The University Of Sheffield.

Phosphorus (P) partitioning among co-occurring plants: competition for P acquisition across different forms of P and through soil microbes

By:

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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Sheffield

Faculty of Science

Department of Animal and Plant Sciences

Abstract

Soils of low phosphorus (P) availability are found globally, and often have high plant species richness despite co-existing species competing for the same limited P resource. How diverse communities maintain co-existence despite limited access to this resource is poorly understood.

This thesis investigated plant P acquisition in P-limited calcareous grasslands and the mechanisms which may sustain species coexistence in these plant communities. For this, a range of calcareous grassland species were used which varied in their methods of P acquisition. Using radiolabelled P-sources, it was shown that interspecific differences in P uptake by plants across a range of chemical P-forms were consistent with contrasting methods of P acquisition. Species with specialist rooting structures and high rates of root exudation acquired the greatest amounts of P from sources which require mobilisation before uptake. This included organic diesters (DNA) and inorganic mineral P (calcium phosphate). However, these species showed consistent reductions in P uptake in response to competition from mycorrhizal species, which maintained or increased P uptake. Comparisons of these competitive interactions in controlled systems showed that this competitive effect was determined by mycorrhizal status.

P uptake by soil microbial communities growing under different plant species monocultures and mixed plant communities was measured from radiolabelled calcium phosphate. While there was some variation in microbial P uptake across plant species monocultures, this did not relate to differences in plant uptake. Microbial P uptake increased significantly in mixed plant communities compared to

monocultures, highlighting the importance of species richness on mobilisation of P from calcium phosphate through microbial uptake.

These findings provide a new perspective on ecological processes which sustain species richness in P-limited plant communities. Given the prevalence of P-limitation throughout terrestrial ecosystems, this could have widespread relevance for improving our understanding of the mechanisms which shape community structure and function.

Acknowledgements

As I write this, I feel humbled to look back at all of the help, kindness and support I have received over the course of my PhD. Firstly I would like to express my appreciation for the dedicated support and guidance I have been given from my supervisors Dr Gareth Phoenix and Professor Duncan Cameron. I would also like to acknowledge the funding provided for this project by NERC and the University of Sheffield, without which this work would not have been possible.

I would like to thank the technical staff in the department, especially Dr Dave Johnson and Irene Johnson, who have provided a helping hand throughout my time in the lab. I would also like to thank Professor Sir David Read, for the time he has spared for advice and discussions, and Professor Jonathan Leake, for technical guidance and constructive criticism.

I would like to thank Alex Williams, Dr Anne Cotton and Alex MacFarlane for providing their time and effort to train me in microbial fingerprinting methods. I would also like to thank Rachel Thorley, Despina Berdeni and Tom Thirkell for tips on experimental techniques and passing on protocols.

There are many other brilliant students and staff that have helped me along the way including, but not limited to: Dr Katie Field, Andy McNally, Sam Amsbury, Robert Caine, Dr Steffi Tille, Dr Chris Hepworth, Dr Heather Walker, Nicola White, Dr Chandra Bellasio, JJ Potter, Scott Davidson, Ingrid Robertson and Lizzy Parker. I would also like to thank Dr Tom Young and Dr Tom Parker for passing on useful research papers and random YouTube videos in equal measure.

To my family, I would like to thank: my Mum, for all of her support and encouragement; my Dad, for his support sustained through regular phone calls

which coincidentally also kept me informed of goals scored by Leicester City FC; and Mam-Mar, who has always driven me to try my best. Lastly, I would like to thank my wife and best friend Caroline, whose love, patience and support has been a huge help and kept me sane through the hard times.

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Introduction: Maintenance of species rich plant communities in P-limited systems

1.1 Resource acquisition and species coexistence

A major question in plant ecology is how coexistence is maintained within plant communities despite co-occurring species needing to secure the same resources necessary for survival (including light, water and nutrients). A species' resource requirements are a defining characteristic of the conditions necessary to sustain a population in the local environment (i.e. their ecological niche). Similarities in resource requirements between species within a community subsequently results in the overlap of 'niche spaces' (May 1974; Pianka 1974).

Overlapping resource requirements leads to competition when that resource is in short supply and it's availability restricts the growth rate of a species (i.e. a 'limiting' resource) (Tilman 1982). According to classic ecological theories, stable coexistence between co-occurring species can only occur below a threshold amount of overlap in requirements of limiting resources (May & MacArthur 1972). However, given the broad similarities in the resource requirements of plants, co-occurring species will inevitably be forced to compete for the same limiting resources. Therefore differences in resource use between species (i.e. niche differentiation) which facilitate coexistence through reduced overlapping resource requirements are of fundamental importance (Silvertown 2004).

Research on coexistence through niche differentiation (i.e. niche complementarity) has focused on resource partitioning, which describes the process where the

acquisition of a limiting resource is divided up within a plant community through interspecific differences in the space, time, and form of acquisition. In theory, this process will promote coexistence among co-occurring species by allowing multiple species to share a limiting resource (Schoener 1974). Indeed, previous studies have already demonstrated this process in the partitioning of resources such as light and water (Kobe 1999; Nippert & Knapp 2007; Kulmatiski & Beard 2013).

Niche differentiation among co-occurring species could also occur through changes in resource acquisition which reduce overlapping niche space (i.e. niche plasticity) (Casper & Jackson 1997). This theory makes a distinction between a species' fundamental and realised niche. The former represents its niche in the absence of interspecific competition whereas the latter occurs in response to interspecific competition (Hutchinson 1957). Accordingly, niche plasticity could facilitate coexistence between species which, despite sharing overlapping resource requirements in their fundamental niche, differentiate in their realised niche.

While our understanding of the ecological processes which govern plant community structure and function is supported by an abundance of research carried out over many years, the majority of this has focused aboveground. Far less progress has been made in understanding the importance of belowground processes on resource acquisition and their influence on plant community structure and function (Schenk 2006; Bardgett & van der Putten 2014).

1.2 Phosphorus availability and species rich plant communities

Belowground, a key factor that regulates plant community structure and function is the availability of nutrients. Many of the world's biodiversity hotspots are located in nutrient-poor habitats - from the highly weathered soils of tropical rainforests to the

ancient soils of Western Australia (Myers et al. 2000). The inverse relationship between nutrient availability and species richness is well documented in plant communities limited by phosphorus (P) (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Ceulemans et al. 2014). Furthermore, these plant communities have also been shown to contain a greater frequency of rare species (Wassen et al. 2005), which are more vulnerable to global change due to reduced investment in sexual reproduction (Fujita et al. 2014). P limitation is widespread throughout terrestrial ecosystems and approaches the extent of nitrogen limitation (Elser et al. 2007). Therefore the understanding of how so many species manage to coexist in P-limited systems is of great importance.

1.3 Phosphorus dynamics in the soil

Phosphorus is essential to all forms of life. It is a vital component of genetic material, found in the phospholipids which make up all cell membranes, and a key constituent of ATP which fuels the vast majority of processes that require energy transfer (Bowler et al. 2010).

Plants acquire P from the soil in the form of orthophosphate, either as $H_2PO_4^-$ or HPO_4^{2-}. This is taken up by the plant via transporters located in the root epidermis (Smith 2002). During P uptake, a diffusion gradient is established which draws orthophosphate and a range of other diffusion-mobile nutrients in solute form to the root surface (Hinsinger et al. 2009). However, the high reactivity of orthophosphate restricts its mobility in the soil which leads to depletion zones developing at the root surface relative to the surrounding substrate (Hinsinger 2001). The incorporation of P in soil solution to biomass and its adherence to other soil constituents means that phosphorus in a plant-available form makes up only a small fraction of the total

amount present in the soil, rarely reaching levels of more than 10 µM (Bieleski 1973).

Different soil P sources are classed as organic or inorganic depending on which soil constituents P is adhered to (Fig 1.1; Hinsinger et al. 2011). Organic forms include phytate, phospholipids and nucleic acids, and account for around 30-65% of total soil P (Harrison 1987). The most abundant forms of soil organic P are phosphomonoesters, which consist largely of phytins such as inositol hexaphosphates (Turner et al. 2002). The strong adherence of P within monoesters resists degradation, and this stability leads to their accumulation in the soil.

Phosphodiesters represent a more transient component of the soil P cycle than monoesters. Diesters are the most abundant source of P in biological tissue (Bieleski 1973), and levels of this organic P form in the soil are maintained through contributions from the turnover of soil organisms and plant litter. Organically bound P in this form (such as nucleic acids and phospholipids) is weakly sorbed and rapidly decomposes, preventing the build-up of this P source in the soil (Bowman & Cole 1978).

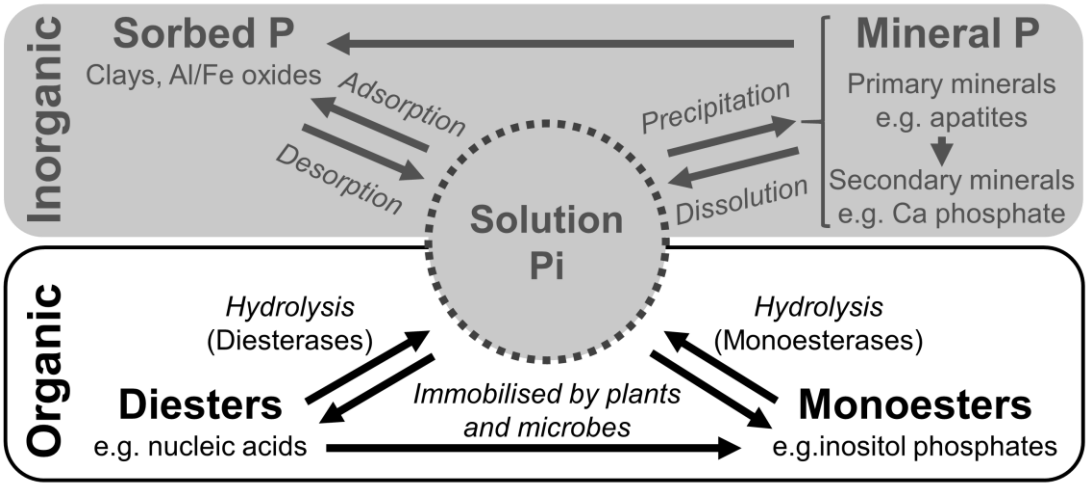

Figure 1.1: Schematic of P dynamics in the soil (adapted from Shen et al. 2011)

Inorganic phosphorus in the soil is found in a range of forms. Primary minerals, such as apatites, are derived from the bedrock and are relatively stable compared to secondary minerals, which form through the precipitation of P to metals including calcium, iron and aluminium (Shen et al. 2011). As well as precipitation reactions, P can also be incorporated into inorganic P forms through adsorption to the surface of clays and aluminium and iron oxides (Hinsinger 2001).

Walker and Syers (1976) developed a model which described the process of soil P cycling over time. In essence, all soil P originates from inorganic primary mineral-bound forms such as calcium apatite. This phosphorus is gradually released into soil solution, where it is either immobilised by plants and soil organisms or adsorbed to other soil constituents. The constant turnover of biota maintains P cycling between organic and inorganic pools (Fig 1.1).

1.4 Plant acquisition of phosphorus

Since plants only acquire P in the form of orthophosphate, a continuous turnover into the soil solution is required for plant growth. Plants can influence this process through a variety of mechanisms.

1.4.1 Root exudation

The root-release of organic acids (e.g. oxalate and citrate) mobilises P from inorganic sources such as calcium phosphate (Jones & Darrah 1994). These low molecular weight chemical compounds act in a number of ways. P mobilisation can occur through the exchange of organic acids with P at the ligand exchange surfaces of metal ions in the soil (Jones 1998). Likewise, complexation of organic acids to

metal ions in the soil's solid phase (such as aluminium, iron and calcium) restricts further immobilisation of P, maintaining a greater amount in solution and available for plant uptake (Parfitt 1978). However, the action of organic acids is highly dependent on conditions in the soil. It has been suggested that the plant production of these compounds is insufficient to reach amounts which have a significant effect on the mobilisation of P through mineral weathering (Drever & Stillings 1997; Ström et al. 2005). Therefore, further focus on their function in natural and semi-natural soils is needed in order to understand their involvement in soil P cycling (Jones et al. 2003; Shen et al. 2011; Duffner et al. 2012)

Plants can also access soil P through the root exudation of extracellular enzymes specific to a range of organic P sources. Phosphorus is mobilised from phosphomonoesters (such as inositol phosphates and phytins) through hydrolysis by phosphomonoesterases (Lee 1988), which are produced in plant roots in response to low P concentrations (Duff et al. 1994). Likewise, the phosphate within phosphodiesters (such as nucleic acids and phospholipids) is released through hydrolysis with phosphodiesterases. While the activity of plant phosphatases in organic phosphorus mineralisation is well documented (Hui et al. 2013), the contribution of various organic P sources to plant P nutrition is poorly understood (Steffens et al. 2010).

1.4.2 Root architecture

Once P is in a form that is accessible to plants, there are a range of plant adaptations which enhance its acquisition. Orthophosphate is taken up via transporters located in the root epidermis (Smith 2002). Subcellular extensions of root epidermal cells (root hairs), are positively related to the acquisition of phosphorus (Itoh & Barber 1983)

and these structures increase P uptake by accessing a larger soil volume and increasing the surface area for plant acquisition. The close involvement of these structures in plant P nutrition has been demonstrated by studies using *Arabidopsis* which have shown that the length and density of root hairs is regulated by the availability of P (Bates & Lynch, 1996; Ma et al. 2001).

Within the plant kingdom, a range of species produce specialised root structures for the acquisition of P consisting of bunched determinate root hairs condensed around distinct points along the root axis. These 'cluster roots' can be found in a number of plant clades - from the proteoid roots found in woody species of Proteaceae (Watt & Evans 1999) to the dauciform roots found in the sedges of Cyperaceae (Fig 1.2; Shane et al. 2004). The production of these cluster roots is greatest under low levels of P supply, and they act to enhance P acquisition by increasing the surface area for nutrient uptake as well as releasing high concentrations of organic acids and phosphatases (Shane & Lambers 2005; Playsted et al. 2006).

1.4.3 Soil microbes

(i) *Mycorrhiza*

Almost all plant species are capable of acquiring P through associations with mycorrhizal fungi. This important group of soil microbes increases the surface area for nutrient acquisition and accesses a larger soil volume through a dense hyphal network which extends beyond the zones of depletion surrounding plant roots and within soil particles that the broader roots of plants cannot access (Smith & Read, 2008). Phosphorus acquired by the fungus is transported through their hyphal network to the host plant, which provides assimilated carbon in return. While this is the characteristic function of the symbiosis between mycorrhizal fungi and plants,

these interactions vary widely within the plant kingdom and across a range of mycorrhizal types. The most common form of this symbiosis is with arbuscular mycorrhizal (AM) fungi, which form associations with the majority of plant species (c. 80%). This type of mycorrhizal fungi are characterised by the formation of arbuscules within the cortical cells of plant roots and these are the structures through which P is transferred to the host plant (Fig 1.3).

The ways in which associations with AM fungi can benefit the P nutrition of host plants are well documented. AM fungi can contribute up to 80% of plant P (Marschner & Dell 1994; Van Der Heijden et al. 2006) and mycorrhizal roots show greater efficiency for phosphorus acquisition compared to non-mycorrhizal roots which have access to equal amounts of soil P (Cui & Caldwell 1996).

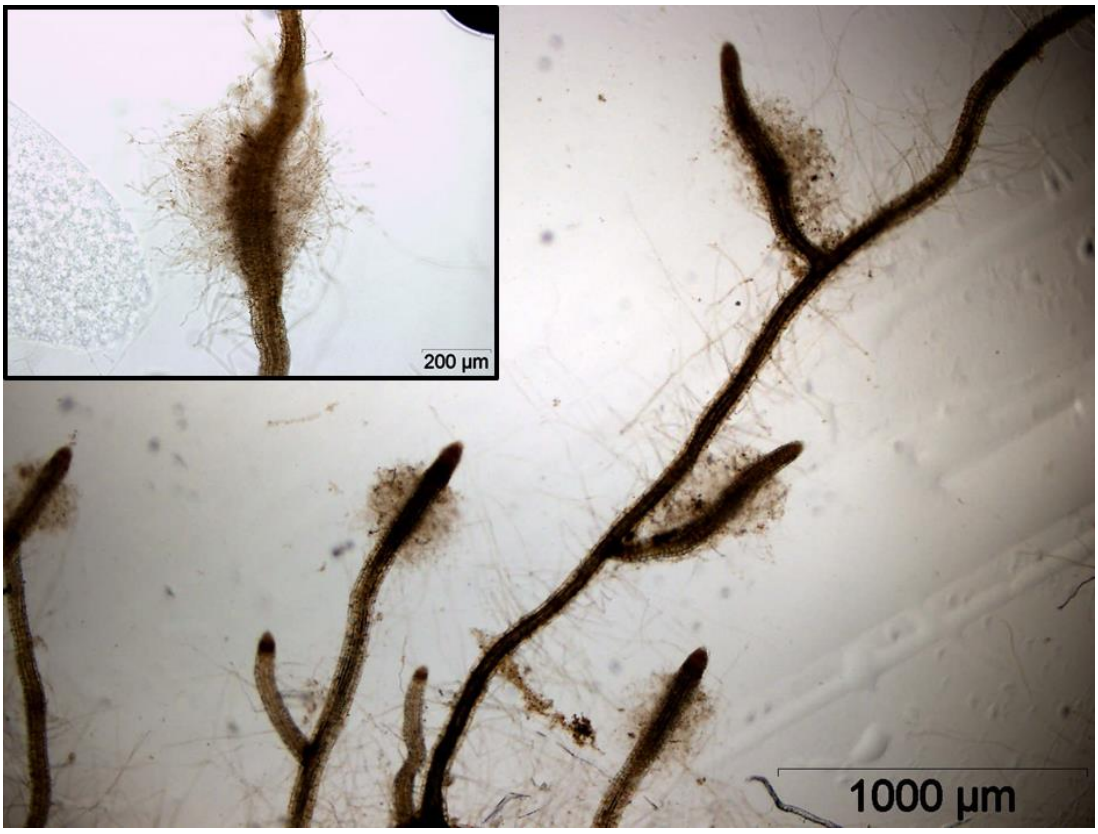

Figure 1.2: Section of root system of the sedge species *Carex caryophyllea* grown on calcareous dune sand. Some lateral roots have developed dauciform roots. Inset: higher magnification of a dauciform root showing densely packed root hairs.

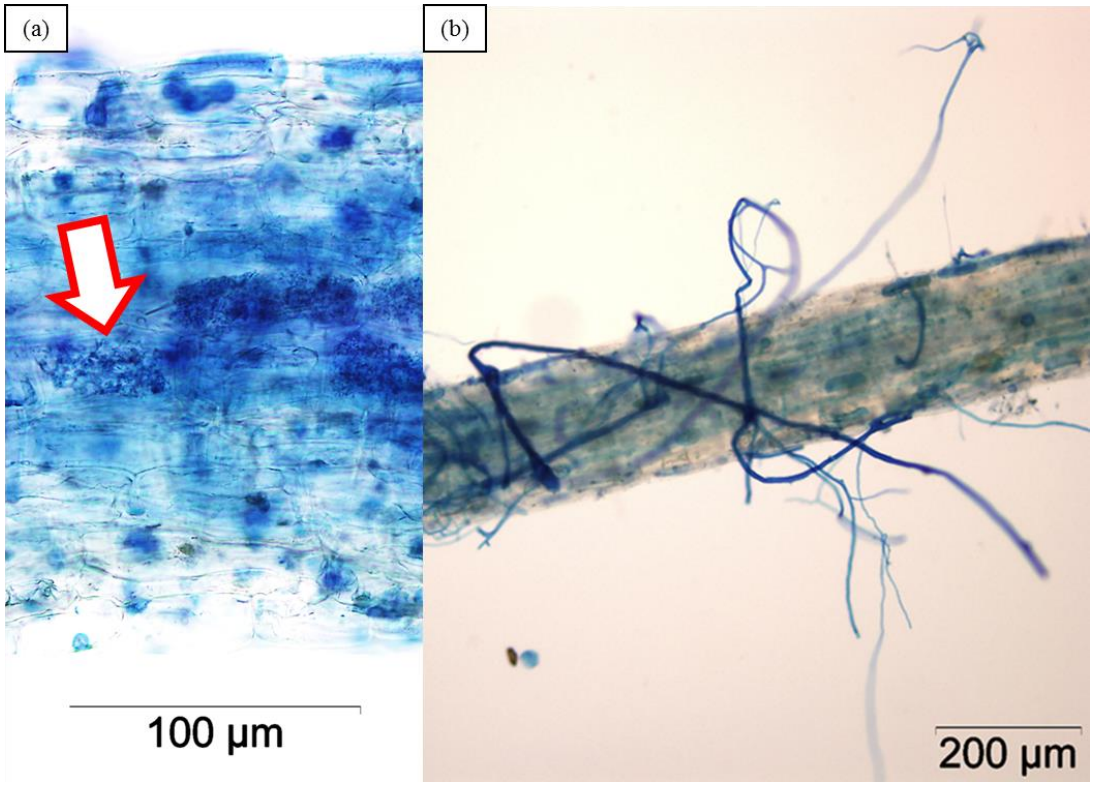

Figure 1.3: Section of *Plantago lanceolata* root system stained using Trypan blue to show colonisation by mycorrhizal fungi. (a) Magnified root section showing arbuscules within the cortical root cells (arrow). (b) Section of root surrounded by external fungal hyphae.

(ii) *Plant growth promoting rhizobacteria*

Other groups of soil microbes also affect plant P acquisition. Phosphate-solubilising bacteria are capable of increasing the availability of P from inorganic and organic sources through the secretion of phosphatases and organic acids (Kim et al. 1998). These bacteria, and other groups which can increase plant productivity, are collectively referred to as plant growth promoting rhizobacteria.

P-solubilising ability has been demonstrated experimentally in bacterial species belonging to a range of genera, including *Actinomycetes*, *Pseudomonas*, and *Bacillus* (Richardson & Simpson 2011). However, there remains uncertainty over the P-solubilising capacity of groups which have been identified *in vitro* without

further testing in field conditions (Gyaneshwar et al. 2002). Likewise, while studies carried out in controlled conditions have demonstrated the beneficial effect of phosphate-solubilising bacteria on plant P nutrition, evidence of this from field studies is not resolved (Pii et al. 2015).

The impact of soil microbes on ecosystem functioning was considered a 'black box' for much of the 20th century (Tiedje et al. 1999). Recent progress in the use of molecular approaches has helped to unravel the complexity of belowground processes involving soil microbes (Bardgett & van der Putten 2014). A focused research effort using these novel methods will help to advance our understanding of the structure and function of soil microbial communities and their effect on plant P nutrition *in situ*.

1.4.4 Plant-microbe interactions and P acquisition

These highlighted mechanisms of plant P acquisition are not isolated from one another. In the soil environment, these processes often interact and the distinction between one method of P acquisition and another can often be hard to distinguish.

For example, while plant root exudates can directly increase the availability of P for plant uptake, these carbon-containing compounds also serve as a substrate for the soil microbial biomass (Baudoin et al., 2003; Lange et al., 2015; Shahzad et al., 2015). Plants in semi-natural systems translocate between 30 and 50% of assimilated carbon below-ground (Kuzyakov & Domanski 2000), and that which is released into the soil can further enhance the mobilisation of P through stimulating soil microbial activity (Hacker et al. 2015).

As well as organic acids and phosphatases, plant root exudates contain a range of rhizodeposits, including mucilage, sugars and amino acids (Dakora & Phillips

2002). The composition of root exudates can vary greatly among species and across environmental conditions (Mimmo et al. 2011). Studies have shown that soil microbes respond to these variations between different species (Zhang et al. 2014) as well as across different components of the rooting system within the same plant (Marschner et al., 2002).

Studies which have investigated how plant stimulation of soil microbial communities can affect nutrient acquisition have focused on nitrogen (N). The term 'priming' refers to the plant input of organic carbon, such as root exudates, into the soil which accelerates the mineralisation of organic matter by soil microbes and subsequently increases the supply of N to the plant (Dijkstra et al., 2013). Root traits are shown to be an important driver of changes in the microbial biomass which stimulates the cycling of N (Legay et al. 2014), however there has been less progress made in our understanding of the effect of plant-microbe interactions on soil P cycling. Therefore, further research is required to elucidate whether plants possess a similar top-down control over their P supply through soil microbial activity (Dijkstra et al. 2013).

As well as the influence of plant-microbe interactions on plant P acquisition, interactions between different groups of soil microbes could also feedback into plant P acquisition. The colonisation of roots by mycorrhizal fungi leads to changes in bacterial community composition (Vestergård et al. 2008). Likewise, 'mycorrhiza helper' bacteria have been shown to enhance root colonisation by AM fungi (Churchland & Grayston 2014). These findings highlight the complexity of below-ground interactions, and the uncertainty that remains over how these processes impact on soil P cycling and plant uptake.

1.5 Mechanisms through which P acquisition influences plant communities

Despite widespread P limitation across terrestrial ecosystems and its connection to diverse plant communities, the mechanisms which maintain coexistence among species competing for the acquisition of this scarce resource are poorly understood. A number of theories have been proposed to explain this relationship.

1.5.1 Niche differentiation through resource partitioning

Diversity could be maintained in P-limited plant communities by species reducing the negative impact of competitive interactions through accessing different sources of P in form, space or time, known as "resource partitioning" or "niche differentiation in resource use" (Turner 2008). The diverse plant communities sustained in P-limited systems appear to contradict Tilman's theory (1982) that coexistence cannot be maintained among species which compete for the same limiting resource. However, resource partitioning could maintain coexistence, despite the low levels of plant available P, if interspecific competition for this limiting resource is avoided through differences in methods of P acquisition which access different sources of P in the soil (Fig 1.4; Turner 2008).

Different methods of plant P acquisition are well-documented, however few studies have investigated whether they access different sources of soil P. In theory, species which form mycorrhizal associations will have a superior foraging capacity, and therefore acquire a greater amount of freely available P within the soil than non-mycorrhizal plants (Smith & Read 2008). On the other hand, species which invest in the production of specialist root structures and high rates of root exudation are better

equipped for the mobilisation of P from poorly accessible sources (Lambers et al. 2006).

Studies which have investigated resource partitioning of nutrients have focused primarily on nitrogen. For instance, McKane et al. (2002) demonstrated differentiation in the timing, depth and chemical form of N uptake among plant species from a tussock tundra community. In line with findings from other N partitioning studies (Kahmen et al. 2006; Felten et al. 2009), this could facilitate co-existence in these communities through reduced competition for the limiting N supply.

