Lancaster Environment Centre

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Intraspecific plant trait variation and grassland ecosystem function

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Abstract

The effects of global environmental change on ecosystem functions, such as carbon (C) and nitrogen (N) cycling, are in part mediated by changes in plant community composition, structure and productivity. Plant traits can serve as easily measurable proxies for plant function, useful for predicting vegetation responses to environmental change and effects of vegetation on ecosystem function. However, many trait-based studies do not take into account intraspecific trait variability (ITV) and it is unclear how much uncertainty this introduces. The overarching aim of this thesis was to improve understanding of the drivers that control ITV as well as the consequences of ITV for ecosystem functions related to C and N cycling in grassland ecosystems. To achieve this, key drivers of ITV including soil properties, neighbouring plants, N addition and drought stress were investigated, as well as consequences of ITV for ecosystem properties and function. A calcareous grassland field biodiversity experiment was used to investigate how neighbouring plants and soil properties affect ITV. A controlled outdoor mesocosm experiment was designed to investigate the effects of plant species interactions and N addition on ecosystem C and N cycling, and whether these effects were mediated by plant trait plasticity. A greenhouse drought experiment with a subsequent litter decomposition essay was conducted to investigate if drought-induced plasticity of root and shoot traits alters their decomposability. Overall, the results indicate that plant species interactions, soil properties, nutrient availability and drought stress contribute to controlling ITV in grasslands, but that the exact patterns of ITV are often species-specific. Phenotypic plasticity in response to these environmental drivers had either weak or no effects on ecosystem functions related to C and N cycling. This suggests that in contexts similar to the ones examined here it may be justified to ignore ITV in trait-based studies and focus on species means. However, particular species

sometimes had disproportionate effects on ecosystem functions relative to their contribution to biomass, which might contribute to explaining why the explanatory power of plant traits for predicting ecosystem functions is often low.

Keywords: Plant traits, intraspecific trait variability, carbon cycling, nitrogen cycling, ecosystem function, grassland

Declaration

I declare that this thesis is my own work and has not been submitted for a degree elsewhere. Contributions from supervisors and collaborators are specifically acknowledged. Many of the ideas in this thesis were the product of discussion with my supervisors Prof. Nick Ostle (Lancaster University), Dr Jeanette Whitaker (UK Centre for Ecology & Hydrology, Lancaster) and Prof. James Bullock (UK Centre for Ecology & Hydrology, Wallingford).

The word length of this thesis is 52545 words including reference lists, and therefore does not exceed the permitted maximum.

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1 General Introduction

Global environmental change driven by human activities, such as land use change, climate change and pollution, is altering terrestrial ecosystems in unprecedented ways (IPBES, 2019). It is vital to understand the effects of this anthropogenic change for ecosystem functions such as carbon (C) and nutrient cycling. Global change drivers have direct effects on ecosystem functions. For example, temperature directly affects soil microbial respiration (Trumbore, 2006). Additionally, effects are indirectly mediated by vegetation, e.g. through changes in plant community composition, structure and productivity, which lead onto altered function (Hooper et al., 2005). The ability to simultaneously predict the response of ecosystems to global change and the resulting effects on ecosystem function using plant traits has been termed a 'Holy Grail' for ecology (Lavorel & Garnier, 2002). Ultimately, an improved understanding of these processes will support the development of better mechanistic models predicting the consequences of global environmental change, such as earth system models (IPCC, 2013).

As will be outlined in the following sections, much research has focused on trait differences between species and their effects on ecosystem function. However,

intraspecific trait variation can also be substantial and less research has considered the effects of this on ecosystem function. This thesis considers the role of intraspecific plant trait variation for grassland ecosystem function. This first chapter provides the scientific background and context for the thesis, followed by an overview of its overarching aim and the questions addressed in each of the four experimental chapters.

1.1 Plant traits

A central approach in the quest for the 'Holy Grail' has been to characterize vegetation in terms of plant traits. Plant traits are defined as 'morphological, physiological or phenological features measurable at the individual level, from the cell to the whole-organism level, without reference to the environment or any other level of organization' (Violle et al., 2007). Advances have been made in this field in recent years through the development of standardized methodologies for trait measurements (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013) which facilitate comparisons across studies and through large global trait databases, for example the TRY database (Kattge et al., 2011, 2020).

Research suggests that plant traits are related to and constrained by ecological strategies and trade-offs, which makes them useful for understanding ecological processes. Evidence for trade-offs has been found in studies comparing multiple traits across species and ecosystems. In the largest study to date, Diaz et al. (2016) found that three quarters of the variation in six aboveground plant traits from a global dataset including over 45 000 vascular plant species was captured by a two-dimensional spectrum. This suggests that throughout the evolution of plant species, certain combinations of traits have proven successful, while others have not. The first major axis of variation identified by Diaz et al. (2016) is related to the size of plants and their organs. The

second axis represents the leaf economic spectrum, which spans from resource-acquisitive leaves with high N content and low leaf mass per area, which tend to have high photosynthetic rates, but short lifespans (Wright et al., 2004), to resource-conservative leaves with the opposite properties. For root traits, patterns of trait covariation and ecological strategies are more complex, as roots need to take up several types of resources (water and various nutrients), they encounter different kinds of physical constraints (e.g. compacted or waterlogged soil) and in some species mycorrhizal associations play a crucial role in resource uptake (Weemstra et al., 2016). Nevertheless, in a recent analysis of a global dataset including four root traits across 1810 species, Bergmann et al. (2020) identified two major axes related to resource acquisition. The first axis is related to the degree of mycorrhizal symbiosis, where species with high specific root length and small root diameter are optimized to take up nutrients without mycorrhizal associations, while species with the opposite traits tend to have stronger associations with mycorrhiza. The second axis of variation is related to resource economics, spanning from acquisitive species with high root N content and low tissue density to conservative species with the opposite traits. In addition to the traits related to these major axes of variation, many other traits can be important to plant and ecosystem functions, both above- and belowground (Laughlin, 2014; Freschet et al., 2021).

1.2 Plant traits and ecosystem function

Plant traits can be used to predict and understand the effect of plant community composition and structure on ecosystem functions. Research has focused on two contrasting, but non-exclusive hypotheses regarding how plant traits affect ecosystem function. The first is the ‘mass ratio hypothesis’ (Grime, 1998), according to which species’ effects on ecosystem functions are relative to their contribution to total biomass

and ecosystem functions can be predicted by community-weighted mean traits (Garnier et al., 2004), i.e. the mean value of traits weighted by each species' contribution to total biomass. The second hypothesis, the 'diversity hypothesis' (Tilman et al., 1996; Hooper et al., 2005) predicts that the diversity of a plant community affects ecosystem function due to complementarity and selection effects (Loreau & Hector, 2001) and that this can be predicted using diversity indices, such as species, phylogenetic or trait functional diversity (Mason et al., 2003).

Support has been found for both hypotheses, in field studies conducted over environmental gradients and in biodiversity experiments (Garnier et al., 2015). For example, in gradient studies of temperate grasslands, community-weighted mean above-ground traits have been found to be correlated with plant biomass, soil microbial community composition, soil N retention and soil C sequestration (de Vries et al., 2012; Grigulis et al., 2013; Manning et al., 2015). In grassland biodiversity experiments, above- and/or belowground biomass often increases with species richness and/or functional diversity (e.g. Barry et al., 2019; Roscher et al., 2013; Tilman et al., 2001; Van Ruijven & Berendse, 2009) and both community-weighted mean traits and functional diversity have been related to ecosystem CO_2 fluxes (Milcu et al., 2014).

Both field gradient and experimental manipulation studies have their own limitations. In gradient studies, ecosystem properties and functions are not only affected by the vegetation, but also by the abiotic factors varying along the gradient. Even though many studies account for some abiotic variables (e.g. de Vries et al., 2012; Manning et al., 2015) these studies remain correlative. Biodiversity experiments can reveal causal links between vegetation and ecosystem properties and functions, but it has been questioned if they are realistic enough to draw conclusions valid in 'real-world' ecosystems

(Wardle, 2016). This problem has been addressed through establishing more realistic biodiversity experiments (e.g. Fry et al., 2018; De Long et al., 2019) and by excluding 'unrealistic' species combinations from analyses (Jochum et al., 2020).

1.3 Intraspecific trait variability (ITV)

1.3.1 Extent and drivers of ITV

While the greatest trait variability is found between species, it has been observed that intraspecific trait variability (ITV) can also be considerable. ITV is defined as "the overall variability of trait values and trait syndromes (sets of trait values including trait trade-offs) expressed by individuals within a species" (Albert et al., 2011). In a global meta-analysis ITV accounted for 25% of total trait variance within plant communities and 32% of total variance between communities (Siefert et al., 2015). ITV observed in the field is jointly caused by genotypic differences within species, phenotypic plasticity and their interaction (Albert et al., 2011).

Genotypic differences are the result of evolutionary processes including mutation, migration, genetic drift and natural selection (Hughes et al., 2008). These processes have been found to occur also at relatively short timescales of about a decade, which makes them relevant to ecological processes (Thompson, 1998). For example, 13-15 years of simulated climate change in a grassland experiment led to within-species genetic differentiation (Ravenscroft et al., 2014, 2015) between treatments and control plots. Evidence for within-species genetic differentiation was also found in a 8-year grassland biodiversity experiment between plants from monocultures and multi-species mixtures (Zuppinger-Dingley et al., 2014). In this case, plants grown from seeds collected from mixtures showed enhanced niche-complementarity when grown in mixtures, compared to plants grown from seeds collected from monoculture, which

indicates local adaptation. It is sometimes assumed that most genotypic variation is adaptive to the local environment, however it has been pointed out that this is not necessarily the case (Ackerly & Monson, 2003). A meta-analysis of reciprocal transplant experiments showed that herbaceous species in temperate regions were locally adapted to their sites of origin in 43.5% of the cases, while in the remaining cases there was no evidence for local adaptation (Leimu & Fischer, 2008).

Phenotypic plasticity is the propensity of a single genotype to produce different phenotypes depending on environmental conditions (Sultan, 2000). This can improve a plant's fitness and thus be adaptive to its local conditions, but also can be non-adaptive (Palacio-López et al., 2015). Non-adaptive plasticity can occur for example if the environmental conditions that induced phenotypic plasticity subsequently change, or if the plasticity was merely a compensatory resource-allocation following plant damage (Valladares et al., 2007). Phenotypic plasticity also includes cross-generational effects, where the environmental conditions of a plant affect its offspring's phenotype, but not through alteration of the genotype (Sultan, 2000).

Results from common garden experiments show that both phenotypic plasticity and genetic variability contribute significantly to ITV along environmental gradients (Read et al., 2014; Lajoie & Vellend, 2018). A range of important environmental drivers of ITV have been identified. For example, a global meta-analysis of studies on ITV in leaf traits of woody and herbaceous plant species across elevational gradients (Midolo et al., 2019) showed common patterns of variation along the gradients, likely related to temperature. However, evidence suggests that other environmental drivers, such as resource availability and plant species interactions can be just as important, if not more so. Several studies have compared the magnitude of ITV across scales, such as between

sites, between plots and between and within individual plants, both for aboveground (Albert et al., 2010b; Messier et al., 2010) and root traits (Weemstra et al., 2021). A common finding in these studies is that while there is variability between sites, there is also considerable and often even larger variability at smaller scales, such as between individuals or plots within the same site, which is likely due to local heterogeneity of the abiotic and biotic environment. This is plausible, as in experimental studies plants exhibit trait plasticity in response to variation in the biotic or abiotic environment, for example the identity or diversity of the neighbouring plants (e.g. Baxendale et al., 2014; Lipowsky et al., 2015; Bennett et al., 2016), nutrient availability (e.g. Fort et al., 2015; Siebenkäs et al., 2015) and drought (de Vries et al., 2016; Lozano et al., 2020).

1.3.2 Consequences of ITV for ecosystem function

While it is known that ITV can be considerable, much less is known about its importance for ecosystem functioning. Often, trait-based studies predicting ecosystem functions use trait values from databases (e.g. the TRY database, Kattge et al., 2011) or measured in monocultures. These trait values are then combined with species composition surveys in the field or experimental plots either by cover (e.g. de Vries et al., 2012) or by contribution to total biomass (e.g. Roscher et al., 2013) to calculate community-weighted mean traits and/or functional diversity. This method reduces the sampling effort compared to sampling traits from each site or treatment and allows one to conduct larger studies. However, a limitation of this approach is that intraspecific trait variability (ITV) is not taken into account and it is uncertain how much error is introduced through this simplification (Funk et al., 2017).

Studies have shown significant effects of genotypic variability in *Populus angustifolia* on soil C and N pools, nitrification and CO_2 efflux (Lojewski et al., 2012; Pregitzer et

al., 2013) genotypic variability in *Arabidopsis thaliana* on litter decomposition (Kazakou et al., 2019). A major difficulty in studying the effects of plasticity and/or local adaptations on ecosystem functions is that environmental biotic and abiotic drivers of ITV (e.g. resource availability or plant community composition) also have direct effects on the ecosystem functions and properties of interest. This is why the most common ecosystem function studies in this context is litter decomposition, as in this case the causes and effects of ITV can be experimentally separated: litter is collected from different locations or experimental treatments and a decomposition assay is then conducted under standardized conditions (e.g. Wardle et al., 1998). In some contexts, ITV has affected litter decomposition, in others it has not. For example, Lecerf & Chauvet (2008) found that decomposability of alder leaves from distantly-separated sites across Europe was strongly affected by ITV. Kazakou et al. (2009) found no effect of experimental N-addition on litter decomposability in Mediterranean herbaceous species. Jackson et al. (2013) found differences in decomposability in litter from 16 co-occurring temperate rain forest plant species along a soil nutrient gradient, but ITV in the traits measured explained the differences poorly. The effect of drought stress, another potentially important driver of ITV, on litter decomposability has not yet been studied.

Another way to investigate the effect of trait plasticity on ecosystem functions are experiments in which plasticity-inducing environmental conditions are manipulated, but in a design simple enough to disentangle direct effects of environmental drivers on ecosystem functions and effects mediated through plant trait plasticity. For example, de Vries et al. (2016) found that the drought effect on C and N cycling in pots with individuals of four temperate grassland species was mediated by phenotypic plasticity in root traits. However, the effect of ITV induced by other key drivers, such as plant

species interactions and nutrient availability, on ecosystem functions has not yet been investigated.

1.4 Investigating C and N cycling in grassland ecosystems

This thesis focuses on C and N cycling in grassland ecosystems. Grasslands cover more than a third of the global land surface (Suttie et al., 2005) and provide many important ecosystem services such as water supply and flow regulation, carbon storage, erosion control, climate mitigation, pollination, and cultural ecosystem services (Bengtsson et al., 2019). Understanding ITV and ecosystem functions in grasslands is therefore of global relevance. Grasslands might also be suitable model ecosystems to test hypotheses that are generalizable to other ecosystems, as grassland species cover a large range of growth strategies (Diaz et al., 2016) and can establish at both high and low levels of resource availability (Craine et al., 2001). Furthermore, high levels of species and functional richness can be reached at relatively small spatial scales (Habel et al., 2013) and grassland ecosystems and plant species are relatively easy to work with in field and experimental studies. Many grassland species grow fast and are small enough to conduct straightforward greenhouse and laboratory experiments, while their size is suitable to measure traits quickly and efficiently. For example, plant height can be measured with a simple ruler, roots and leaves can be scanned using an A4 scanner, and grinding a relatively small amount of plant material is sufficient to obtain representative measures of plant chemical traits. All these considerations make grasslands ideal systems to study the effects of variation in plant traits on ecosystem functions.

The coupled C and N cycles are key to many important ecosystem services delivered by grasslands, such as soil C storage, fodder production and nutrient retention. Quantifying the most relevant aspects of C and N cycling in a plant-soil system is

challenging, as many inter-related components (e.g. plants, litter, soil biota, soil organic matter) and complex processes are involved (Cortois & de Deyn, 2012). Also, the plant-soil system goes through big changes throughout the year, which means that to detect long-term changes, e.g. in soil C sequestration, long-term data over several growing seasons is needed (Poeplau et al., 2011).

In this thesis, a combination of field, outdoor mesocosm, greenhouse and laboratory experiments was used to assess different aspects of C and N cycling (see Fig.1.1). In the field experiment, the measurements included above- and belowground biomass, as well as soil properties related to nutrient cycling and microbial properties. In the outdoor mesocosm experiment, CO_2 fluxes to and from the atmosphere could be measured easily without disturbing the soil by clipping a flux chamber directly on the mesocosm pots. The measurements included photosynthesis, respiration, short-term C dynamics using a ^{13}C tracer approach, as well as similar plant, soil and microbial properties as the ones measured in the field. The greenhouse/laboratory experiment focused on litter decomposition.

1.5 Thesis aims and objectives

The overarching aim of this thesis was to improve understanding of the drivers that control ITV as well as the consequences of ITV for ecosystem functions related to C and N cycling in grassland ecosystems.

To achieve this, key drivers of ITV including soil properties, neighbouring plants, N addition and drought were investigated, as well as consequences of ITV for ecosystem properties and function (see Fig. 1.1). Specifically, the four experimental chapters address the following questions:

Chapter 2: Which above- and belowground drivers affect intraspecific plant trait variability in calcareous grasslands?

The drivers of ITV observed at local scales are poorly understood. In this chapter, a 4-year-old calcareous grassland biodiversity experiment on the Salisbury Plain was used to test the hypothesis that plants exhibit intraspecific trait variability which is related to traits of the surrounding plant community due to differences in resource availability (e.g. light and soil nutrients).

Chapter 3: How do species interactions, N-addition and plant trait plasticity affect carbon and nitrogen cycling?

In Chapter 2, neighbouring plants and N availability were identified as potentially important drivers of ITV. In this chapter, a controlled mesocosm experiment with monocultures and two-species mixtures was set up to investigate: how neighbouring species affect plant trait plasticity; how interactions between plant species from different functional groups affect ecosystem properties and functions; and if these effects are modified by N addition.

Chapter 4: How do interactions between plant species and plant trait plasticity alter the fate of recently assimilated carbon?

Interactions between plant species are known to affect a variety of ecosystem functions, but the effect on short-term C dynamics has not been investigated. In this chapter, a sub-set of the mesocosms analysed in Chapter 3 was used to compare short-term C dynamics between monocultures of two grassland species and their mixture using a ^{13}C pulse-labelling approach.

Chapter 5: Does drought-induced plasticity of root and shoot traits alter their decomposability?

Water-availability is another potentially important driver of ITV identified in Chapter 2. In this chapter, a drought experiment including three grassland species from contrasting functional groups and a subsequent litter decomposition assay were used to assess the effects of this on shoot and root litter decomposability. It was hypothesized that the effects of drought on shoot and root traits vary between grassland plant functional groups and that drought affects root and shoot litter decomposability due to its effect on traits.

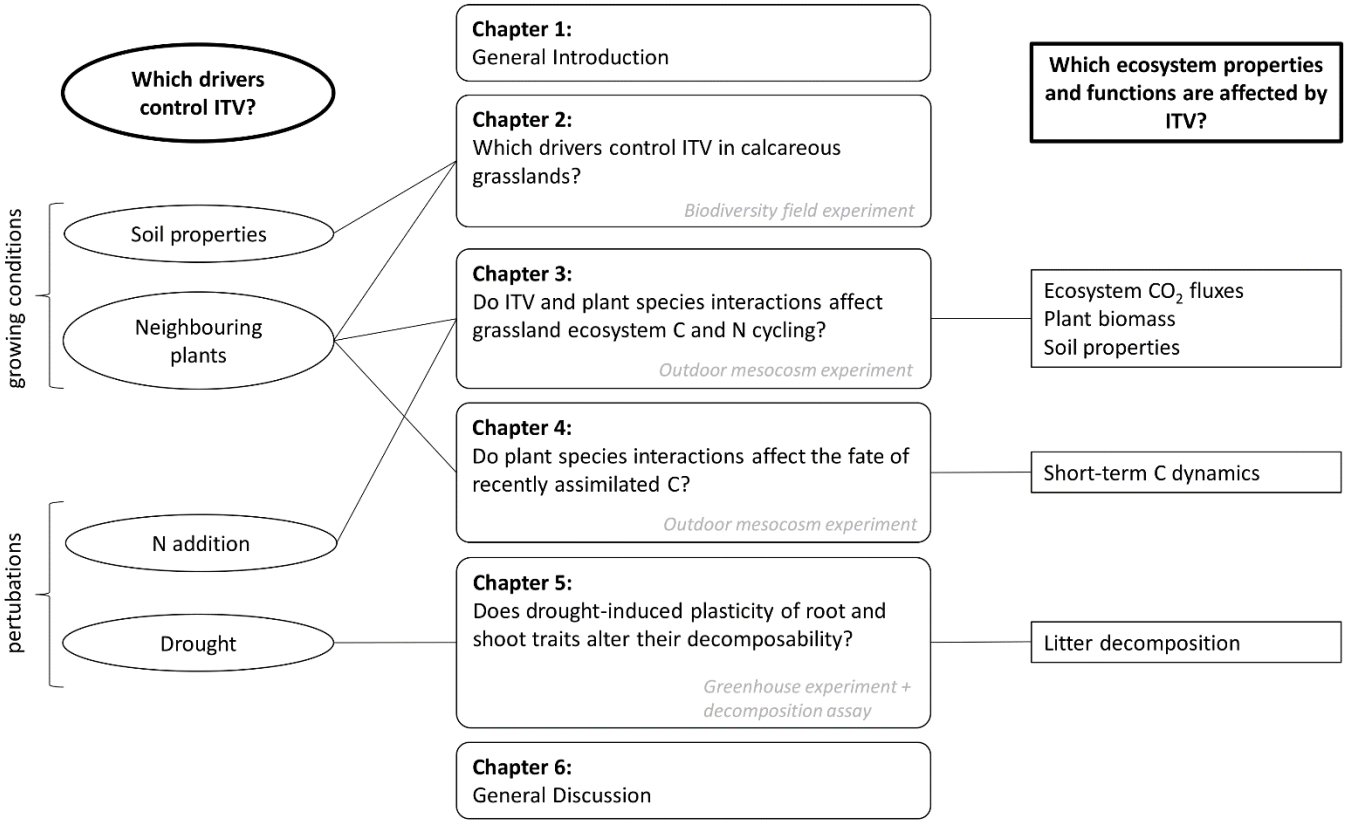

Figure 1.1: Conceptual diagram of the thesis structure, showing the drivers of intraspecific trait variation (ITV) and the ecosystem properties investigated in each chapter. C – carbon, N – nitrogen.

2 Above- and belowground drivers of intraspecific plant trait variability in calcareous grasslands

Abstract

Plant traits have been found to vary considerably within species within the same site. However, the mechanisms behind this variation are poorly understood. Here, a 4-year calcareous grassland biodiversity experiment on the Salisbury Plain was used to test the hypothesis that plants exhibit intraspecific trait variability depending on traits of the surrounding plant community due to differences in resource availability (e.g. light and soil nutrients). Focal individuals from three forb species were sampled from plots with differing (trait-based) functional group composition. For each focal individual, aboveground traits were measured along with properties of the surrounding plant community and soil. In two of the focal species, traits varied between functional group treatments, which could be linked to differences in resource availability caused by the surrounding plant community. In addition, variation in plant traits in all three focal species was correlated with a range of properties of the surrounding plant community and soil, for example sward height, above- and belowground plant biomass and stoichiometry, soil nitrogen availability and pH, as well as soil microbial properties.

These results show that plant community traits as well as vegetation and soil properties determine the magnitude of intraspecific trait variability in diverse calcareous grasslands. In these systems there is potential that including intraspecific trait variability as a response to these factors may improve models and predictions of ecosystem functioning.

Keywords: Plant traits, intraspecific trait variabilitye, plasticity, plant-soil interactions, soil microbial properties

2.1 Introduction

Plant traits can serve as easily measurable proxies for plant function, useful for predicting vegetation responses to environmental change, vegetation community processes, and effects of vegetation on ecosystem function (Garnier et al., 2015). Often, trait-based studies use mean values for plant species, based on the assumption that intraspecific trait variability (ITV) is negligible compared to interspecific variability (Grime, 1979; Shipley et al., 2016). However, it has been observed that ITV can be considerable. For example, in a French valley covering a gradient in temperature and radiation, ITV accounted for approximately 30 % of overall trait variability in plant species from different life forms including grasses, forbs and trees (Albert et al., 2010a). In a global meta-analysis ITV accounted for 25% of total trait variance within plant communities and 32% of total variance between communities (Siefert et al., 2015). ITV has also been shown to affect plant species' tolerance to environmental change (Valladares et al., 2014; Wright et al., 2016), community processes (Violle et al., 2012) and ecosystem functions (Lecerf & Chauvet, 2008; de Vries et al., 2016; Kazakou et al., 2019).

Accounting for ITV usually requires increased sampling effort, as traits of more individuals need to be measured. There are cases where it may be justified to neglect ITV, depending on the spatial scale and the aims of the study (Albert et al., 2011). Overall, the importance of ITV relative to interspecific variation decreases with increasing spatial scale (Siefert et al., 2015) and some traits (e.g. tissue nutrient concentrations) tend to be more variable than others (e.g. phenological traits) (Des Marais et al., 2013).

To determine when ITV needs to be considered, it is necessary to identify its dominant controls. Controls on ITV include genotypic differences, phenotypic plasticity and differences in age/growth stage. Genotypic differences are the result of evolutionary processes including mutation, migration, genetic drift and natural selection (Hughes et al., 2008). Phenotypic plasticity is the propensity of a single genotype to produce different phenotypes depending on environmental conditions (Sultan, 2000). There are a multitude of factors in the abiotic and biotic environment of a plant that interact to shape its phenotype, such as climate, availability of water and nutrients, shading, herbivory, grazing, disturbance, interactions with neighbouring plants and microbes, pathogens and many more (Valladares et al., 2007). Standardized protocols for trait measurements aim to minimize differences in age and growth stage by conducting measurements on "mature and healthy individuals" (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013), but some variability remains. Also, trait values measured on "mature and healthy" plants are unlikely to be representative of the majority of plants within a community, especially in managed and grazed grassland ecosystems.

