration efforts. The overall aims were to develop a mechanistic understanding of the processes hindering native grass establishment and to improve the effectiveness of techniques used in the restoration of native grasslands.

Two glasshouse experiments (chapters 2 and 3) were designed to investigate whether soil microbial communities present in old-fields hinder native plant establishment and allow exotic plants to dominate. The results indicate that native grasses performed better in the presence of soil microbes from remnant grassland. However, these microbial effects were heavily influenced by nutrient availability in the soil. Characterisation of the microbial communities, using molecular barcoding, revealed that they differed between old-field sites and remnant grassland. Differences in soil physiochemical properties between soil types, as well as the presence of different plant species, appear to explain the observed differences in microbial community composition. In turn, these changes in microbial communities affected plant performance, particularly when soil nutrient availability was low.

High nutrient availability in old-fields from past farming practices usually results in dominance of fast-growing annual exotic plants. Reducing soil fertility is therefore seen as an effective approach to restoration. I trialled four methods (carbon supplements, slashing, burning, and scalping; chapter 4) to 1) reduce biomass of exotic species, 2) reduce

soil nutrients, and 3) increase biomass of native grasses. Overall, scalping was the only method to achieve all three aims whereas carbon supplements and slashing reduced exotic biomass with no apparent benefit to native species. Both carbon supplements and scalping resulted in changes to the soil microbial community. Given the importance of plant-soil interactions, the implications of these result for future restoration works are discussed.

One strategy to promote resistance to invasion in a revegetated community is to plant species that use resources in a complementary way, i.e. planting a diversity of functional groups. In a field trial (chapter 5), grass species from complementary functional groups (chosen based on phenology) were grown in different combinations and densities to test whether native communities are more resistant to invasion if resources are utilised all year round (niche saturation). Overall, high density planting was most effective at lowering exotic biomass. Planting C3 (winter-growing) and C4 (summer-growing) grasses together did not reduce invasibility, in contrast to my predictions. Instead, planting C3 plants alone was effective at reducing exotic biomass, providing evidence that planting functional groups that match the functional group of potential invaders could be an effective strategy for restoration.

Findings presented in this thesis demonstrate the importance of soil amendments, both abiotic and biotic, and planting arrangements in ecological restoration. Greater consideration of these should lead to more successful and sustainable restoration outcomes in grassland habitats.

Chapter 1. General introduction

The Green Army setting up an experiment at Para Woodlands Reserve

1.1 Restoration frameworks

The vast environmental impact humans have had on native ecosystems has led to a shift in conservation efforts from the preservation of intact systems to the restoration of degraded systems (Dobson *et al.* 1997; Suding 2011). In general, restoration aims to restore a degraded site into an ecosystem which provides ecosystem services and is aesthetically appealing or with conservation values such as habitat for particular animals (Hobbs and Norton 1996). It is also important that the desired community composition and ecosystem function is self-sustainable in the long term with minimum input after establishment (Hobbs and Norton 1996). However, in practice restoration efforts often produce inconsistent or unwanted results (Hobbs and Harris 2001). This has been attributed to using a simplistic understanding of ecological processes when aiming to restore the historic features of the system (Suding *et al.* 2004). Often the feedbacks between biotic and abiotic factors that have developed in the degraded state are ignored (Suding *et al.* 2004). Failures in restoration has led to the need of concerted research in restoration ecology that is solidly grounded in ecological theory.

Traditionally, restoration ecologists and practitioners have focussed on a simplistic succession-based approach to restoration where disturbed systems return to native vegetation states via re-establishing historic abiotic conditions (Clements 1916; Prach *et al.* 2001; Walker and del Moral 2009). This approach can be considered a linear continuous model where changes in the environment leads to a proportional change in species composition along one trajectory (Suding and Hobbs 2009). Under such a model, reversing the cause of land degradation may be enough to return the system to its historical state or restoration may be used to speed up the process of succession. However, success is usually limited to sites where disturbance is minor, a native seed bank persists, or the site is surrounded by native vegetation (reviewed in Prach and Pysek 2001 and; Suding *et al.*

2004). Therefore, in most cases, a more comprehensive model is needed for successful restoration. For instance, a solid succession approach should consider priority effects produced by early pioneer species (Alford and Wilbur 1985), biotic and abiotic legacy effects from past use or undesired species (Facelli and Pickett 1990) and the possibility of alternative pathways (Gleason 1939), including inhibition (Connell and Slatyer 1977).

The more sophisticated model of alternative stable states recognises that a given environment can support two or more distinct states, i.e. assemblages of species (Beisner *et al.* 2003; Suding *et al.* 2004; Suding and Hobbs 2009). In contrast to what is predicted by a continuous model, environmental change may initially lead to very little change in species composition or function until a critical threshold is crossed. At this point, abrupt change can shift the system between two or more states. How resilient a system is, i.e. the amount of disturbance or stress that system can withstand before changes in processes or structure occurs, determines when a transition among these states occurs (Gunderson 2000). The new state can then be reinforced by complex interactions, often in the form of positive feedback, allowing it to persist and become resistant to change. Therefore, these models have uses in both predicting when a system might suddenly collapse as a result of environmental change (Beisner *et al.* 2003; Nyström *et al.* 2000) but also provide a management framework for restoring systems that have already collapsed to a degraded state (Cramer *et al.* 2008; Hobbs and Norton 1996; Suding *et al.* 2004; Suding and Hobbs 2009).

A degraded state that has crossed particular thresholds (e.g. with abiotic conditions severely changed, or persistent biological legacy, such as, an exotic seed bank or changes in microbial species) may appear as a 'novel ecosystem' different in species composition and function from any historical system (Cramer *et al.* 2008; Suding and Hobbs 2009). This can happen when pioneer species are introduced species, as is often the case in today's

successional systems. The organisms in the novel ecosystem can create positive feedback through a number of mechanisms (e.g. changes in nutrient cycling, plant-soil feedbacks) that favours the growth and establishment of those early established species and prevents the establishment of other species (inhibition pathway in Connell and Slatyer 1977). Therefore, releasing the system from human-induced pressures (e.g. farming, overgrazing) does not necessarily result in the re-establishment of the original system (Suding *et al.* 2004). Overcoming constraints created by positive feedback then becomes a priority for successful restoration.

Frameworks for restoration using models of alternative stable states are particularly useful where multiple constraints to restoring a degraded system occur (Suding *et al.* 2004). It is important to identify and prioritise the constraints before restoration work begins. Given that managers do not have the time or funds to make these assessments on a site-by-site basis it is clear that experimentation and mechanistic understanding of the constraints involved in these feedbacks is vitally important. If the constraints are not overcome, the system may resist restoration efforts or, when subject to environmental fluctuations, shift abruptly back into the degraded state or into an entirely new state. Therefore, it is also important to understand how to reinstate positive feedbacks in the target community so that the desired state persists and is resilient to change.

1.2 Old-fields as alternative stable states

The classical model of old-field succession was pioneered by studies from north-eastern North America where vegetation change after land abandonment followed a predictable, successional pattern of recovery (reviewed in Hobbs and Walker 2007). In some cases, early exotic colonisers can be replaced by native perennial grasses or woody plants after 10–20 years of succession (Collins 1990; Hermy and Verheyen 2007; Inouye *et al.* 1987). However, this pattern of recovery is limited to sites inherently suitable for

agriculture, e.g. with naturally high soil fertility and high rainfall, and where few amendments are needed to support crop production (Cramer *et al.* 2008). Therefore, the soil conditions are likely to resemble those in the historical state, i.e. no abiotic thresholds are crossed, and the native community can successfully recolonise and be competitive against the exotic colonisers if a seed source is available (Bellemare *et al.* 2002; Cramer *et al.* 2008; Seabloom *et al.* 2003; Wijdeven and Kuzee 2000).

In geologically old landscapes, like those in Australia, which have naturally nutrient-poor soil with low water retention, recovery is less likely to follow a successional pattern because both biotic (e.g. destruction of native seed bank, changes in soil microbe assemblages) and abiotic (e.g. soil chemistry, water fluxes) thresholds are crossed (Bever 1994; Cramer *et al.* 2008; Standish *et al.* 2008). Farming practices in these landscapes can alter environmental conditions so severely that the degraded state may persist for decades or even hundreds of years (Dambrine *et al.* 2007; Foster *et al.* 2003; McLauchlan 2006). For example, fertilizer legacies can give invasive exotic species a competitive edge over native species as they are better at exploiting the high nutrient conditions and tend to have earlier germination and faster growth than native species (Standish *et al.* 2006; Wainwright *et al.* 2012). The exotic species can then maintain higher nutrient levels (via fast growth and rapid decomposition of litter) or form positive plant-soil feedbacks with the microbial community (Dyer and Rice 1999; Klironomos 2002). Therefore, old-fields stuck in a persistent, degraded state (used as the definition of old-fields hereafter) present an important challenge for the practice of ecological restoration and an interesting model of alternative stable state in action.

1.3 Study system

In Australia, the majority of the lowland temperate grasslands and open grassy woodlands have been destroyed, mainly due to the suitability of the land for agriculture.

Now only degraded fragments remain and they are mostly dominated by annual introduced grasses. Consequently, these areas have been described as Australia's most threatened terrestrial ecosystems (Kirkpatrick 1995). This is worrying considering the plant diversity they can contain, the number of native animal species that rely on these grasslands, and the valuable ecological services native grasslands provide, such as water processing in catchments, salinity reduction and soil stabilization (Kirkpatrick 1995). Therefore, it is not surprising that there has been substantial interest in restoring these systems in recent years (Dorrough *et al.* 2006; Lindsay and Cunningham 2011; Morris and de Barse 2013; Prober *et al.* 2002; Suding 2011).

Para Woodlands Reserve, South Australia, was chosen as the field site for this project because it is an active restoration site with areas previously used for cereal cropping for many decades (Rosser 2013). Livestock grazing and regular fertilizer application also occurred in these areas until farming ceased in 2004 and the available soil nutrient levels remains higher than in nearby remnant areas (Hughes 2005). The old-field sites are now dominated by exotic winter-growing annual grasses, in particular *Avena barbata* (Pott ex Link)*, Lolium rigidum* (Gaud.) and *Bromus* species. The main aim for the reserve is to be restored to a functioning, self-sustained grassy woodland. However, difficulties have arisen in restoring the old-field areas since the planted community has low survival and the sites become reinvaded by exotic plants. Given the level of environmental change experienced on the property it is likely that both abiotic and biotic thresholds have been crossed, therefore, adopting an alternative stable states model may assist in the restoration process. This could help provide a greater understanding of the best approaches to build resilient native communities.

Areas of remnant vegetation in comparatively good condition are also present on the property. Although grazing by livestock occurred in these areas prior to 2004, these

remnant sites have been useful as reference systems for determining restoration goals and have been used as soil collection sites for this project. These remnant areas are classified as open grassy woodlands with an understory dominated by *Austrostipa* species S.W.L. Jacobs & J. Everett, *Rytidosperma* species Syn. Pl. Glum., *Aristida behriana* F. Muell. and *Themeda triandra* (R.Br.) Stapf with an over-story of *Eucalyptus camaldulensis* var. *camaldulensis* (Dehnh.), *E. porosa* F. Muell. ex Miq., *E. odorata* (Behr) and *E. leucoxylon* var. *leucoxylon* F. Muell.

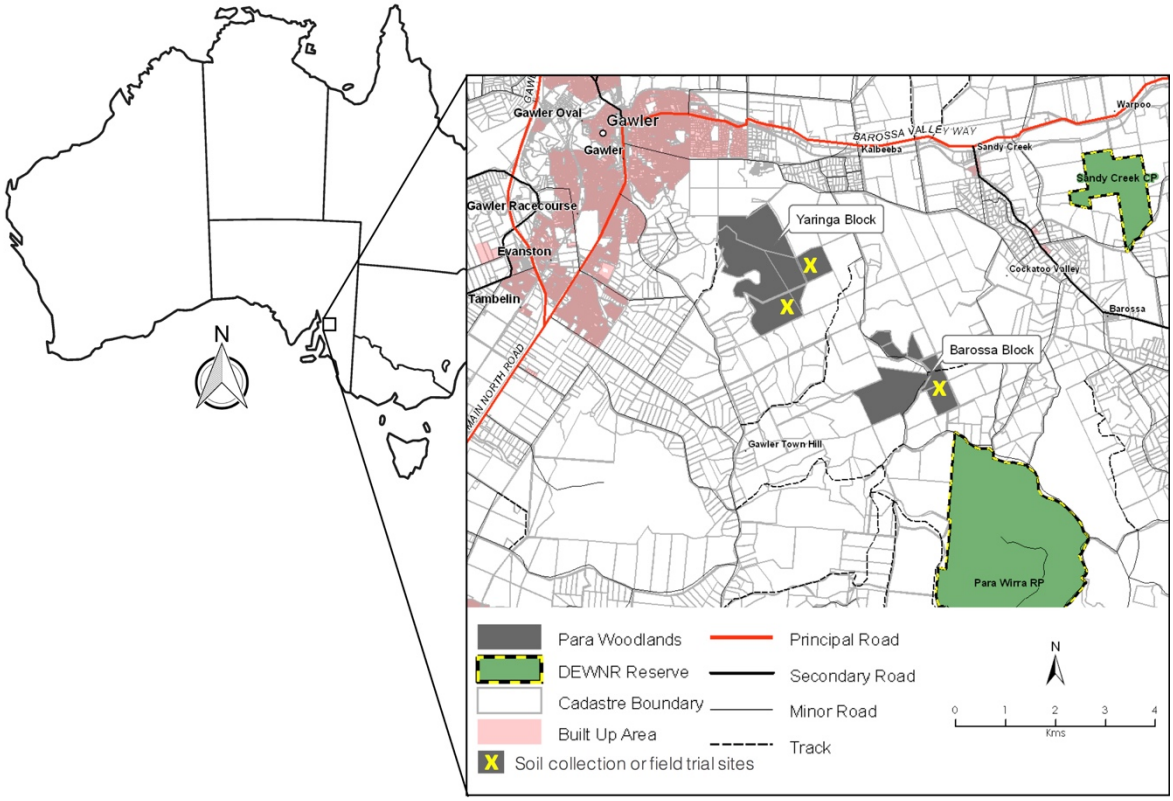

Figure 1.1. Location of the two areas of Para Woodlands Reserve, labelled Yaringa and Barossa blocks, and experimental sites and soil collection sites used during this project (adapted from Rosser 2013).

1.4 Overall aim and thesis outline

Old-field restoration can be complex because of the intense changes caused by farming practises. Currently, the amount of research on this type of restoration is severely lacking in Australian systems, which is concerning as plant responses to possible

constraints can be very species specific and dependant on environmental conditions. In addition, much of the research undertaken fails to provide insights into the underlying mechanisms, limiting the possibility of drawing general conclusions and reducing the ability to extrapolate the results to a wide range of conditions. While it is unrealistic to expect every local species to be studied, it is important to gain a clear understanding of the mechanisms behind possible constraints to establishment of the main species to be able to give restoration practitioners advice on a broader scale. Therefore, I have chosen to focus on grass species because grassland restoration is a main aim at Para Woodlands reserve due to major losses of these systems in the area and because annual grasses cause a major threat in the area.

I propose to conceptualise old-fields as alternate stable states, with internal feedbacks that reinforce the degraded state. This should allow us to understand the environmental conditions that need to be modified to shift communities from ones made up entirely of exotic species to resilient communities containing native species. This approach has guided the methods in this project, which includes experiments looking at possible feedbacks in degraded versus remnant areas, trials of different methods to overcome constraints to restoration, and an exploration of resilience in revegetated communities.

The overall aims of this project were to develop a mechanistic understanding of the processes hindering native grass establishment and to improve the effectiveness of techniques used for restoration of native grasslands. In this thesis, I describe my use of field and glasshouse experiments to explore different aspects of old-field restoration concentrating on below-ground processes. In particular, I focus on plant-soil biota interactions (chapters 2 and 3), reducing nutrients via site preparation techniques (chapter 4), and resource use and community resilience (chapter 5). Finally, a general discussion

(chapter 6) will tie these themes together and provide insights for future work on this topic and propose approaches for restoration of old-fields.

1.5 Chapter summaries

Soil microbial community composition and activity can be driven by soil resource availability (Fierer *et al.* 2012; Leff *et al.* 2015; Ramirez *et al.* 2010), cultivation history and cropping sequence (Buckley and Schmidt 2001; Calderón *et al.* 2000), and/or grazing intensity (Bardgett *et al.* 2001; Lopez-Sangil *et al.* 2011). Therefore, it is not surprising that old-fields have been shown to support very different soil microbial communities than native systems (Araujo *et al.* 2014; Gellie *et al.* 2017; Wong 2013). In addition, soil microbes can affect plants either positively or negatively through pathogenic effects, herbivory, aeration of soils, and controlling nutrient cycles. Symbiotic relationships with microbes play essential roles in plant growth and development, with around 80% of vascular plants relying on soil microbes to aid nutrient uptake in exchange for organic matter to feed on (Ferrazzano and Williamson 2013; Wolfe and Klironomos 2005). Therefore, shifts in community structure of soil microbes has the potential to favour particular species over others (Bever 1994) and change outcomes of restoration practices. Therefore, a glasshouse study (**chapter 2**) was implemented to investigate whether soil microbes from remnant areas can aid the restoration of old-fields by improving the growth of native grasses, and whether soil microbes from an old-field encourage further invasive species establishment.

Plant species can support different soil microbial communities, (e.g. via differences in the organic matter they produce, root exudates they release, and root structure) which, in turn, can lead to a change in the performance of that species (Bever *et al.* 2010). Negative feedback, i.e. when performance is decreased, can favour plant species coexistence and diversity (Kardol *et al.* 2007; Mills and Bever 1998). Positive feedback, i.e. when

performance is increased, can lead to dominance of a single species (Kardol *et al.* 2007; Reynolds *et al.* 2003). These feedbacks have the potential to reinforce an alternative stable state or drive systems along unexpected successional trajectories. The complexity of plant-soil interactions is further influenced by local abiotic conditions, including soil characteristics and physiochemical properties. Understanding how plant-soil interactions can change in different soil types can have important implications for predicting plant community structure, as soils differ due to anthropogenic change. In a reciprocal transplant experiment (**chapter 3**), using soil from old-field and remnant areas the aim was to better understand the complex interactions between soil microbes, soil abiotic conditions and plant species.

In old-fields where restoration is difficult, finding the most suitable site preparation technique prior to revegetation is crucial to create suitable environmental conditions for the target community. It has been suggested that a combination of techniques is the best way to achieve this because there are often multiple constraints that need to be overcome (Fuhlendorf and Engle 2004; Gibson-Roy *et al.* 2010). In particular, high nutrient availability from past fertiliser use can persist long after farming has ceased and usually results in dominance of fast-growing annual exotic plants (Standish *et al.* 2006). A trail of four methods of site preparation: fire, top-soil removal (scalping), slashing (followed by removal of plant biomass), and carbon supplements, aimed to compare their effectiveness at reducing exotic biomass and improve native grass establishment (**chapter 4**). This trial was also set up to understand the mechanisms underpinning each method, in particular, quantify any changes in soil nutrient availability and soil bacterial community composition associated with these methods.

For a revegetated community to persist with minimal ongoing maintenance it will need to be resilient to the invasion of exotic plant species (D'Antonio and Meyerson 2002;

Kulmatiski 2006). The likelihood of invasion is dependent on the amount of resources (including water, light, nutrients and bare ground) left unconsumed by the resident plants (Davis *et al.* 2000). One way to improve resilience may be by matching the functional group of the planted species to the exotic species likely to invade, as they would use similar resources (Fargione *et al.* 2003). However, if the exotic species has a competitive edge over the native species, such as an earlier start to the growing season, revegetating with a mixture of functional groups may be more advantageous (Roscher *et al.* 2013). Therefore, a field trial tested the hypothesis that a community with a mixture of species growing at different times would be more effective at competing with invasive species than communities where resident species are actively growing over a narrower time period (**chapter 5**).

Finally, the results of the four data chapters are summarised in the general discussion (**chapter 6**) including the implications for restoration ecology. The future directions that can be informed by the findings of this thesis are also discussed.

This thesis serves to improve our understanding on plant-plant and plant-microbe-soil interactions in old-fields and grassy ecosystems of south-eastern Australia. By using a mechanistic approach to understand these linkages the results discussed here will have implications for restoring old-fields into self-sustaining native grasslands with relevance to other systems with Mediterranean-type climates. Overall, this project aims to improve our understanding by using novel approaches and emergent technologies to test fundamental questions in community ecology and restoration ecology.

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Chapter 2. Evidence for species-specific plant responses to soil microbial communities from remnant and degraded land provides promise for restoration

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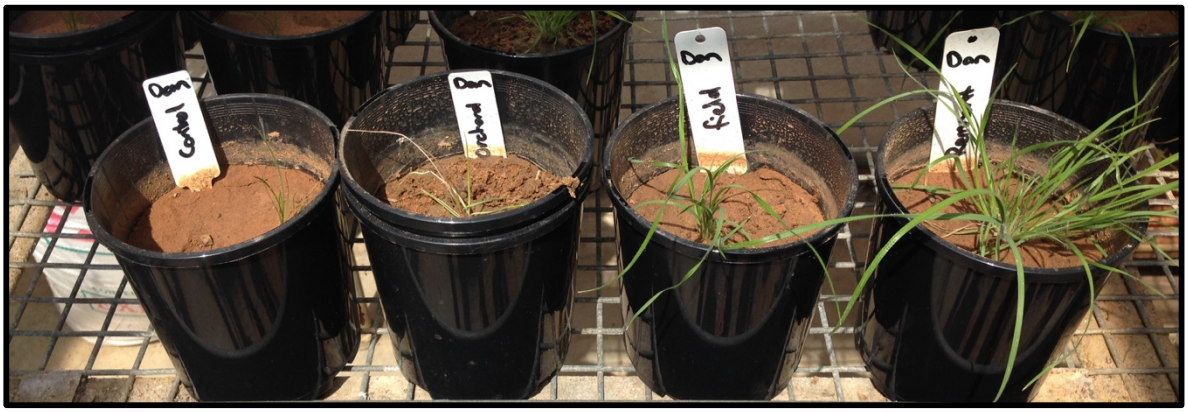

Examples of *Rytidosperma auriculatum* plants at the time of harvest in each soil treatment; from left to right, sterile control, inoculated with whole soil from a native seed orchard, an old-field and a remnant grassland.

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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Evidence for species-specific plant responses to soil microbial communities from remnant and degraded land provides promise for restoration

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Abstract Below-ground interactions between soil microbial communities and plants play important roles in shaping plant community structure, but are currently poorly understood. Understanding these processes has important practical implications, including for restoration. In this study, we investigated whether soil microbes from remnant areas can aid the restoration of old-fields, and whether soil microbes from an old-field encourages further invasive establishment. In a glasshouse experiment, we measured growth and survival of two native grasses (*Austrostipa nodosa* and *Rytidosperma auriculatum*) and an invasive grass (*Lolium rigidum*) grown in sterile soil inoculated with whole soil from three locations: an old-field, a remnant grassland, and a seed orchard planted with native grasses 7 years ago. Plants grown in sterile, non-inoculated soil acted as controls. The orchard inoculant was included to test whether soil microbes from an area cultivated with native grasses induced plant responses similar to remnant areas. The remnant treatment resulted in the highest biomass and no mortality for *R. auriculatum*. All inoculant types increased the biomass of the invasive species equally. The native grass, *A. nodosa*, was the most sensitive to the addition of inoculum, whereas the invasive *L. rigidum* suffered very low mortality across all treatments. Overall, mortality was highest in the old-field treatment at 42.9%. These results give insights into how soil microbes can affect community structure and dynamics, e.g. the high mortality of natives with old-field inoculant may be one mechanism that allows invasive species to dominate. Poorer performance of native species with the orchard inoculant suggests it would not make a suitable replacement for remnant soil; therefore, more work is needed to understand the requirements of target species and their interactions before this technique can be exploited to maximum benefit.

Key words: *Austrostipa nodosa*, grassland restoration, *Lolium rigidum*, old-field, plant-soil interaction, *Rytidosperma auriculatum*.

INTRODUCTION

Plant-soil microbial feedbacks have a major influence on plant community structure (van der Heijden *et al.* 1998; Wardle 2002). This could have important practical implications, such as in restoration of native habitats where it is yet to be incorporated systematically into frameworks. Soil microbial communities are made up of a suite of microorganisms including bacteria, fungi, and nematodes, among others. These communities are capable of causing both positive and negative effects on plant growth and survival (Johnson *et al.* 1997; Reynolds *et al.* 2003; Bever *et al.* 2010). Effects on plant performance can often be species specific and be important drivers of patterns of abundance, diversity and coexistence in plant communities (Bever *et al.* 2010; Reinhart 2012).

Although often overlooked in mainstream ecology, our understanding of plant-soil interactions has grown recently. For instance, it is well documented that plants can influence the microbial composition through these interactions (reviewed in Bever *et al.* 2010; Kulmatiski *et al.* 2008), sometimes quite rapidly in glasshouse conditions (Kulmatiski & Kardol 2008) but can take longer in field conditions (Gellie *et al.* 2017). A change in microbial composition can decrease plant performance generating negative feedbacks that reduce species abundance (Bever *et al.* 2010). Negative feedbacks favour plant species coexistence and diversity or may lead to successional replacement (Mills & Bever 1998; Kardol *et al.* 2007). Conversely, positive feedback occurs when the performance of a plant is increased after changes to microbial composition are made (Reynolds *et al.* 2003; Kardol *et al.* 2007). This is one mechanism by which invasive plants can maintain dominance in a system, thus slowing or preventing successional

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replacement (Miller *et al.* 1995; Corbin & D'Antonio 2004; Jordan *et al.* 2008).

Restoration can be particularly challenging in abandoned farmland (old-fields) because past farming practices have often altered environmental conditions so severely that both biotic (e.g. destruction of native seed bank, reduction in beneficial soil microbes) and abiotic (e.g. soil chemistry, water fluxes) thresholds are crossed, shifting the system to an alternative stable state (Cramer *et al.* 2008). Dominance of exotic annual grasses often occurs in old-fields due to their early germination and fast growth compared with native species. In addition, once established they can reinforce the degraded state of old-fields by changing nutrient cycles, soil biota composition and creating a thick layer of litter (Facelli & Facelli 1993; Cramer *et al.* 2008; Blank *et al.* 2016). While it is common for a single or few invasive species to dominate an old-field through these mechanisms, little is known about how these changes may enable other invasive species (previously absent or less dominant) to also invade the system.

Given the difficulties with restoring old-fields, one restoration 'tool' that holds potential is the use of microbial inoculants to facilitate the recovery of soil mutualists and therefore assist the restoration of native plants (Rowe *et al.* 2007; Emam 2016). A recent meta-analysis concluded that using whole soil from remnant areas to inoculate restoration sites may be more effective than commercial products and that the benefits of using remnant inocula could last for several years (Maltz & Treseder 2015). However, as these benefits become better known, the demand for remnant sources of inocula may negatively impact the, already threatened, remnant areas. Therefore, suitable sources of inoculant, such as established restoration sites or native seed orchards, may need to be explored as alternatives to remnant areas.

Here, we report results of a glasshouse-based experiment in which we compared the performance of two native and one invasive grass species grown in the presence of three microbial communities: one found in soil from an old-field dominated by invasive grasses, another from a remnant grassland dominated by *Austrostipa spp.* and *Rytidosperma spp.* and the third from a seed orchard where native plants were grown in monoculture blocks. The orchard soil was included to test whether it would make a suitable alternative inoculant for restoration. Given that the orchard had been established for 7 years which was within the timeframe that other studies have shown plant-induced changes in microbial communities (Kulmatiski & Kardol 2008; Gellie *et al.* 2017). We aimed to address three questions:

1. Do microbial communities from a remnant grassland benefit native grass growth and establishment?
2. Does an invasive grass, *Lolium rigidum*, benefit from the soil microbial changes found in an old-field?
3. Do microbial communities in an orchard affect plant performance in similar ways to those in remnant areas?"

We hypothesised that because microbial communities present in old-field and remnant soils would be different, plant growth and establishment would vary in response to these differences. Specifically, we predicted the invasive species to do better in old-fields where it has invaded, whereas the two native species were expected to perform better in the presence of the microbial communities with which they naturally occur (remnant areas).

METHODS

Soil and seed collection

Soil and seed collection occurred within Para Woodlands Reserve, South Australia, (34.608°S, 138.784°E). The region has a Mediterranean-type climate with winter-dominated rainfall of 450 mm/annum on average. The reserve was a cereal and sheep farm which received regular fertilizer application until farming ceased in 2004. Areas of intact remnant vegetation are present and have lower soil nutrients than the neighbouring old-field areas (Rosser 2013), though grazing likely occurred in these areas prior to 2004. These remnant areas are classified as open grassy woodlands dominated by *Austrostipa* and *Rytidosperma* species with an over-story of *Eucalyptus camaldulensis* and *E. leucoxylon*. To meet the demands of restoration efforts a seed orchard of native plants, planted in blocks, was constructed in 2007 with invasive species suppressed using plastic matting.

We collected soil from three sites: an old-field, remnant grassland and seed orchard (see Appendix S1 for further information). At the old-field site soil was collected from five locations (1 m^2 quadrats) within three 20 × 20 m plots, removing vegetation. At the remnant site, soil was also collected in a 20 × 20 m plot from the base of native grass species to avoid collecting from underneath invasive plants that were present between the tussocks. First plant material and debris was removed from around the surface and then a hand trowel was used to collect soil from around the native grass roots. The orchard soil was collected from the base of *Austrostipa nodosa* plants, which were grown in a monoculture covering approximately six square metres. All soil was collected to a depth of 10 cm and stored at 4°C until further processing. Collected soil was used as inoculum to reflect natural field conditions, following the 'natural experiment' approach of Kulmatiski and Kardol (2008).

We chose two perennial C_3 native grasses, *Rytidosperma auriculatum* and *A. nodosa*, that are both common in the

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remnant area and are widely used for restoration at Para Woodlands and surrounding areas. Seeds of these species were collected from the orchard in spring 2013. For the invasive grass, we chose a species that was present in the old-field at a lower abundance (*L. rigidum*, 17% cover) compared with the dominant species (*Avena barbata*, 100% cover). Seed of *L. rigidum*, an annual C_3 grass, was collected from the soil seed bank in the old-field site in May 2014.

Glasshouse-based experiment

This experiment had four soil treatments including three inoculated treatments and a control. The inoculated treatments were created with 3.5 parts autoclaved (at 121°C for 1 h) sandy loam and one part fresh homogenised soil from one of the field sites, i.e. orchard, old-field and remnant. The control was the sterile sandy loam but with no inoculum added. Soil analysis was carried out on five replicate samples of each soil treatment to measure nitrate nitrogen, ammonium nitrogen, plant-available (Colwell) phosphorus, potassium (Colwell), sulphur, organic carbon, conductivity and pH ($CaCl_2$), at CSBP laboratories (Bibra Lake, WA). Nutrient availability was low for all soil treatments, particularly ammonium which was below detection levels in some cases (Table 1). Salinity was low and pH was neutral for all treatments (Table 1).

Seeds were germinated on trays of vermiculate and paper towel and kept moist. All trays were kept in a germination cabinet at 12°C with total darkness for two weeks. Following this, 40 seedlings of similar size from each species were selected. There were ten replicate 1 L pots for each soil treatment per species, however due to misidentification four *L. rigidum* were not included so there were only eight replicates for the old-field treatment and nine for the orchard and remnant treatments ($N = 116$). All pots received 900 mL of the corresponding soil, watered to saturation and planted with one seedling. The pots were then randomly placed in the greenhouse under natural light conditions and re-randomised every 3 weeks. Pots were watered equal amounts every 2–3 days so that water was not limiting (Bever 1994) and non-target seedlings that emerged from the soil seedbank were removed.

Harvest was timed to phenology of species, using first flowering as the trigger, to account for differential responses to AM due to plant ontogeny (Bethlenfalvay *et al.* 1982; Ronsheim 2012; Miller *et al.* 2014). This was required by the use an annual and two perennial species. When the inflorescences began to appear, 97 days for *L. rigidum*, and 122 days for *R. auriculatum* and *A. nodosa*, the shoots were cut at the base and roots were rinsed separately to remove adhering soil. Final mortality of the experimental plants was recorded at the time of harvest. Small root samples (between 30 and 90 mg fresh mass) were collected, weighed and stored in 50% ethanol to investigate formation of arbuscular mycorrhizas (AM). Fresh mass of the remaining roots were recorded before they were oven dried with the shoots at 70°C for at least 48 h. The fresh to dry weight ratios of the bulk of the roots were used to estimate the dry weights of the small samples and these were added to the total dry weights.

The root samples were prepared based on a clearing and staining technique described by Vierheilig *et al.* (1998). First they were rinsed thoroughly with tap water to remove ethanol and then submerged in 10% KOH for 4 days at room temperature. Roots were then rinsed, submerged in 5% white vinegar for three minutes, rinsed again and submerged in 5% ink solution (Schaeffer black ink) and placed in a 90°C water bath for five minutes. To de-stain the roots they were rinsed until the water ran clear, submerged in 5% vinegar and left over night. All samples were stored in 50% glycerol. The grid intersect method (Giovannetti & Mosse 1980) was used to quantify formation of AM.