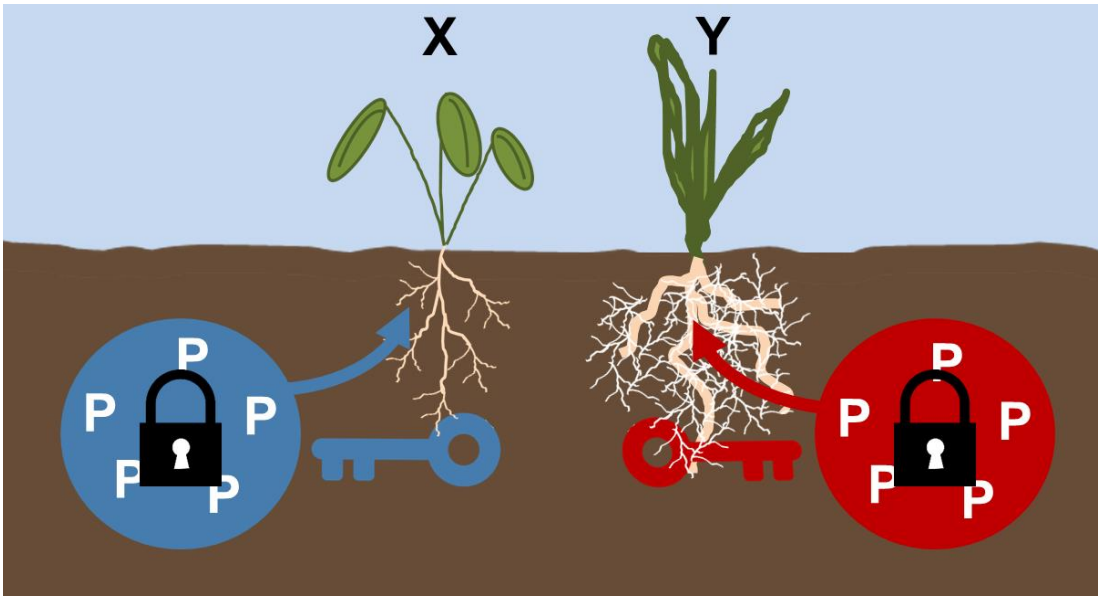

Figure 1.4: Schematic representation of resource partitioning of phosphorus. Different methods of P acquisition are symbolised as 'keys' which unlock P from a source of corresponding colour. For resource partitioning, it is proposed that coexistence is maintained through interspecific differences in resource acquisition. This is illustrated by different coloured keys possessed by species X and Y (blue and red keys respectively) which unlock P from different sources of soil P.

In comparison, there has been little work done to investigate the potential for resource partitioning of P. From the studies which have tested this theory, there is indirect evidence to show that plant species differ in their uptake of P from a range of sources in the soil (Fransson et al. 2003), and P uptake among competing plant species is greatest when supplied with a variety of P sources (Ahmad-Ramli et al. 2013). The first direct evidence of P partitioning came through the use of radioactive tracers that demonstrated interspecific differences in P uptake of contrasting chemical forms of organic, inorganic and mineral P among commonly co-occurring grassland plants (Phoenix et al., unpublished data). An improved understanding of different methods of plant P acquisition will aid future consideration of the capacity for plant communities to partition P sources in the soil.

1.5.2 Enhanced niche differentiation through niche plasticity

Building on the theory of niche differentiation through resource partitioning, niche plasticity proposes that species are capable of shifting niche space in response to neighbouring species in order to reduce interspecific competition (Fig 1.5). These shifts could occur in at least two ways depending on the plasticity of dominant or subordinate plant species. That is, subordinate species could shift to a less-used resource in response to a superior species (niche pre-emption) or dominant species maintain competitive dominance by switching between resources (dominant plasticity) (McNaughton & Wolf 1970; May & MacArthur 1972).

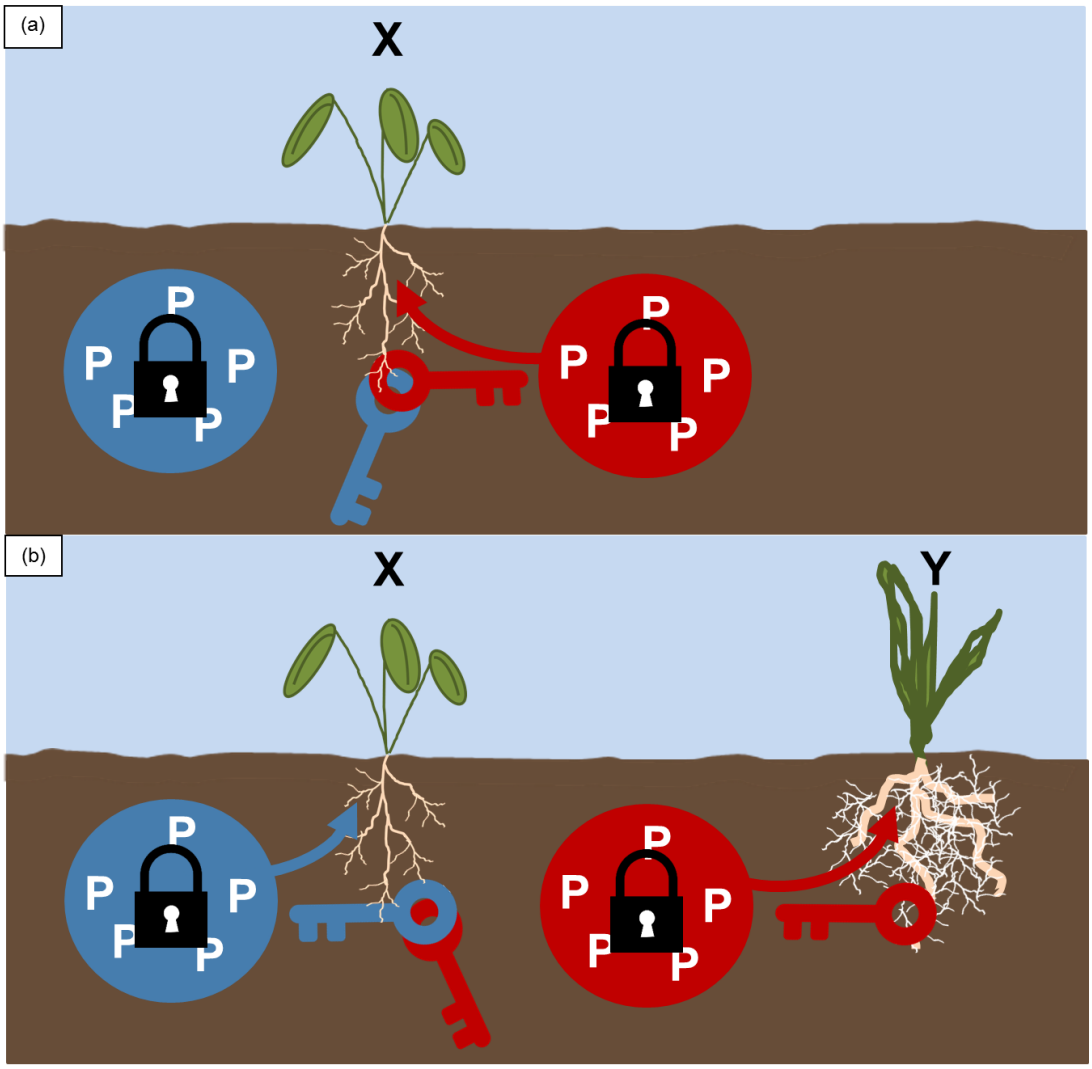

Figure 1.5: Schematic representation of niche plasticity in plant P acquisition. A distinction is made between a species' resource acquisition in (a) the absence of interspecific competition (i.e. fundamental niche) and (b) in response to interspecific competition (i.e. realised niche). In the first instance (a), species X acquires P from the 'red' source (fundamental niche). However, species Y also accesses P from this source. Therefore, in response to interspecific competition from species Y (b), species X switches to P acquisition from the 'blue' source (realised niche). Subsequently, niche overlap between species X and Y is reduced which reduces competition for P acquisition and facilitates coexistence.

Complimentary niche shifts have been shown to occur for N, in studies which have demonstrated species-specific switches in uptake between N forms in response to competition in mixed communities (Ashton et al. 2010). Despite evidence of interspecific differences in the acquisition of P from a range of soil sources, it is not known whether co-occurring species are capable of reducing overlapping resource requirements of P through niche plasticity in response to competition.

1.5.3 Interactions between contrasting methods of P acquisition

Competitive interactions between species which differ in their methods of P acquisition could offer a potential mechanism that sustains species richness in P-limited plant communities. Lambers et al. (2008) divided species into 'scavengers' or 'miners' depending on their method of P acquisition. 'Scavenger' species were defined by their superior foraging capacity for freely accessible P due to the dense and extensive hyphal network of their mycorrhizal fungal partners (see section 1.4.3). On the other hand, 'mining' species were adapted for the mobilisation of P from poorly accessible sources in the soil through the production of specialist rooting structures and release of root exudates (see sections 1.4.1 and 1.4.2). Lambers et al. (2008) proposed that scavenging species which are poorly suited to soils where P is predominantly locked up in sources of low bioavailability could survive by acquiring P which has been mobilised by their P-mining neighbours (Fig 1.6).

There have been a number of studies which have demonstrated how interactions between species with these contrasting methods of P uptake can influence P acquisition. These have predominantly focused on the beneficial impacts of intercropping on P nutrition of crop species in agricultural systems (Li et al. 2014).

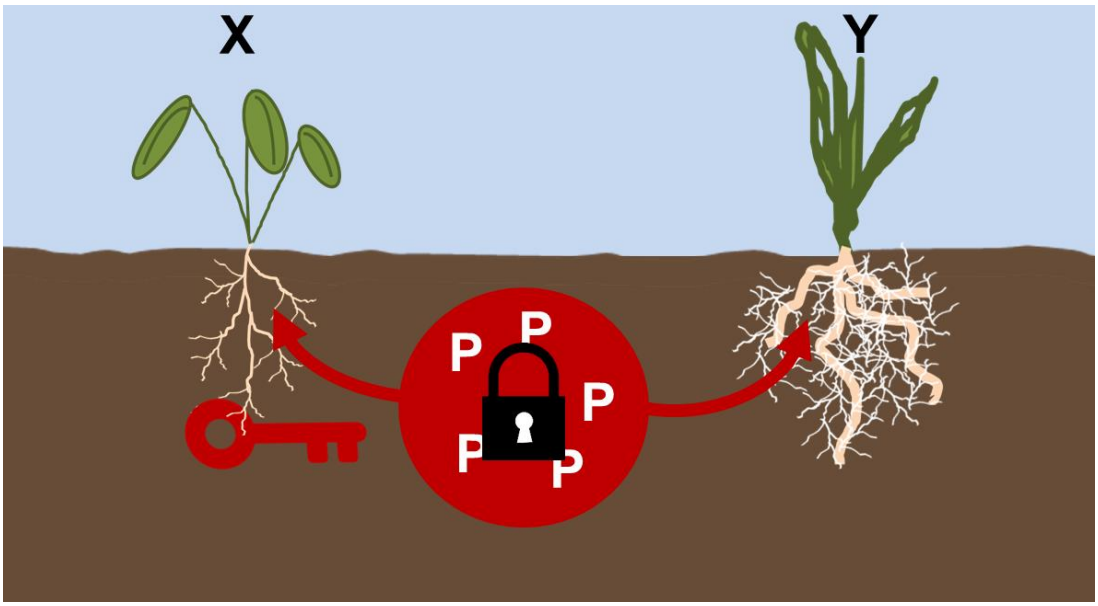

Figure 1.6: Schematic representation of plant P acquisition through scavenger-miner interactions. In this scenario, only species X possesses the key to access the 'red' soil P source (i.e. specialist adaptation for P acquisition) which allows both species X and Y to acquire the liberated P.

For example, wheat plants showed increased yield and P uptake from an organic P source when intercropped with P-solubilising chickpea plants (Li et al. 2003). Similar yield responses have also been demonstrated in field conditions when wheat and maize plants were intercropped with P-solubilising faba bean (Li et al. 2016; Zhang et al. 2016).

Muler et al. (2014) investigated scavenger-miner interactions outside of an agricultural context and showed that the productivity of scavenging species increased when in competition with a mining species. However, it has yet to be shown whether scavenging species acquire poorly accessible P in greater amounts when in competition with a mining species.

Observations of plant communities from the P-impoverished soils of Western Australia provide indirect evidence for how scavenger-miner interactions sustain species richness in P-limited plant communities. Lambers et al. (2011) observed a

range of plant communities and found that the highest levels of diversity were sustained in plant communities which contained a significant proportion of mining species. However, further work is required in natural and semi-natural systems which can determine whether mining species can actually mobilise P for scavengers, and therefore sustain co-existence and biodiversity in P-limited plant communities.

1.6 Calcareous grasslands

In order to investigate the relationship between high levels of species richness and low levels of P availability, the calcareous grasslands of Western Europe were used as a model system. These plant communities commonly contain more than 30 plant species per $50m^2$ (Willems 1978), making them among the most diverse in the world on such a scale (Fig 1.7).

The shallow Rendzina-type soils associated with calcareous grasslands characteristically contain low levels of extractable inorganic phosphorus (Phoenix et al. 2003). This is caused by the binding of freely available P in soil solution to calcium ions derived from the limestone bedrock. The lower layers of these soils can contain more than 70% calcium carbonate (Putten et al. 2000), and the accumulation of calcium phosphates represents a major component of total soil P in calcareous soils (Zhang et al. 2014).

The diverse array of grasses, forbs and sedges found in these plant communities have a range of different methods of P acquisition. Grasses and the majority of forbs form symbioses with arbuscular mycorrhizal fungi (section 1.4.3), whereas other species of forb instead rely on high rates of organic acid exudation for P uptake (Tyler & Ström 1995; Fransson et al. 2003) (section 1.4.1). Likewise, sedge species

form specialist root structures called dauciform roots (section 1.4.2) rather than relying on mycorrhizal associations for P acquisition.

The value of calcareous grasslands is demonstrated with their widespread designation as 'Sites of Special Scientific Interest' across the UK (JNCC 2010). As well as supporting a diverse range of plant species, these ecosystems provide a range of important services, from a forage resource for pollinator species (Carvell 2002) to a net sink for carbon (Janssens et al. 2005). However, the area of semi-natural grasslands in the UK suffered a steep decline throughout the 20th century (Fuller 1987), mostly through conversion to arable land (Newton et al. 2012). Therefore, as well as providing an ideal study system for the community dynamics of P-limited plant communities, further research into the ecology of calcareous grasslands will aid their future conservation and restoration.

Figure 1.7: Photograph of a diverse plant community from a calcareous grassland at Wardlow Hay Cop, Derbyshire (53°15'44"N, 1°43'52"W; photographed 03/06/2013). A range of grasses, forbs and sedges can be seen, as well as flowering orchids.

1.7 Aims

The link between low phosphorus availability and high species richness is well documented. Despite this, the mechanisms which maintain coexistence in diverse communities with low P supply are poorly understood. This thesis sets out to investigate a range of theories which have been proposed as an explanation for this relationship through the following research questions:

1. How do plant P uptake patterns across a range of soil P sources vary between species with contrasting methods of P acquisition?
2. What are the mechanisms which underlie changes in P uptake in response to interspecific competition?
3. How do plant species with different methods of P acquisition influence microbial P uptake in P-limited plant communities? Do changes in microbial biomass P influence interspecific differences in plant P uptake?

This thesis addresses these questions using calcareous grassland communities as a model. These investigations included the use of radio-isotope labelled P which made it possible to quantify P uptake in natural soils from supplied sources separately from pre-existing soil P sources. An overview and objectives for each research area are provided below.

1.7.1 Interspecific differences in P uptake patterns of co-occurring plants

Few studies have investigated the role of P acquisition in structuring plant communities, but there is indirect evidence of interspecific differences in P acquisition from those studies which have. It has been shown that P uptake among competing plant species is greatest when supplied with a variety of P sources

(Ahmad-Ramli et al. 2013). Furthermore, interspecific differences in P uptake have been correlated to the size of different soil P fractions (Fransson et al. 2003). The first direct evidence of interspecific differences in P uptake came through the use of radioactive tracers of chemical forms of organic, inorganic and mineral P that showed different uptake patterns among commonly co-occurring grassland species (Phoenix et al., unpublished data). This study also demonstrated changes in P uptake between individuals grown in monocultures and mixed communities. However, it has not been shown whether these changes occur in response to the distinct P uptake preferences and acquisition adaptations of neighbouring species.

Species with contrasting methods of P acquisition may also influence the uptake of neighbouring species by increasing the availability of P from poorly accessible sources (Lambers et al. 2006; Lambers et al. 2008). While this mechanism is documented in studies on intercropping in agricultural systems (Li et al. 2014), its potential function in natural and semi-natural systems is not known. Positive impacts on the biomass of neighbouring species have been demonstrated (Muler et al. 2014) but it is not known whether this is the result of increased access to P caused by co-occurring species.

The study described in **Chapter 3** supplied chemical P sources to a range of calcareous grassland species. P was supplied as orthophosphate as well as organic diester (DNA) and inorganic mineral (calcium phosphate) forms, representing pre-existing soil pools of contrasting bioavailability. Likewise, the selected species varied in their methods of P uptake, from mycorrhizal associations to the production of specialist root structures and root exudation. This allowed the measurement of how P uptake patterns varied between species with contrasting methods of P

acquisition across a range of chemical P sources, and how P uptake was influenced by co-occurring species.

1.7.2 The mechanisms underlying changes in P uptake in response to interspecific competition

Theories which have been proposed to explain how diverse plant communities are maintained in P-limited systems, such as niche differentiation through resource partitioning, were based on studies which have investigated these mechanisms through N acquisition in N-limited communities. However, there are far fewer studies which have investigated the relationship between community structure and P acquisition. Likewise, the influence of competitive interactions on the P uptake of species with contrasting methods of acquisition has been extensively tested in agricultural systems, but not in natural and semi-natural systems. Therefore further work is required in order to understand these mechanisms within the context of the fundamental ecological processes which govern plant communities.

The study described in **Chapter 3** measured the impact of interspecific competition on plant P uptake using a paired design. This made it possible to see whether there were changes in patterns of P uptake which corresponded to the method of P acquisition of a co-occurring species.

While the benefits of mycorrhizal associations to plant P nutrition are well documented (Smith & Read 2008), how these benefits are influenced by interactions with species which possess contrasting methods of P acquisition is poorly understood. The study described in **Chapter 4** investigated how interactions between a mycorrhizal 'scavenger' species and a non-mycorrhizal, cluster-root producing 'mining' species were mediated by mycorrhizal status. Mycorrhizal status

was controlled through substrate sterilisation and inoculum. This made it possible to measure the influence of mycorrhizal associations in the competitive interactions between species with contrasting methods of P acquisition.

The study described in **Chapter 5** moved from measuring P uptake in paired species to mixed communities. This made it possible to investigate the impact of interspecific competition on plant P uptake within a multi-species assemblage. These conditions provided a closer reflection of field conditions, where competitive interactions can include numerous species at any one time.

1.7.3 Plant-microbe interactions and P acquisition in P-limited plant communities

Interactions between plants and soil microbes have significant effects on plant community structure and function (Van Der Heijden et al. 2008). Despite this, the majority of research on plant community dynamics has focused aboveground, and less progress has been made in understanding the importance of belowground interactions (Bardgett & van der Putten 2014).

The influence of soil microbes on P acquisition in plant communities ranges from direct associations with mycorrhizal fungi and phosphate-solubilising bacteria, to indirect effects of microbial turnover and nutrient cycling in the soil. The study described in **Chapter 5** investigated the influence of plant species on microbial P uptake and whether this reflected interspecific differences in plant P uptake. Plant monocultures and mixed communities were supplied with radio-isotope labelled calcium phosphate and P uptake was measured in soil microbial communities associated with different plant communities.

Plants are capable of influencing microbial activity in the soil through the release of root exudates (section 1.4.4). Increased microbial P uptake can then feedback into plant P uptake, either directly through increased P mobilisation by phosphate-solubilising bacteria or indirectly through turnover of the microbial biomass (Vanveen et al. 1987; Macklon et al. 1997; Richardson et al. 2001; Achat et al. 2010; Marschner et al. 2011; Turner et al. 2012). Depending on interspecific differences in plant-microbe interactions, a positive relationship between plant and microbial P uptake could therefore contribute to the partitioning of P sources.

Different bacterial species have been shown to vary in their capacity to mobilise P from a range of soil P sources (Rodríguez & Fraga 1999; Richardson 2001; Pii et al. 2015). Therefore differences in the P uptake of microbial communities associated with different plant species could be related to selective enhancement of microbes which are more effective at mobilising soil P (Marschner et al. 2011). This was investigated in **Chapter 5** through the use of microbial fingerprinting techniques which measured changes in microbial community composition and species richness.

The effect of combining species monocultures into a mixed plant community on microbial P uptake was also investigated in **Chapter 5**. Previous studies have shown that plant species richness stimulated the activity and biomass of soil microbes (Lange et al. 2015; Thakur et al. 2015). This could increase mobilisation of P from organic sources (Hacker et al. 2015), however mineral-bound P sources such as calcium phosphate represent a significant source of soil P in calcareous soils which have not been investigated (Zhang et al. 2014). Increased microbial P uptake from this source, driven by increased species richness, could have important effects on soil P cycling and P availability to associated plant communities. Subsequent benefits in plant P uptake could cause a positive feedback which maintains co-

existence within increasingly diverse plant communities through increased access to this limiting soil resource.

Production of radio-isotope labelled calcium phosphate and its use in tracer studies

2.1 Summary

The use of radio-isotope phosphorus (P) labelled compounds makes it possible to measure plant P uptake directly from a supplied source, and distinguish this P uptake separately from that derived from pre-existing P-pools in the soil. A range of ^{33}P-labelled chemical compounds can be purchased directly or produced following a standardised protocol from commercial kits, but many important chemical P forms found in the soil cannot be obtained in these ways. Given the use of calcareous soils in this thesis, mineral-bound P in the form of calcium phosphate (CaP) was of great importance. However, this P source is not commercially available in a radio-isotope labelled form, so it was necessary to synthesise ^{33}P-labelled CaP for use in tracer experiments. Additionally, while a protocol exists for the production of ^{33}P-CaP, its solubility at soil relevant pH ranges and optimisation for specific activity has not been investigated. Therefore, it is unclear whether this synthesised CaP remains relatively insoluble (as therefore can be used to represent soil CaP) or liberates P at soil pHs (and so instead would represent a much more immediately available P source not representative of soil CaP). Furthermore, the protocol has not previously been manipulated to alter specific activity. This means that use of the original protocol to produce CaP with low specific activity involves addition of larger amounts of total P in the tracer. This could potentially negate one of the main benefits of using ^{33}P tracers, in that very small and realistic amounts of P can be added to the system. The amount of P released from CaP was measured in suspensions where pH had been manipulated to cover a soil-relevant range. This

showed that the original protocol produced a P source which was stable in the pH range of test soils. Therefore, P would not be immediately released from CaP upon supply to the soil, meaning the synthesised CaP was suitable to represent this P source in the soil. Specific activity was manipulated by changing the amounts of initial reactants used in the labelling process, which resulted in an order-of-magnitude increase in specific activity. This meant that the same amount of ^{33}P-CaP could be supplied at a higher specific activity allowing a smaller total amount of P to be supplied. However, there were no differences in plant ^{33}P uptake in response to the CaP sources of differing specific activity. This indicates that the differing total amount of P supplied using these different specific activities did not influence the ability of plants to acquire ^{33}P. Therefore, this means that experiments using either low or high specific activity CaP are comparable. These findings show that the synthesised CaP, at both low and high specific activities, is suitable for use in tracer studies alongside other commercially available radio-isotope labelled P sources.

2.2 Introduction

The shallow Rendzina-type soils associated with calcareous grasslands characteristically contain low levels of extractable inorganic phosphorus (Johnson et al. 1998; Phoenix et al. 2003). This is caused by the binding of freely available P in soil solution to calcium ions derived from the limestone bedrock (Tunesi et al. 1999). The lower layers of these soils can contain more than 70% calcium carbonate (Putten et al. 2000). Therefore, the accumulation of calcium phosphate represents a major P source, which has been estimated to reach over 55% of total P in calcareous soils (Zhang et al. 2014).

Previous studies have measured the effects of supplying calcium phosphate to plants established on field-collected soils. For example, Vogelsang et al. (2006) supplied a range of P sources, including calcium phosphate, to plant communities established in mesocosms with field-collected soil. However, there was no effect of different P sources on plant productivity or diversity. In a separate study, Lange Ness & Vlek (2000) showed that P uptake of maize plants was increased in soils with added calcium phosphate and mycorrhizal inoculum. A drawback of these studies is that, when measuring plant P uptake through P additions to a soil substrate, it is not possible to distinguish whether the source of P fixed in plant tissue was naturally occurring or experimentally added.

This ambiguity can be overcome by restricting plant access to a single source of P. For example, Akhtar et al. (2009) grew *Brassica* cultivars hydroponically with calcium phosphate as a P source and showed increasing amounts of plant P uptake in solutions with lower pH. However, an issue with this approach is that experimental conditions in these simplified study systems are far-removed from field conditions.

This is critical in terms of gaining a realistic understanding of plant P acquisition, which is influenced by a complex variety of interrelated soil conditions, from microbial associations to mineral weathering (Smith & Read 2008; Shen et al. 2011; Pii et al. 2015).

Measuring P uptake through the use of radio-isotope labelled P sources provides a solution to the drawbacks of both of these methods. This technique provides a direct measure of plant uptake from a specific P source applied to soil so that P uptake can be studied in conditions representative of the field.

Radio-isotope compounds have been used in ecological studies for many years, and are utilised to measure a range of parameters, such as photosynthesis, respiration and decomposition, as well as nutrient acquisition (Cameron et al. 2006; Kritzler & Johnson 2009; Field et al. 2012). Given the importance of P in the form of calcium phosphate (CaP) in calcareous soils, it was necessary to synthesis radio-labelled CaP for tracer experiments in this thesis. Calcium phosphate has been used as a radio-isotope-labelled P source previously (Leake et al. 2009). However, the solubility of this synthesised CaP at soil pH, and whether the specific activity of the CaP could be improved, have not previously been investigated.

While P transformations occur naturally through soil P cycling, it was important for P to remain fixed in synthesised CaP (as occurs with natural CaP pools in the soil). For calcium phosphate, one of the major factors which controls the equilibrium between precipitation and dissolution of P in these compounds is pH (Tunesi et al. 1999). The solubility of P in CaP increases at lower pH levels (Hinsinger 2001). However, it is not known whether the synthesised ^{33}P-CaP is soluble within a soil relevant pH range, in which case it would not be suitable in tracer studies to

represent this relatively recalcitrant mineral-bound soil P source. Therefore, the solubility of the synthesised CaP was measured across a soil-relevant pH range in order to see how much P would be released upon introduction to conditions in the soil.

In addition, the original protocol for the production of ^{33}P-CaP had not previously been manipulated to increase specific activity. Therefore, the original protocol produced CaP with a specific activity which was relatively low compared to other commercially available ^{33}P-labelled compounds. Specific activity is a measure of the activity of a radio-isotope per unit mass of the element present. Supplying a P source at a higher specific activity means a smaller amount of total P is supplied, as the ratio of radioactive P to non-radioactive P in the compound is higher. The benefit, therefore, of high specific activity is that realistic and very small amounts of P can be supplied that are less likely to disrupt plant-soil P dynamics (i.e. not saturating the system with P, or avoiding inadvertently stimulating P uptake mechanisms). To investigate this, the protocol was adjusted to produce higher specific activity CaP. These (original) low and (new) high specific activity sources were then tested in a plant uptake tracer experiment to see whether they influenced the amount of plant ^{33}P uptake.