Due to the range of interacting factors influencing ITV it is difficult to tease apart the most important controls on ITV in the field. Several studies have found that while there

is variability between sites, there is also considerable and often even larger variability between individuals within the same site (Albert et al., 2010b; Messier et al., 2010). However, the controls on local variability and their relative importance have not been studied in detail. A potentially important factor at the local scale is the interaction with the surrounding plant community and microhabitat. Plants can adjust their traits plastically to react directly to competition by neighbouring plants by avoiding them (e.g. by growing taller stems to avoid shade), by confronting and suppressing them (e.g. by increased shoot or root allocation to compete for light or nutrients/water) or by tolerating their competitive effects (e.g. by increasing specific leaf area to tolerate shade) (Novoplansky, 2009). Plants in pot experiments exhibit considerable trait plasticity as a response to neighbour species (e.g. Baxendale et al., 2014; Bennett et al., 2016). Additionally, plant community traits are known to affect soil properties such as nutrient cycling and physical properties (Grigulis et al., 2013; Gould et al., 2016; Fry et al., 2018), which could in turn induce phenotypic plasticity. Plants in pot experiments exhibit plasticity as a response to manipulations in soil properties, such as Nitrogen (N), Phosphorus (P) and water availability (Fort et al., 2015; Siebenkäs et al., 2015). Additionally, specific microhabitats might favour better adapted genotypes. On the other hand, ITV can have other reasons which are not related to the local environment, such as e.g. differences in age (especially in perennials) and growth stage, damage by pathogens or differences in genotype caused by neutral processes like dispersal (Hubbell, 2001). Also, adaptive plasticity can be limited when plants experience limitation of several resources at once (Freschet et al., 2015), as well as in stressful or unpredictable environments (Valladares et al., 2007). It is not understood well to what extent phenotypic and genotypic adaptations to the surrounding plant community and microhabitat play a role in controlling ITV at a local scale in the field.

The aim of this study was to investigate whether the magnitude of ITV in individual grassland species is determined by plant community traits of the surrounding vegetation through changes in resource availability. The study was conducted in a calcareous grassland experiment where plant communities consisting of three trait-based plant functional groups and their combinations had been sown to assess their effect on soil functions and drought tolerance (Fry et al., 2018). The functional groups differed mainly in rooting architecture (complex vs. simple), rooting depth (shallow vs. deep), plant height (tall vs. small) and resource strategy (acquisitive vs. conservative) (Fry et al., 2018). Three forb species were selected as focal species and sampled along with the surrounding plant community and soil to test the following hypotheses:

1. The magnitude of ITV depends on traits of the surrounding plant community due to differences in resource availability (e.g. light and nutrients).
    a. Plants grow taller and with higher SLA when growing in communities with higher sward height and biomass due to competition for light.
    b. Plants exhibit higher tissue N-content when growing in communities with more complex root architecture and a resource-acquisitive strategy due to higher N availability in the soil.
2. Communities with complex root architecture and a more resource-acquisitive strategy increase availability of nutrients in the soil compared to communities with a simple root architecture and more resource-conservative strategy. This is due to faster litter turnover, potentially higher rates of root exudation, increased microbial niche heterogeneity and better foraging ability.

2.2 Methods

2.2.1 Experimental system

The study was conducted on a 4-year grassland biodiversity-ecosystem function experiment (Fig. 2.1, see Fry et al. (2018) for more details) set up in 2013 in a former arable field on the Salisbury Plain, in Wiltshire, southern England (50.5988° N, 2.0709° E, 260 m above sea level) with calcareous soil and an organic layer of about 10 cm. The soil was bare at the beginning of the experiment.

The experiment consisted of plant functional group (FG) treatments composed of three FGs that were hypothesized to differ in their effects on soil functions. The plant species used in the experiment came from the CG3a plant community type (*Bromus erectus* grassland with typical sub-community according to the UK National Vegetation Classification (Rodwell, 1992)). Species were classified into one of the three FGs using a cluster analysis based on database trait values of all species. The traits used for classification were rooting depth, rooting architecture, height, specific leaf area and life history strategy. Each FG contained 15 to 20 species of grasses, forbs and legumes. Their key attributes are shown in Table 2.1. As described in Fry et al. (2018), species of FG 1 on average had deep tap roots (simple root architecture), tall height and relatively low SLA (rather resource conservative strategy). FG 2 consisted of on average smaller plants with coarse, but shallow roots (simple root architecture), low SLA and higher perenniality than the other two groups (resource conservative strategy). FG 3 consisted of on average tall species with fibrous, complex root architecture and higher SLA than the other two groups (resource acquisitive strategy).

The experiment was set up in a random factorial block design of 42 plots, 8 x 8 m in size, with spaces of 2 m between the plots. FGs were sown separately and in 2 and 3

way-combinations, resulting in seven treatments: FG 1, FG 2, FG 3, FG 1&2, FG 1&3, FG 2&3, FG 1,2&3. The treatments were randomly arranged in rows which were replicated six times, with each block consisting of a row of plots along the same height of the slope.

Figure 2.1: Aerial image of the experiment taken on 26/5/2017.

2.2.2 Sampling

Three forb species common in the experiment, one from each FG, were chosen. From the previous year's vegetation survey species were selected that occurred in each experimental plot of the relevant treatment. In addition, all three species were flowering at the time of sampling to aid identification. *Daucus carota* (common name: wild carrot) was chosen from FG 1, *Clinopodium vulgare* (common name: wild basil) from FG 2 and *Leucanthemum vulgare* (common name: ox-eye daisy) from FG 3 (see Table 2.1). Field sampling was carried out on 11th and 12th July 2017. One healthy-looking mature individual of each species was selected in each of the plots in which their FG occurred, resulting in 3 species x 4 treatments x 6 replicates = 72 individuals. Turves (25x25 cm)

turves were sampled beneath focal plants to the depth of the bedrock (approximately 10 cm). The turves were transported to the laboratory in plastic bags within two days. In each turf, traits of the focal individuals as well as vegetation community and soil properties were measured, as described in the following sections.

During the measurements of traits and aboveground vegetation properties, the samples were stored in a shaded space outdoors for one week and watered daily due to hot weather. After harvesting aboveground biomass, the turves were stored in the dark at 4°C while processing root and soil samples.

Table 2.1: Key attributes of each functional group (FG) (Fry et al., 2018) and focal species chosen in each FG.

	FG 1	**FG 2**	**FG 3**
Root architecture	simple (tap roots)	simple	complex (fibrous)
Rooting depth	deep	shallow	shallow
Plant height	tall	small	tall
Resource strategy	rather conservative	conservative	acquisitive
Perenniality	low	high	medium
Focal species	*Daucus carota*	*Clinopodium vulgare*	*Leucanthemum vulgare*

2.2.3 Traits of focal individuals

The height of each focal individual was measured as the distance between the top of the photosynthetic tissue and the soil surface. Then, shoots of the focal individuals were cut at the base and re-hydrated overnight at 4 °C in wet tissue paper, as plants in the field may be dehydrated to an unknown extent and this makes the measurements more comparable (Pérez-Harguindeguy et al., 2013). Shoots were weighed. 2-8 mature and healthy-looking leaves per individual were weighed and scanned using an EPSON flatbed scanner. Leaf area was analysed using the software WinRhizo (Regent Instruments Inc., Sainte-Foy-Sillery-Cap-Rouge, QC, Canada). Leaf and shoot dry

weight were determined after drying for 48 hours at 65 °C. Specific leaf area (SLA) was calculated as the ratio of leaf area to leaf dry weight. Leaf dry matter content (LDMC) was calculated as leaf dry weight divided by leaf fresh weight. Dried leaves were ground in a ball mill and 15 mg used to analyse leaf C and N content in an elemental analyser (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

2.2.4 Aboveground vegetation properties

For an estimation of sward height, the height of the 5th highest shoot (excluding the focal shoot) was measured in each turf to obtain a measure relevant to shading of the focal plant. Aboveground biomass was cut at the base in an area of 15×15 cm in the middle of the turf, dried at 65°C for 48 hours, weighed and ground in a ball mill with 15 mg sub-samples analysed for C and N content in the elemental analyser.

2.2.5 Belowground vegetation properties

A 10 × 10 cm sub-sample of soil was cut out of the middle of each turf. Soil was shaken-off and collected for soil analyses and roots were carefully washed, weighed, and stored in 50% Ethanol at 4°C until needed for analysis. Roots were sorted into coarse roots of >1mm diameter and fine roots of <1mm diameter. Fine roots were cut into 2 cm long segments and 4 sub-samples per sample were weighed and scanned using an EPSON flatbed scanner. Scans were analysed for root length and diameter using the software Winrhizo. All roots were oven-dried at 65°C for 48h and weighed. Specific root length (SRL) was calculated as length per dry biomass. Root tissue density (RTD) was calculated as root volume per dry biomass. Root dry matter content (RDMC) was calculated for fine roots as dry divided by fresh mass. The fine root sub-samples were ground in a ball mill and 15 mg were analysed for C and N content in the elemental analyser.

2.2.6 Soil properties

Soil was passed through a 2mm sieve. For pH measurements, 10 g of fresh soil was mixed with 25 ml deionized water, passed in the shaker for 30 minutes and left to rest for another 30 minutes. Soil pH was measured using a pH meter (Mettler Toledo, Salford, UK). Soil moisture content was determined by calculating the mass loss of soil after oven-drying at 105 °C for 48 hours. For soil C and N content, oven-dried soil was ball-milled with 30 mg sub-sample analysed in the elemental analyser. Olsen P, a proxy for plant available phosphate in soil, was measured by mixing 5 g of fresh soil with 100ml 0.5M sodium bicarbonate. The extract was frozen until analysis on an autoanalyser for phosphate content (Bran and Luebbe, Northampton, UK). Microbial biomass C and N were measured using the chloroform fumigation-incubation method (Brookes et al., 1985). Sub-samples (5 g) of fresh soil were fumigated with chloroform for 24 hours before extraction with 25 ml of 0.5 M K_2SO_4. Another set of sub-samples (5 g) of fresh soil were extracted with K_2SO_4 in the same way without fumigation. The extracts were analysed for microbial C and N using a TOC analyser (5000A, Shimadzu, Milton Keynes, UK). Microbial C and N were calculated as the difference between fumigated and unfumigated soil multiplied by adjustment factors $k_C = 0.35$ (Sparling et al., 1990) and $k_N = 0.54$ (Brookes et al., 1985). K_2SO_4-extractable N was used as a proxy for plant available N (Jones & Willett, 2006). Microbial community composition was assessed using phospholipid fatty acid (PLFA) analysis. PLFAs were extracted from 1.5 g freeze-dried soil (Bligh & Dyer, 1959; White et al., 1979), fractionated using un-bonded silica columns (ISOLUTE SI, Biotage, Sweden) and analysed on an Agilent 6890 gas chromatograph (Agilent, US). Gram positive PLFA was identified using the biomarkers i15:0, a15:0, i16:0, 7Me17:0, i17:0 and a17:0, gram negative PLFA using the biomarkers 16:1ω7, 16:1ω5, cy17:0, 18:1ω7 and cy19:0 and bacterial PLFA using

all of these biomarkers. Fungal PLFA was identified using the biomarkers 18:1ω9 and 18:2ω6,9. Total PLFA were identified using all biomarkers mentioned before and additionally 14:0, 16:1, 16:0, 17:1ω8, br17:0, br18:0, 18:1ω5, 18:0 and 19:1. Additionally, gram positive to negative and fungal to bacterial ratios were calculated. All measures conducted on fresh soil were converted to units per gram dry soil.

2.2.7 Statistical Analyses

Statistical analyses were conducted in R version 3.6.1 (R Core Team, 2019) and figures were produced using the *ggplot2* package (Wickham, 2016). Variables were $\log_{10}$- or square root- transformed when necessary to fulfil model assumptions.

Plant FG treatment effects on traits of focal plants (Hypothesis 1a and b) were tested using Analysis of variance (ANOVA), separately for each species. First, linear models with focal traits as response variable and FG treatment as explanatory variable were fit without random effects using the function lm(). When a plot of model residuals against block showed a block-dependent pattern upon visual examination, linear mixed models were fit using the lmer()-function of the *lme4* package (Bates et al., 2015). In these cases, block was included in the model as a random effect and FG treatment as a fixed effect. This approach was chosen because some response variables were not affected by block and in these cases fitting a model with random effects resulted in overfitting ("singular fit"). Table 2.2 shows the model structure including random effects and transformations for each response variable. For linear models without random effects, the significance of treatment effects was calculated using ANOVA. For linear mixed models, the significance of treatment effects was calculated using likelihood ratio tests (with restricted maximum likelihood). Models were compared to null models with only

random effects using the function anova(model, null model). Tukey post hoc tests were performed using the glht()-function of the *multcomp* package (Hothorn et al., 2008).

Additionally, the effects of presence/absence of each FG (except the FG that the focal species belonged to, as this FG was present in all treatments) on the same response variables were tested. This was done because plots of the data suggested that while ANOVA did not detect significant differences between treatments, there were trends related to the presence/absence of specific FGs. For this, models were constructed using the same transformations and random effect structure as for the first set of models (see Table 2.2), but with presence/absence of one of the FGs as a fixed effect. Significance of presence/absence of a specific FG was calculated as for the first set of models, using either likelihood ratio tests or ANOVA. Additionally, when presence/absence of one of the FGs had a significant effect, models were compared to a full model including presence/absence of both FGs as fixed effects using likelihood ratio tests or ANOVA. This was to ensure that the models were not missing effects of the other FG. However, there was no case where the full model fit significantly better than the model including presence/absence of only one FG, which means that the models including presence/absence of only one FG were suitable to analyse this data.

To test Hypothesis 2, a similar procedure was used as for testing Hypothesis 1, this time using all data, not separated by focal species. FG treatment effects on vegetation and soil properties were again first modelled using the lm()-function without random effects. When a plot of model residuals against block or focal species identity showed a block- and/or species- dependent pattern upon visual examination, linear mixed models were fit using the lmer()-function. In these cases, block and/or focal species were included in the model as (crossed) random effect(s) and FG treatment as fixed

effect. The significance of treatment effects was again determined using either ANOVA or likelihood ratio tests. Model structures are shown in Table 2.3. Effects of presence/absence of a specific FG were tested using the same procedure as for Hypothesis 1, but this time including all three FGs. When presence/absence of one of the FGs had a significant effect, models were compared to a full model including presence/absence of all three FGs as fixed effects using likelihood ratio tests or ANOVA. Again, there was no case where the full model fit significantly better than the model including presence/absence of only one FG.

As supporting analysis, correlation matrices were computed between traits of focal individuals and soil/vegetation properties to explore the mechanisms potentially controlling ITV. This was done for each focal species separately. Even though this analysis has a risk of false-positive results due to multiple testing, no correction procedure (e.g. Bonferroni) was applied as these may be over-penalizing (Moran et al., 2003). Instead, the correlation matrices were interpreted with caution: where only one trait was significantly correlated with only one vegetation or soil property, the p-value was only slightly lower than 0.05 and there did not seem to be a biologically meaningful interpretation (e.g. in the case of the correlation between leaf N in *Daucus* with root C of the surrounding plant community), the pattern may have arisen by chance. Only if either (i) the p-value was highly significant, if (ii) a trait was correlated with several inter-related vegetation/soil properties, or if (iii) several traits were correlated with the same vegetation/soil property, it was considered likely that there was an underlying biological cause (Moran et al., 2003).

As a supplementary analysis, coefficients of variation were computed for each trait of each focal species.

Table 2.2: Model structure for (mixed effect) ANOVA testing for the effects of functionals group (FG) combination as well as the effects of FG presence/absence on focal traits. *y* stands for response variables, *x* for fixed effects and (1|block) for block as a random effect. C – carbon, N – nitrogen.

variable	**model structure**		
	Daucus	*Clinopodium*	*Leucanthemum*
Height	$y \sim x + (1\|block)$	$y \sim x$	$y \sim x + (1\|block)$
Shoot dry weight	$\log_{10}(y) \sim x$	$y \sim x$	$\log_{10}(y) \sim x + (1\|block)$
Height/shoot dry weight	$\log_{10}(y) \sim x$	$\log_{10}(y) \sim x$	$\log_{10}(y) \sim x + (1\|block)$
Specific leaf area	$\log_{10}(y) \sim x + (1\|block)$	$\log_{10}(y) \sim x + (1\|block)$	$\log_{10}(y) \sim x + (1\|block)$
Leaf dry matter content	$y \sim x$	$\log_{10}(y) \sim x + (1\|block)$	$y \sim x + (1\|block)$
Leaf C	$y \sim x + (1\|block)$	$y \sim x$	$y \sim x$
Leaf N	$\log_{10}(y) \sim x + (1\|block)$	$\log_{10}(y) \sim x + (1\|block)$	$\log_{10}(y) \sim x + (1\|block)$
Leaf C : N ratio	$y \sim x + (1\|block)$	$\log_{10}(y) \sim x + (1\|block)$	$y \sim x$

Table 2.3: Model structure for (mixed effect) ANOVA testing for the effects of functional group (FG) combination and FG presence/absence on soil/vegetation properties. *y* stands for response variables, *x* for fixed effects and (1|block) and (1|species) for block/focal species as random effect. PLFA - phospholipid fatty acids, C – carbon, N – nitrogen.

variable	**model_structure**
Aboveground biomass	log10(y) ~ x + (1|block)
Sward height	y ~ x + (1|block)
Aboveground biomass C	1/log10(y) ~ x + (1|species)
Aboveground biomass N	log10(y) ~ x + (1| block)
Aboveground biomass C : N ratio	y ~ x + (1|block)
Total root dry weight	log10(y) ~ x + (1|species)
Fine root dry weight	log10(y) ~ x + (1|species)
Coarse root dry weight	sqrt(y) ~ x
Root to shoot ratio	sqrt(y) ~ x + (1|species)
Mean root diameter	1/(y) ~ x + (1|species)
Specific root length	log10(y) ~ x
Root tissue density	log10(y) ~ x
Root dry matter content	y ~ x
Total fine root length	log10(y) ~ x + (1|block) + (1|species)
Root C	y ~ x + (1|species)
Root N	y ~ x + (1|block)
Root C : N ratio	log10(y) ~ x + (1|block)
Soil pH	y ~ x + (1|species)
Soil C	log10(y) ~ x
Soil N	y ~ x + (1|block)
Soil C : N ratio	log10(y) ~ x + (1|block)
Olsen phosphorus	sqrt(y) ~ x + (1|block)
K_2SO_4-extractable N	log10(y) ~ x + (1|block) + (1|species)
Microbial C	y ~ x + (1|block) + (1|species)
Microbial N	y ~ x + (1|block) + (1|species)
Microbial C : N ratio	y ~ x + (1|block)
Fungal PLFA	log10(y) ~ x + (1|species)
Bacterial PLFA	y ~ x + (1|species)
Fungal to bacterial ratio	log10(y) ~ x
Gram negative PLFA	y ~ x + (1|species)
Gram positive PLFA	y ~ x + (1|species)
Gram positive to negative ratio	y ~ x
Total PLFA	y ~ x + (1|species)

2.3 Results

2.3.1 Effects of FG composition and presence/absence of FGs on trait variability of focal plants

Treatment effects on traits of focal plant individuals (Hypothesis 1) were tested using ANOVA that included random effects as needed (Table 2.2).

In *Daucus*, FG combination significantly ($p < 0.05$) affected height of the focal individuals (Fig. 2.2 A, Table S 2.4 a), $p = 0.011$). Tukey post hoc testing revealed that the height of individuals growing in FG 1 plots was less than the height of individuals growing in FG 1&3 and FG 1,2&3 plots (Table S 2.4 a). The presence of FG 3 also affected a range of traits in focal individuals (Table S 2.4 b), increasing height by 19% ($p = 0.002$) and leaf N content by 20% ($p = 0.017$) and decreasing C : N ratio by 16% ($p = 0.016$). The presence of FG 3 also increased shoot dry weight by 66%, albeit not significantly ($p = 0.082$).

In *Clinopodium*, FG combination significantly affected LDMC of the focal individuals (Fig. 2.2 B, Table S 2.5 a, $p = 0.024$). Tukey post hoc testing revealed that individuals growing in FG 1&2 plots had higher LDMC than individuals growing in FG 1 plots, but presence/absence of a specific FG did not have any effect on LDMC (see Table S 2.5). Presence of FG 3 significantly increased the height of focal individuals by 21% or 9.3 cm ($p = 0.029$).

In *Leucanthemum*, there was a significant effect of FG combination on leaf C (Fig. 2.2 C, Table S 2.6 a, $p = 0.033$). However, Tukey post hoc testing revealed no significant differences between any of the groups.

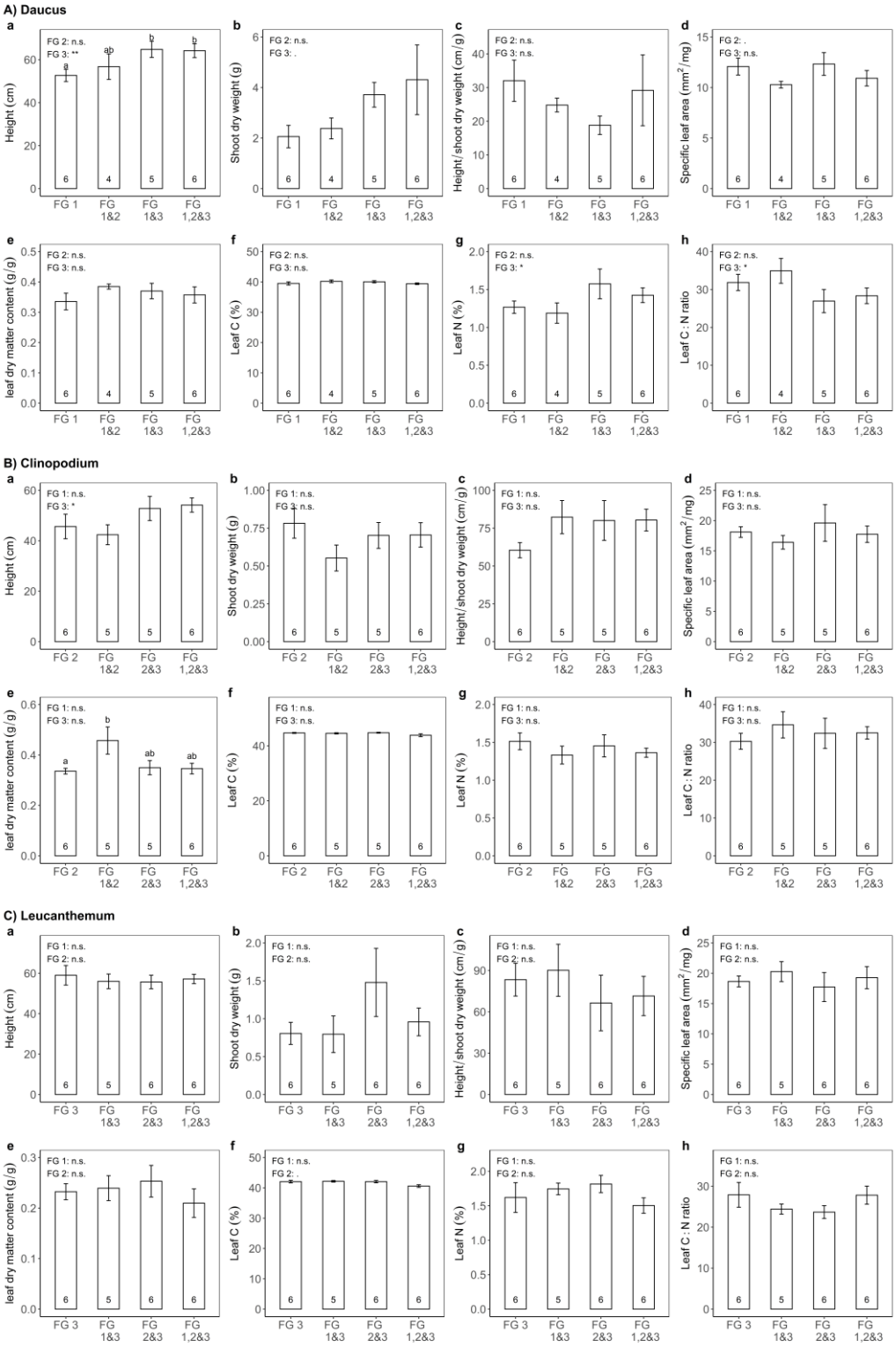

Figure 2.2: Effects of functional group (FG) combination and presence/absence on focal traits of *Daucus* (A), *Clinopodium* (B) and *Leucanthemum* (C) (mean +/- standard error). Different letters on top of bars indicate significant ($p < 0.05$) differences between treatments tested with ANOVA (random effect structure see Table 2.2) and subsequent Tukey post hoc test. Effects of FG presence/absence are indicated in the top left corner of each bar plot. Number of samples in each group is indicated at the bottom of each bar ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*, $p < 0.1$·). C – carbon, N – nitrogen.

2.3.2 Effects of FG composition and presence/absence of FGs on plant community properties

To determine FG treatment effects on vegetation properties (Hypothesis 2), ANOVA were conducted, that included random effects as needed (Table 2.3). Sward height was the only aboveground vegetation property significantly affected by FG combination (Fig. 2.3, Table S 2.1, $p = 0.015$). However, a wider range of properties were affected by the presence/absence of specific FGs. The presence of FG 3 increased sward height by 40% or 16 cm on average ($p<0.001$) with a non-significant increase in aboveground biomass by 23 % ($p = 0.098$), while the presence of FG 2 decreased aboveground biomass N by 10% ($p = 0.007$) and increased aboveground biomass C : N ratio by 10% (Table S 2.2, $p = 0.01$). The only belowground vegetation properties affected by FG combination were root to shoot ratio ($p = 0.008$) and RDMC (Fig. 2.4, Table S 2.1, $p = 0.010$). However, the presence of specific FGs had significant effects on a range of belowground vegetation properties (Fig. 2.4, Table S 2.2). The presence of FG 1 decreased RDMC by 7% ($p = 0.001$). The presence of FG 2 increased total root dry weight by 55% ($p = 0.021$), fine root dry weight by 64% ($p = 0.007$) and RTD by 52% ($p = 0.043$). The presence of FG 3 decreased root C by 1% (Table S 2.2, $p = 0.049$).