Data analysis

Total biomass was analysed as a function of plant species and soil treatment with a linear model. Data was transformed using square root transformations to meet parametric assumptions. The overall mortality was analysed using a generalised linear model (GLM) using the 'glm' function in the base package of R, where the response was binomial (0 = alive, 1 = dead) and tested as a function of plant species and soil treatment. The significance of the interaction and main effects was assessed using a chi-squared test. The presence of AM in the roots was also treated as binomial (0 = not present, 1 = present) however a mixed-effects model using the 'glmer' function in the R package 'lme4' with an observation-level random effect included was needed to account for over-dispersion in the GLM (Bates

Table 1. Mean (±SE) of soil physicochemical properties for the soil treatments ($n = 5$)

		Inoculant source		
Soil physiochemical properties	Control	Old-field	Orchard	Remnant
NO_3^- -N (mg kg^{-1})	5.4 ± 0.2	5.6 ± 0.2	8.3 ± 0.3	6.2 ± 0.2
NH_4^+ -N (mg kg^{-1})	<1	<1	1.8 ± 0.2	<1
P Cowell (mg kg^{-1})	4.2 ± 0.2	5.2 ± 0.2	5.6 ± 0.2	4.2 ± 0.2
K (mg kg^{-1})	117.8 ± 1.8	176.6 ± 7.2	168.6 ± 6.9	124 ± 1.9
S (mg kg^{-1})	5.6 ± 0.1	4.8 ± 0.1	5.1 ± 0.2	5.6 ± 2.2
Organic C (%)	0.2 ± 0.01	0.3 ± 0.05	0.3 ± 0.01	0.4 ± 0.04
Conductivity (dS m^{-1})	0.1 ± 0.001	0.1 ± 0.001	0.1 ± 0.01	0.1 ± 0.01
pH (1:5 $CaCl_2$)	7.3 ± 0.02	7.3 ± 0.05	7.3 ± 0.04	7.2 ± 0.04

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et al. 2014). Control data were removed from the analysis because AM presence was very low (see Results). Where significant interactions were detected with analysis of variance (ANOVA) tests, *post hoc* tests of pairwise comparisons were made using the 'glht' function in the R package 'multcomp' (Hothorn *et al.* 2008). The 'glht' function allows multiple comparisons based on generalised linear models by using *post hoc* Tukey's honest significant difference (HSD) tests. All models were graphically checked for their error distributions and homogeneity of variances. All statistics were performed in R version 3.1.1 (R Core Team 2017).

RESULTS

Mortality

Out of 116 plants, 26 had died by the end of the experiment. There was no significant interaction between soil treatment and species for the number of deaths ($\chi^2 = 6.9$, $P = 0.33$). However, there was a significant species effect ($\chi^2 = 12.96$, $P = 0.002$) and soil treatment effect ($\chi^2 = 12.33$, $P < 0.01$). Furthermore, there was a significant difference between *A. nodosa*, which had the highest mortality (37.5%), and *L. rigidum*, which had the lowest mortality (5.6%) (Fig. 1). Neither of these species differed significantly from the native *R. auriculatum* (22.5% mortality). Between soil treatments the highest mortality was produced by old-field inoculum (42.9%) which was significantly higher than the control (6.7%) but no significant differences occurred for orchard and remnant compared with the control (Fig. 1).

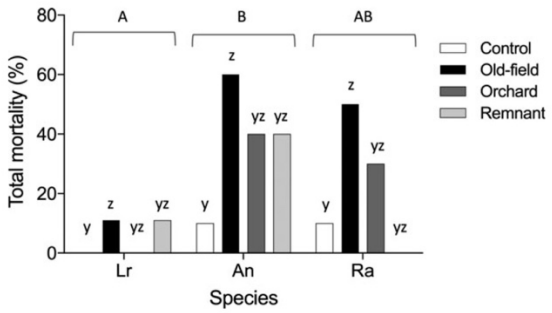

Fig. 1. Total mortality (%) of three species, invasive grass *Lolium rigidum* (Lr) and native grasses *Austrostipa nodosa* (An) and *Rytidosperma auriculatum* (Ra), at the end of the experiment grown with control soil with no inoculum added or inoculated with either old-field soil, orchard soil or remnant soil. Sample size varied for each soil treatment: control $n = 30$; old-field $n = 28$; orchard $n = 29$; remnant $n = 29$. Tukey's post hoc test found statistical significance between soil treatments (represent by lowercase letters) and between species (represent by capital letters).

Biomass

The three species responded differently to the soil treatments as indicated by a significant interaction for total biomass (ANOVA, $F = 5.923$, $P < 0.001$; Fig. 2). Post-hoc comparisons indicated that *L. rigidum* had greater growth in all inoculated treatments than in the control (old-field $P < 0.001$, orchard $P = 0.025$, remnant $P = 0.017$), indicating some benefit from soil microbes generally (Fig. 2). Compared to the control, both native grasses had increased growth with remnant inoculum (*R. auriculatum* $P < 0.001$, *A. nodosa* $P = 0.025$) but no difference with the orchard inoculum (Fig. 2). *R. auriculatum* also showed significantly greater growth in the old-field inoculum ($P < 0.001$, Fig. 2).

Presence of AM

The control soil had significantly less formation of AM in the roots than the inoculated soil for all species (per cent AM present: *A. nodosa* 0.06 ± 0.06%, *R. auriculatum* 0.16 ± 0.15% and *L. rigidum* 2.43 ± 1.5%; pairwise comparison compared with other treatments $P < 0.001$), therefore controls were left out of further analysis. There was a significant interaction between species and soil treatment (chisq = 14.35, $P = 0.006$). Post hoc comparisons found that AM formation in the remnant treatment was significantly higher than the orchard treatment

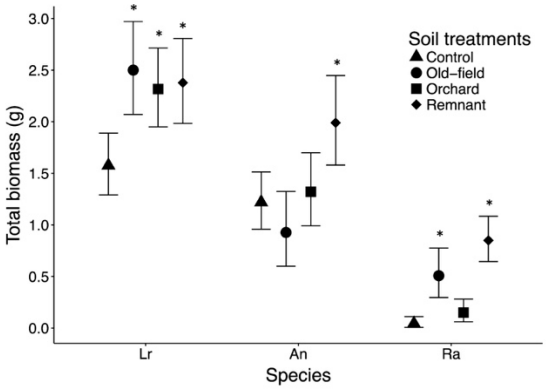

Fig. 2. Mean total biomass of three species, invasive grass *Lolium rigidum* (Lr) and native grasses *Austrostipa nodosa* (An) and *Rytidosperma auriculatum* (Ra), grown with control soil with no inoculum added (triangles) or inoculated with either old-field soil (circles), orchard soil (squares) or remnant soil (diamonds). Error bars are 95% confidence intervals. Asterisks indicate significant differences in biomass for inoculated soil compared with the control for each species separately. Analysis was carried out on square-root transformed data and then results were squared for interpretation.

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for all species (*A. nodosa* z value = 3.12 and $P = 0.011$, *R. auriculatum* z value = 4.09 and $P < 0.001$, and *L. rigidum* z value 5.021 and $P < 0.001$; Fig. 3). AM formation only differed between remnant and old-field treatments for *A. nodosa* (z value = 4.3, $P < 0.001$), where AM formation was greater in the remnant soil treatment (Fig. 3).

DISCUSSION

We found that grass responses to the soil microbiota of different sites were not only species specific, but depended on the demographic parameter considered. We found some evidence that supports our hypothesis that native plant species perform better with inoculum sourced from their site of origin with the highest biomass with remnant microbes for both species. However, survival was only greater in the remnant inoculum for *R. auriculatum* and not for *A. nodosa*. There was no evidence to support that the soil microbes in the old-field benefits the invasive species as *L. rigidum* had greater growth in all inoculant types compared with the sterile control.

Native species responses

Both native species had positive growth responses to the remnant inoculum compared with the control and *R. auriculatum* did not suffer any mortality in this treatment. Comparatively, growth was quite stunted in the control soil for both native species, particularly *R. auriculatum*. One explanation for increased performance could be from positive effects of forming AM; plants were essentially non-mycorrhizal in the control soil (between 0% and 0.2% roots with AM) and AM formation was much higher in the remnant treatment (23.3 ± 3.9% and 27.1 ± 4.1%) for both native species. However, *A. nodosa* had 40% total mortality in the remnant treatment indicating a negative response to this inoculation treatment.

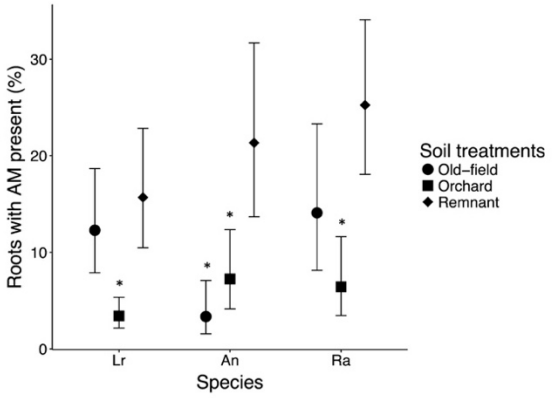

Fig. 3. Per cent of arbuscular mycorrhizas (AM) present in roots of three species, invasive grass *Lolium rigidum* (Lr) and native grasses *Austrostipa nodosa* (An) and *Rytidosperma auriculatum* (Ra), grown with either old-field inoculum (circles), orchard inoculum (squares) or remnant inoculum (diamonds). Controls were removed from analysis because AM presence was too low. Error bars are 95% confidence intervals. Asterisks indicate significant differences in AM formation compared with the remnant treatment after Tukey contrast analysis.

Negative feedbacks often occur when pathogens build up in the soil in which the plants are growing, and is thought to be a mechanism leading to plant coexistence and diversity maintenance, and involved in successional replacement (Bever *et al.* 1997, 2010; Herzberger *et al.* 2015). If pathogens did cause the high mortality of *A. nodosa,* the relatively large growth of surviving plants suggests that the beneficial effects of mycorrhizal fungi in these plants may have outweighed the negative effects of pathogens. Alternatively, this may reflect different effects of mycorrhizas at different developmental stages (Ronsheim 2012), i.e. the AM associations were potentially a carbon drain in the early stages but increased phosphorus availability in the surviving adult plants. Further investigation, such as identifying the soil microbial community composition or quantifying pathogen loads, is required to understand the underlying mechanisms involved.

The old-field inoculum resulted in the highest mortality reaching up to 60% in native species. This is somewhat contradictory to the findings of Herzberger *et al.* (2015), where native perennial grasses performed better with agricultural soil inoculum than remnant soil in tallgrass prairies in North America. However, in our study, *R. auriculatum* had greater growth in treatments receiving the old-field inoculum than in the control, suggesting that some beneficial microbes were present in the old-field inoculum. The results also suggest that *R. auriculatum* is less sensitive to changes in soil microbial communities than *A. nodosa*, which experienced nearly double the mortality overall and the lowest biomass with old-field microbes. This, along with differences in mortality in the remnant soil, indicates that it is not possible to make generalisations about the effects of various microbial communities on species based in coarse functional groups (i.e. native perennial C3 *vs.* annual invasive grasses). Instead, effects appear to be more species specific or may depend on subtler ecological properties of species. Further investigation on whether effects are species specific or can be generalised at some functional group level are warranted.

Interestingly, responses to the different sources of inoculum differed between life history stages of the native species, with increased growth but reduced establishment (i.e. higher mortality), with inoculated

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soil compared with the control. While the addition of inoculum likely increased pathogens, and thus reduced survival, the greater growth of the surviving plants compared to the control is suggestive of the plants gaining some advantage from the presence of microbes, whilst also overcoming any pathogenic effects (Bever 1994; Klironomos 2002). It is not possible to determine the final balance between the effects of mortality and growth in a community context because this experiment did not incorporate density dependent effects. However as different combinations of density and per capita reproduction are possible (Goldberg *et al.* 2001), further assessments are required. Restoration outcomes can be strongly affected by these differential effects on numbers and performance. More importantly, by understanding how soil microbial communities affect different life history stages, we may begin to understand the processes underpinning community structure.

Invasive species response

Given the non-specialist nature of invasive species and that they are less likely than native species to have a negative feedback to soil microbial communities (Klironomos 2002; Hawkes *et al.* 2013), the positive growth response of *L. rigidum* to all three inoculants in this study is, perhaps, not unexpected. This could be because *L. rigidum* has escaped pathogens from its home range (Maron *et al.* 2014), a theory that warrants more investigation. In addition, invasive species are hypothesised to be generally more plastic than native species, allowing them to adapt to environments more effectively (Davidson *et al.* 2011). If this is true for *L. rigidum*, then it may go some way to explaining why it had much lower mortality than the two native species.

Orchard inoculant

We included inoculant from the native seed orchard to see whether this can be a viable alternative to using remnant soil for harvesting beneficial microbial communities. However, we found no evidence to support this approach because the native plants grown with orchard inoculant had no increase in biomass compared to the control, and very low percentage of roots with AM fungi. This was surprising given that the *A. nodosa* plants in the orchard appear healthy and have a high seed production (M. E. Smith, pers. observation, 2015). However, there may be other benefits to the grasses in the orchard beyond soil microbes, such as reduced competition and increased moisture from weed matting and increased nutrients from past farming practices.

The seemingly negative response to orchard inoculum could be because planting in a monoculture is not suitable for re-establishing symbiotic relationships with microbes, or that plant-specific pathogen loads may have a negative impact (Klironomos 2002). Time could also be a factor because our source orchard has been established for 7 years and it has been shown that it takes at least 8 years for microbial communities in restored areas to shift towards a composition similar to remnant areas (Gellie *et al.* 2017). However, this is unlikely given only one-year difference and that changes in the microbial communities have been shown to occur in a matter of months in glasshouse experiments (Kulmatiski & Kardol 2008). In addition, changes in microbial communities can be non-linear through time and depend on many external factors including soil type and properties and functional diversity of plants (Kulmatiski & Kardol 2008; Cavagnaro *et al.* 2016; Dassen *et al.* 2017). More research is warranted to better understand the complex relationships between plant and microbes before building 'inoculant orchards' for restoration practices.

Future research directions

Here we build on the mounting evidence that responses to soil microbes are species specific and depend on many factors, including the requirements of the plants, susceptibility to pathogens and life history stage (Kulmatiski & Kardol 2008). In this study, we did not attempt to measure differences in species composition of the microbial communities and so we were unable to assess whether species responses were dependent on particular microbial species. Future research would benefit from using sequencing based tools to identify the microbial community to help identify species or functional groups that are important for native grass establishment or any pathogens that may threaten restoration success. Future studies should also consider the pot size required for adequate growth of the test species. While the plants in our study did not have pot bound roots and 1 L pots are commonly used for these species, or closely related species (Allcock 2002; Simarmata *et al.* 2005; Tran & Cavagnaro 2010), growth may have been effected if the soil had higher nutrients or if the experiment had a longer time period.

Conclusions and wider implications

Understanding the processes underpinning plants community structure is likely to increase the success of restoration outcomes. This study highlights

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the need to restore the soil microbial community as well as the plant community for restoration projects to be successful and sustainable. In particular, the high mortality of native species in the presence of old-field microbes suggests that any attempt to remediate soil microbial communities should result in better restoration outcomes in old-field systems. However, we conclude that the specialised relationships of target plants and their soil microbial communities requires further research before large scale inoculation can take place for restoration. While our study is limited to only two native species, the different results observed between these species means that it is unlikely to be a one-size-fits-all inoculant for restoration and restoration goals need to be considered when designing an appropriate inoculum. In addition, our results show that soil microbial amendments may have no effect on invasive species but including symbionts that help make native species more competitive warrants further investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Characterisation of soil collection sites at Para Woodlands Reserve.

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Chapter 3. Interactions between soil properties, soil microbes and plants in remnant-grassland and old-field areas: a reciprocal transplant approach

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This experiment was carried out in the glasshouse facilities at the Waite Campus of the University of Adelaide. This photo was taken on the day of the *Avena barbata* harvest, 31st August 2015.

3.1 Statement of authorship

Title of Paper	Interactions between soil properties, soil microbial communities and plants in remnant grassland and old-field areas using a reciprocal transplant approach
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Principal Author

Name of Principal Author (Candidate)	Monique E. Smith		
Contribution to the Paper	Designed the study, carried out field collections and glasshouse work, analysed data, wrote manuscript as principal author		
Overall percentage (%)	85 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24-10-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Timothy R. Cavagnaro		
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Contribution to the Paper	Advised on study design; advised on the analysis and interpretation of the data; advised on and edited the manuscript		
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REGULAR ARTICLE

Interactions between soil properties, soil microbes and plants in remnant-grassland and old-field areas: a reciprocal transplant approach

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Abstract
Background and aims The importance of plant-soil feedback is becoming widely acknowledged; however, how different soil conditions influence these interactions is still relatively unknown. Using soil from a degraded old-field and a remnant grassland, we aimed to explore home-field advantages in plant-soil feedbacks and plant responses to the abiotic and biotic soil conditions. We quantified the soil bacterial and fungal community from these sites and their responses to soil conditions and plant species.
Methods Sterilized old-field and remnant-grassland soil was inoculated with home or away soil in a reciprocal transplant experiment using a native grass, *Rytidosperma auriculatum,* and an invasive grass, *Avena barbata,* as test species. The soil fungal and bacterial communities were characterised using high throughput sequencing.
Results Plants had a greater growth response to microbes when an inoculant was added to its home soil. However, this relationship is complex, with microbial communities changing in response to the plant species and soil type.
Conclusion The apparent home-field advantage of the soil microbes shown in this study may restrict the utility of inoculants as a management tool. However, since we inoculated sterile soil, future work should focus on understanding how the inoculated microbial community interacts and competes with resident communities.

Keywords Bacterial community · eDNA · Fungal community · Invasive annual grass · Native perennial grass · Old-fields · Remnant grasslands · Home-field advantage

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Introduction

Both the magnitude and direction of plant-soil feedbacks can affect above- and below-ground community structure, trajectories of successional change, and the success of invasion and restoration processes (Bever et al. 1997; Callaway et al. 2004; Herzberger et al. 2015; Kardol et al. 2006; Wardle 2002). Soil microbial communities can influence plant performance directly, e.g. via aiding nutrient acquisition or pathogenic effects, or indirectly, e.g. via influencing nutrient cycling or decomposition processes (Ayres et al. 2009; Packer and Clay 2000; van der Heijden et al. 2006; van der Putten et al. 2001). Plants can also influence the microbial community in a variety of ways. For example, differences in root structures, root exudates and leaf

litter quality can shape microbial community composition (reviewed in Bever et al. 2010; Kulmatiski et al. 2008). Many feedback studies are plant-centric and either do not characterise the soil biota, group taxa to a coarse level, or focus on a limited number of associated soil microbes (Bever et al. 2012; Hoeksema et al. 2010; Kardol et al. 2015; Kulmatiski and Kardol 2008). However, recent advances in DNA sequencing technologies are providing new approaches for investigating these interactions in finer detail than before.

The complexity of plant-soil interactions is further influenced by local abiotic conditions, including soil characteristics and physiochemical properties (Bezemer et al. 2006; Johnson et al. 2010; Ronsheim 2012; Rua et al. 2016; van der Putten et al. 2016). It is well known that plants can adapt to their local soil conditions, sometimes referred to as home-field (or home-site) advantage (see Leimu and Fischer 2008 for meta-analysis). In studies testing for the presence of a home-field advantage in plants, reciprocal transplant designs are often employed, whereby plants are transplanted between areas where they were present (home soil) and where they did not exist (away soil; Montalvo and Ellstrand 2000; Pregitzer et al. 2010; Smith et al. 2012). In such studies, any home-field advantage is often attributed to adaptation to the local environment as a whole and the relative contributions of the biotic (e.g. microbial) and abiotic components (e.g. soil chemistry) of the soil to this response are not ascertained (Kawecki and Ebert 2004). Understanding how plant-soil interactions can change in different soil types can have important implications for species and habitat management, such as whether inoculating with mutualistic symbionts is beneficial to enhance ecosystem services (Ohsowski et al. 2012; Yuan et al. 2016).

In attempts to restore abandoned farm land (old-fields) plant-soil interactions may be particularly important as abiotic and biotic conditions are often greatly altered from their remnant state (Cramer et al. 2008). The effects of past farming practices, particularly cultivation and fertilization, in old-fields can leave long lasting soil legacies (Drenovsky et al. 2010; Standish et al. 2006; Wong 2013). Once established, exotic species can reinforce the degraded state of old-fields by changing nutrient cycles and soil biota composition and by creating a thick layer of litter (Ba et al. 2018; Cramer et al. 2008; Facelli and Facelli 1993). However, soil inoculation could be an effective restoration tool at such sites (Neuenkamp et al. 2018), whereby the inoculant source can steer the restoration outcome to a desired plant community (Wubs et al. 2016). By using a reciprocal transplant approach, where both soil microbes and plant species are transplanted into the old-field and remnant-grassland (home and away) soil, we are able to separate out the abiotic and biotic soil legacies in old-fields compared to remnant areas. This will help to gain a better understanding of the mechanisms leading to exotic species dominance and the requirements needed for successful restoration of native communities.

While previous studies have found evidence of positive plant responses to their local soil microbial community and home soil environment, a recent meta-analysis suggested that this is often not the case (Rua et al. 2016). Responses could depend on the type of plant being investigated. For example, negative feedbacks can be common in grasslands and is a mechanism for maintaining species diversity and promoting successional replacements (Kulmatiski et al. 2008; Kardol et al. 2006). Additionally, invasive species may be more likely to adapt to foreign soil than native species because they are more plastic in their responses (Davidson et al. 2011), have less reliance on symbionts or have escaped pathogens from their home range (Hawkes et al. 2013; Klironomos 2002; Mitchell and Power 2003). Therefore, the use of reciprocal transplant experiments that consider responses of both native and invasive species should be particularly enlightening in the context of old-field restoration.

Here we present results of a study in which we attempt to separate the abiotic and biotic components of both old-field and remnant soil of South Australian grasslands and measure their impact on plant performance of two grass species: native perrenial *Rytidosperma auriculatum* (J. M. Black) Connor & Edgar and invasive annual *Avena barbata* Pott ex Link. We undertook a glasshouse reciprocal transplant experiment where whole-soil inoculant and sterile control soil from an old-field and a remnant-grassland were transplanted into home and away (sterilized) soil in a fully crossed factorial experiment. By growing the two species separately in each of eight soil treatments we sought to answer the following questions 1) do the plant-soil interactions show a home-field advantage when inoculants and plants are added to their home soil? and 2) do the native and invasive species respond differently, in terms of growth and survival, to the abiotic and biotic conditions in the old-field and remnant-grassland areas? Further, by using DNA sequencing

techniques to characterize the bacterial and fungal communities we were able to ask the following questions: 3) do microbial communities differ between the inoculant sources (old-field and remnant-grassland)? and 4) do the communities change depending on which bulk soil they are added to or which plant species they are exposed to? We also documented the formation of arbuscular mycorrhizas (AM) by the plant roots for a more direct link between the plants and a specific group of soil microbes known to play an important role in plant ecosystem dynamics (Ba et al. 2018). Overall, this study aims to have applied outcomes by building on our understanding of mechanisms involved in invasive species dominance and the requirements of native species for restoration projects.

Materials and methods

Soil and seed collection

Soil and seed collection was carried out at Para Woodlands Reserve, South Australia, (34.608°S, 138.784°E). The reserve lies in a region with a Mediterranean-type climate with mean annual (winter dominated) rainfall of 450 mm, and a mean annual air temperature of 23.6 °C (Bureau of Meteorology 2017). The reserve is an active restoration site with some areas degraded by previous farming practices, mainly cereal cropping, livestock grazing and regular fertilizer application until farming ceased in 2004. Areas of remnant vegetation in comparatively good condition are present nearby and have lower soil nutrients than the neighbouring old-field areas (Rosser 2013), though grazing likely occurred in these areas prior to 2004. These remnant areas are classified as open grassy woodlands dominated by grasses (Rosser 2013), such as, *Rytidosperma* species Steud., *Austrostipa* species S.W.L. Jacobs & J. Everett or *Themeda triandra* (R.Br.) Stapf with an over-story of *Eucalyptus camaldulensis* Dehn. and *E. leucoxylon* F. Muell.

Soil was collected during June 2016 (Austral Winter) from two locations: an old-field and a remnant-grassland (hereafter remnant; see Table S1 for further information). Soil collection occurred within three 20 × 20 m plots at each site, taking soil from the base of either invasive (old-field) or native (remnant) grasses. All soil was collected to a depth of 10 cm, sieved (< 3 mm) and stored at 4 °C until further processing (10 days).

The perennial C_3 grass, *Rytidosperma auriculatum* was chosen for this experiment because it is an Australian native, common in the region and is widely used for restoration at Para Woodlands and surrounding areas (Smith et al. 2018). Its performance was compared to the annual C_3 grass, *Avena barbata*, which is originally from central Asia and the Mediterranean and now invasive worldwide. *Avena barbata* has been shown to be a strong competitor in this region, reducing species richness and the occurrence of *Rytidosperma* species (Lenz et al. 2003) and it was the dominant species in the old-field site as indicated by 100% cover in a pilot study (data not shown). Seed collection occurred in Spring 2015 from Para Woodland's seed orchard (*R. auriculatum)* or from the old-field site (*A. barbata*).

Glasshouse-based microcosm experiment

In order to assess the impact of different microbial communities on the growth and establishment of our target species we undertook reciprocal inoculation of sterilized bulk soil, from the old-field and remnant areas, with two inoculant types, either unsterilized (referred to as 'live' hereafter) or sterilized (referred to as 'mock' hereafter) inoculum from both field sites (referred to as 'inoculant sources' hereafter). This gave a total of eight inoculation treatments (2 bulk soils × 2 inoculant types × 2 inoculant sources). Soils were sterilised by twice-autoclaving for 1 h at 121 °C, and inoculation treatments were established by mixing at a rate of 85% bulk soil and 15% inoculant soil (on a dry weight basis). From these mixtures five replicate samples were taken and analysed for nitrate nitrogen, ammonium nitrogen, total nitrogen, plant-available (Colwell) phosphorus, total carbon, conductivity and pH ($CaCl_2$), at CSBP laboratories (Bibra Lake, WA, www.csbp-fertilisers.com.au). Soil samples for genetic analysis were stored frozen at −20 °C for later analysis (referred to as 'pre-experimental' samples hereafter).

From the homogenised soil mixtures, 898 g of soil (based on dry weights) was added to individual plastic bags, i.e. 10 replicates for each inoculant treatment and plant species combination. The same moisture content was established, based on the moisture content observed in the field (0.13 g water/g dry soil), in each bag by adding Reverse Osmosis (RO) water as required. Soil was mixed homogenously and allowed to equilibrate in the bags for 2 weeks. Following this, the soil was added to 1 L plastic pots at a bulk density of 1 g/cm^3. The field

capacity of each inoculant treatment was determined using methods from Cavagnaro (2016). Briefly, soil was packed into a sintered glass funnel (bulk density = 1) connected to a 100 cm water column ($\Psi m = -10$ kPa). The soil was then saturated with water, allowed to drain for 48 h and weighed before being oven dried at 105 °C for 48 h and the gravimetric moisture content calculated. The gravimetric moisture content at field capacity ranged between 0.22 and 0.28 g water/g dry soil.

Seeds were germinated in the dark on trays of vermiculate and paper towel in a germination cabinet at 12 °C, with regular watering over a period of two weeks. All pots were planted with one seedling of equal size and any seedling that died in the first two weeks was replaced. The pots were then arranged in a randomised block design in the greenhouse, with one replicate from each treatment combination per block. Pots were watered to 75% field capacity thrice weekly.

To account for the differential responses to AM (Bethlenfalvay et al. 1982; Miller et al. 2014; Ronsheim 2012; Smith et al. 2018), harvest was timed to phenology of species, using first flowering as the trigger. This differed between the annual *A. barbata* (57 days) and the perennial *R. auriculatum* (81 days). The shoots were cut at the base and soil cores (10 mm diameter by 70 mm deep) were collected (sterilizing equipment between each sample collection) and frozen (−20 °C) for genetic analysis (see below). Roots were then rinsed separately to remove adhering soil. Small root samples (between 30 and 90 mg fresh mass) were collected, weighed and stored in 50% ethanol (*V*/V) to assess the formation of AM. To account for different sizes in the small root samples the dry weights were estimated using the fresh to dry weight ratios of the main root samples and these values were added to the total dry weights. Plant material was oven dried at 70 °C for at least 48 h before being weighed.

The root samples set aside to investigate formation of AM were prepared following the clearing and staining technique described by Vierheilig et al. (1998). First, roots were cleared in 10% potassium hydroxide (KOH) and stained in 5% ink solution (Schaeffer black ink) at 90 °C for 5 min. All samples were stored in 50% glycerol until formation of AM was quantified using the grid intersect method (Giovannetti and Mosse 1980).

Statistical analysis – soil and plant material

All statistics were performed in in R version 3.1.1 (R Core Team 2017). To determine how soil properties at the beginning of the experiment were explained by main effects, i.e. the different bulk soils, inoculant sources and inoculant types, principal component analysis (PCA) was performed using the 'princomp' function from the base statistics package and plotted using the *FactoMineR* package in R (Le et al. 2008). PCA plots provide a visual representation of the similarity of groups and help to identify properties (and the correlation of properties) that separate groups from each other (Bruckner and Heethoff 2017). In addition, linear models were also carried out on each soil property using the 'lm' function in the base R and the main effects, bulk soil, inoculant source and inoculant type, included.

Out of 160 plants only four died during the experiment, all of which were *R. auriculatum,* therefore no conclusions could be made regarding the effect of soil treatment on plant survival. As a result, the following analyses were carried out on 156 plants. The presence of AM in the roots, treated as binomial (0 = not present, 1 = present), was analysed as a function of plant species, bulk soil type and inoculant source using a generalized-linear, mixed-effects model with block (location of replicate) included as a random effect. In addition, an observation-level random effect, i.e. individual roots, was included to account for some of the unexplained error causing over-dispersion in the model. Analysis was carried out using the 'glmer' function in the R package *lme4* (Bates et al. 2014). Mock-inoculant data was removed from the analysis because AM presence was very low (see results).

Total biomass was analyzed as a function of plant species, bulk soil type, inoculant source and inoculant type (autoclaved or not) using a linear mixed model with block as a random factor. The 'lmer' function was used to run the model and statistical significance was tested using the 'Anova' function, both from the *lme4* package (Bates et al. 2014). Root-to-shoot ratios were also calculated and analysed but not included due to high variation and very few differences between factors.

Microbial growth responses (MGR) were calculated using the individual total dry biomass of the live-inoculated plants and the mean total dry biomass of the corresponding mock-inoculated plants (Eq. 1; following Watts-Williams and Cavagnaro 2012). Values

above zero indicate higher growth when plants are grown with the live-inoculant and values below zero indicate less growth with the live-inoculant, zero indicates no difference. The effects of plant species, bulk soil type and inoculant source on the MGR were then tested for using the 'lmer' function with block as a random factor.

$$\%\mathrm{MGR} = \frac{\text{dry weight (live)} - \text{mean dry weight (mock)}}{\text{mean dry weight (mock)}} \times 100 \quad (1)$$

For all models, where significant differences were detected with analysis of variance (ANOVA) tests, we made planned pairwise comparisons (i.e. carried out a few targeted comparisons of interest between levels of the factors; henceforth planned comparisons) in the interests of testing our research questions, rather than every possible combination. These comparisons were made using the 'glht' function in the R package *multcomp* (Hothorn et al. 2008). The glht function allows multiple comparisons based on Tukey's contrast analysis for generalized linear models. All models were assessed graphically for their error distributions and homogeneity of variances. Where appropriate, we have presented the data by box and whisker plots to show the distribution, whereby, the median is represented by the line inside the boxes, the boxes show the 25th and 75th percentiles, the whiskers show 1.5 x interquartile range and the circles are points outside this range.

DNA extraction, amplification and sequencing

We used Illumina sequencing to characterise the soil microbial community. Our interest was on determining any differences in community composition between the soil inoculation sources and whether these communities changed after exposure to the different grass species or bulk soils, therefore, only samples from the live-inoculum treatments were used. This included three subsamples of the pre-experimental soil mixtures and five randomly selected samples from the experimental pots at the time of harvest from both plant species ($N = 52$).

The whole soil samples were sent to the Australian Genome Research Facility (AGRF, Adelaide, Australia) for DNA extractions, PCR amplification and sequencing. DNA was extracted using the PowerSoil Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) following the manufacturer's protocol (https://www.qiagen.com/us/resources/, accessed February 13, 2018). Two regions of ribosomal DNA were amplified to assay the bacterial and fungal communities namely, 16S and ITS (using the forward and reverse primers 341F - 806R and ITS1F - ITS2R respectively). The primary PCR amplification was carried out under the following conditions: 7 min activation at 95 °C, followed by 29 cycles of 30 s at 94 °C, 60 s at 50 °C, 60 s at 72 °C and 7 min of final extension at 72 °C, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia). Amplicons were indexed using a secondary PCR performed with TaKaRa Taq DNA Polymerase (Clontech) and then measured by florometry (Invitrogen Picogreen). The normalised PCR products were then pooled and sequenced on an Illumina MiSeq platform (San Diego, CA, USA) with 2 × 300 base pairs paired-end chemistry.

Bioinformatics

The paired-end reads were assembled and trimmed using PEAR (version 0.9.5; Zhang et al. 2014) and then quality filtered in USEARCH (version 7.1.1090; Edgar 2010) using a maximum expected errors threshold of 0.5 and a minimum read length of 150 bp. The files were converted from FASTQ to FASTA format and pooled using Quantitative Insights into Microbial Ecology (QIIME 1.8; Caporaso et al. 2010). Using USEARCH, full length duplicate sequences were removed and remaining sequences were sorted by abundance and singletons or unique reads in the data set were discarded. A set of Operational Taxonomic Units (OTUs) representative sequences from the reads was constructed using the UPARSE-OTU algorithm in UPARSE (Edgar et al. 2011), and chimeric sequences were discarded using the UCHIME algorithm. Remaining sequences were clustered using "rdp_gold" and "Unite" databases as the references. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using Greengenes (version 13_8; DeSantis et al. 2006) and Unite (version 7.1; Kõljalg et al. 2005) databases.