2.3 Methods

2.3.1 Production of ^{33}P-labelled calcium phosphate

^{33}P-labelled calcium phosphate was synthesised using the method of Leake et al. (2009). 1 MBq aqueous ^{33}P-labelled orthophosphate (Perkin Elmer, UK) was added

to a reaction mixture containing 1 mL each of calcium nitrate (0.05 M) and ammonium hydrogen orthophosphate (0.05 M). The reaction was sped up by raising pH with the addition of 0.5 mL 32% ammonium solution.

Reaction mixtures were centrifuged and 1 mL of unprecipitated reactants were removed. This was followed by resuspension of the reaction mixture in 5 mL distilled water and repeated cycles of centrifugation, removal of supernatant, and resuspension. The amount of ^{33}P removed was measured through liquid scintillation counting (Packard Tri-carb 3100TR, Isotech) and used to calculate the amount of ^{33}P incorporated into the newly synthesised calcium phosphate.

2.3.2 P solubility from synthesised calcium phosphate across a pH range

Solubility of the newly synthesised calcium phosphate was measured over a pH range (2.4-8.35) encompassing that found in field conditions. This was done by adding HCl in varying amounts (10-70 uL) to calcium phosphate suspended in 6 mL distilled water. The suspension was centrifuged to form a pellet and an aliquot of solution above the pellet was removed and measured for soluble phosphorus content. This was done using the colorimetric Murphy-Riley method (Murphy & Riley 1962).

2.3.3 Increasing specific activity through reduced reactants

Specific activity of ^{33}P-labelled calcium phosphate was increased by altering the amount of initial reactants supplied to the reaction mixtures prior to the incorporation of ^{33}P. The volume of the reaction mixture was reduced in proportion with these changes, however the amount of ^{33}P supplied to the reaction mixture was maintained. Details of alterations to the original protocol, and subsequent differences in the production of calcium phosphate are detailed in Table 2.1.

Table 2.1: Amount of reactants used per reaction mixture in the original protocol compared to the modified protocol for increasing specific activity. The outcome of differences in the amount of reactants is then shown for the newly synthesised calcium phosphate.

	Reactants		Product		
Protocol	Calcium Nitrate 0.05 M (µl)	Ammonium Hydrogen Orthophosphate 0.05 M (µl)	Amount of CaP produced (mg)	Incorporation of ^{33}P (%)	Specific Activity (MBq.g-1)
Original	1000	1000	4.76±0.16	52.2±0.7	594.6±20.1
Reduced Reactants	72	72	0.41±0.05	74.3±0.5	9969.3±1092.7

2.3.4 Growth conditions for plants receiving calcium phosphate

Mesocosms (7x7x8 cm) containing monocultures of four different calcareous grassland species (*Agrostis capillaris, Plantago lanceolata, Rumex acetosa* and *Carex caryophyllea*) were established on Rendzina soil (pH 6.5) collected from a calcareous grassland field site at Wardlow Hay Cop, Derbyshire (53°15'44"N, 1°43'52"W). Seeds of *A. capillaris, P. lanceolata,* and *R. acetosa* (Emorsgate, Kings Lynn, UK) were sown directly into the mesocosms while *C. caryophyllea* individuals were collected from Wardlow Hay Cop and transplanted into the pots at the same time as when seeds were sown. Eight replicates of each monoculture were prepared.

Mesocosms were established over a period of 20 weeks in a climate controlled greenhouse with conditions set at 16 hours daylight (with supplementary light when necessary), day/night temperatures of 20°C/15°C, and regular watering.

After 19 weeks, mesocosms were supplied with 0.8 MBq ^{33}P-labelled calcium phosphate either at the original specific activity or at the increased specific activity

from the modified protocol. These equated to 1.35 mg and 0.08 mg of P respectively. Compared to total amounts of soil P, this represents a contribution of around 2% and 0.1% respectively.

This was supplied in 10 mL solution of distilled water and dispensed through a syringe loaded with a two-sideport needle to a depth of 8 cm at four injection points spread evenly across the mesocosm. At each injection point, the needle was fully inserted and 2.5 mL of the ^{33}P solution was released as it was withdrawn gradually up through the soil profile.

2.3.5 Measurement of plant ^{33}P uptake

Acid digestion was used for analysis of ^{33}P content within the samples. Above-ground biomass was harvested 7 days after ^{33}P application and then freeze-dried, ground and homogenized. 20-30 mg of plant material was digested in 1 ml of concentrated sulphuric acid. To ensure complete digestion, the samples were heated to 350°C for a period of 15 minutes.

After this step, 200 µl of hydrogen peroxide was supplied to each sample and, following 1 minute at 350°C, the samples were completely digested. Any samples which were not digested completely by this point received further additions of hydrogen peroxide and reheating.

Digested samples were diluted up to 10 ml with distilled water. Of this amount, a 2 ml aliquot was added to 10 ml of liquid scintillant (Emulsifier Safe, Perkin-Elmer). ^{33}P content of the samples was then measured with a scintillation counter (Packard Tri-carb 3100TR, Isotech), and subsequently corrected for decay.

2.3.6 Statistical Analysis

Data for P solubilisation from calcium phosphate was transformed reciprocally. A quadratic relationship provided the best fit of the data, which was analysed using Spearman's rank correlation.

To measure the effects on plant tissue ^{33}P concentration from calcium phosphate with altered specific activity, a two-way ANOVA was used with species and calcium phosphate as factors. Tukey HSD tests were then carried out to show where significant differences occurred between each species and specific activity treatment. All analyses were carried out using Minitab (Minitab Inc., State College, PA, USA).

2.4 Results

2.4.1 P solubility across a pH range

There was a significant relationship between pH and the solubilisation of P from calcium phosphate (Fig 2.1; r_s=0.917, n=9, P=0.001). Ca-bound P remained stable between pH 8.4 and 5.6, with less than 2% of P solubilised. Lower pH levels led to increased amounts of solubilised P. However, the shape of the relationship shows that major increases in solubility did not occur until below pH 3.5, with more than 25% of Ca-bound P solubilised at pH 2.4.

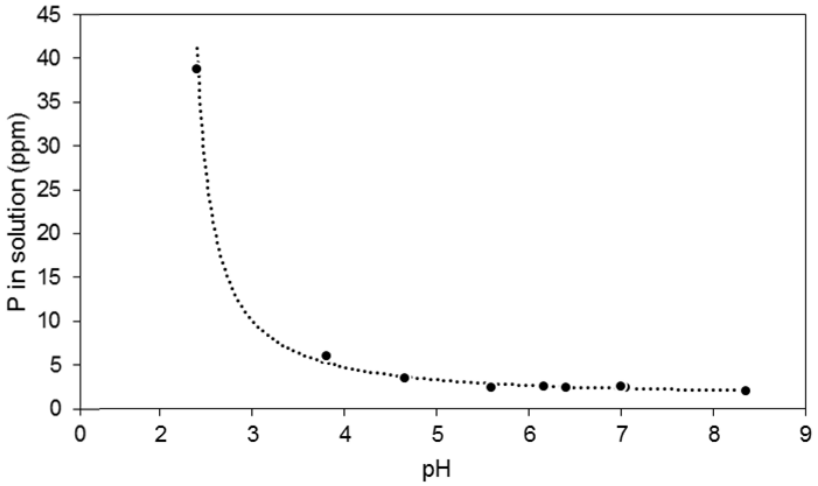

Figure 2.1: Amount of P solubilised from calcium phosphate suspended in solutions of varying pH levels.

2.4.2 Plant ^{33}P uptake

There was no significant effect of differing specific activity on plant uptake of P from calcium phosphate (Fig 2.2; df=1, 31, F=0.24, $P>0.05$). There was a significant effect of species identity on P uptake (df=3,31, F=253.86, $P<0.001$), with significantly higher tissue concentrations of ^{33}P in *R. acetosa* (Tukey HSD, $P<0.05$). There was no interaction between species and calcium phosphate specific activity (df=3,31, F=2.12, $P>0.05$).

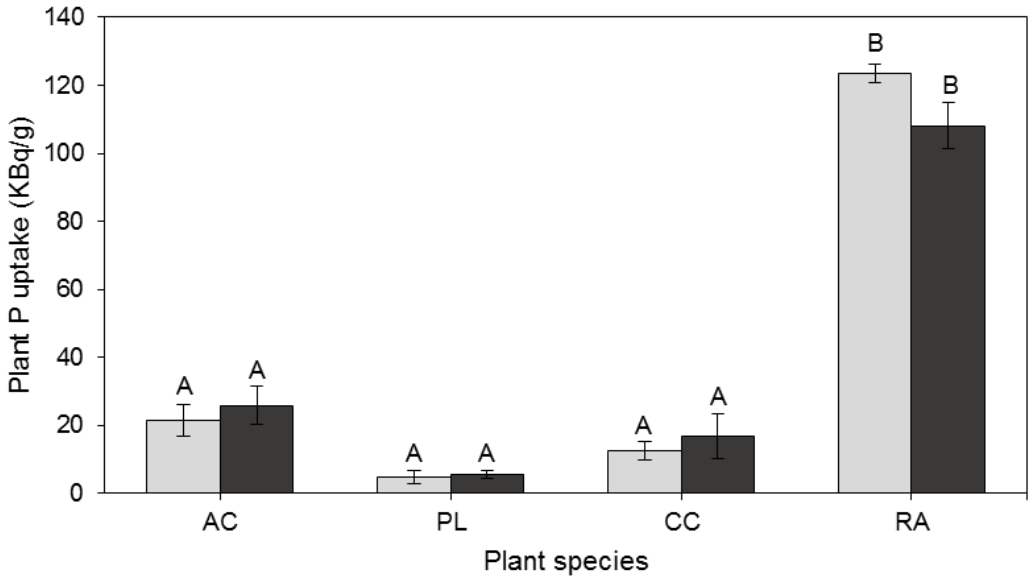

Figure 2.2: Tissue concentration of ^{33}P for each species (*Agrostis capillaris*, AC; *Plantago lanceolata*, PL; *Carex caryophyllea*, CC; *Rumex acetosa*, RA) supplied with calcium phosphate. Grey bars and black bars represent mesocosms supplied with calcium phosphate produced following the original protocol and the increased specific activity protocol respectively. Means are shown ± 1 s.e.m. Means with the same letter do not differ significantly from each other (Tukey HSD). See text for statistics.

2.5 Discussion

Currently, radio-isotope labelled calcium phosphate is not commercially available, and there is no standardised protocol for its production. Therefore it was necessary to investigate important characteristics that could influence its experimental use. The findings from this study show that ^{33}P-labelled calcium phosphate produced following the original protocol (Leake et al. 2009) remained stable in pH conditions representing those found in calcareous soils and will only show appreciable solubility in pH ranges found in very acidic soils. Furthermore, increasing specific activity of P within calcium phosphate had no impact on plant uptake of ^{33}P, which

suggests that changing the total amount of P supplied to the system within the ranges used here did not affect the ability of plants to access this P source.

Soil pH is a major abiotic factor which controls the equilibrium between precipitation and dissolution of Ca-bound P (Tunesi et al. 1999). The measured pH of the soils used in this study was around 6.5 and precipitated P in calcium phosphate was shown to remain stable in a pH range between 8.4 and 5.6. At lower pH, increasing amounts of P are released, reflecting reduced sorption in calcium phosphate mineral phases (Hinsinger 2001). The stability of synthesised calcium phosphate shown here is in line with previous studies which have measured P solubilisation from synthesised hydroxyapatite (Bell et al. 1978).

The low solubility of CaP around the pH range of soils used in this thesis demonstrates that experimental systems are not flushed with plant available P when supplied with this P source (i.e. the synthesised CaP is appropriately recalcitrant). From this it can be considered that ^{33}P uptake from synthesised CaP provides a good representation of plant P derived from calcium phosphate in field conditions, although it may go through multiple transformations before plant acquisition.

There was a significant effect of species on ^{33}P uptake from calcium phosphate, driven by the higher tissue concentration of ^{33}P in *R. acetosa* (differences in plant ^{33}P uptake are investigated in more detail in Chapter 3). Most importantly however, there was no significant effect of changing the specific activity of calcium phosphate on plant ^{33}P uptake across all species. Therefore, the amount of calcium phosphate supplied to the system in this experiment did not influence the ability of plants to acquire ^{33}P from this source. This indicates that the experimental treatment did not saturate the system with P or inadvertently stimulate ^{33}P uptake. CaP of either

specific activity is therefore suitable for uptake tracer studies and results using either can be considered comparable.

As shown by Putten et al. (2000), calcareous grassland soils contain large amounts of calcium carbonate derived from the limestone bedrock. The release of calcium ions leads to the build-up of calcium phosphate as it binds to phosphorus in soil solution (Tunesi et al. 1999). The accumulation of calcium phosphate represents a major P source, which has been estimated to reach over 55% of total soil P in calcareous soils (Zhang et al. 2014). The amount of P supplied to mesocosms as ^{33}P-CaP was equivalent to less than 2% of total soil P. Given that calcium phosphate is a major component of calcareous soils this amount may represent a negligible contribution to pre-existing soil pools from the plant's perspective and therefore represents a tracer that can be supplied with minimal impact on soil P availability.

Niche shifts in phosphorus uptake from different P chemical forms in response to competition: increasing similarity between species with contrasting methods of acquisition.

3.1 Summary

Low phosphorus (P) availability is linked to high species richness and the abundance of rare species. Despite this, the mechanisms which maintain coexistence in diverse plant communities with low P supply are poorly understood. Co-existence may be maintained through resource partitioning of phosphorus whereby co-occurring species avoid interspecific competition by acquiring P from different chemical forms in the soil. This could be further enhanced by shifting patterns of P uptake which result in reduced overlap in resource use (niche plasticity) between co-occurring species. In theory, reducing the negative impact of interspecific competition will promote coexistence among species within the plant community. Furthermore, interactions between species with contrasting methods of P acquisition may help maintain diversity. For instance, species specialised in mobilising P from poorly accessible sources may sustain neighbouring species with a superior foraging capacity for liberated P. These hypotheses are yet to be tested in P-limited systems. In this study, experimental microcosms were supplied with radio-isotope labelled organic, inorganic and mineral P sources which varied in their bioavailability. The microcosms contained paired species and monocultures of forbs, grass, and sedge species, which commonly co-exist in calcareous grasslands. These species varied in their methods of P acquisition – from mycorrhizal associations through to specialist root structures and root exudation. In the absence of interspecific competition, there

were differences in P uptake across species, with non-mycorrhizal species taking up greater amounts of P from poorly accessible sources. However in response to competition, interspecific differences were diminished by the competitive advantage of mycorrhizal species which reduced P acquisition of non-mycorrhizal species while their own uptake was maintained. These findings provide evidence to support the hypothesis that mycorrhizal species, despite acquiring smaller amounts of poorly accessible P, could benefit from and survive alongside non-mycorrhizal species which make these sources of P available for uptake.

3.2 Introduction

Understanding plant interactions and their effects on biodiversity is an integral part of plant ecology. How so many species are capable of coexisting in diverse plant communities, despite competing for the same limiting resources has been a long-standing source of debate (Silvertown 2004).

Nutrient limitation is a common characteristic of species rich plant communities. This is particularly apparent in systems of low P supply, where multiple studies have demonstrated the inverse relationship between soil P availability and diversity (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Ceulemans et al. 2014). Under P-limited conditions, plant communities have also been shown to contain a greater frequency of rare species (Wassen et al. 2005).

Many of the world's biodiversity hotspots are located in nutrient-poor habitats - from the highly weathered soils of tropical rainforests to the ancient soils of Western Australia (Myers et al. 2000). In these nutrient-poor conditions, competition for limiting resources leads to co-occurring species having a greater impact on one another's survival than in environments which are resource-rich (Goldberg et al. 1999). Despite widespread significance, how coexistence is maintained under a limited supply of P is poorly understood.

A number of theories have been proposed to explain how so many species are capable of coexisting in resource-poor conditions. Resource acquisition underlies each of these theories, but they differ in the emphasis placed on species interactions. Diversity could be maintained in resource-poor habitats by co-occurring species avoiding the negative impact of competitive interactions by accessing different P sources in form, space or time, which is known as "resource partitioning" or "niche

differentiation in resource use" (McKane et al. 2002; Turner 2008). Furthermore, interactions between species with contrasting methods of P acquisition could facilitate coexistence if the action of one species made resource conditions more favourable for another (Lambers et al. 2008).

The majority of studies on resource partitioning have focused on nitrogen (N). These have shown differentiation in the timing, depth and chemical form of N uptake between species (McKane et al. 2002; Kahmen et al. 2006; Felten et al. 2009), reducing competition for the limiting N supply and so facilitating co-existence. However, there has been very little research which has investigated the potential for resource partitioning of P. This is despite the fact that P approaches N in the extent to which it is limiting in terrestrial ecosystems (Elser et al. 2007), and the demonstrated importance of P as a limiting resource in species rich communities (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Ceulemans et al. 2014).

Resource partitioning of soil phosphorus has been proposed to occur through various methods of plant P acquisition acting on a range of P sources present in the soil (Turner 2008). In support of this, there is indirect evidence which shows that plant species differ in their uptake of P from a range of sources (Fransson et al. 2003) and P uptake among competing plant species is greatest when supplied with a variety of P sources (Ahmad-Ramli et al. 2013). Direct evidence of P partitioning came through the use of radioactive tracers, which demonstrated interspecific differences in P uptake of contrasting chemical forms of organic, inorganic and mineral P among co-occurring grassland species (Phoenix *et al.*, unpublished data). However, it is not known whether co-occurring species are capable of reducing overlapping resource requirements by niche plasticity in response to competition.

Niche plasticity describes the reductions in niche overlap which occur in response to competition when co-occurring species shift their patterns of resource uptake. For N, it has been demonstrated that there are species-specific switches in uptake between N forms in response to competition in mixed communities (Ashton et al. 2010). However, it has not been investigated whether shifts in P acquisition are a species-specific response to the uptake patterns of neighbouring species. Investigation of these interactions will provide an important insight on the processes which could maintain species coexistence through P partitioning.

Resource partitioning and niche plasticity place emphasis on the reduction of interspecific competition through differences in resource use. However, competitive interactions between co-occurring species may also allow more species to persist in the unfavourable conditions of P-limited soils (Lambers et al. 2006; Lambers et al. 2008; Li et al. 2014).

Lambers et al. (2008) divided plant species from the nutrient poor soils of Western Australia into two groups depending on their method of P acquisition. 'Scavenging' species form symbiotic associations with arbuscular mycorrhizal (AM) fungi which provide the host plant with P from the soil, in return for carbohydrates. On the other hand, 'mining' species are more effective at acquiring their own P from poorly accessible soil sources through the production of specialist root structures and exudation of organic acids and phosphatases.

Observations of plant communities across a range of soil ages have shown that not only are plant communities most diverse on the oldest soils but, also, they contain a significant proportion of 'mining' species (reflecting the severely depleted amounts of freely available P in the soil) (Lambers et al. 2011). The success of mining

species was attributed to specialist adaptations for P acquisition, and it was suggested that these species were also responsible for sustaining other species in these plant communities and therefore maintaining higher levels of diversity. In theory, species which scavenge plant-available P are poorly suited to conditions where P is predominantly sorbed onto soil particles, but these species could survive by acquiring the P which had been mobilised by their P-mining neighbours. Muler et al. (2014) provided evidence for the beneficial effect of scavenger-miner interactions which showed a greater yield in scavenging species when in competition with a mining species (compared to monoculture). However, it has yet to be shown whether mining species increase access to recalcitrant P forms in co-occurring scavenging species. This mechanism is consistent with studies on intercropping in agricultural systems, which have shown that crop species can increase the P acquisition of neighbouring plants (Li et al. 2007; Li et al. 2012; Zhang et al. 2016).

In this chapter, we used calcareous grasslands as a study system to investigate niche plasticity of resource partitioning in a P-limited system. Some of the most diverse plant communities in Europe are found in calcareous grasslands, consisting of a diverse array of grasses, forbs and sedges (Roem & Berendse 2000; Critchley et al. 2002; Ceulemans et al. 2014). Furthermore, calcareous grassland soils are characteristically limited by the availability of P.

A range of different methods of P acquisition can be found within calcareous grassland plant communities. Grasses and the majority of forbs form symbioses with arbuscular mycorrhizal fungi. The hyphal network of these fungal symbionts enhances host plant P acquisition through uptake beyond zones of depletion surrounding the roots and within soil particles which the broader roots of plants

cannot access (Smith & Read 2008). Alternatively, other species of forb rely on the release of large amounts of root exudates for P uptake (Tyler & Ström 1995; Fransson et al. 2003). Organic acids are an important component of these root exudates, which mobilise P from mineral sources such as calcium phosphate and increase the amount of P available for plant uptake (Jones & Darrah 1994; Tyler & Ström 1995; Shen et al. 2002). Furthermore, non-mycorrhizal species of sedge form specialist root structures called dauciform roots. These dense proliferations of root hairs perform a similar function to 'proteoid' (or 'cluster') roots found in Proteaceae, and increase the plant's capacity for P uptake from poorly accessible sources through increased root surface area and exudation of organic acids and phosphatases (Playsted et al. 2006; Shane et al. 2006).

This study investigated the effect of competition on P uptake between species with contrasting methods of P acquisition by separating competitive interactions into a paired design. To do this, species were used which differ in their methods of P acquisition through either root exudation and specialist root structures, or mycorrhizal associations – representing both 'mining' and 'scavenging' methods of P acquisition respectively (Lambers et al. 2008). This included *Agrostis capillaris* (mycorrhizal grass), *Plantago lanceolata* (mycorrhizal forb), *Rumex acetosa* (non-mycorrhizal forb) and *Carex caryophyllea* (non-mycorrhizal sedge with dauciform roots). Each species was grown in monoculture as well as in pair-wise competitive interactions.

Phosphorus was supplied in the form of orthophosphate, calcium phosphate or DNA. These respectively represent inorganic, mineral and organic sources of P in the soil and vary in their bioavailability, from the freely available orthophosphate

through to calcium phosphate and DNA, which require solubilisation and hydrolysis before their associated P can be acquired.

It was hypothesised that (a) there would be interspecific differences in P uptake from the three supplied P sources among the test species when grown in monoculture (i.e. niche complementarity in their fundamental niches). Furthermore, these differences would be consistent with the method of P acquisition of each species, with the mycorrhizal species taking up more of the supplied orthophosphate, and the non-mycorrhizal species acquiring more P from DNA and calcium phosphate.

This study also investigated three potential mechanisms for the maintenance of species coexistence in response to interspecific competition. If resource partitioning is a mechanism which maintains coexistence in P-limited plant communities, we hypothesised that (b) interspecific differences in P uptake would be maintained in response to interspecific competition. Furthermore, to investigate whether test species showed niche plasticity in P uptake, we hypothesised (c) an increase in interspecific differences in response to competition.

In line with the scavenger-miner hypothesis, differences in P uptake between species may be reduced in competition (i.e. a reduction in niche differentiation). In this case, we hypothesised that (d) scavenging species would sustain (or increase) P uptake mobilised from poorly accessible sources by mining species. In addition, it was hypothesised that (e) the negative effect of interspecific competition would lead to a reduction in P uptake from the same sources in mining species, providing evidence that mining species liberate P for the benefit of scavengers.

3.3 Method

3.3.1 Experimental set-up

Four plant species were selected to represent the range of P acquisition strategies and functional groups which are commonly found in calcareous grasslands. These were *Agrostis capillaris* (mycorrhizal grass), *Plantago lanceolata* (mycorrhizal forb), *Rumex acetosa* (non-mycorrhizal forb) and *Carex caryophyllea* (non-mycorrhizal sedge with dauciform roots).

The plants were grown in polypropylene tubes (height: 11 cm, diameter: 4 cm) with 30 µm mesh covering the base. These contained Rendzina soil (pH 6.5) collected from a calcareous grassland field site at Wardlow Hay Cop, Derbyshire (53°15'44"N, 1°43'52"W). The soils associated with calcareous grasslands are commonly P-limited, and this has been previously documented at this site (Phoenix et al. 2003). For collection, soil was removed to bed-rock, air-dried and sieved (2 mm).

Seeds of *A. capillaris*, *P. lanceolata*, and *R. acetosa* (Emorsgate, Kings Lynn, UK) were sown directly into the microcosms. Because of its low growth rate and difficulties in germination from seed, *C. caryophyllea* individuals were collected from Wardlow Hay Cop and transplanted into the pots at the same time as when seeds were sown. A previous pilot study determined sowing and planting densities which led to a similar biomass for each species at time of harvest. The selected species were grown separately in monoculture, as well as being paired with each of the other species. All monocultures and species pairs were replicated 15 times.

Test species were grown for 12 weeks in a climate controlled greenhouse with conditions set at 16 hours daylight (with supplementary lighting when necessary), day/night temperatures of 20°C/15°C, and regular watering.

3.3.2 Production of ^{33}P-labelled P sources

Microcosms were supplied with one of three radioactively-labelled P sources: orthophosphate (inorganic), calcium phosphate (inorganic mineral) and DNA (organic diester). ^{33}P-labelled orthophosphate is commercially available (Perkin Elmer, UK), however ^{33}P-labelled DNA and calcium phosphate required synthesis in the laboratory. ^{33}P-labelled calcium phosphate was synthesised using the approach previously outlined in section 2.3.1.

Production of ^{33}P-labelled DNA was carried out with a random primed DNA labelling kit (Roche applied science, West Sussex, UK), as per the supplier's methods developed by Feinberg and Vogelstein (1983). Template DNA (λDNA 12.5 µg/mL) was denatured by heating to 95 ºC in a water bath for 10 minutes and then transferred to an ice bath to chill rapidly. A mixture of nucleotides were then added containing dCTP, dGTP, dTTP and dATP, the latter of which was ^{33}P-labelled (Perkin Elmer, UK). Following this, a hexanucleotide primer mixture (representing the vast majority of all sequence combinations) and Klenow enzyme were supplied. The mixture was incubated for 30 minutes at 37 ºC. During this time, complimentary strands were synthesised through hybridization of primers to single-stranded DNA fragments and incorporation of nucleotides using Klenow enzyme. The reaction was stopped by adding EDTA (0.2 M, pH 8.0). Unincorporated nucleotides were removed using Sephadex G-50 quick spin columns (Roche Applied Science, West Sussex, UK). The incorporation of ^{33}P-labelled dATP into the newly synthesised

DNA strand was then confirmed by scintillation counting (Packard Tri-carb 3100TR, Isotech).

3.3.3 ^{33}P treatments

After 10 weeks, each microcosm was supplied with one of three radioactively-labelled P sources (orthophosphate, DNA, or calcium phosphate). The 15 replicates of each species combination were divided into three groups of five, each receiving 0.2 MBq of one ^{33}P source. This represented a negligible contribution to the pre-existing P-pools in the soil, amounting to less than 1 ng of ^{33}P.

The P sources were each supplied to plant pots in 10 mL solution of distilled water. These were dispensed through a syringe fitted with a two-sideport needle to a depth of 8 cm at the injection point located at the centre of the pot. To achieve an even distribution, the needle was withdrawn gradually up through the soil profile during the release of the ^{33}P solution.

3.3.4 ^{33}P uptake analyses

Following the supply of radioactively-labelled P sources, the duration of the labelling period lasted seven days. This length of time was based on previous work which indicated that plant P uptake was accurately reflected over that period (Phoenix *et al.*, unpublished data).