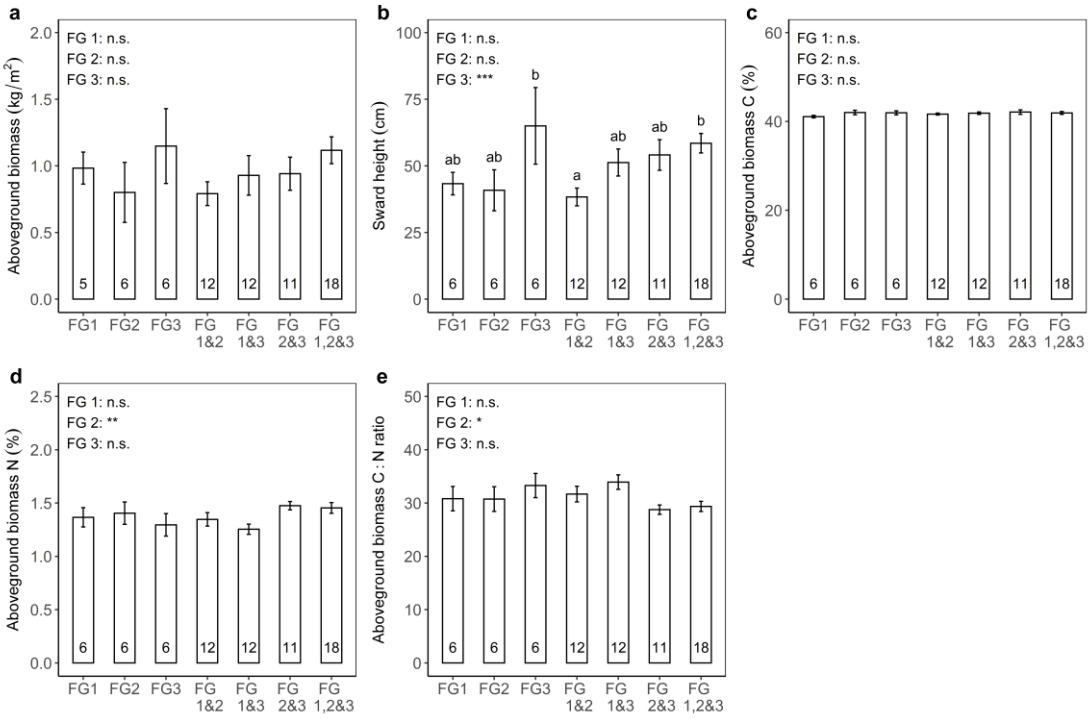

Figure 2.3: Effects of functional group (FG) combination and presence/absence on aboveground vegetation properties (mean +/- standard error). Different letters on top of bars indicate significant ($p < 0.05$) differences between FG combinations tested with ANOVA (random effect structure see Table 2.3) and subsequent Tukey post hoc test. Different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$). Effects of FG presence/absence are indicated in the top left corner of each bar plot ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*, $p < 0.1$·). Number of samples in each group is indicated at the bottom of each bar. C – carbon, N – nitrogen.

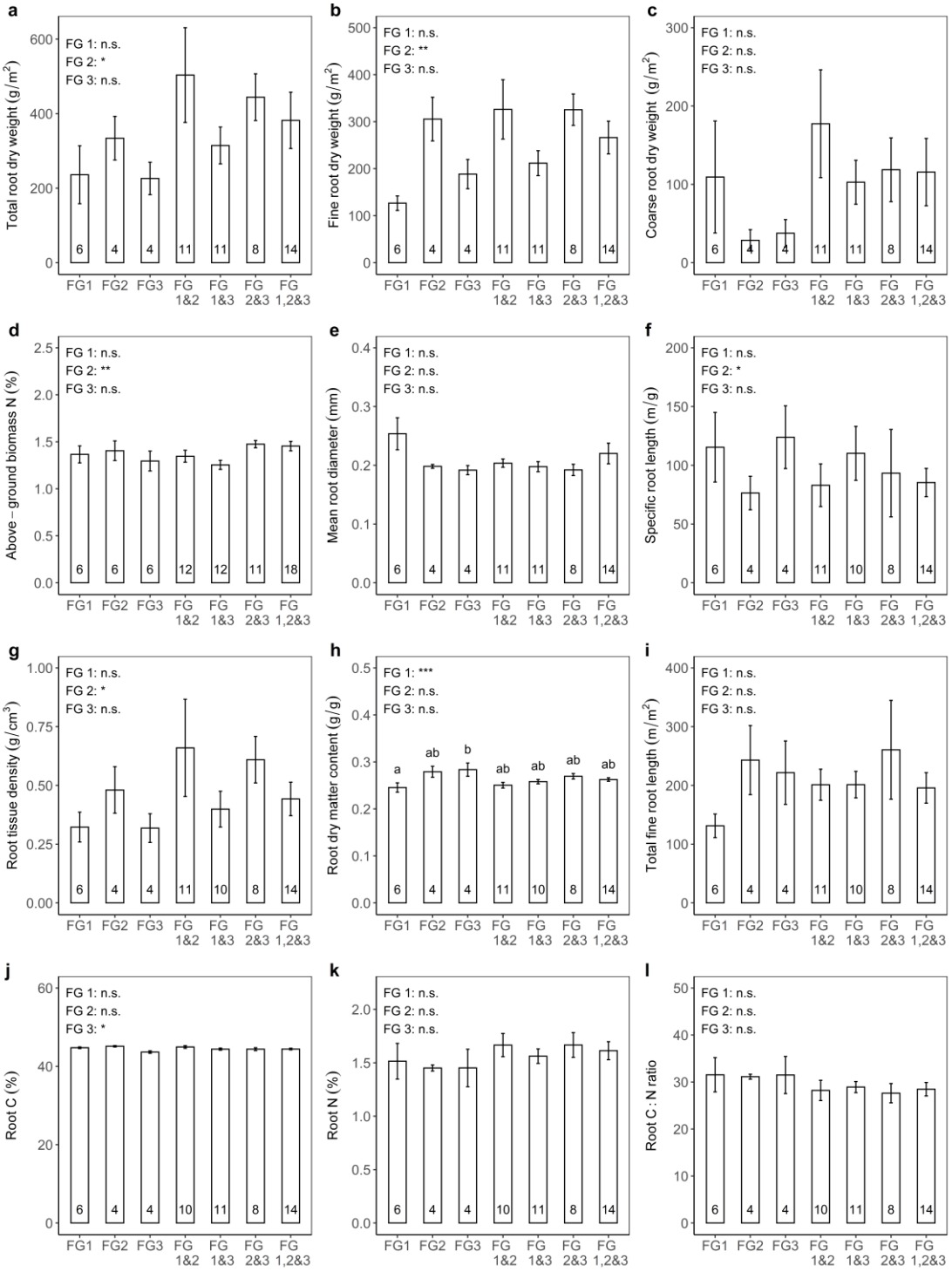

Figure 2.4: Effects of functional group (FG) combination and presence/absence on belowground vegetation properties (mean +/- standard error). Different letters on top of bars indicate significant ($p < 0.05$) differences between FG combinations tested with ANOVA (random effect structure see Table 2.3) and subsequent Tukey post hoc test. Different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$). Effects of FG presence/absence are indicated in the top left corner of each bar plot ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*, $p < 0.1$·). Number of samples in each group is indicated at the bottom of each bar. C – carbon, N – nitrogen.

2.3.3 Effects of FG composition and presence/absence of FGs on soil properties

Treatment effects on soil properties (Hypothesis 2) were tested using ANOVA that included random effects as needed (Table 2.3). FG combination did not significantly affect any soil properties (see Fig. 2.5 and 2.6, Table S 2.1). However, the presence of FG 1 decreased soil C : N ratio on average by 8% ($p = 0.028$) and Olsen P by 33% ($p = 0.048$); FG 3 increased K_2SO_4-extractable N by 68% ($p = 0.002$) and gram positive to negative bacterial ratio by 3% ($p = 0.015$).

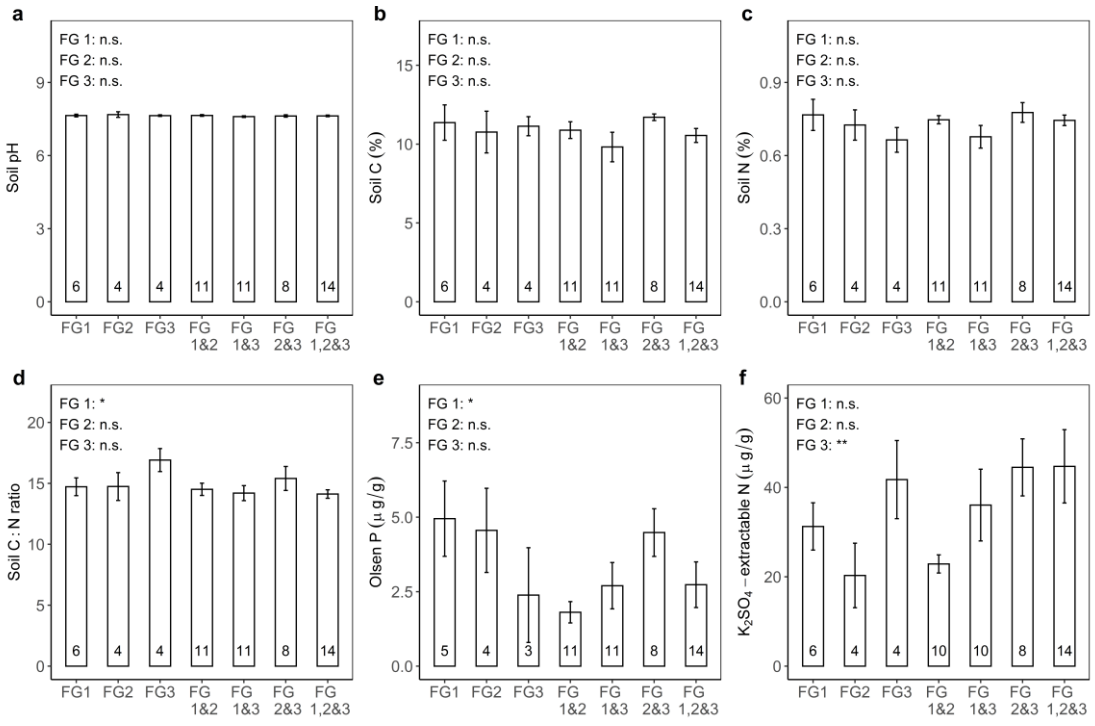

Figure 2.5: Effects of FG combination and presence/absence on soil properties (mean +/- standard error). Different letters on top of bars indicate significant ($p < 0.05$) differences between FG combinations tested with ANOVA (random effect structure see Table 2.3) and subsequent Tukey post hoc test. Different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$). Effects of FG presence/absence are indicated in the top left corner of each bar plot ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). Number of samples N in each group is indicated at the bottom of each bar. C – carbon, N – nitrogen.

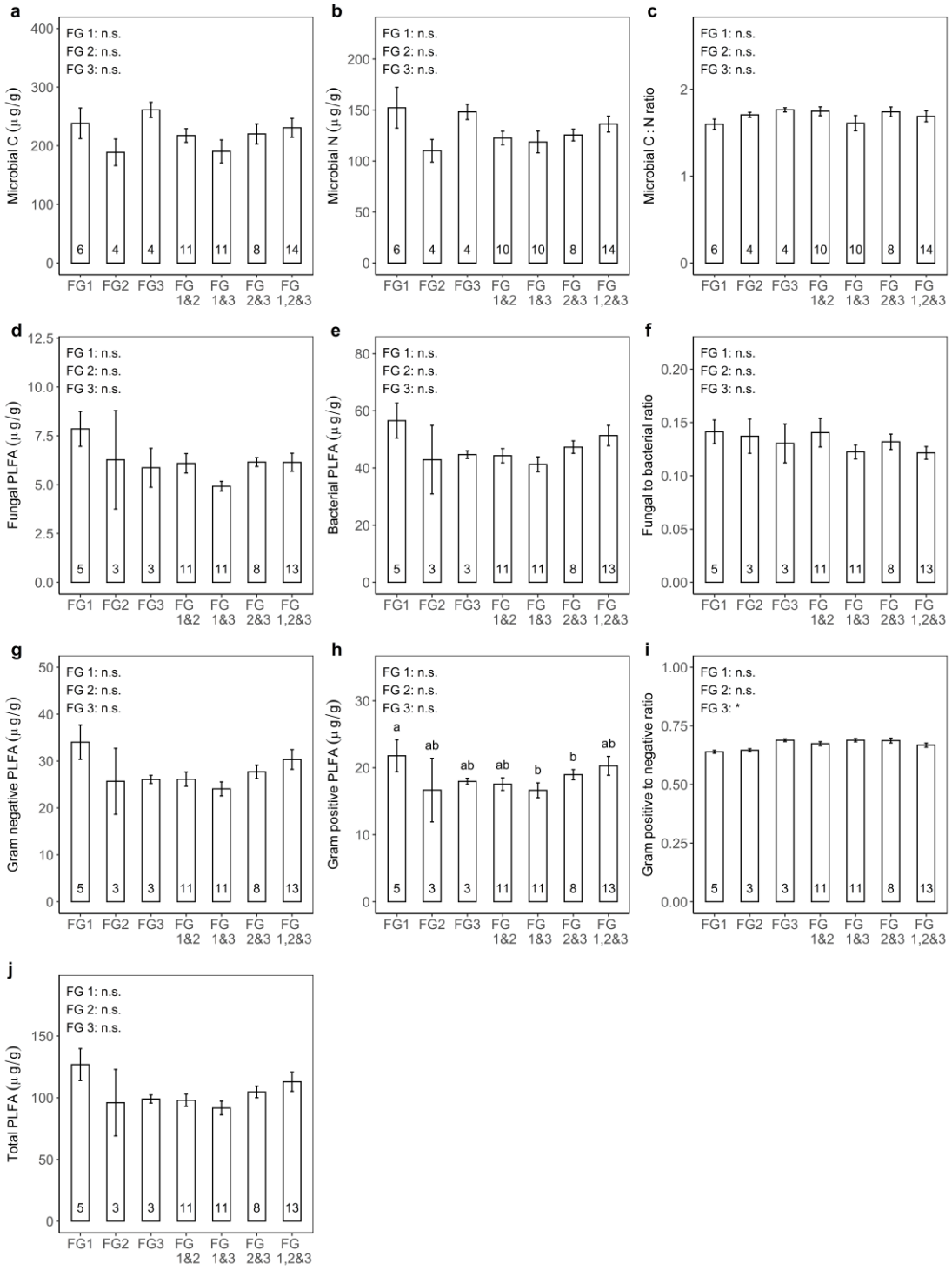

Figure 2.6: Effects of FG combination and presence/absence on soil microbial properties (mean +/- standard error). Different letters on top of bars indicate significant ($p < 0.05$) differences between FG combinations tested with ANOVA (random effect structure see Table 2.3) and subsequent Tukey post hoc test. Effects of FG presence/absence are indicated in the top left corner of each bar plot ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). Number of samples N in each group is indicated at the bottom of each bar. C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

2.3.4 Correlations between traits of focal individual plants and surrounding plant and soil properties

As an exploratory analysis of the mechanisms potentially controlling ITV, pairwise correlations were conducted between traits of focal individuals and vegetation/soil properties. Correlations with $p < 0.05$ were considered significant. However, as explained in the Methods section, only if either (i) the p-value was highly significant, if (ii) a trait was correlated with several inter-related vegetation/soil properties, or if (iii) several traits were correlated with the same vegetation/soil property, it was considered likely that there was an underlying biological cause (Moran et al., 2003) and only these cases will be mentioned in the following sections.

In *Daucus* (Fig. 2.7, Table 2.4), height was positively correlated with extractable N ($r = 0.66$). Also, height was negatively correlated with fungal, bacterial, gram negative and total PLFA ($r = -0.53$ to -0.67) and positively with gram positive to negative ratio ($r = 0.60$). These PLFA measures were also co-correlated. This is of interest, because the co-correlation points towards a common underlying mechanism, while the absence of correlation points towards several underlying mechanisms. None of the PLFA measures were significantly correlated with extractable N. Shoot dry weight was negatively correlated with microbial C and fungal, bacterial, gram negative and total PLFA ($r = -0.50$ to -0.56), which were all co-correlated. The ratio of height/shoot dry weight was positively correlated with microbial C ($r = 0.48$). SLA was not significantly correlated with any of the soil or vegetation properties. LDMC was correlated with a range of root properties: positively with total and coarse root biomass, root to shoot ratio and root N ($r = 0.52$ to 0.62) and negatively with SRL ($r = -0.54$) and root C : N ratio ($r = -0.57$). Total and coarse root biomass, root to shoot ratio and SRL were all co-correlated. Root N and root C : N ratio were correlated and there was a non-significant

correlation between root N and SRL ($p<0.1$). Leaf C was positively correlated with aboveground biomass C : N ratio ($r = 0.44$). Leaf N was negatively correlated with root C ($r = -0.50$). It was also positively correlated with K_2SO_4-extractable N, albeit not significantly ($r = 0.36$, $p>0.1$). Leaf C : N ratio was positively correlated with root C ($r = 0.49$).

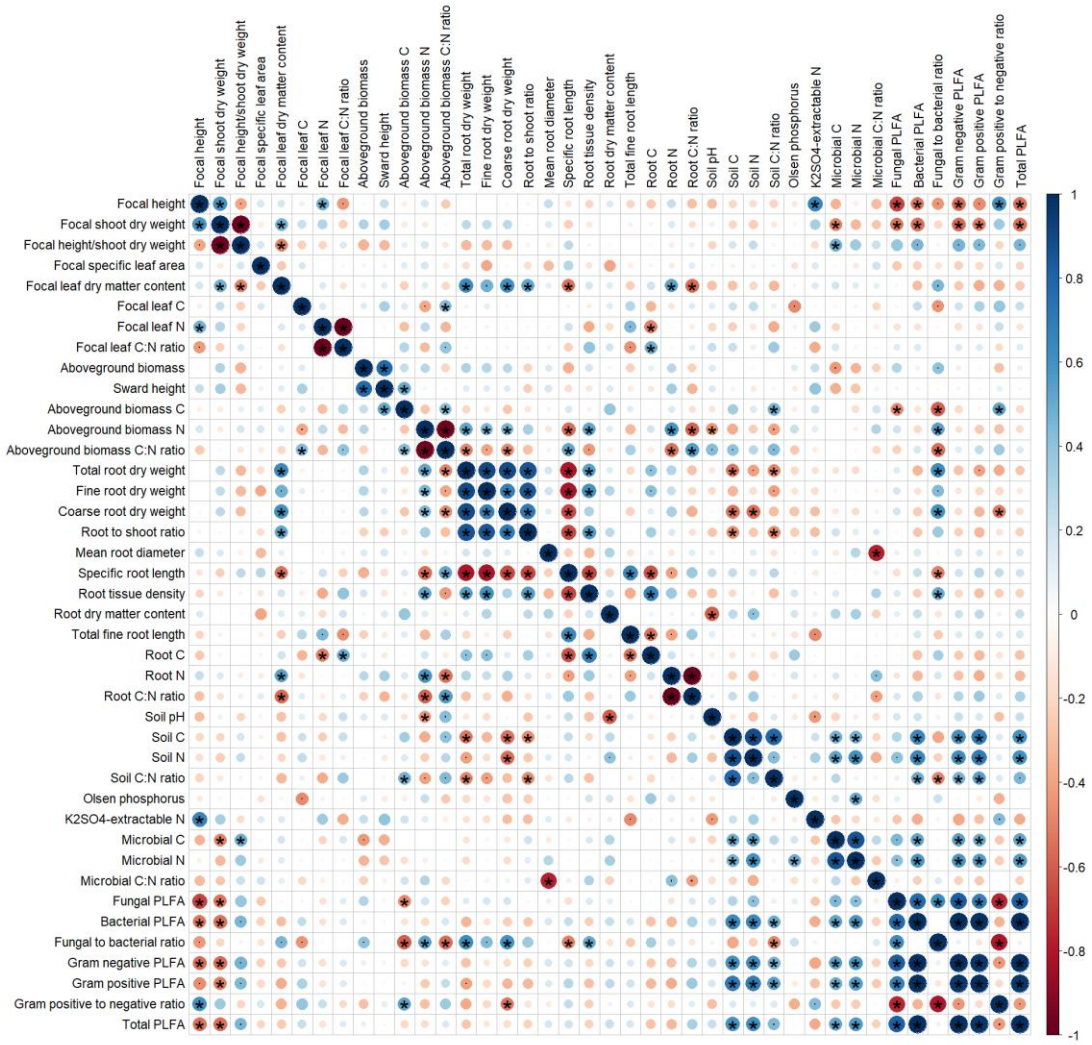

Figure 2.7: Pairwise correlations between traits of focal individuals in *Daucus* and vegetation/soil properties. Colour and size of circles indicate Pearson correlation coefficients. Significant correlations ($p < 0.05$) are marked with "*", correlations with $p<0.1$ are marked with "·". C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

Table 2.4: Pearson correlation coefficients between traits of focal individuals and vegetation/soil properties in *Daucus*. Significance of correlation is indicated as: p < 0.001***, p < 0.01**, p < 0.05*, p < 0.1·. C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

	Height	**Shoot dry weight**	**Height/ shoot dry weight**	**Specific leaf area**	**Leaf dry matter content**	**Leaf C**	**Leaf N**	**Leaf C : N ratio**
Aboveground biomass	0.03	0.31	-0.35	-0.06	0.09	0.14	-0.01	0.02
Sward height	0.23	0.34	-0.32	0.08	0.13	0.32	0.04	0.00
Aboveground biomass C	-0.08	-0.10	0.10	0.13	-0.23	0.15	-0.29	0.29
Aboveground biomass N	0.17	-0.12	0.18	0.17	0.13	-0.38·	0.25	-0.31
Aboveground biomass C : N ratio	-0.26	0.05	-0.12	-0.10	-0.21	**0.44***	-0.34	0.40·
Total root dry weight	-0.01	0.26	-0.32	-0.17	**0.62****	-0.08	-0.03	0.04
Fine root dry weight	-0.01	0.25	-0.31	-0.38	0.47·	0.12	0.06	-0.03
Coarse root dry weight	0.05	0.27	-0.30	-0.04	**0.60***	-0.05	-0.04	0.06
Root to shoot ratio	-0.06	0.04	-0.07	-0.17	**0.52***	-0.01	-0.04	0.05
Mean root diameter	0.23	0.15	-0.09	-0.32	0.11	-0.01	0.04	-0.07
Specific root length	-0.14	-0.24	0.25	0.29	**-0.54***	0.05	0.20	-0.24
Root tissue density	-0.21	-0.12	0.06	-0.13	0.19	-0.10	-0.38	0.40
Root dry matter content	0.15	0.02	0.03	-0.39	0.13	0.13	-0.19	0.19
Total fine root length	-0.22	-0.08	0.02	-0.09	-0.24	0.29	0.44·	-0.46·
Root C	-0.27	-0.06	-0.02	-0.06	0.20	-0.33	**-0.50***	**0.49***
Root N	0.24	0.15	-0.10	-0.11	**0.53***	-0.13	0.02	-0.03
Root C : N ratio	-0.29	-0.16	0.09	0.12	**-0.57***	0.13	-0.10	0.11
Soil pH	-0.30	0.09	-0.21	0.06	-0.29	0.12	-0.19	0.23
Soil C	-0.21	-0.21	0.19	-0.08	-0.29	0.22	-0.24	0.23
Soil N	-0.17	-0.25	0.25	-0.07	-0.20	0.25	-0.12	0.10
Soil C : N ratio	-0.22	-0.11	0.06	-0.06	-0.32	0.10	-0.37	0.36
Olsen phosphorus	-0.11	-0.05	0.02	-0.16	0.06	-0.48·	-0.08	0.03
K_2SO_4-extractable N	**0.66****	0.34	-0.19	0.14	0.14	-0.04	0.36	-0.36
Microbial C	-0.34	**-0.50***	**0.48***	-0.04	0.18	-0.05	-0.22	0.17
Microbial N	-0.09	-0.32	0.36	-0.10	0.13	0.06	0.03	-0.05
Microbial C : N ratio	-0.32	-0.28	0.22	0.21	0.11	-0.21	-0.08	0.04
Fungal PLFA	**-0.67****	**-0.54***	0.38	-0.27	0.05	-0.05	-0.16	0.13
Bacterial PLFA	**-0.53***	**-0.55***	0.46·	-0.22	-0.30	0.24	-0.16	0.15
Fungal to bacterial ratio	-0.43·	-0.20	0.06	-0.15	0.46·	-0.45·	-0.04	0.00
Gram negative PLFA	**-0.57***	**-0.56***	0.46·	-0.23	-0.26	0.20	-0.17	0.16
Gram positive PLFA	-0.46·	**-0.51***	0.45·	-0.20	-0.37	0.31	-0.14	0.14
Gram positive to negative ratio	**0.60***	0.37	-0.21	0.17	-0.35	0.38	0.23	-0.19
Total PLFA	**-0.55***	**-0.55***	0.45·	-0.22	-0.25	0.22	-0.14	0.14

In *Clinopodium*, height was positively correlated with aboveground biomass (Fig. 2.8, Table 2.5, r = 0.67), sward height (r = 0.60) and negatively with aboveground biomass C : N ratio (r = -0.46) and root to shoot ratio (r = -0.51). Aboveground biomass, sward height and root to shoot ratio were co-correlated. Aboveground biomass C : N ratio was significantly correlated with sward height and non-significantly with aboveground biomass ($p<0.1$). Shoot dry weight was negatively correlated with aboveground biomass C : N ratio (r = -0.43) and positively with RDMC (r = 0.48), which were as well co-correlated. The ratio of height/shoot dry weight was not significantly correlated with any of the soil or vegetation properties. SLA was negatively correlated with mean root diameter (r = -0.51) and root N (r = -0.62) and positively with root C : N ratio (r = 0.65) and soil N (r = 0.56). Root N and root C : N ratio were correlated with each other, but mean root diameter and soil N were not correlated with any of the other properties. LDMC was positively correlated with root to shoot ratio (r = 0.52) and fungal to bacterial ratio (r = 0.56), which were also co-correlated. Leaf C was not significantly correlated with any of the soil or vegetation properties. Leaf N was positively correlated with soil N (r = 0.51) and leaf C : N ratio negatively (r = -0.49).