Statistical analysis – microbiome

All sample singletons and any rare OTUs that had a total abundance less than 10 were removed (Bálint et al. 2015). When using high-throughput sequencing the

sequencing depth typically varies in order of magnitude between samples, and there are multiple methods to account for this technical bias (Bálint et al. 2016; McMurdie and Holmes 2014; Weiss et al. 2017). We chose to rarefy OTU abundance to the technical replicate with the lowest number of reads using the 'single_rarefaction.py' function in QIIME (Weiss et al. 2017). With the rarefied data, we used linear models to explain OTU richness and Pielou's evenness with bulk soil type, inoculant source and plant species included as factors. These data were treated the same as the plant responses data above.

To select a set of core OTUs to use for the community composition analysis we used OTU incidence-abundance relationships to define an incidence threshold, as outlined in Bálint et al. (2015). Briefly, generalized additive models on the logarithms of average read abundance of OTUs against the number of soil samples were produced and the core OTUs were identified as those present in more than 10 samples. We then used these core OTUs to test how the experimental factors shaped the bacterial and fungal community composition using multispecies generalized linear models (GLMs). GLMs explicitly model the mean-variance relationship characteristic of ecological counts, and is therefore recommended over distance-based methods such as ordination or PERMANOVA (Warton et al. 2012). Models were fitted using the 'manyglm' function in the *mvabund* package (Wang et al. 2012) with a negative binomial probability distribution. The explanatory variables bulk soil, inoculant source and plant species were considered and significance tests were carried out using the 'anova.manyglm' function using likelihood-ration tests (ANOVA, pit-fall resampling, 300 bootstraps). This function also provided univariate tests for each OTU where *P*-values were adjusted for multiple testing.

To answer our third question ("are the microbial communities different between the inoculant sources?"), all models, i.e. linear models for evenness and richness and GLMs for community composition, were run using only the pre-experimental samples. To assess whether the soil communities changed over the course of the experiment (question four) models were rerun with all the data and the pre-experimental samples were included as a level of the factor 'species' and compared directly to the soil exposed to each plant species throughout the experiment. If changes were apparent, models were then rerun separately with the pre-experimental samples excluded to make the comparisons between plant species.

To visualise the results of the multispecies GLMs carried out on the core OTUs, we performed two-dimensional nonmetric multidimensional scaling (NMDS) with the 'metaMDS' function in the *vegan* package in R (Oksanen et al. 2017). This method is useful for exploratively finding groupings of the data and does not require normality (Bruckner and Heethoff 2017). The NMDS was performed on Bray-Curtis dissimilarities calculated from log-transformed data and the subsequent 95% intervals around the inoculant types were calculated using the 'veganCovEllipse' function. Note, the NMDS only serves for the visualisation of the statistically tested GLM results (as per Bálint et al. 2015).

Results

Soil properties

Differences in soil physiochemical properties were mostly explained (70.7%) by PC1 which separated the two bulk soil types (Fig. 1). Old-field samples had higher total nitrogen, plant-available (Colwell) phosphorus, total carbon, conductivity and pH ($CaCl_2$) compared with remnant bulk soil samples (Fig. 1, Table 1). Along this axis there was also separation between the inoculant sources for the mock-inoculants within each bulk soil (Fig. 1). PC2 explained a further 22.8% of variation and this axis separated samples depending on the inoculant-type (Fig. 1). This variation was explained mostly by nitrate nitrogen which was higher in the live-inoculants, the opposite to ammonium nitrogen which was higher in the mock-inoculants (Fig. 1, Table S2). PC2 also separated inoculant sources for the live-inoculants within each bulk soil, again explained by differences in nitrate nitrogen concentrations (Fig. 1; Table 1).

Formation of AM

The roots of *A. barbata* and *R. auriculatum* were essentially non-mycorrhizal (0% and 0.4 ± 0.9% AM formation respectively) in mock-inoculated soils, and so were omitted from further analysis of patterns of AM formation (Fig. 2a). In the live-inoculated treatments, the three-way interaction was not significant; however, all

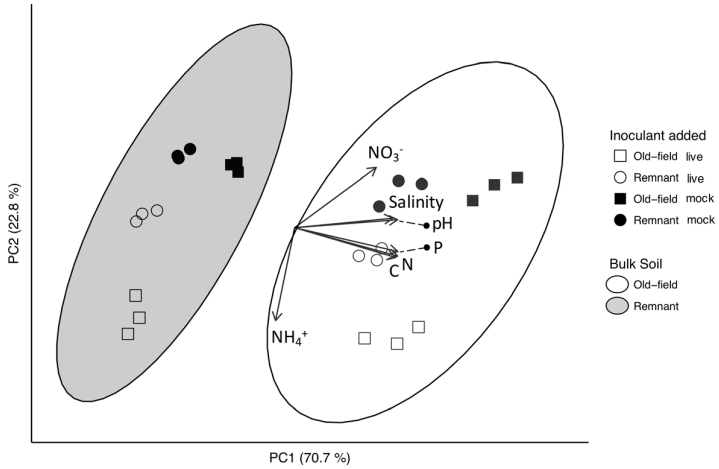

Fig. 1 Principal coordinates analysis of soil treatments after two weeks' incubation ($N = 40$). Total variation explained by principal component (PC) one and two is 93.5%. Ellipses (black ovals) represent 95% confidence intervals around the group mean

factors, i.e. bulk soil, inoculant source and plant species were involved in significant two-way interactions (Table 2). Overall, old-field inoculant produced more AM in *A. barbata* roots compared with *R. auriculatum* (z value = 5.4, $P < 0.001$) and when compared with remnant inoculant (for *A. barbata* roots and old-field bulk soil only, z value = 3.6, $P < 0.001$, Fig. 2a). There was also higher AM formation in remnant bulk soil than in old-field bulk soil when remnant microbes were added (z value = −5.8, $P < 0.001$, Fig. 2a).

Plant responses

The only plant response variable we found evidence for home-field advantage (question one) was MGR. Planned comparisons (see Table 2 for significant interactions) found that regardless of species, a positive MGR was more likely when live-inoculant was added to its home soil (old-field z value = 3.8, $P < 0.001$, remnant z value = −3.2, $P = 0.003$, Fig. 2b). In addition, when plants were grown in remnant bulk soil, *R. auriculatum* had a more positive MGR than *A. barbata,* regardless of inoculant source (z value = −3.6, $P < 0.001$, Fig. 2b).

There were two significant three-way interactions for the total (plant) biomass data (Table 2). One indicates differences in growth between the soil treatments (bulk soil x inoculant source x inoculant type) and results of planned comparisons are represented by different letters in Fig. 3. The other three-way interaction (plant species x bulk soil x inoculant type) goes towards answering our second question, i.e. whether the two species responded differently, in terms of total biomass, to the soil treatments. Planned comparisons for levels of this interaction show that *A. barbata* grew

Table 1 Mean ± SD soil physiochemical properties of the eight inoculation treatments, mixed as 85% bulk soil with 15% inoculum ($N = 40$)

Bulk soil	Inoculum *source*	Inoculum *type*	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	Plant available (Cowell; mg/kg)	Conductivity (dS/m)	pH (1:5 $CaCl_2$)	Total N (%)	Total C (%)
Old-field	Old-field	Live	42.3 ± 1.2^{Ay}	10.7 ± 1.2^{ay}	29.0 ± 1.0^{Aa}	0.07 ± 0.02^{Ay}	6.0 ± 0.0^{y}	0.27 ± 0.00^{Aa}	3.0 ± 0.0^{A}
		Mock	56.7 ± 2.3^{Az}	2.0 ± 0.0^{z}	30.7 ± 0.6^{Aa}	0.10 ± 0.01^{Az}	6.1 ± 0.1^{az}	0.27 ± 0.00^{Aa}	3.0 ± 0.0^{Aa}
	Remnant	Live	46.7 ± 2.1^{Ay}	5.7 ± 0.6^{by}	24.3 ± 0.6^{Aby}	0.07 ± 00.0^{Ay}	5.9 ± 0.1	0.26 ± 0.00^{Ab}	2.9 ± 0.0^{A}
		Mock	51.7 ± 4.9^{Az}	2.0 ± 0.0^{z}	28.0 ± 1.0^{Abz}	0.08 ± 0.01^{Az}	6.0 ± 0.1^{b}	0.25 ± 0.01^{Ab}	2.9 ± 0.0^{Ab}
Remnant	Old-field	Live	30.7 ± 2.3^{Bay}	10.7 ± 1.2^{ay}	14.0 ± 0.0^{Ba}	0.05 ± 0.01^{By}	5.6 ± 0.1^{y}	0.22 ± 00.0^{Ba}	2.6 ± 0.1^{B}
		Mock	46.3 ± 0.6^{Bz}	2.0 ± 0.0^{z}	12.7 ± 0.6^{Ba}	0.06 ± 0.00^{Bz}	5.8 ± 0.1^{az}	0.22 ± 0.00^{Ba}	2.7 ± 0.0^{Ba}
	Remnant	Live	39.3 ± 2.1^{Bby}	4.7 ± 0.6^{by}	11.0 ± 1.0^{Bb}	0.05 ± 0.01^{By}	5.6 ± 0.1	0.21 ± 0.01^{Bb}	2.6 ± 0.0^{B}
		Mock	47.0 ± 1.7^{Bz}	2.0 ± 0.0^{z}	11.3 ± 0.6^{Bb}	0.05 ± 0.01^{Bz}	5.7 ± 0.1^{b}	0.21 ± 0.00^{Bb}	2.6 ± 0.0^{Bb}

Different letters illustrate statistically significant differences between planned comparisons; uppercase A-B = bulk soil, lowercase a-b = inoculant source, lowercase y-z = inoculant type

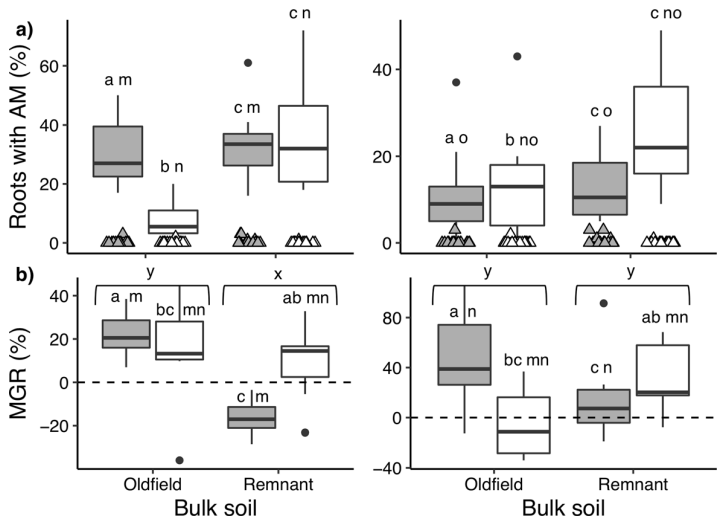

Fig. 2 Percent of arbuscular mycorrhizas (AM) present in roots (**a**) and the Microbial Growth Response (MGR; **b**) of *Avena barbata* (exotic; graphs left) and *Rytidosperma auriculatum* (native; graphs right). Plants were grown with either old-field inoculum (grey) or remnant inoculum (white) added to autoclaved bulk soil from either the old-field or remnant sites ($N = 77$). For AM formation, mock-inoculated samples (triangles) were removed from analysis because AM presence was negligible. See Eq. 1 for MGR explanation, however briefly, positive values indicate increased growth and negative values indicate reduced growth when soil microbes are present (compared with sterile controls). Different letters indicate significant differences between means (Tukey, $P > 0.05$) following significant interactions between bulk soil and inoculant source (**a**–**c**), inoculant source and plant species (m-o) and bulk soil and plant species (x-z; graph b only). See Table 3 for ANOVA results

larger with old-field bulk soil, with live-inoculant (z value = 8.6, $P < 0.001$) and mock-inoculant (z value = 4.3, $P < 0.001$), than in remnant bulk soil, but there was no effect of bulk soil for *R. auriculatum,* with either live- or mock- inoculated soil (z value = 0.8, $P = 0.850$ and z value = 1.2, $P = 0.655$ respectively), regardless of inoculant source (Fig. 3). Total biomass was higher with live-inoculant compared with mock-inoculant for *A. barbata* when in old-field bulk soil (z value = 3.6, $P = 0.002$) and also in the old-field bulk soil with old-field sourced inoculant compared with remnant sourced inoculant (z value = 3.1, $P = 0.009$, Fig. 3). Total biomass of *R. auriculatum,* on the other hand, did not differ between inoculant types or inoculant sources.

Microbial sequence data quality and summary

Sequencing ITS1 and 16S amplicons from 52 soil samples yielded 7,038,737 and 8,040,510 paired-end Illumina MiSeq reads, respectively. After quality-filtering 5,241,536 ITS1 and 4,971,384 16S reads could be assigned to 836 fungal or 7461 bacterial OTUs (taxa outside of these groups were not included) and a range in sampling depth of between 34,923 and 167,003 for ITS1 and between 50,386 and 156,750 for 16S reads per sample. After rarefying to 50,000 for 16S and 34,500 for ITS (see rarefaction curves Fig. S1) and removing the sample singletons and rare OTUs (< 10 reads), there were 269 fungal and 3468 bacterial OTUs remaining for further analysis. The abundance distribution of fungal OTUs was strongly skewed with the 10 most abundant OTUs representing 85.8% of all reads (Fig. 4a). Nearly all reads (98.8%) were from the phyla Ascomycota and the genus *Penicillium* (Fig. 4a). The reads in the bacterial communities were spread over many more OTUs but looking at the class level 86.3% of reads were represented by 10 dominant classes (Fig. 4b). These 10 classes represented five phyla including Actinobacteria, Proteobacteria, Bacteroidetes, Gemmatimonadetes and Firmicutes with Acidobacteria and Chloroflexi representing a further 6.7% of reads.

For both fungal and bacterial communities, there were more unique OTUs in remnant inoculant compared with old-field inoculant and with *A. barbata* than with *R. auriculatum* (Table 3). There were more unique fungal OTUs in remnant bulk soil but more unique bacterial OTUs in old-field bulk soil (Table 3). In most cases the unique OTUs were rare with very low abundances (< 50 reads); however, a few OTUs were found in high

Table 2 Results from generalized linear models for responses, arbuscular mycorrhiza (AM) formation and plant growth, represented as Microbial Growth Response (MGR) and total biomass ($N = 77$)

Factor	AM formation	MGR	Biomass
Bulk soil (BS)	**<0.01**	**0.04**	**<0.01**
Inoculant source (IS)	0.26	0.76	0.09
Plant species (PS)	**<0.01**	**<0.01**	**<0.01**
Inoculant type (IT)	N/A	N/A	0.07
BS x IS	**<0.01**	**<0.01**	0.06
BS x PS	0.27	**0.02**	**<0.01**
IS x PS	**<0.01**	**0.02**	0.19
IT x PS	N/A	N/A	0.32
BS x IT	N/A	N/A	0.65
IS x IT	N/A	N/A	0.56
BS x IS x PS	0.1	0.06	0.16
BS x IS x IT	N/A	N/A	**0.02**
BS x IT x PS	N/A	N/A	**0.03**
IS x IT x PS	N/A	N/A	0.13
BS x IS x IT x PS	N/A	N/A	0.87

The models included bulk soil (old-field and remnant), inoculant source (old-field or remnant), plant species (*Avena barbata* and *Rytidosperma auriculatum)* and inoculant type (biomass only; live or mock). Results of inoculant type (including interaction) are not applicable (N/A) for AM formation and MGR. Significant ($P < 0.05$) factors are shown in bold, df = 1 in all cases

abundance and thus were potentially important. For example, fungal OTUs with reads above 500 included unidentified Ascomycota sp. (remnant inoculant; OTU_34) and *Trichoderma ghanense* (remnant bulk; OTU_42), and bacterial OTUs with reads above 1000 included unidentified TM7–3 sp. (remnant inoculant; OTU_91), *Sphingobacterium multivorum* (*A. barbata*; OTU_392) and an unidentified *Sphingobacteriaceae sp.* (*A. barbata*; OTU_2832; Table 3).

Microbial community – pre-experimental samples

Using the pre-experimental samples only, our models identified clear differences between the inoculant sources at the beginning of the experiment (question three; see Table 4a for ANOVA results). Firstly, using the rarefied abundances of the core OTUs there were differences in community composition depending on bulk soil and inoculant source, identified from the multivariate GLMs ($P = 0.003$ for bacteria and fungi, Fig. 5). In addition, using the rarefied data with all OTUs, there was higher fungal OTU richness in soil with remnant inoculant (73.8 ± 4.3 SD) than with old-field inoculant (66.5 ± 2.1 SD; $F = 20.1$, $P = 0.002$). Pielou's evenness of fungal communities was higher when the inoculants were added to their away soil (F value = 170.1. $P < 0.001$). However, there was less evidence when looking at the bacterial communities with only a weak difference for OTU richness ($F = 4.6$, $P = 0.065$; fewer OTUs in old-field inoculant when in old-field bulk soil) and no differences for Pielou's evenness.

Microbial community – all samples

Using the rarefied abundances of the core OTUs it was clear that the soil communities at the end of the experiment were different to the pre-experimental samples (question four) regardless of which plant species they were exposed to ($P = 0.003$ for bacteria and fungi), according to the multivariate GLMs and the nMDS plots (Table 4b; Fig. 5). Overall, the biggest differences in community composition for fungi and bacteria were due to the inoculant source with no points overlapping in multivariate space in the nMDS plots (Fig. 5).

Further analysis without the pre-experimental samples (i.e. to compare the effect of plant species) on the core fungal communities found a significant three-way interaction between the plant species, bulk soil and inoculant source ($P = 0.010$). Planned comparisons found that the composition of the old-field fungal communities began to differ depending on which plant species they were exposed to ($P = 0.030$, Fig. 5a). Fungal community composition was also different depending on which bulk soil they were added to for both inoculant sources ($P = 0.020$ for remnant and old-field inoculants), especially when exposed to *A. barbata* ($P = 0.052$, Fig. 5a). After controlling for multiple testing, the abundances of 27 fungal OTUs could be explained by inoculant source, of which, four were in high abundance (> 500 reads) and either higher in old-field inoculant, *Metarhizium marquandii* (OTU_8) or in remnant, *Humicola sp.* (OTU_18), an unidentified *Ascomycota* sp. (OTU_29) and an unidentified *Ascomycota* sp. (OTU_34). All five fungal OTUs explained by bulk soil, *Penicillium polonicum* (OTU_4), *Coniochaeta spp.* (OTU_21 and OTU_31), *Chaetomium atrobrunneum* (OTU_11) and *Cryptococcus terreus*

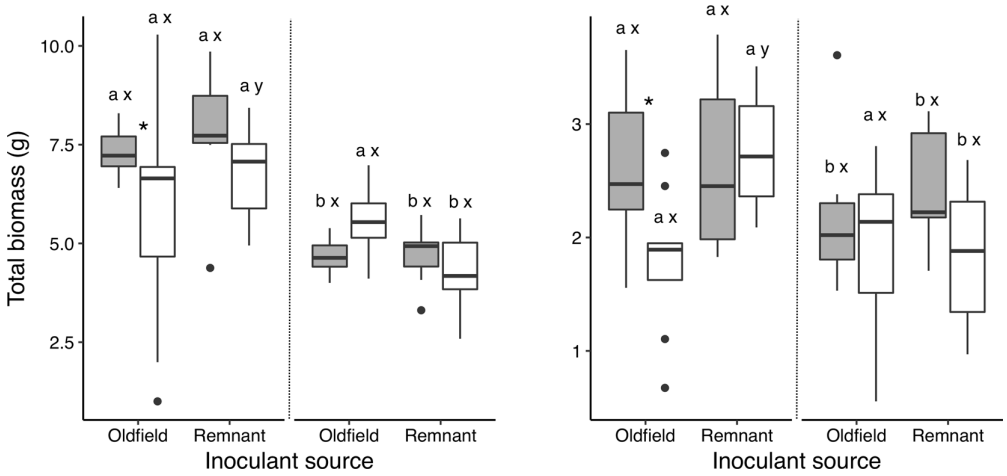

Fig. 3 Total biomass of *Avena barbata* (exotic; left graph) and *Rytidosperma auriculatum* (native; right graph) when grown in autoclaved bulk soil from either the old-field (left of dotted line) or remnant sites (right of dotted line) which was inoculated with either live- (grey) or mock (white) inoculants from old-field and remnant sources ($N = 156$). Different letters indicate significant differences between means (Tukey, $P > 0.05$) for planned comparisons following a significant three-way interaction between bulk soil, inoculant source and inoculant type. Planned comparisons were between bulk soils (a, b), inoculant sources (x-y) and inoculant types (significant differences are indicated by an asterisk) for each level of the interaction. See Table 3 for ANOVA results and see text for planned comparisons regarding the three-way interaction between plant species, bulk soil and inoculant type

(OTU_19), were higher in remnant bulk soil and had a total abundance over 500 reads. No fungal OTUs were explained by species.

For the bacterial communities (without pre-experimental samples) there was also a significant three-way interaction ($P = 0.007$). Further analysis found that community composition was different depending on which plant species they were exposed to for each level of bulk soil and inoculant combination ($P = 0.04$–0.049), except for remnant inoculant in old-field bulk soil ($P = 0.056$, Fig. 5b). Bacterial communities were also different depending on which bulk soil they were added to ($P = 0.031$), except when old-field inoculant was added to pots with *R. auriculatum* ($P = 0.070$, Fig. 5b). After controlling for multiple testing 10 bacterial OTU abundances could be explained by species, of which three were found in high abundances (> 1000 reads): an unidentified *Solirubrobacterales* sp. (OTU_10) and *Rhodoplanes sp.* (OTU_65) which were higher with *R. auriculatum* and an unidentified *Sphingomonadaceae* sp. (OTU_133) which was higher with *A. barbata*. Bulk soil explained the abundance of 101 bacterial OTUs, 47 of which were in high abundance (Table S4), and inoculant source explained 710 bacterial OTU abundances, 134 of which were in high abundance (Table S5).

Microbial community richness and evenness – all samples

Analysis of the rarefied OTU abundances, using all samples, found some evidence for changes to the microbial community over time (question four) but mostly for the bacterial communities (Table 4b). The number of bacteria OTUs was higher at the end of the experiment, than in the pre-experimental soil samples ($F = 27.9$, $P < 0.001$) but irrespective of plant species (t value = 0.3, $P = 0.936$). There were also more bacterial OTUs present when remnant inoculant was added to old-field bulk soil rather than remnant bulk soil (t value = 3.0, $P = 0.018$). Planned comparisons, after significant interactions were identified (Table 4b), show that Pielou's evenness was always higher in bacterial communities at the end of the experiment compared with the pre-experimental samples (all $P < 0.004$). In addition, when remnant inoculant was added to remnant bulk soil Pielou's evenness was lower with *A. barbata* than *R. auriculatum* (t value = −3.5, $P = 0.011$). Pielou's evenness was higher in remnant bulk soil compared with old-field when old-

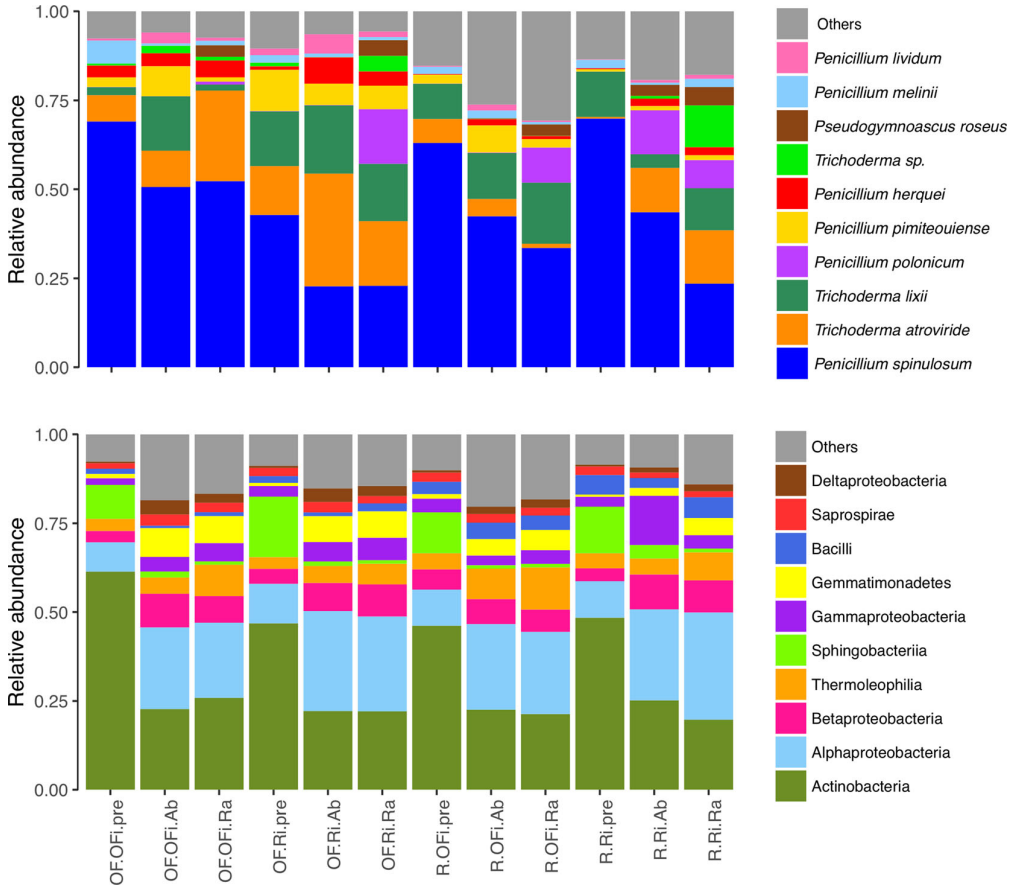

Fig. 4 The relative abundance of the 10 most abundant (**a**) fungal OTUs and (**b**) bacterial classes and rare OTUs are represented as 'others' ($N = 52$). Each inoculant treatment is represented on the x-axis: OF = old-field bulk soil, R = remnant bulk soil, OFi = old-field-sourced inoculant, Ri = remnant-sourced inoculant, pre = pre-experimental samples, Ab = *Avena barbata* (exotic) and Ra = *Rytidosperma auriculatum* (native)

field inoculant was added (t value = −2.8, $P = 0.028$) and in old-field inoculant compared with remnant-inoculant when added to remnant bulk soil (t value = 3.3, $P = 0.007$).

For the rarefied fungal communities, there were very little differences in the planned comparisons, after a significant three-way interaction was identified (Table 4b); however, more fungal OTUs were present when old-field inoculant was exposed to *A. barbata* and added to remnant bulk soil compared with old-field bulk soil (t value = −3.4, $P = 0.016$). Pielou's evenness of the fungal community was not affected by the factor 'species' (including the pre-experimental samples; Table 4b), and the planned comparisons investigating the significant interaction between bulk soil and inoculant source (Table 4b) did not find any statistically significant results.

Discussion

In answer to our first question, we found some support for home-field advantage for plant-soil interactions. In particular, all plants responded more positively to microbes when inoculant was added to their home soil. In addition, when grown in remnant bulk soil the native *R. auriculatum* had a more positive MGR than the exotic *A. barbata* regardless of inoculant origin. Regarding question two, we found that overall *A. barbata* had faster growth than *R. auriculatum* and the total biomass

Table 3 Fungal (top) and bacterial (bottom) OTUs found exclusively in each inoculant origin at the beginning of the experiment (pre-experimental) and unique OTUs in each level of the main factors at the end of the experiment (compared to the other levels of that factor and regardless of the other factors; $N = 52$)

Inoculant source		Bulk soil		Species*	
Old-field (51, 270)	Remnant (74, 460)	Old-field (17, 77)	Remnant (24, 40)	*A. barbata* (37, 162)	*R. auriculatum* (17, 84)
Bullera sp. (OTU_45), *Aquamyces chlorogonii* (OTU_59), Myriangiales sp. (OTU_73), *Penicillium oxalicum* (OTU_75), *Bullera spp.* (OTU_109, OTU_129), Lasiosphaeriaceae sp. (OTU_112), *Paraconiothyrium variabile* (OTU_74), Sordariaceae_sp (OTU_88), *Trichoderma longibrachiatum* (OTU_115)	Ascomycota sp. (OTU_34), *Mortierella sp.* (OTU_43), *Purpureocillium lavendulum* (OTU_46), *Bullera spp.* (OTU_50, OTU_57), *Claviceps truncatispora* (OTU_66), Sordariomycetes sp. (OTU_64), Pleosporaceae sp. (OTU_100), Chaetothyriales sp. (OTU_92), *Cladophialophora sp.* (OTU_77)	*Aquamyces chlorogonii* (OTU_59), *Trichoderma asperellum* (OTU_775), Ascomycota sp. (OTU_110), Chaetomiaceae sp. (OTU_96), *Neostagonospora sp.* (OTU_118), Dothideomycetes sp. (OTU_141), Trechisporales sp. (OTU_135), *Bullera spp.* (OTU_117, OTU_164), Pleosporales sp. (OTU_172)	*Trichoderma ghanense* (OTU_42), *Neurospora terricola* (OTU_71), *Trichoderma sp.* (OTU_372), *Trichoderma longibrachiatum* (OTU_115), *Coniochaeta sp.* (OTU_101, OTU_733), Sordariomycetes sp. (OTU_107), *Cryptococcus friedmannii* (OTU_151), *Bullera sp.* (OTU_244), Ascomycota sp. (OTU_146)	Hypocreaceae sp. (OTU_67), *Alternaria kulundii* (OTU_63), *Paraconiothyrium variabile* (OTU_74), *Tomentella sp.* (OTU_90), Chytridiomycota sp. (OTU_98), Sordariomycetes sp. (OTU_107), *Bullera sp.* (OTU_133), Chaetomiaceae sp. (OTU_96), Orbiliaceae sp. (OTU_121), Ascomycota sp. (OTU_168)	*Penicillium polonicum* (OTU_861), *Tricladium alaskense* (OTU_153), Ascomycota sp. (OTU_254), Neostagonospora sp. (OTU_118), *Entoloma callichroum var. callichroum* (OTU_163), Myriangiales sp. (OTU_219), *Sclerostagonospora phragmiticola* (OTU_215), *Pyrenopeziza brassicae* (OTU_306), Dothideomycetes sp. (OTU_298), Herpotrichiellaceae sp. (OTU_250)
Ellin5290 sp. (OTU_203), Ellin5301 sp. (OTU_1962, OTU_7436), Legionellales sp. (OTU_339), iii1–15 sp. (OTU_442), TM7–3 sp. (OTU_564), Oxalobacteraceae sp. (OTU_563), Microbacteriaceae sp. (OTU_2312), Solirubrobacterales sp. (OTU_4835), Gemmatimonadetes sp. (OTU_6634)	TM7–3 sp. (OTU_91), Oxalobacteraceae sp. (OTU_267), *Flavobacterium sp.* (OTU_308, OTU_629), Sphingobacteriaceae sp. (OTU_296), Ellin5301 sp. (OTU_380), Chitinophagaceae sp. (OTU_6301), Ellin6067 sp. (OTU_2310), Kouleothrixaceae sp. (OTU_518), *Burkholderia sp.* (OTU_3553)	Myxococcales sp. (OTU_934), Cytophagaceae sp. (OTU_4322, OTU_855), *Pedobacter sp.* (OTU_1207), Haliangiaceae sp. (OTU_7030), Deltaproteobacteria spp. (OTU_1248, OTU_1408), Myxococcaceae sp. (OTU_1542), DS-18 sp. (OTU_2038), Burkholderiales sp. (OTU_7102)	TM7–1 sp. (OTU_615), Microbacteriaceae sp. (OTU_1691), Stramenopiles sp. (OTU_1405), Oxalobacteraceae sp. (OTU_1970), Nocardioidaceae sp. (OTU_1441), N1423WL sp. (OTU_1442), Sphingobacteriaceae sp. (OTU_5717, OTU_7781), *Solibacillus sp.* (OTU_5120), Legionellaceae sp. (OTU_2377)	*Sphingobacterium multivorum* (OTU_392), *Flavobacterium spp.* (OTU_1590, OTU_1153, OTU_308), Sphingobacteriaceae sp. (OTU_2286, OTU_2832, OTU_7101, OTU_720, OTU_867), *Pseudomonas sp.* (OTU_6241)	*Paenibacillus sp.* (OTU_1429), Legionellaceae sp. (OTU_742), Ellin5301 sp. (OTU_2568), TM7–1 sp. (OTU_1081, OTU_4964), Gemm-5 sp. (OTU_1739), Coxiellaceae sp. (OTU_1105, OTU_1823), *Aquicella sp.* (OTU_1329), SJA-4 sp. (OTU_1227)

OTUs only found in the pre-experimental samples have also been included. The 10 most abundant OTUs are listed and the numbers in brackets indicate the total number of unique OTUs found for fungi and bacteria respectively

*_Avena barbata_ = exotic, _Rytidosperma auriculatum_ = native

Table 4 Soil fungal and bacterial community results from linear models (for Pielou's evenness and species richness) and multivariate GLM (community composition) including bulk soil (old-field and remnant), inoculant source (old-field and remnant) and species (*Avena barbata, Rytidosperma auriculatum* and pre-experimental samples)

Factor	df	Richness	Evenness	Community	Richness	Evenness	Community
a) Pre-experimental		Fungi			Bacteria		
Bulk soil (BS)	1	0.1	**> 0.01**	**0.01**	0.23	0.06	**0.01**
Inoculant source (IS)	1	**> 0.01**	**0.01**	**0.01**	0.07	0.28	**0.01**
BS x IS	1	0.14	**> 0.01**	**0.03**	0.09	0.07	**0.03**
b) All samples		Fungi			Bacteria		
Bulk soil (BS)	1	**> 0.01**	0.27	**> 0.01**	0.08	0.78	**> 0.01**
Inoculant source (IS)	1	**0.04**	0.6	**> 0.01**	0.95	0.05	**> 0.01**
Species (S)	2	0.26	0.07	**> 0.01**	**> 0.01**	**> 0.01**	**> 0.01**
BS x IS	1	0.21	**0.01**	**> 0.01**	**> 0.01**	**> 0.01**	**> 0.01**
BS x S	2	0.82	0.11	**0.01**	0.13	**> 0.01**	**0.01**
IS x S	2	0.28	0.61	**> 0.01**	0.16	**> 0.01**	**> 0.01**
BS x IS x S	2	**> 0.01**	0.34	0.14	0.37	0.18	**> 0.01**

Significant ($P < 0.05$) factors are shown in bold, $N = 52$

was affected by the bulk soil, inoculant type and inoculant source whereas there were very little differences in total biomass between these factors for *R. auriculatum*. Concerning questions three and four, the microbial communities differed significantly between old-field and remnant inoculant and the communities changed in composition over the course of the experiment depending on which bulk soil they were added to and which plant species they were exposed to.