After seven days, the aboveground biomass of the microcosms was harvested, sorted by species, freeze-dried and then weighed. Post-harvest, colonisation of mycorrhizal plant roots (*A. capillaris* and *P. lanceolata*) was confirmed through Trypan blue root staining (Phillips & Hayman 1970) and the roots of *C. caryophyllea* were inspected

for the presence of dauciform roots. Acid digestion and liquid scintillation counting was used for analysis of ^{33}P content within the samples, as outlined in section 2.3.5.

3.3.5 Statistical analyses

A two-way ANOVA was used, with species and competitor identity (including the species own identity when in monoculture) as factors, to measure the effects on plant tissue ^{33}P concentration for each P source. Tukey HSD tests were then carried out to show where significant differences occurred within species treatments. Data were $\log_{10}$ transformed in order to achieve normality and homogeneity of variances. The same patterns in ^{33}P uptake were seen when expressed either as total tissue ^{33}P content or tissue concentration, indicating that any differences in ^{33}P uptake were not confounded by differences in plant biomass.

The competitive response of each species was measured across P sources. This was expressed as the percentage difference in tissue ^{33}P concentration between a species when grown in monoculture and in interspecific competition. Dunnett's post hoc test was used to determine whether the change in tissue ^{33}P concentration in response to each competitor differed significantly from monoculture. All analyses were carried out using the statistical packages Minitab (Minitab Inc., State College, PA, USA) and R 3.2.2 (R Core Team 2015).

3.4 Results

3.4.1 Plant uptake of radio-isotope labelled P sources

In monoculture, non-mycorrhizal species (*C. caryophyllea* and *R. acetosa*) showed the greatest uptake of ^{33}P across the supplied P sources. Specifically, *R. acetosa* showed the greatest ^{33}P uptake when supplied with orthophosphate and calcium phosphate, while *C. caryophyllea* showed the greatest ^{33}P uptake from DNA. Tissue concentrations of ^{33}P were lowest in A. *capillaris* monocultures for each source.

Across all species, uptake of ^{33}P from the supplied orthophosphate showed significant effects of species identity and interspecific competition (Table 3.1, Fig 3.1). In *A. capillaris,* tissue ^{33}P concentration was lowest of all species and there were no significant differences across competitive interactions. *Plantago lanceolata*, *C. caryophyllea* and *R. acetosa* all showed a significant reduction in ^{33}P tissue concentration in competition with *A. capillaris* (Tukey HSD, $P<0.05$).

There were significant effects of species identity and interspecific competition in ^{33}P uptake from calcium phosphate (Table 3.1, Fig 3.2). *Rumex acetosa* showed the highest tissue concentrations of ^{33}P, followed by *C. caryophyllea,* then *A. capillaris* and *P. lanceolata*. Apart from *A. capillaris,* each species showed shifting patterns of tissue ^{33}P concentration in response to interspecific competition. In *P. lanceolata,* tissue ^{33}P concentration remained the same when in competition with *R. acetosa* and showed a slight reduction when in competition with *A. capillaris*. However, there was a significant increase in tissue ^{33}P when in competition with *C. caryophyllea*. In *C. caryophyllea* and *R. acetosa*, there were significant reductions in tissue ^{33}P concentration when in competition with *A. capillaris* and *P. lanceolata* (Tukey HSD, $P<0.05$).

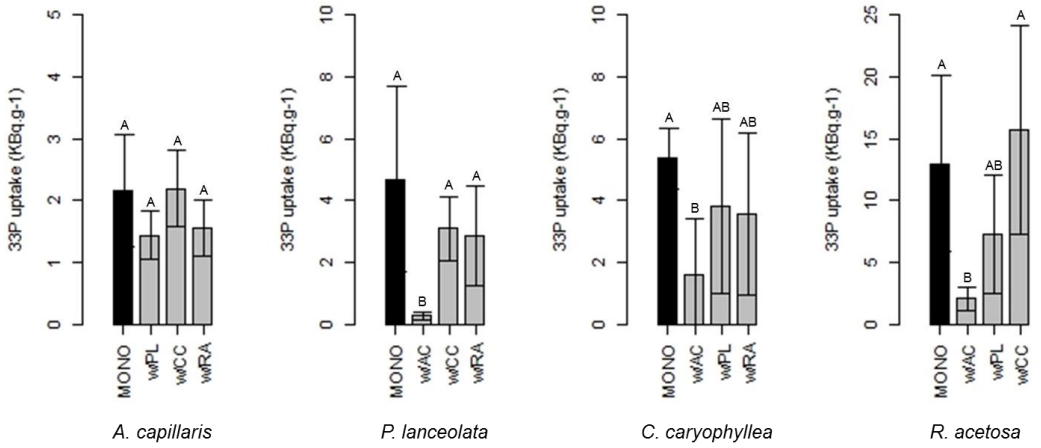

Figure 3.1: Tissue ^{33}P concentration for each species across competitive interactions for microcosms supplied with radio-isotope labelled orthophosphate. Black bars and grey bars represent species monocultures and paired species respectively (AC, *A. capillaris;* PL, *P. lanceolata;* CC, *C. caryophyllea;* RA, *R. acetosa*). Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 3.1 for statistics.

Uptake of ^{33}P from DNA showed significant effects of species identity and interspecific competition on tissue ^{33}P concentrations (Table 3.1, Fig 3.3). *Carex caryophyllea* showed the highest tissue concentrations of ^{33}P, followed by *R. acetosa*. There was no effect of interspecific competition on *A. capillaris* or *P. lanceolata*. However, there were significant reductions in tissue ^{33}P concentration of *C. caryophyllea* and *R. acetosa* when in competition with *A. capillaris* and *P. lanceolata* (Tukey HSD, $P<0.05$).

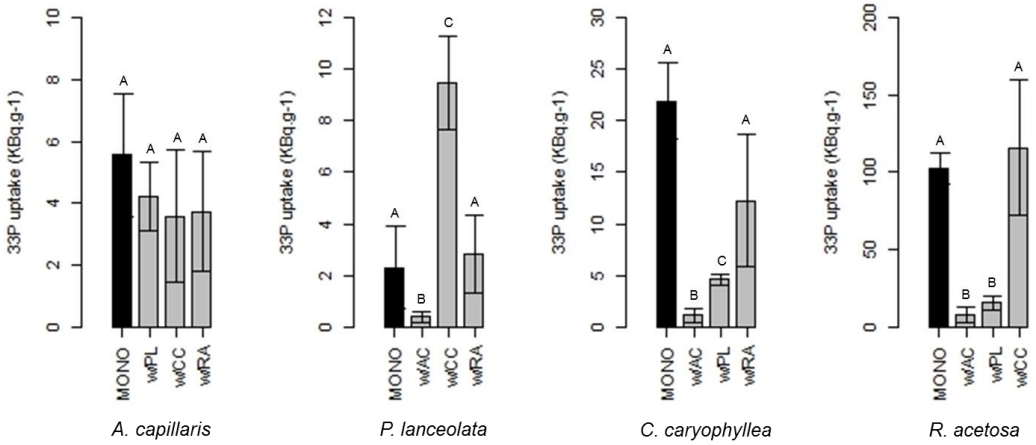

Figure 3.2: Tissue ^{33}P concentration for each species across competitive interactions for microcosms supplied with radio-isotope labelled calcium phosphate. Black bars and grey bars represent species monocultures and paired species respectively (AC, *A. capillaris;* PL, *P. lanceolata;* CC, *C. caryophyllea;* RA, *R. acetosa*). Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 3.1 for statistics.

Table 3.1: Two-way ANOVA results for the impact of species identity and interspecific competition on plant tissue 33P concentration.

P source	Factor	df	*F*	*P*
Orthophosphate	Species	3,76	25.98	<0.001
	Competitor	3,76	21.19	<0.001
	Species*competitor	9,76	2.93	0.006
Calcium phosphate	Species	3,73	130.3	<0.001
	Competitor	3,73	55.69	<0.001
	Species*competitor	9,73	20.06	<0.001
DNA	Species	3,79	67.85	<0.001
	Competitor	3,79	51.95	<0.001
	Species*competitor	9,79	13.51	<0.001

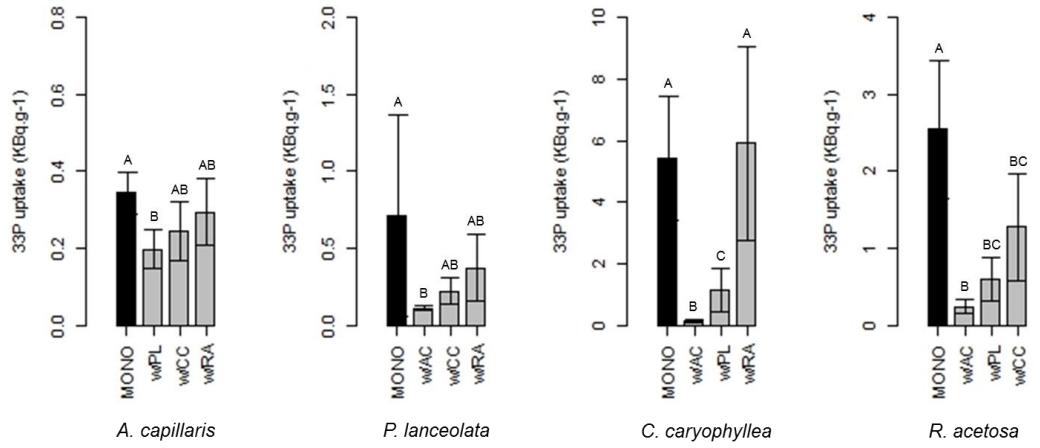

Figure 3.3: Tissue ^{33}P concentration for each species across competitive interactions for microcosms supplied with radio-isotope labelled DNA. Black bars and grey bars represent species monocultures and paired species respectively (AC, *A. capillaris;* PL, *P. lanceolata;* CC, *C. caryophyllea;* RA, *R. acetosa*). Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 3.1 for statistics.

3.4.2 Competitive responses across P sources

The change in ^{33}P uptake in response to interspecific competition for all species across each P source was predominantly negative (Table 3.2). The largest reductions in ^{33}P uptake were in response to *A. capillaris* and *P. lanceolata*. On the other hand, the circumstances where ^{33}P uptake was maintained or increased (relative to monocultures) were consistently in response to *C. caryophyllea* and *R. acetosa*.

For *A. capillaris*, there was no significant effect of competitor identity or changing P source on competitive response. The mean change in tissue ^{33}P concentration was consistently negative in response to all species when compared to monoculture. However, the only significant reduction (43%; Dunnett's $P<0.05$) occurred in response to competition with *P. lanceolata* when supplied with DNA.

Table 3.2: Competitive response of *Agrostis capillaris*, *Plantago lanceolata*, *Carex caryophyllea* and *Rumex acetosa* across each ^{33}P-labelled P source. See text for statistics. Competitive response was expressed as the percentage difference in tissue ^{33}P concentration between a species when grown in monoculture and in interspecific competition. Dunnett's post hoc test was used to determine whether the change in tissue ^{33}P concentration in response to each competitor differed significantly from monoculture (* = P<0.05; ** = P<0.01; *** = P<0.001).

^{33}P source	Species	Competitor			
		Agrostis	*Plantago*	*Carex*	*Rumex*
Orthophosphate	*Agrostis*		-33.64%	+0.98%	-28.53%
	Plantago	-94.15%***		-34.18%	-38.98%
	Carex	-69.80%*	-28.77%		-33.42%
	Rumex	-83.96%*	-44.13%	+20.95%	
Calcium Phosphate	*Agrostis*		-35.51%	-24.18%	-32.85%
	Plantago	-71.24%**		+110.06%***	+14.16%
	Carex	-94.67%***	-79.09%***		-44.10%*
	Rumex	-92.31%***	-84.74%***	+13.57%	
DNA	*Agrostis*		-42.56%*	-28.62%	-14.25%
	Plantago	-84.06%*		-68.70%	-47.34%
	Carex	-97.06%***	-84.42%***		+21.38%
	Rumex	-90.52%***	-76.50%***	-49.68%*	

In *P. lanceolata*, there was a significant effect of P source and competitor on competitive response (two-way ANOVA, P source: df=2,43, F=50.08, P<0.001; competitor: df=2,43, F=57.24, P<0.001). Across all P sources, ^{33}P uptake was significantly reduced when in competition with *A. capillaris* (71-94%; Dunnett's P<0.05). There were no significant reductions in ^{33}P uptake in competition with *C. caryophyllea* and *R. acetosa* in the orthophosphate and DNA treatments. In the calcium phosphate treatment, ^{33}P uptake increased significantly when in competition with *C. caryophyllea* (110%; Dunnett's P<0.05) and also increased with *R. acetosa* (14%).

The competitive response of *C. caryophyllea* was significantly affected by P source but not competitor identity (two-way ANOVA, P source: df=2,40, F=11.74, $P<0.001$). For the orthophosphate treatment there were reductions in ^{33}P uptake in response to all species, but this was only significant in response to *A. capillaris* (70%; Dunnett's $P<0.05$). There were significant reductions in ^{33}P uptake in response to each competitor in the calcium phosphate treatment (Dunnett's $P<0.05$). A similar response to *A. capillaris* and *P. lanceolata* was seen in the DNA treatment. However, ^{33}P uptake increased in response to competition from *R. acetosa* (9%), but this was not significant.

There was a significant effect of competitor and P source on the competitive response of *R. acetosa* (Table 3.2; two-way ANOVA, competitor: df=2,42, F=5.12, $P=0.011$; P source: df=2,42, F=28.35, $P<0.001$). In the orthophosphate treatment, ^{33}P uptake was reduced in response to *P. lanceolata* (44%), and *A. capillaris* (84%) which was significant (Dunnett's $P<0.05$). ^{33}P uptake was maintained in response to *C. caryophyllea*. The same pattern was shown in the calcium phosphate treatment, although the reduction in ^{33}P uptake in response to *P. lanceolata* was also significant in this instance (85%; Dunnett's $P<0.05$). *Rumex acetosa* ^{33}P uptake in the DNA treatment showed significant reductions in response to each species (Dunnett's $P<0.05$).

3.5 Discussion

This is the first study to investigate how competition between paired species affects P uptake directly and how these competitive responses differ across contrasting P sources. The interspecific differences in P uptake shown in monocultures are consistent with acquisition adaptations for each species. Importantly, these differences are reduced when in competition with species with contrasting methods of P acquisition. This leads to a reduction in niche differentiation (i.e. an increased overlap of P uptake patterns and less P partitioning) and supports the hypothesis that species specialised in mobilising P from poorly accessible sources may sustain neighbouring species with a superior foraging capacity for available P.

3.5.1 Interspecific differences in uptake of ^{33}P across supplied sources

In the absence of interspecific competition, there were differences in ^{33}P uptake between species across P sources. *Rumex acetosa* and *C. caryophyllea* acquired the most ^{33}P from calcium phosphate. Both of these species belong to families which exhibit P-mining acquisition strategies that are adapted for the mobilisation of P from mineral sources, such as calcium phosphate, through high rates of organic acid exudation (Tyler & Ström 1995; Shane et al. 2006). Organic acids release plant available P from calcium phosphate through lowering rhizosphere pH and the chelation of calcium (Jones 1998).

In this study, DNA was the other supplied P source which is not directly available for plant uptake, and requires hydrolysis before releasing P. As in the calcium phosphate treatments, *R. acetosa* and *C. caryophyllea* both showed the greatest levels of ^{33}P uptake from this organic P source. This pattern could also be linked to the high rates of root exudation which are characteristic of the respective families of

R. acetosa and *C. caryophyllea* (Tyler & Ström 1995; Shane et al. 2006). Root exudates (such as organic acids, amino acids and sugars) provide an easily degradable source of organic carbon which acts as a substrate for the microbial biomass in the soil (Baudoin et al. 2003; Shahzad et al. 2015). A greater input of root exudates will therefore sustain larger populations of fungi and bacteria in the rhizosphere (Lange et al. 2015). This can increase plant P uptake as soil microbes are capable of mobilising P from organic sources through the production of phosphatases (Spohn et al. 2013; Hacker et al. 2015), making it directly available for plant uptake, or indirectly through the subsequent turnover of soil microbes (Vanveen et al. 1987; Macklon et al. 1997; Achat et al. 2010; Turner et al. 2012).

High levels of P uptake in *R. acetosa* were also demonstrated by Orwin et al (2010), who showed higher leaf P content in this species when compared to a range of other grassland species. Likewise, this was linked to increased soil P cycling, as the soils associated with *R. acetosa* showed comparatively high levels of available P.

It was hypothesised that mycorrhizal species in monoculture would acquire more ^{33}P-labelled orthophosphate (a directly accessible P source) due to the superior foraging capacity of their associated mycorrhizal fungal symbionts. However, the findings shown here do not support this, with ^{33}P uptake of orthophosphate in monoculture being greatest in the non-mycorrhizal *R. acetosa,* and lowest in the mycorrhizal *A. capillaris*. This could be caused by the immobilisation of orthophosphate when supplied to the microcosms, through microbial uptake and adherence to soil particles for example. This could therefore restrict access to ^{33}P in mycorrhizal species compared to non-mycorrhizal species, whose superior acquisition of ^{33}P from poorly accessible sources is demonstrated in the other supplied P forms.

3.5.2 Changing ^{33}P uptake patterns in response to interspecific competition

The results from this study show consistent changes in ^{33}P uptake across species in response to interspecific competition. These shifting patterns of ^{33}P uptake did not lead to reductions in niche overlap, as species with contrasting methods of P uptake became more similar under conditions of interspecific competition. This reduction in niche complementarity is in contrast to similar studies which have shown how niche shifts in nitrogen acquisition could be a mechanism which facilitates co-existence (Ashton et al. 2010). The observed changes in ^{33}P uptake were associated with differences in plant P acquisition strategy, as uptake in non-mycorrhizal species was consistently reduced when in competition with mycorrhizal species.

The competitive advantage of mycorrhizal species shown here could be due to the enhanced foraging capacity of their mycorrhizal symbionts, which is consistent with the scavenger-miner coexistence hypothesis (Lambers et al. 2006; Lambers et al. 2008; Li et al. 2014). Likewise it was also proposed that ^{33}P uptake of mycorrhizal 'scavenger' species would be maintained (or increased) in competition with non-mycorrhizal 'mining' species due to scavengers acquiring P which has been liberated by mining species.

For *P. lanceolata*, in the calcium phosphate treatment there were increases in ^{33}P uptake when in competition with non-mycorrhizal species (statistically significant in response to *C. caryophyllea* but not *R. acetosa*). There were reductions in the ^{33}P uptake of *P. lanceolata* in response to non-mycorrhizal species for the other P sources, but these were not significant. *Agrostis capillaris* showed the smallest response to interspecific competition of all species across P sources. The only

significant shift in ^{33}P uptake was a reduction in response to *P. lanceolata* (another mycorrhizal species). The most significant reductions in ^{33}P uptake of non-mycorrhizal species (*C. caryophyllea* and *R. acetosa*) were consistently in response to mycorrhizal species, across all P sources. This is the first study to provide direct evidence of such changes in ^{33}P uptake through the use of radio-isotope labelled P sources on semi-natural soils.

These findings are supported by Muler et al. (2014), who showed reduced P uptake in a cluster-root producing species (*Banksia attenuata*) when in competition with a mycorrhizal species (*Scholtzia involucrata*). This is also in line with studies on P acquisition in intercropped species in agricultural systems. Li et al. (2003) measured P uptake from phytate, a poorly accessible organic P source, in intercropped wheat and chickpea plants. It was shown that, though chickpea was more efficient at mobilising P from phytate, it's P uptake was reduced in response to the competitively dominant wheat plants.

The competitive advantage of mycorrhizal species shown in this study has been attributed to the dense hyphal network of their fungal symbionts, which extends beyond zones of depletion surrounding the roots and within soil particles which broader plant roots cannot access (Smith & Read 2008). Previous studies have shown that the presence of mycorrhizal fungi can have profound effects on plant communities, where the productivity of individual species varies in response to manipulations of mycorrhizal status (Klironomos et al. 2011). Francis and Read (1995) established plant communities which varied in their reliance on mycorrhizal associations. As expected, *P. lanceolata* showed a positive response in yield to the introduction of mycorrhizal fungi to the system. Whereas *R. acetosella* (a sister species of *R. acetosa*), and a range of other species from non-host families, were

hindered by the presence of mycorrhizal fungi. Van der Heijden *et al*. (1998) established calcareous grassland communities in mesocosms which were inoculated with a range of AM species. *Carex flacca* (closely related to *C. caryophyllea*) was the only non-mycorrhizal species in the experiment, and showed the biggest decline in biomass upon the introduction of mycorrhizal fungi to the mesocosms.

The shifts in productivity in response to mycorrhizal fungi treatments have been shown to have mixed effects on plant species diversity. Grime et al. (1987) showed that *R. acetosa* and *Arabis hirsuta* (both non-mycorrhizal species) succumbed to neighbouring mycorrhizal species when communities were inoculated with AM fungi. However, the dominant mycorrhizal species, *Festuca ovina*, also suffered in response to mycorrhizal colonisation. This favoured the subordinate mycorrhizal species within these communities and resulted in a significant increase in diversity. On the other hand, increases in plant species diversity have also been shown to occur in response to the suppression of mycorrhizal fungi, favouring subordinate species which were less reliant on mycorrhizal associations than the dominant species within the community (Hartnett & Wilson 1999).

For investigation of the drivers of community structure and function, belowground interactions with mycorrhizal fungi and other soil microbes have received less attention than aboveground ecological factors (Bardgett & van der Putten 2014). Therefore, while a number of studies have measured the influence of mycorrhizal associations on plant communities, uncertainty remains over their influence compared to other ecological factors (Klironomos et al. 2011).

In theory, the competitive advantage of mycorrhizal species within a calcareous grassland community (as shown here) could lead to a reduction in species richness

through the exclusion of subordinate non-mycorrhizal species. However, the effects of nutrient limitation have been shown to be most severe for dominant species within grassland communities (Jumpponen et al. 2005), and the specialist adaptations of non-mycorrhizal species means that they are well equipped to persist in soils which are nutrient depleted (Shane et al. 2006). Furthermore, the competitive environment created in the microcosms used in this experiment may have been more intense than the conditions found in the field. The characteristic Rendzina soils of calcareous grasslands are rocky and shallow, offering pockets of soil which are absent of root competition from neighbouring species. This would therefore give non-mycorrhizal species the opportunity to persist in spatially discrete conditions in spite of the competitive advantage of mycorrhizal species within the community. Other drivers, such as grazing pressure (Maccherini & Santi 2012; Smith et al. 2014), have also been shown to influence diversity in these grasslands. The findings from this study can be integrated into a theoretical framework which will provide a greater understanding of the maintenance of high species richness in calcareous grasslands, and also other P-limited systems which often have high species richness and a greater frequency of rare species.

3.5.3 Conclusions

This study has highlighted interspecific differences in P uptake across a range of chemical forms among species with contrasting methods of P acquisition. However, these differences were reduced in response to interspecific competition. The greater capacity for P uptake from calcium phosphate and DNA sources in non-mycorrhizal 'mining' species (*C. caryophyllea* and *R. acetosa*) was consistently reduced in response to the competitive advantage of mycorrhizal 'scavenging' species (*A. capillaris* and *P. lanceolata*). On other hand, there were no significant reductions in

P uptake of scavenging species when in competition with mining species. In fact, *P. lanceolata* showed increased P uptake in these interactions. This supports the hypothesis that mycorrhizal species, despite being poorly suited to P-depleted soils, could survive by acquiring P which has been liberated by neighbouring non-mycorrhizal mining species. Given the prevalence of P limitation across terrestrial ecosystems, and its relationship to diversity in plant communities, the role of competitive interactions demonstrated here could be globally important in sustaining the many species rich plant communities in P-limited ecosystems.

Mycorrhizal status alters competition between species with contrasting methods of P acquisition

4.1 Summary

Competition between species with contrasting methods of phosphorus (P) acquisition could play an important role in sustaining species rich plant communities on P-limited soils. It has been proposed that mycorrhizal 'scavenger' species are sustained by co-occurring 'mining' species with specialist root structures which increase the availability of P sources that are otherwise not directly accessible. Though earlier findings support this (Chapter 3), a mechanistic understanding of how these competitive interactions can sustain diverse plant communities is lacking. To investigate this, we manipulated the mycorrhizal status of a scavenger species (*Plantago lanceolata*) and measured the outcome of competition for P uptake from calcium phosphate, a mineral-bound P source, with a non-mycorrhizal mining species (*Carex caryophyllea*) which produces dauciform roots as a specialist adaptation for the acquisition of P from poorly accessible sources. *Plantago lanceolata* individuals colonised by mycorrhizal fungi had the highest levels of P uptake and caused significant reductions in the P uptake of *C. caryophyllea*. Alterations to the mycorrhizal status of *P. lanceolata* therefore demonstrated their reliance on fungal symbionts for a competitive advantage over mining species. Furthermore, despite possessing specialist adaptations for P acquisition, *C. caryophyllea* did not show consistently higher levels of P uptake from calcium phosphate than *P. lanceolata*. This could be an artefact resulting from the removal

of soil microbes and may therefore indicate their importance in optimising *C. caryophyllea'*s dauciform root adaptation for P acquisition. This study provides an important insight into the mechanisms underlying scavenger-miner competitive interactions and demonstrates the competitive advantage provided by mycorrhizal associations. Furthermore, it highlights the need to further investigate the potential importance of associations with an established soil microbial community for P acquisition in mining species.

4.2 Introduction

Many of the world's most diverse plant communities are found in nutrient-poor habitats (Myers et al. 2000). In these communities, co-occurring species are capable of maintaining coexistence despite intense competition for limiting resources. Furthermore, the link between nutrient limitation and high species richness has been clearly demonstrated for phosphorus (P) (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Wassen et al. 2005; Ceulemans et al. 2014). Despite the prevalence of P limitation in terrestrial ecosystems, which approaches nitrogen limitation in its global extent (Elser et al. 2007), the mechanisms that lead to high species diversity under low soil P availability are poorly understood.

A number of theories have been proposed to explain how so many species are capable of coexisting in P-limited conditions. Turner (2008) proposed that co-existence could be facilitated by P partitioning, whereby co-occurring species, with different methods of P acquisition, show preference for different chemical forms of P in the soil and hence reduce interspecific competition for P. Based upon observations of plant communities on the nutrient-poor soils of Western Australia, Lambers et al. (2008) divided plant species into two groups depending on their method of P acquisition. The first of those being 'scavenging' species, which form symbiotic associations with mycorrhizal fungi that enhances their acquisition of freely available P from the soil. The second group, 'mining' species, are more effective at acquiring P which is not directly accessible and requires mobilisation through the production of specialist root structures and exudation of organic acids and phosphatases. It was hypothesised that diversity was sustained in P-limited plant communities because scavengers acquired the otherwise unavailable P which had

been mobilised by their P-mining neighbours (Lambers et al. 2006; Lambers et al. 2008; Li et al. 2014).

Findings from the previous chapter provide support for this 'scavenger-miner' hypothesis as it was shown that interactions between mycorrhizal and non-mycorrhizal species directly affected P uptake patterns in a manner which could sustain mycorrhizal species under conditions of P limitation. P uptake from poorly accessible sources was highest in non-mycorrhizal mining species, but competition with mycorrhizal scavenger species led to changes in P acquisition and reduced differences between species (i.e. reduced niche differentiation). The acquisition of P by non-mycorrhizal species decreased while mycorrhizal species maintained or increased their uptake. This is in line with Muler et al. (2014), who showed that there was a greater yield in a mycorrhizal, scavenging species (*Scholtzia involucrata*) when in competition with a cluster-root producing, mining species (*Banksia attenuata*) when compared to monoculture. These findings demonstrate the predicted response to competition between scavenger and mining species, however the mechanisms behind this outcome are poorly understood.