Figure 2.8: Pairwise correlations between traits of focal individuals in *Clinopodium* and vegetation/soil properties. Colour and size of circles indicate Pearson correlation coefficients. Significant correlations ($p < 0.05$) are marked with "*", correlations with $p < 0.1$ are marked with "·". C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

Table 2.5: Pearson correlation coefficients between traits of focal individuals and vegetation/soil properties in *Clinopodium*. Significance of correlation is indicated as: p < 0.001***, p < 0.01**, p < 0.05*, p < 0.1·. C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

	Height	**Shoot dry weight**	**Height/ shoot dry weight**	**Specific leaf area**	**Leaf dry matter content**	**Leaf C**	**Leaf N**	**Leaf C : N ratio**
Aboveground biomass	**0.67*** **	0.29	0.21	0.38.	-0.26	-0.04	0.31	-0.30
Sward height	**0.60** **	0.25	0.20	0.23	-0.34	-0.20	0.22	-0.21
Aboveground biomass C	-0.17	-0.20	0.09	0.06	-0.26	0.09	-0.30	0.28
Aboveground biomass N	0.38·	0.41·	-0.15	-0.34	0.25	-0.25	-0.10	0.07
Aboveground biomass C : N ratio	**-0.46***	**-0.43***	0.11	0.30	-0.28	0.27	0.04	-0.01
Total root dry weight	0.17	-0.12	0.26	0.05	0.28	-0.10	0.22	-0.22
Fine root dry weight	0.05	-0.19	0.26	-0.09	0.28	-0.17	0.11	-0.11
Coarse root dry weight	0.41·	0.17	0.11	0.00	0.34	-0.20	0.27	-0.28
Root to shoot ratio	**-0.51***	-0.32	-0.03	-0.33	**0.52***	-0.04	-0.03	0.02
Mean root diameter	-0.20	0.10	-0.25	**-0.51***	0.16	-0.04	-0.40	0.40
Specific root length	-0.02	-0.26	0.26	0.31	-0.16	0.20	0.02	-0.02
Root tissue density	0.05	0.19	-0.15	-0.13	0.05	-0.19	0.05	-0.04
Root dry matter content	0.24	**0.48***	-0.35	-0.23	-0.03	-0.36	0.02	-0.06
Total fine root length	0.02	-0.43·	0.46·	0.28	0.01	0.11	0.10	-0.10
Root C	-0.09	0.14	-0.23	-0.02	-0.16	0.11	0.06	-0.09
Root N	-0.21	0.23	-0.40	**-0.62** **	0.36	-0.09	-0.45·	0.42·
Root C : N ratio	0.23	-0.23	0.41	**0.65** **	-0.38	0.11	0.46·	-0.42·
Soil pH	0.00	0.07	-0.07	0.17	-0.13	0.11	0.06	-0.08
Soil C	-0.17	-0.20	0.09	0.26	-0.03	0.22	0.34	-0.30
Soil N	0.01	-0.37	0.39	**0.56***	-0.22	0.16	**0.51***	**-0.49***
Soil C : N ratio	-0.23	0.10	-0.27	-0.27	0.18	0.10	-0.13	0.17
Olsen phosphorus	0.00	0.12	-0.13	0.03	-0.07	0.18	-0.04	0.10
K_2SO_4-extractable N	0.31	-0.09	0.31	0.35	-0.26	0.05	0.35	-0.31
Microbial C	0.09	-0.03	0.09	0.16	0.07	0.06	0.25	-0.26
Microbial N	0.16	0.03	0.06	-0.01	0.06	-0.12	0.16	-0.17
Microbial C : N ratio	-0.02	-0.06	0.07	0.37	0.09	0.35	0.35	-0.33
Fungal PLFA	0.00	-0.31	0.39	0.03	0.21	-0.25	0.11	-0.11
Bacterial PLFA	0.17	-0.02	0.17	0.30	-0.26	-0.09	0.31	-0.33
Fungal to bacterial ratio	-0.18	-0.37	0.31	-0.25	**0.56***	-0.19	-0.09	0.12
Gram negative PLFA	0.13	-0.05	0.17	0.28	-0.26	-0.12	0.29	-0.32
Gram positive PLFA	0.22	0.02	0.16	0.34	-0.28	-0.06	0.33	-0.34
Gram positive to negative ratio	0.45.	0.35	-0.05	0.22	-0.04	0.34	0.13	-0.07
Total PLFA	0.15	-0.05	0.19	0.30	-0.21	-0.10	0.32	-0.34

In *Leucanthemum*, height was positively correlated with aboveground biomass (Fig. 2.9, Table 2.6, r = 0.49), sward height (r = 0.51) and aboveground biomass C (r = 0.54) and negatively with soil C : N ratio (r = -0.47). Aboveground biomass, sward height and aboveground biomass C were also co-correlated and soil C : N ratio was correlated with aboveground biomass C. Shoot dry weight was positively correlated with soil pH (r = 0.49). The ratio of height/shoot dry weight was negatively correlated with soil pH (r = -0.52), and also SLA was negatively correlated with soil pH (r = -0.54). LDMC was positively correlated with soil pH (r = 0.61) and fungal to bacterial ratio (r = 0.75) and negatively with a range of microbial properties, such as microbial C, N and C : N ratio and bacterial, gram negative, gram positive and total PLFA (r = -0.52 to -0.68). Soil pH was correlated only with microbial C : N ratio, but not the other microbial properties. Microbial properties were co-correlated. Leaf C was positively correlated with soil pH (r = 0.50) and negatively with microbial C and N and bacterial, gram positive, gram negative and total PLFA. Leaf N was positively correlated with aboveground biomass N (r = 0.52) as well as negatively with aboveground biomass C : N ratio (r = -0.47) and mean root diameter (r = -0.48). Leaf C : N ratio was negatively correlated with aboveground biomass N (r = -0.52) and positively with aboveground biomass C : N ratio (r = 0.47) and RDMC (r = 0.49).

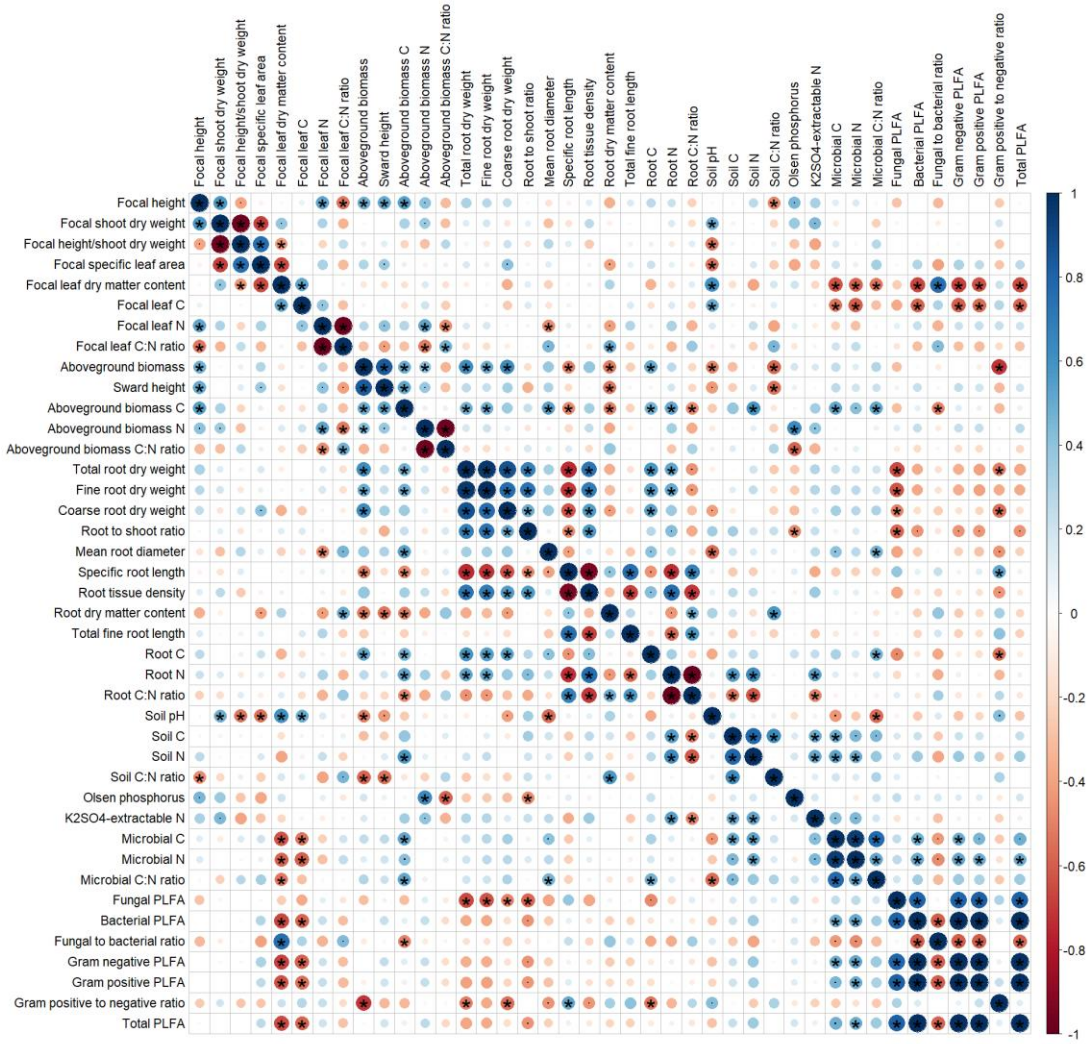

Figure 2.9: Pairwise correlations between traits of focal individuals in *Leucanthemum* and vegetation/soil properties. Colour and size of circles indicate Pearson correlation coefficients. Significant correlations ($p < 0.05$) are marked with "*", correlations with $p < 0.1$ are marked with "·". C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

Table 2.6: Pearson correlation coefficients between traits of focal individuals and vegetation/soil properties in *Leucanthemum*. Significance of correlation is indicated as: p < 0.001***, p < 0.01**, p < 0.05*, p < 0.1·. C – carbon, N – nitrogen, PLFA - phospholipid fatty acids.

	Height	**Shoot dry weight**	**Height/ shoot dry weight**	**Specific leaf area**	**Leaf dry matter content**	**Leaf C**	**Leaf N**	**Leaf C : N ratio**
Aboveground biomass	**0.49***	0.03	0.09	0.30	-0.12	-0.06	0.28	-0.29
Sward height	**0.51***	0.00	0.12	0.36·	-0.16	-0.03	0.40·	-0.40·
Aboveground biomass C	**0.54****	0.32	-0.21	0.09	-0.16	-0.15	0.28	-0.30
Aboveground biomass N	0.40·	0.35·	-0.30	-0.08	0.15	0.20	**0.52***	**-0.52***
Aboveground biomass C : N ratio	-0.31	-0.31	0.27	0.08	-0.16	-0.25	**-0.47***	**0.47***
Total root dry weight	0.31	0.14	-0.07	0.16	-0.10	0.00	0.15	-0.17
Fine root dry weight	0.28	0.19	-0.13	0.10	-0.05	0.06	0.17	-0.17
Coarse root dry weight	0.24	-0.14	0.22	0.40·	-0.35	-0.25	0.06	-0.09
Root to shoot ratio	-0.04	0.12	-0.14	-0.05	0.12	0.16	0.06	-0.02
Mean root diameter	-0.15	-0.30	0.29	0.13	-0.23	-0.15	**-0.48***	0.45·
Specific root length	-0.08	-0.15	0.14	0.01	0.04	0.07	0.10	-0.05
Root tissue density	0.13	0.26	-0.25	-0.07	0.08	0.02	0.07	-0.11
Root dry matter content	-0.36	-0.09	-0.01	-0.40·	0.30	0.02	-0.42·	**0.49***
Total fine root length	0.15	-0.03	0.08	0.10	0.01	0.16	0.30	-0.23
Root C	0.27	0.00	0.08	0.22	-0.35	-0.16	0.07	-0.10
Root N	0.22	0.16	-0.11	0.10	-0.15	0.15	0.31	-0.34
Root C : N ratio	-0.23	-0.17	0.12	-0.09	0.12	-0.18	-0.35	0.37
Soil pH	0.05	**0.49***	**-0.52***	**-0.54***	**0.61****	**0.50***	0.15	-0.08
Soil C	-0.05	-0.07	0.07	-0.04	-0.17	0.06	-0.06	0.09
Soil N	0.27	0.05	0.02	0.16	-0.40	-0.07	0.22	-0.23
Soil C : N ratio	**-0.47***	-0.21	0.10	-0.25	0.18	0.13	-0.39	0.45·
Olsen phosphorus	0.45·	0.36	-0.28	-0.39	0.20	-0.02	0.11	-0.12
K_2SO_4-extractable N	0.33	0.45·	-0.40	-0.28	-0.20	-0.06	-0.10	0.06
Microbial C	0.13	-0.10	0.15	0.21	**-0.62****	**-0.53***	-0.24	0.20
Microbial N	0.15	0.00	0.04	0.10	**-0.59***	**-0.59****	-0.30	0.25
Microbial C : N ratio	0.05	-0.23	0.27	0.33	**-0.52***	-0.30	-0.05	0.05
Fungal PLFA	-0.28	-0.06	-0.01	0.04	-0.25	-0.38	0.11	-0.13
Bacterial PLFA	-0.01	-0.02	0.02	0.29	**-0.67****	**-0.58***	0.22	-0.31
Fungal to bacterial ratio	-0.33	-0.10	0.02	-0.40	**0.75*****	0.30	-0.35	0.44·
Gram negative PLFA	-0.01	-0.04	0.05	0.32	**-0.68****	**-0.59***	0.21	-0.30
Gram positive PLFA	-0.02	0.01	-0.01	0.26	**-0.64****	**-0.56***	0.23	-0.32
Gram positive to negative ratio	-0.26	0.19	-0.27	-0.27	0.21	0.29	0.23	-0.18
Total PLFA	-0.04	-0.02	0.01	0.27	**-0.65****	**-0.58***	0.20	-0.29

2.3.5 Plant trait coefficients of variation

Coefficients of variation were computed for each trait of each focal species to compare their variability (Fig. 2.10). Coefficients of variation for height, SLA, LDMC, leaf N and leaf C : N ratio ranged around 0.2 for all three species. For leaf C the Coefficient of variation was substantially smaller, around 0.02, for all three species. Shoot dry weight and the ratio of height/shoot dry weight had higher coefficients of variation; around 0.3 for *Clinopodium* and between 0.5 and 0.7 for *Daucus* and *Leucanthemum*.

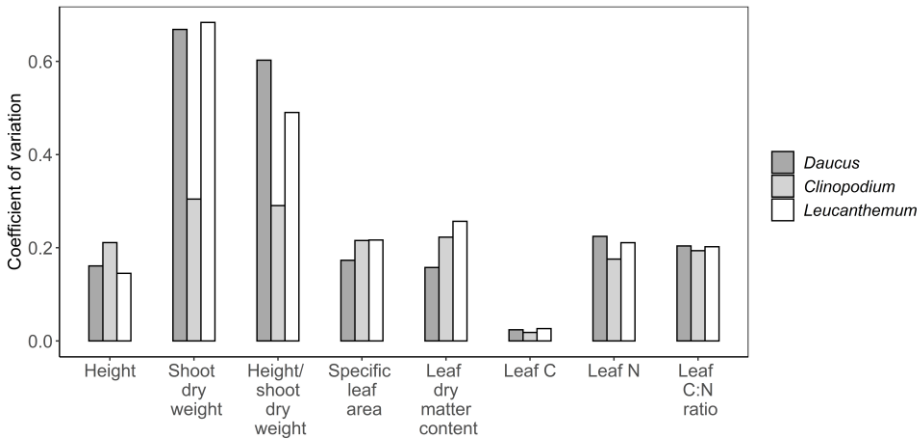

Figure 2.10: Coefficients of variation for focal traits of *Daucus, Clinopodium* and *Leucanthemum*. C – carbon, N – nitrogen.

2.4 Discussion

The aim of this study was to investigate whether plant community traits determine the magnitude of ITV in individual grassland species through changes in resource availability. The experimental FG treatments differed in vegetation and soil properties between the experimental treatments, mostly depending on presence/absence of one of the three FGs. ITV in two of the three focal species was significantly affected by FG treatment, which can be attributed to differences in their plant community traits and soil properties. Notably, treatments containing FG 3 (tall, resource-acquisitive plants with fibrous roots) had higher sward height and soil N availability and also ITV in *Daucus*

and *Clinopodium* was significantly affected by presence of FG 3. Additionally, ITV in all focal species was correlated with soil and vegetation properties that did not differ significantly between experimental treatments, like pH and microbial properties. Overall, a significant proportion of ITV was related to local differences in soil and vegetation properties. However, patterns differed strongly between the three study species, indicating that mechanisms of ITV are species-specific.

2.4.1 Above and belowground drivers of ITV

Hypothesis 1 proposed that the magnitude of ITV would depend on traits of the surrounding plant community due to differences in resource availability (e.g. light and nutrients). This could be confirmed for some traits. FG presence/absence had significant effects on some traits in *Daucus* and *Clinopodium*, but not in *Leucanthemum* (see Fig. 2.2). In all three species there were a number of significant correlations between traits and vegetation/soil properties, which can point towards possible mechanisms controlling ITV (see Fig. 2.7 – 2.9 and Table 2.4 - 2.6).

Daucus growing in the presence of FG 3 was taller, with higher biomass and higher leaf N content. Height (and also leaf N, although not significantly) was highly correlated with K_2SO_4-extractable N, indicating that this pattern may be caused by higher nutrient availability facilitating growth in the presence of FG 3, supporting hypothesis 1b. None of the traits in *Daucus* was significantly correlated with sward height or aboveground biomass, so light availability did not seem to play an important role in determining ITV in height, rejecting hypothesis 1a for *Daucus*. Additionally, height and shoot dry weight were negatively correlated with a range of microbial properties. This could indicate resource competition between plants and microorganisms. Competition between plants and microbes for nutrients is suspected to be uncommon over an extended time scale

due to temporal niche differentiation (Kuzyakov & Xu, 2013), however it could be possible in a nutrient poor calcareous grassland with shallow soil. LDMC was correlated with a range of root properties: positively with root biomass and root N and negatively with SRL. These root properties and LDMC might both be related to water availability (which was not measured in this study) (Pérez-Harguindeguy et al., 2013; de Vries et al., 2016), so variation in LDMC might be related to differences in water availability between micro-habitats.

Clinopodium was tallest in the presence of FG 3, but no other traits were affected by the presence/absence of any FG. Height was strongly positively correlated with aboveground biomass and sward height, but not with any soil or root properties. This could mean that in *Clinopodium* variability in height was caused by aboveground competition for light in accordance with Hypothesis 1a. SLA and leaf N were positively correlated and leaf C : N ratio negatively correlated with total soil N. Total soil N might reflect a more long-term N availability than K_2SO_4-extractable N, which might be especially important for the perennial species of FG 2 like *Clinopodium*.

In *Leucanthemum*, no traits were significantly affected by FG composition or the presence/absence of any FG, in contrast to Hypotheses 1a and b. The height of *Leucanthemum* was positively correlated with aboveground biomass, sward height and aboveground biomass C, which it may have been driven by light availability. Interestingly, a range of traits were correlated with pH: plants had higher biomass, higher LDMC, lower ratio of height/shoot dry weight and lower SLA with higher soil pH. Shoot dry weight, the ratio of height/shoot dry weight and SLA were not significantly correlated to any other vegetation/soil property. LDMC and leaf C were correlated to a range of microbial properties, but none of them were significantly

correlated with soil pH. This indicates that there may have been a direct effect of pH on these traits, rather than one mediated by microbial properties or nutrient availability. It is surprising that plants had a higher biomass with higher soil pH as generally the pH at the site was alkaline (between 7.3 and 7.9) and high compared to the range for optimal plant growth, which generally lies between 6 and 7.5 (Ramírez-Rodríguez et al., 2007). An explanation could be that the sampling included several genotypes with differing traits, some of which could germinate and establish better at high pH. Alternatively, the high pH may have had a more negative effects on competitors than on *Leucanthemum*, giving it a competitive advantage. However, this seems unlikely as pH was not correlated with above- or belowground biomass of the surrounding plant community. LDMC and leaf C were negatively correlated with microbial C/N, bacterial and total PLFA. This could potentially be related to water availability, as good water availability might both decrease LDMC (Pérez-Harguindeguy et al., 2013) and be beneficial for microbial growth.

The height of both *Daucus* and *Clinopodium* increased in the presence of FG 3, which also was associated to a higher sward height of the surrounding plant community. The height of *Leucanthemum* was positively correlated with sward height. Thus, ITV occurred in the same direction in all species. This is consistent with results from the Jena experiment, where it was found that species richness and/or legume presence generally increased shoot height in both grasses and legumes due to intensified light competition (Gubsch et al., 2011; Lipowsky et al., 2015)

For the other traits, patterns of ITV differed between the three species. Leaf traits were correlated with soil and root properties, rather than with aboveground vegetation properties. Even SLA, which is related to light acquisition (Pérez-Harguindeguy et al.,

2013) and varied depending on light availability in other studies (Gubsch et al., 2011; Lipowsky et al., 2015) was not affected by aboveground properties. Unexpectedly, leaf N was never significantly correlated with K_2SO_4 extractable N, even though there was a non-significant positive correlation for Daucus. In contrast, Gubsch et al. (2011) found increased levels of leaf N as a response to legume presence and Guiz et al. (2018) observed decreased levels on leaf N with increased species richness in an experiment that did not include legumes. Both of these results were likely related to plant-available N. It seems that in the stressed environment of the shallow, only recently restored chalk soil other factors were more important than N-availability in determining ITV in leaf traits, such as water availability, soil pH and microbial properties.

Correlation coefficients between traits of focal individuals and vegetation/soil properties mostly ranged between 0.5 and 0.7 (see Table 2.4 – 2.6), indicating that in these cases a single microenvironmental factor could explain 25 to 49% of the variation in focal traits. This suggests that adaptation (either genotypic or phenotypic) to local microenvironment is relevant to ITV, and ITV was not purely due to random factors such as differences in plant age or random dispersal of different genotypes. Consequently, ITV could potentially influence ecosystem function in a systematic way, especially if traits of different species vary on average in the same direction, as was the case for height in this study. When ITV occurs in species-specific directions it is not clear if CWM would be affected. However, functional diversity could be affected which might enhance or decrease niche partitioning (Gubsch et al., 2011), which might in turn have effects on ecosystem functioning.

2.4.2 FG treatment effects on vegetation and soil properties

Hypothesis 2 proposed that communities with complex root architecture and a more resource-acquisitive strategy (i.e. FG 3) would increase availability of nutrients in the soil compared to communities with a simple root architecture and more resource-conservative strategy (i.e. FG 2). This hypothesis had already been tested similarly in the same experiment (planted in 2013) in 2015 (Fry et al., 2018), but with samples from random locations within each plot, rather than associated with specific focal species. It was tested again here, in 2017, to understand how plant-soil interactions have changed vegetation and soil properties over time and to investigate if the same effects could be observed in the altered sampling design.

Here, Hypothesis 2 was supported. FG 3 presence was associated with a large (68%) increase in K_2SO_4-extractable N (see Fig. 2.5), a proxy for plant available N. Faster rates of N cycling as a response to species with resource-acquisitive traits have been observed in other studies (e.g. Orwin *et al.*, 2010; Grigulis *et al.*, 2013). In contrast, Fry et al. (2018) found different results in July 2015. In that study, the strongest pattern observed was that community weighted mean (CWM) plant height (which was highest in FG 3) had a negative effect soil N cycling, and additionally there was a weaker positive effect of root architectural complexity (which was also highest in FG 3) on soil N cycling. They suggested that exploitative species may have been poorly adapted to the nutrient poor chalk soil and may have depleted soil N quickly. Even though Fry et al. (2018) used a wider range of methods for quantifying soil N cycling than here, this shows that the dynamics of N cycling have changed over time. While in 2015 the exploitative species may have depleted soil N resources by July, in 2017 the soil may have had a higher organic content and more N may have entered the system due to N-fixating legumes, enabling the exploitative species to contribute to faster N cycling.

Most microbial properties on the other hand (such as microbial biomass C/N and fungal to bacterial ratio) were not affected by FG composition or presence/absence of any FG (see Fig. 2.6). This is consistent with the findings from 2015 of Fry et al. (2018) and potentially explained by the fact that microbial associations with plants can take many years to form (Morriën et al., 2017). However, in this study there was a positive effect of FG 3 presence on bacterial gram positive to negative ratio. This might be because the microbial community in plots containing FG 3 may have been exposed to higher drought stress in the relatively dry environment of the chalk, based on results by Fry et al. (2018). In that study, also the effect of drought shelters on ecosystem properties was investigated. Root biomass in plots containing FG 3 was more adversely affected by drought and resilience of ecosystem respiration was lower in plots with tall plants (like FG 3). The microbial community may have responded through a shift to a higher gram positive to negative ratio, as gram positive bacteria tend to be more drought tolerant due to their thicker cell walls (Schimel et al., 2007; Fuchslueger et al., 2014).

The vegetation properties measured in this study were rarely significantly different between the seven FG treatments, but treatments with presence/absence of specific FGs differed in some properties (see Fig. 2.3 and 2.4). Treatments with presence of FG 3 had higher sward height and biomass, which is consistent with a higher CWM height (calculated based on a vegetation survey and trait database values) and also a trend of higher aboveground biomass in plots with FG 3 in 2015 (Fry et al., 2018). Root C was decreased in treatments with presence of FG 3, which may be consistent with a more complex root architecture. However, none of the other root traits were significantly affected by presence/absence of FG 3. This might be because none of the traits measured accurately reflected the "complexity" of the root system, because root traits were expressed differently in the field or because in the sampling design of this study root

traits may be more strongly affected by the identity of the focal species rather than the treatment. Treatments where FG 2 was present had higher fine and total root biomass, lower SRL and higher RTD, which may be due to the fact that FG 2 species had a higher perenniality and thus the root systems remained in the soil and kept growing for several years. Treatments with FG 2 presence also had higher aboveground biomass N content and lower C : N ratio, which is surprising as FG 2 is the group with the lowest SLA and a resource-conservative strategy. Possibly, FG 2 species had less investment in C-rich structural components such as stems due to their smaller statue, which could have decreased the overall biomass C : N ratio (Abbas et al., 2013). Plots with presence of FG 1 had a lower RDMC which may be due to the fact that the deeper taproots can access and store water from deeper soil layers than the other two shallow-rooted species.

The altered sampling design of this study compared to Fry et al. (2018), sampling close to focal species rather than randomly in each plot, did affect the results of this study. As described in the Methods section, plots of the residuals of linear models of vegetation and soil properties as a response to FG treatments against focal species identity showed a species-dependent pattern for some properties (see Table 2.3). This indicates that many vegetation and soil properties were affected not only by the FG treatments but also by the focal species identity, for example aboveground biomass C and several root, soil and microbial properties. This could mean that either the focal plants had a measurable effect on the surrounding plant community and soil properties, or, more likely, that each focal species needed a different set of local conditions to germinate and establish. Even though focal species identity was included as a random effect in the models to account for this effect, this means that differences in vegetation and soil properties between the seven treatments might have been more pronounced with a random sampling design.

Overall, vegetation and soil properties were mostly affected by presence/absence of specific FGs, but rarely by interactions between FGs, as in the seven FG treatments (see Fig. 2.3 – 2.6). This might indicate that particular FGs were driving changes in these properties. Other studies have found that presence of 'traditional' functional groups such as legumes, grasses and forbs are important in driving different sets of ecosystem functions (Fornara et al., 2009; Allan et al., 2013). The results of this study indicate that the same might be true for trait-based functional groups.