Soil physiochemical properties

The bulk soil types differed significantly in their physiochemical properties, which is consistent with the sites having different land-use histories. Old-fields are known to retain high levels of nutrients long after farming has ceased (Drenovsky et al. 2010; Standish et al. 2006; Wong 2013). In this case, a decade after farming had ceased, phosphorus and potassium concentrations were at least twice as high in the treatments with old-field

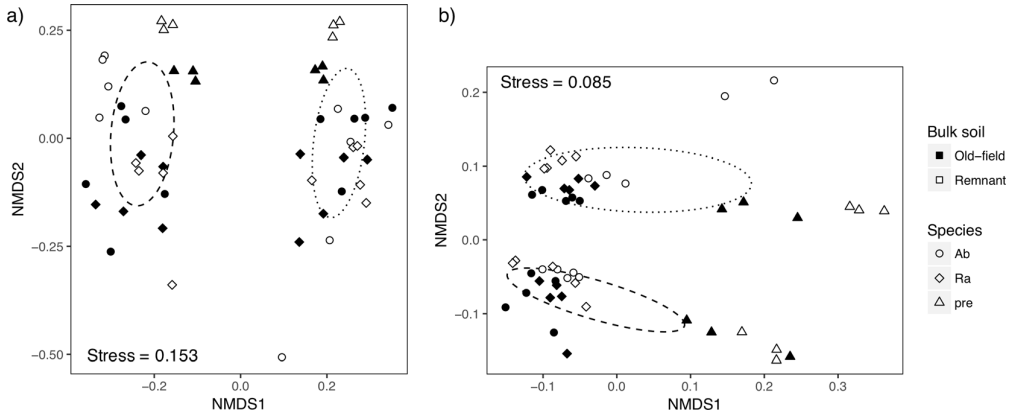

Fig. 5 Nonmetric multidimensional scaling (NMDS) ordination of soil (**a**) fungal and (**b**) bacterial communities from live-inoculated experimental samples ($N = 52$). Inoculant sources are separated by NMDS1 for fungi and NMDS2 for bacteria, where old-field inoculant points are less than zero in both cases. Ellipses (ovals) represent the 95% confidence intervals of the group means for each inoculant source; old-field = dashed and remnant = dotted. Shapes represent the plant species grown in the soil treatments, *Avena barbata* (Ab) and *Rytidosperma auriculatum* (Ra), and the pre-experimental samples (pre)

bulk soil than remnant. Given that both bulk soils were treated in the same way these differences should reflect field conditions. However, since they were autoclaved twice, the actual values likely vary from those in the field so caution is needed when making inferences from these results (Skipper and Westermann 1973; Warcup 1957). There were also subtle differences between live and mock inoculated soils, most likely due to autoclaving the mock soil. Given that the inoculant only contributed to 15% of the total soil mixtures and the differences in soil physiochemical properties were small, we concluded that differences in plant growth were more likely due to microbial effects rather than changes in physiochemical properties (Smith and Smith 1981).

Home-field advantages of plant-soil interactions

Evidence for home-field advantage in the form of greater MGRs when inoculant was added to its home soil, regardless of plant species, demonstrated that the soil biota provides the most benefit to their hosts in their native soils. Further supporting this, we found higher AM formation when remnant inoculant was added to remnant bulk soil than when added to old-field bulk soil. This is in line with other studies that have found higher AM colonization, external hyphae and plant biomass when AM fungal communities were added to their home soil (Ji et al. 2013; Johnson et al. 2010; Lambert et al. 1980; Weinbaum et al. 1996). There was also lower community evenness in the home soil for both fungi (pre-experimental samples only) and bacterial communities. This could be due to some taxa being highly adapted to their home soil and more likely to dominate (thus lowering community evenness; Hillebrand et al. 2008) or perhaps a more 'level playing field', at least to start with, in the away soil. A longer-term study is needed to determine how these communities change over time in the different soil types as we may have measured during a transition between communities.

Home-field advantage of soil biota, as demonstrated here, can have implications for ecological restoration because it highlights the importance of establishing soil conditions suitable for both the desired plant community and their mutualist soil microbial community. The current practice of using soil from a target ecosystem to inoculate a degraded system (Harris 2009) may not have the desired impact if they are not adapted to the local environment (Emam 2016). More work is needed to understand how the inoculated soil community may compete with the resident soil community and how restoration practices may shift the balance in favour of the former (Smith 2018).

Species-specific plant responses

Given the differences between the plant species studied here (perennial native vs annual exotic), and that other studies have found invasive species less likely to exhibit negative feedback from soil biota (Ba et al. 2018; Hawkes et al. 2013; Klironomos 2002; Smith et al. 2018), we expected that the species would respond quite differently to the soil treatments. However, only subtle differences were found. Firstly, *A. barbata* responded rapidly to the higher nutrients in the old-field soil (as bulk soil and an inoculant), whereas *R. auriculatum* did not. This was not surprising given that it is common for annual plants to respond more rapidly to higher nutrient (and other resource) availability than perennial species (Tilman 1988). We also found that *R. auriculatum* had a more positive MGR than *A. barbata* when grown in remnant bulk soil, regardless of inoculant origin. This may suggest that the native species had a stronger reliance on soil microbes and/or is better adapted to the lower nutrient availability of remnant bulk soil than the exotic species (Abraham et al. 2009; Walker and Reddell 2007).

Other differences in species responses to these treatments may also exist in variables that were not measured, such as reproductive output and growth of future generations (Brinkman et al. 2010; Jordan et al. 2008). Longer-term studies that cover multiple generations could help determine more differences and are warranted given that we demonstrated that the microbial communities differed depending on the plant species they were exposed to (discussed under microbial responses below). Also, we are limited with only two plant species and including more in future work would help decipher differences in responses between exotic and native plants.

Other plant responses

The total biomass of both plant species was only significantly different between the live- and mock-inoculants in one treatment where growth was higher when microbes were present. This contrasts with a previous study in which *R. auriculatum* and a different annual invasive grass, *Lolium rigidum* Gaud., were grown

using the same sources of soil inoculant as here (Smith et al. 2018). It was found that both species had greater growth in the presence of live-inoculants regardless of origin, but more so for *R. auriculatum* which had very stunted growth in the sterile control soil. One major difference between these studies is the available nutrients in the soil treatments which was greater in the current study. This is consistent with studies that have found that soil microbes, particularly mycorrhizal fungi, are important at low nutrient availability and become less important at high availability (Son and Smith 1988). The low rates of AM formation in our study compared with Smith et al. (2018) also supports this.

Intriguingly, the differences in total biomass we found in this study between the live- and mock-inoculated soil were only present when plants were grown in old-field bulk soil, the soil treatment with the highest nutrient availability. This is contrary to our above argument and we expected to see greater dependence on soil microbes at lower nutrient availability, i.e. in the remnant bulk soil. Reasons for increased growth in the old-field bulk soil with old-field live-inoculated may include enhanced nutrient mineralization (Kuzyakov et al. 2000), micronutrient limitation (Lambers et al. 2009), high nutrients helping to overcome the effects of antagonistic microbes (Chaparro et al. 2012) or the high rates of AM formation compared with remnant inoculant (Graham et al. 1982; Koide and Li 1990).

A recent study found that the addition of roots of *A. barbata* caused a strong reduction in the growth of *R. caespitosum* (Gaud.) Connor & Edgar, a grass species closely related to our native test species, in line with the theory that invasive species induce changes to the soil biota in ways that favour themselves, either directly by creating positive feedback or by disadvantaging other species (Hawkes et al. 2013; Klironomos 2002). Our study does not support this as old-field inoculant gave no advantage to *A. barbata* or any negative effect on the native species. Given our study included the wider soil community, i.e. whole soil inoculant, we conclude that the success of *A. barbata* as an invader in this area is more strongly from a size advantage and direct competition for resources (Lenz et al. 2003) rather than from advantages from interactions with AM fungi.

Microbial responses

Our results show that the old-field and remnant inoculants contained distinct soil microbial communities. This was supported by the NMDS plots and the presence of unique OTUs, which demonstrated higher OTU richness and more unique OTUs in the remnant inoculated soil for both bacterial and fungal communities. This finding is consistent with other studies where differences in the microbiomes of old-field and remnant areas have been found (Araujo et al. 2014; Gellie et al. 2017; Steenwerth et al. 2002; Wong 2013), and reflects what we know about the land-use histories of these sites.

Fungal and bacterial community composition differed between the pre-experimental samples and those collected at harvest. We did not include pots without plants to look at the microbial community changes under greenhouse conditions (Bulgarelli et al. 2015); however, there were clear differences depending on the bulk soils and plant species present, indicating that these factors influenced the communities over time. The differences observed between the bulk soils were most likely due to the differences in soil physiochemical properties. Varying levels of nutrient availability have been associated with unique soil microbiomes (Fierer et al. 2012; Leff et al. 2015; Ramirez et al. 2010) and in our case, we found a substantial number of unique OTUs in each bulk soil. Out of the OTUs that were affected by bulk soil type, the majority of bacterial OTUs were found in greater abundances in old-field than in remnant bulk soil whereas all fungal OTUs had greater abundances in the remnant bulk soil. This concurs with several studies that have found fungi to be generally more sensitive to increased nutrients and prefer higher C:N ratios (Busse et al. 2009; Fierer et al. 2009).

Differences in microbial communities also appeared to depend on the plant species they were exposed to. Our findings support the theory that native and invasive plants can alter soil microbial communities in different ways (Klironomos 2002; Stinson et al. 2006). For instance, there was an increase in fungal richness when old-field inoculant was exposed to *A. barbata* and there were more unique bacterial and fungal species in the presence of this species. Other studies have attributed an increase in microbial diversity to exotic species and this is one mechanism by which they can dominate a system, particularly if there is an increase in pathogens, which inhibit native plant growth or establishment (Lekberg

et al. 2013; Mangla and Inderjit 2008). Of the OTUs found in higher abundances when exposed to *A. barbata* we could not identify any plant pathogens. Often, very little information on the function of OTUs was available or classification was too coarse. This highlights that, while genetic tools show a lot of promise for expanding our knowledge on soil microorganisms, there is a need for better links between description and function of microorganisms before these tools can be utilized to their full potential. Nevertheless, the results show that the two grass species are associated with distinct microbial communities. More work is needed to determine whether the apparent increase in diversity with the invasive species is sustained over a longer period or if it is an artefact of the microbial communities shifting from one composition to another.

Conclusions

Home-field advantage played an important role in modulating plant and soil microbial community interactions in this study. However, this relationship is complex, with microbial communities changing in response to the plant species and soil type. Understanding these complicated relationships between plants, microbes and soils has wide practical implications such as whether inoculation of soils with local mutualistic symbionts is beneficial to enhance ecosystem services (Rua et al. 2016). Our results suggest that using remnant soil as an inoculant for old-field restoration may not promote the growth of the desired community, at least over the time period in this study, and that the revegetated plants may be able to promote changes in the microbial community over time anyway (Gellie et al. 2017). While the approach used in this study, i.e. using sterilized bulk soil, is unlikely to match exactly field conditions and processes, this was a necessary step to separate microbial effects from soil physiochemical effects. More work is needed to better understand how the inoculated microbial community interacts and competes with the resident community as soil inoculation becomes more utilized.

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Chapter 4. Decreasing soil nutrient availability and removing exotic annuals to promote native grass establishment

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Experimental plots in an old-field at Para Woodlands Reserve with a scalped treatment in the foreground.

4.1 Statement of authorship

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Principal Author

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Contribution to the Paper	Designed the study, carried out field and laboratory work, analysed data, wrote manuscript as principal author
Overall percentage (%)	80 %
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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4.2 Abstract

In severely degraded systems active restoration is required to overcome legacies of past land use and to create conditions that promote the establishment of target communities. In old-fields these legacies can include high soil fertility and dominance of exotic species. We trialed four methods of site preparation: fire, scalping (removal of top 50 mm of soil), slashing (vegetation cut to 30 mm above the ground followed by removal of plant biomass) and carbon (as sugar and saw dust) addition, and compared their effectiveness to reduce exotic plant biomass and improve native grass establishment. Our main aim was to understand the mechanisms underpinning the effects of each method, thus, we quantified any changes in soil nutrient availability and soil bacterial community after application. Scalping was the most effective technique used due to a reduction in both available nutrients and competition from exotic species. In comparison, the remaining methods had little or no effect on exotic biomass, native grass establishment or soil nutrient availability and could not be recommended for ecological restoration of old-fields, at least as a once off application used here. Both scalping and carbon addition resulted in changes in the soil bacterial community. These changes have the potential to alter plant community assembly in many ways (e.g. via nutrient acquisition, pathogenic effects, nutrient cycling, decomposition). Therefore, we recommend that restoration practitioners consider how their techniques may influence the soil biota, and in turn, affect restoration outcomes.

Key words: Annual weed control, burning, carbon supplements, microbial nutrient immobilisation, perennial grass establishment, slashing, top-soil removal

4.3 Introduction

Control of invasive plants is a significant challenge for restoration and often has limited success in practice (Kettenring and Adams 2011, Munson and Lauenroth 2012).

While there is extensive literature on the control of exotic plants, many studies simply look at the direct effects of different manipulations on invasive species without considering indirect effects and often manipulations lack benefits for native plant communities (Kettenring and Adams 2011). Site preparation before planting is perhaps the most important step in any restoration project to overcome the challenge of invasive species (Hobbs 2007). It is during this stage that structural properties and ecosystem processes must be manipulated to favor the desirable species and ensure replanted communities are resilient and self-sustaining (Hobbs 2007). This is particularly important in systems that are persistent and resilient to change (Suding et al. 2004).

The model of alternative stable states recognises that a given environment can support two or more distinct states, i.e. assemblages of species (Beisner et al. 2003, Suding et al. 2004, Suding and Hobbs 2009). How resilient a system is, i.e. the amount of disturbance or stress that system can withstand before changes in processes or structure occurs, determines when a transition among these states occurs (Gunderson 2000). Degraded sites that are in an alternative stable state often need multiple factors to be manipulated in order to shift them to a desirable state (Suding et al. 2004). Examples of these can be found in previously cultivated landscapes (old-fields) which have experienced fertilization, tillage, changes in hydrology and the replacement of perennial vegetation with exotic annual grasses (Corbin and D'Antonio 2004, Brown et al. 2006, Standish et al. 2007). This often alters environmental conditions so severely that both biotic (e.g. destruction of native seed bank, changes in soil microbial community) and abiotic (e.g. soil chemistry, water fluxes) thresholds are crossed (Suding et al. 2004, Cramer et al. 2008). More work is needed to identify mechanisms involved in, and complex interactions induced by, different site preparation techniques to increase our ability to manage the system towards desirable outcomes.

One of the most important hindrances to restoring old-fields is high nutrient availability that can persist long after farming has ceased and usually favors the dominance of fast-growing annual exotic plants (Standish et al. 2006). Therefore, reducing soil fertility is often a main aim in restoration activities and a number of ways to achieve this have been developed (reviewed in Corbin et al. 2004). One method often touted as effective is adding a carbon source (e.g. sugar or saw dust) to the soil to facilitate microbial nutrient immobilization: microbial activity is stimulated and soil nutrients become temporally stored as microbial biomass. In systems that have been invaded by annual grasses, it has been demonstrated that the addition of carbon generally enhances native plant establishment and reduces exotic plant cover (Blumenthal et al. 2003, Prober et al. 2005, Kardol et al. 2013, Morris and de Barse 2013). However, this method would not be suitable on its own in old-fields that have a history of cultivation. In these systems, the thick growth of exotic plants would also need to be controlled in order to tip the balance in favor of the desired community.

The removal of above-ground biomass is not only useful for reducing competition, it can also reduce the nutrients present in the soil by preventing the incorporation of plant material, and the nutrients it contains, into the soil (Schelfhout et al. 2017). It has been demonstrated that grazing and burning are both able to reduce available nitrogen (N), although fire produces an initial N flush. However, it may take some years of repeated treatments to achieve the desired outcome (Perry et al. 2010). Even so, these two techniques are widely used because they are relatively cheap and effective at reducing biomass and could be used in combination with carbon addition. A more extreme method that removes exotic biomass, and reduces available nutrients at once, is top-soil removal (or scalping). This technique removes the topsoil (and everything above it) to a depth usually between five and ten centimeters and thus is also effective in reducing the exotic seedbank (Jobbágy

and Jackson 2001, Verhagen et al. 2001, Buisson et al. 2008). In comparison with other site preparation methods scalping is often the most effective at eradicating exotic species and minimizing post-seeding management (Corbin et al. 2004, Gibson-Roy et al. 2010a); however, this method is expensive and may have adverse effects on other environmental factors such as soil structure, soil microbial communities and water holding capacity (Kardol et al. 2008) as well as landscape effects or ongoing management if the soil is not removed from site.

Differences in soil microbial communities can have strong influences on restoration outcomes because they can affect plant communities in many ways (e.g. nutrient acquisition, pathogenic effects, nutrient cycling, decomposition; Packer and Clay 2000, van der Putten et al. 2001, van der Heijden et al. 2006, Ayres et al. 2009). Bacteria play a major role in nutrient cycling and mutualistic bacteria, such as rhizobia, have even been shown to improve restoration outcomes when introduced to a site (Thrall et al. 2005, Terrazas et al. 2016). Bacterial communities are very dynamic and have been shown to change depending on the plant species present and respond to changes in soil nutrients and thus can vary greatly between old-field and remnant sites (Ramirez et al. 2010, Araujo et al. 2014, Piper et al. 2015, Smith et al. 2018a). Therefore, it is often assumed that site preparation methods that aim to change soil nutrient availability (as outlined above) could result in changes in the soil bacterial communities; however, this is rarely quantified (but see Kardol et al. 2008) and comparisons of the effectiveness of the methods are scarce.

Here we report results of a field experiment where we compared the effectiveness of four methods of site preparation to help restore old-fields to native grasslands in southern temperate Australia. We applied treatment of: fire, scalping (removal of top 50 mm of soil), slashing (vegetation brushcut to 30 mm, biomass removed), and carbon (saw dust and sugar

mixed) to an old-field dominated by annual grasses. Control plots were left untreated. Our main aim was to understand the mechanisms underpinning each method to help determine why some techniques are effective and to help to determine, on the basis of factual information, what are likely results under different environmental conditions. To achieve this, our research questions included, 1) which technique was most efficient at reducing competition from exotic species, measured as their total biomass per plot and, 2) how is the establishment and growth of native grasses affected by the different techniques. It was critical to look at indirect effects so we included two additional questions, 3) which techniques were effective at reducing soil nutrient availability and 4) are there any differences in soil bacterial community composition associated with these techniques.

4.4 Methods

Study site and sampling design

The study was undertaken in an abandoned agricultural field at Para Woodlands Reserve, South Australia, (34.628 °S, 138.785 °E). The region has a Mediterranean-type climate with a mean rainfall of 450 mm/annum and a mean annual air temperature of 23.6 °C (BOM 2017). The study site was a cereal and sheep farm and received regular fertilizer application until farming ceased in 2004. The soil has been characterized as deep brown and grey cracking clays (but see results for physiochemical properties; Rosser 2013). All plant species present at the site were invasive species, dominated by winter-growing annual grasses, in particular *Avena barbata* Pott ex Link*, Lolium rigidum* Gaud. and *Bromus spp.* (100 % and both 17 % cover respectively; Appendix S1; Fig. S1).

In May 2015 (Austral Autumn), the experiment was set up in an area of the old-field that was relatively homogeneous in floristic composition and topography and fenced to exclude livestock and kangaroos. We established 24 plots (3 m x 3 m), separated by a 1

m buffer, and randomly assigned one of four treatments (control, burn, slash and scalp) to each plot, resulting in six replicates per treatment (however one burnt plot was excluded due to an error in set up) in a fully randomized design. The burnt treatment used fire to remove the litter layer and expose bare soil. The slashed treatment saw vegetation brushcut to a height of 30 mm and the litter removed with a rake. In the scalped treatment, a shovel was used to remove the top 50 mm of soil and all vegetation and litter above. Within the center of each plot two subplots (1 m x 2 m) were established, separated by a buffer of 0.5 m, and randomly assigned to receive either a carbon (C) addition or not (22 replicates each after the removal of the burnt plot mentioned above). We used an equal part mixture of sucrose (white sugar) and saw dust applied to the soil surface at 0.42 kg C m^{-2} immediately after the application of the other manipulations (Blumenthal et al. 2003, Prober et al. 2005).

Each subplot in turn was divided into two (1 m x 1 m) and each was used to test two planting materials of native grasses: seeding or tubestock planting, randomly allocated to a side (see Appendix S1; Fig. S2 for full layout). *Rytidosperma caespitosum* Gaud. seed was used for the seeding method at a rate of 1.5 g m^{-2} (approximately 1480 seeds) and 1 L of water was added to each area before and after sowing to reduce loss due to wind and to promote germination. For tubestock planting, *R. racemosum* R. Br. was grown from seed in the previous winter at South Para Nursery (Kersbrook Landcare Group Inc., Kersbrook, South Australia). A mechanical auger was used to make 25 evenly spaced holes and the tubestock plants were planted by hand. Two different species were utilized due to limited seed availability; however, these species are closely related and are both winter-growing perennial grasses, native to the region. This was considered a suitable compromise for the purposes of this experiment because no direct comparisons were made between seeded and planted plots. Plot sizes of 1 m x 1 m were adequate to answer our aims given the size and

density of vegetation at the site and that similar studies have used comparable plot sizes (Buisson et al. 2008, Gibson-Roy et al. 2010a).

Data collection

Emergence of native grass seedlings was recorded within seeded subplots every fortnight. To make detailed measurements it was necessary to restrict counting to sampling quadrats (30 cm x 30 cm). Four quadrats were outlined in each seeded subplot, located 10 cm from the edge of the subplots to reduce edge effects, and 20 cm from each other, and two were randomly chosen for seedling counts (Appendix S1; Fig. S2). After peak emergence 30 randomly selected individual seedlings within each of those quadrats were marked and monitored for survival over the season (note that one scalped plot only had 29 seedlings and two control plots had 25 and 15 seedlings). Mortality of tubestock plants was also recorded fortnightly and dead plants were replaced. All above-ground biomass was harvested after peak plant growth, i.e. in November 2015, including all seedlings within the quadrats, surviving tubestock plants and all exotic plants in each subplot (samples from the seeded sides were collected separately from the tubestock sides). All plant material was dried at 60 °C for 24 hours before being weighed. Exotic plants harvested from three replicates on the seeded side were randomly selected and material was divided into grass or broadleaf material, as a coarse measure of composition, and weighed separately.

Two soil cores (10 cm diameter x 10 cm deep) were collected and homogenized on two occasions; 1) one week after treatments were applied and planting had occurred (i.e. late austral-autumn; hereafter initial samples; two samples taken from the center of subplots), and 2) at the time of harvest (i.e. late austral-spring; hereafter harvest samples; two subsamples taken from both the seeded and tubestock subplot at random points). Care was taken to use sterilized equipment, cleaning and resterilizing between sample collection. For microbial genomic analysis, a representative 50 g sample of soil was collected from the

homogenized soil samples collected at harvest. Soil samples were stored on ice, then sent to commercial laboratories for analysis (see details below). Analyses for physiochemical properties were carried out at CSBP Limited (Bibra Lake, Western Australia) to measure nitrate nitrogen, ammonium nitrogen, plant-available (Colwell) phosphorus, potassium (Colwell), sulphur, organic carbon, conductivity and pH ($CaCl_2$).

Bacterial data preparation

DNA extractions, PCR amplification and sequencing were performed by the Australian Genome Research Facility (AGRF, Adelaide, Australia). DNA was extracted using the PowerSoil Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) following the manufacturer's protocol. For the primary PCR, the 16S DNA was amplified using the forward 341F and reverse 806R primers under the following conditions: 7 min activation at 95 °C, followed by 29 cycles of 30 s at 94 °C, 60 s at 50 °C, 60 s at 72 °C and 7 min of final extension at 72 °C, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia). A secondary PCR was performed, indexing the amplicons with TaKaRa Taq DNA Polymerase (Clontech) and measured by fluorometry (Invitrogen Picogreen). The normalized PCR products were then pooled and sequenced on an Illumina MiSeq platform (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry.

The paired-end reads were assembled and trimmed using PEAR (version 0.9.5; Zhang et al. 2014). Assembled reads were quality filtered in USEARCH (version 7.1.1090; Edgar 2010) using a maximum expected errors threshold of 0.5 and a minimum read length of 150bp and pooled using Quantitative Insights into Microbial Ecology (QIIME 1.8; Caporaso et al. 2010). Sequences were sorted by abundance and any full length duplicate sequences, singletons or unique reads in the data set were discarded using USEARCH. A set of representative sequences for each Operational Taxonomic Unit (OTU) were constructed from the reads using the UPARSE-OTU algorithm in UPARSE (Edgar et al.

2011), and chimeric sequences were discarded using the UCHIME algorithm. Sequences were clustered followed by chimera filtered using "rdp_gold" and "Unite" databases as the references. Reads were mapped back to OTUs with a minimum identity of 97% to obtain the number of reads in each OTU. Taxonomy was assigned using Greengenes (version 13_8; DeSantis et al. 2006) and Unite (version 7.1; Kõljalg et al. 2005) databases and any sequences not assigned to bacteria were discarded, as were sample singletons and rare OTUs that had a total abundance less than ten (Bálint et al. 2015).

Statistical analysis - plant and soil

The accumulated emergence of the native seedlings was compared over time with a linear mixed model using the 'lmer' function from the *lme4* package in R (Bates et al. 2014). The full model included fixed effects weeding treatment (including control, burn, slash and scalp), carbon addition (add C and no C) and time (days since seeding). An individual plot identification number was included as a random effect to account for the repeated measures taken over time. Seedling mortality was analyzed using parametric survival regression model with a Weibull distribution using the 'survreg' function in the R package *Survival* (Therneau 2015). Final mortality (i.e. proportion of dead plants by day 141), was also compared using a generalized linear model (GLM) using the 'glm' function in the base package of R where the response was binomial (0 = alive, 1 = dead) and tested as a function of weeding treatment and carbon addition.

Linear models were used to analyze all biomass data where native tubestock and seedling biomass was analyzed as a function of weeding treatment and carbon addition and, in addition, the models for exotic biomass included planting material (of native plants). Soil physiochemical properties were also analyzed using linear models and the two time-periods were analyzed separately because the initial samples did not include planting material but the samples taken at harvest did. Differences between samples were visualized using

Principal Component Analysis (PCA) plots. PCA plots are a useful tool to provide information about the similarity/dissimilarity of groups and to identify properties (and the correlation of properties) that separate groups from each other (Bruckner and Heethoff 2017).

For all models, data were transformed using square root or log transformations where necessary to meet parametric assumptions. Where significant differences were detected with analysis of variance (ANOVA) tests, *post hoc* tests of pairwise comparisons were made using the 'glht' function in the R package *multcomp* (Hothorn et al. 2008). The 'glht' function allows multiple comparisons for linear models, GLMs, linear mixed effects models, and survival models. All models were graphically checked for their error distributions and homogeneity of variances. All statistics were performed in R version 3.1.1 (R Core Team 2017).

Statistical analysis - bacterial community

To account for differences in the sequencing depth we rarefied OTU abundance to the technical replicate with the lowest number of reads using the 'single_rarefaction.py' function in QIIME (Weiss et al. 2017). With the rarefied data, we calculated OTU richness, i.e. the number of unique OTUs in each sample, and Pielou's evenness (Pielou 1966), i.e. the relative abundance of different OTUs in each sample. These two diversity measures are complementary and have been found to respond differently to environmental factors (Wang et al. 2017). Pielou's evenness (J') was calculated as equation 1,

$$J' = \frac{H'}{\log(\text{richness})} \quad (1)$$

where H' (known as Shannon-Wiener diversity) is calculated using equation 2.

$$H' = -\sum p_i \ln p_i \quad (2)$$

We then used linear models to explain OTU richness and Pielou's evenness with weeding treatment and carbon addition included. These data were treated the same as the plant biomass data above.

To test how the experimental factors shaped the bacterial and community composition we used multispecies GLMs. GLMs explicitly model the mean-variance relationship characteristic of ecological counts, and are therefore recommended over distance-based methods such as ordination or PERMANOVA (Warton et al. 2012). Models were fitted using the 'manyglm' function in the *mvabund* package (Wang et al. 2012) with a negative binomial probability distribution. The explanatory variables weeding treatment and carbon addition were considered and significance tests were carried out using the 'anova.manyglm' function using likelihood-ratio tests (ANOVA, pit-fall resampling, 300 bootstraps). This function also provided univariate tests for each OTU where *P*-values were adjusted for multiple testing.

To visualize the results of the multispecies GLMs, we performed two-dimensional nonmetric multidimensional scaling (NMDS) with the 'metaMDS' function in the *vegan* package in R. (Oksanen et al. 2017). The NMDS was performed on Bray-Curtis dissimilarities calculated from log-transformed data and the subsequent 95 % intervals around the inoculant types were calculated using the 'veganCovEllipse' function. The NMDS only serves for the visualization of the statistically tested GLM results (as per Bálint et al. 2015), because this method is useful to exploratively find groupings of the data that does not require normality (Bruckner and Heethoff 2017).

4.5 Results

Exotic plant biomass

All main effects, namely, weeding treatment, carbon addition and planting material, acted independently on the biomass of the exotic plants (Table 1). Exotic plant biomass was lower in the plots with carbon added, compared to no carbon, and in the plots with native tubestock plants, than in the seeded plots (Fig. 1a, b). *Post-hoc* comparisons found that all the weeding treatments were statistically significantly different from each other and the control (all $P < 0.01$) except for the burnt plots which did not differ from the control ($P = 0.29$; Fig. 1a, b). In the subset of samples from the seeded side of plots, the proportion of exotic forbs to grasses changed depending on the weeding treatment ($P < 0.01$) but not with the addition of carbon ($P = 0.40$). In the slashed and control plots the exotic biomass was nearly entirely made up of grasses but the proportion of exotic forbs increased in the burnt and scalped plots (Fig. 1c).

Table 1. Results from the linear model for total above-ground biomass of exotic plants as explained by weeding treatment (control, burn, scalp and slash), carbon addition (add C and no C) and planting material of native plants (seeds of tubestock). Significant ($P < 0.05$) factors are shown in bold ($N = 92$).

Factor	df	*P* value
Weeding treatment (WT)	3	**<0.01**
carbon addition (CA)	1	**<0.01**
Planting material (PM)	1	**<0.01**
WT x CA	3	0.77
WT x PM	3	0.28
CA x PM	1	0.69
WT x CA x PM	3	0.53

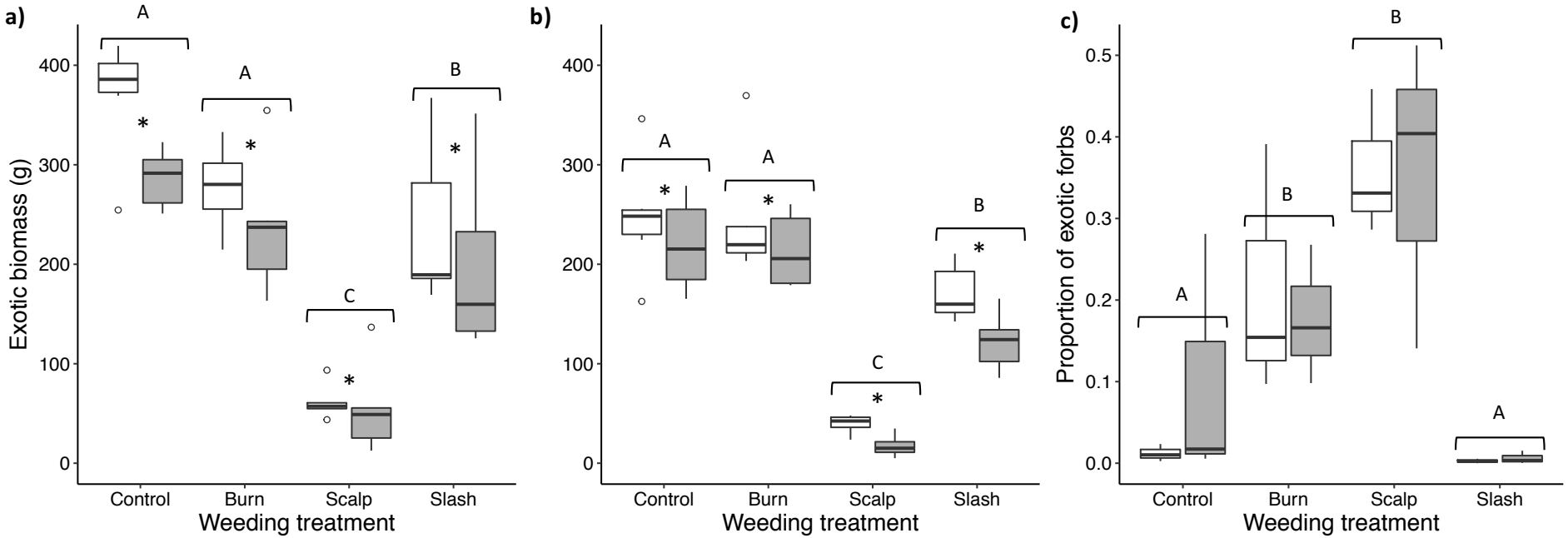

Figure 1. Above-ground biomass of all exotic plants present at time of harvest in experimental plots which had native plants added as either (a) seeds or (b) tubestock plants ($N = 46$ for both). In addition, (c) the proportion of exotic biomass, in a subset of the seeded plots ($N = 24$), that was made up of forbs. Asterisks indicate significant differences in biomass for plots with carbon added (grey) and those with no carbon added (white). The median is represented by the line inside the boxes, the boxes show the 25th and 75th percentiles, the whiskers show 1.5 x interquartile range and open circles are points outside this range. Different letters indicate a significant difference among means between weeding treatments according to Tukey HSD test ($P < 0.05$).