The ability of mining species to increase P availability from sources in the soil which are not directly accessible to other plants relies on the production of specialist root structures and high rates of root exudation. Specialist root structures, known as 'cluster roots', are found in a range of plant families (e.g. Proteaceae, Fabaceae and Cyperaceae) and are characterised by dense proliferations of root hairs (Shane & Lambers, 2005). These are produced in response to low levels of soil P availability, and enhance P uptake through the release of large amounts of root exudates (Playsted et al. 2006). Organic acids are an important group of these exudates, which chelate calcium and other metal cations (such as iron and aluminium), leading to the

release of mineral-bound P. Furthermore, the binding of organic acids to P-sorbing sites blocks immobilisation of P, maintaining a greater amount in solution and available for plant uptake (Parfitt 1978).

For scavenger species, Lambers et al. (2008) proposed that their ability to acquire the P which has been made available for plant uptake by mining species was due to their enhanced foraging capacity gained through mycorrhizal associations. Arbuscular mycorrhizal (AM) fungi acquire P through their dense hyphal network, which extends beyond zones of depletion surrounding the roots and within soil particles which the broader roots of plants cannot access (Smith & Read 2008). Despite the prevalence of mycorrhizal associations in the plant kingdom, which are found in over 80% of species, few studies have measured the impact of these interactions on community structure and function when compared to the attention that other ecological factors have received (Klironomos et al. 2011).

Towards the end of the last century, early evidence of the effect of mycorrhizal associations on plant communities came from a small number of studies which showed how changes in the productivity of individual species within a community differed in response to manipulations of mycorrhizal status. Francis and Read (1995) established plant communities which varied in their reliance on mycorrhizal associations. *Plantago lanceolata*, a species dependent on mycorrhizal fungi, predictably showed a positive response in yield to the introduction of mycorrhizal fungi to the system. However, species which did not rely on mycorrhizal associations for nutrient uptake were hindered by the presence of mycorrhizal fungi. Likewise, Grime et al. (1987) showed that the productivity of non-mycorrhizal species was reduced by neighbouring mycorrhizal species when plant communities were inoculated with mycorrhizal fungi. However, this also reduced the biomass of

the dominant mycorrhizal species, instead favouring the subordinate mycorrhizal species within these communities, which resulted in a significant increase in diversity. More recently, an increasing amount of studies have demonstrated how mycorrhizal associations can influence the structure and function of plant communities, however further work must be done to integrate these findings with other ecological factors (Klironomos et al. 2011).

The scavenger-miner hypothesis proposes that mycorrhizal scavengers acquire the P which has been mobilised by their non-mycorrhizal mining neighbours (Lambers et al. 2006; Lambers et al. 2008; Li et al. 2014). This provides a potential mechanism for how mycorrhizal fungi could mediate interspecific competition in order to sustain diverse plant communities on P-limited soils. This study directly investigated this by manipulating the mycorrhizal status of a scavenger species and measuring how this affected competition for a mineral-bound P source with a non-mycorrhizal mining species. The non-mycorrhizal mining species was *Carex caryophyllea*, a cluster-root producing sedge, and the scavenger species was *Plantago lanceolata*, which relies on mycorrhizal associations for P acquisition. Results from the previous chapter showed that in monocultures P uptake from calcium phosphate (a mineral-bound P source which requires solubilisation before associated P can be acquired by plants) was higher in *C. caryophyllea* than *P. lanceolata*. In line with the scavenger-miner hypothesis, when in competition with each other P uptake of the scavenger species (*P. lanceolata*) increased while P uptake of the mining species (*C. caryophyllea*) decreased. In this study, P uptake was measured from radioactively-labelled calcium phosphate and microcosms were established with species monocultures or a combination of *P. lanceolata* and *C. caryophyllea*, and with or without mycorrhizal inoculum.

Given the reliance of scavenger species on mycorrhizal associations for P uptake, it was hypothesised that (a) P uptake of *P. lanceolata* would be higher when colonised by mycorrhizal fungi. Furthermore, it was predicted that the competitive advantage of scavenger over mining species could be attributed to their mycorrhizal associations. Therefore it was hypothesised that (b) the increase in the P uptake of *P. lanceolata* when in competition with *C. caryophyllea* should only occur in the mycorrhizal treatments. It was also hypothesised that (c) the reduction in P uptake observed in *C. caryophyllea* should only occur in response to mycorrhizal *P. lanceolata* and (d) in the absence of mycorrhizal colonisation of *P. lanceolata*, *C. caryophyllea* would maintain P uptake.

4.3 Method

4.3.1 Experimental set-up

Test species were grown in polypropylene tubes (height: 11 cm, diameter: 4 cm) with 30 µm mesh covering the base. The substrate within the microcosms consisted of sterilised calcareous dune sand (pH 7.8) collected from Aberfraw, Anglesey, North Wales (53°11'07"N, 4°27'08"W). As well as being P depleted, the low organic matter content of this substrate meant that it could be sterilised through heat treatment (1 hour at 120°C) without substantial leaching of nutrients.

Prior to the experiment, a stock population of *C. caryophyllea* was established from individuals which had been collected from a calcareous grassland field site at Wardlow Hay Cop, Derbyshire, UK (53°15'44"N, 1°43'52"W). After collection, their roots were thoroughly washed in distilled water to remove remaining soil

particles before being transferred to trays containing sterilised calcareous dune sand. After a period of 10 weeks, successfully established individuals within the stock population were selected and, following another thorough root washing in distilled water, transferred to the freshly prepared microcosms. *Plantago lanceolata* seeds (Emorsgate, Kings Lynn, UK) were surface sterilised and germinated in petri dishes before individuals were transferred to each microcosm. Mycorrhizal treatments received 15 g of inoculum (Root Grow, UK) which was added in a layer 2 cm below the surface of the substrate.

Plantago lanceolata and *C. caryophyllea* were grown in separate microcosms, each containing two individuals, as well as being paired together in the same microcosm with one individual of each species. There were ten replicates of each species combination, with half of these receiving the mycorrhizal treatment.

The plants were grown in a controlled environment growth chamber (Conviron BDR16, Conviron, Canada) over a period of 12 weeks. Conditions in the growth chamber were 18 °C/15 °C day/night temperatures with a day length of 12 hours. Irradiance was set at 200 μmol m^{-2} s^{-1}, and CO_2 at 400 ppm. Plants were watered on alternate days throughout the growth period.

4.3.2 ^{33}P supply and uptake analyses

After 11 weeks, each microcosm was supplied with 0.2 MBq of ^{33}P-labelled calcium phosphate. This was synthesised and supplied using the methods outlined in section 2.3.1 and 2.3.4 respectively. In line with previous chapters, the duration of the labelling period was seven days. Following this, plants were harvested and separated into above- and below-ground biomass, freeze-dried and weighed prior to analysis of ^{33}P content.

Post-harvest, successful colonisation of *P. lanceolata* roots receiving the mycorrhizal inoculum was confirmed through Trypan blue root staining (Phillips & Hayman 1970) and the roots of *C. caryophyllea* individuals were inspected for the presence of dauciform roots. Acid digestion and liquid scintillation counting was carried out as outlined in section 2.3.5.

4.3.3 Statistical analyses

A two-way ANOVA was used to measure the effect of mycorrhizal inoculation and interspecific competition on ^{33}P uptake for *P. lanceolata* and *C. caryophyllea*. This was expressed as either tissue concentration or content and separated into above- and below-ground biomass. Tukey HSD tests were then carried out to show where significant differences in the uptake of ^{33}P occurred across competitive interactions. All analyses were carried out using the statistical packages Minitab (Minitab Inc., State College, PA, USA) and R 3.2.2 (R Core Team 2015).

4.4 Results

4.4.1 Tissue concentration of ^{33}P

In *P. lanceolata*, there was a significant effect of mycorrhizal inoculation on tissue concentration of ^{33}P in both above- and below-ground biomass (Table 4.1; Fig 4.1). Above-ground ^{33}P tissue concentration of *P. lanceolata* was significantly higher in mycorrhizal than non-mycorrhizal plants by ten- and seven-fold in monocultures and mixed microcosms respectively (Tukey HSD, $P<0.05$; Fig 4.1a). However, the response to interspecific competition within both mycorrhizal and non-mycorrhizal treatments was not significant.

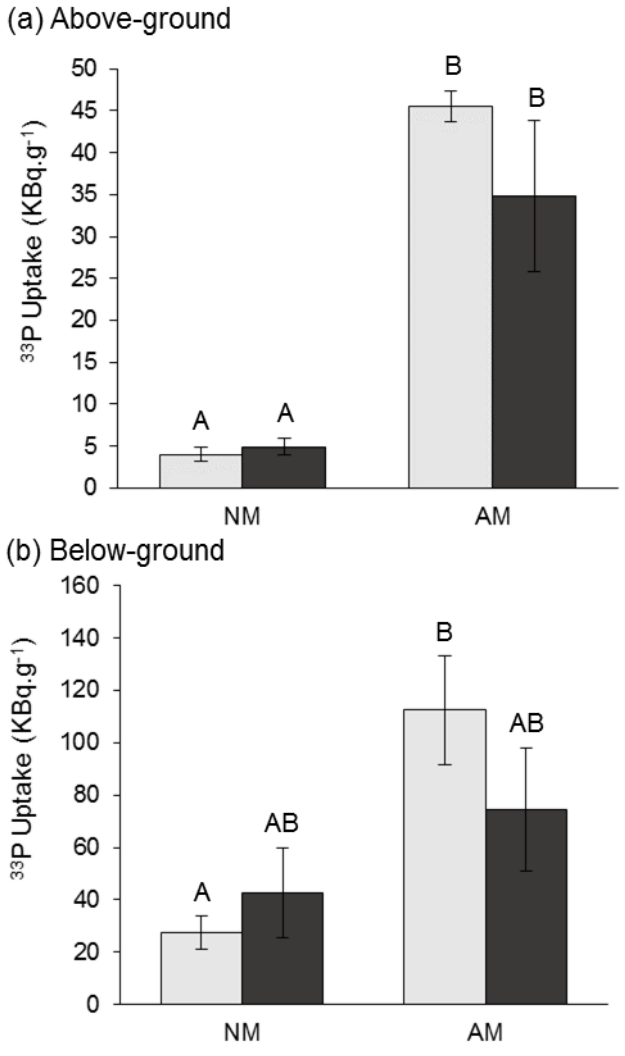

Figure 4.1: *Plantago lanceolata* tissue ^{33}P concentration separated into (a) above-ground biomass and (b) below-ground biomass. Grey bars and black bars represent monocultures and mixed species microcosms respectively. 'AM' treatments received mycorrhizal inoculum, whereas 'NM' treatments did not. Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 4.1 for statistics.

Table 4.1: Two-way ANOVA results for the impact of mycorrhizal inoculation and interspecific competition on plant tissue ^{33}P concentration. Significant effects ($p \leq 0.05$) are shown in bold.

		Factor	df	F	*P*
Plantago lanceolata	Above-ground	Mycorrhizal inoculation	1,16	67.2	**<0.001**
		Interspecific competition	1,16	1.23	0.288
		Myc*Comp	1,16	1.76	0.208
	Below-ground	Mycorrhizal inoculation	1,16	11.58	**0.005**
		Interspecific competition	1,16	0.45	0.516
		Myc*Comp	1,16	2.37	0.148
Carex caryophyllea	Above-ground	Mycorrhizal inoculation	1,18	3.45	0.083
		Interspecific competition	1,18	0.74	0.402
		Myc*Comp	1,18	7.45	**0.016**
	Below-ground	Mycorrhizal inoculation	1,18	0.31	0.584
		Interspecific competition	1,18	3.67	0.075
		Myc*Comp	1,18	0.01	0.917

Below-ground, there was a significant difference between mycorrhizal and non-mycorrhizal treatments of *P. lanceolata* monocultures, with tissue concentration of ^{33}P in mycorrhizal plants three times higher than non-mycorrhizal plants (Tukey HSD, $P<0.05$; Fig 4.1b). However, tissue concentrations of ^{33}P in *P. lanceolata* individuals exposed to interspecific competition were not significantly different between mycorrhizal or non-mycorrhizal treatments, and also did not differ from the monocultures of mycorrhizal and non-mycorrhizal plants.

There was no significant effect of mycorrhizal treatment or interspecific competition across above- and below-ground measurements of tissue ^{33}P concentration in *C. caryophyllea* (Table 4.1; Fig 4.2). However, in the above-ground biomass of *C. caryophyllea* there was a significant interaction between mycorrhizal treatment and interspecific competition (Fig 4.2a). Above-ground tissue ^{33}P concentration of *C. caryophyllea* exposed to interspecific competition was significantly reduced by 85% in the microcosms which received mycorrhizal inoculum compared to those that did

not (Tukey HSD, $P<0.05$). There was no difference between *C. caryophyllea* monocultures under the mycorrhizal and non-mycorrhizal treatments. Below-ground, there were no significant differences across treatments, although there was a trend for higher concentration of ^{33}P in *C. caryophyllea* monocultures (Fig 4.2b).

4.4.2 Tissue content of ^{33}P

The concentration of ^{33}P was an order magnitude greater in below-ground biomass compared to above-ground for both *P. lanceolata* and *C. caryophyllea*. Therefore, P uptake at the whole plant level was expressed as biomass ^{33}P content, rather than tissue concentration.

There was a significant effect of mycorrhizal inoculation on ^{33}P content of *P. lanceolata* at the whole-plant level as well as when separated into above- and below-ground biomass (Table 4.2). Above-ground, ^{33}P content was significantly higher in mycorrhizal treatments, with an almost twenty fold increase between monocultures and seven fold increase between mixed microcosms (Tukey HSD, $P<0.05$; Fig 4.3a). There was a similar trend for higher ^{33}P content in mycorrhizal *P. lanceolata* below-ground, but there were no significant differences across treatments (Fig 4.3b). At the whole-plant level, the significant difference between mycorrhizal treatments was maintained in mycorrhizal *P. lanceolata* monocultures, whose tissue ^{33}P content was six times greater than non-mycorrhizal monocultures (Tukey HSD, $P<0.05$; Fig 4.3c). However, reductions in the ^{33}P content of mycorrhizal *P. lanceolata* in response to interspecific competition meant that, in these treatments, ^{33}P uptake was not significantly different between mycorrhizal and non-mycorrhizal *P. lanceolata* (Fig 4.3c).

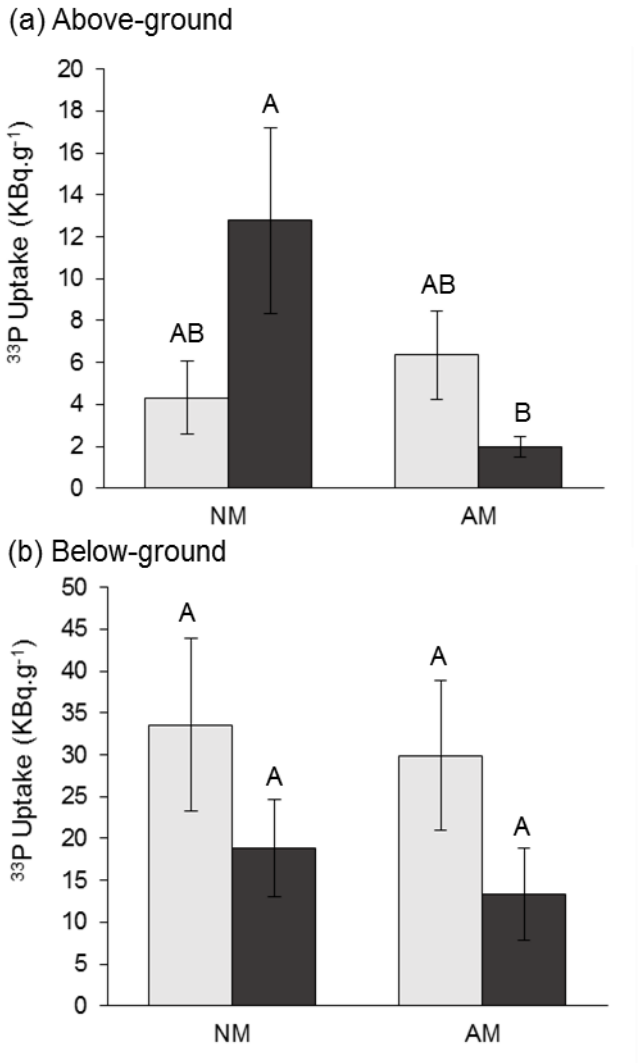

Figure 4.2: *Carex caryophyllea* plant tissue ^{33}P concentration separated into (a) above-ground biomass and (b) below-ground biomass. Grey bars and black bars represent monocultures and mixed species microcosms respectively. 'AM' treatments received mycorrhizal inoculum, whereas 'NM' treatments did not. Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 4.1 for statistics.

Table 4.2: Two-way ANOVA results for the impact of mycorrhizal inoculation and interspecific competition on plant tissue ^{33}P content. Significant effects ($P<0.05$) are shown in bold.

		Factor	df	F	*P*
Plantago lanceolata	Above-ground	Mycorrhizal inoculation	1,16	31.16	**<0.001**
		Interspecific competition	1,16	0.32	0.58
		Myc*Comp	1,16	0.71	0.415
	Below-ground	Mycorrhizal inoculation	1,16	5.06	**0.042**
		Interspecific competition	1,16	0.30	0.595
		Myc*Comp	1,16	2.15	0.167
	Total	Mycorrhizal inoculation	1,16	17.29	**0.001**
		Interspecific competition	1,16	0.49	0.56
		Myc*Comp	1,16	1.56	0.234
Carex caryophyllea	Above-ground	Mycorrhizal inoculation	1,18	2.20	0.158
		Interspecific competition	1,18	0.02	0.882
		Myc*Comp	1,18	2.69	0.122
	Below-ground	Mycorrhizal inoculation	1,18	0.00	0.945
		Interspecific competition	1,18	2.48	0.136
		Myc*Comp	1,18	0.05	0.826
	Total	Mycorrhizal inoculation	1,18	0.08	0.781
		Interspecific competition	1,18	2.04	0.174
		Myc*Comp	1,18	0.20	0.664

There were no significant effects of mycorrhizal treatment or interspecific competition on the ^{33}P content of *C. caryophyllea* biomass (Table 4.2). However, there was a general trend for reduced ^{33}P content in response to interspecific competition (Fig 4.4). Though not significant, whether inoculated or not, this was the same at a whole plant level as well as below-ground, with the exception of the above-ground biomass of *C. caryophyllea* in microcosms without added inoculum (where ^{33}P content remained the same).

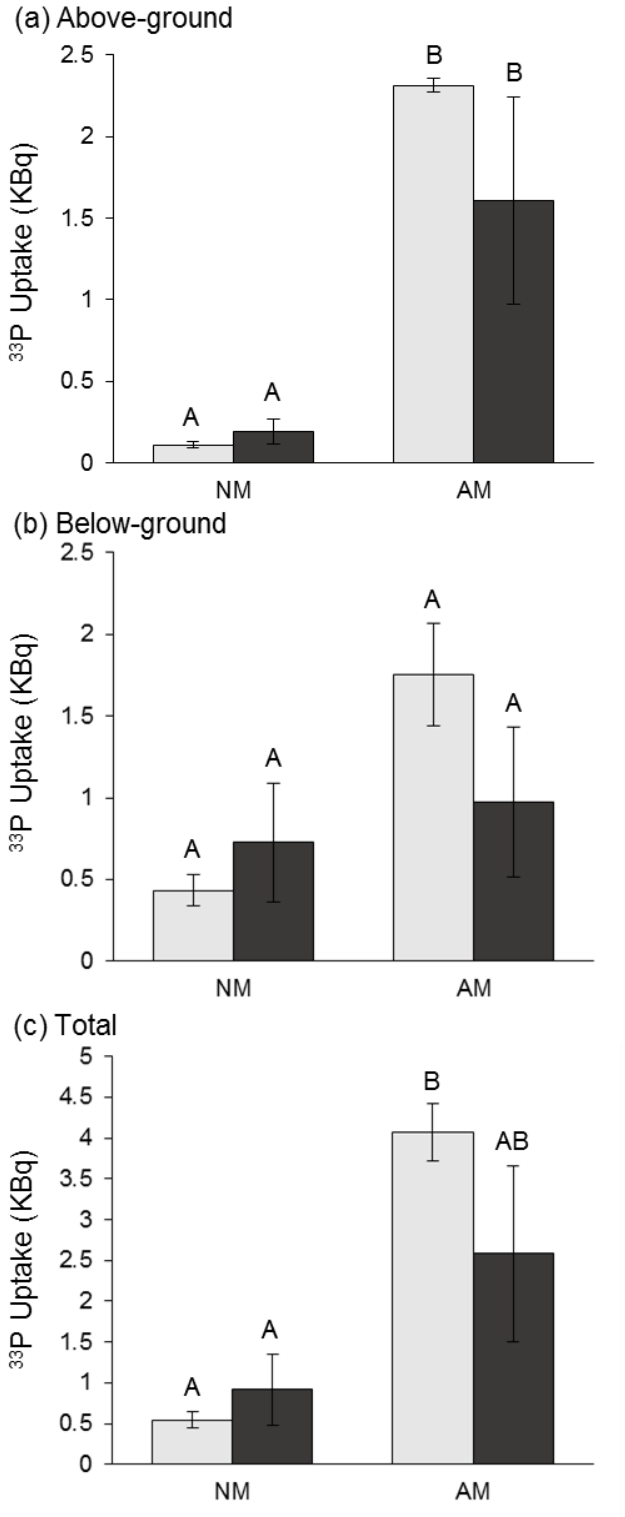

Figure 4.3: *Plantago lanceolata* plant tissue ^{33}P content separated into (a) above-ground biomass, (b) below-ground biomass and combined into (c) total biomass. Grey bars and black bars represent monocultures and mixed species microcosms respectively. 'AM' treatments received mycorrhizal inoculum, whereas 'NM' treatments did not. Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 4.2 for statistics.

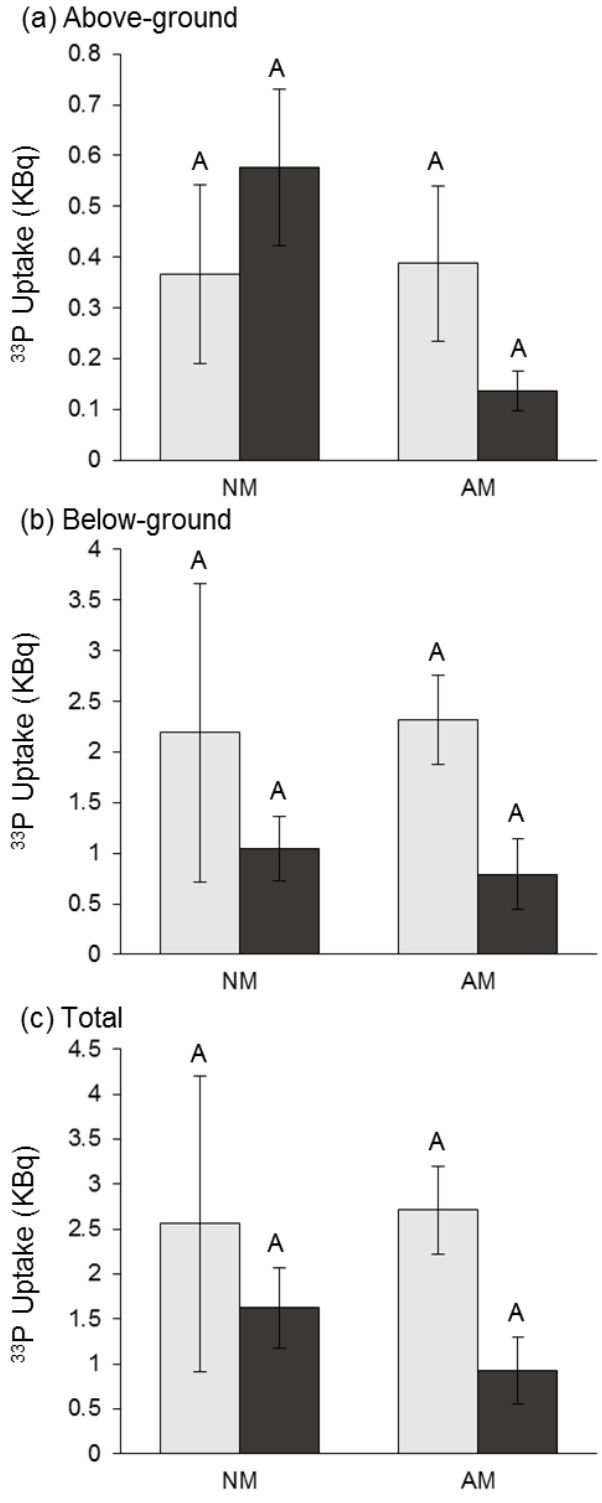

Figure 4.4: *Carex caryophyllea* plant tissue ^{33}P content separated into (a) above-ground biomass, (b) below-ground biomass and combined into (c) total biomass. Grey bars and black bars represent monocultures and mixed species microcosms respectively. 'AM' treatments received mycorrhizal inoculum, whereas 'NM' treatments did not. Means are shown ± 1 s.e.m. Tukey HSD tests were carried out to show where significant differences occurred within species treatments. Means with the same letter do not differ significantly from each other. See Table 4.2 for statistics.

4.5 Discussion

This study investigated how mycorrhizal associations directly affect P uptake between competing species with contrasting methods of P acquisition. The effect of mycorrhizal associations and interspecific competition were measured separately and in combination. As expected, mycorrhizal inoculation had a significant effect on the P uptake of *P. lanceolata* but not *C. caryophyllea*, due to the former's reliance upon mycorrhizal associations for nutrient acquisition and a lack of this reliance in the latter. Of greater significance is the absence of a response to interspecific competition in both species without mycorrhizal inoculation. This shows the importance of mycorrhizal fungi in mediating competition between scavenger and mining species, as demonstrated in the reduction in P uptake by the non-mycorrhizal *C. caryophyllea* only when *P. lanceolata* was mycorrhizal. These findings provide a mechanistic explanation for how competition between species with contrasting methods of P acquisition can cause changes in P uptake which influence community dynamics in P-limited systems.

4.5.1 Impact of mycorrhizal colonisation on ^{33}P acquisition

Mycorrhizal treatments resulted in clear differences in the ^{33}P uptake of *P. lanceolata*. Tissue concentration of ^{33}P was consistently higher in mycorrhizal than non-mycorrhizal *P. lanceolata*, in both monocultures and mixed microcosms. The difference in the amount of ^{33}P uptake in *P. lanceolata* between mycorrhizal treatments highlights the reliance upon mycorrhizal associations for P acquisition in this species. In the absence of mycorrhizal fungi, the simple rooting system of *P. lanceolata* (consisting of coarse roots with few root hairs) was poorly equipped for the acquisition of ^{33}P from the supplied mineral-bound P source. In comparison,

mycorrhizal *P. lanceolata* acquired significantly more ^{33}P from calcium phosphate. This also suggests that the inoculated AM fungi were capable of mobilising mineral-bound P, since any other P-solubilising soil microbes within the native microbial community would have been removed through sterilisation prior to the experiment. This is in line with previous studies which have demonstrated mineral weathering by AM fungi in the field (Koele et al. 2014).