2.4.3 Future work

It would be interesting to explore multivariate models to see how much ITV could be explained by a combination of microenvironmental factors, which could not be realized here due to the small sample size for each species. Also, it would be of interest to include ITV of root traits, to study a wider range of species and to include the effect of water availability and trait-distances between focal and surrounding species.

2.4.4 Conclusion

This study shows that diverse calcareous grassland plant communities with differing community traits induce intraspecific plant trait variability in focal species through changes in soil properties and light availability. Additionally, variation in plant traits of focal individuals was related to differences in properties of the surrounding plant community and the soil, for example sward height, above- and belowground plant biomass and stoichiometry, soil nitrogen availability and pH, as well as soil microbial properties. These results show that plant community traits as well as vegetation and soil properties determine the magnitude of ITV in diverse calcareous grasslands. In these systems there is potential that including ITV as a response to these factors may improve models and predictions of ecosystem functioning.

3 The effect of plant trait plasticity and species interactions on carbon and nitrogen cycling in grasslands

Abstract

Plant traits and diversity indices have been shown to explain variation in ecosystem properties and functions across ecosystems. However, there is uncertainty regarding the role of intraspecific trait variation and interactions between species. This study explored the effects of pairwise interactions between four common temperate grassland species on plant traits and ecosystem properties in plant-soil mesocosms. Ecosystem properties and functions related to carbon and nitrogen cycling, as well as plant traits of each species were compared between monocultures and two-species mixtures. In addition, a nitrogen addition treatment corresponding to an 18 % increase in atmospheric deposition was applied to explore if increased resource availability modifies plant species interactions. Phenotypic plasticity in shoot dry weight indicated that neighbouring species generally had beneficial or neutral effects on one another, but plasticity in the other traits was mostly limited and did not appear to affect ecosystem properties or functions. Nitrogen addition did not significantly modify any of the species

interactions and only affected community-weighted mean leaf chemical traits and CO_2 fluxes. The interactions between plant species affected ecosystem properties and functions in idiosyncratic ways, depending on the particular ecosystem property or function and sometimes the species. Compared to monocultures, the effects of the species in mixtures were sometimes additive, sometimes synergistic and sometimes one of the component species had a disproportional effect relative to its biomass. This suggest that the usefulness of metrics for predicting ecosystem properties and functions from plant traits (such as community-weighted mean traits and diversity indices) is context-dependent.

Keywords: plant species interactions, ecosystem function, plant functional traits, intraspecific trait variation

3.1 Introduction

Global change caused by human activities, such as land use change, climate change and an increase in atmospheric nitrogen (N) deposition, is drastically altering terrestrial ecosystems, leading to species losses and changes in species distributions (IPBES, 2019). In turn, changes in plant community composition and diversity can have significant effects on ecosystem functions such as carbon (C) and N cycling and the emission of greenhouse gases (Cardinale et al., 2012).

Plant traits are morphological, anatomical, physiological or phenological features measurable at the individual level (Violle et al., 2007). Their study has allowed improved understanding of the mechanisms by which the species composition of plant communities affects ecosystem properties and functions (Garnier et al., 2015). Evidence is growing to support the 'mass ratio hypothesis' (Grime, 1998), which predicts that ecosystem functions are related to community-weighted mean (CWM; Garnier et al.,

2004) traits. The mechanisms by which plant traits affect ecosystem properties and functions are often related to their growth strategy (fast- vs. slow-growing), which can be broadly characterized using leaf traits like specific leaf area (SLA) and leaf N content (Reich, 2014; Diaz et al., 2016). For example, Grigulis et al. (2013) found that CWM aboveground traits explained a significant fraction of variation in various ecosystem properties across three European grassland sites, each including a range of management types. Fast-growing species were associated with faster rates of ecosystem C and N cycling, higher plant biomass, lower soil fungal to bacterial ratio, lower N retention and lower C sequestration than slow-growing species. De Vries et al. (2012) found that CWM aboveground traits explained microbial community composition across 160 grassland sites in England. Again, fast-growing species were associated with lower soil fungal to bacterial ratio than slow-growing species. Also, root traits can affect a number of soil processes like soil C and N cycling, microbial properties and soil structural properties (Bardgett et al., 2014).

In diverse communities, beyond the effects of individual plant species on ecosystem functions, synergistic effects can arise from species interactions. For example, there is evidence for the 'diversity hypothesis' (Tilman et al., 1996; Hooper et al., 2005), according to which diversity indices, such as species, phylogenetic or trait functional diversity (FD; Mason et al., 2003) can predict changes in ecosystem function. Support for the diversity hypothesis has been found in several grassland biodiversity experiments. The majority of studies focused on above- and sometimes also belowground biomass, which often increased with species richness and/or FD (e.g. Barry et al., 2019; Roscher et al., 2013; Tilman et al., 2001; Van Ruijven & Berendse, 2009). Also, both CWM traits and FD have been shown to explain ecosystem CO_2 fluxes (Milcu et al., 2014) and species richness has been found to increase soil microbial

biomass and activity as well as soil C storage (Lange et al., 2014, 2015) in grassland biodiversity experiments.

Several mechanisms have been proposed for how plant species interactions can increase ecosystem productivity, for example: (i) Plants can be complementary in their above- or belowground resource use, e.g. in space, time or N forms (Loreau & Hector, 2001). (ii) Selection effects can occur, meaning that in plant mixtures more competitive larger or faster growing plants species become dominant. (iii) In mixtures, species-specific pathogens may be diluted, leading to reduced negative plant-soil feedbacks and better growth for some species (Hendriks et al., 2013). (iv) Some species, e.g. legumes, can facilitate others through nutrient enrichment (e.g. legumes, Vitousek et al., 2013) or through an amelioration of microclimatic conditions, (e.g. through increased shading of the soil surface in times of drought, Wright et al., 2015). (vi) Additionally, the composition and diversity of the surrounding plant community can induce intraspecific trait variability (Gubsch et al., 2011; Guiz et al., 2018). This can in turn modify any of the mechanisms mentioned above. For example, alpine herbaceous plant species were observed to enhance resource complementarity by shifting their uptake pattern of different N forms depending on their neighbouring species (Ashton et al., 2010). Also, plasticity can make a species more competitive with respect to its neighbours (Novoplansky, 2009), enhancing selection effects.

While the relative importance of these mechanisms is still being debated and tested (e.g. Jesch et al., 2018), the effects of plant species interactions on ecosystem C and N cycling are even more complex and less well-studied (Lange et al., 2019). Increased plant biomass has direct effects on C and N cycling, e.g. on net ecosystem exchange (NEE) and ecosystem respiration (R_{eco}) (Milcu et al., 2014). Additionally, increased biomass,

diversity, root exudation and altered microclimate may increase soil microbial biomass and diversity, which can alter soil C and N cycling (Lange et al., 2014, 2015). Also, intraspecific trait variability (see (vi)) can affect C and N cycling. For example, drought-induced plant trait plasticity has been found to affect soil N availability (de Vries et al., 2016) and litter decomposition (see Chapter 5).

Interactions between neighbouring plants also depend on the soil nutrient status. For example, increased N deposition has been linked to a loss in species richness by inhibiting sensitive species, or by favouring fast-growing species which outcompete slower-growing ones (Stevens et al., 2010). Also, diversity effects on plant biomass can be either increased or decreased by addition of nutrients (Reich et al., 2001; Pontes et al., 2012; Siebenkäs et al., 2016).

Even though metrics like CWM traits and diversity indices are often correlated with ecosystem properties and functions, their explanatory power is often low (van der Plas et al., 2020). A reason for this might be that they do not capture the mechanisms of interactions between species fully. However, in complex biodiversity experiments with many species it is difficult to disentangle the various interactions between component species and their effects on ecosystem properties and function.

This study explored the effects of pairwise interactions between plant species on ecosystem properties and function. This was achieved using simple plant-soil mesocosms consisting of monocultures and two-species mixtures of four functionally distinct, common grassland species: a fast and a slow-growing grass, as well as a fast- and a slow-growing forb. A N addition treatment corresponding to a moderate (18%) increase in atmospheric deposition (2 kg of N/ha) was applied to explore if this modified the interactions between plant species and their ecosystem effects, for example by

benefitting fast-growing species. Above- and below-ground plant traits and biomass were measured in each mesocosm to assess phenotypic plasticity between treatments and thus provide information about the types of interactions between species in mixtures. A range of soil properties related to C and N cycling, as well as CO_2 fluxes were measured to characterize ecosystem C and N functions.

In particular, the following questions were addressed:

1. How do neighbouring species affect plant trait plasticity?
2. How do interactions between plant species from different functional groups affect ecosystem properties and functions?
3. Are these effects modified by N addition?

3.2 Methods

3.2.1 Experimental design and mesocosm establishment

The mesocosm experiment was set up in June 2018 at Hazelrigg field station in northern England (54°10N, 2°460W). The site has a mean annual temperature of 9 °C and a mean annual precipitation of 1050 mm. Atmospheric N deposition in the years 2016 to 2018 was around 11.2 kg N/ha/year (Levy et al., 2020). Mesocosm pots (38 x 38 cm, 40 cm deep) were filled with a 10 cm layer of chippings and a 20 cm layer of mesotrophic grassland soil (pH ~ 6 (De Vries et al., 2015; Barneze et al., 2020)) collected from the surrounding grassland and sieved to 1 cm to remove stones and roots. The experiment was set up in a two-way randomized block design and comprised four blocks. Each block contained 22 mesocosm pots with 10 different species composition treatments and one bare soil control treatment either with or without N addition. This resulted in a total of 88 mesocosm pots. The study included four common grassland species from two functional groups with distinct growth strategies (relative growth rates (RGR) taken

from Grime & Hunt (1975)): a faster-growing grass (*Dactylis glomerata*, RGR = 1.31 g/g/week), a slower-growing grass (*Anthoxanthum odoratum*, RGR = 0.94 g/g/week), a faster-growing rhizomatous forb (*Plantago lanceolata*, RGR = 1.40 g/g/week) and a slower-growing tap-rooted forb (*Rumex acetosa*, RGR = 1.36 g/g/week). Seeds were from purchased from Emorsgate Seeds (King's Lynn, Norfolk, UK). All four species were planted in monocultures and in all possible two-species combinations. Seedlings were germinated in plug trays in the greenhouse using compost (John Innes No. 2) for four weeks before transplanting to mesocosms. The compost was then rinsed from the roots and seedlings were transplanted into the mesocosms in a grid of 6 x 6 = 36 seedlings. In two-species mixture treatments, 18 seedlings of each species were planted alternately. In early May 2019, 2 kg of N/ha was added using a watering can to the N addition treatment mesocosms as NH_4NO_3 dissolved in water. The same amount of water was added to the pots without N addition treatment. All mesocosm pots were watered throughout the summer months of 2018 and 2019 and weeded as required to remove extraneous species.

3.2.2 Net ecosystem exchange and Ecosystem respiration

CO_2 flux measurements were conducted on 10th, 14th, 17th and 20th June 2019 using flux chambers connected to an infrared gas analyser (EGM 4, PP Systems, Herts, UK) in a closed loop gas circuit. Net ecosystem exchange (NEE) was measured using custom-made transparent flux chambers (Orwin et al., 2014). They were constructed by fitting a frame made from a mesocosm pot with acrylic windows fit on all sides and sealing tape along the edges to be connected to the planted mesocosms. Ecosystem respiration (R_{eco}) was measured using the same type of chamber, but darkened with black plastic sheet. Flux chambers were clipped to the rim of the mesocosm pots during measurement. Fluxes were measured for 2 minutes in the light and dark.

Simultaneously, soil moisture was recorded with a ThetaProbe meter (Delta-T Devices, Cambridge, *UK*), soil temperature using a Thermamite 1 thermometer (ETI Ltd, Worthing, UK) and photosynthetically active radiation (PAR) using a PAR sensor (Skye Instruments, Powys, UK).

3.2.3 Plant traits and vegetation properties

Aboveground plant traits were measured in early July for each species in each pot. Height was measured in five randomly selected individuals along a diagonal transect within the pot as the distance between the top of the photosynthetic tissue and the soil surface. Five mature and healthy-looking leaves including petioles were collected per pot and species. They were rehydrated in bottles with de-ionised-water for 24 hours in the dark at 4 °C, as plants may be dehydrated to an unknown extent and this makes the measurements more comparable (Pérez-Harguindeguy et al., 2013). Leaves were then blotted dry, weighed and scanned using an EPSON flatbed scanner. Leaf area (LA) was analysed using the software WinRhizo (Regent Instruments Inc., Sainte-Foy-Sillery-Cap-Rouge, QC, Canada). Leaf dry weight was determined after drying for 72 hours at 65 °C. Specific leaf area (SLA) was calculated as the ratio of leaf area to leaf dry weight. Leaf dry matter content (LDMC) was calculated as leaf dry weight divided by leaf fresh weight. Dried leaves were ground in a ball mill and 15 mg used to analyse leaf C and N content in an Elementar Analyser (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany). On 8th to 10th July, aboveground biomass was cut at the base, sorted by species, dried at 65°C for 72 hours and weighed. Shoot dry weight per individual was determined by dividing aboveground biomass by 36 in monocultures and by 18 in two-species mixtures. Community-weighted mean (CWM) traits were computed for each pot using the aboveground biomass m_i and trait value $trait_i$ of each species i present:

$$\mathrm{CWM} = \sum_{i=1}^{n} m_i \times trait_i$$

To determine root biomass and community traits, a core with 5.8 cm diameter and 15 cm depth was sampled in the centre of each pot on 13th July. The cores were stored at 4°C until needed for analysis. Roots were carefully washed, weighed and then stored in 50% Ethanol at 4°C until further processing. Samples from the mixture mesocosms were not separated into species as it is difficult to do this accurately. Fine roots (<1mm) were cut into 2 cm long segments and two sub-samples per sample were weighed and scanned using an EPSON flatbed scanner. Scans were analysed for root length and diameter using the software Winrhizo. All roots were oven-dried at 65°C for 48h and weighed. Specific root length (SRL) was calculated as length per dry biomass. Root dry matter content (RDMC) was calculated as dry divided by fresh mass. The fine root sub-samples were ground in a ball mill and 15 mg were analysed for C and N content using the Elementar Analyser.

3.2.4 Soil properties

From each mesocosm, four soil cores of 3 cm diameter were collected to a depth of 10 cm at the end of the experiment on 13th July. The soil was passed through a 2 mm sieve to break it up and remove large roots and mixed thoroughly. For pH measurements, 10 g of fresh soil was mixed with 25 ml deionized water, placed in a shaker for 30 minutes to homogenize the soil solution and left to rest for another 30 minutes. Soil pH was measured using a pH meter (Mettler Toledo, Salford, UK). Soil moisture content was determined by calculating the mass loss of soil after oven-drying at 105 °C for 48 hours. KCl extractable NO_3^- and NH_4^+ was measured as a proxy for plant available N, by

mixing 5 g of fresh soil with 25ml of 1.0 M KCl. The extract was frozen until analysis on an autoanalyser (Bran and Luebbe, Northampton, UK) for NO_3^- and NH_4^+ content.

The activity of five extracellular enzymes in the soil (Phosphatase, β-glucosidase, N-acetyl-glucosaminidase (NAG), Leucine-amino-peptidase (LAP) and Phenol-oxidase) was measured. Phosphatase converts unavailable organic P into plant-available phosphate. β-glucosidase is involved in the degradation of cellulose into glucose. NAG is involved in release of N from chitin and bacterial cell walls. LAP is involved in hydrolysis of amino acid residues (N- terminus of peptides and proteins). Phenol-oxidase is involved in lignin and tannin degradation. A modified version of the method described by Saiya-Cork et al. (2002) was used. Samples were frozen at -80°C until analysis. 1 g of defrosted soil per sample was blended for 1 minute with 125 ml of 50 mM Sodium acetate buffer (pH 5.00). All enzymes except Phenol-oxidase were assayed fluorometrically. Fluorescing 4-methylum-belliferone (MUB) or 7-amido-4-methylcoumarin (7-AMC) was used to tag the substrates (4-MUB-phosphate for phosphatase, 4-MUB-ß-D-glucoside for β-glucosidase, 4-MUB-N-acetyl-ß-D-glucosaminide for NAG, L-Leucine-7-AMC for LAP). The soil suspensions were pipetted onto 96-well plates. For each sample and each enzyme, a sample-well (sample suspension + substrate) was replicated 8 times, as well as two types of standard wells to account for background fluorescence of soils (sample suspension + buffer) and MUB/MC (sample suspension + MUB/MC). Additionally, for each enzyme, a set of reference standard wells (standard + buffer) and negative control wells (substrate + buffer) were replicated eight times. The microplates were incubated in the dark at 15°C for 2-3 hours with fluorometric measurements on a Cytation 5 plate reader with Gen5 software (BioTek, Winooski, U.S.) every 30 minutes. After correcting for negative controls, quenching and background fluorescence, enzyme activities were expressed in

units of µmol/g/h. The activity of phenol-oxidase was measured spectrophotometrically using the substrate 3,4-Dihydroxy-L-phenylalanine (L-DOPA). In 96-well plates, 8 replicates of sample wells (soil suspension + L-DOPA + hydrogen peroxide), background wells (soil suspension + buffer) and a set of standard wells (buffer + L-DOPA)/(buffer + L-DOPA + hydrogen peroxide) were prepared. The well plates were incubated in the dark at 15°C for 24 hours and then assayed using the plate reader. After correcting for backgrounds and standards, enzyme activities were expressed in units of µmol/g/h. All measures conducted on fresh soil were converted to units per gram dry soil.

3.2.5 Statistical Analyses

Statistical analyses were conducted in R version 3.6.1 (R Core Team, 2019) and figures were produced using the *ggplot2* package (Wickham, 2016). Variables were $\log_{10}$-transformed when necessary to fulfil model assumptions.

Treatment effects on plant trait plasticity (Question 1 and 3) were assessed separately for each species and each trait using mixed effect two-way analysis of variance (ANOVA), using the *nlme* package (Pinheiro et al., 2020). Models included species composition (including monocultures and all mixtures containing the species in question), N addition and their interaction as fixed effects and block as random effect. Tukey post hoc tests were performed using the *emmeans* package (Lenth et al., 2020).

Similarly, treatment effects on above- and belowground biomass, CWM aboveground traits, community root traits and soil properties (Question 2) were determined using mixed effect ANOVAs. Models included species composition (four monocultures, six mixtures and bare soil control treatment), N addition and their interaction as fixed effects and block as a random effect. Treatment effects on CO_2 fluxes were assessed

using mixed effect ANOVAs that included FG combination, N addition, their interaction, PAR and soil temperature as fixed effects. Pot identity was included as a random effect to account for repeated measures. Block was not included in these models as examination of the data indicated no block-effects on the response variables. For CO_2 fluxes, outliers were removed when their model residuals were below or above three times the interquartile range (three values for NEE and eight values for R_{eco}) due to presumed measurement error and to ensure that residual distributions met model assumptions. As bare soil CO_2 fluxes were much lower than for all other treatments and only of interest to provide a control to ensure instrument functioning, statistical analyses were repeated excluding bare soil treatments. However, the results did not differ considerably from the results including bare soil treatments.

3.3 Results

3.3.1 Effects of species composition, N addition and their interaction on plant trait plasticity

Plantago had the highest overall shoot dry weights of around 10 to 20 g per shoot (where 'shoot' refers to the total aboveground parts of a plant), while the other three species' shoot dry weights lay around 2.5 to 7.5 g per shoot. Despite this, growing with *Plantago* did not lead to a significant decrease in shoot dry weight for any of the other species ($p > 0.05$). *Dactylis*, *Anthoxanthum* and *Plantago* all had the highest shoot dry weight when growing with *Rumex* ($p < 0.05$), approximately double compared to in monoculture (Fig. 3.1). The shoot dry weight of *Rumex* was not significantly affected by species composition. Additionally, *Anthoxanthum* had higher shoot dry weight when growing with *Dactylis* than in monoculture ($p < 0.001$).

Shoot height varied depending on neighbour identity for the grasses, but not for the forbs ($p < 0.05$, Fig. 3.1). *Dactylis* had lower shoot height when growing with *Anthoxanthum* than in the other species compositions. *Anthoxanthum* was taller when growing with *Rumex*.

Leaf morphological traits of *Anthoxanthum* (SLA, LA and LDMC) were affected by species composition ($p < 0.05$, Fig. 3.2). Its SLA was higher and LDMC lower when growing with *Plantago* than in monoculture. Its LA was lower when growing with *Dactylis* than in the other treatments. *Rumex* had lower LA when growing with *Plantago* than when growing with *Anthoxanthum*. Leaf morphological traits of *Dactylis* and *Plantago* were not significantly affected by neighbour identity.

Leaf chemical traits (leaf C, N and C : N ratio) were only affected by species composition in one instance (Fig. 3.3): *Plantago* had higher leaf C ($p < 0.05$) when growing with *Anthoxanthum* than in monoculture.

N addition and its interaction with species compositions had significant effects on some traits ($p < 0.05$, Fig. 3.1-3.3). N addition increased LA in *Rumex*, leaf C in *Anthoxanthum* and decreased leaf C in *Rumex* and leaf C : N ratio in *Plantago*. The interaction between species composition and N addition affected shoot height in *Dactylis*.

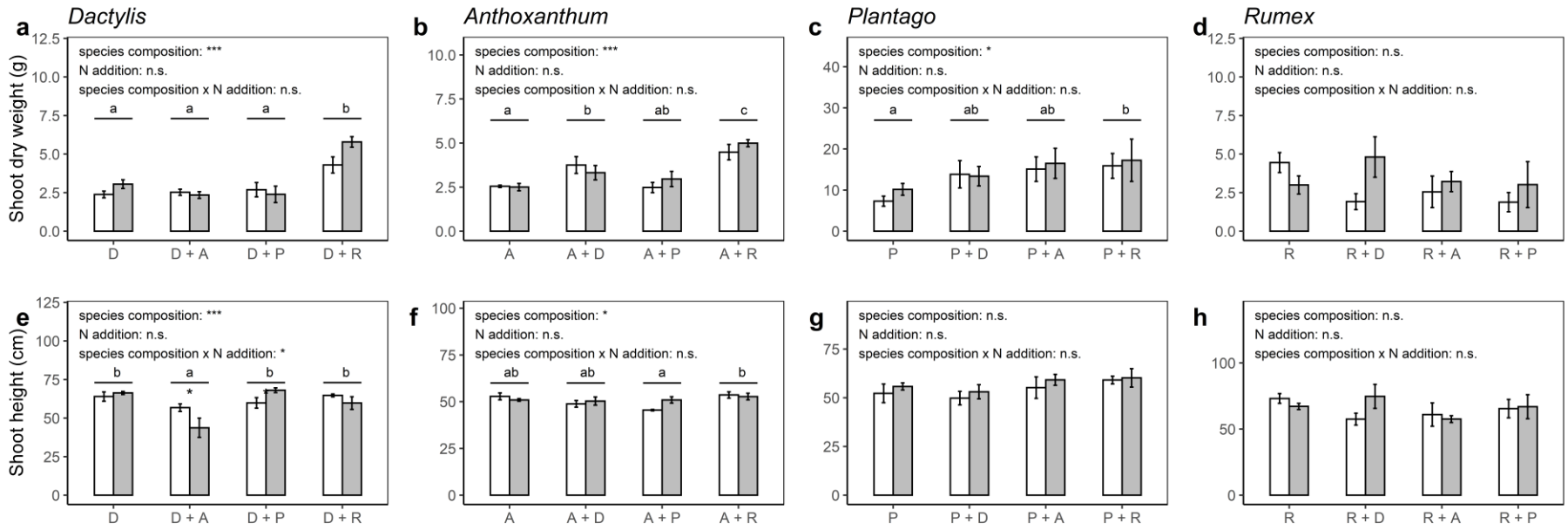

Figure 3.1: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on shoot dry weight and height (mean +/- standard error) in *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: $p < 0.001$***, $p < 0.01$**, $p < 0.05$*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Stars on top of bars indicate significant interactions between species composition and N addition.

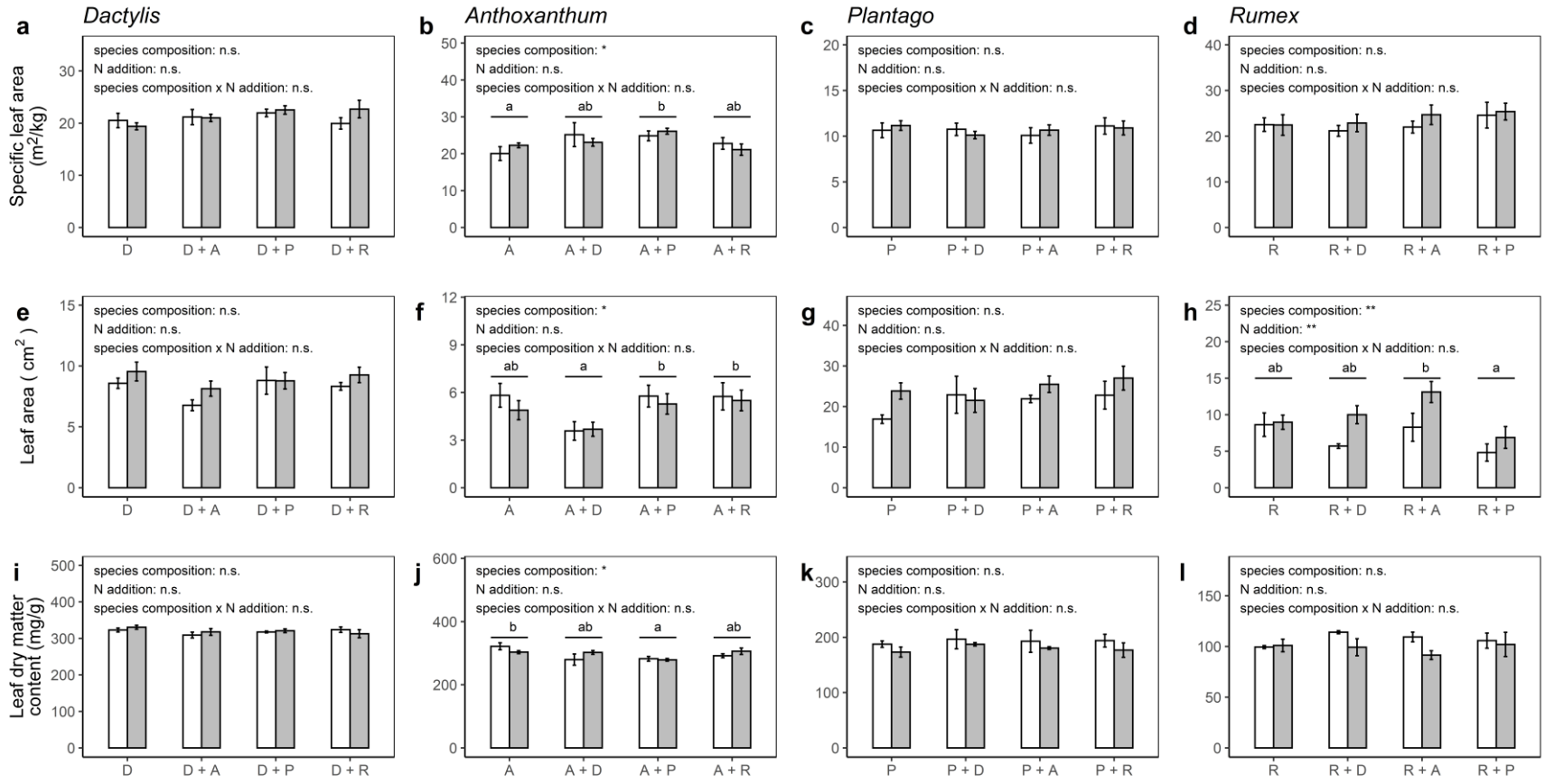

Figure 3.2: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on leaf morphological traits (mean +/- standard error) in *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R). Significance of main and interactive effects were assessed using ANOVA, significance indicated as p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant (p < 0.05) differences between species compositions tested with Tukey post hoc test.