Native seedling emergence and mortality

The rates of seedling emergence and mortality were both affected by weeding treatment ($P < 0.01$ and $P < 0.01$ respectively) but not carbon addition ($P = 0.26$ and $P = 0.10$ respectively; Fig. 2a, b; see Table S1 and S2 for results of full model). The burnt and slashed plots always had higher emergence than the controls particularly towards the end of the season (Fig. 2a; $P < 0.01$). At day 30 these treatments had on average just over twice as many seedlings emerge than the control ($P = 0.05$ and $P = 0.04$ for burnt and slashed respectively) and this difference reached nearly three times as many seedlings by day 111 ($P < 0.01$ for both burnt and slashed; Fig. 2a). Similarly, the difference between scalped plots and the control plots changed over time, from scalped plots having just a few seedlings less on day 30 ($P = 1$) to nearly double the control by day 111, the only time at which this difference reached marginal statistical significance ($P = 0.06$; Fig. 2a). Seedling mortality was most rapid in the control plots with only around 25 % surviving by day 70 (Fig. 2b). Seedlings had the highest rate of survival in the scalped plots with the average surviving never dropping below 87 % (Fig. 2b). While the rate of mortality increased rapidly after day 111 in the burnt and slashed plots, the final proportion of surviving seedlings was higher in all weeding treatments compared to the control (Fig. 2b; $P < 0.01$ for all treatments).

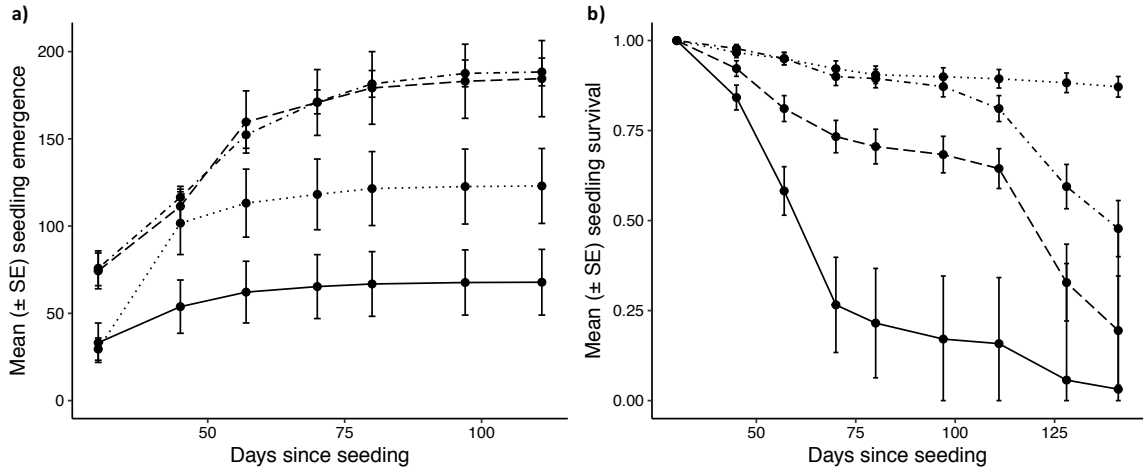

Figure 2. *Rytidosperma caespitosum* seedling a) emergence (N = 168) and b) survival (N = 367) rates during the experiment. Seeds were applied to plots on 2nd June 2015 (beginning of winter). Line type indicates the weeding treatment; solid = control, dashed = burnt, dotted = scalp and dot-dash = slash. Note standard deviations do not appear below zero.

Native plant biomass

Both carbon addition and weeding treatment influenced the growth of the native tubestock plants but these two factors were independent of each other (Table 2). Overall, adding carbon resulted in lower biomass of native plants and scalping alone resulted in higher biomass ($P < 0.01$), all other treatments did not differ from the control (Fig. 3a). Seedling biomass, on the other hand, was affected by the weeding treatment but not carbon addition (Table 2). Scalping again resulted in the highest biomass but all weeding treatments resulted in higher biomass than the control (all $P < 0.01$; Fig. 3b).

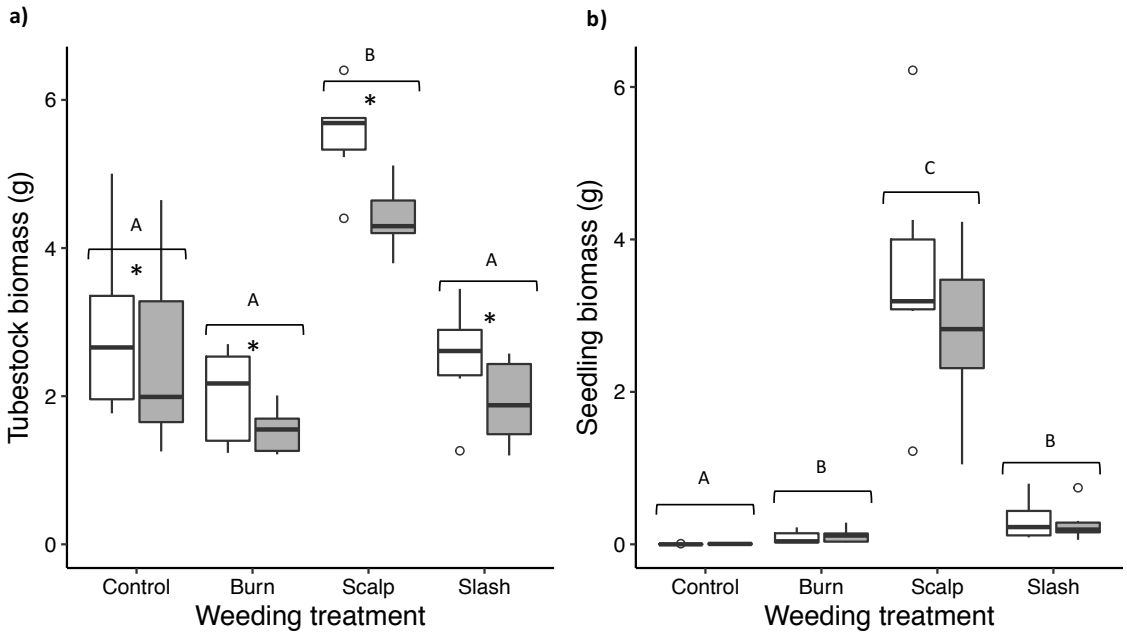

Figure 3. Above-ground biomass of (a) *Rytidosperma racemosum* tubestock plants and (b) *Rytidosperma caespitosum* seedlings the at time of harvest. Asterisks indicate significant differences in biomass for plots with carbon added (grey) and those with no carbon added (white). The median is represented by the line inside the boxes, the boxes show the 25th and 75th percentiles, the whiskers show 1.5 x interquartile range and open circles are points outside this range, $N = 46$ for both graphs. Different letters indicate a significant difference among means between weeding treatments according to Tukey HSD test *(*$P < 0.05$).

Table 2. Results of linear models for *Rytidosperma racemosum* tubestock plants and *Rytidosperma caespitosum* seedlings, including weeding treatment (control, burn, scalp and slash) and carbon addition (add C and no C). Significant ($P < 0.05$) factors are shown in bold. $N = 46$ for both.

Factor	df	Tubestock	Seedling
Weeding treatment (WT)	3	**<0.01**	**<0.01**
Carbon addition (CA)	1	**<0.01**	0.91
WT x CA	3	0.58	0.64

Soil physiochemical properties

Overall, there were very little differences in electrical conductivity and pH between the treatments with conductivity ranging from 0.11 to 0.24 dS/m and pH(1:5 $CaCl_2$) between 5.2 and 6.5 over the course of the experiment (Appendix S1; Table S3). In the

initial soil samples (after treatments were applied and before plants were added) differences in soil physiochemical properties were mostly explained by PC1 (45 %) which separated the subplots with carbon added to those without (Fig. 4a). This separation was attributed to a reduction in nitrate-nitrogen when carbon was added (Fig. 4a, $P < 0.01$) and this difference was three-fold in the control plots and up to nine-fold in the scalped plots (Appendix S1; Table S3). A further 23 % of variance was explained by PC2 which separated the weeding treatments (Fig. 4a). A reduction in phosphorus in the scalped plots was a main driver along this axis with one-to-two thirds less phosphorus in the scalped plots than the controls, without and with carbon added respectively (Appendix S1; Table S3, $P < 0.01$). There was also a higher concentration of ammonium-nitrogen in the burnt plots than the control ($P < 0.01$) but minimal difference between slashed plots and the controls (Fig. 4a).

The amount of variance explained by PC1 and PC2 for the harvest samples was very similar to the initial soil samples but the samples were arranged differently (Fig. 4b). The separation between carbon added and no carbon plots was more along PC2 but again explained by a reduction in nitrate-nitrogen in carbon added plots across all treatments (Fig. 4b, Appendix S1; Table S3, $P < 0.01$). There was also an increase in organic carbon with the addition of carbon whereas, scalping resulted in less organic carbon (Fig. 4b, Appendix S1; Table S3, $P < 0.01$ for both). PC1 separated the scalped samples from all other treatments (including controls) which again was explained by a reduction in phosphorus (Fig. 4b, Appendix S1; Table S3, $P < 0.01$). There was also a reduction in potassium due to scalping an increase due to burning evident along this axis (Fig. 4b, Appendix S1; Table S3, $P < 0.01$). There were very little differences between the planting materials; however, overall planting tubestock increased potassium (Fig. 4b, Appendix S1; Table S3, $P < 0.01$). See Appendix S1, Table S4 for ANOVA results.

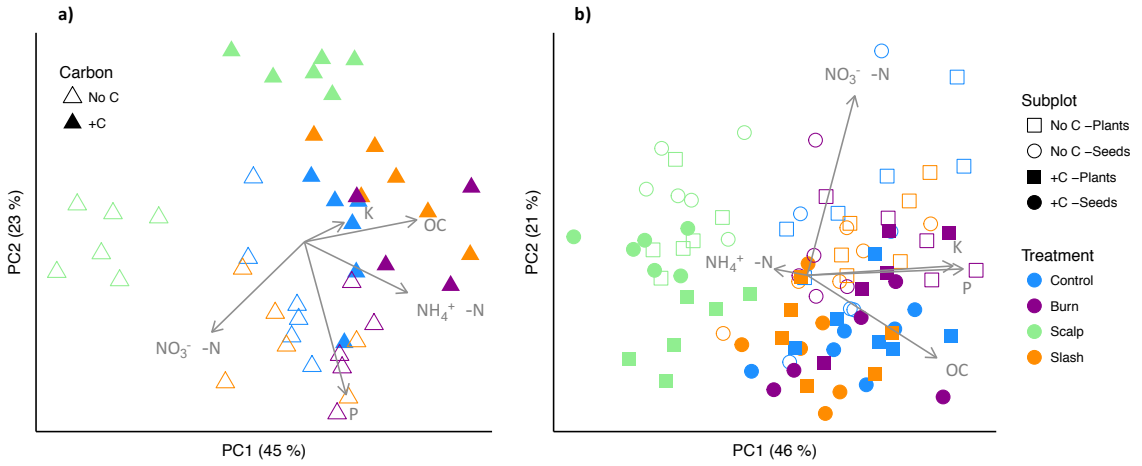

Figure 4. Principal coordinates analysis of soil physiochemical properties of each weeding treatment and carbon addition at (a) the beginning of the experiment (triangles; $N = 46$) and (b) at the time of harvest ($N = 92$). Planting material is represented by the shapes (graph b only; squares = tubestock plants, circles = seeds), open shapes = no carbon, closed shapes = carbon addition and weeding treatments are represented by the colors (see legend).

Bacterial sequence data quality and summary

Sequencing 16S amplicons from 40 soil samples yielded 2,886,387 paired-end Illumina MiSeq reads, respectively. After quality-filtering 1,674,591 16S reads could be assigned to 5,971 bacterial OTUs (taxa outside of bacteria were not included) and a range in sampling depth of between 20,559 and 97,728 per sample. After rarefying to 20,000 reads (see rarefaction curves in Appendix S1: Fig. S3) and removing the sample singletons and rare OTUs (< 10 reads), there were 2,324 bacterial OTUs remaining for further analysis. There were 20 phyla represented in the rarefied data set of which eight were dominant (> 1.5 % of reads) including Actinobacteria (50 % of reads), Proteobacteria (18 %), Acidobacteria (10 %), Firmicutes (7.8 %), Chloroflexi (6.6 %), Gemmatimonadetes (3 %), Verrucomicrobia (1.6 %) and Bacteroidetes (1.5 %). There were only subtle differences in the relative abundances of these phyla between the weeding treatment and carbon addition combinations (Appendix S1: Fig. S4).

Bacterial community composition

The mean number of OTUs per sample ranged from 955 ± 52 to 1,131 ± 73 and there was a statistically significant interaction between weeding treatment and carbon addition (Table 3). Further analysis revealed that the only difference between the carbon treatments was in the scalp treatment (t = -2.7, $P = 0.05$) where there were fewer OTUs when carbon was added. There were no differences between treatments when no carbon was added; however, for the plots which received carbon OTU richness was highest in the slash treatment than in the other treatments: control (t = -3.1, $P = 0.04$), burn (t = -3.1, $P = 0.04$) and scalp (t = -4.9, $P < 0.01$). Overall, there was not a substantial difference between Pielou's evenness per sample (between 0.79 ± 0.02 and 0.83 ± 0.02); however, there was a weeding treatment effect (Table 3), whereby the evenness of the control was significantly higher than burn (t = 2.9, $P = 0.03$) and scalp (t = 4.2, $P < 0.01$) and evenness of slashed plots was higher than scalped plots (t = -3.6, $P = 0.01$).

The multivariate GLM found that the soil bacterial community composition was slightly different between the carbon added plots and the no carbon plots and there was a significant effect of treatment but there was no interaction between the two factors (Table 3). Planned comparisons between the treatments found that the bacterial communities in the scalped plots were significantly different to the other treatments ($P = 0.026$) but there were no other differences (Fig. 5). After controlling for multiple testing the abundances of 35 OTUs could be explained by the main effects but only 12 were found in high abundance (> 1,000 reads). Two of these OTUs, *Agromyces sp.* (OTU_15) and *Cellulomonas sp.* (OTU_10), had higher abundances when carbon was added to plots ($P = 0.05$ and $P < 0.01$ respectively). Eleven of these OTUs were affected by weeding treatment. Of them, four were in lower abundance in the scalped plots than the control, namely *Cellulomonas sp.* (OTU_10), unidentified Kineosporiaceae (OTU_40), *Actinomycetospora sp.* (OTU_98)

and unidentified Ellin6529 (OTU_44). A further seven OTUs were found in higher abundance in the scalped plots than the controls; *Bacillus spp.* (OTU_1 and OTU_18), unidentified Gaiellaceae (OTU_21, OTU_123 and OTU_188), unidentified Solirubrobacterales (OTU_41) and unidentified Oxalobacteraceae (OTU_241).

Table 3. Soil bacterial community results from linear model (for Pielou's evenness and species richness) and multivariate GLM (community composition) including weeding treatment (control, burn, scalp and slash) and carbon addition (add C and no C). Significant ($P < 0.05$) factors are shown in bold.

Factor	df	Evenness	Richness	Composition
Weeding treatment (WT)	3	**<0.01**	**<0.01**	**<0.01**
Carbon addition (CA)	1	0.15	**0.04**	**0.05**
WT x CA	3	0.53	**0.03**	0.48

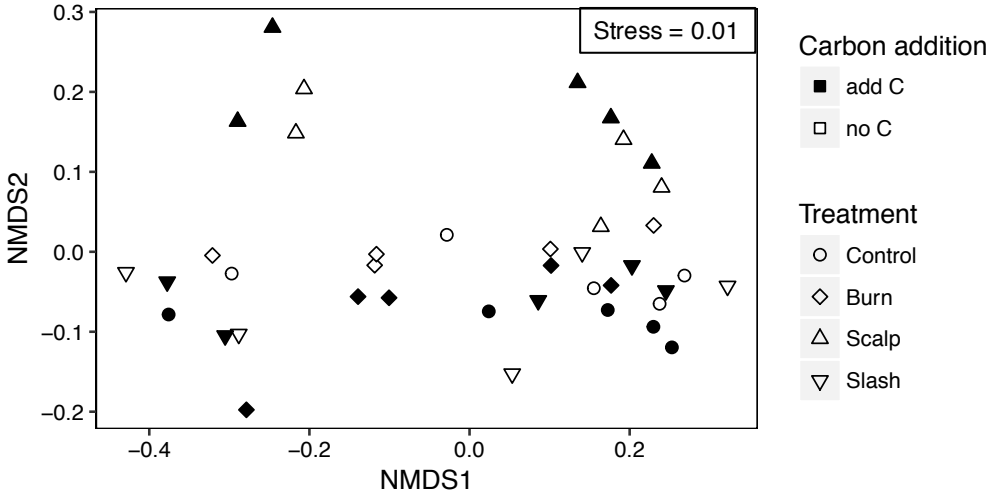

Figure 5. Nonmetric multidimensional scaling (NMDS) ordination of soil bacterial communities from experimental samples collected from seeded sides of subplots ($N = 40$). Open shapes = no carbon, closed shapes = carbon addition and weeding treatments are represented by the shapes (see legend).

4.6 Discussion

Weeding techniques

We found that scalping was the most effective weeding technique because not only was the exotic biomass reduced 2-5-fold compared to the other treatments and the control

(question one), but this technique was also the most effective at promoting native grass growth (question two). Growth of the native tubestock plants was around twice as large as the control and there was very low mortality of the native seedlings after scalping. We attribute the success of scalping to a reduction in both available nutrients (question three) and competition from exotic species. Further, we found clear differences in soil bacteria communities compared to the control and other techniques (question four; discussed below). Other studies have found scalping to be a successful site preparation technique in grasslands (Cole et al. 2005, Buisson et al. 2006, Gibson-Roy et al. 2010b), drained fens (Hedberg et al. 2014) and tropical areas (Bai et al. 2012). However, our study also found that scalping performed poorly in terms of seedling emergence with only a weak advantage over the control. This result suggests that scalping can create a harsh environment (i.e. more exposed to the elements, possible with less friable soil) or removes soil or organic matter containing the nutrients and water required for seedling establishment (Kardol et al. 2008).

Compared to scalping, both slashing and burning resulted in nearly double the number of seedlings emerging and nearly three times more than the control. However, the rate of mortality towards the end of the experiment was alarming and the low biomass of seedlings at harvest made it doubtful that many would have survived the dry summer (Lamont et al. 1993). A longer-term study is certainly warranted to assess survival to the next growing season (Austral winter). Further, we suggest that other methods, such as soil ripping, may need to be used in conjunction with burning and slashing to promote seedling survival (Commander et al. 2013). There were no differences between burn and control plots in the biomass of exotic or native plants thus indicating no benefit of using this method in restoring this site. This is consistent with a meta-analysis of 84 invasive plant control studies which found that overall burning reduced native biomass and increased invasive biomass because a reduction in competition encouraged new exotic invasions (Kettenring

and Adams 2011). However, some studies have found it to be an effective technique, especially in combination with carbon supplements (Prober et al. 2005, Morris and de Barse 2013). In our study, the weeding treatments and carbon supplements acted independently.

Carbon supplements

Overall, adding carbon resulted in a reduction in nitrate-nitrogen, particularly in the scalped plots where there was up to a 9-fold difference. This occurred rapidly, i.e. a week after application (initial samples), and was sustained over the growing season. Many other studies have reported the same result (Blumenthal et al. 2003, Prober et al. 2005, Kardol et al. 2008, Perry et al. 2010, Morris and de Barse 2013) and attributed this to microbial nutrient immobilization. We documented an increase in abundance of two OTUs from genera known to be decomposers, *Cellulomonas* and *Agromyces* (Stackebrandt and Kandler 1979, Zgurskaya et al. 1992). The dominance of these OTUs could explain why OUT richness was less when carbon was added (effect of carbon addition in scalped plots only; Veech et al. 2003); however, evenness did not change with carbon addition as it would be expected when few species dominate a community (Wang et al. 2017).

We expected that a reduction in available nutrients would reduce the biomass of invasive plants (Morghan and Seastedt 1999, Alpert and Maron 2000, Prober et al. 2005) which would reduce competition pressure and, in turn, increase the growth of native plants (Blumenthal et al. 2003, Prober et al. 2005). However, this study found a reduction in biomass of both invasive and native plants even though soil nutrients were comparable to similar native grasslands (Prober et al. 2005, Cole et al. 2017). The responses of native species to carbon supplementing are varied between studies (reviewed in Perry et al. 2010) but positive effects have been recorded at higher rates of carbon application (Blumenthal et al. 2003) and forbs appear to be less affected than grasses (Alpert and Maron 2000).

Bacterial communities

Recent studies have shown that active restoration can have strong influences on the microbial community (Araujo et al. 2014, Gellie et al. 2017) but few have looked into how specific techniques can influence this change (Kardol et al. 2008). This study found evidence that carbon supplements and scalping, in particular, can change bacterial community structure, at least in the short term. The abundance of four OTUs (out of those with total abundance greater than 1,000) was reduced in scalped plots, including two possible cellulose metabolizers *Cellulomonas sp.* (Stackebrandt and Kandler 1979) and an unidentified Kineosporiaceae (Schellenberger et al. 2010). However, there were no differences in OTU richness between scalped and control plots and, contrary to Kardol et al. (2008), seven OTUs were actually found in higher abundance in scalped plots (see Appendix S1; Table S5 for details). The differences in abundance could be explained by the different soil profile measured in the scalped plots compared to the other treatments (i.e. due to top-soil removal) or the OTUs could have flourished after less competition from particular OTUs removed in the top soil - which could explain the reduction in community evenness.

Many studies have shown that a change in bacterial community can result in changes in plant performance (Smith et al. 2018b; Packer and Clay 2000, Ayres et al. 2009); however, future work is needed to determine whether the changes recorded in this study could have affected plant performance. Inoculation of mutualist soil biota is becoming more widely used in restoration (Neuenkamp et al. 2018) and the effectiveness of this technique may be compromised or enhanced by the site preparation techniques utilized. For example, scalping followed by inoculation has been shown to improve restoration outcomes compared to inoculation without site manipulation (Wubs et al. 2016). Future work is

needed to explore this interaction between soil properties, soil biota and plant communities (Smith et al. 2018b).

Recommendations for restoration

We found evidence that active restoration is needed to restore old-fields into native grassland communities by the sheer lack of seedling emergence or survival in the control plots. This supports the literature on alternative stable states which suggests that certain thresholds need to be crossed in order for a target community to thrive in these systems (Suding et al. 2004, Cramer et al. 2008). However, more work is needed to determine what those thresholds may be at sites which differ in soil fertility or invasive species cover and how the timing or rates of application of the techniques used here can change their effectiveness at a range of thresholds. For instance, the small reductions in soil nutrients and exotic species biomass from carbon supplements and slashing did not provide satisfactory conditions for native grasses but perhaps higher doses or frequencies of applications are needed to return this old-field back into a desirable (for native grasses) state (Blumenthal et al. 2003, Corbin et al. 2004, Perry et al. 2010).

Scalping resulted in the most severe reduction in invasive species biomass, soil fertility and soil bacterial community and perhaps, these effects were so drastic that the system was pushed into a more desirable state or the combination of system changes was required. It is important to note that this method may not be suitable at all sites, particularly where soil depth is limiting, therefore we should consider the mechanisms that have explained the success to find suitable alternatives. In particular, a reduction in exotic seed bank was probably a major contributor to reduced exotic biomass in scalped plots and would be suitable to target a wide range of exotic plants (Verhagen et al. 2001). Grazing and burning at the right time (i.e. before exotic species set seed) has shown to reduce exotic seed banks (Hastings and DiTomaso 1996, Stromberg and Kephart 1996); however, success

may depend on the extent of exotic seed banks, seed longevity and require repeated follow-up treatment, sometimes for several years (D'Antonio and Meyerson 2002).

It is also worth mentioning that the composition of exotic species changed from nearly all grasses to around 40-50 % forbs in the scalped plots which could also relieve competition pressure on the native grasses as studies have shown plants exert more competitive effects on species from the same functional group (Fargione et al. 2003). However, this effect was likely much less important than the overall reduction in exotic biomass and nevertheless could affect the long-term management of the site.

In conclusion, even though carbon addition was effective at reducing soil nutrients and exotic biomass, the reduction in native biomass leaves us unable to recommend this technique for restoration practices of old-fields. Scalping, on the other hand, was far superior to the other methods in terms of nutrient and exotic reduction and improved the growth of native species. The lack of native seedling emergence would need to be overcome; however, and this could possibly be achieved by ploughing before seeding to loosen the soil and reduce the loss of seeds due to wind or seed predators. Burning and slashing had little or no effect on exotic biomass, native grass establishment, soil nutrient availability and therefore are not suitable in ecological restoration of old-fields, at least as a once off application such as used here. In addition, scalping and carbon addition both prompted changes in the soil bacterial community and, given how important the plant-soil interactions have shown to be, further consideration is needed on how these may affect plant growth and community structure.

4.7 References

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Chapter 5. Resource pre-emption, rather than increasing functional group complexity, reduced invasion by exotic species in a grassland field experiment

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Experimental plots at Para Woodlands after the first planting event in June 2015

5.1 Statement of authorship

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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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5.2 Abstract

Understanding the factors that influence community resistance to exotic invasion is an important goal for community ecology with implications for ecological restoration. In Mediterranean-type systems, invasive C3 annual grasses appear early in the season and can pre-empt resources and attain a competitive dominance over native perennial grasses. Here, we tested two mechanisms of invasion resistance, 1) increasing the functional group complexity (i.e. including C3 and C4 native plants together so that resources are being used over a longer period) and 2) increasing plant density. Overall, C3 native grasses were the superior competitors against both invasive C3 grasses and native C4 grasses. Higher density communities were successful at reducing exotic biomass; however, there was a trade-off with reduced individual performance among the native plants. This could be because the C3 native plants were planted earlier than the C4 native plants due to the differences in phenology and therefore likely pre-empted resources and gained a size advantage. Otherwise, the C4 native and C3 invasive species likely coexisted because the system was not at equilibrium given that the climatic conditions during the experiment did not favour C4 growth and the native communities only had one season to establish. Future work should include different planting times, i.e. introduce the native C4 plants first, and longer-term studies should be implemented to explore these mechanisms, i.e. functional diversity and planting density, in greater detail.

Keywords: Annual grasses, community assembly, competition, invasibility, life-history, Mediterranean-type climates, niche partitioning, perennial grasses, South Australia

5.3 Introduction

New species arrivals can impact ecosystems in dramatic ways (D'Antonio and Meyerson 2002; Kulmatiski 2006). Therefore, using community ecology theory to

understand the mechanisms influencing community resistance to exotic invasion can have profound implications for management and restoration of ecological systems (Shea and Chesson 2002). Davis *et al.* (2000) suggested that a biotic community is more susceptible to invasion when resource availability is greater than resource use for a significant period. Therefore, communities with a high density of plants could be more resistant to invasion if density increases the proficiency of resource use. In restored systems, high density planting has been shown to reduce the overall biomass of invaders. However, this strategy can also cause strong competition which could exclude less competitive reintroduced species, thus reducing species diversity (Antonovics and Levin 1980; Cuda *et al.* 2015; Kimball *et al.* 2014). Greater community diversity has also been suggested to increase invasion resistance because plant species that use resources in a variety of ways can deplete a broad spectrum of resources, thus increasing the chance of niche overlap with potential invaders (Elton 2000; Gooden and French 2015). Since density and diversity increase resource use in different ways, understanding how these mechanisms interact could be important to reduce invasibility of communities, particularly in a restoration context.

Increasing the functional diversity, rather than species diversity, of planting has been shown to reduce invaders in a number of trials (Dukes 2001; Fargione *et al.* 2003; Pokorny *et al.* 2005; Tilman *et al.* 1997). However, criteria for grouping plants into functional groups can vary depending on the project aim or type of community being studied (Wilson 1999). Given that the ability of a species to capture resources is strongly influenced by the seasonal timing of life-history events (germination, growth and reproduction), incorporating phenological differences into a system may be another way to reduce invasibility (Cleland *et al.* 2013; Godoy and Levine 2014). Given that, density-dependent effects are often stronger in intraspecific competition than with interspecific competition (niche complementarity; Abrams 1983; Chesson 2000; Gooden and French

2015; Tilman 1982), higher density of target plants may result in less intraspecific competition in phenologically diverse plantings. Interestingly, this possibility has been seldom explored experimentally (but see Connolly *et al.* 1990; Tilman *et al.* 1997).

Species that initiate their growth early in a season can pre-empt resources and thus influence the establishment and growth of later-emerging species, a process known as priority effects (Alford and Wilbur 1985; Grman and Suding 2010; Schantz *et al.* 2015; Shulman *et al.* 1983). Through this mechanism invasive species may be able to establish dominance in a system. In Mediterranean-type climates where water is a limiting resource, such as those found in south eastern Australia and California, the highest productivity occurs over the winter and spring months due to winter dominated rainfall. Priority effects from winter-growing (C3) invasive annual grasses are common in these areas because these species appear earlier in the season - i.e. when autumn rain breaks the summer drought - than C3 native perennial grasses, and thus the invasive plants attain competitive dominance via a seasonal priority advantage (Wainwright *et al.* 2012). Native perennial species have been shown to be superior competitors against annual invaders if they can overcome recruitment limitations and can gain a size advantage by establishing earlier (Grman and Suding 2010; Perry *et al.* 2003; Seabloom *et al.* 2003). However, the highly disturbed or nutrient-rich conditions commonly found in sites targeted for restoration (e.g. abandoned fields) means that the pre-emption of resources often needs to be overcome to suppress invasive species (Kardol *et al.* 2013).

In a field trial in Minnesota, resident functional groups were more effective at inhibiting the establishment of invaders from the same functional group; however, summer-growing (C4) plants stood out as consistent superior competitors against all functional groups included in the study (Fargione *et al.* 2003). This study, and others which

incorporate C3 and C4 grasses in similar trials, have been carried out in continental or semi-tropical climates where C4 species actively grow in mid-season (Fargione *et al.* 2003; Symstad 2000; Tilman *et al.* 1997). In Mediterranean-type climates, C4 plants are active late in the season as their emergence occurs after that of the C3 plants and they are the last to set seed before the soil dries out mid-summer. Suppression of early-emerging species by inter-seasonal effects from late-emerging species is uncommon but not unheard of (Facelli and Facelli 1993; Lenz *et al.* 2003). Grazing trials have demonstrated that when C4 perennial grasses decline in tall-grass prairies the resulting community becomes dominated by C3 annual grasses (Smith and Knapp 1999). However, further investigations are needed to determine whether C4 plants can be competitive across seasons in Mediterranean-type climates where they are an important component of grassland communities (Cole *et al.* 2017). This climatic differentiation is important because the pattern of water availability in Mediterranean-type climates is not ideal for C4 plants unless a substantial summer rainfall occurs or their deep roots can tap into water sources so that they are less dependent on seasonality of rain (Cole and Lunt 2005; Lodge 1981).

As the literature cited herein suggests, there is the potential for C4 plants to use resources over the summer and early autumn, leaving fewer available for the early-arriving invasive species (Figure 1). However, this could also leave a 'resource gap' in winter, when the C4 plants are not actively growing (Figure 1) that invasive species could exploit. A more effective restoration approach could be to incorporate species with phenological differences to the C4 plants resulting in resource use across a greater time period and thus increasing functional diversity while doing so. In other words, by incorporating a temporal and a diversity element to the idea by Davis *et al.* (2000), i.e. reducing available resources in order to reduce invasibility, should, in theory, be effective at resisting invasion of exotic species. In Mediterranean-type grasslands, this could involve planting C3 and C4 grasses

together, resulting in native species actively growing across multiple seasons (late-autumn to mid-summer).

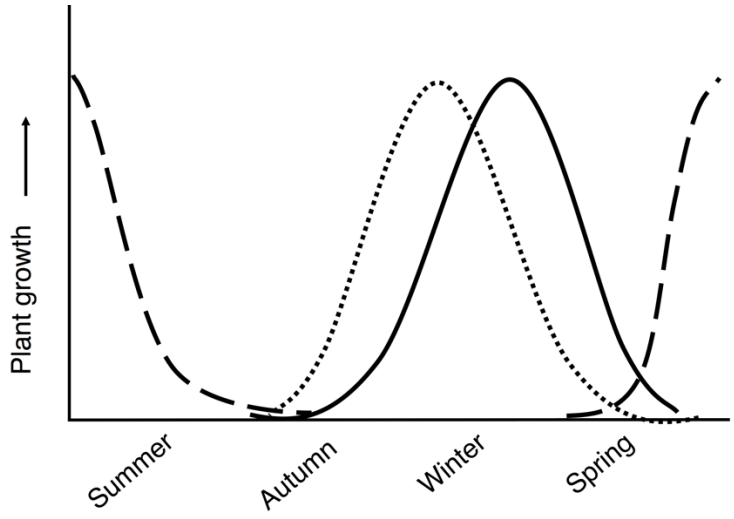

Figure 1. Schematic diagram of the active growing times (thus resource use) for winter growing (C3, solid line) and summer growing (C4, long-dashed line) native perennial grasses and invasive annual grasses (C3, dotted line) throughout the four seasons in a Mediterranean-type climate

We tested the hypothesis that a community with a mixture of species growing at different times would be more effective at competing with invasive species than communities where resident species are actively growing over a narrower time period. To achieve this, we planted different densities of C4 and C3 perennial grasses on their own (single functional group) and together (mixed functional groups). We used the biomass of exotic species to determine effectiveness of the various combinations of native grasses to supress invasive annual grasses. We also investigated whether there was a trade-off between reduced invasion of exotics and the health of resident species in high density communities. Understanding how the complex interactions between intra-functional and inter-functional groups and between native and invasive species was important to understand the dynamics of each community therefore we incorporated a weeded treatment to act as a baseline to measure the planted community's performance without the added

impact of exotics. We also measured levels of available resources (soil water and soil nutrients) to test whether the responses of exotic species correspond to patterns of resource availability created by the resident community.