The mycorrhizal treatment had no effect on the ^{33}P uptake of *C. caryophyllea*, which was the expected outcome of a species that relies on dauciform root production as a specialist adaptation for P acquisition rather than forming symbiotic mycorrhizal associations.

4.5.2 Impact of interspecific competition on ^{33}P acquisition

There was no significant effect of interspecific competition on the P acquisition of *P. lanceolata.* In non-mycorrhizal treatments, plants acquired relatively small amounts of ^{33}P which showed no discernible response to competition from *C. caryophyllea*. In mycorrhizal plants, the lack of response to interspecific competition may be due to the superior foraging capacity of their fungal partners which was capable of maintaining high levels of ^{33}P uptake in the presence of *C. caryophyllea*. Likewise, *C. caryophyllea* showed no significant effect of interspecific competition on P acquisition in both the mycorrhizal and non-mycorrhizal treatments.

4.5.3 Interaction of mycorrhizal associations and interspecific competition on ^{33}P acquisition

It was hypothesised that when in competition with *C. caryophyllea*, ^{33}P uptake would increase only in mycorrhizal *P. lanceolata.* However, there was no significant

interaction between mycorrhizal treatment and interspecific competition in *P. lanceolata*. While ^{33}P uptake was higher in mycorrhizal plants, the absence of a further increase when in competition with *C. caryophyllea* could be due to the relatively low levels of ^{33}P uptake of the sedge competitor. In the previous chapter, *C. caryophyllea* showed high levels of ^{33}P uptake from calcium phosphate. In Chapter 3, increased availability of P from this source due to the liberating action of *C. caryophyllea* roots (i.e. the mining species) could have subsequently increased ^{33}P uptake of co-occurring *P. lanceolata* (i.e. the scavenging species). However, in contrast to the results from Chapter 3, ^{33}P uptake from calcium phosphate of mycorrhizal *P. lanceolata* in this experiment was greater than *C. caryophyllea*. Therefore, in this chapter, an apparent reduced capacity of *C. caryophyllea* to liberate ^{33}P from calcium phosphate may have caused the absence of a clear effect on the ^{33}P uptake of co-occurring *P. lanceolata* under interspecific competition (discussed further in section 4.5.4).

The interaction between mycorrhizal inoculation and interspecific competition had a significant effect on P acquisition of *C. caryophyllea.* In mixed microcosms, ^{33}P uptake by the sedge was significantly reduced by competition from mycorrhizal *P. lanceolata.* This could be because the hyphae of mycorrhizal fungi, when compared to the roots of *C. caryophyllea*, are better equipped for P acquisition due to their smaller diameter which increases their surface area to volume ratio and allows them to forage within soil particles which the broader roots of plants cannot access (Smith & Read, 2008). This supports the hypothesis that *P. lanceolata* would only possess a competitive advantage over *C. caryophyllea* when they were able to form mycorrhizal associations.

Changes in ^{33}P uptake in the current experiment represent short-term competitive responses. If these differences in P uptake were maintained over a longer time-scale, we would expect to see increased productivity in mycorrhizal *P. lanceolata* at the expense of *C. caryophyllea*. This is in line with studies which have measured changes in the productivity of individual species within a plant community and have shown that mycorrhizal species are favoured at the expense of non-mycorrhizal species (Francis & Read 1995; van der Heijden et al. 1998).

Only one of the two species in this study rely on mycorrhizal associations for P uptake (*P. lanceolata*). Previous studies have investigated how AM fungi interact with competition between multiple mycorrhizal species which differed in their dominance within the plant community. Mariotte et al. (2013) investigated the competitive interactions between mycorrhizal species in similar experimental conditions. Inoculation with AM fungi was shown to have a detrimental effect on the growth of plant species, with a greater impact on dominant species that subsequently favoured subordinate species. At the community level, Grime et al. (1987) also showed that, as well as non-mycorrhizal species, the dominant mycorrhizal species suffered in response to the addition of mycorrhizal fungi. This favoured the subordinate mycorrhizal species within these communities and subsequently increased plant species diversity. However, in communities where subordinates were less reliant on mycorrhizal associations than the dominant species, increased plant community diversity was brought about instead by the suppression of mycorrhizal fungi (Hartnett & Wilson 1999; Smith et al. 1999). These varying impacts of mycorrhizal associations on plant communities highlight the complexity of belowground interactions. Despite this, the focus of research on plant community dynamics has largely remained aboveground, and less progress has

been made in understanding the influence of belowground interactions on community structure and function (Bardgett & van der Putten 2014).

It has been proposed that mycorrhizal associations could help to maintain species richness in P-limited plant communities as they would allow scavenger host-plants to acquire P which had been mobilised by their P-mining neighbours (Lambers et al. 2006; Lambers et al. 2008; Li et al. 2014). Furthermore, the dominance of mycorrhizal scavengers in this scenario would be limited by their reliance on co-occurring mining species due to the scavenger's comparatively low capacity for the acquisition of P from poorly accessible sources (demonstrated in Chapter 3). This is supported by findings which show that the effects of nutrient limitation are most severe for dominant species within grassland communities (Jumpponen et al. 2005).

4.5.4 Differences between contrasting methods of P acquisition

This study investigated the influence of mycorrhizal associations and interspecific competition on the ^{33}P uptake of plant species with contrasting methods of P acquisition. We focused on interspecific differences in the response to these factors, rather than differences in ^{33}P uptake *per se*. None-the-less, the results show that ^{33}P uptake was broadly similar between *P. lanceolata* and *C. caryophyllea*, while the tissue concentration of ^{33}P in the above-ground biomass of mycorrhizal *P. lanceolata* was higher than *C. caryophyllea*. These findings are in contrast to those of Chapter 3 which showed that ^{33}P uptake from calcium phosphate of *C. caryophyllea* was ten times higher than *P. lanceolata* (when in monoculture).

Differences in experimental conditions may be a cause of the apparent disparity between the results for ^{33}P uptake of *C. caryophyllea* in the current and previous chapter. The substrate in the current experiment was low in organic matter and

sterilised to allow manipulation of mycorrhizal status, which would have also removed any other microbes. In contrast, the microcosms in Chapter 3 contained soil gathered from the field with the microbial biomass still intact.

In the absence of an established soil microbial community in this study, *C. caryophyllea* would have relied upon the direct effect of root exudation of organic acids for the mobilisation of ^{33}P from calcium phosphate. However, it has previously been suggested that the plant production of organic acids alone has little impact on P mobilisation in calcareous soils (Jones 1998). This is because the concentration of supplied organic acids needed to have a noticeable effect on P availability is larger than any reported values for rhizosphere and bulk soils (Staunton & Leprince 1996; Ström et al. 2005). Therefore, this could partly explain why P acquisition in *C. caryophyllea* was relatively low compared to *P. lanceolata.*

It is also possible that the removal of the native microbial community could have had a knock-on effect on the ability of *C. caryophyllea* to acquire P from calcium phosphate. The characteristic high rates of root exudation in cluster-root producing species could indirectly increase soil P availability through the stimulation of microbial activity. Plant root exudates, such as amino acids, organic acids and other sugars, provide an easily degradable source of organic carbon which acts as a substrate for soil microbes (Shahzad et al. 2015; Baudoin et al. 2003). A high rate of root exudation, as seen from the production of dauciform roots (Playsted et al. 2006), could therefore sustain a larger population of fungi and bacteria in the rhizosphere (Lange et al. 2015). This can enhance plant P uptake through direct increases in the availability of soil P due to the activity of phosphate-solubilising bacteria, as well as indirectly through the release of nutrients due to turnover of the

microbial biomass (Vanveen et al. 1987; Macklon et al. 1997; Richardson et al. 2001; Achat et al. 2010; Marschner et al. 2011; Turner et al. 2012).

Interactions between plants and soil microbes could be a significant factor in the maintenance of diversity in P-limited plant communities which therefore requires further consideration (Bardgett et al. 2014). It has been shown that plant communities with greater levels of diversity also had significantly increased biomass and activity within the soil microbial community (Zak et al. 2003; Lange et al. 2014; Hacker et al. 2015). This relationship may be linked to species-specific effects on microbial communities which can occur through root exudation (Zhang et al. 2014). Grayston et al. (1998) isolated microbial communities from a range of plant species and showed clear differences in their utilisation of carbon sources analogous to different root exudates.

The differences in microbial communities between species, and within rooting systems, could be intrinsically linked to P acquisition. Plant functional traits have been associated with changes in microbial communities (de Vries et al. 2012; Legay et al. 2014) that have knock-on effects on rhizosphere processes which influence the plant acquisition of P (Wardle et al. 2004). However, further work is required to understand how the diversity of plant communities could be mediated by interactions with soil microbes. Associations with the microbial community could be a key component of *C. caryophyllea*'s method of P acquisition. The removal of the native soil microbial community may therefore explain the altered ^{33}P uptake patterns of *C. caryophyllea* relative to *P. lanceolata* in this experiment when compared to findings of the previous chapter.

4.5.5 Conclusion

This study focused on the impact of mycorrhizal inoculation and competitive interactions on P uptake between species with contrasting methods of P acquisition. As expected, only the mycorrhizal scavenger species showed a positive response to the introduction of AM fungi. Interspecific competition in the absence of mycorrhizal inoculation did not affect P acquisition, but with mycorrhizal inoculum, the ^{33}P uptake of non-mycorrhizal mining species (*C. caryophyllea*) was reduced in response to the mycorrhizal scavenging species (*P. lanceolata*). This indicates that the competitive advantage of scavenger species requires mycorrhizal associations, which provide a superior foraging capacity to host plants through the dense and extensive hyphal network in the soil. Mycorrhizal species could therefore be sustained in species-rich P-limited plant communities, where they scavenge P among other species that may have adaptations better suited to obtaining less bioavailable P. These findings demonstrate the importance of interactions between plants and soil microbes and highlights the need to include them among the other factors which shape species richness in the many diverse plant communities around the world that are exposed to P-limitation.

Responses of P acquisition by soil microbes to plant species with different methods of P acquisition

5.1 Summary

Belowground interactions with soil microbes influence plant P acquisition in a range of ways - from direct associations with mycorrhizal fungi and phosphate-solubilising bacteria, to indirect effects of microbial turnover and nutrient cycling in the soil. So, the impact of plant-microbe interactions on P acquisition in P-limited soils could play an important role in mediating plant competition and co-existence. There is a need, therefore, to understand how microbial P uptake is affected by plant species. In this study we measured the impact of plants on microbial P in soils with mixtures and monocultures of four P-limited calcareous grassland plant species. This included species of grass, sedge and forb that have contrasting root adaptations for P acquisition, including mycorrhizal associations, specialist root structures and root exudation. Each monoculture and four-species mixed community mesocosm was supplied with ^{33}P-labelled calcium phosphate, a mineral-bound P source which represents a major portion of P in calcareous soils. ^{33}P acquired from this P source was subsequently measured in the soil microbial biomass and plants. Changes in microbial P uptake could be driven by microbial species which differ in their capacity to mobilise P. Therefore the influence of plant monocultures and mixed communities on microbial community structure and species richness was measured using terminal restriction fragment length polymorphism (TRFLP) analysis. There was limited evidence of differences in microbial ^{33}P uptake in response to different

plant species, and this was not consistent with differences in plant ^{33}P uptake (i.e. plant species with higher ^{33}P uptake were not associated with greater amounts of microbial ^{33}P). This implies that microbial ^{33}P uptake and turnover did not contribute to demonstrated differences in plant ^{33}P uptake from calcium phosphate. Combining all four species into mixed plant communities significantly increased microbial ^{33}P uptake compared to microbial uptake under plant monocultures. Associated plants did not appear to benefit from this in their tissue concentration of ^{33}P, which could be due to increased microbial competition for P. However, the short time-scale of this study offered little opportunity for plants to access ^{33}P through subsequent microbial turnover. Beyond the timescale considered here, microbial turnover could provide associated plant communities with indirect access to this limiting resource, such that greater microbial ^{33}P may ultimately lead to greater plant ^{33}P. These results show that mixed plant communities play an important role in stimulating the release of ^{33}P from calcium phosphate through microbial uptake, compared to microbes associated with plant monocultures. Given the high levels of calcium phosphate in calcareous soils, mobilising P from this important soil source could provide a vital long-term supply of P which sustains diverse plant communities limited by the availability of this resource.

5.2 Introduction

Interactions between plants and soil microbes can have significant effects on plant community structure and function (Van Der Heijden et al. 2008). This can range from direct impacts, such as mutualistic associations with mycorrhizal fungi or the detrimental impact of soil pathogen infection, to indirect effects through regulation of ecosystem processes, such as decomposition, nutrient retention and nutrient cycling (Balser & Firestone 2010; Klironomos et al. 2011; Wagg et al. 2014; Pii et al. 2015). Despite this, the majority of research on plant community dynamics has focused aboveground, and less progress has been made in understanding the importance of belowground interactions (Bardgett & van der Putten 2014).

Nutrient availability is an important regulator of plant communities, which can it itself be influenced by interactions with soil microbes. Microbial activity stimulated by plants drives the mineralization of soil organic matter and increases N and P availability across a range of soil types and plant communities (Dijkstra et al. 2009; Dijkstra et al. 2013; Brzostek et al. 2013; Shahzad et al. 2015). However, the influence of plant-microbe interactions on nutrient cycling from inorganic sources has received less attention. This could play an important role in P-limited calcareous grasslands, where large portions of soil P are contained in calcium phosphates (Zhang et al. 2014).

Despite low P availability, high levels of plant species richness are sustained in calcareous grassland communities (Janssens et al. 1998; Critchley et al. 2002; Ceulemans et al. 2014). Coexistence between species competing for this limited resource could be maintained through partitioning of soil P sources through contrasting methods of plant P acquisition (Turner 2008). Furthermore, coexistence

could also be sustained by species increasing P availability for co-occurring species (Lambers et al. 2008).

Investigation of the mechanisms which sustain plant species richness in P-limited communities have focused largely on plant-plant interactions. However, plant-microbe interactions could also play an important role in providing access to soil P for species competing for this limited resource. Plant P uptake is influenced by a diverse range of soil microbes - from mycorrhizal fungi, which effectively forage for P through their dense and extensive hyphal networks supported by their host plants (Smith & Read 2008), to free-living phosphate-solubilising bacteria which mobilise P from poorly accessible sources through the release of organic acids and phosphatases (Kim et al. 1998; Vessey 2003).

Studies on the effects of plant diversity which have focused below-ground have shown that increased P mobilisation in species rich plant communities is driven by soil microbes (Hacker et al. 2015). Higher levels of microbial P uptake could feedback into plant P uptake, either directly through increased P mobilisation by phosphate-solubilising bacteria or indirectly through turnover of the microbial biomass (Vanveen et al. 1987; Macklon et al. 1997; Richardson et al. 2001; Marschner et al. 2011). Increasing plant access to P from relatively recalcitrant sources could subsequently reduce interspecific competition for plant P acquisition. Therefore, a positive feedback between microbial and plant P uptake may play a key role in sustaining species richness in calcareous grassland communities. However, the influence of plants on microbial P uptake in these systems is poorly understood. While the effect of plant-microbe interactions on accessing P from organic sources has been demonstrated (Hacker et al. 2015), mineral-bound P sources such as

calcium phosphate represent a significant source of soil P in calcareous soils which have not been investigated (Zhang et al. 2014).

The rhizosphere describes the layer of soil surrounding plant roots which provides the interface for interactions with soil microbes. Plants influence microbial activity through the exudation of compounds such as organic acids, amino acids and sugars from their roots. These are an easily degraded source of organic carbon (C) which act as a substrate for the soil microbial biomass (Baudoin et al. 2003; Shahzad et al. 2015). Increased root exudation influences microbial P uptake by stimulating microbial activity and sustaining a larger microbial biomass (Hamilton & Frank 2001). This is seen in studies which have demonstrated increased microbial activity in diverse plant communities where carbon inputs to the rhizosphere are increased (Lange et al. 2015; Thakur et al. 2015). Interspecific differences between plant species in the rate of root exudation may also contribute to differences in P uptake of the associated microbial biomass. This could drive interspecific differences in plant P uptake, if species which stimulate microbial activity through root exudation subsequently gain increased access to P through microbial turnover and P mobilisation.

Previous studies have demonstrated how soil microbes respond to interspecific differences in root exudates (Zhang et al. 2014), and species-specific differences in associated microbial communities have been shown across a range of different plant species (Kuske et al. 2002; Costa et al. 2006; Burns et al. 2015). This sensitivity has been demonstrated down to the level of different rooting zones of the same plant species (Marschner et al. 2004). These microbial responses could have knock-on effects on P uptake, since bacterial species have been shown to differ in their capacity to mobilise P from a range of soil P sources (Rodríguez & Fraga 1999;

Richardson et al. 2001; Pii et al. 2015). Interspecific differences in the composition of microbial communities driven by plant species could lead to selective enhancement of microbes which vary in their ability to acquire soil P (Reynolds et al. 2003; Marschner et al. 2011). Plant-driven changes in microbial communities have shown reciprocal influences on the cycling of other important soil resources such as nitrogen (Zak et al. 2003). However, similar relationships between microbial community composition and P cycling have not been investigated.

In this study we investigated the response of soil microbial communities to mixtures and monocultures of plant species with contrasting root adaptations for P acquisition – from mycorrhizal associations through to specialist root structures and root exudation. For this we measured microbial and plant P derived from a radio-isotope labelled mineral-bound P source (calcium phosphate) injected into the soil. Changes in microbial community composition and levels of microbial species richness between the different plant monocultures and the mixed community were also investigated using terminal restriction fragment length polymorphism (TRFLP) analysis. This made it possible to see whether changing patterns of microbial P uptake across plant communities were matched with changes in microbial community composition.

We used natural soil collected from a P-limited calcareous grassland and established the following species: *Agrostis capillaris* (mycorrhizal grass), *Plantago lanceolata* (mycorrhizal forb), *Rumex acetosa* (non-mycorrhizal forb) and *Carex caryophyllea* (non-mycorrhizal sedge with dauciform roots).

We hypothesised that (a) higher levels of microbial P uptake would match higher levels of plant P acquired from calcium phosphate. Also (b) microbial P uptake

would respond positively to mixed plant community mesocosms. Lastly, we hypothesised that (c) differences in soil microbial communities would reflect differences in microbial P uptake.

5.3 Method

5.3.1 Experimental set-up

The selected plant species consisted of *Agrostis capillaris* (mycorrhizal grass), *Plantago lanceolata* (mycorrhizal forb), *Rumex acetosa* (non-mycorrhizal forb) and *Carex caryophyllea* (non-mycorrhizal sedge with dauciform roots). These were established in mesocosms (7x7x8 cm) containing Rendzina soil (pH 6.5) collected from a calcareous grassland field site at Wardlow Hay Cop, Derbyshire (53°15'44"N, 1°43'52"W). The soil associated with calcareous grasslands is commonly P-limited, and P-limitation has been previously documented in field measurements at this site (Phoenix et al. 2003). For collection, soil was removed to bedrock, air-dried and sieved (2 mm). Each species was grown separately in monoculture as well as being combined into a mixed community of all four species. Four replicates of each monoculture and mixed community were prepared. In addition, mesocosms containing soil but no plant species were set-up to serve as a control soil-only treatment for microbial analyses.

Seeds of *A. capillaris*, *P. lanceolata*, and *R. acetosa* (Emorsgate, Kings Lynn, UK) were sown directly into the mesocosms while *C. caryophyllea* individuals were collected from Wardlow Hay Cop and transplanted into the pots at the same time as when seeds were sown. Mesocosms were established over a period of 20 weeks in a

climate controlled greenhouse with conditions set at 16 hours daylight (with supplementary light when necessary), day/night temperatures of 20°C/15°C, and regular watering.

5.3.2 Microbial community analyses

After 18 weeks, three replicate soil samples were collected from mixed community mesocosms, each species monoculture and the control soil treatment. This was done by inserting a corer through the soil profile which yielded 1-2 g of soil (fresh weight). For each replicate, four subsamples were collected and homogenised to create the replicate sample.

From each sample, 0.25g of soil was taken for DNA extraction using a PowerSoil DNA isolation kit (MoBio). In the DNA extracts, 16s rRNA gene sequences were targeted from a broad range of bacteria (including *Pseudomonas* spp) with primers 799F (AACMGGATTAGATACCCKG) and 1193r (ACGTCATCCCCACCTTCC) which avoided amplification of subunit rRNA genes derived from plant chloroplasts. A 0.75 uL aliquot of each DNA sample was added to a PCR master mix consisting of 35 uL reaction buffer (5mM dNTPs, 15mM $MgCl_2$, stabilizers and enhancers), 1 uL of each primer in 20 uM solution and 0.5 uL of myTaq DNA polymerase (Bioline, London, United Kingdom) diluted to a final volume of 50 uL with nuclease free water. The thermocycler program started with denaturation at 94 ºC for 2 minutes, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 57 ºC for 30 s and elongation at 72 ºC for 30 s, and ended on a final extension of 72 ºC for 7 min. An aliquot of the amplified PCR product for each sample was stained with ethidium bromide and visualized using UV radiation on a 1% agarose gel.

PCR products were purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. Preliminary tests were carried out with two different restriction enzymes (aluI and cfoI) to determine which yielded the most fragments. Digestion of PCR products was subsequently carried out using restriction enzyme cfoI in a reaction mixture consisting of 1.5 uL reaction buffer, 0.2 uL acetylated bovine serum albumin, 0.5 uL restriction enzyme (Promega) and 1 uL PCR product. Samples were diluted to a final volume of 20 uL with sterile, deionised water and then incubated at 37 ºC for 3 hours. Afterwards, digest products from each sample were desalted by precipitation with 13.125 uL ice cold 95% ethanol and 0.525 uL 3M sodium acetate (pH 5.2) with 0.25µl glycogen (20 mg/ml) as a carrier. Samples were centrifuged at 14000 x *g* for 20 minutes at 4 °C to form a pellet and washed twice in 70% (v/v) ethanol.

For TRFLP analysis, the desalted digest products were resuspended in 10 uL deionised formamide with 0.5% GeneScan 500 ROX internal size standard (Applied Biosystems). Samples were then denatured by heating at 95 ºC for 5 minutes and cooled on ice. Fragment size analysis was carried out using capillary electrophoresis on an ABI 3730 PRISM capillary DNA analyser (Applied Biosystems). The terminal restriction fragments (T-RFs) from each sample produced electropherograms which were analysed using Peak Scanner v2.0 (Thermo Fisher Scientific). For each sample, fragment profiles were expressed as peak area and aligned with a confidence interval of 0.7 bp using T-REX software (Culman et al. 2009). Fragments with peak heights less than 50 arbitrary units of fluorescence were removed to reduce noise.

For every sample, the relative abundance of each T-RF was calculated by dividing its peak height by the total area of all T-RFs in the electropherogram. Fragments

with a relative abundance of 0.5% of the total area were removed which reduced the effect of electropherogram variations potentially caused by differences in the quantity of DNA analysed. This was combined with binary data recording the presence or absence of individual peaks and exported to Excel (Microsoft, Redmond, WA) for further analysis.

5.3.3 Analyses of ^{33}P uptake

After 19 weeks, each mesocosm was supplied with 0.8 MBq of ^{33}P-labelled calcium phosphate, synthesised using the approach previously outlined in section 2.3.1.

The radio-isotope labelled calcium phosphate was supplied to each mesocosm in 10 mL solution of distilled water. This was dispensed through a syringe loaded with a two-sideport needle to a depth of 8 cm at four injection points spread evenly across the mesocosm. At each injection point, the needle was fully inserted and 2.5 mL of the ^{33}P solution was released as it was withdrawn gradually up through the soil profile.

After the supply of radioactively-labelled calcium phosphate, the duration of the labelling period lasted seven days. Following this, plants were harvested and mixed communities were separated by species. These were then freeze-dried and weighed prior to analysis of ^{33}P content. Post-harvest, colonisation of mycorrhizal plant roots (*A. capillaris* and *P. lanceolata*) was confirmed through Trypan blue root staining (Phillips & Hayman 1970) and the roots of *C. caryophyllea* were inspected for the presence of dauciform roots. Fresh soils were stored at 5 ºC for immediate analysis of microbial ^{33}P.

^{33}P content in plant samples was measured by acid digestion and liquid scintillation counting as outlined in section 2.3.4. Microbial ^{33}P in soil samples was measured

using a modified method originally developed by Brookes et al. (1982) and Hedley & Stewart (1982). Following chloroform fumigation, phosphorus is released from lysed microbial cells within fresh soil samples. Soil samples were mixed with an extractant solution ($NaHCO_3$, 0.5 *M*) and passed through Whatman 42 filter paper. ^{33}P content in the solutions was measured using a scintillation counter (Packard Tri-carb 3100TR, Isotech). The amount of extractable ^{33}P present in the soil prior to chloroform fumigation is measured by mixing fresh unfumigated soil samples with extractant solution in the same manner. Microbial ^{33}P was then measured by calculating the difference between the amounts of ^{33}P extracted from fresh soil which had been fumigated and fresh soil which had not.

When using this technique, a proportion of the phosphorus released from lysed cells as a result of chloroform fumigation would have sorbed onto soil colloids. To account for this, a subset of fresh soil samples were spiked with 0.25 MBq of carrier-free ^{33}P. Recovery efficiency was then determined by calculating the amount of ^{33}P recovered after extraction as a proportion of that which was initially supplied as a spike. This figure was then used to adjust measurements of the amount of ^{33}P recovered from fumigated soils.

Previous studies have made the assumption that fumigation releases 40% of the P contained within the microbial biomass, and it is commonplace to adjust microbial P measurements accordingly (Brookes et al. 1982). Here we focus on the relative amounts of ^{33}P in microbial biomass (measured in KBq) across mesocosms rather than absolute amounts in the soil (measured in ug). Therefore applying a correction factor across all measurements would not alter any differences between mesocosms.

5.3.4 Statistical analyses

Differences in the microbial communities derived from soil samples were measured using a principal component analysis of the relative abundance data. Samples were plotted using principal components which allowed visualisation of any clustering among replicates and treatments.

Microbial community diversity measures were made using presence/absence data of fragment peaks. Diversity was expressed as species richness, which was calculated using the number of fragment peaks within the electropherogram of each sample. Differences among soils associated with each plant monoculture and mixed plant communities were tested using a one-way ANOVA.

Microbial ^{33}P was expressed per g of soil dry weight and plant dry weight. Data were Log_{10} transformed to achieve normality and homogeneity of variances and differences between plant communities were measured using a one-way ANOVA, followed by Tukey HSD.

To measure the effects of interspecific competition on plant tissue ^{33}P concentration, a two-way ANOVA was used with species identity and monoculture/mixed community as factors, followed by Tukey HSD tests to show where significant differences occurred across species treatments. Data were log_{10} transformed in order to achieve normality and homogeneity of variances. All analyses were carried out using the statistical packages Minitab (Minitab Inc., State College, PA, USA) and R 3.2.2 (R Core Team 2015).

5.4 Results

5.4.1 Differences in microbial communities

The TRF profiles consisted of 58 fragments within the range of 41 to 420 bp, eight of these were present in no more than one treatment while 18 were found across them all. A principal components analysis (PCA) of the data from the TRF profiles of each sample showed no clear separation among samples from each monoculture and the mixed communities (Fig 5.1). The first principal component (PC1) explained 62.4% of the variation in the data set, the second (PC2) explained 19.9% and the third (PC3) explained 6.5%.