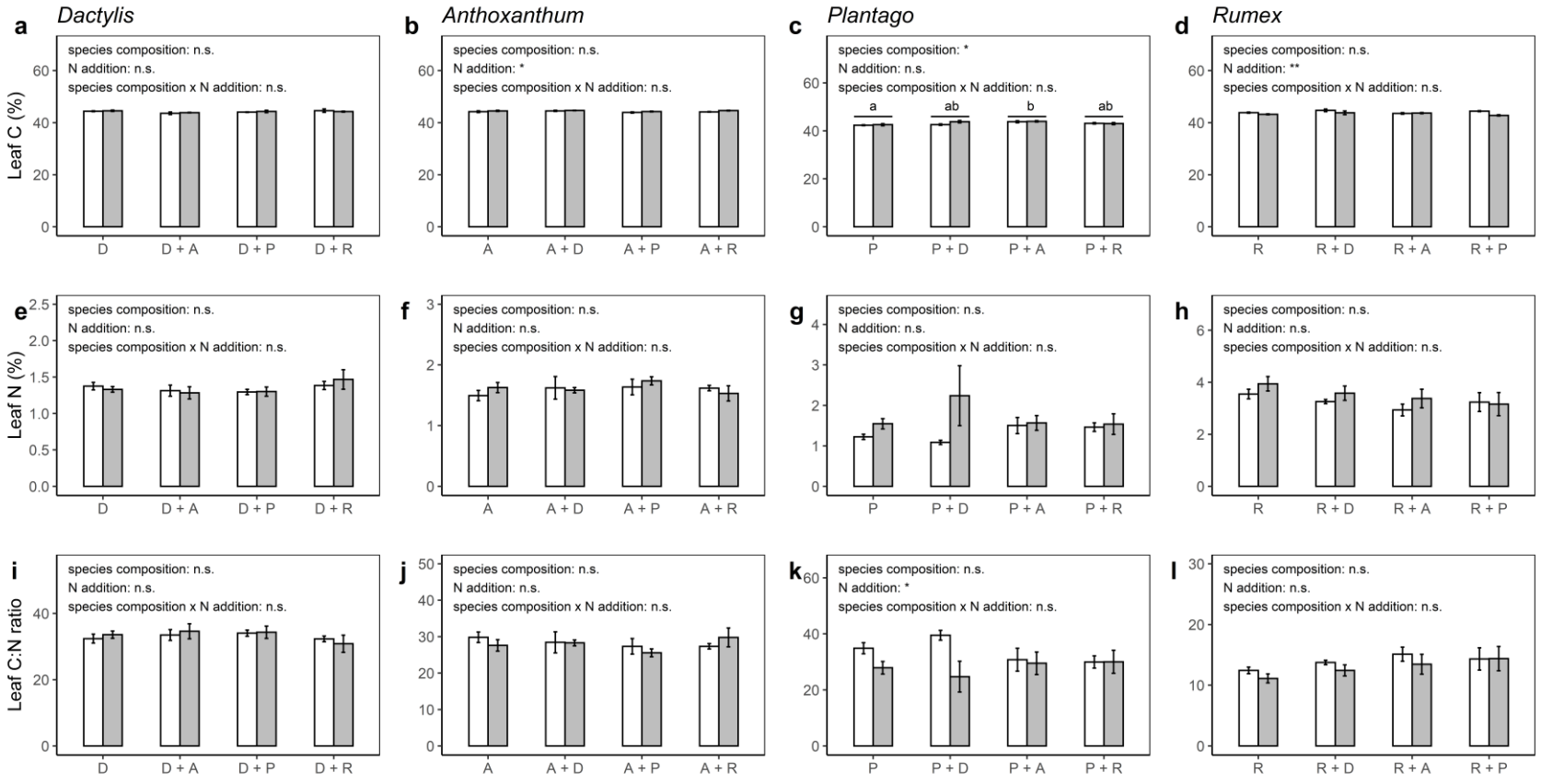

Figure 3.3: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on leaf chemical traits (mean +/- standard error) in *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R). Significance of main and interactive effects was assessed using ANOVA: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant (p < 0.05) differences between species compositions tested with Tukey post hoc test. C – carbon.

3.3.2 Effects of species composition, N addition and their interaction on above- and belowground biomass

Aboveground biomass and root : shoot ratio varied significantly according to presence/absence of *Plantago* ($p < 0.001$, Fig. 3.4); there were no other significant differences between species composition treatments. Aboveground biomass was more than doubled when *Plantago* was present and root : shoot ratio was tripled when *Plantago* was absent. There was a non-significant trend that mixtures had a higher mean aboveground biomass than the means of the monocultures of both component species in most mixtures, i.e. *Dactylis*/*Anthoxanthum*, *Dactylis*/*Rumex*, *Anthoxanthum*/*Plantago*, *Anthoxanthum*/*Rumex* and *Plantago*/*Rumex*. Root biomass was about two times higher in *Rumex* monocultures than in the other monocultures, which did not differ significantly between one another. In the mixtures, root biomass was never significantly different from either of the monocultures of the two component species. Mostly, the mean lay at intermediate values between the monocultures of the two component species. For the *Dactylis*/*Plantago* and the *Anthoxanthum*/*Plantago* mixture it was higher, even though the difference was non-significant.

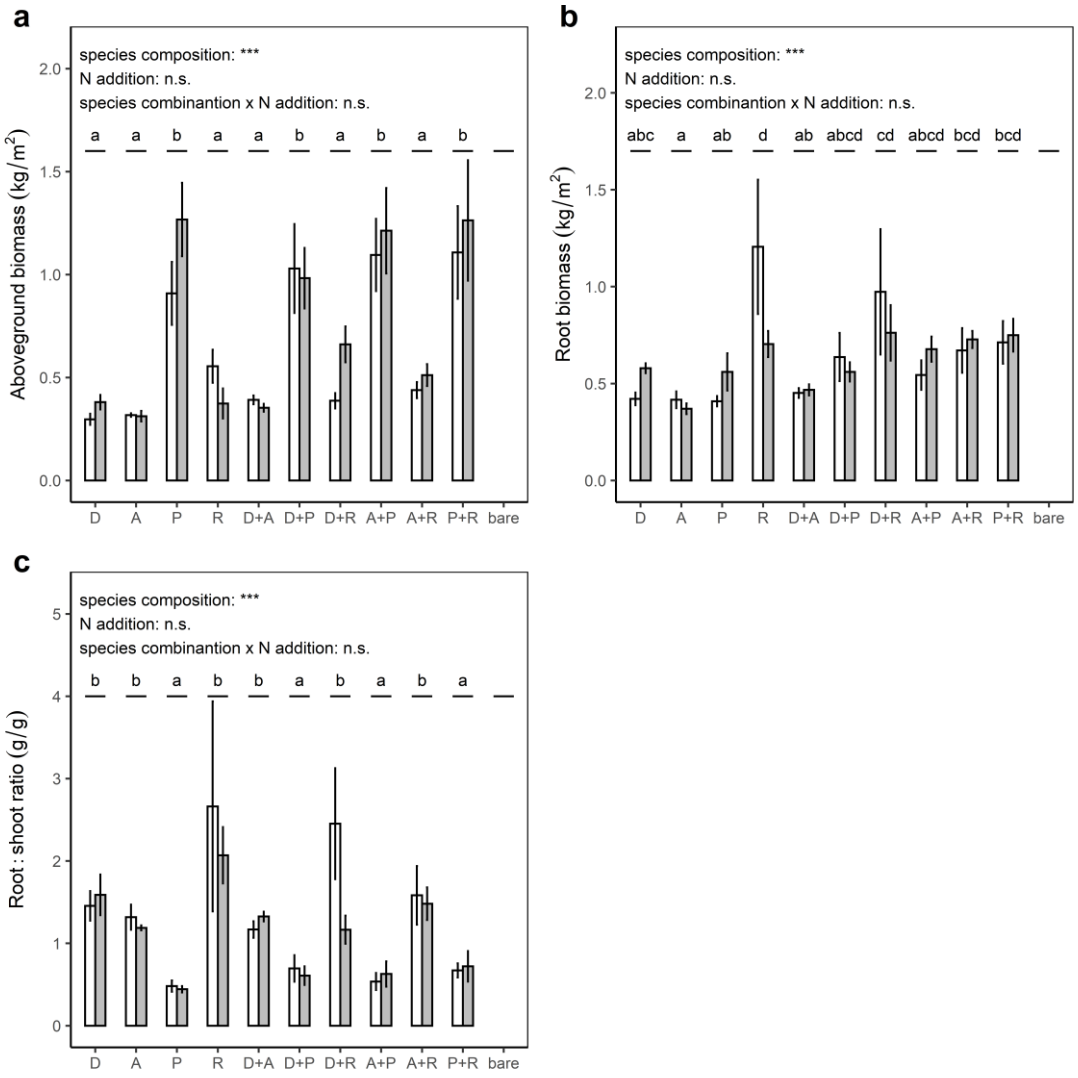

Figure 3.4: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on aboveground biomass, root biomass and root : shoot ratio (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

3.3.3 Effects of species composition, N addition and their interaction on aboveground CWM traits

Aboveground CWM traits were significantly affected by species composition ($p < 0.05$, Fig. 3.5 and 3.6). In monocultures, *Rumex* was the tallest species, with the lowest LDMC and leaf C : N ratio and with highest leaf N content. *Plantago* had the lowest SLA (about half compared to the other species) and the highest LA (about doubled compared to the other species).

Both morphological (Fig. 3.5) and chemical (Fig. 3.6) aboveground CWM traits generally did not differ significantly between *Plantago* monocultures and mixtures containing *Plantago* ($p > 0.05$). The only exception was CWM leaf C, which was significantly higher in mixtures of *Anthoxanthum*/*Plantago* mixtures than in monocultures, analogue to the increase of leaf C in *Plantago* in the mixture (see Fig. 3.3c). Aboveground CWM traits in mixtures containing *Plantago* were often significantly different to monocultures of the second species in the mixture. For treatments not containing *Plantago*, aboveground CWM trait values of mixtures tended to lie at intermediate values between the monocultures of the same species. An exception to this were *Anthoxanthum*/*Dactylis* mixtures, in which CWM height and LA corresponded to *Anthoxanthum* monocultures, but differed from *Dactylis* monocultures.

N addition significantly increased CWM LA and CWM leaf N and decreased CWM leaf C : N ratio ($p < 0.05$, Fig. 3.5 and 3.6).

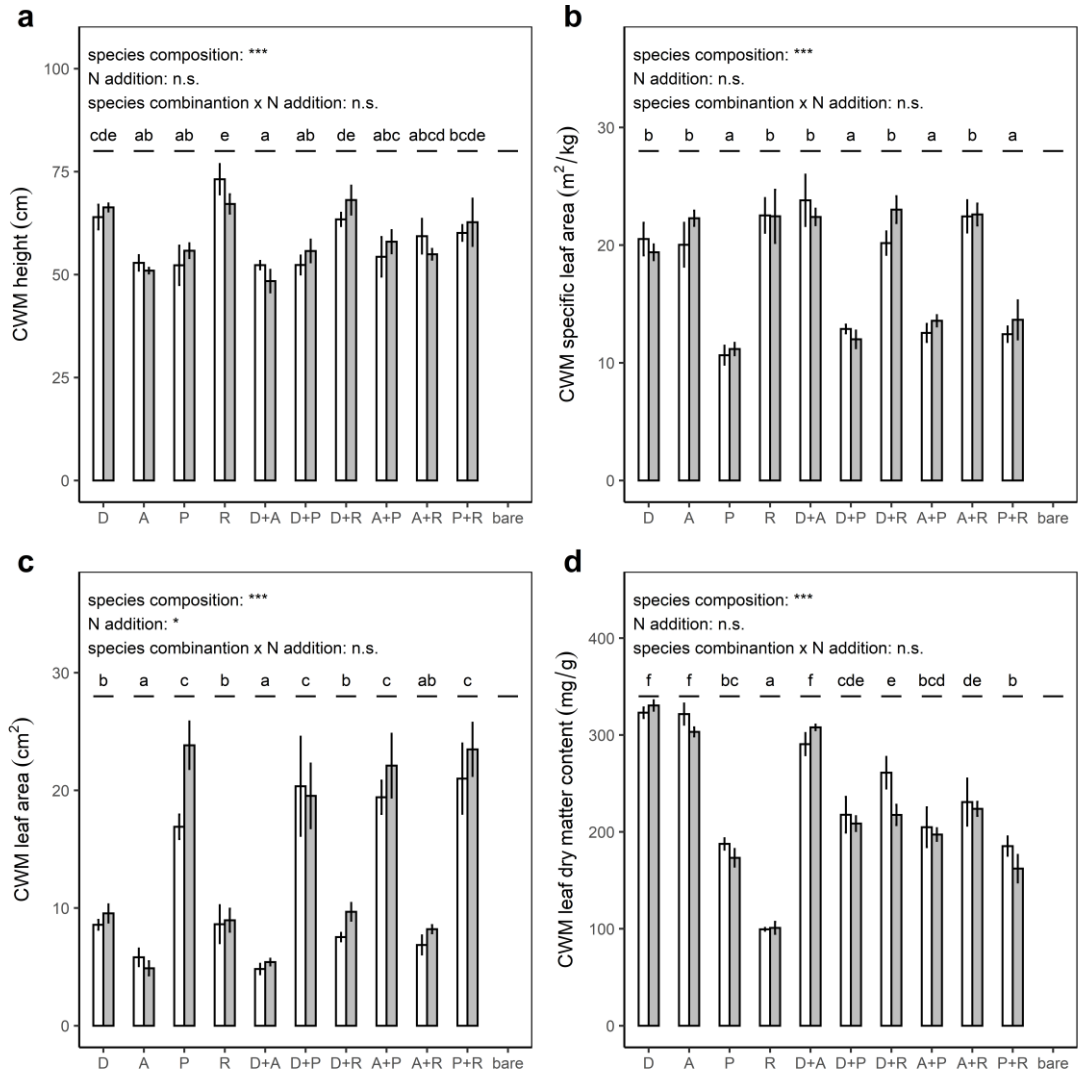

Figure 3.5: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on community-weighted (CWM) morphological aboveground traits (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

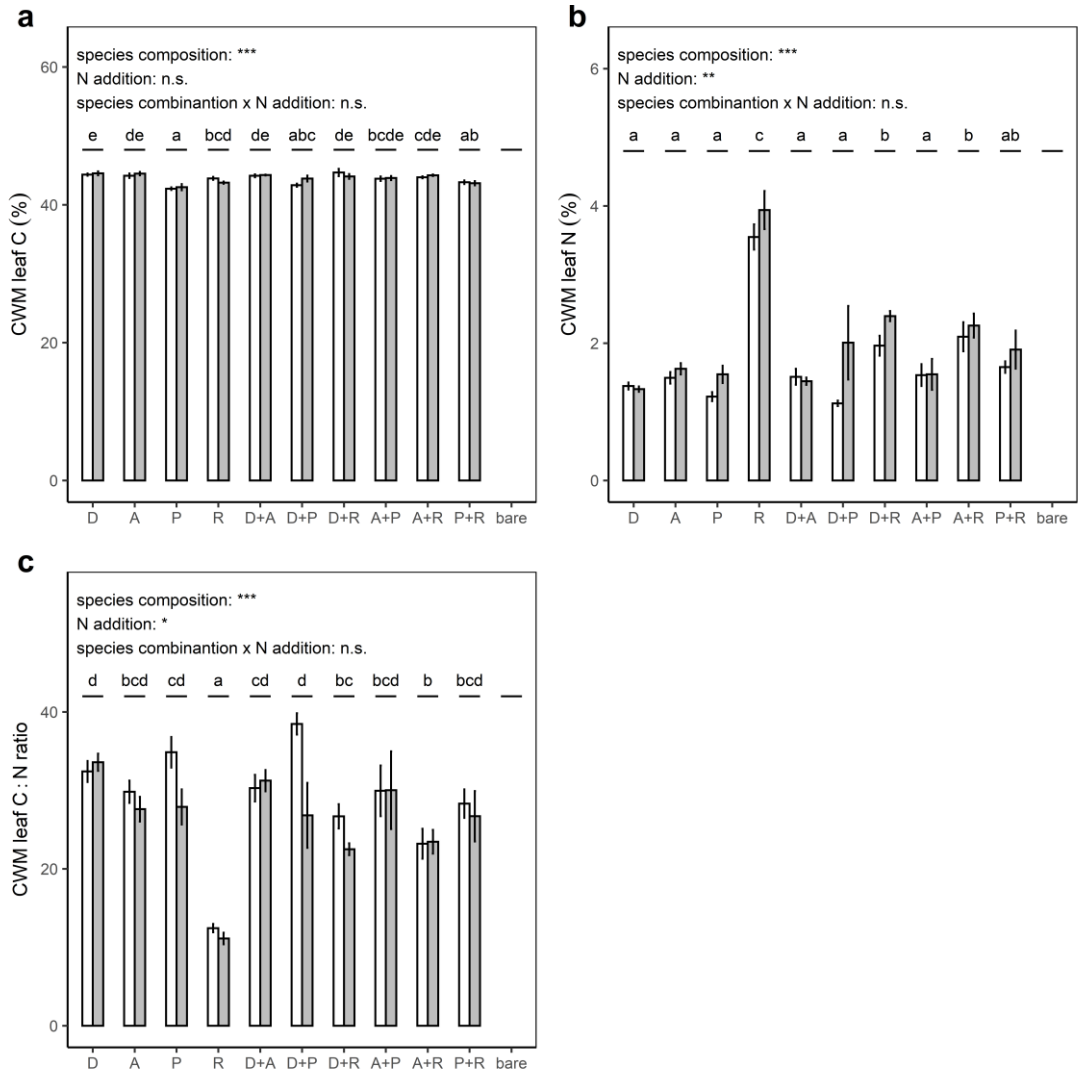

Figure 3.6: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on community-weighted (CWM) leaf chemical traits (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R). C- carbon.

3.3.4 Effects of species composition, N addition and their interaction on community root traits

Root traits were in general significantly affected by species composition ($p < 0.05$, Fig. 3.7). In monocultures, *Rumex* had the highest RDMC, high root diameter and low SLA. *Plantago* also had high root diameter and low SLA, but the overall lowest RDMC. The two grasses did not differ significantly in their root traits, having intermediate RDMC, low root diameter and high SLA.

Community root traits in mixtures generally lay at intermediate values between the monocultures of the same two species. Unlike for aboveground traits, treatments containing *Plantago* were no exception to this pattern. N addition and its interaction with species composition did not affect any of the root traits.

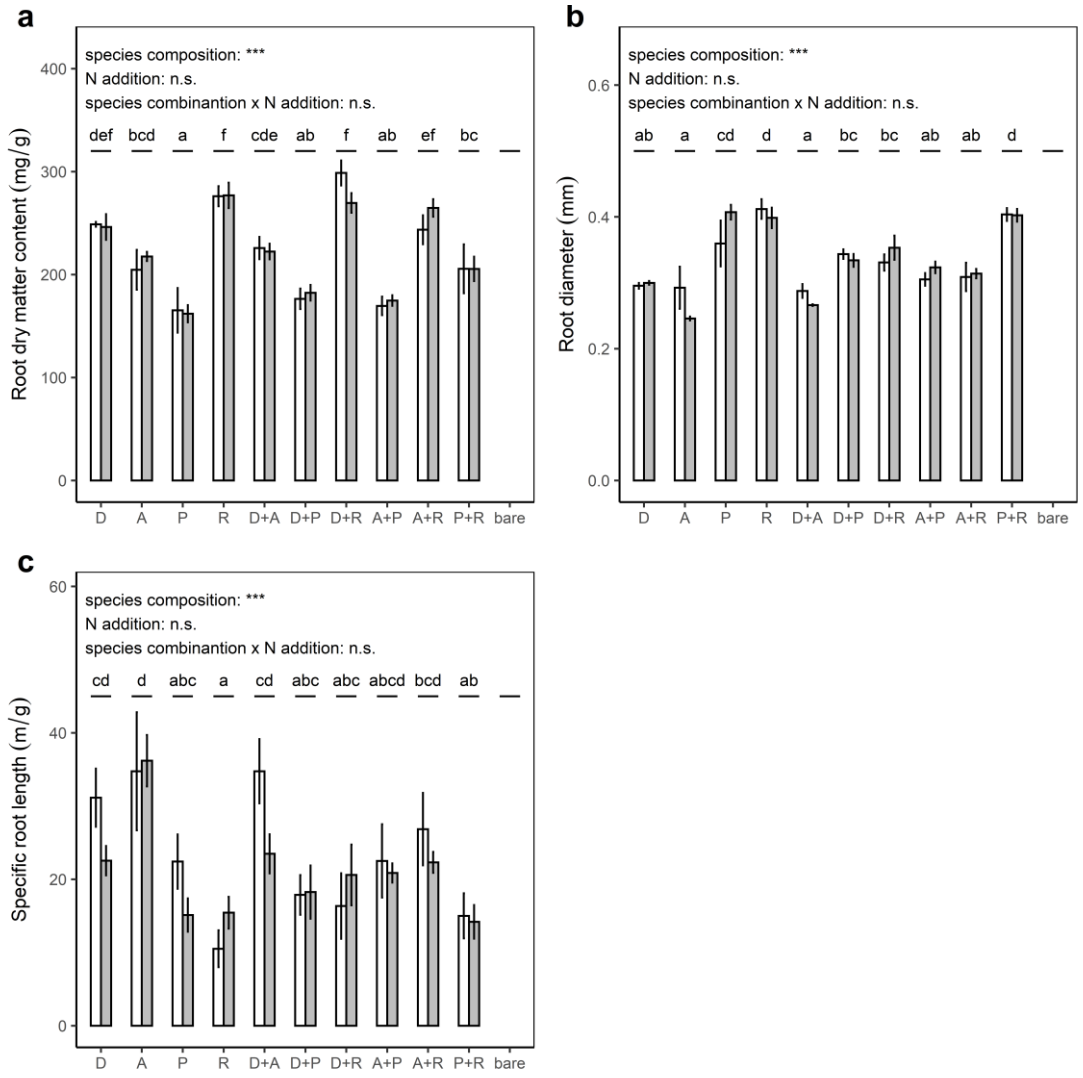

Figure 3.7: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on root morphological traits (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

3.3.5 Effects of species composition, N addition and their interaction on soil properties

Soil abiotic properties (pH, moisture, NH_4^+, NO_3^-, Fig. 3.8) were all affected by species composition, but not by N addition or their interaction. pH was lowest in bare soil, higher in treatments containing *Plantago* and highest in treatments where *Plantago* was absent. Soil moisture was highest in bare soil, lowest where *Plantago* was present and intermediate in treatments without *Plantago*. Plant-available soil NH_4^+ was highest in *Dactylis* monocultures and bare soil treatments, lowest in *Plantago* monocultures and *Anthoxanthum*/*Plantago* mixtures and intermediate in all other treatments. Plant-available soil NO_3^- was significantly different between all of the monocultures: lowest in *Plantago*, higher in *Anthoxanthum*, higher in *Dactylis* and highest in *Rumex*. In mixtures, levels of NO_3^- corresponded to the monocultures of the species with the lower NO_3^- level out of the two component species. In bare soil, NO_3^- was about three times as high as in *Rumex* monocultures.

Four of the five enzyme activities studied were affected by species composition (Phosphatase, β-glucosidase, NAG and Phenol-oxidase, $p < 0.05$, Fig. 3.9), with LAP unaffected by species composition. Phosphatase was highest in *Anthoxanthum* monocultures, *Anthoxanthum/Dactylis* mixtures and bare soil and lowest in mixtures containing *Plantago*. β-glucosidase was low in *Dactylis* and *Plantago* monocultures, in mixtures containing *Plantago* and in bare soil. It was highest in *Anthoxanthum*, *Rumex* and mixtures containing one of these species, but not *Plantago*. NAG exhibited the largest variability. It was lowest in Rumex monocultures and bare soil, highest in *Anthoxanthum* monocultures and *Anthoxanthum/Dactylis* mixtures and intermediate in the remaining treatments. Phenol-oxidase was highest in bare soil but not significantly

different between the planted treatments. N addition and its interaction with species composition did not affect enzyme activity significantly.