5.4 Methods

Study site and species

The study was undertaken at Para Woodlands Reserve, South Australia, (34.628 °S, 138.785 °E) in an abandoned field that was previously a cultivated cereal crop. The region has a Mediterranean-type climate with a mean annual air temperature of 23.6 °C. The rainfall pattern is winter-dominated with an annual average of 450 mm (BOM 2017). The study site received regular fertilizer application until farming ceased in 2004. The soil can be characterised as deep brown and grey cracking clays (results for physiochemical properties analyses are provided below). A vegetation survey was carried out in spring 2014 to determine the dominant species at the site (methods reported in supplementary material). All plants present at the site were invasive species, dominated by winter-growing annual grasses, mainly *Avena barbata* (Pott ex Link), *Lolium rigidum* (Gaud.) and *Bromus sp.* (Scop.), with some annual broadleaf species such as *Medicago sp.* (L.) and *Raphanus raphanistrum* (L.). also present in low abundances (Table S1).

We chose four native grass species that are common in Para Woodlands Reserve and surrounding areas for this study. These species represented two functional groups: C3 grasses, *Rytidosperma caespitosum* (Gaud.) Connor & Edgar and *Austrostipa flavescens* (Labill.) S.W.L. Jacobs & J. Everett, or C4 grasses, *Enneapogon nigricans* (R.Br.) P. Beauv. and *Themeda triandra* (R.Br.) Stapf. All species are perennial grasses; however, their tussocks vary in size (Jessop *et al.* 2006). The largest, *T. triandra* can grow up to 150 cm tall and 50 cm wide with flat leaf-blades, 10–30 cm long and 1–8 mm wide and flowers

in summer. The smallest, *E. nigricans,* on the other hand only grows to 55 cm tall and 20 cm wide with leaves 1–5 mm wide and 30 cm long and also flowers in summer. Both C3 species flower in spring and *R. caespitosum* grows up to 90 cm tall and 40 cm wide with leaf blades 15-35 cm long and 1-4 mm wide whereas, *A. flavescens* grows up to 140 cm tall and 40 cm wide with leaf blades 9–54 cm long and 2–7 mm wide.

Experimental design and implementation

The experiment included three levels of functional group complexity, including two single functional group treatments - C3 species planted together (single-C3 treatment hereafter) or C4 species planted together (single-C4 treatment hereafter) - and a mixed functional group treatment (all C3 and C4 species planted together, mixed treatment hereafter). Two densities of planting where also used, high density (44 plants/m^2) and low density (20 plants/m^2). The combinations of functional complexity and density of planting will herewith be referred to as community assemblages. The community assemblages were repeated in 12 randomly selected plots, half of which were weeded by hand regularly while the other half remained unweeded throughout the experiment. Overall, there were six replicates for each combination of functional complexity, density and weeding, resulting in a total of 72 plots (3 functional complexities x 2 densities x 2 weeding x 6 replicates). After an establishment period (May to September 2015), monitoring and weeding occurred during the experimental phase (12 months) until all above-ground biomass was harvested in October 2016.

The experimental plots (1 x 1 m) were established in May 2015 in a randomised block design with six blocks, each containing one replicate of the 12 treatment combinations, to account for the differences in soil characteristics (see Table S2 and Figure S1 for details) and topography. Plots were situated three metres apart and were hand-weeded twice before planting. The native grasses were grown from seed during

winter/spring 2014 at South Para Nursery, which regularly produces plants using standard practice for restoration of this site, and thus planted as tubestock plants. Due to the different phenology of the C3 and C4 species, planting was timed to suit the environmental conditions required by each group, i.e. C3 species were planted in May right after the first substantial rains and C4 species in August 2015. A template was used to ensure the plants were evenly spaced and the species were placed at randomised positions within the template. All plots were hand weeded for one month after the C4 species were planted to allow the native plants to establish. Afterwards half of the plots (weeding treatments) were weeded monthly or as required.

Data collection

Survival of planted grasses was recorded every three weeks and dead plants were replaced in June (winter) and October (spring) 2015 and again in May 2016 using the same cohort of tubestock plants. The summer (December – February) was too dry to justify replanting and supplement watering - 20 litres per plot - was required on two occasions to maintain the experimental plants alive, once on January 21st and again on February 19th. Above-ground biomass was harvested between October 5th and 25th (spring) 2016 and all samples were dried for 48 hours at 60 °C and then weighed. Replants from 2016 were not used in biomass analysis because the glasshouse conditions would have affected growth differently to those plants in the field. Native plants were weighed individually whereas exotic biomass was considered as all standing material, after the native plants were removed, in the unweeded plots. In addition, we weighed the biomass of exotic grasses and forbs separately from a subset of four randomly chosen replicates to characterise the functional group composition of the exotic component of the plant community.

Soil samples (10 cm diameter x 10 cm deep) were collected at the beginning of the experiment, to measure any possible differences in soil properties between experimental

blocks (see Table S1), and at the time of harvest, to measure changes in soil conditions that could reflect differences of resource use in each treatment combination. The samples were collected from two random locations within the central section of each plot and combined as a single sample. Physiochemical analyses were carried out at CSBP laboratories (Bibra Lake, WA) to measure nitrate nitrogen, ammonium nitrogen, plant-available (Colwell) phosphorus, potassium (Colwell), organic carbon, conductivity and pH ($CaCl_2$).

Long-term (1885-2016) daily precipitation and temperature data was collected from the Roseworthy Bureau Station (BOM 2017) and summarized to compare with conditions during the study. In three randomly selected experimental blocks (one, five and six, Figure S1), soil volumetric water content at ten to fifteen cm (%) was collected every 2 hours using Decagon 5TM soil moisture sensors (Decagon Devices Inc., Pullman, WA, USA) from September 2015 to October 2016 (only shown 7th May to 5th October 2016).

Data analysis

All analyses were conducted in R version 3.3.3 (R Core Team 2017). For the exotic species, total above-ground biomass and broadleaf:grass ratio were analysed with linear mixed models (LMMs) with functional complexity (all three levels) and density as fixed effects and block as a random effect, using the 'lmer' function from *lme4* package (Bates *et al.* 2015).

Analysis of the survival and biomass of the native grasses had to be done separately for the C3 and C4 species because the design was not balanced, i.e. C3 species were never present in single-C4 treatment and vice versa. Therefore, there were only two levels of functional complexity for this analysis per functional group, single and mixed treatment. To analyse survival data, we fitted parametric survival models using the 'survreg' and 'frailty' functions in the *survival* package (Therneau and Grambsch 2000) with functional complexity, density, weeding and species as fixed effects and block as a random effect. We

fitted five models with different survival functions, namely Weibull, Gaussian, exponential, extreme and logistic, and compared the AIC values to determine which model best described the survival. The biomass of native plants, based on individual plant biomass without the 2016 replacements, was also analysed using LMM and the same factors as the survival analysis. The total biomass of all plants per plot at the time of harvest (including 2016 replacements and exotic plants) were analysed using linear models ('lm' function) with the same fixed factors as individual biomass. The same was done for the total biomass per species at the time of harvest.

Similarly, soil physiochemical properties were analysed separately as a function of functional complexity, density and weeding with block as a random factor. To determine differences in soil volumetric water content between community assemblages the daily precipitation data collected at Roseworthy bureau station was used to determine drying-off periods (DPs). Six DPs were chosen as a single day during the 2016 growing season (winter, May 7th to October 5th) that followed five consecutive days of low rainfall, i.e. less than two mm for DP1-DP4 or less than six mm for DP5 and DP6 (Figure 6b). Volumetric water content was then analysed as a function of functional complexity, density and weeding using linear models. However, due to technical difficulties there was never a time when all probes were working therefore at each DP at least one treatment only had two replicate measurements. A Bonferroni correction was applied to all *P* values to account for multiple testing.

With all analyses, data were transformed using square root where necessary to meet parametric assumptions but raw data are used for presentation. Where significant differences were detected with analysis of variance (ANOVA) tests, *post hoc* tests of pairwise comparisons were made using the 'glht' function in the package *multcomp* (Hothorn *et al.* 2008). The glht function allows multiple comparisons for mixed models

using *post hoc* Tukey's honest significant difference (HSD) tests. All models were graphically checked for their error distributions and homogeneity of variances.

5.5 Results

Exotic biomass

Density and functional complexity of native communities affected the total exotic biomass in the unweeded plots ($p < 0.001$ for both; Figure 2) but there was no statistically significant interaction ($p = 0.816$) between them. Overall, exotic biomass was lowest when native plants were planted in high density and in the single-C3 treatment (Figure 2). Exotic biomass was highest in the single-C4 treatment (Figure 2). On average, exotic grasses made up around three quarters of the above-ground exotic biomass in each plot (76.6 % ± 17.5 SD) with the remainder being exotic forbs. Functional complexity and density of planting did not affect the composition.

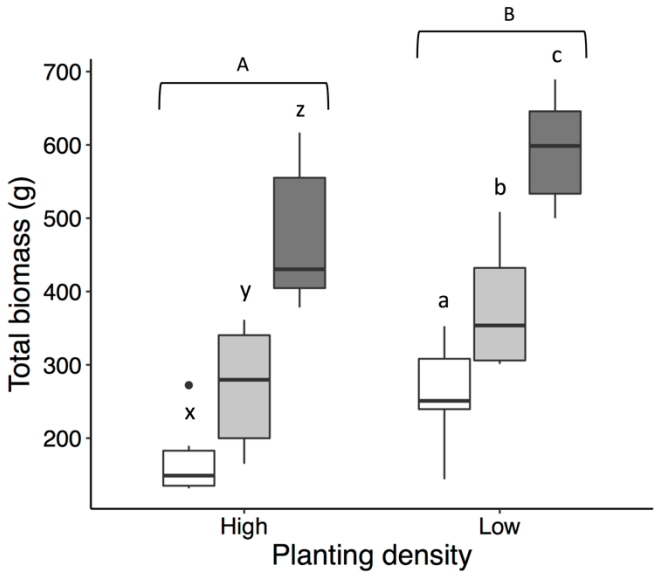

Figure 2. Total above-ground biomass of exotic plants present in experimental plots where native grasses were planted at high or low density or in different functional groups (white = single-C3, light grey = mixed, dark grey = single-C4). Significant differences in biomass are indicated by the different letters, upper case for density and lower case for functional complexity.

Native plant survival

Out of 3,004 native plants originally planted five were unaccounted for, and thus removed from analysis, and 304 died; one *R. caespitosum,* 134 *A. flavescens*, 69 *E. nigricans* and 100 *T. triandra.* For both C4 species, survival was negatively impacted when planted in the mixed treatment ($p < 0.001$; Figure 3) but functional complexity was not involved in any interactions. However, there were two significant two-way interactions, one between species and density ($p < 0.001$) and another between species and weeding treatment ($p = 0.002$). *Post hoc* comparisons found that only *T. triandra* was affected by density with lower survival in high density plots ($p = 0.041$) and only *E. nigricans* was affected by weeding with higher survival when weeded ($p < 0.001$; Figure 3). The two C4 species differed in their survival over time but only in weeded plots ($p = 0.009$) and in high density plots ($p < 0.001$) where survival of *E. nigricans* was higher in both cases (Figure 3). There were no significant interactions for the C3 species however, survival was higher for *R. caespitosum* ($p < 0.001$), and in low density plots ($p < 0.001$) and when planted in the mixed treatment ($p < 0.001$; Figure 3). There was no effect of weeding ($p = 0.583$).

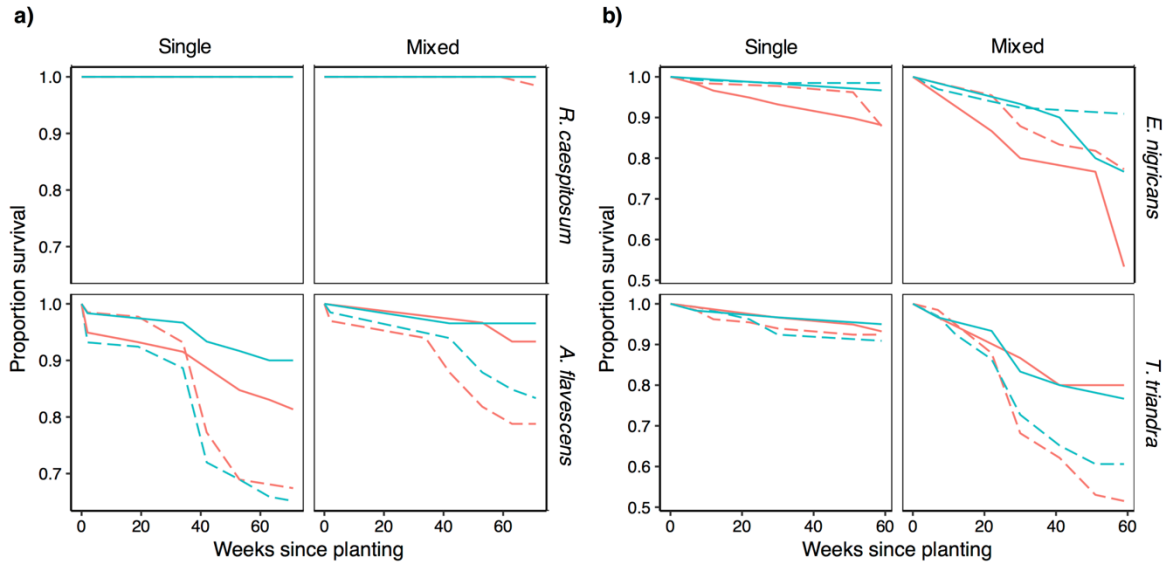

Figure 3. The proportion of survival for a) C3 (*Rytidosperma caespitosum* and *Austrostipa flavescens*) and b) C4 (*Enneapogon nigricans* and *Themeda triandra*) plants over the course of the experiment (in weeks since planting; C3 plants on 28/5/2015, C4 plants on 19/8/2015). The left panels show survival when the plants were grown with their own functional group (single treatments) and the right panels shows survival in mixed functional group treatments. Planting density is represented by line type (dashed = high, solid = low), and line colour represents weeding treatment (red = unweeded, blue = weeded).

Native plant biomass

Individual biomass was always higher for both C3 and C4 species when planted at low density ($p < 0.001$, Figure 4). The difference in growth between species was also significant for both C3 (*R. caespitosum* was larger than *A. flavescens)* and C4 (*T. triandra* was larger than *E. nigricans*) species ($p < 0.001$ for both; Figure 4). For the C4 species there was a significant interaction between functional complexity and weeding ($p < 0.001$) and further analysis found that the individual biomass was always larger in the single treatment than in the mixed treatment ($p < 0.001$) and weeding only resulted in higher biomass in the single treatment ($p < 0.001$) and not in the mixed treatment ($p = 0.379$; Figure 4). For the C3 species we considered the three-way interaction between functional complexity, density and weeding as marginally significant and worth investigating via *post hoc* comparisons ($p = 0.054$). This was based on the grounds that disregarding an important

interaction is more conducive to misunderstanding of the system than considering an unimportant one (Facelli and Facelli 2002; Fowler 1990) and that there were three significant two-way interactions suggesting that these factors are impacting on each other in complex ways. Overall, the individual biomass of C3 species was larger in the weeded plots except when planted on their own (single treatment) at low density ($p = 0.651$; Figure 4). Similarly, C3 species grew larger in the mixed treatment except when grown in low density unweeded plots ($p = 0.063$; Figure 4).

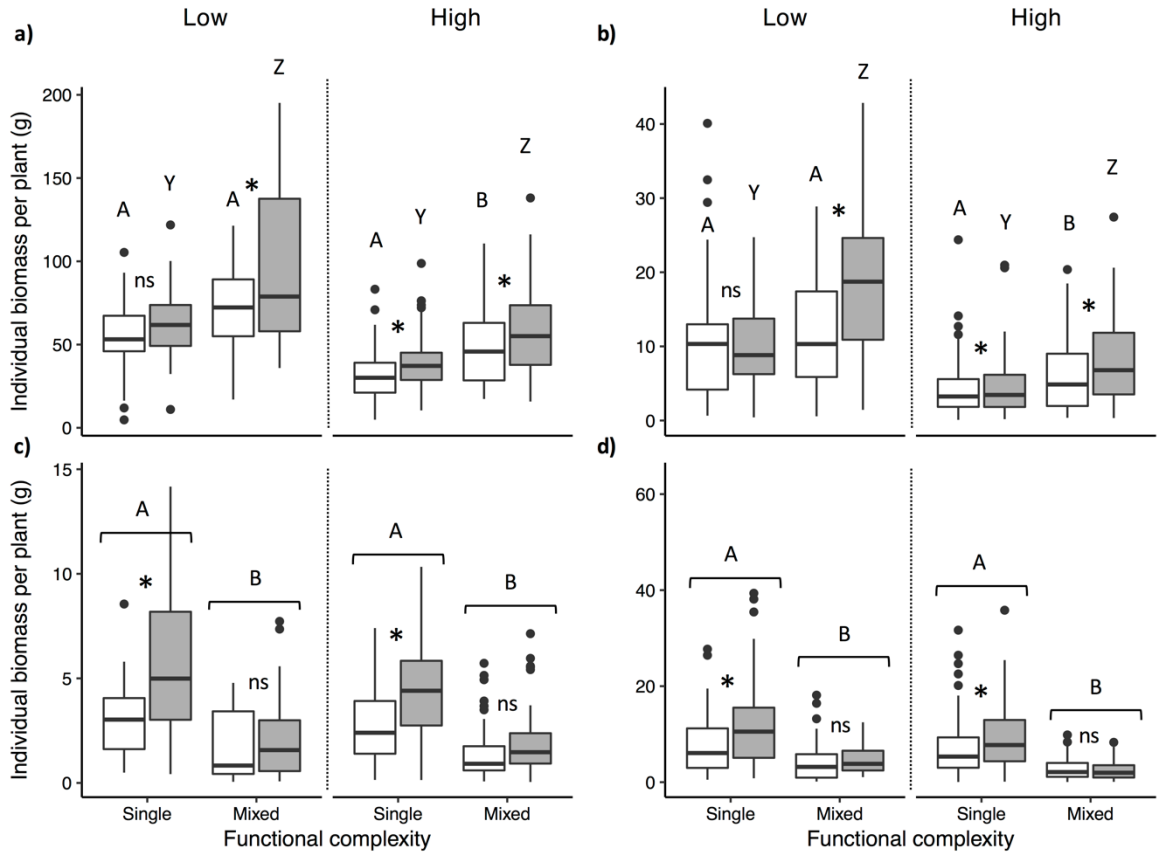

Figure 4. Individual above-ground biomass of C3, a) *Rytidosperma caespitosum, b) Austrostipa flavescens,* and C4, c) *Enneapogon nigricans* and d) *Themeda triandra* plants when grown with either their own functional group (single treatments) or in mixed functional groups and at different densities (low = left of dotted line; high = right of dotted line). Unweeded plots are in white and weeded plots are in grey. The median is represented by the line inside the boxes, the boxes show the 25th and 75th percentiles and the whiskers show 1.5 x interquartile range. Asterisks indicate significant differences between weeded and unweeded plots after Tukey contrast analysis, ns = non-significant. Differences in biomass between functional complexity is indicated by different letters: letters in plots a

and b indicate differences in biomass between functional complexity treatments within weeding treatments (AB for unweeded; YZ for weeded). Plots c and d show differences in biomass between functional complexity regardless of weeding treatment. Analysis was performed of square-root transformed data.

There was a significant interaction between functional complexity and weeding for the total biomass of all plant material per plot ($p < 0.001$). Planned comparisons found that the total biomass was higher in the unweeded plots ($p < 0.01$) except in the C3-single plots where there was no difference ($p = 0.21$, Figure 5). Over both levels of weeding treatment, total biomass was lower in the C4-single plots than with the mixed and C3-single plots ($p < 0.01$ for all, Figure 5). There was also a significant interaction between density and wedding treatment ($p < 0.01$). Total biomass was always lower in the weeded plots for both high and low-density communities ($p < 0.01$). Total biomass was lower in low-density plantings in weeded plots ($p < 0.01$) but not in the unweeded plots ($p = 0.813$, Figure 5).

In the analysis of total biomass per plot for the native species separately, we found that the total biomass of C3 native plants was always higher in weeded plots ($p < 0.001$, Figure 5). There was a significant interaction between species and functional complexity ($p = 0.005$) and between species and density ($p <0.001$) and on both occasions total biomass of *R. caespitosum* was always larger than *A. flavescens* ($p < 0.001$ for all comparisons, Figure 5). Further comparisons found that total biomass was lower in mixed functional treatments and low-density planting for *R. caespitosum* ($p < 0.001$ for both) but not for *A. flavescens* ($p = 0.606$ and $p = 0.281$ respectively, Figure 5). For the total biomass of C4 plants, there were three significant two-way interactions each involving functional complexity and another main effect, including species ($p < 0.001$), density ($p = 0.028$) and weeding ($p = 0.031$). Most *post hoc* comparisons were highly significant ($p < 0.001$) indicating that total biomass was always higher for *T. triandra* than *E. nigricans,* in high density than in low density plots and in the single functional treatment than in the mixed

functional treatment (Figure 5). Weeding resulted in higher total biomass in the single treatment ($p < 0.001$) but not the mixed treatment ($p = 0.838$, Figure 5).

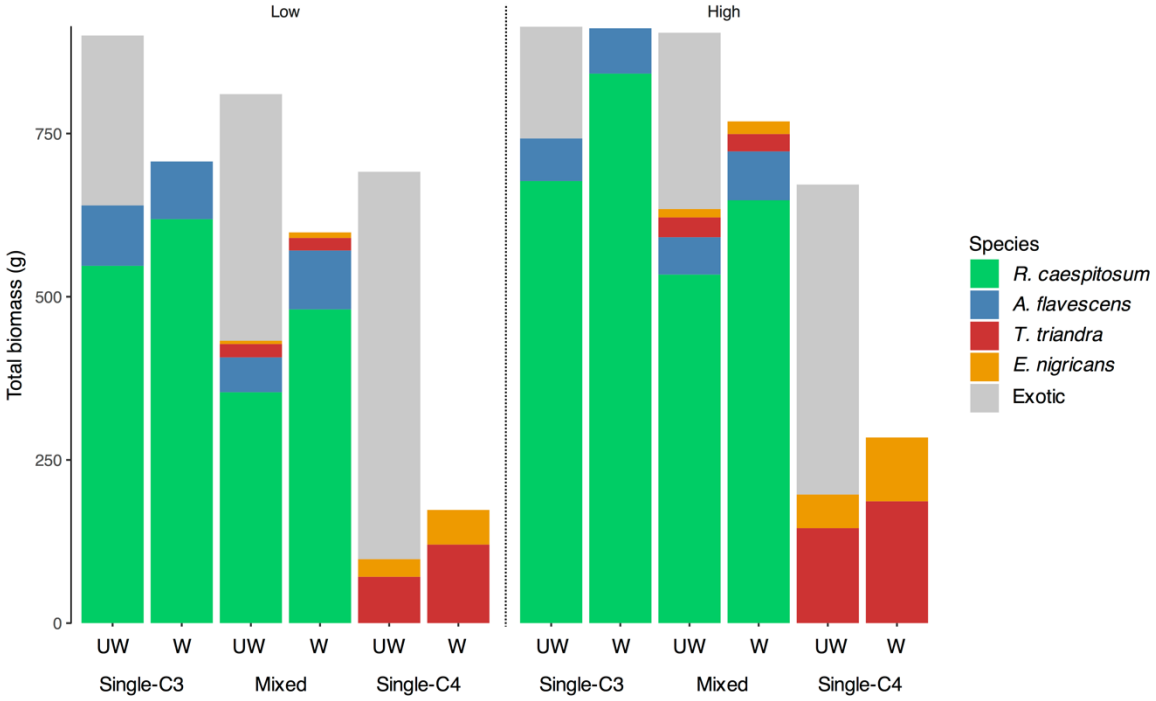

Figure 5. Mean total biomass of all standing plant material at the time of harvest in each community type; high and low density (low = left of dotted line; high = right of dotted line), weeded (W) and unweeded (UW) and single or mixed functional groups. Colours represent the mean total biomass of each native species and grey represents the total biomass of exotic plant material in the unweeded plots.

Soil properties

Out of the seven soil physiochemical properties measured at the time of harvest three were not affected by the treatment combinations, namely, ammonium nitrogen, potassium and conductivity (Table 1). Nitrate nitrogen and organic carbon were higher in high density plots ($p = 0.005$ and $p < 0.001$ respectively), whereas available phosphorus was higher in weeded plots ($p = 0.004$) and in the mixed treatment than in the single-C3 treatment ($p < 0.001$; Table 1). There was a statistically significant three-way interaction for pH ($p = 0.037$) and post hoc comparisons found that pH was lower in unweeded plots

but only in high density single treatments (p = 0.007 and p = 0.037 respectively). In addition, there was lower pH in the high density C3 unweeded plots ($p < 0.001$).

Generally, there was less variation in soil volumetric water content in the weeded plots however there was not a significant difference after p-adjustments at any of the DPs. There was a significant difference between functional complexity treatments on three occasions mid-season, DP2 (June 4th), DP3 (July 21st) and DP4 (August 8th), and the single-C3 treatment was lowest on each occasion (*P-adjusted* < 0.001 at each DP, Figure 6). Density of planting was also important in determining volumetric water content on three occasions mid-season, DP3 (July 21st), DP4 (August 8th) and DP5 (September 7th) where generally values were higher in high density plantings, particularly in the unweeded plots (*P-adjusted* < 0.001 at each DP, Figure 6). There were no significant interactions after p-adjustments.

Table 1. Mean (± SD) of soil physicochemical properties for the different community assemblages (n = 6) at the time of harvest. For the weeded treatment UW = unweeded and W = weeded. Differences between planting treatments are indicated by different letters (ab for density, yz for functional complexity, lm for weeding).

Functional complexity	Density	Weeding	NH4+ -N (mg/kg)	NO3- -N (mg/kg)	Plant available P (Cowell; mg/kg)	K (mg/kg)	Organic C (%)	Conductivity (dS/m)	pH (1:5 CaCl2)
Single-C3	High	UW	5.0 ± 0.9	8.7 ± 2.3^{a}	19.3 ± 2.3^{l,y}	822.3 ± 28.8	2.8 ± 0.5^{a}	0.09 ± 0.03	6.6 ± 0.6^{l,a}
Single-C3	High	W	5.2 ± 1.5	9.8 ± 4.2^{a}	20.2 ± 4.1^{m,y}	814.0 ± 78.3	3.0 ± 0.4^{a}	0.12 ± 0.02	7.1 ± 0.4^{m}
Single-C3	Low	UW	5.0 ± 2.2	8.2 ± 1.5^{b}	16.7 ± 3.4^{l,y}	776.3 ± 81.5	2.6 ± 0.2^{b}	0.11 ± 0.01	7.2 ± 0.4^{b}
Single-C3	Low	W	5.0 ± 1.8	7.5 ± 2.1^{b}	17.0 ± 4.5^{m,y}	769.2 ± 47.8	2.4 ± 0.3^{b}	0.10 ± 0.03	7.0 ± 0.5
Mixed	High	UW	5.7 ± 1.4	9.3 ± 2.7^{a}	22.7 ± 9.2^{l,z}	795.8 ± 82.3	2.7 ± 0.4^{a}	0.11 ± 0.03	7.0 ± 0.7
Mixed	High	W	4.2 ± 0.4	7.8 ± 1.2^{a}	26.3 ± 6.6^{m,z}	822.0 ± 80.4	2.9 ± 0.4^{a}	0.11 ± 0.04	7.2 ± 0.5
Mixed	Low	UW	4.7 ± 1.9	7.2 ± 1.5^{b}	22.2 ± 6.8^{l,z}	843.3 ± 116.3	2.5 ± 0.4^{b}	0.09 ± 0.03	7.0 ± 0.5
Mixed	Low	W	4.5 ± 1.2	7.8 ± 1.3^{b}	26.3 ± 9.5^{m,z}	822.0 ± 59.5	2.5 ± 0.3^{b}	0.10 ± 0.03	7.2 ± 0.5
Single-C4	High	UW	5.8 ± 1.7	10.2 ± 2.0^{a}	18.3 ± 6.5^{l,yz}	788.3 ± 54.2	2.7 ± 0.1^{a}	0.10 ± 0.03	6.9 ± 0.7^{l}
Single-C4	High	W	4.8 ± 1.5	9.3 ± 2.7^{a}	24.2 ± 9.7^{m,yz}	756.5 ± 51.6	2.6 ± 0.3^{a}	0.11 ± 0.03	7.3 ± 0.5^{m}
Single-C4	Low	UW	4.2 ± 1.0	8.2 ± 1.0^{b}	16.2 ± 3.4^{l,yz}	749.2 ± 85.5	2.4 ± 0.2^{b}	0.11 ± 0.04	7.3 ± 0.4
Single-C4	Low	W	4.5 ± 1.8	8.7 ± 3.0^{b}	24.2 ± 4.9^{m,yz}	820.3 ± 62.1	2.3 ± 0.1^{b}	0.10 ± 0.03	7.2 ± 0.6

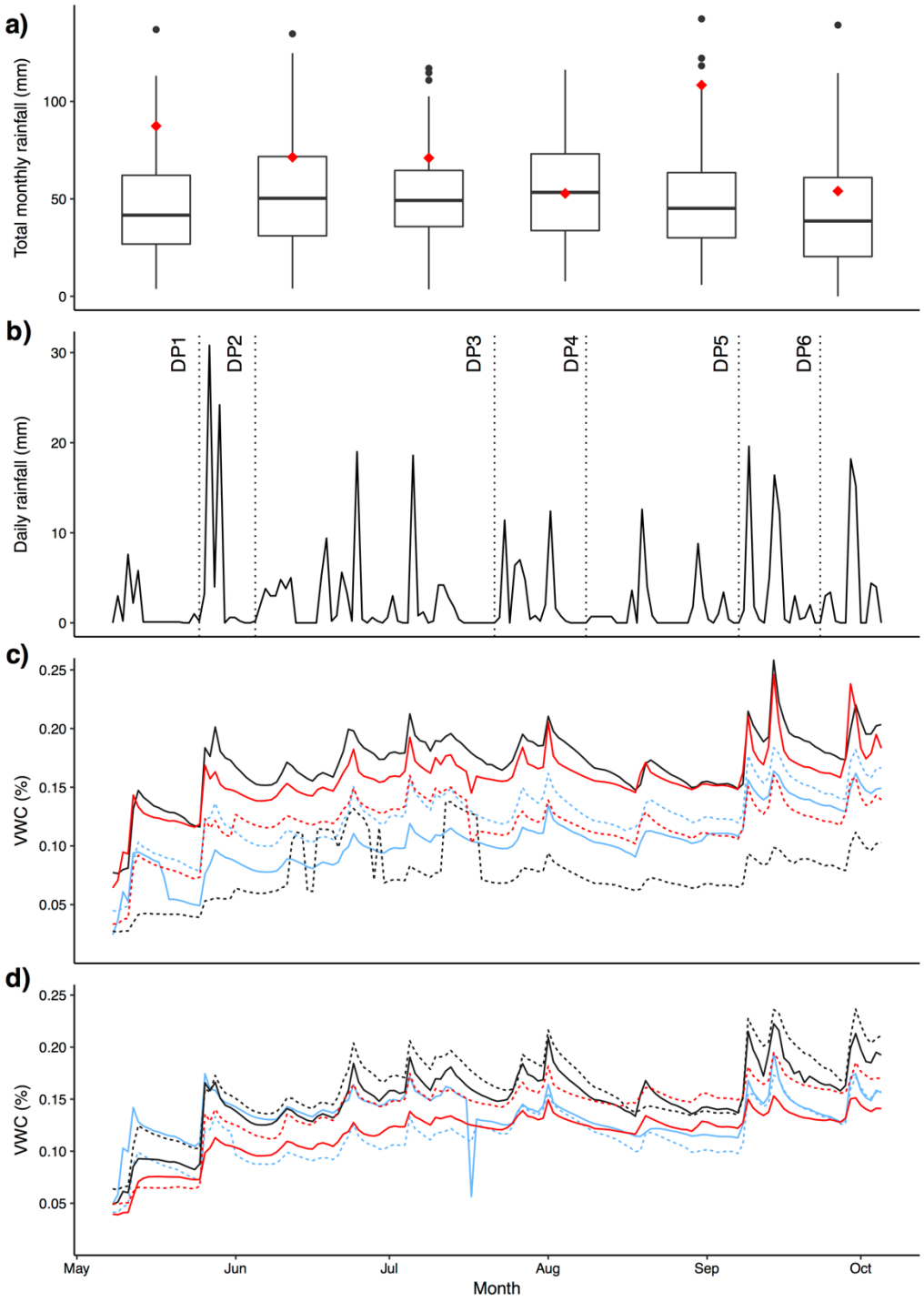

Figure 6. Rainfall and mean soil volumetric water content (VWC) from May to October (winter months). Monthly rainfall (a) from 1885 to 2016 is characterized by box and whisker plots, where the boxes show the 25th and 75th percentiles and the whiskers show 1.5 x interquartile range. Red diamonds represent the monthly rainfall during 2016. Daily rainfall (b) data during 2016 were used to select drying-off periods (DP) throughout the season to use for comparisons in soil moisture in unweeded (c) and weeded (d) plots. Colour of lines represent the functional complexity (red = single-C4, blue = single-C3, black = mixed treatment) and line type represents density (solid = high, dashed = low). Monthly and daily data are from Roseworthy weather station (BOM 2017).