The number of different-sized fragments measured in each sample ranged from 12 to 40 (Fig 5.2). Using this as a measure for microbial species richness, there was no significant effect of plant monocultures and mixed communities on the number of microbial species (Fig 5.2; one-way ANOVA, $P>0.05$). However, the highest levels of species richness were in soils from the monocultures of the two mycorrhizal species (*A. capillaris* and *P. lanceolata*).

5.4.2 Microbial biomass phosphorus derived from ^{33}P-labelled calcium phosphate

^{33}P content of soil microbial biomass showed a significant response to differences in plant community (Fig 5.3; one-way ANOVA, df = 5, 23, F = 10.83, $P<0.001$). Microbial ^{33}P from the soils of mixed plant community mesocosms was significantly higher than the other soil treatments (Tukey HSD, $P<0.05$). From plant monocultures, microbial ^{33}P was in the region of two to four times less than in mixed community mesocosms, whereas the soil control was over seven times less.

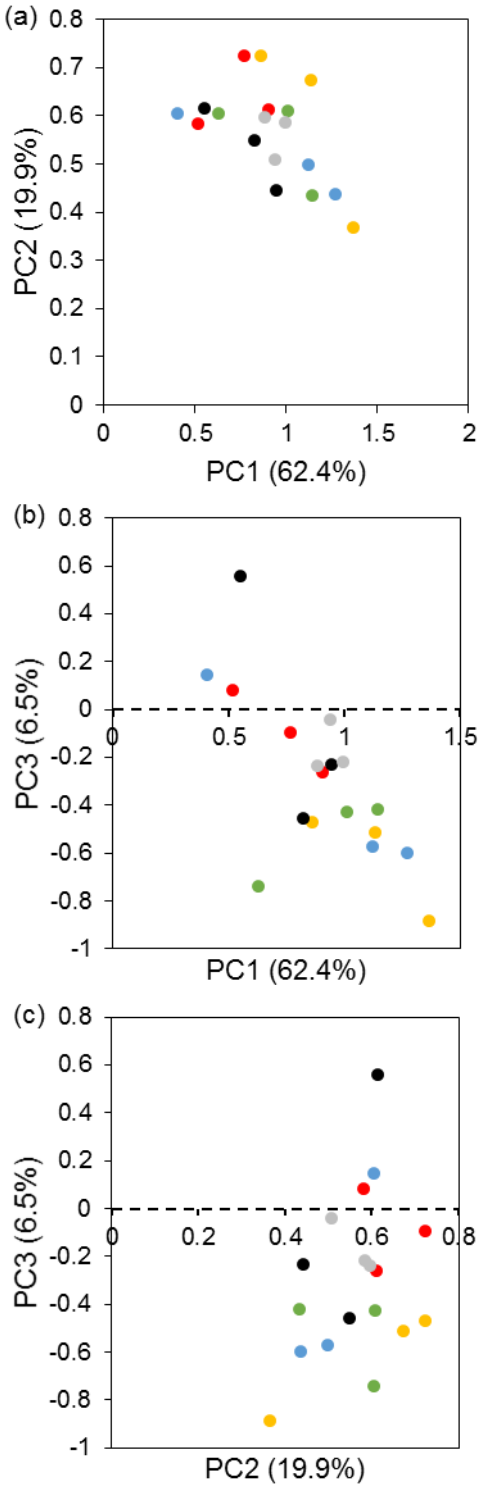

Figure 5.1: Principal component analyses for TRF profiles from microbial communities in soils of different plant communities. Samples represented by colours as follows: ● *Agrostis capillaris*, ● *Plantago lanceolata*, ● *Carex caryophyllea*, ● *Rumex acetosa*, ● Mixed community, ● Control soil. (a) PC1 vs. PC2, (b) PC1 vs. PC3, (c) PC2 vs. PC3.

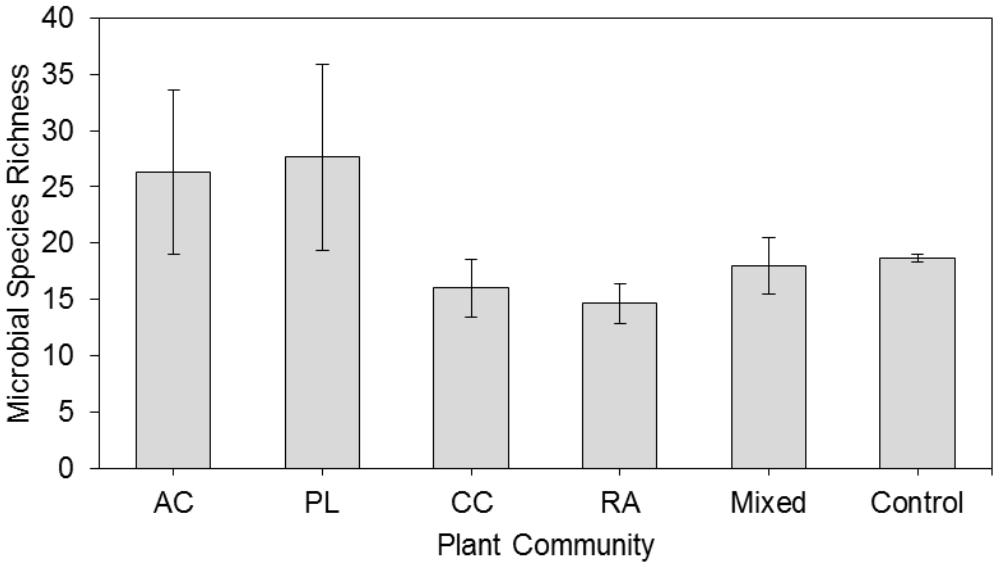

Figure 5.2: Average species richness (no. of different sized TRFs) of microbial communities from each soil treatment: *Agrostis capillaris* (AC), *Plantago lanceolata* (PL), *Carex caryophyllea* (CC), *Rumex acetosa* (RA), four-species mixture, and soil-only control. Means are shown ± 1 s.e.m. There were no significant differences between treatments (Tukey HSD, $P>0.05$).

Compared to the soil control, there was a three-fold increase in microbial ^{33}P from *C. caryophyllea* monocultures (Tukey HSD, $P<0.05$), but there was no significant difference from other plant monocultures.

There was a significant effect of plant species on microbial ^{33}P across monocultures and mixed communities when microbial ^{33}P was expressed per g of plant biomass (Fig 5.4a; one-way ANOVA, df = 4, 19, F = 8.14, $P=0.001$). These differences were driven by plant identity rather than biomass differences as there was no significant differences in plant biomass across monocultures and mixed communities (Fig 5.4b; one-way ANOVA, $P>0.05$). Soils from mixed plant community mesocosms showed the highest levels of microbial ^{33}P per g plant biomass, which differed significantly from monocultures of *A. capillaris* and *P. lanceolata* (Tukey HSD, $P<0.05$). The intermediate amounts of microbial ^{33}P per plant g in *C. caryophyllea* monocultures

were not significantly different from mixed community mesocosms or *P. lanceolata* monocultures (Tukey HSD, $P>0.05$), but were four-fold greater on average than *A. capillaris* monocultures (Tukey HSD, $P<0.05$). Microbial ^{33}P in *R. acetosa* monocultures showed no significant differences from other plant communities (Tukey HSD, $P>0.05$), although the difference from mixed communities was only marginally non-significant (Tukey HSD, $P<0.10$).

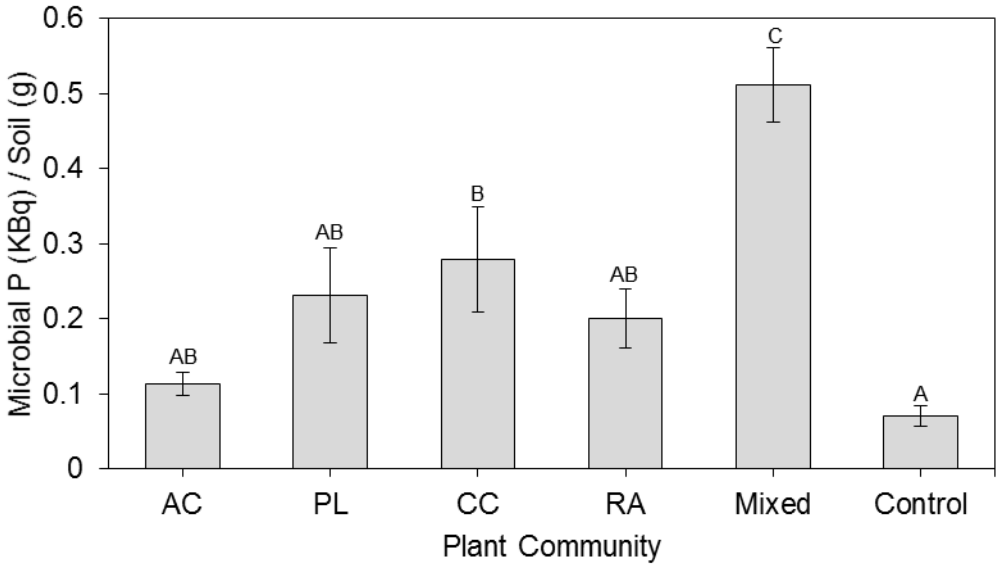

Figure 5.3: Microbial ^{33}P (KBq) measured per gram of soil from each soil treatment (*Agrostis capillaris*, AC; *Plantago lanceolata*, PL; *Carex caryophyllea*, CC; *Rumex acetosa*, RA; four-species mixture, and soil-only control). Means are shown ± 1 s.e.m. Means with the same letter do not differ significantly from each other (Tukey HSD, $P>0.05$).

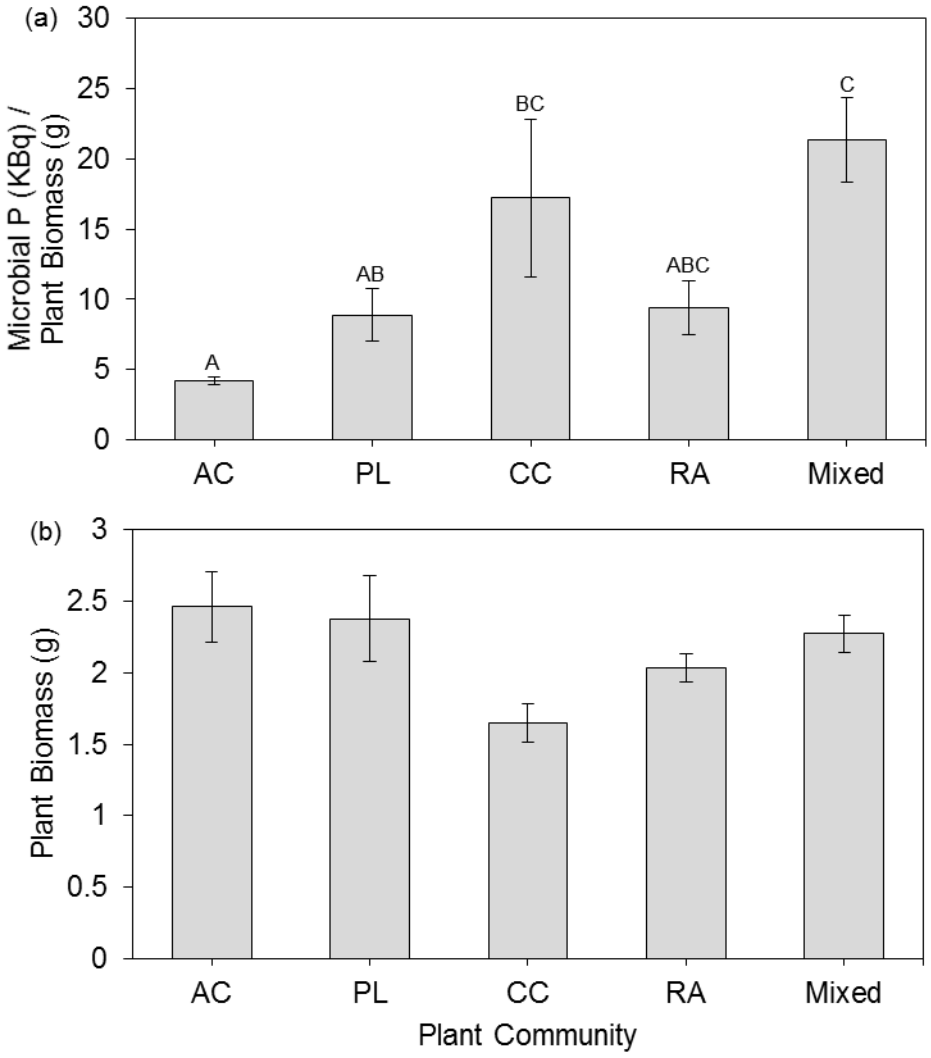

Figure 5.4: (a) Microbial ^{33}P (KBq) measured in proportion to plant biomass for each plant community (*Agrostis capillaris*, AC; *Plantago lanceolata*, PL; *Carex caryophyllea*, CC; *Rumex acetosa*, RA; four-species mixture) supplied with radio-isotope labelled calcium phosphate. Means are shown ± 1 s.e.m.. Tukey HSD tests were carried out to show where significant differences occurred across treatments. Means with the same letter do not differ significantly from each other (Tukey HSD, $P>0.05$). (b) Plant dry weight (g) from mesocosms. Means are shown ± 1 s.e.m. There were no significant differences between treatments (Tukey HSD, $P>0.05$).

5.4.3 Plant ^{33}P uptake

There were significant effects of species identity, competition (whether in monoculture or mixed community) and their interaction on plant tissue concentration of ^{33}P (Fig 5.5, Table 5.1). In monocultures, the highest tissue concentration of ^{33}P was in *R. acetosa* which was at least five times greater than the other species (Tukey HSD, $P<0.05$). ^{33}P acquisition was lowest in *P. lanceolata* monocultures, which differed significantly from *A. capillaris* as well as *R. acetosa* (Tukey HSD, $P<0.05$).

In mixed communities, tissue concentration of ^{33}P was significantly reduced in all species (*A. capillaris,* 87%; *C. caryophyllea,* 83%; *R. acetosa,* 61%; Tukey HSD, $P<0.05$) except *P. lanceolata,* which maintained a similar concentration to when in monoculture (Tukey HSD, $P>0.05$). While ^{33}P uptake in *R. acetosa* remained significantly higher than other species in mixed communities (Tukey HSD, $P<0.05$), there were no significant differences between *A. capillaris*, *P. lanceolata* and *C. caryophyllea* (Tukey HSD, $P>0.05$).

Table 5.1: Two-way ANOVA results for the impact of species identity and interspecific competition on plant tissue concentration of 33P.

Factor	df	*F*	*P*
Species	3,31	46.41	<0.001
Competition	3,31	42.17	<0.001
Species*Competition	3,31	4.18	0.016

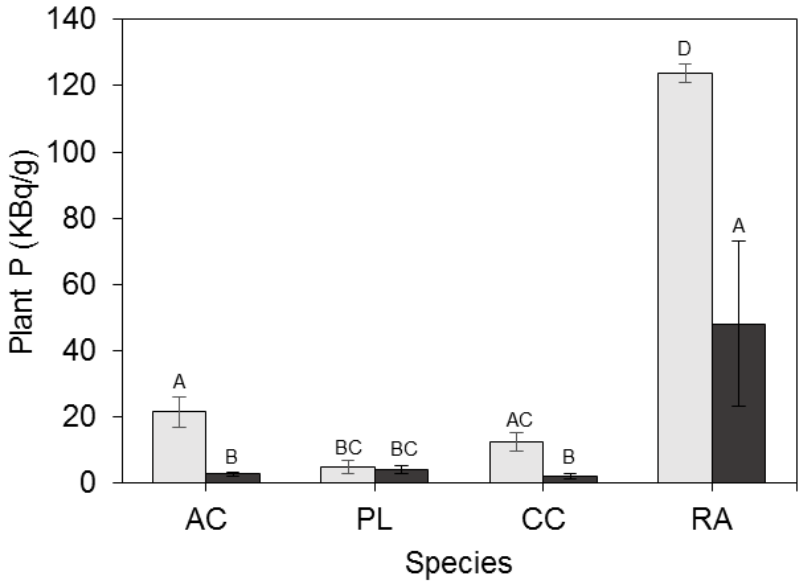

Figure 5.5: Tissue concentration of ^{33}P for each species (*Agrostis capillaris,* AC; *Plantago lanceolata,* PL; *Carex caryophyllea,* CC; *Rumex acetosa,* RA) supplied with calcium phosphate. Grey bars and black bars represent species in monoculture and mixed communities respectively. Means are shown ± 1 s.e.m. Means with the same letter do not differ significantly from each other (Tukey HSD). See Table 5.1 for statistics.

5.5 Discussion

This study investigated the impact of plant community composition (contrasting plant species, and mixed communities vs monocultures) on microbial P uptake from a mineral-bound source, alongside uptake of P by plants. The results show some differences in microbial P uptake in response to different plant monocultures, but these were relatively small when compared to the large increase in microbial P uptake in mixed plant community mesocosms. Therefore, mixed plant communities may have important effects on the mobilisation of P from mineral-bound sources through stimulating microbial uptake, and this effect may be considerably larger than differences driven by individual plant species. There was no evidence of changes in the structure and richness of microbial communities in any treatments,

hence these could not be responsible for differences in microbial P uptake. This suggests that higher levels of ^{33}P in microbial biomass were driven by quantitative increases in their biomass rather than compositional changes in species of phosphate-solubilising bacteria. There was also no evidence that increased microbial P uptake led to greater plant P uptake from the calcium phosphate source, indicating that greater microbial liberation of P from calcium phosphate may not immediately benefit plant P uptake. However, the short timespan of this experiment would have restricted plant access to ^{33}P through microbial turnover.

5.5.1 The impact of plant species on microbial ^{33}P uptake

Mixed plant community mesocosms had significantly greater microbial ^{33}P uptake derived from calcium phosphate (compared to monocultures). These findings are supported by previous studies which have shown that increasing plant species richness has positive effects on microbial activity and can stimulate P cycling from soil organic matter (Eisenhauer et al. 2010; Hacker et al. 2015). They do, however, contrast with previous studies of calcareous grasslands that have shown that neither soil respiration or plant productivity increases with greater plant diversity (Johnson et al. 2008; Phoenix et al. 2008). Those studies were conducted on vastly more mature plant communities (8 years old, compared to 20 weeks in this chapter), which adds an element of caution in extrapolating the short-term nature of the findings from this chapter to the longer term.

The positive relationship between plant species richness and microbial activity has been shown to be driven by increased carbon inputs from plants to the surrounding rhizosphere (Lange et al. 2015), consistent with the ecological theory that biodiversity increases function (Hector et al. 1999; Isbell et al. 2011). The release of

root exudates (such as organic acids, amino acids and sugars) provides an easily degradable source of organic carbon that stimulates activity in the soil microbial biomass (Baudoin et al. 2003; Shahzad et al. 2015). However, in monocultures, this contrasts with observations of ^{33}P content in microbial biomass associated with *R. acetosa*. Microbial ^{33}P uptake in these mesocosms showed no significant differences from other species monocultures despite this species being part of a family which characteristically releases large amounts of root exudates (Tyler & Ström 1995).

Previous studies which have demonstrated increased microbial activity in diverse plant communities showed that this relationship was also driven by increased plant productivity (Liu et al. 2008). In this chapter there were no significant differences in plant biomass across monocultures and mixed communities. However, microbial ^{33}P uptake measured in proportion to plant dry weight showed increased amounts in *C. caryophyllea* monocultures which were more similar to the amount of microbial ^{33}P uptake in mixed community mesocosms than the other plant monocultures. This evidence of increased activity in soil microbes associated with *C. caryophyllea* is in line with increased ^{33}P uptake from DNA demonstrated in Chapter 3. P in this form requires decomposition before it can be acquired, and soil microbes are important drivers of P mineralization in grassland soils (Bünemann et al. 2012). Previous studies have also demonstrated increased bacterial activity in soils associated with sedges (Johnson et al. 2003). This could be due to specialist adaptations for P acquisition in sedges, which produce dauciform roots structures that are characterised by dense proliferations of root hairs and the release of large amounts of root exudates (Shane & Lambers 2005; Playsted et al. 2006). The function of these specialist root structures is similar to that of the cluster roots found in species of Fabaceae (Shane et al. 2006). Plant-microbe interactions measured in white lupin

(*Lupinus albus*) showed that spatial variability in bacterial communities were driven by the production of cluster roots. These findings suggest that influence of *C. caryophyllea* on microbial ^{33}P uptake is driven by the production of dauciform roots. Therefore, as well as the direct effect on P mobilisation from recalcitrant sources of these specialist root structures through the exudation of carboxylates and phosphatases (Playsted et al. 2006), they may also increase P mobilisation through stimulating microbial activity.

The role of root exudates in plant-microbe interactions and stimulating P cycling requires further study. However, the rapid decomposition of root exudates in the field restricts the conditions in which their function can be tested. Nevertheless, the development of novel techniques for harvesting root exudates and analysing their contents (Vranova et al. 2013; Ernst et al. 2014) promises to improve our understanding of how plants can influence microbial activity and chemical conditions in the soil.

5.5.2 The impact of plant species on soil microbial communities

It was hypothesised that differences in microbial P uptake associated with different plant species would be matched with differences in microbial community composition. However, microbes which acquired greater amounts of ^{33}P from calcium phosphate (mixed community mesocosms and to a lesser extent *C. caryophyllea* monocultures) showed no differences from the microbial communities associated with other plant species.

For soil N cycling, plant-driven changes in the function of soil microbes are matched by changes in their composition (Zak et al. 2003). While previous studies have shown that plant diversity effects on P mobilisation were driven by soil microbes

(Hacker et al. 2015), how this relates to the composition of microbial communities has not been investigated.

Changes in microbial community composition could have important effects on microbial P uptake given that different species can vary in their capacity to mobilise P from a range of soil P sources (Rodríguez & Fraga 1999; Richardson 2001; Pii et al. 2015). Plant-mediated interspecific differences in the composition of microbial communities could lead to selective enhancement of microbes which differ in their ability to acquire soil P (Reynolds et al. 2003; Marschner et al. 2011). Therefore it was hypothesised that differences in microbial P uptake could be related to differences in the composition of species in microbial communities. However, this was not supported by the findings from this study. There were no clear differences in microbial community structure across soil treatments, including the mesocosms with higher levels of microbial ^{33}P.

Diversity in soil microbial communities has been shown to increase nutrient cycling, through influences on associated ecological processes including decomposition, turnover and leaching (Wagg et al. 2014). Therefore, it was hypothesised that microbial species richness would be higher in mesocosms which showed increased microbial ^{33}P uptake from calcium phosphate. However, there were no significant differences in species richness across the microbial communities from monocultures and mixed plant community mesocosms. This therefore shows that changes in microbial P uptake can occur without changes in species richness. Assuming consistent concentrations of ^{33}P in the microbial biomass across mesocosms (Cleveland & Liptzin 2007), this suggests that increases in microbial ^{33}P uptake were caused by quantitative changes in the soil microbial biomass rather than compositional changes in microbial community.

There was, however, a trend towards greater species richness in microbial communities associated with mycorrhizal monocultures (*A. capillaris* and *P. lanceolata*). While this did not affect microbial ^{33}P uptake, the higher levels of species richness are consistent with previous findings which have shown increased numbers of bacterial species in response to AM root colonisation (Andrade et al. 1997; Vestergård et al. 2008). AM fungi form species-specific interactions with free-living soil microbes (Secilia & Bagyaraj 1987), which could further raise the species richness of soil microbial communities associated with mycorrhizal plants. However, uncertainty remains over the nature of these belowground interactions, as previous studies have also shown that the diversity and activity of free-living soil microbes is hindered by mycorrhizal plant species and their associated fungal partners (Ravnskov et al. 1999; Johnson et al. 2003).

While the time period of this study was sufficient for plant-microbe interactions to cause differences in ^{33}P uptake in the microbial biomass, a longer period of time could be necessary for differences in the composition of soil microbial communities to arise. Previous studies which have measured changes in microbial communities over time have shown that differences were only established over a period of at least one to two years (Smalla et al. 2001; Eisenhauer et al. 2010), compared to the 20 week period used here.

In this study, primer pairs were selected which amplified 16s rRNA sequences centred on bacterial species including phosphate-solubilising bacteria (*Pseudomonas* spp). Other components of the microbial community (such as *Penicillum* spp and *Trichoderma* spp) have also been shown to affect P mobilisation and plant uptake (Wakelin et al. 2007; Yadav & Tarafdar 2011; Maity et al. 2014; Garcia-Lopez et al. 2015). When amplifying soil DNA extracts, targeting these fungal groups with

different primers could provide further opportunities to explore how plants can influence microbial community composition and P cycling in the soil. However, in this study it is unlikely that changes in microbial ^{33}P uptake occurred in response to shifts in fungal communities (at least for mycorrhizal species) given the broad similarities in microbial ^{33}P uptake between the monocultures of plant species which sustain mycorrhizal associations (*A. capillaris* and *P. lanceolata*) and those that do not (*C. caryophyllea* and *R. acetosa*).

5.5.3 Plant P uptake compared to associated soil microbes

It was hypothesised that increased plant ^{33}P uptake from calcium phosphate would be matched by higher levels of microbial ^{33}P uptake. This could arise from positive feedback between plant stimulation of microbial activity through root exudation, and microbial processes such as increased P mobilisation by phosphate-solubilising bacteria or turnover of the microbial biomass (Vanveen et al. 1987; Macklon et al. 1997; Richardson et al. 2001; Marschner et al. 2011). However, mesocosms with greater plant ^{33}P uptake did not show greater microbial ^{33}P.

Rumex acetosa showed the highest tissue concentrations of ^{33}P across species in monoculture and mixed communities. The high levels of ^{33}P uptake in *R. acetosa* (shown here and in Chapter 3) could be due to the release of large amounts of organic acids characteristic of *Rumex* spp (Tyler & Ström 1995). Plant access to P from calcium phosphate is enhanced through the production of organic acids which release plant available P through lowering rhizosphere pH and the chelation of calcium (Jones 1998). It was hypothesised that this method of P acquisition would also increase microbial ^{33}P uptake as root exudates provide a substrate for the soil microbial biomass (Baudoin et al. 2003; Shahzad et al. 2015), and therefore

stimulate microbial activity and sustain a larger microbial biomass (Hamilton & Frank 2001). This is supported by studies which have demonstrated increased microbial activity in diverse plant communities with increased carbon inputs to the rhizosphere (Lange et al. 2015; Thakur et al. 2015) and increased P mobilisation capacity (Hacker et al. 2015). However, compared to soil microbes from other species monocultures, ^{33}P uptake from calcium phosphate was not increased in soil microbes associated *R. acetosa* monocultures. This suggests that diversity in the range of species releasing root exudates (in mixed community mesocosms) is important for stimulating microbial as well as total C input to the rhizosphere.