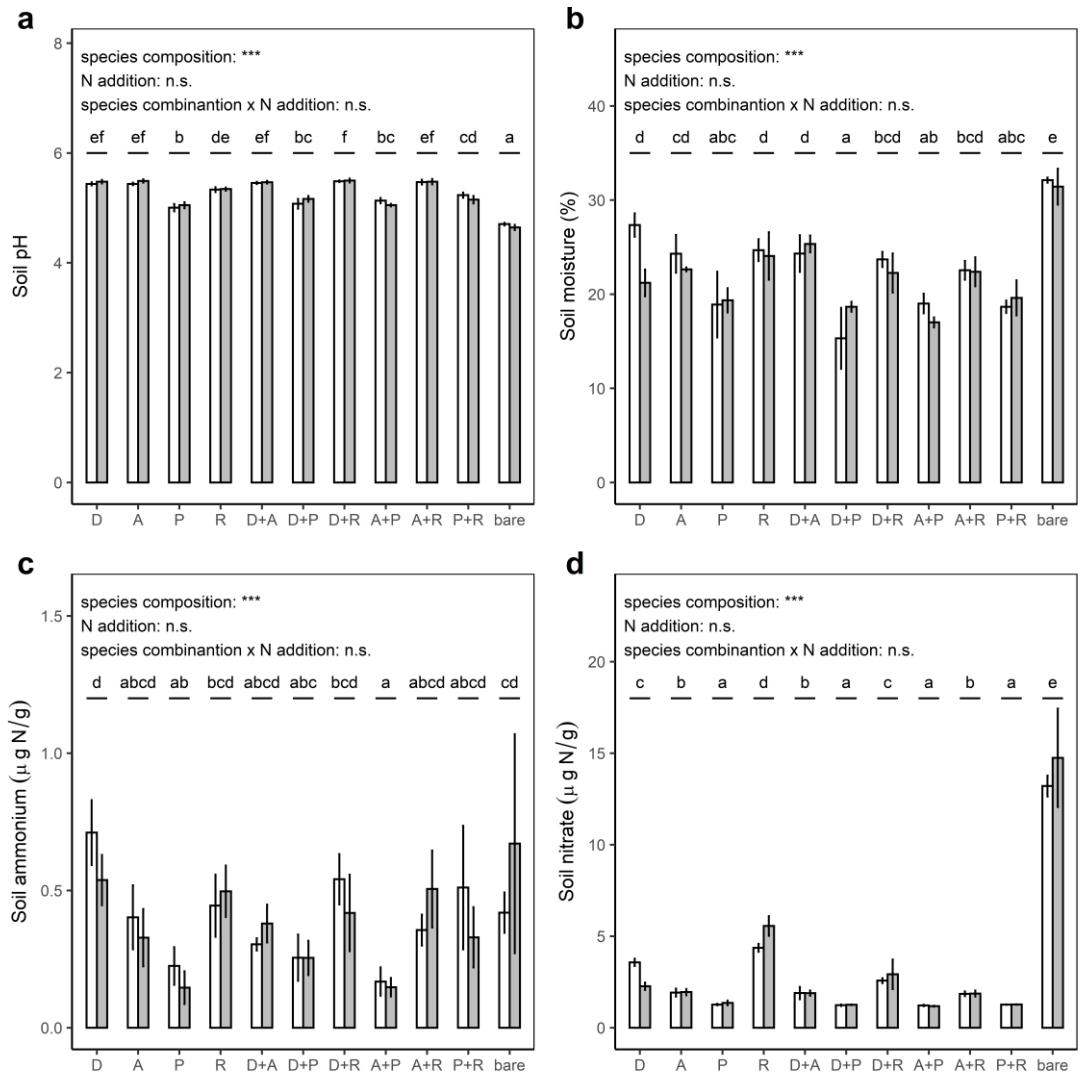

Figure 3.8: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on soil properties (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

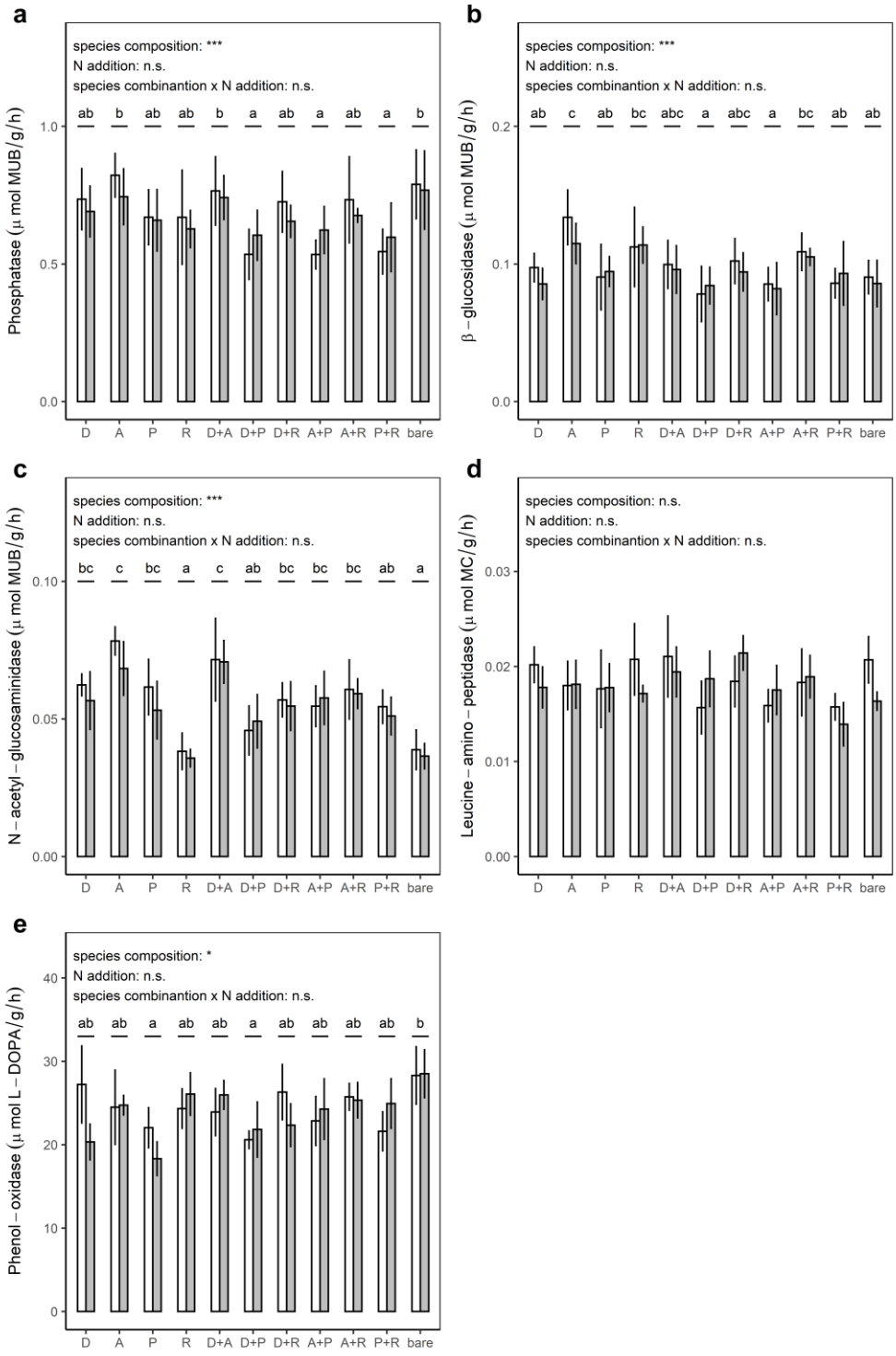

Figure 3.9: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on soil enzyme activities (mean +/- standard error). Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

3.3.6 Effects of species composition, N addition and their interaction on ecosystem CO_2 fluxes

NEE differed between species compositions and was also increased significantly by N addition ($p < 0.05$, Fig. 3.10a), but there was no significant interaction between species composition and N addition. Of the monocultures, NEE was lowest in *Anthoxanthum* and *Rumex*. *Plantago* monocultures had the highest NEE, even though the difference to *Dactylis* monocultures was non-significant. Mixtures containing *Plantago* did not differ significantly in their NEE from *Plantago* monocultures, but it was always higher than the monocultures of the other component species. Mixtures that did not contain Plantago showed different patterns: The *Anthoxanthum*/*Rumex* and *Dactylis*/*Rumex* mixture had an NEE higher than monocultures of both component species, even though the difference was not significant. The NEE of the *Dactylis*/*Anthoxanthum* mixture lay in between the values of the monocultures of both component species.

R_{eco} and photosynthesis differed between species compositions, photosynthesis was also overall significantly increased by N addition and in both fluxes there were significant interactions between species composition and N addition ($p < 0.05$, Fig. 3.10b and c).

In monocultures, R_{eco} was lowest in *Dactylis* and *Anthoxanthum*, higher in in *Rumex* and highest in *Plantago*. In mixtures, R_{eco} generally corresponded to the monocultures of the component species with the higher R_{eco} and was significantly higher than the one with the lower R_{eco}. Photosynthesis was lowest in *Anthoxanthum* monocultures, higher in *Dactylis* and *Rumex* monocultures and highest in *Plantago* monocultures. The magnitudes of photosynthesis in mixtures followed the same pattern as observed for NEE. N addition significantly decreased both R_{eco} and photosynthesis only in *Rumex* monocultures. In some mixtures containing *Rumex*, N addition increased R_{eco} and

photosynthesis (*Dactylis*/*Rumex* for both and additionally *Anthoxanthum*/*Rumex* for photosynthesis). The other species composition treatments were not significantly affected by N addition.

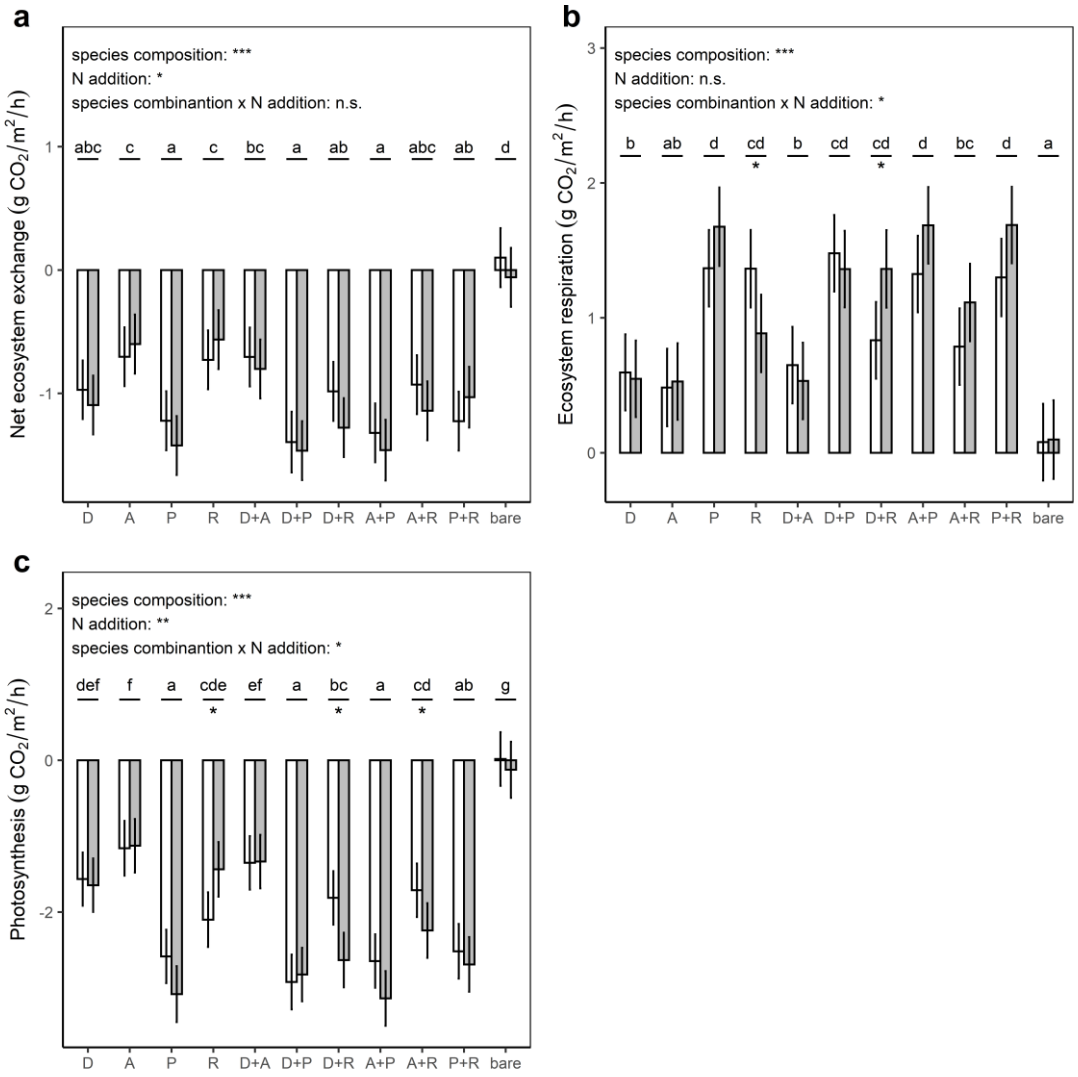

Figure 3.10: Effects of species composition, nitrogen (N) addition (grey = N added, white = control) and their interaction on CO_2 fluxes. Bars represent estimated marginal means taking into account photosynthetically active radiation, soil temperature and day of measurement, error bars represent standard errors. Significance of main and interactive effects were assessed using ANOVA with significance indicated as: p<0.001***, p<0.01**, p<0.05*. Different letters on top of bars indicate significant ($p < 0.05$) differences between species compositions tested with subsequent Tukey post hoc test. Stars on top of bars indicate significant interactions between species composition and N addition. Species compositions consist of *Dactylis* (D), *Anthoxanthum* (A), *Plantago* (P) and *Rumex* (R).

3.4 Discussion

The aim of this study was to explore the effects of interactions between plant species on ecosystem properties and functions, and whether they are modified by N addition corresponding to an increase in atmospheric deposition (2 kg/ha). The dry weight of individual shoots was generally either increased or not affected by growing in mixtures, but other above-ground traits were rarely affected. Ecosystem properties and functions were idiosyncratically affected by growing in mixture. Above- and belowground biomass, NEE and photosynthesis were on average higher in mixtures compared to monocultures, even though this effect was not statistically significant, suggesting synergistic effects. Other ecosystem properties of mixtures lay either in between the monoculture values for the two component species, suggesting additive effects, or were disproportionally affected by one of the component species. N addition did not significantly modify any of the species interactions, but affected CWM leaf chemical traits and CO_2 fluxes.

3.4.1 Phenotypic plasticity as response to neighbouring plant species and N addition

The four plant species varied in their patterns of plasticity, with few general trends. The effects of neighbouring species on phenotypic plasticity in plant traits (Question 1) point to several types of interactions occurring between the species.

Shoot dry weight was the most variable trait overall. It sometimes increased in mixtures in comparison to monocultures and sometimes stayed the same, but never decreased (Fig. 3.1). This indicates that facilitation or complementary resource use was more important in the interaction between species than competition. This finding contrasts with results of competition experiments where competitive intensities increased with larger trait differences between plant neighbours (Fort et al., 2014; Kraft et al., 2014;

Bennett et al., 2016). However, these experiments were conducted in smaller pots and only over 3-4 months, while here the plants were grown in larger pots for two growing seasons. A possible mechanism is that plant-microbial feedbacks take time to develop (Morriën et al., 2017). Also, experiments conducted over longer time can allow temporal complementarity to unfold (Wagg et al., 2017). On the other hand, an even longer duration of the experiment might have intensified competition for nutrients and light as the plants would have grown larger and nutrients could have been depleted further (Trinder et al., 2012; Bezemer et al., 2018).

All species approximately doubled shoot dry weight in mixtures with *Rumex*, while *Rumex* shoot dry weight never decreased in mixtures. *Rumex* monocultures had the highest available soil NO_3^- concentrations measured in the top 10 cm soil layer (Fig. 3.8), even though they had the same aboveground biomass as *Dactylis* and *Anthoxanthum* monocultures and the overall highest root biomass and CWM leaf N content (Fig.3.4 and 3.6). An explanation for this could be vertical resource complementarity, as the *Rumex* taproots could have obtained nutrients from deeper soil layers than the other species, leaving more nutrients in the upper layers. Spatial below-ground resource partitioning has often been assumed to be a major mechanism for biodiversity effects. While little evidence for this has been found in long-term grassland biodiversity experiments (Barry et al., 2020), it may still be important in some plant communities.

The shoot dry weight of *Plantago*, the fastest-growing species, almost doubled in all mixtures compared to monocultures, which results in roughly the same *Plantago* total aboveground biomass. This might be due to strong intraspecific competition for light and/or nutrients in monocultures, as *Plantago* had a much higher shoot dry weight than

the other species and as treatments containing *Plantago* had the lowest availability of water and NO_3^- (Fig. 3.8). The "law of constant final yield" predicts that in monocultures, after a certain time, the total biomass will be the same irrespective of the planting density (Shinozaki & Kira, 1956; Weiner & Freckleton, 2010). Thus, the doubled aboveground biomass of *Plantago* in mixtures corresponds to what would be expected if the plants of other species in the mixture were not present. Surprisingly, despite this, growing in mixture with *Plantago* did not decrease shoot dry weight in any of the other species. As light, nutrient and water availability were less in treatments containing *Plantago*, there might be a compensating factor at play favouring the other species, e.g. reduced levels of species-specific pathogens or spatial or temporal resource partitioning.

Plasticity in the other aboveground traits was more limited (Fig. 3.2 and 3.3) and seemed to be driven by several factors. *Anthoxanthum* showed plasticity in response to species composition in all morphological traits, but not in chemical traits and the other three species only in one other morphological or chemical shoot trait respectively. Sometimes, plasticity was related to shoot dry weight, such as for shoot height in *Anthoxanthum* and leaf C in *Plantago*. *Anthoxanthum* had higher SLA and lower LDMC when growing in mixture with *Plantago* than in monoculture, which could be an adaptation to shading from the dense leaves of *Plantago* (Siebenkäs et al., 2015).

The LA of *Rumex* was higher in the N addition treatment and this was the only occasion in the study that a morphological trait was significantly affected by N addition (Question 3). Despite having a lower RGR and much lower shoot dry weight, *Rumex* had a higher SLA than *Plantago*, the overall lowest LDMC and the overall highest leaf N content. Also, it had the highest root biomass, RDMC and lowest SRL in monoculture. This

indicates that while the roots and possibly stems were more slow-growing, it had fast-growing, short-lived leaves that turn over and regenerate quickly. This might explain why LA increased only in *Rumex* with the relatively recent N addition (2 months before measurement). LA of *Rumex* was also higher in *Rumex*/*Anthoxanthum* mixtures than in *Rumex*/*Plantago* mixtures, which could be related to differences in N availability.

Several traits differed from monocultures in *Anthoxanthum*/*Dactylis* mixtures. *Anthoxanthum* shoot dry weight was significantly higher in the mixture than in monoculture while *Dactylis* biomass was not affected. NO_3^- availability in the mixtures was the same as in *Anthoxanthum* monocultures and lower than in *Dactylis* monocultures (Fig.3.8), so it is possible that *Anthoxanthum* could take up more N per planted individual in the mixture, leading to the increased shoot dry weight. Additionally, the LA of *Anthoxanthum* was lower in the mixture. Together with the increase in shoot dry weight this could mean that *Anthoxanthum* grew more tillers per tussock with a higher number of smaller leaves in the mixture. Clonal reproduction (e.g. in tussocks) can improve the competitive ability of plants (Liu et al., 2016), so it is possible that the presence of another tussock-forming species (*Dactylis*) triggered an increase in ramet formation. Also, *Dactylis* shoots were less tall in these mixtures than in monoculture. As the height and shoot dry weight of *Anthoxanthum* in the mixtures was not particularly high or low compared to the other species in mixture with *Dactylis* (Fig. 3.1), it is unlikely that this decrease in height was caused by a change in light availability. Thus, it is possible that also *Dactylis* expanded more horizontally than vertically due to the presence of another tussock-forming grass.

3.4.2 Effects of species interactions and N addition on plant community and ecosystem properties

The effects of species interactions in the mixtures on plant community and ecosystem properties (Question 2, Fig. 3.4 to 3.10) fell into roughly four categories: With respect to the monoculture values of the two component species, the mixture values lay either (i) in between, (ii) above both, (iii) at the same level as one of the monocultures, but different from the other, or (iv) at varying levels depending on the particular mixture.

(i) The mixture values lay mostly in between the monoculture values for the two component species for root biomass (except in treatments including *Rumex*), root : shoot ratio, aboveground CWM traits (except in treatments containing Plantago), root traits and soil moisture (except in treatments containing Plantago). For the aboveground and root traits this indicates that there was not sufficient phenotypic plasticity to shift community traits strongly from what would be expected from their monocultures. For root biomass and soil moisture this gives support to the mass-ratio hypothesis and suggests that no strong synergistic interactions took place between the species.

(ii) The complementarity and/or facilitation suggested by the phenotypic plasticity in shoot dry weights did not lead to a significant increase in total aboveground biomass of a mixture compared to both of its two component species' monocultures, but there was a non-significant trend for most mixtures, i.e. *Dactylis*/*Anthoxanthum*, *Dactylis*/*Rumex*, *Anthoxanthum*/*Plantago*, *Anthoxanthum*/*Rumex* and *Plantago*/*Rumex*. The same non-significant trend could be observed for NEE and photosynthesis, which are strongly linked to aboveground biomass (De Long et al., 2019). For root biomass there was a corresponding non-significant trend in treatments not containing *Rumex*. This trend is consistent with observations from biodiversity experiments and might become stronger over time (Meyer et al., 2016).

(iii) CWM aboveground traits and most soil properties in mixtures containing *Plantago* did not differ significantly from *Plantago* monocultures, but often differed significantly from the monocultures of the second species in the mixture. For soil NO_3^- and R_{eco}, the mixtures had the same effect as the monocultures with the stronger effect. For NO_3^-, the most influential species was *Plantago*, followed by *Anthoxanthum*, *Dactylis* and *Rumex*. Interestingly, this order was not related to growth strategy, as *Anthoxanthum* had the lowest RGR and the second-lowest levels of soil NO_3^-, while *Rumex*, had the second-highest RGR and the highest levels of soil NO_3^-. The more influential species always had a higher contribution to total aboveground biomass compared to the second species in a mixture. *Plantago* always had a 6-8 times higher contribution to total aboveground biomass than the other species in the mixture, so this is consistent with the mass-ratio hypothesis in treatments containing *Plantago*. In treatments without *Plantago*, however, the difference was much smaller (1.5-2 times higher contribution to biomass). This suggests that the species had an effect that was disproportional relative to its contribution to biomass. Disproportional effects of particular species to ecosystem function have been observed in other studies, particularly for legumes (e.g. Lange et al., 2014). This is a factor that might explain why CWM traits have been found to predict ecosystem function better in monocultures than in mixtures (De Long et al., 2019). For R_{eco}, the most influential species was *Plantago*, followed by *Rumex*, followed by *Anthoxanthum* and *Dactylis* at the same level. For *Plantago* this is probably due to its high aboveground biomass and for *Rumex* due to its high below-ground biomass, combined with the facilitation of the other species in the mixtures.

(iv) Soil pH, NH_4^+ and enzyme activities did not fall in either of the categories discussed above, but showed differing patterns depending on species and mixtures. Soil pH decreased compared to the field pH of around 6. Bare soil had the lowest pH, probably

due to leaching (Bleam, 2016). The plants may have inhibited leaching, but at the same time acidified the soil by taking up nutrients, which may be why treatments containing *Plantago* and thus the highest biomass had the second-lowest pH levels. The differences in enzyme activities (Fig. 3.9) were mostly subtle and hard to interpret. There was no common pattern among all enzymes, which indicates that the differences between treatments were not purely driven by the amount of microbial biomass. NAG, which is involved in the degradation of chitin, was the most variable enzyme activity between species composition treatments. Contrary to findings of other studies (Olander & Vitousek, 2000; Sinsabaugh et al., 2008) it did not appear related to soil N availability or pH (Fig. 3.8), but rather to particular plant species, being highest in treatments including *Anthoxanthum* and lowest in *Rumex* monocultures.

RDMC and root diameter were the only properties that varied significantly between *Plantago* monocultures and mixtures containing *Plantago*. This is consistent with the observation that *Plantago* monocultures did not have higher root biomass than other species, on the contrary *Rumex* had higher root biomass. Studies suggest that some soil properties may be more correlated with root traits than with aboveground traits (Legay et al., 2014), but this study provides an example where this is not the case. Slow-growing species might have a high contribution to root biomass and thus community root traits, while fast-growing species may have a smaller contribution to root biomass, but still control ecosystem properties.

N addition did not alter species interactions significantly. The only ecosystem properties affected by N addition or its interaction with species composition were NEE and photosynthesis as well as CWM leaf N and C : N ratio. Leaf N and shoot dry weight were not significantly affected by N addition at the species level, and leaf C : N ratio

only for one species, so the significant effect on CWM leaf chemical traits were mediated by a combination of non-significant changes in biomass and in leaf chemical traits at the species level. N addition did not modify interactions between species measurably.

3.4.3 Conclusion

The results of this study show that interactions between plant species affect ecosystem properties and functions in idiosyncratic ways, depending on the particular ecosystem property or function and sometimes the species. This suggest that the usefulness of metrics for predicting ecosystem properties and functions from plant traits (such as CWM traits and diversity indices) depends on the particular context. Phenotypic plasticity in shoot dry weight indicated that neighbouring species generally had either beneficial or neutral effects on one another, resulting in a non-significant trend of increased aboveground biomass, NEE and photosynthesis in mixtures compared to monocultures. This suggests synergistic effects and gives support to the diversity hypothesis. Plasticity in the other traits was mostly limited and did not appear to affect ecosystem properties or functions. For some ecosystem properties in mixtures (e.g. soil moisture), the comparison with the corresponding monocultures suggested that both species had additive effects relative to their biomass, giving support to the mass-ratio hypothesis. For some ecosystem properties and function (e.g. plant available NO_3^-, R_{eco} and NAG activity) one of the component species in mixtures appeared to have an effect disproportional relative to its contribution to total biomass. This mechanism has the potential to limit the explanatory power of both CWM traits and diversity indices. Finally, N addition did not significantly modify any of the species interactions and only affected CWM leaf chemical traits and CO_2 fluxes.

4 Effects of interactions between grassland plant species on the fate of recently assimilated carbon

Abstract

Vegetation plays a crucial role in controlling terrestrial ecosystem carbon (C) cycling and storage, but the mechanisms are not fully understood, for example the role of interactions between plant species. Here, a ^{13}C pulse-labelling approach was used to explore how interactions between two temperate grassland species (*Plantago lanceolata* and *Dactylis glomerata*) affect short-term C dynamics. Monocultures and mixtures of the two species grown in mesocosms were pulse-labelled with $^{13}CO_2$. Throughout the following week, levels of ^{13}C were measured in leaf, root and soil samples, as well as in respired CO_2. Results showed that *Plantago* and *Dactylis* monocultures differed in their effect on short-term C dynamics, and synergistic effects were found in the mixtures. In mixtures, almost twice as much ^{13}C was allocated to root biomass than in both monocultures. Additionally, the temporal pattern of ^{13}C allocated to roots and soil differed between mixtures and monocultures. These findings suggests

that the interaction between grassland species altered the fate of recently assimilated C, which could potentially affect long-term C dynamics such as soil C storage.