5.6 Discussion

We found little evidence to suggest that planting perennial C3 and C4 grasses together is an effective method to reduce exotic C3 species invasions. While this planting strategy was more effective at reducing exotic biomass than planting C4 grasses on their own, C3 native grasses, particularly *R. caespitosum,* were the superior competitors and were more effective when planted on their own. While higher density planting was successful at reducing exotic biomass as expected, there was a trade-off with reduced individual performance among the native plants and this result was consistent across all levels of functional complexity. Interestingly, this size advantage in low-density communities did not result in equal biomass of the whole community in weeded plots suggesting that density limits the growth of the native community. Native C3 species were strong competitors against the C4 species as shown by reduced survival and biomass of C4 plants in the mixed treatments than in the single treatments.

C3 native plants effective against invasive plants

There are a few explanations why the native C3 species were the most effective competitors against the exotic C3 species. Firstly, many studies have found that resident species inhibit invaders from their own functional group more strongly than other functional groups (Dukes 2001; Emery 2007; Fargione *et al.* 2003; Gooden and French 2015). The mechanisms for this competitive dominance can vary but most relate to niche overlap between similar species (limiting similarity, Abrams 1983). Given that the C3 invasive species were actively growing at the same time as the C3 natives it is likely that they were inhibited by an overlap in resource use. However, a meta-analysis found that limiting similarity occurred with forbs but not grass species (Price and Partel 2013).

The single-C3 treatment reduced two soil resources, phosphorus and water content, to the lowest levels indicating that the native C3 species are strong belowground

competitors. This could be because they were given a head start as they were planted as one-year-old tubestock plants and plots were weeded to allow establishment in the first season. Therefore, the negative effect on invasive species could also be due to a size advantage, gained through earlier growth, which suppressed the growth of smaller individuals (size-asymmetric competition, Perry *et al.* 2003; Weiner 1985). In other words, the C3 natives were able to pre-empt resources and outcompete the invading exotic plants.

Perennial grasses have been shown to be superior competitors against annual invaders and this effect can increase as the perennial grasses grow larger in each successive year after planting (Corbin and D'Antonio 2004; Lulow 2006). One explanation for this advantage is that annual species tend to allocate fewer resources to roots and more resources to leaf and seed production (Grime and Hunt 1975; Jackson and Roy 1986), which results in faster growth above-ground but weaker competitors for belowground resources than perennials (Garnier 1991; Tilman 1982). A head start in growth and a well-established root system would allow perennials to take advantage of this difference and, in particular, exploit deeper water resources (Dyer and Rice 1999; Seabloom *et al.* 2003).

In addition, plant-soil feedbacks may have been an important contributor of competitive dominance, but was not tested here. For example, the accumulation of functional-group-specific herbivores or pathogens could prevent the establishment of invaders or by planting the native grasses earlier and allowing them to become established may have resulted in positive plant-soil feedback, giving them a competitive advantage (Grman and Suding 2010; Klironomos 2002; Smilauerova and Smilauer 2016).

C4 native plants were not good competitors

Limiting similarity could also explain why the community assemblages that included native C4 species (single-C4 or mixed functional treatments) were less effective

than the single-C3 treatment at resisting invasion. Essentially, if competition between species with overlapping niche requirements, in space and time, is more intense than between those with different requirements then it is possible that the C4 grasses were not competing for the same resources, at least at the same time, as the C3 invasive species, thus making them less effective competitors. Given that the C4 grasses were actively growing later in the season, it is likely that they were using limiting resources, such as water, at a different time to the invasive species which appeared earlier in the season (Figure 1). It is thought that niche complementarity is one of the main reasons for species coexistence (Grime 2006; Tilman 1982) so it is possible that this mechanism can allow these two functional groups to coexist. However, this theory assumes that the environment is constant, uniform or at equilibrium and therefore is unlikely, particularly in newly established communities like the ones in this study (Grubb 1977; Pickett 1980). An alternative view, known as non-equilibrium coexistence, recognises that biotic and abiotic events can prevent the process of competitive exclusion by creating resource gaps that the less dominant species can exploit (Chesson 2000; Pickett 1980).

The establishment time of the C4 species may not have been sufficient to make them successful competitors. A study in a climatically similar region in Victoria, Australia, found that *T. triandra,* the dominant C4 species, took longer than other species to become established (Gibson-Roy *et al.* 2009). In our study, we attempted to overcome this delay by planting mature tubestock plants rather than using direct seeding. This meant our native grasses would have been more mature than those in a study by Cole *et al.* (2017) who found that *Austrostipa* (C3 species) swards with successful recruitment of C4 grasses, including *T. triandra,* suppressed exotic annuals more than the *Austrostipa*-only. Alternatively, the climatic conditions may not have favoured the C4 species and thus made them less competitive. For example, these species may depend on summer rains to become

established or build up their individual tussock sizes (Cole *et al.* 2017; Lodge 1981) whereas our site had below average rainfall over summer (we watered to the average rainfall to aid establishment). In addition, the very wet spring of 2016 (200 mm above average, Figure 6) may have provided ideal conditions for the C3 plants (native and exotic) and gave them a competitive edge over the C4 species. While the C4 species would have been actively growing at this time they have a higher water use efficiency and therefore might not be as competitive in these conditions (Ehleringer and Monson 1993). These conditions and the fact that the invasive species established earlier (i.e. priority effects) could explain why the exotic species reduced the biomass of both C4 native grasses and the survival of one species (*E. nigricans*).

Density effects

High-density planting reduced the biomass of both the exotic and native plants and reduced the survival of *T. triandra* and *A. flavescens* thus demonstrating a trade-off between a desirable effect on exotic species but an undesirable impact on native species. Interestingly, there were no reductions in soil moisture or nutrients in high density plantings suggesting other resources, such as light availability, may have been more important (Dyer and Rice 1999; Kardol *et al.* 2013; Young *et al.* 2011). The increased growth of individual native plants in low-density communities did not result in equal biomass of the whole community in weeded plots suggesting that density is limiting the native community, at least within the timeframe used here. There was no difference between total biomass of high and low-density communities in the unweeded plots therefore the exotic species appear to be using up the remaining resources in the low-density communities, as evident by the increase in biomass of exotic species in these communities.

We predicted that competition at high density would be more intense in the single functional treatment than the mixed treatments due to niche partitioning; however, there

was no interaction between functional complexity and density for native plant biomass showing that these mechanisms acted independently. We planted the native species in higher densities than they are found naturally in Para Woodlands Reserve and nearby areas (Lenz and Facelli 2005; Rosser 2013); however, these species (or species from the same genus) have been recorded to occur at much higher densities, particularly after fire (Morgan 1999). Perhaps we would see a greater impact on invasive species at higher densities. Alternatively, we may see a greater density effect if we looked at recruitment in the following seasons due to the Janzen-Connell hypothesis which predicts that recruitment is reduced near conspecific adults or where conspecific seed density is greatest (Connell 1971; Janzen 1970; Wright 2002).

Natives vs. natives

Out of the four-native species planted, *R. caespitosum* grew the largest, had the lowest mortality (only one plant died) and had overall the strongest negative effect on the other species, making it the superior competitor. For instance, biomass and survival of *A. flavescens* was lower in the single treatment than in the mixed treatment. This indicates that *R. caespitosum* was a stronger competitor than both C4 species thus allowing *A. flavescens* to take advantage of unused resources in the mixed treatment before the C4 species can use them. This difference was so great that the total biomass per plot of *A. flavescens* was not different between the single and mixed treatments which is surprising given that the number of individual plants per species doubled in the single treatment and replanting ensured that these treatments did not converge. This suggests that, in this system, species identity is more important than functional groups (C3 and C4 namely) in that plants that are intrinsically larger dominate and those weaker are more likely to be dominated, or suffer high mortality.

Individual biomass of C4 plants were always higher in single treatments, i.e. without the native C3 species. Similarly, weeding only resulted in higher biomass (individual and total) in the single treatment thus the C3 plants were able to extract unused resources as effectively as the exotic species in the unweeded plots. This is surprising given that these species, or at least species from the same genera, have been shown to coexist in artificial communities of similar trials (Cole *et al.* 2017; Gibson-Roy *et al.* 2009) and in remnant areas of Para Woodlands Reserve (Rosser 2013) and within the surrounding region (Hattersley 1983; Hyde 1995; Kirkpatrick *et al.* 1995). In our communities however, it appears that priority effects control the community structure. That is, the C3 native grasses probably had a growth advantage as they were planted at the start of winter and C4 natives at the end of winter, to match the phenology of the species, thus the C3 natives had three months to become established before the C4 natives were introduced.

Implications and future work

This study has focussed on the first season after the establishment of a native community which has shown to be important in determining resistance to invasion of exotics (Gibson-Roy *et al.* 2009). The next stage would be to expand the timeframe to determine how sustainable the community assemblages are and whether longer establishment times allow the C4 species to become more competitive. Future work could also benefit from incorporating higher diversity. We focussed on grasses because during a pilot study, exotic grasses made up majority of biomass with three exotic forbs only present in low abundances (< 1 % cover). However, at the time of harvest forbs made up 25 % of the biomass. This increase could have been caused by a number of factors such as disturbance from planting or weeding or for climatic reasons, particularly higher rainfall.

Here we presented results that show planting density and functional group complexity can have profound effects on both the resident community and new arrivals and

interestingly, these two factors did not interact. The mixed functional treatment was not as effective at reducing exotic biomass as we had predicted. Rather the evidence suggests that matching functional groups to potential invaders and/or size asymmetric competition is more important for community resilience. The somewhat unexpected results also highlight the need to conduct these types of studies in different climates. Currently a lot of literature comes from the northern hemisphere and C4 grasses were effective competitors in systems where they actively grow in mid-season (Fargione *et al.* 2003) but perhaps across-seasonal effects are less common. In addition, it appears that processes of pre-emption are important in the assembly of these communities as seen by the competitive dominance of the C3 native plants on those that arrived or were planted after, i.e. both the C4 native and C3 invasive species. Changing the planting time so that the native C4 were introduced first will help to decipher whether this is the case or if the climatic reasons mentioned above have more impact.

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Chapter 6. General discussion

Field work with volunteers at Para Woodlands Reserve

6.1 Thesis summary

The main aim of this thesis was to contribute to the mechanistic understanding of the constraints in old-field restoration and processes required to establish resilient native grass communities.

The recovery of old-fields is highly varied with some sites returning to a state that resembles the diverse native community that existed prior to farming with little or no interference (Collins 1990; Hermy and Verheyen 2007; Inouye *et al.* 1987), while others persist in a degraded state for many decades and are very difficult to restore (Cramer *et al.* 2008; Standish *et al.* 2008). In the former case, seed limitation is often listed as a constraint that once relieved, can allow the degraded system to follow a successional pathway towards the historical state (Kardol *et al.* 2008; Seabloom *et al.* 2003; Standish *et al.* 2007). However, at the old-field site in this project when no other active restoration was applied (i.e. control plots in chapter 4) the native grass seedlings had very low emergence and survival over the growing season and were outcompeted by the exotic species. This confirmed our predictions that an alternative stable state model is more appropriate than a simplistic successional model for restoring this site because there are likely multiple constraints preventing the successional trajectory towards the historical state. The following discussion considers the constraints investigated in this study, particularly plant-soil interactions, soil nutrient availability and competition with exotic species.

6.2 The role of plant-soil interactions in ecological restoration

The importance of plant-soil feedback in community assembly is becoming more recognised (Bever *et al.* 1997; Callaway *et al.* 2004; Herzberger *et al.* 2015; Kardol *et al.* 2006; Wardle 2002), with implications for restoration ecology. Past farming practices and

subsequent changes in environmental conditions (e.g. increased soil nutrient availability and a shift from perennial to annual plant communities) in old-fields are likely to have altered soil microbial communities from the historical state (Araujo *et al.* 2014; Gellie *et al.* 2017). Therefore, this thesis included a glasshouse study (chapter 2) aimed, in part, to see whether changes in the microbial community could be a constraint to restoring old-fields. The most significant finding was that the native species experienced 50-60 % mortality when exposed to soil microbes from the old-field, compared with 10 % in the exotic species. The native species also had lower growth in this treatment compared with remnant microbes, whereas the exotic species grew equally well in all inoculated soil. These two pieces of evidence suggest that change in the soil microbial community in old-fields is one mechanism which gives exotic species a competitive edge over native grasses.

One ecological restoration 'tool' that has recently gained momentum in the literature is the use of microbial inoculants (Emam 2016; Rowe *et al.* 2007; Wubs *et al.* 2016). The idea here is to give native plants an advantage over exotic species by facilitating the recovery of soil mutualists. Different origins of soil inocula have even been shown to drive plant community succession towards different target communities from grassland to heathland vegetation (Wubs *et al.* 2016). Given the negative impact of old-field soil microbes evident in chapter two this could be one tool to assist restoration at this site. The positive growth and survival of the native grasses when exposed to remnant microbes indicates that it could be a good inoculant source. A recent meta-analysis found that using whole soil from remnant areas can provide benefits in restored areas that last for several years (Maltz and Treseder 2015). However, given the need to protect remnant areas it was also important to trial different sources of inocula. Therefore, soil from a native grass seed orchard was included in this trial (chapter 2). Unfortunately, poorer performance of native species with the orchard inoculant suggests it would not make a suitable replacement for

remnant soil even though plants at the source site were healthy and supplied viable seeds. Future work is needed to understand the requirements of target species and their interactions with different soil biota before this technique can be implemented to maximum benefit.

In chapter 2, sterile, commercial grade soil was inoculated with small amounts of soil from the different locations to minimise the differences in soil properties between treatments. While this was necessary to tease apart the microbial effects from soil chemistry effects the results led to further questions. In particular, how do these plant-soil interactions differ when the abiotic conditions of old-fields and remnant areas are considered? The reciprocal transplant approach in chapter 3 allowed us to address this question. Interestingly, microbial effects on the plants were not as prominent using this approach with very little mortality, only subtle growth responses to inocula and low rates of formation of arbuscular mycorrhizas (AM). It was concluded that the higher nutrient availability in the second experiment meant that symbiotic relationships with AM fungi became less important for the test species (Son and Smith 1988). This finding put into question the importance of soil microbes at the sites, at least, in comparison with the findings from the first experiment and with the plant species tested.

Despite these findings, there was evidence that the plant species supported different soil microbial communities, even after the short experimental time, thus supporting recent work suggesting that native and exotic plants can alter soil microbial communities in different ways (Klironomos 2002; Stinson *et al.* 2006). This is thought to be one mechanism through which exotic species can maintain dominance in a system, particularly if there is an increase in pathogens that inhibit native plant growth or establishment (Lekberg *et al.* 2013; Mangla *et al.* 2008). If this was the case, we would expect to see a positive growth response when the exotic grass, *Avena barbata,* was exposed to old-field microbes because

it has been the dominant species at that site for at least a decade (Kulmatiski and Kardol 2008; Rosser 2013). However, this was not observed and therefore, the results failed to show support for plant-soil feedbacks as a reason for a persistent degraded state at our site, at least between *A. barbata* and old-field microbes under glasshouse conditions.

Another interesting result from chapter 3 was the evidence for local adaptation for the plant-soil interactions. For example, the microbial growth response (MGR) of the plants, regardless of species, was more likely to be positive when the inoculant was added to its home soil. This could imply that adding remnant inocula to old-fields may not have the desired benefits to the native community unless the abiotic conditions are also amended. This was further supported by influence of bulk soil type on the soil microbial communities. When grown in remnant bulk soil the native grass, *Rytidosperma auriculatum* had a higher positive MGR than *A. barbata*, regardless of inoculant origin. This may suggest that the native species had a stronger reliance on soil microbes and/or is better adapted to the lower nutrient availability of remnant bulk soil than the exotic species (Abraham *et al.* 2009; Walker and Reddell 2007). Therefore, reinstating the abiotic and biotic conditions of a remnant grassland may help give native grasses a competitive edge against exotic grasses. However, this relationship is dynamic, with microbial communities changing in response to the plant species and soil type.

Glasshouse studies that use whole soil inocula, such as chapters 2 and 3, act as an intermediate between highly controlled single microbial species mesocosms and field trials. Therefore, they act as important stepping stones in increasing our mechanistic understanding of the functioning of specific soil biota in their natural complex biotic and abiotic environment (Cortois and De Deyn 2012). However, these experiments have limitations to real world situations. As such, future work should focus on broadening the experiments to incorporate more plant species and field conditions. Similarly, longer-term

studies would help track how the observed changes in microbes are maintained and allow us to look into other impacts of the plants, such as fecundity. These developments would help to gain more realistic insights in to the interactions discussed here, in particular how resident and inoculated microbes interact and what effects the microbes have on plant-plant interactions.

Recent studies have shown that active restoration, without microbial inoculation, can drive the soil microbial community from an old-field community to one that resembles communities observed in remnant areas (Araujo *et al.* 2014; Gellie *et al.* 2017). Our findings suggest two possible mechanisms for this change. First, the microbial communities were strongly influenced by restoration techniques, i.e. carbon supplements and particularly scalping (top-soil removal; chapter 4). Secondly, the plant species can also support different microbes (chapter 3). Therefore, the applicability of microbial inoculants is brought into question. However, given the recent success of microbial inoculants in driving heathland and grassland plant communities (Wubs *et al.* 2016) it is clear that further studies are justified using field conditions and more diverse plant communities. There may also be other benefits such as introducing soil microbes that help with drought resistance or are specialised symbionts with rare or keystone plant species (Ferrazzano and Williamson 2013; van der Putten *et al.* 2016).

As discussed above, by using genetic tools in this project we were able to demonstrate clear changes in the soil microbial community composition after interactions with different plant species, soil properties and restoration techniques in finer detail than before. Advances in genetic tools, such as the use of environmental DNA (eDNA), therefore, provide a lot of promise for expanding our knowledge on soil microorganisms. However, the interpretation of the data was limited by a lack of knowledge on the different functions of the microbes affected by these interactions. Future work would benefit from

better links between description and function in microorganisms before these genetic tools can be utilized to their full potential.

6.3 Overcoming abiotic and biotic constraints in old-field restoration

In geologically old and nutrient poor landscapes like Australia, fertiliser legacies in old-fields give invasive exotic species a competitive edge over native species which are adapted to the naturally low nutrient conditions (Standish *et al.* 2006; Walker and Reddell 2007). Therefore, chapter 4 aimed to determine which site preparation techniques, out of carbon supplements, burning, slashing (with biomass removal) and scalping, was the most effective at reducing soil nutrients and competition from exotics. In terms of their impact on these two factors, the techniques ranged from having no effect (burning), subtle effects (slashing and carbon supplements) to strong effects (scalping). Interestingly, it was only scalping that significantly improved the growth and establishment of the native grasses. This suggests that, as we predicted, the level of environmental modification needed to restore this old-field is quite high.

Other studies have found scalping to be a successful site preparation technique (Buisson *et al.* 2006; Cole *et al.* 2005; Gibson-Roy *et al.* 2010); however, the success of scalping could be due to the strong effects it had on soil nutrients and exotic biomass and/or because it acted on multiple processes at once. Therefore, the other techniques, such as carbon supplements and slashing, may also be successful if the intensity or number of applications of the technique result in particular thresholds being crossed. Future work should include trials using different levels of soil nutrients or invasion intensity to determine what those thresholds may be and how the timing or rates of application of the techniques used here can change their effectiveness at a range of thresholds.

6.4 Rebuilding resilient native communities

The final piece of the restoration puzzle, after exploring possible constraints in old-fields and techniques for overcoming constraints, is to determine how to rebuild resilient native communities. This project focussed on building resilience against new species arrivals because they can impact ecosystems in dramatic ways (D'Antonio and Meyerson 2002; Kulmatiski 2006). Results from chapter 5 show that planting density and functional group complexity can have profound effects on both the resident community and new arrivals and interestingly, these two factors did not interact. We predicted that a community with a mixture of species growing at different times would be more effective at outcompeting exotic species than communities where resident species are actively growing over a narrower time period; however, this was not the case. Rather the evidence suggests that matching functional groups to potential invaders, pre-emption of resources, and/or size asymmetric competition is more important for community resilience (Dukes 2001; Emery 2007; Fargione *et al.* 2003; Perry *et al.* 2003; Weiner 1985).

To build on this finding, future work should include higher diversity. We focussed on grasses because during a pilot study, exotic grasses made up the majority of biomass with only three exotic forbs present in low abundances (< 1 % cover). However, at the time of harvest, forbs made up 25 % of the biomass. This increase could have been caused by a number of factors such as disturbance from planting or weeding or for climatic reasons, particularly higher rainfall. Chapter four also found that restoration techniques can shift the functional composition of exotic species. Therefore, to have greater application for restoration more functional groups should be included.

Our finding that the C4 native plants were negatively impacted by both C3 native and exotic species is somewhat contradictory to other studies which have found C4 grasses to be superior competitors (Fargione *et al.* 2003; Symstad 2000; Tilman *et al.* 1997). These

studies have been carried out in continental or sub-tropical climates where C4 species actively grow mid-season but perhaps across-seasonal effects are less common. It is likely that the pre-emption of resources by the C3 species impacted the C4 plants and reversing the order of planting, i.e. C4 plants first, may completely change the outcome of the project.

Future work would benefit from expanding the timeframe of the study to determine how sustainable the community assemblages are and investigate the impacts of the community assemblages on recruitment of native species. A long-term study would also help to understand what conditions the C4 species need to become more competitive against exotic species.

6.5 Conclusions

- Soil fungal and bacterial communities are affected by the soil conditions, plant species present and restoration techniques; however, the effects these changes have on plants are impacted more by the soil conditions than microbial community itself. The very complicated interaction can be better understood with better linkages between the description and function of microorganisms.
- Old-field restoration in southern Australia can be improved if site preparation techniques can cross particular thresholds. Scalping was the only technique successful at doing this with great reductions in available nutrients, exotic biomass and changes in the bacterial community.
- The community assembly and competition between species was controlled by niche complementarity, pre-emption of resources and/or size asymmetric competition. Therefore, if appropriate site techniques are used and the native community is able to become established then the invasibility of the community may be reduced, particularly if it includes functional groups that match those of potential invaders.

The use of the alternative stable state model was found to be appropriate for old-field restoration in this region. By using this approach, this project gained insights into potential constraints to restoration, techniques for overcoming those constraints and mechanisms involved in rebuilding resilient communities.

6.6 References

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Appendix 1. Supplementary material for chapter 2

Table A1. Characterisation of soil collection sites at Para Woodlands Reserve

Site	Soil type	Land-use history	Dominant vegetation	GPS coordinates
Old-field	Deep brown and grey cracking clays	Cultivation and grazing, received regular fertilization until 2004	Annual invasive grasses particularly *Avena barbata*	34.628°S, 138.785°E
Remnant	Gravely sandy loam over clay	Regular sheep grazing until 2004	Open grassy woodland dominated by *Austrostipa spp.* and *Rytidosperma spp.*	34.624°S, 138.791°E
Orchard	Deep brown and grey cracking clays	Quarry in-filled with soil from the old-field site then became a seed orchard	Soil collected from under *Austrostipa nodosa* plants	34.642°S, 138.817°E

Appendix 2. Supplementary material for chapter 3

Table S1. Characterisation of soil collection sites at Para Woodlands Reserve

Site	Soil type	Land-use history	Dominant vegetation	GPS coordinates
Old-field	Deep brown and grey cracking clays	Cultivation and grazing, received regular fertilization until 2004	Annual invasive grasses particularly *Avena barbata*	34.628°S, 138.785°E
Remnant	Gravely sandy loam over clay	Regular sheep grazing until 2004	Open grassy woodland dominated by *Themeda triandra* and *Rytidosperma spp.*	34.796°S, 138.805°E

Table S2. The weights and contribution of the soil nutrients along the first two principal components.

	Loadings		Contribution	
	PC1	PC2	PC1	PC2
NH_4^+ -N	0.337	0.497	0.134	0.238
NO_3^- -N	-0.079	-0.767	0.031	0.367
P Cowell	0.426	-0.198	0.169	0.095
Conductivity	0.405	0.078	0.161	0.037
pH	0.424	0.066	0.169	0.031
Total N	0.425	-0.235	0.169	0.113
Total C	0.417	-0.247	0.166	0.118

Table S3. Mean ± SD of richness and evenness of microbial communities (pre = pre-experimental samples, Ab = *Avena barbata*, invasive and Ra = *Rytidosperma auriculatum*, native.

Bulk soil	Inoculum source	Species	Bacteria evenness	Bacterial richness	Fungal evenness	Fungal richness
Old-field	Old-field	pre	0.54 ± 0.06	1157 ± 134	0.31 ± 0.01	77 ± 6
		Ab	0.80 ± 0.02	1697 ± 165	0.35 ± 0.06	65 ± 14
		Ra	0.79 ± 0.03	1618 ± 147	0.36 ± 0.06	64 ± 12
	Remnant	pre	0.62 ± 0.03	1356 ± 65	0.44 ± 0.03	84 ± 3
		Ab	0.78 ± 0.02	1797 ± 147	0.38 ± 0.08	88 ± 16
		Ra	0.79 ± 0.00	1722 ± 58	0.42 ± 0.03	74 ± 12
Remnant	Old-field	pre	0.64 ± 0.02	1308 ± 25	0.36 ± 0.01	83 ± 3
		Ab	0.80 ± 0.02	1727 ± 83	0.44 ± 0.11	96 ± 13
		Ra	0.79 ± 0.02	1636 ± 57	0.44 ± 0.11	72 ± 19
	Remnant	pre	0.62 ± 0.04	1311 ± 92	0.28 ± 0.01	94 ± 7
		Ab	0.71 ± 0.06	1424 ± 331	0.38 ± 0.09	76 ± 24
		Ra	0.78 ± 0.01	1599 ± 135	0.43 ± 0.07	95 ± 14

Table S4. Abundant bacterial OTUs (abundance >1000 reads) explained by bulk soil and their total abundances in each soil type

OTU ID	Taxa	Old-field	Remnant
OTU_238	Acidimicrobiales	837	379
OTU_85	Acidobacteriaceae	1665	2815
OTU_34	*Bacillus*	2381	7120
OTU_4	*Bacillus*	9234	36437
OTU_172	*Balneimonas*	9144	4949
OTU_6992	Bradyrhizobiaceae	920	426
OTU_269	*Burkholderia*	500	773
OTU_43	Burkholderiaceae	3312	5255
OTU_97	*Candidatus Koribacter*	726	1480
OTU_204	Chitinophagaceae	851	361
OTU_80	Chitinophagaceae	2449	1234
OTU_198	Cytophagaceae	882	217
OTU_316	Cytophagaceae	732	320
OTU_71	Cytophagaceae	2216	834
OTU_83	Ellin5290	3209	950
OTU_130	Ellin5301	1634	616
OTU_146	Ellin5301	2865	1297
OTU_149	Ellin5301	1002	384
OTU_3243	Ellin5301	1884	338
OTU_35	Ellin5301	6102	2437

OTU_594	Ellin5301	730	357
OTU_82	Ellin5301	3933	2768
OTU_1636	Ellin6067	1269	652
OTU_451	Gaiellaceae	1336	2041
OTU_23	Gemmatimonadetes	6782	2370
OTU_231	Haliangiaceae	1605	772
OTU_294	Haliangiaceae	1009	347
OTU_473	Haliangiaceae	1160	414
OTU_118	*Lysobacter*	3015	1362
OTU_152	*Lysobacter*	1027	172
OTU_50	*Lysobacter*	2657	196
OTU_95	*Lysobacter*	1566	405
OTU_605	*Mycobacterium*	594	1036
OTU_70	*Mycobacterium*	1763	2829
OTU_107	Myxococcales	1350	573
OTU_158	Myxococcales	2780	681
OTU_174	Nocardioidaceae	1316	632
OTU_387	*Opitutus*	1470	673
OTU_66	*Paenibacillus*	2087	3897
OTU_292	*Paenibacillus chondroitinus*	457	939
OTU_6335	*Phenylobacterium*	593	1018
OTU_51	*Ramlibacter*	3517	1590
OTU_1	*Streptomyces*	148062	98329
OTU_134	Xanthomonadaceae	1737	1066
OTU_2366	Xanthomonadaceae	3016	1354
OTU_26	Xanthomonadaceae	6761	2528
OTU_40	Xanthomonadaceae	7965	2499

Table S5. Abundant bacterial OTUs (abundance >1000 reads) explained by inoculant source and their total abundances in each inoculant

OTU ID	Taxa	Old-field inoculant	Remnant inoculant
OTU_101	Acetobacteraceae	2738	987
OTU_217	Acetobacteraceae	654	1665
OTU_367	Acetobacteraceae	650	1532
OTU_171	Acidobacteriaceae	1054	1862
OTU_85	Acidobacteriaceae	1341	3139
OTU_160	Actinomycetales	2899	1829
OTU_103	*Agromyces*	1733	337
OTU_470	AKIW781	897	178
OTU_236	*Asticcacaulis*	262	800
OTU_172	*Balneimonas*	9788	4305
OTU_41	*Balneimonas*	2296	3924

OTU_3	*Bradyrhizobium*	19081	28599
OTU_148	*Burkholderia*	42	1809
OTU_892	*Burkholderia*	29	1220
OTU_43	Burkholderiaceae	5572	2995
OTU_986	Burkholderiaceae	234	2113
OTU_253	C0119	339	1091
OTU_210	C111	1370	491
OTU_743	*Candidatus Solibacter*	689	1651
OTU_89	Caulobacteraceae	647	2200
OTU_123	Chitinophagaceae	98	1291
OTU_202	Chitinophagaceae	1309	387
OTU_315	Chitinophagaceae	1212	367
OTU_77	Chitinophagaceae	2634	1433
OTU_905	Chitinophagaceae	1313	754
OTU_126	Comamonadaceae	1195	1913
OTU_532	Comamonadaceae	1499	393
OTU_175	Conexibacteraceae	1298	616
OTU_1877	Conexibacteraceae	1237	747
OTU_2256	Cupriavidus	93	1549
OTU_53	DS-18	3151	135
OTU_1091	DS-19	943	124
OTU_213	Ellin5290	119	1276
OTU_86	Ellin5290	2268	977
OTU_32	Ellin5301	6829	1198
OTU_690	Ellin5301	345	937
OTU_82	Ellin5301	5198	1503
OTU_116	Ellin5302	2248	12
OTU_240	Ellin5303	1186	412
OTU_87	Ellin5304	411	2180
OTU_35	Ellin5305	2126	6413
OTU_149	Ellin5306	1259	127
OTU_1636	Ellin6067	1586	335
OTU_4600	Ellin6067	2483	1276
OTU_7509	Ellin6068	840	239
OTU_62	Ellin6069	1191	2607
OTU_766	Ellin6075	2099	1199
OTU_2007	Ellin6529	1663	490
OTU_242	Ellin6529	2035	984
OTU_196	Ellin6530	908	319
OTU_350	Ellin6531	991	267
OTU_110	Ellin6532	1726	649
OTU_58	Erythrobacteraceae	1687	2953
OTU_246	*Flavisolibacter*	1407	238
OTU_464	*Flavisolibacter*	724	1347
OTU_168	Gaiellaceae	5197	2085

OTU_256	Gaiellaceae	1506	178
OTU_345	Gaiellaceae	1002	408
OTU_144	Gemmatimonadetes	2853	453
OTU_194	Gemmatimonadetes	775	261
OTU_143	*Geodermatophilus*	6107	2498
OTU_275	iii1-15	1676	402
OTU_322	iii1-15	1346	644
OTU_422	iii1-15	899	211
OTU_4043	Intrasporangiaceae	557	994
OTU_42	Intrasporangiaceae	6774	841
OTU_49	JG30-KF-CM45	4321	535
OTU_111	JG30-KF-CM46	1757	109
OTU_375	JG30-KF-CM47	1281	493
OTU_108	JG30-KF-CM48	1409	514
OTU_4173	*Kribbella*	2377	832
OTU_223	*Labrys*	547	926
OTU_188	*Legionella*	1034	197
OTU_118	*Lysobacter*	1062	3315
OTU_425	*Methylobacterium*	1927	1207
OTU_44	*Methylobacterium*	1242	2633
OTU_221	Micromonosporaceae	54	987
OTU_559	Micromonosporaceae	914	204
OTU_112	*Mycobacterium*	1251	3089
OTU_605	*Mycobacterium*	373	1257
OTU_107	Myxococcales	1505	418
OTU_1656	Nocardioidaceae	1604	237
OTU_55	Nocardioides	5517	2090
OTU_140	*OR-59*	1244	220
OTU_371	Oxalobacteraceae	812	308
OTU_46	Oxalobacteraceae	2070	10558
OTU_292	*Paenibacillus chondroitinus*	327	1069
OTU_69	*Pedobacter*	1682	5965
OTU_6335	*Phenylobacterium*	357	1254
OTU_334	*Pseudonocardia*	389	927
OTU_212	Pseudonocardiaceae	229	1432
OTU_14	*Ramlibacter*	2994	9684
OTU_109	Rhizobiaceae	1272	2424
OTU_37	Rhizobiales	4157	7577
OTU_131	Rhodospirillaceae	451	2177
OTU_64	Rhodospirillaceae	537	3658
OTU_113	*Rubrobacter*	1936	399
OTU_7225	*Rubrobacter*	2326	564
OTU_79	*Rubrobacter*	7567	738
OTU_280	SC-I-84	947	142
OTU_288	*Skermanella*	1178	133