There was no evidence of a positive relationship between plant and microbial ^{33}P uptake arising from mixed communities, which showed increased microbial ^{33}P but reductions in plant ^{33}P when compared to monocultures (except for *P. lanceolata*). Previous studies have shown that turnover of the microbial biomass can provide a significant source of P for associated plant communities (Achat et al. 2010; Turner et al. 2012). Therefore increased P content of the microbial biomass could increase soil P inputs and provide greater access to associated plants (Marschner et al. 2011). While there was no evidence to support this, turnover of the microbial biomass has been shown to range from 3 to 59 days (Vanveen et al. 1987). Extending the labelling period of the current study would have provided more time for microbial turnover to occur and made it possible to measure whether this process contributed to plant ^{33}P uptake.

Plantago lanceolata was the only species which maintained similar levels of ^{33}P uptake between monocultures and mixed community mesocosms, with the other species consistently showing lower ^{33}P uptake in mixed communities compared to monocultures. The competitive ability of *P. lanceolata,* considered both in terms of

supressing ^{33}P uptake of neighbouring species and resisting suppression themselves, has been demonstrated in previous chapters. Mycorrhizal associations play an important role, as the results from Chapter 4 showed that the competitive advantage of *P. lanceolata* was not observed in the absence of mycorrhizal fungi.

Hence, the maintenance of ^{33}P uptake between monoculture and mixed communities in *P. lanceolata* could be due to associations with mycorrhizal fungi and their interactions with the free-living soil microbial community. It has been shown that mycorrhizal plants can effectively acquire P which has been mobilised by bacteria from poorly accessible sources (Toro et al. 1997). The relatively higher levels of microbial ^{33}P in mixed community mesocosms (compared to *P. lanceolata* monocultures) could have provided a source of ^{33}P for *P. lanceolata*, either directly through their AM fungal partners or indirectly through interactions with phosphate-solubilising bacteria in the associated microbial community. This would have enabled this species to maintain P uptake levels despite interspecific competition from co-occurring species. This could provide a mechanistic explanation for how species which rely on mycorrhizal associations for P acquisition such as *P. lanceolata* are sustained in diverse plant communities in these P-limited systems.

On the other hand, *A. capillaris*, which also forms mycorrhizal associations, did not show the same competitive response as *P. lanceolata*, instead showing significant reductions in ^{33}P uptake in mixed communities compared to monoculture. This is hard to explain as results from Chapter 3 showed that P uptake patterns were similar between *P. lanceolata* and *A. capillaris* across paired competitive interactions. However, the nature of interactions with mycorrhizal fungi can differ between plant species depending on the reliance of the host plant on their fungal partner. Previous studies have shown that AM fungi direct more resources to a host which is a better

'cooperator' (i.e. provides more carbon) (Kiers et al. 2011). Among grassland species, *A. capillaris* releases relatively low amounts of C into the rhizosphere (Cotrufo & Gorissen 1997). Furthermore, the comparatively simple rooting network of *P. lanceolata* illustrates their greater reliance on mycorrhizal fungi (Hetrick et al. 1988). Therefore, *P. lanceolata* may have been a better 'cooperator' than *A. capillaris* within the common mycelial network of mixed community mesocosms, which led to a greater supply of ^{33}P to *P. lanceolata.* This is in line with previous studies which have shown that P transfer to mycorrhizal roots of plants sharing a common mycelial network is stimulated in response to increased carbon supply (Bucking & Shachar-Hill 2005) and this is preferentially allocated to the hosts which are greater carbon sources (Fellbaum et al. 2014).

The high levels of microbial ^{33}P in mixed plant community mesocosms highlights how interactions between above- and below-ground ecosystem components can have important influences on ecological processes (Wardle et al. 2004). Large amounts of soil P are locked up in calcium phosphates in calcareous grasslands (Zhang et al. 2014). Given the limiting amounts of P in soil solution in these systems, this represents an important P source for plant communities. While there was no clear evidence of plant ^{33}P uptake reflecting higher levels of microbial ^{33}P over the period of this current study, it would be interesting to see whether plant uptake increased over longer timescales, given the greater opportunity for turnover of microbial biomass P (Vanveen et al. 1987). Positive feedback between plant and microbial P uptake, driven by plant species richness, could provide a mechanism which maintains co-existence within increasingly diverse, P-limited, plant communities. A wider range of species would stimulate increased P mobilisation from calcium phosphate and uptake in soil microbes. Associated plant communities

could then access this fixed P source through subsequent turnover of the microbial biomass (Marschner et al. 2011). Competition for this limited resource would therefore be reduced among co-existing plant species in more diverse communities which sustain a microbial biomass with greater amounts of P. Though speculative, this process could explain how high levels of species richness are sustained on soils of low P availability (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Ceulemans et al. 2014).

5.5.4 Conclusion

This study investigated the influence of P-limited plant communities on microbial P uptake and consequences for plant P uptake. There was some evidence of changes in microbial ^{33}P uptake in response to different species in monoculture, but these differences did not appear to relate to differences in plant ^{33}P uptake. Combining each species into a mixed community had by far the biggest influence on microbial ^{33}P uptake, which increased significantly in these mesocosms compared to monocultures. There was no evidence of changes in microbial communities in relation to increased ^{33}P uptake, indicating that higher levels of ^{33}P in the microbial biomass were driven by quantitative increases in their biomass rather than compositional changes in species of phosphate-solubilising bacteria. The positive effect of mixed plant communities on microbial ^{33}P uptake suggests that mixed plant communities can have an important impact on the mobilisation of P from calcium phosphate in calcareous soils through stimulating microbial uptake. Calcium phosphate represents a significant proportion of P in calcareous soils, therefore this process could provide an important long-term supply of P which sustains diverse plant communities in P-limited calcareous grasslands.

General Discussion

Despite widespread P limitation across terrestrial ecosystems and its connection to high species richness in plant communities, the mechanisms which maintain coexistence among species competing for the acquisition of this scarce resource are poorly understood. This chapter draws together the findings from this thesis and incorporates them into what is currently known about the relationship between plant P acquisition and community structure and function in P-limited plant communities. This thesis identified gaps in understanding and addressed these through the following research questions:

1. How do plant P uptake preferences for a range of soil P sources vary between species with contrasting methods of P acquisition?

2. Do those preferences change in response to interspecific competition and, if so, what are the mechanisms which underlie those changes?

3. How do plant species with different methods of P acquisition influence microbial P uptake in P-limited plant communities? Do changes in microbial biomass P influence interspecific differences in plant P uptake?

These research questions were investigated with the use of P-limited calcareous grassland as a model, which are recognised for sustaining species rich plant communities on low levels of soil P (Janssens et al. 1998; McCrea et al. 2001;

Critchley et al. 2002; Ceulemans et al. 2014). Throughout this thesis, P acquisition was measured with the use of radio-isotope labelled P-sources (Chapter 2). This made it possible to directly measure P uptake across a range of chemical P forms. In Chapter 3, the uptake of P in calcareous grassland species with various methods of P acquisition was investigated. This also included a measurement of the effects of interspecific competition, through comparisons between species pairs and monocultures. Differences between species with contrasting methods of P uptake were investigated further in controlled conditions, and the effect of mycorrhizal status on competition for P uptake was measured (Chapter 4). Plant-microbe interactions were investigated in Chapter 5 by measuring the plant influence on microbial P uptake, in response to both mixtures and monocultures of calcareous grassland species with contrasting methods of P uptake.

6.1 Differences in P uptake across a range of chemical forms between species with contrasting methods of P acquisition

The study described in Chapter 3 supplied a range of chemical P sources to microcosms containing species pairs and monocultures. This consisted of grassland species which varied in their methods of P uptake, from mycorrhizal associations to the production of specialist root structures and root exudation. P was supplied in the form of orthophosphate, organic diester (DNA) and inorganic mineral (calcium phosphate). Each of these represent naturally occurring soil P pools of contrasting bioavailability.

The results showed interspecific differences in P uptake across monocultures, with P uptake from DNA and calcium phosphate greater in *C. caryophyllea* and *R. acetosa,* than *A. capillaris* and *P. lanceolata*. Both of these chemical P forms require mobilisation before P can be taken up, and the demonstrated differences in plant uptake can be explained through differences in their methods of P acquisition. *Carex caryophyllea* and *R. acetosa* both belong to families with characteristic high rates of root exudation of organic acids (Tyler & Ström 1995; Shane et al. 2006). This can directly influence soil phosphorus availability by releasing P from calcium phosphate through lowering rhizosphere pH and the chelation of calcium (Jones 1998; Hinsinger 2001).

The influence of root exudates on soil P cycling can also occur indirectly through stimulation of microbial activity. Root exudates consist of a range of organic compounds including mucilage, sugars and amino acids (Dakora & Phillips 2002; Bertin et al. 2003). These provide an easily degradable source of organic carbon which acts as a substrate for the microbial biomass in the soil (Baudoin et al. 2003; Shahzad et al. 2015). A greater input of root exudates sustains a larger population of fungi and bacteria in the rhizosphere (Lange et al. 2015). This can feedback into plant P uptake through microbial mobilisation of P from organic sources (such as DNA) through the production of phosphatases (Spohn et al. 2013; Hacker et al. 2015), making it directly available for plant uptake, or indirectly through the subsequent turnover of soil microbes (Vanveen et al. 1987; Achat et al. 2010; Marschner et al. 2011; Turner et al. 2012).

In plant monocultures, mycorrhizal species (*A. capillaris* and *P. lanceolata*) did not demonstrate a greater capacity for P uptake from orthophosphate, the directly accessible P source, when compared to non-mycorrhizal species. This is despite *A.*

capillaris and *P. lanceolata* possessing a superior foraging capacity through associations with mycorrhizal fungi. Uptake of orthophosphate was greatest in non-mycorrhizal *R. acetosa*, and lowest in the mycorrhizal *A. capillaris*. When supplied with this P source, the low levels of P uptake in mycorrhizal species may reflect the rapid fixation of orthophosphate in the soil, through microbial uptake or adherence to soil particles for example (Tunesi et al. 1999; Jonasson et al. 1999). As shown in the uptake of P from DNA and calcium phosphate, the ability of mycorrhizal plants to access this P when it is not directly accessible is restricted (compared to non-mycorrhizal species).

The demonstrated interspecific differences in P uptake across a range of chemical forms shown in Chapter 3 provide supporting evidence for partitioning of P forms in P-limited plant communities (Turner 2008). Similar interspecific differences in P uptake have been demonstrated previously among species from a P-limited calcareous grassland community across a range of P sources (Phoenix et al., unpublished data). Ahmad-Ramli et al. (2013) also provided evidence of interspecific differences in P uptake among competing plant species which increased P uptake when supplied with a variety of P sources, while Fransson et al. (2003) showed that interspecific differences in P uptake correlated to the size of different soil P fractions.

6.2 Mechanisms underlying competitive interactions between species with contrasting methods of P acquisition

The study presented in Chapter 3 showed that interspecific differences in P uptake are influenced by competition between species with contrasting methods of P

acquisition. P uptake in non-mycorrhizal species was reduced in response to co-occurring mycorrhizal species, while uptake of mycorrhizal species was maintained (or increased). The changes in plant P uptake subsequently led to a reduction in niche differentiation (i.e. an increased similarity in the amount of uptake from the various P sources).

These findings differ from studies on N acquisition which showed that competing species may switch uptake between forms of N to reduce niche overlap (Ashton et al. 2010). Instead, the contrasting competitive responses between mycorrhizal and non-mycorrhizal species supported the scavenger-miner hypothesis (Lambers et al. 2008; Lambers et al. 2011; Li et al. 2014). Accordingly, mining species (specialised in mobilising P from poorly accessible sources) could sustain neighbouring scavenger species with a superior foraging capacity for mobilised P gained through mycorrhizal associations.

The study described in Chapter 4 investigated the mechanisms underlying contrasting competitive responses between species with different methods of P acquisition. Specifically, mycorrhizal status was experimentally manipulated in microcosms and species were grown in monoculture or paired with a species which differed in their method of P acquisition. The effects of mycorrhizal status and interspecific competition were measured on P uptake from a mineral-bound P source (calcium phosphate).

The findings showed that the competitive effect of mycorrhizal species demonstrated in Chapter 3 was reliant upon their mycorrhizal status. The P uptake of a cluster-root producing sedge species (*C. caryophyllea*) was significantly lower only when competing for P with *P. lanceolata* in the presence of mycorrhizal fungi

(compared to mesocosms without mycorrhizal inoculum). These findings are in line with studies which have shown that mycorrhizal species gain a competitive advantage over non-mycorrhizal species in response to the introduction of mycorrhizal fungi (Grime et al. 1987; Francis & Read 1995; van der Heijden & Horton 2009).

Muler et al. (2014) investigated competitive interactions between a cluster-root producing 'mining' species (*Banksia attenuata*) and a mycorrhizal 'scavenger' species (*Scholtzia involucrata*). Consistent with this thesis, that work also showed a negative effect of a co-occurring scavenger on P uptake of the mining species. Few other studies have investigated scavenger-miner competitive interactions and the effects on P acquisition in natural and semi-natural plant communities. However, these interactions have received far more attention in agricultural systems, through investigation of the potential benefits of intercropping on P nutrition and plant productivity. Many studies have demonstrated facilitative effects on the P uptake of crop plants when grown alongside species which exhibit 'mining' traits such as high rates of root exudation of organic acids and phosphatases (Li et al. 2014). Reflecting results in natural and semi-natural plant communities, these have also shown the negative effect on P uptake in co-occurring mining species (Li et al. 2003).

Outside of an agricultural context, this thesis provides the first investigations of how scavenger-miner interactions directly affect P acquisition. This could provide a mechanism which sustains species richness in P-limited plant communities through mining species increasing access to P sources which are not directly accessible for neighbouring scavenger species. Therefore, this may allow a greater range of species to persist in soils with an otherwise limited supply of P.

6.3 The influence of plants on microbial P uptake in P-limited plant communities

The findings from Chapters 3 and 4 indicated that soil microbes could play an important role in P acquisition in the plant communities of calcareous grasslands. Mycorrhizal fungi caused a direct increase in the competitive effect of *P. lanceolata*, which could explain why non-mycorrhizal species showed consistent reductions in P uptake when in competition with mycorrhizal species.

The study described in Chapter 5 investigated the influence of plant species on microbial P uptake. The potential contribution of soil microbes to plant P partitioning was considered by observing whether microbial P uptake matched interspecific differences in plant P uptake. This investigates the question of whether microbes might influenced plant P partitioning by liberating P for plants (either directly or through their uptake and subsequent turnover). Alternatively, plant species could be outcompeted through immobilisation of P into the microbial biomass. Monocultures and mixed communities were supplied with radio-isotope labelled calcium phosphate and P uptake was measured in soil microbial communities associated with different plant communities. Microbial fingerprinting techniques were used to see whether any effects of plant-microbe interactions on P uptake were reflected in differences in microbial community composition.

The findings showed some evidence of variation in P uptake of microbes associated with different plant species in monoculture, but these differences were not consistent with differences in plant P uptake. This was most apparent in *R. acetosa* monocultures, where plant P uptake from calcium phosphate was highest but

associated microbial communities showed no differences in P uptake compared to microbes established under other plant species monocultures.

Microbial P uptake can benefit plant P uptake, through increased P mobilisation by phosphate solubilising bacteria or indirectly through turnover of the microbial biomass (Vanveen et al. 1987; Macklon et al. 1997; Richardson et al. 2001; Marschner et al. 2011). However, these results suggest that microbial P uptake from calcium phosphate did not contribute to demonstrated differences in plant P uptake from this P source.

The biggest plant influence on microbial P uptake was when each species was combined into mixed communities. In these conditions, microbial P uptake was considerably increased (with a two- to four-fold increase when compared to species monocultures). There was no evidence of changes in microbial communities in relation to increased P uptake. This suggests that greater amounts of P in the microbial biomass from mixed plant communities were driven by quantitative increases in microbial biomass rather than compositional changes in communities of phosphate-solubilising bacteria.

Previous studies have also shown that increasing plant species richness has a positive effect on microbial activity, and stimulates P cycling from soil organic matter (Eisenhauer et al. 2010; Hacker et al. 2015). This could be caused by increased carbon inputs from plants to the surrounding rhizosphere (Lange et al. 2015), providing a substrate which stimulates activity in the soil microbial biomass (Baudoin et al. 2003; Shahzad et al. 2015).

The findings from Chapter 5 suggest that species richness could have an important influence on the mobilisation of P from calcium phosphate through microbial

uptake. However, previous studies have shown that the composition of plant species, rather than diversity *per se*, regulates microbial activity (Johnson et al. 2008). The study in Chapter 5 did not distinguish between these two factors. In order to do so, it would be necessary to further increase the number of species present in mixed community mesocosms and measure the effect on microbial P uptake.

It was predicted that increased amounts of microbial P would increase P availability in the soil and benefit plant uptake through subsequent turnover of the microbial biomass (Macklon et al. 1997; Richardson et al. 2001; Marschner et al. 2011). However, there was no evidence of plant P uptake benefiting from increased microbial P uptake in mixed communities. None-the-less, the short time in which P uptake was measured in Chapter 5 offered only a narrow window for microbial activity to influence plant P uptake, and turnover of the microbial biomass has been shown to vary over a period of 3 to 59 days (Vanveen et al. 1987). This might also explain the absence of relationships between microbial P and plant P uptake between the various plant monocultures.

Measuring P uptake over longer time scales would provide more time for microbial turnover to occur and the contribution of this process to plant P uptake could then be more accurately measured. This would make it possible to investigate whether increasing species richness may drive a positive feedback in P-limited calcareous grasslands. Given that a large proportion of phosphorus in calcareous soils is fixed in calcium phosphate (Zhang et al. 2014), this could provide an important P supply in these plant communities. To speculate, increased microbial P uptake in diverse plant communities could provide an indirect P supply to plants which reduces interspecific competition and may therefore help to maintain coexistence.

6.4 Future Directions

Despite the prevalence of P limitation in terrestrial ecosystems, there are few studies which have investigated the role of P partitioning in shaping plant community structure and function. The findings from this thesis have highlighted the importance of different methods of P acquisition in competitive interactions and the uptake of P from different sources. A greater understanding of the physiological differences between species with contrasting methods of P acquisition is an important area of future research. For example, the release of root exudates plays a key role in plant P acquisition, and the amount and composition of these compounds can vary widely - whether comparisons are made between different plant species or along the length of a single root (Gahoonia et al. 2007; Marschner et al. 2011; Mimmo et al. 2011). However, much of what is known about the function of these compounds comes from studies carried out in controlled conditions (i.e. plants grown on sterilised substrates or in hydroponic systems). This is largely on account of the rapid decomposition of root exudates in the field, which restricts the conditions where their function can be tested. Nevertheless, the development of novel techniques for harvesting and analysing the contents of root exudates could overcome these limitations (Vranova et al. 2013; Ernst et al. 2014).

In the context of this thesis, these approaches would provide further insight into the mechanisms which underlie P partitioning between species with contrasting methods of P acquisition. Interspecific differences in the release of root exudates have been inferred based on characteristic traits of closely related species. For example, *Rumex* spp. (Tyler & Ström 1995) and *Carex* spp. (Shane et al. 2006). However, measurement of root exudate production in these species would directly answer whether differences in plant P uptake were related to this trait.

Another key area for future research concerns the influence of soil microbes on plant P acquisition and community structure and function. The impact of soil microbes on ecosystem functioning was considered a 'black box' for much of the 20th century (Tiedje et al. 1999). However, recent progress in the use of molecular approaches has made it possible to unravel the complexity of belowground processes involving soil microbes. From sequencing soil microbial communities to measuring gene expression, plant-microbe interactions can now be investigated on a spatial scale ranging from centimetres down to nanometres (Marschner et al. 2011; Bardgett & van der Putten 2014).

The ability of soil microbes to solubilise P has been demonstrated for a range of soil sources, from various forms of organic monoesters and diesters to inorganic mineral-bound P forms (Richardson & Hadobas 1997; Yadav & Tarafdar 2011; Garcia-Lopez et al. 2015). Further investigation should consider whether plants form species-specific associations with soil microbes which mobilise P from different soil sources. This could provide a partitioning mechanism which mediates plant competition for P acquisition through associations with soil microbes (Reynolds et al. 2003).

6.5 Applications in calcareous grasslands

The focus of this study was on the ecological processes surrounding the acquisition of P in calcareous grasslands. This system was used as a model due to high levels of species richness sustained on soils characterised by low P availability (Janssens et al. 1998; McCrea et al. 2001; Critchley et al. 2002; Ceulemans et al. 2014). As well as supporting a diverse range of plant species, these ecosystems provide a range of

valuable services, including grazing pasture for livestock, a forage resource for pollinator species and a net sink for carbon (Carvell 2002; Janssens et al. 2005). Despite this, these ecosystems are at risk due to the steep decline in the area of semi-natural grasslands in the UK (Fuller 1987), mostly through conversion to arable land (Newton et al. 2012).

Efforts have been made to prevent further loss of these valuable ecosystems with their widespread designation as Sites of Special Scientific Interest (SSSIs) across the UK (JNCC 2010). However, re-establishment requires an in-depth understanding of ecological processes which sustain the many species found in these plant communities (Janssens et al. 1998). This must involve a consideration of the complex interactions which occur among species from these P-limited plant communities, as highlighted by this thesis.

A range of other ecological factors have been shown to have important impacts on calcareous grassland communities. For example, disturbance through livestock grazing has a beneficial effect on plant species richness by reducing vegetation cover and restricting the dominance of a small number of grass species (Jacquemyn et al. 2011; Maccherini & Santi 2012). Therefore, the findings from this thesis must be integrated into the current knowledge of the ecological processes which influence community structure and function in calcareous grasslands.

Climate change is predicted to have a widespread impact on the structure of plant communities (Jägerbrand et al. 2009; Yang et al. 2011; Dieleman et al. 2015). However, calcareous grassland plant communities have shown resilience in response to the simulated effects of climate change. The long-lived, slow-growing species in

these unproductive habitats maintain relative abundance when exposed to drought conditions and temperature fluctuations (Grime et al. 2008).

The resilience shown in these plant communities could be driven by other forms of belowground resource partitioning, such as rooting depth and water uptake. This process has been demonstrated in tallgrass prairie, where drought conditions induced interspecific differences in water uptake from different soil depths (Nippert & Knapp 2007). When the availability of water was limited, subordinate species shifted water uptake to deeper layers, avoiding competition from dominant species. While the Rendzina soils which underlay calcareous grasslands are relatively shallow, they none-the-less offer opportunities for partitioning of rooting depth in microsites of deeper soil. Previous studies on limestone grassland plant communities have shown that these microsites provide a refuge for species which are less tolerant of drought, hence maintaining stability in these plant communities at a whole-plot level despite microsite variation (Fridley et al. 2011). In a similar way, spatial variation in P sources could also help sustain species richness. Partitioning could occur with depth (as seen in the arctic tundra work of McKane et al. (2002) or horizontally over small distances that could facilitate the relatively fine scale species co-existence of calcareous grasslands.

As well as climate change, N deposition is another anthropogenic effect which could influence community structure and function in calcareous grasslands. Simulated soil inputs of pollutant N have shown that loading the soil with N exacerbates P limitation. The knock-on effects of this on P acquisition have been demonstrated through the increased activity of phosphatases in plants and microbes (Johnson et al. 1999; Phoenix et al. 2003). This suggests that plant and soil microbes will have an increased reliance on P uptake from organic sources in response to N deposition.

Further investigation of P uptake from other soil sources is necessary in order to measure the potential impact of N deposition on plant P partitioning in calcareous grassland communities.

6.6 Applications for arable agriculture and wider relevance

This thesis focused on plant competition and P acquisition in a semi-natural ecosystem. However, understanding the ecological processes which influence plant communities in these conditions is a pre-requisite for finding relevant applications in a wider context, such as agricultural systems.

The demonstrated impact of competitive interactions between species which possess contrasting methods of P acquisition on plant P uptake could be of relevance for intercropping techniques. The findings from this thesis are in line with other studies which have demonstrated how plant-plant interactions can enhance P nutrition through intercropping in agricultural systems (Li et al. 2014). Furthermore, enhancing plant P nutrition through associations with phosphate-solubilising bacteria has led to investigations of whether these associations can be harnessed in an agricultural context (Vessey 2003; Pii et al. 2015).

As well as in P-limited calcareous grasslands, P nutrition is of great importance in arable agriculture. Current conventional farming practices rely on excessive use of phosphate fertilisers. In the US for example, each year farmers apply over 4 million tons of P fertilisers to the soil (USDA 2013). Only a fraction of this supplied P is acquired by crop plants. The rest of this is either incorporated into residual soil P pools (Sattari et al. 2012) or washed into water systems which causes damaging environmental impacts such as algal blooms and ‘dead zones’ (Tirado & Allsopp

2012). The negative effects of these human activities have left the P cycle at the limits of a safe operating space for the planet (Rockström et al. 2009). Assessing the capacity of plants to acquire P from pre-existing sources in the soil will aid the development of sustainable agricultural practices which can increase P-use efficiency and reduce the reliance on application of fertilisers.

The current excessive use of artificial P fertilisers reflects the poor attitude towards soil conservation in general. Over the latter half of the 20th century, it was estimated that soil erosion led to the loss of almost one-third of the world's arable land (Pimentel et al. 1995). This trend is likely to continue, with current erosion rates in arable and intensively grazed lands far exceeding their rate of formation (FAO & ITPS 2015). Underlying this issue is a lack of understanding of the complex processes in the soil which govern their function. It was Leonardo Da Vinci who stated in the 16th century that "*we know more about the movement of the celestial bodies than about the soils underfoot*". In many ways, this observation still holds true today, with overwhelming estimates of soil diversity reporting over 50,000 bacterial species and tens of metres of fungal hyphae per gram of soil (Leake et al. 2004; Roesch et al. 2007). It is hoped that the knowledge gained of belowground interactions on ecosystem processes from this thesis will contribute to advances in soil science research which can be translated into tools and techniques that lead to the adoption of sustainable land management practices and reverse the global trend of soil erosion (Doran 2002).

6.7 Conclusion

This thesis set out to investigate the ability of plants to access P from a range of chemical forms, and how this related to interactions with co-occurring species and soil microbes. For this, calcareous grasslands were used as a model system, which contain diverse plant communities on soils with low P availability.

Using a range of radio-isotope labelled P sources, it was possible to directly measure differences in plant P uptake between species with contrasting methods of P acquisition. Interspecific differences in P uptake across a range of chemical forms were consistent with contrasting methods of P acquisition, providing evidence of P partitioning between these species.

Changes in P uptake in response to competition supported the hypothesis that species richness could be sustained on P-limited soils through mycorrhizal species acquiring P which has been mobilised by co-occurring P-solubilising 'mining' species. Under controlled conditions, it was shown that mycorrhizal 'scavenger' species relied on their mycorrhizal associations for their competitive advantage over mining species.

Microbial P uptake in calcareous soils was measured from radio-isotope labelled calcium phosphate. While there was some variation in microbial P uptake by microbes associated with different plant species monocultures, this did not reflect differences in plant uptake. This suggests that microbial P uptake did not influence plant acquisition of P from calcium phosphate.

Microbial P uptake increased significantly in association with mixed communities compared to monocultures, highlighting the importance of species richness on the mobilisation of P from calcium phosphate through microbial uptake. While this did

not benefit plant P uptake, future consideration of the long-term effect of microbial turnover on P acquisition is warranted.

These findings provide a new perspective on the ecological processes which sustain high levels of species richness in P-limited plant communities and highlight the importance of below-ground interactions on the acquisition of this limiting resource. Given the prevalence of P-limitation in terrestrial ecosystems, the findings from this study could have widespread significance.

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