OTU_257	Solibacteraceae	823	3088
OTU_211	Solirubrobacterales	1396	406
OTU_2148	Solirubrobacterales	963	42
OTU_5158	Solirubrobacterales	767	402
OTU_611	Solirubrobacterales	1808	521
OTU_117	Sphingobacteriaceae	16	1332
OTU_121	Sphingobacteriaceae	1967	444
OTU_2419	Sphingobacteriaceae	10266	806
OTU_290	Sphingobacteriaceae	114	1178
OTU_967	Sphingobacteriaceae	303	756
OTU_3822	*Sphingomonas yabuuchiae*	928	1723
OTU_225	Spirobacillales	133	1678
OTU_227	Sva0725	1118	68
OTU_127	TM7-1	1363	188
OTU_141	TM7-1	1256	106
OTU_170	TM7-1	912	90
OTU_91	TM7-3	0	1389
OTU_92	TM7-3	1287	245
OTU_6415	*Variovorax paradoxus*	1042	2228
OTU_222	WD2101	986	334
OTU_248	Xanthomonadaceae	1148	96
OTU_216	*Yonghaparkia*	8	1070
OTU_13	*Aeromicrobium*	13740	6334
OTU_52	Gemm-1	2548	793
OTU_527	*Modestobacter*	507	1252
OTU_31	Nocardioidaceae	1919	5237
OTU_528	Nocardioidaceae	798	446
OTU_67	Patulibacteraceae	3695	1602
OTU_99	*Rhodoplanes*	1713	2965
OTU_193	*Rubrobacter*	2977	881
OTU_138	Solibacterales	3318	909
OTU_377	Solibacterales	189	819
OTU_68	Solibacterales	1409	4303

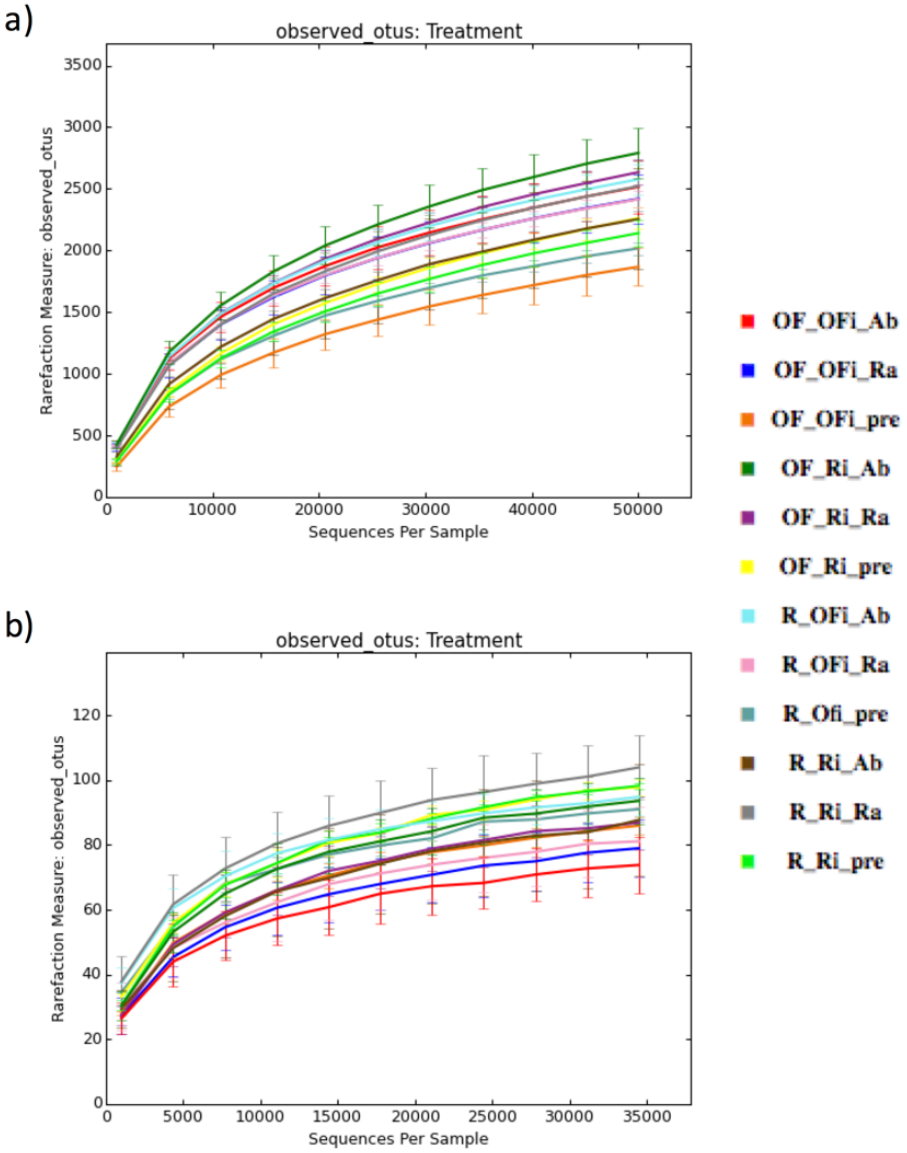


Figure S1. Rarefaction curves for a) bacterial b) fungal communities

Appendix 3. Supplementary material for chapter 4

Table S1. Results of generalised linear model for seedling emergence including all possible factors, weeding treatment (control, burn, scalp and slash), carbon addition (add C and no C) and time (days). Carbon addition was excluded from the final model as it was not influence seedling emergence (see Table 2 for results). Significant (P <0.05) factors are shown in bold (N = 168).

Factor	df	P value
Intercept	1	**<0.01**
Weeding treatment (WT)	3	**0.03**
Carbon addition (CA)	1	0.13
Time (T)	6	**<0.01**
WT x CA	3	0.15
WT x T	18	**<0.01**
CA x T	6	0.85
WT x CA x T	18	1

Table S2. Results of a parametric survival regression model with a Weibull distribution for native seedling survival including all possible factors, weeding treatment (control, burn, scalp and slash), carbon addition (add C and no C). Carbon addition was excluded from the final model as it was not influence seedling emergence (see Table 3 for results). Significant (P < 0.05) factors are shown in bold (N = 367).

Treatment	df	P value
Weeding treatment (WT)	3	**<0.01**
Carbon addition (CA)	1	0.1
WT x CA	3	0.07

Table S3. Mean (± SE) of soil physicochemical properties for each treatment (*n = 6* except for burn *n* = 5).

Weeding treatment	Carbon Addition	Planting method/time*	NH_4^+ -N (mg/kg)	NO_3^- -N (mg/kg)	P Cowell (mg/kg)	K (mg/kg)	Organic C (%)	Conductivity (dS/m)	pH (1:5 $CaCl_2$)
Control	No C	Initial	6.2 ± 0.4	3.2 ± 0.2	33.3 ± 3.9	834.3 ± 19.8	2.8 ± 0.0	0.12 ± 0.01	6.3 ± 0.2
Control	No C	Tubestock	6.2 ± 0.5	10.7 ± 1.1	34.7 ± 3.6	939.0 ± 35.6	2.9 ± 0.05	0.19 ± 0.01	6.3 ± 0.2
Control	No C	Seeding	5.8 ± 0.3	8.7 ± 2.0	31.2 ± 2.0	900.7 ± 14.2	3.0 ± 0.11	0.19 ± 0.03	6.0 ± 0.1
Control	Add C	Initial	6.7 ± 0.6	0.9 ± 0.3	29.7 ± 2.8	826.5 ± 33.8	3.2 ± 0.11	0.16 ± 0.02	6.2 ± 0.2
Control	Add C	Tubestock	7.3 ± 0.6	5.8 ± 0.7	30.5 ± 2.6	965.8 ± 30.2	3.4 ± 0.13	0.16 ± 0.00	6.1 ± 0.2
Control	Add C	Seeding	6.2 ± 0.5	5.5 ± 0.6	29.0 ± 2.9	899.7 ± 20.0	3.5 ± 0.09	0.22 ± 0.01	6.0 ± 0.1
Burn	No C	Initial	8.2 ± 0.5	5.8 ± 1.1	35.8 ± 1.9	937.8 ± 20.0	3.1 ± 0.09	0.12 ± 0.01	6.5 ± 0.1
Burn	No C	Tubestock	6.2 ± 0.2	8.6 ± 0.5	31.8 ± 2.5	1060.0 ± 29.3	3.0 ± 0.14	0.18 ± 0.03	6.6 ± 0.1
Burn	No C	Seeding	4.8 ± 0.4	8.6 ± 1.2	27.0 ± 2.1	873.0 ± 4.9	3.0 ± 0.09	0.17 ± 0.01	6.3 ± 0.1
Burn	Add C	Initial	8.8 ± 1.0	1.3 ± 0.6	27.5 ± 2.7	915.0 ± 36.2	3.5 ± 0.06	0.18 ± 0.03	6.4 ± 0.2
Burn	Add C	Tubestock	7.4 ± 0.2	7.4 ± 1.0	31.2 ± 3.9	996.2 ± 11.8	3.2 ± 0.07	0.19 ± 0.02	6.5 ± 0.2
Burn	Add C	Seeding	6.4 ± 1.0	5.2 ± 1.3	27.6 ± 3.2	927.0 ± 15.3	3.5 ± 0.33	0.17 ± 0.02	6.2 ± 0.1
Scalp	No C	Initial	3.3 ± 0.3	9.3 ± 0.8	18.0 ± 1.1	829.5 ± 5.7	2.2 ± 0.10	0.11 ± 0.01	6.3 ± 0.2
Scalp	No C	Tubestock	5.7 ± 0.3	7.2 ± 0.7	14.3 ± 0.6	829.2 ± 17.0	2.0 ± 0.07	0.14 ± 0.01	6.4 ± 0.2
Scalp	No C	Seeding	3.8 ± 0.5	9.5 ± 0.7	13.7 ± 1.2	820.3 ± 22.9	2.1 ± 0.05	0.11 ± 0.02	6.4 ± 0.2
Scalp	Add C	Initial	5.8 ± 0.5	0.6 ± 0.3	9.0 ± 1.4	883.3 ± 18.3	3.1 ± 0.14	0.24 ± 0.02	5.2 ± 0.3
Scalp	Add C	Tubestock	7.8 ± 0.3	3.8 ± 0.7	13.0 ± 0.9	837.7 ± 18.7	2.5 ± 0.09	0.11 ± 0.01	6.2 ± 0.1
Scalp	Add C	Seeding	14.3 ± 4.4	6.7 ± 0.3	14.7 ± 1.2	764.7 ± 9.9	2.2 ± 0.12	0.13 ± 0.02	6.3 ± 0.1
Slash	No C	Initial	6.8 ± 0.5	5.7 ± 0.9	34.7 ± 2.1	809.5 ± 25.9	3.0 ± 0.07	0.17 ± 0.01	6.5 ± 0.3
Slash	No C	Tubestock	5.5 ± 0.2	8.7 ± 0.7	32.2 ± 2.2	1004.3 ± 21.7	2.8 ± 0.03	0.15 ± 0.01	6.5 ± 0.3
Slash	No C	Seeding	5.8 ± 1.0	8.3 ± 0.9	27.8 ± 3.1	882.3 ± 31.1	3.2 ± 0.11	0.14 ± 0.01	6.3 ± 0.2
Slash	Add C	Initial	7.3 ± 0.5	0.4 ± 0.2	24.2 ± 2.2	849.2 ± 21.8	3.6 ± 0.14	0.23 ± 0.02	6.0 ± 0.3

Slash	Add C	Tubestock	6.2 ± 0.6	4.7 ± 0.4	27.0 ± 2.5	936.0 ± 19.1	3.0 ± 0.15	0.15 ± 0.01	6.4 ± 0.2
Slash	Add C	Seeding	5.3 ± 0.3	4.7 ± 0.8	26.3 ± 1.0	871.8 ± 15.2	3.2 ± 0.14	0.14 ± 0.01	6.4 ± 0.2

* Samples collected initially were taken from the middle of the subplots before plants were added and the tubestock and seeded samples were taken at the time of harvest from the centre of the 1 m^2 halves of each respective subplot

Table S4. P values for ANOVA run separately for the soil properties as response variables (*n = 6* except for burn *n* = 5).

	NH_4^+ -N (mg/kg)	NO_3^- -N (mg/kg)	P Cowell (mg/kg)	K (mg/kg)	Organic C (%)	Conductivity (dS/m)	pH (1:5 $CaCl_2$)
Initial samples							
Weeding treatment (WT)	**<0.001**	**0.017**	**<0.001**	**<0.001**	**<0.001**	**0.010**	**0.049**
Carbon addition (CA)	**0.002**	**<0.001**	**<0.001**	**0.037**	**<0.001**	**<0.001**	**0.006**
WT x CA	**0.010**	**<0.001**	**0.012**	0.362	0.070	**0.028**	0.146
Harvest samples							
WT	0.357	0.3	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.046**
CA	**<0.001**	**<0.001**	0.169	0.198	**<0.001**	**0.032**	0.426
Planting material (PM)	**0.009**	0.853	0.14	**<0.001**	**0.009**	0.542	0.084
WT x CA	**<0.001**	0.584	0.793	0.345	0.07	0.691	0.972
WT x PM	0.748	**0.018**	0.612	**0.028**	0.106	**0.036**	0.461
PM x CA	0.329	0.853	0.246	0.445	0.253	0.167	0.611
WT x PM x CA	**0.044**	0.555	0.892	**0.021**	0.247	0.524	0.994

Table S5. Bacterial OTUs, with abundances greater than 1,000, found in higher abundance in the scalped plots than the controls

Taxa	Function (if known)	References*
Bacillus spp. (OTU_1 and OTU_18)	Genera includes both free-living (nonparasitic) and parasitic pathogenic species	(Turnbull and Bacillus 1996)
Unidentified Gaiellaceae (OTU_21, OTU_123 and OTU_188) Unidentified Solirubrobacterales (OTU_41)	Abundance changes depending on carbon-to-nitrogen ratios	(Hermans *et al.* 2017)
Unidentified Oxalobacteraceae (OTU_241)	Gram-negative, highly abundant on AMF hyphae	(Scheublin *et al.* 2010)

***References for Table S5**

Hermans S. M., Buckley H. L., Case B. S., Curran-Cournane F., Taylor M. & Lear G. (2017) Bacteria as Emerging Indicators of Soil Condition. *Applied and Environmental Microbiology* **83**, 13.

Scheublin T. R., Sanders I. R., Keel C. & van der Meer J. R. (2010) Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi. *Isme Journal* **4**, 752-63.

Turnbull P. & Bacillus S. B. (1996) Barron's Medical Microbiology. University of Texas Medical Branch, Galveston.

Methods of pilot vegetation survey

In spring of 2014, a vegetation survey was carried out at Para Woodlands Reserve to determine the dominant species at the field site. Fifteen 1 m^2 quadrats were randomly placed within a 100 x 100 m plot. The percent cover of each species was determined by counting the presence in 100 cells (10 cm x 10 cm).

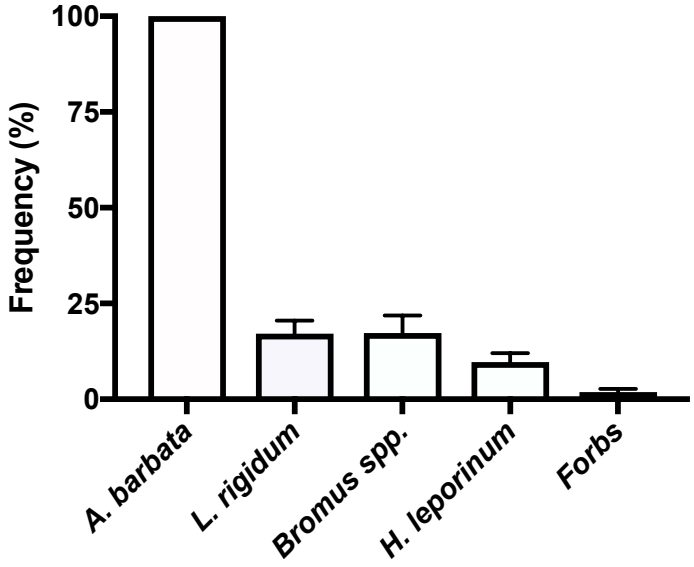


Figure S1. Mean (± SE) percent cover of plant species during a pilot study at the field site

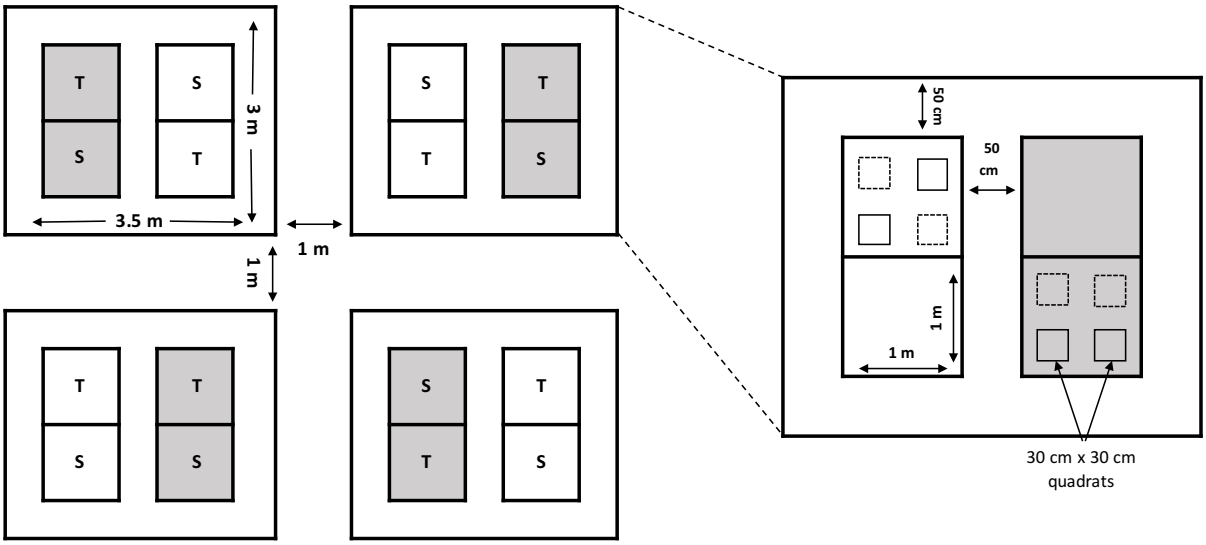


Figure S2. Example layout of four (out of 24) experimental plots which were randomly assigned as a control, burnt, slash or scalped treatment. Subplots within these received either a carbon addition (shaded) or were left bare (white). The sub plots were split in two with one side receiving native seeds (S) and the other receiving native tubestock (T) plants. The inset is a close up of one plot to demonstrate that the subplots were separated by a 50 cm buffer and seeds were counted in two out of four possible quadrats.

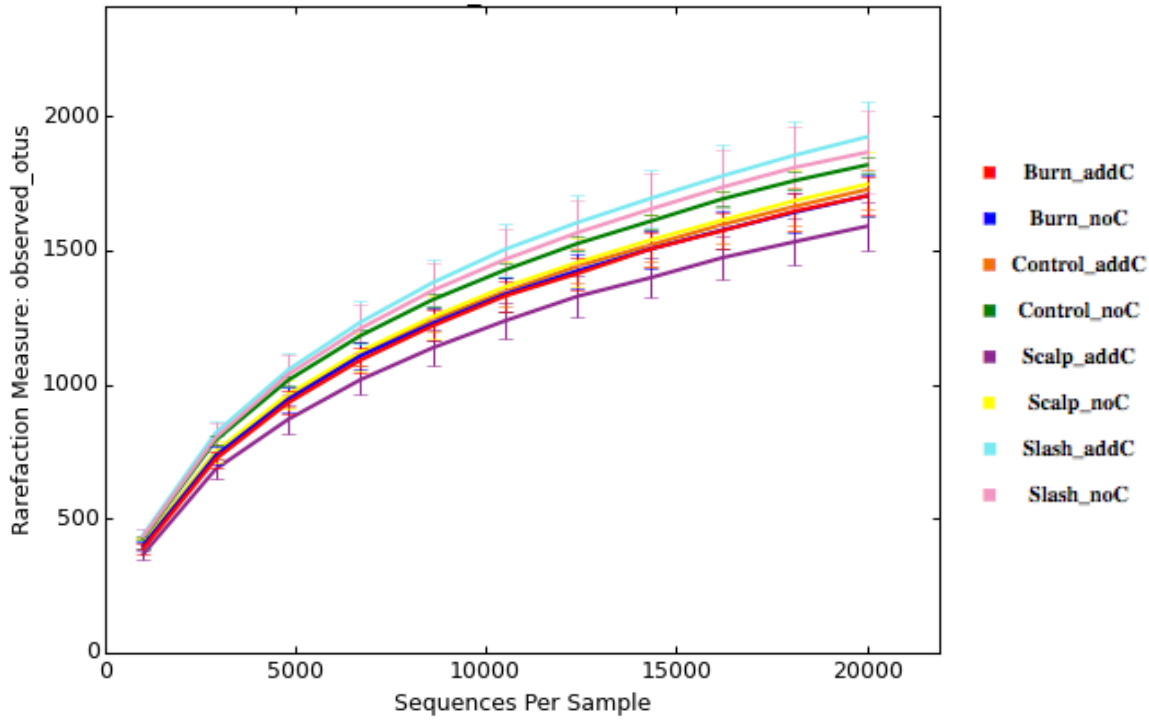


Figure S3. Rarefaction curves for bacterial OTU richness for each treatment combination

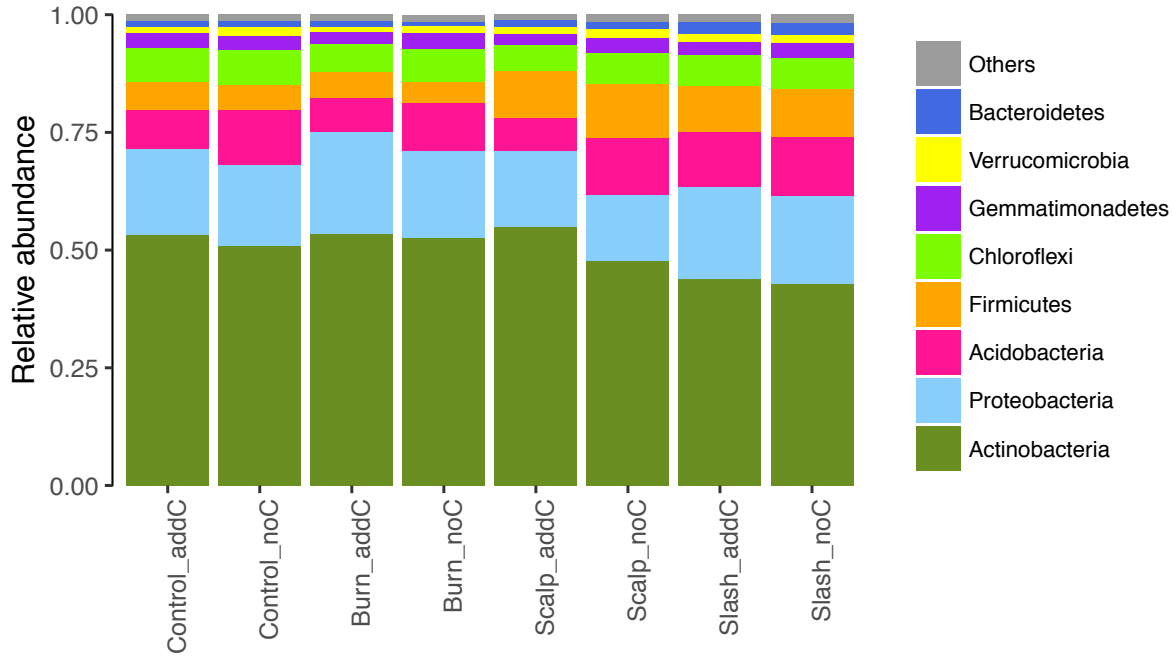


Figure S4. The relative abundance of the bacterial phyla and rare phyla (< 1.5 % reads) represented as 'others'. Weeding treatments and carbon addition combinations are represented on the x-axis.

Appendix 4. Supplementary material for chapter 5

Methods of vegetation survey

In spring of 2014 (a year before planting) a vegetation survey was carried out at Para Woodlands Reserve to determine the dominant species at the field site. Fifteen 1 m^2 quadrats were randomly placed within a 100 x 100 m plot. The percent cover of each species was determined by counting the presence in 100 cells (10 cm x 10 cm).

Table S1. Mean (± SD) percent cover of plant species during a pilot study at the field site

Avena barbata	*Lolium rigidum*	*Bromus sp.*	*Hordeum leporinum*	*Raphanus raphanistrum*	*Medicago sp.*	*Kickxia spuria*
100 ± 0	17 ± 13	17 ± 18	10 ± 9	1 ± 2	1 ± 1	1 ± 3

Methods for pre-planting soil sampling

The samples were collected from six locations within each block then homogenised before three samples were used for analysis. Physiochemical analysis was carried out at CSBP laboratories (Bibra Lake, WA) to measure nitrate nitrogen, ammonium nitrogen, plant-available (Colwell) phosphorus, potassium (Colwell), organic carbon, conductivity and pH ($CaCl_2$).

Table S2. Mean (± SD) of soil physicochemical properties for the experimental blocks (*n* = 3) at the time of planting.

Soil Physiochemical Properties	1	2	3	4	5	6
NH4+ -N (mg/kg)	5.7 ± 0.6^{ab}	4.3 ± 0.6^{a}	4.7 ± 0.6^{ab}	5.3 ± 0.6^{ab}	4.3 ± 0.6^{a}	6.0 ± 0.0^{b}
NO3- -N (mg/kg)	4.3 ± 0.6^{a}	5.0 ± 0.0^{ab}	6.7 ± 0.6^{c}	5.3 ± 0.6	4.0 ± 0.0^{a}	5.0 ± 0.0^{ab}
P Cowell (mg/kg)	41.7 ± 3.2^{b}	28.7 ± 2.1^{a}	37.7 ± 1.5^{ab}	39.0 ± 5.2^{b}	42.0 ± 4.4^{b}	36.0 ± 4.6^{ab}
K (mg/kg)	885.3 ± 107.9	890.7 ± 66.3	832.3 ± 38.4	778.0 ± 62.2	818.3 ± 23.0	760.3 ± 14.6
Organic C (%)	3.1 ± 0.2^{ab}	2.9 ± 0.1^{a}	3.0 ± 0.2^{ab}	3.3 ± 0.2^{b}	3.0 ± 0.0^{ab}	3.3 ± 0.2^{b}
Conductivity (dS/m)	0.07 ± 0.01^{a}	0.10 ± 0.02^{ab}	0.16 ± 0.01^{e}	0.13 ± 0.01^{cd}	0.12 ± 0.00^{bc}	0.15 ± 0.00^{de}
pH (1:5 CaCl2)	6.3 ± 0.1^{a}	6.9 ± 0.1^{b}	7.3 ± 0.1^{c}	7.5 ± 0.1^{c}	7.0 ± 0.2^{b}	7.5 ± 0.1^{c}

Appendix 5. Photos of experiments

Chapter 2

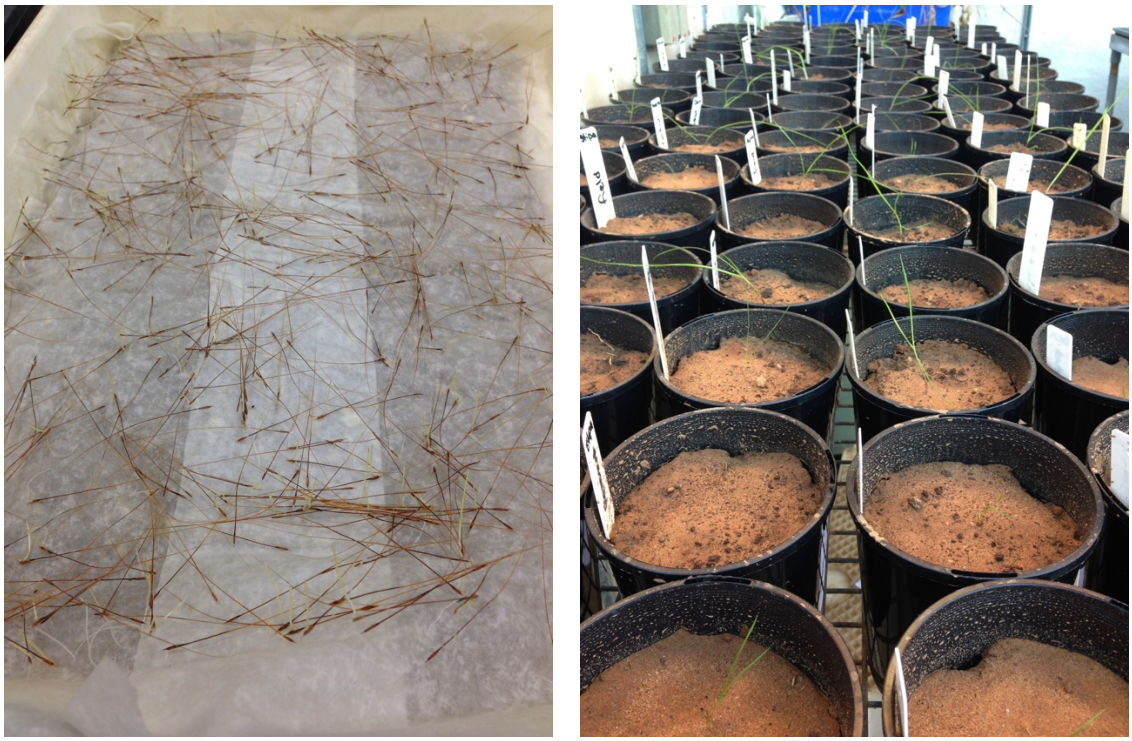


Figure A5.1 Seeds were pre-germinated in a temperature and light controlled germination cabinet, on trays of vermiculate and paper towel, before being planted into pots and placed in the glasshouse.

Chapter 3

Figure A5.2 Glasshouse setup and the lab at the *Avena* harvest 2015.

Chapter 4

Figure A5.3 Examples of each weeding treatment, slashing (top-left) burning (top-right), scalping (bottom-left), control (bottom-right)

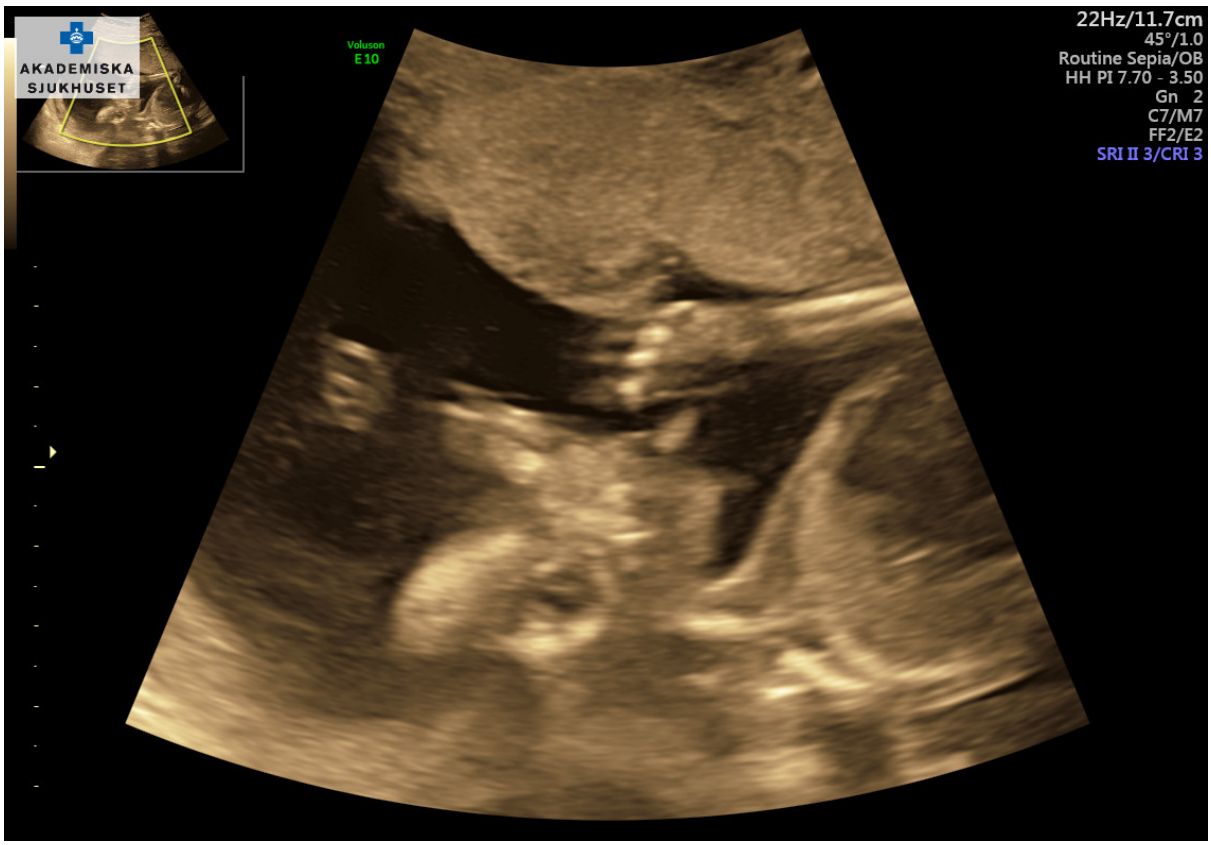
Figure A5.4 Seedling emergence and mortality was recorded regularly, top = scalped plots, bottom left = slashed plot, bottom right = control plot

Chapter 5

Figure A5.5 High density single-C3 (left) and single-C4 (right) plots

Figure A5.6 Low density single-C3 plots at the time of harvest in unweeded (left) and weeded (right) plots

Appendix 6. The next chapter



Baby Christmas due 15th March 2018

Uppsala, Sweden