Keywords: ^{13}C pulse labelling, plant species interactions, carbon allocation

4.1 Introduction

Terrestrial ecosystems store approximately 2000-3000 Pg of Carbon (C) in vegetation and soils, about three times as much as the atmosphere (IPCC, 2013). In the face of global change, it is therefore critical to understand biotic controls on ecosystem C dynamics, such as C sequestration, storage and the emission of greenhouse gases. Temperate grasslands hold about 13% of terrestrial C stocks, predominantly in the soil (Royal Society, 2009). These C stocks are controlled by climate, abiotic soil properties, management intensity and also vegetation composition (Manning et al., 2015; Ward et al., 2016).

Vegetation can affect ecosystem C cycling through several mechanisms. A very important factor controlling photosynthesis and ecosystem respiration is the amount of above-ground biomass (De Long et al., 2019). Also, the growth strategy of the plants can play a role, as fast-growing species tend to take up more carbon per unit biomass, but also respire more (Reich et al., 1998). Another important factor is the quantity and quality (i.e., chemical composition) of shoot and root litter. Both labile and recalcitrant litter fractions can contribute to forming stable soil organic matter (SOM) (Cotrufo et al., 2015). Labile compounds are efficiently incorporated by microbes and their residues can form stable SOM (Kallenbach et al., 2016). Recalcitrant compounds decompose slowly and small fragments of litter can remain in the soil due to their recalcitrance and/or through physical protection in soil aggregates (Cotrufo et al., 2015). Generally, root litter contributes more to stable SOM than shoot litter due to its high recalcitrance,

the physical protection in deep soil layers, increased soil aggregate formation through mycorrhiza and root hairs and chemical interactions with metal irons (Rasse et al., 2005). Rhizodeposition, in the form of root exudation and transfer to root-associated mycorrhizal fungi, is another factor that can influence soil C cycling, as it can stimulate microbial activity and alter microbial community composition (Lange et al., 2015; Baumert et al., 2018), speed up or slow down mineralization of existing SOM (Kuzyakov, 2010; Henneron et al., 2020) and contribute to the formation of soil aggregates (Baumert et al., 2018).

How vegetation affects C cycling through these mechanisms is affected by the traits of the species present, especially the most dominant (Roscher et al., 2019). In addition, synergistic effects can arise from the interactions between plant species. For example, diverse systems often have increased above- and below-ground biomass compared to less diverse systems, for example due to complementarity effects (e.g. Barry et al., 2019; Roscher et al., 2013; Tilman et al., 2001; Van Ruijven & Berendse, 2009), leading to increased photosynthesis, respiration and litter inputs. Competition for light, nutrients or water between plant species can also lead to altered root : shoot ratios (e.g. Mommer et al., 2010). Neighbouring plants can also induce phenotypic plasticity in above- and belowground traits, for example through altered nutrient, water or light availability, which could in turn affect C cycling. For example, shading by neighbouring plants can decrease specific leaf area and N availability alter tissue N content (Siebenkäs et al., 2015), which are both related to photosynthetic capacity as well as plant respiration rates (Reich et al., 1998). Also, rates of root exudation could be affected by interactions between species, for example by affecting water and nutrient availability, which have been shown to affect root exudation rates (Carvalhais et al., 2011; de Vries et al., 2019).

Changes in soil organic carbon generally occur slowly over decades (Poeplau et al., 2011), which makes it challenging to study their controlling factors. Measurements of above- and belowground biomass and CO_2 fluxes contribute to understanding, but a limitation is that they are integrative measures influenced by various processes and highly variable throughout the year. Above- and below ground biomass are regulated by plant growth and allocation, but also by mortality (Mommer et al., 2015) and herbivory (McNaughton et al., 1989). CO_2 flux chamber measurements of ecosystem respiration with opaque chambers consist of ecosystem-level plant and soil microbial respiration, measurements of net ecosystem exchange with transparent chambers additionally include photosynthesis. ^{13}C pulse-labelling approaches allow to study the transfer and allocation of recently assimilated C, which can further improve the understanding of ecosystem C cycling (Ostle et al., 2000). It has been shown in tracer studies that short-term C dynamics are affected by plant species, functional groups and species diversity (Ward et al., 2009; de Deyn et al., 2012). However, the effects of interactions between plant species on the fate of recently assimilated C remain poorly studied.

The aim of this study was to investigate whether a pair-wise interaction between grassland plant species has an effect on short-term C cycling. Two functionally distinct common grassland species (the rhizomatous forb *Plantago lanceolata* and the fine-rooted grass *Dactylis glomerata*) were grown in monocultures and a mixture. A $^{13}CO_2$ pulse-labelling approach was used to investigate C assimilation through photosynthesis, as well as its transfer to roots, soil and respired CO_2. It was hypothesized that short-term C dynamics would differ between monocultures and mixtures, with interactions having an additive or synergistic effect.

4.2 Methods

4.2.1 Experimental design and mesocosm establishment

The experiment was conducted on a subset of pots from the mesocosm experiment described in Chapter 3. The mesocosm experiment was set up in June 2018 at Hazelrigg field station (54°10N, 2°460W). The site has a mean annual temperature of 9°C and a mean annual precipitation of 1050 mm. Mesocosm pots (38 x 38 cm, 40 cm deep) were filled with a 10 cm layer of chippings and a 20 cm layer of mesotrophic grassland soil (pH around 6 (De Vries et al., 2015; Barneze et al., 2020)) collected from the surrounding grassland and sieved to 1 cm to remove roots and rocks.

Two species common to European temperate grasslands were selected for the experiment, which belong to different functional groups and are functionally distinct with different above-below ground growth forms: *Plantago lanceolata*, a rhizomatous fast-growing forb with a relative growth rate (RGR) of 1.40 g/g/week (Grime & Hunt, 1975) and *Dactylis glomerata*, a relatively slower-growing grass with fibrous roots and a RGR of 1.31 g/g/week (Grime & Hunt, 1975). The experiment was set up in a randomized block design and comprised four blocks, each including monocultures of the two species, their mixture and a bare soil treatment. Seeds were from purchased from Emorsgate Seeds (King's Lynn, Norfolk, UK). Seedlings were germinated in plug trays in the greenhouse using compost (John Innes No. 2) for 4 weeks before transplanting to mesocosms. The compost was then rinsed off the roots and seedlings were transplanted into the mesocosms in a grid of 6 x 6 = 36 seedlings. In the mixture treatment seedlings of two species were planted alternately. The mesocosm pots were watered throughout the summer months of 2018 and 2019 and weeded as required.

4.2.2 $^{13}CO_2$ pulse labelling

A $^{13}CO_2$ pulse chase experiment was performed to investigate the short-term C dynamics in the plant-soil systems, following the approach by Ostle et al. (2003, 2007). Mesocosms were labelled with 98 atom % ^{13}C enriched CO_2 on 27th June 2019 over a period of 3.5 hours from 11:30 am to 15:00pm. Wooden stakes were placed in the four corners of each mesocosm to ensure that plants would not be damaged and transparent plastic bags were placed over the stakes and sealed onto the rim of the mesocosm pot while labelling. 20 ml of $^{13}CO_2$ were injected into each plastic bag using a syringe, and repeated after 15 minutes. After 30 min, the bags were removed to allow the systems to ventilate and cool for 5 minutes. This same course of 30 minutes of labelling followed by 5 minutes of ventilation was repeated 6 times in total. A total of 260 ml of $^{13}CO_2$ was injected for each mesocosm pot (including one accidental injection of 40 ml).

4.2.3 CO_2 flux and ^{13}C enrichment measurements

CO_2 flux and ^{13}C enrichment measurements were conducted using the N8 mobile GasLab (Gladiss) of the University of Manchester.

Measurements were conducted on 25th and 26th June (to establish a natural abundance baseline before labelling), and again on 27th June, 30 minutes after labelling was completed (day 0), on 28th June (day 1), 30th June (day 3), 1st July (day 4), 2nd July (day 5) and 3rd July (day 6). Simultaneously photosynthetically active radiation (PAR) was recorded using a PAR sensor (Skye Instruments, Powys, UK) and air temperature by the meteorological station of the mobile GasLab.

Net ecosystem exchange was measured using custom-made transparent and opaque flux chambers (Orwin et al., 2014). They were constructed by fitting a frame made from an mesocosm pot with acrylic windows fit on all sides and sealing tape along the edges to

be connected to the planted mesocosms. Ecosystem respiration was measured using the same type of chamber, but darkened with black plastic sheet. These chambers were clipped to the rim of the mesocosm pots during measurements. Soil respiration was measured using a chamber built from drain pipe (6 cm diameter, 30 cm height) that was held against the soil surface by hand during measurements.

For each measurement, the chambers were placed on the mesocosm pot for 4 minutes and connected with tubes to a Picarro isotope analyser G2201-I (Picarro Inc., USA) inside the mobile GasLab in a closed loop gas circuit. The analyser was conducting continuous measurements (on average 1.2 measurements per second) of $^{12}CO_2$ and $^{13}CO_2$ concentration (in ppm) in the chamber. Fluxes were calculated using concentrations from minute 3 and 4 to ensure that the CO_2 had enough time to circulate through the entire measurement system. $^{12}CO_2$ and $^{13}CO_2$ fluxes in g CO_2-C/m^2/h were calculated in R version 3.6.1 (R Core Team, 2019) using a modified version of the the *conc_to_flux* function from the *ecoFlux* package (Shannon, 2018) to account for the different in molar masses of $^{12}CO_2$ and $^{13}CO_2$. The fluxes were calculated as follows:

$$CO_2 flux \left[\frac{\mathrm{g\,C}}{\mathrm{m^2\,h}}\right] = slope * \frac{V_{chamber}}{A_{chamber}} * 10^{-6} * Cf \qquad Eq.\ 1$$

Slope is the CO_2 change in ppm per time calculated by linear regression. $V_{chamber}$ and $A_{chamber}$ are the volume and area of the flux chamber in m^2 and m^3. 10^{-6} is a conversion factor from µg to g. *Cf* is a conversion factor that uses the ideal gas law to convert ppm CO_2 to µg CO_2-C/m^3:

$$Cf = \frac{P * m_{molar}}{R * T} \qquad Eq.\ 2$$

Where *P* is the air pressure (Pa), m_{molar} is the molar mass of C (12 for ^{12}C and 13.003 for ^{13}C), *R* is the ideal gas constant (8.314 $m^3Pa/K/mol$) and *T* is the air temperature

(K). The total CO_2 flux was calculated as the sum of $^{12}CO_2$ and $^{13}CO_2$ flux. The Picarro also put out a value of $\delta^{13}C$ (see Eq. 3 in section 4.2.5) for each concentration measurement and their mean value was calculated corresponding to each flux measurement from minute 3 to 4.

4.2.4 ^{13}C enrichment in leaves, roots and soil

Leaf, root and soil samples were taken before labelling to establish a natural abundance baseline, and after labelling on the same days as flux measurements. On each day, 5 leaves were collected from each species from the monocultures and mixtures. Also, a soil core with 1 cm diameter and 10 cm depth was collected from each pot on each day. On day 0, samples were taken directly after labelling and on the following days at the same hour of day. Leaves and soil cores were immediately frozen at -20 °C and later oven-dried at 60°C to constant mass. Roots were picked out of the dried soil using tweezers. Dried leaves, roots and soil were ball-milled and 4mg analysed in an the Picarro isotope analyser G2201-I (Picarro Inc., USA), coupled with a Picarro combustion module (A0201).

4.2.5 Calculations of ^{13}C excess and pulse-derived ^{13}C per area

The output for leaf, root, soil and respired C isotopic composition was given by the isotope analyser in units of $\delta^{13}C$, which is calculated as follows:

$$\delta^{13}C = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \right) * 1000 \qquad \textit{Eq. 3}$$

Where $\left(\frac{^{13}C}{^{12}C}\right)_{standard}$ is the isotopic ratio of the standard material PDB, which is 0.011237.

From this, the atom % was calculated for each sample, which is the ratio of ^{13}C atoms relative to the total number of C atoms:

$$atom\ \% = \frac{^{13}C}{^{12}C + {}^{13}C} = \frac{100 * \left(\frac{^{13}C}{^{12}C}\right)_{standard} * \left(\frac{\delta^{13}C}{1000} + 1\right)}{1 + \left(\frac{^{13}C}{^{12}C}\right)_{standard} * \left(\frac{\delta^{13}C}{1000} + 1\right)} \qquad Eq.\ 4$$

The ^{13}C excess representing the ^{13}C derived from the pulse was calculated by subtracting the natural abundance atom % from the atom % of samples taken after labelling:

$$^{13}C\ excess\ =\ atom\ \%_{sample} -\ atom\ \%_{baseline} \qquad Eq.\ 5$$

Where $atom\ \%_{baseline}$ was calculated from samples taken before labelling, and $atom\ \%_{sample}$ from samples taken after labelling.

For shoot and roots, the pulse-derived ^{13}C per area allocated to roots and shoots was extrapolated using the ^{13}C excess, the molar mass of ^{13}C ($m_{^{13}C}$), the pot area, biomass dry weight ($m_{biomass}$) and biomass C%:

$$pulse - derived\ ^{13}C\ \left[{}^{g}/_{m^2}\right] =$$

$$\frac{^{13}C\ excess\ * m_{^{13}C}\ * m_{biomass} * C\%_{biomass}}{\left((100 -\ ^{13}C\ excess) * m_{^{12}C} +\ ^{13}C\ excess\ * m_{^{13}C}\right) * pot\ area} \qquad Eq.\ 6$$

The pulse pulse-derived shoot ^{13}C per area in mixtures was calculated as the sum from both species.

4.2.6 Above-ground/root biomass and shoot dry weight

Above-ground biomass was cut at the base on 8th to 10th July, sorted by species, dried it at 65°C for 72 hours and weighed. Shoot dry weight per individual was determined by dividing above-ground biomass dry weight by 36 in monocultures and by 18 in the

mixture. To determine root biomass and community traits, a core with 5.8 cm diameter and 15 cm depth was sampled in the centre of each pot on 13th July. The cores were stored at 4°C until needed for analysis. Roots were carefully washed, oven-dried at 65°C for 48h and weighed.

4.2.7 Statistical analyses

Statistical analyses were conducted in R version 3.6.1 (R Core Team, 2019) and figures were produced using the *ggplot2* package (Wickham, 2016). All analyses consisted of linear mixed effect models using the package *lme4* (Bates et al., 2015). Significance of fixed effects was determined using likelihood ratio testing (LRT), comparing models with and without the variable of interest. In case of significance, Tukey post hoc tests were conducted using the *emmeans* package (Lenth et al., 2020). Variables were $\log_{10}$- or square root-transformed when necessary to fulfil model assumptions.

The effect of treatment on shoot and root biomass as well as on root : shoot ratio was assessed by analysis of variance (ANOVA) using linear mixed effect models, including treatment as a fixed and block as a random effect. The effect of treatment, species and their interaction on individual shoot dry weight was assessed using a mixed effect model that included treatment, species and their interaction as fixed effects and block as a random effect.

The effect of treatment on CO_2 fluxes (ecosystem respiration, net ecosystem exchange, photosynthesis and soil respiration) was assessed by repeated measures ANOVA using linear mixed effect models that included treatment as a fixed effect and mesocosm pot and block as crossed random effects. Photosynthesis was estimated by subtracting ecosystem respiration from net ecosystem exchange. The bare soil treatment was excluded from the analysis. Mesocosm pot was included in all models as a random

effect to account for repeated measures. All models included air temperature as fixed effect and the models for net ecosystem exchange and photosynthesis additionally included PAR fixed effect to account for variability in respiration and photosynthesis due to variability in solar irradiation and air temperature throughout the days and between measurement days.

The time course of ^{13}C excess leaf, root, soil and respiration, as well as the pulse-derived ^{13}C per area was analysed by repeated measures 2-way ANOVA using linear mixed effect models. The models included treatment, timepoint and their interaction as categorical fixed effects and mesocosm pot and block as crossed random effects. Time was analysed as a categorical rather than continuous variable because the evolution of the response variables over time had various non-linear shapes. For the soil samples, three outliers with model residuals below or above three times the interquartile range were removed due to presumed measurement error and to ensure that residual distributions met model assumptions.

4.3 Results

4.3.1 Root and shoot biomass

Shoot and root biomass, as well as root : shoot ratio were significantly affected by the mixture/monoculture treatments ($p<0.05$, Fig. 4.1 a-c – these results are a subset of the results already presented in Chapter 3, Fig. 3.1 and 3.4). Tukey post hoc testing revealed that shoot biomass was significantly higher in the *Plantago* monoculture and in the mixture than in the *Dactylis* monoculture ($p<0.001$, Fig. 4.1), but there was no significant difference between *Plantago* monoculture and mixture ($p>0.05$). Root biomass was on average about 50% higher in the mixture than in the monocultures, however post-hoc Tukey testing revealed that these differences were not statistically

significant (p=0.091 for the contrast between *Dactylis* monoculture and mixture and p=0.073 for the contrast between *Plantago* monoculture and mixture). The dry weight of each individual shoot was, on average, about four times higher in *Plantago* than in *Dactylis* (p<0.001, Fig. 4.1 d), but it did not differ significantly between the monocultures and mixture (i.e. p=0.108 for treatment and p = 0.214 for species x treatment effect).

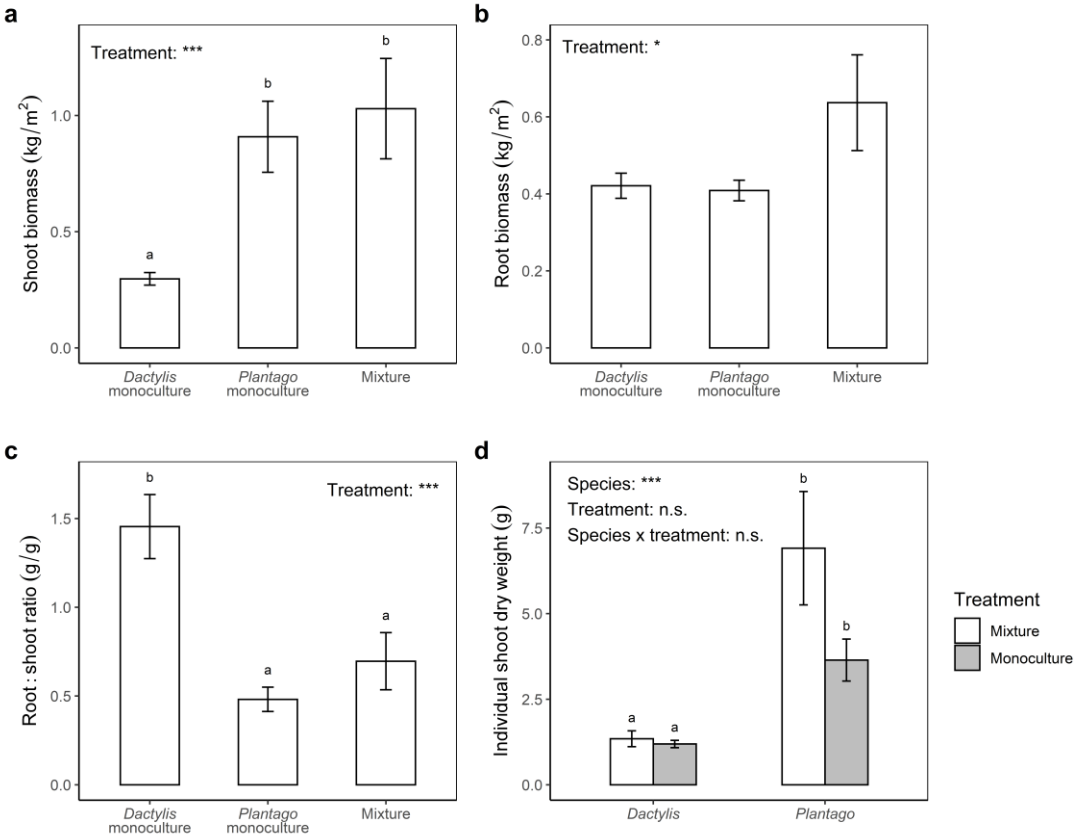

Figure 4.1: Effects of monoculture and mixture treatments on shoot biomass (a), root biomass (b), root : shoot ratio (c) and individual shoot dry weight (d). Data represent mean +/- 1 standard error. Significance of main/interactive effects of treatment (a-d) and species (only d) were assessed using mixed-effect ANOVA and likelihood ratio testing. Significance is indicated as: p < 0.001***, p < 0.01**, p < 0.05*. Different letters indicate significant differences identified by Tukey post hoc test (p < 0.05).

4.3.2 CO_2 fluxes

Ecosystem respiration, net ecosystem exchange and soil respiration were significantly positively affected by air temperature ($p < 0.05$, Fig. 4.3 a, b, e, Table 4.1). Net ecosystem exchange was significantly negatively affected by PAR ($p = 0.001$, Table 4.1, Fig. 4.3 c does not clearly show this negative effect as it is presumably masked by the effect of air temperature). Photosynthesis was significantly negatively affected by PAR ($p < 0.001$, Fig. 4.3 d, Table 4.1), but not by air temperature ($p > 0.05$, Table 4.1). Ecosystem respiration and photosynthesis differed significantly between treatments in analyses excluding the bare soil treatment ($p < 0.05$, Fig. 4.2 a, c, Table 4.1). Tukey post hoc testing revealed that ecosystem respiration was significantly higher in *Plantago* monoculture and mixture than in *Dactylis* monoculture, with no significant difference between *Plantago* monoculture and mixture. Photosynthesis was significantly higher in mixtures than in *Dactylis* monocultures. Net ecosystem exchange and soil respiration were not significantly affected by the treatments ($p > 0.05$, Fig. 4.2 b, d, Table 4.1).

Table 4.1: Main effects of treatment (monocultures vs. mixture), air temperature and photosynthetically active radiation (PAR) on ecosystem respiration, net ecosystem exchange, photosynthesis and soil respiration, tested by likelihood ratio tests (LRT). df - degrees of freedom.

	Treatment			**Air temperature**			**PAR**		
	df	**LRT**	**p**	**df**	**LRT**	**p**	**df**	**LRT**	**p**
Ecosystem respiration	**2**	**16.56**	**<0.001**	**1**	**100.6**	**<0.001**			
Net ecosystem exchange	2	3.479	0.176	**1**	**38.91**	**<0.001**	**1**	**10.46**	**0.001**
Photosynthesis	**2**	**9.040**	**0.010**	1	0.449	0.503	**1**	**24.67**	**<0.001**
Soil respiration	2	1.214	0.545	**1**	**4.057**	**0.044**			

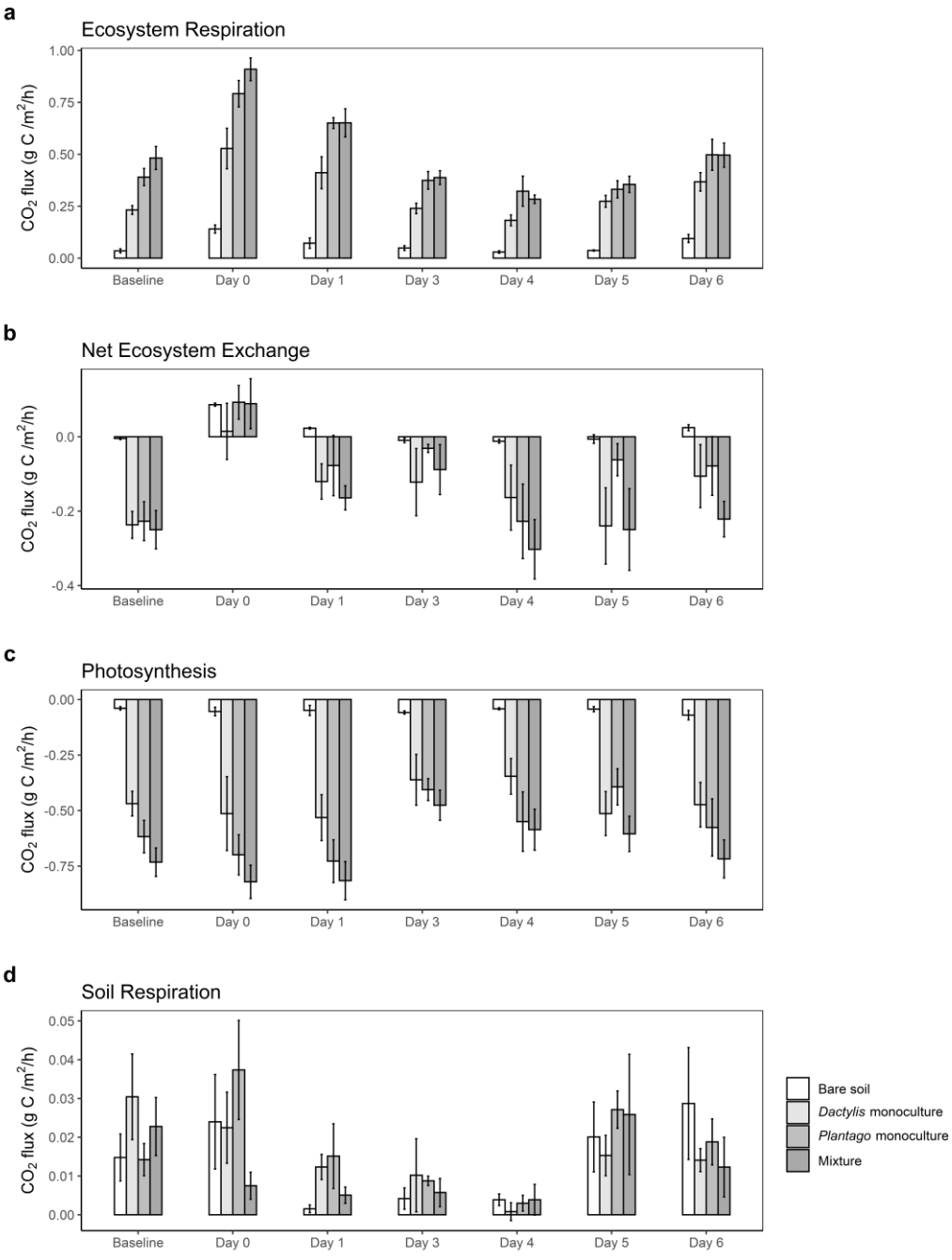

Figure 4.2: Ecosystem respiration (a), net ecosystem exchange (b), photosynthesis (c) and soil respiration (d) in monoculture, mixture and bare soil treatments over the course of the pulse-chase study. Data represent mean +/- 1 standard error. ^{13}C labelling was carried out on day 0 before flux measurements.

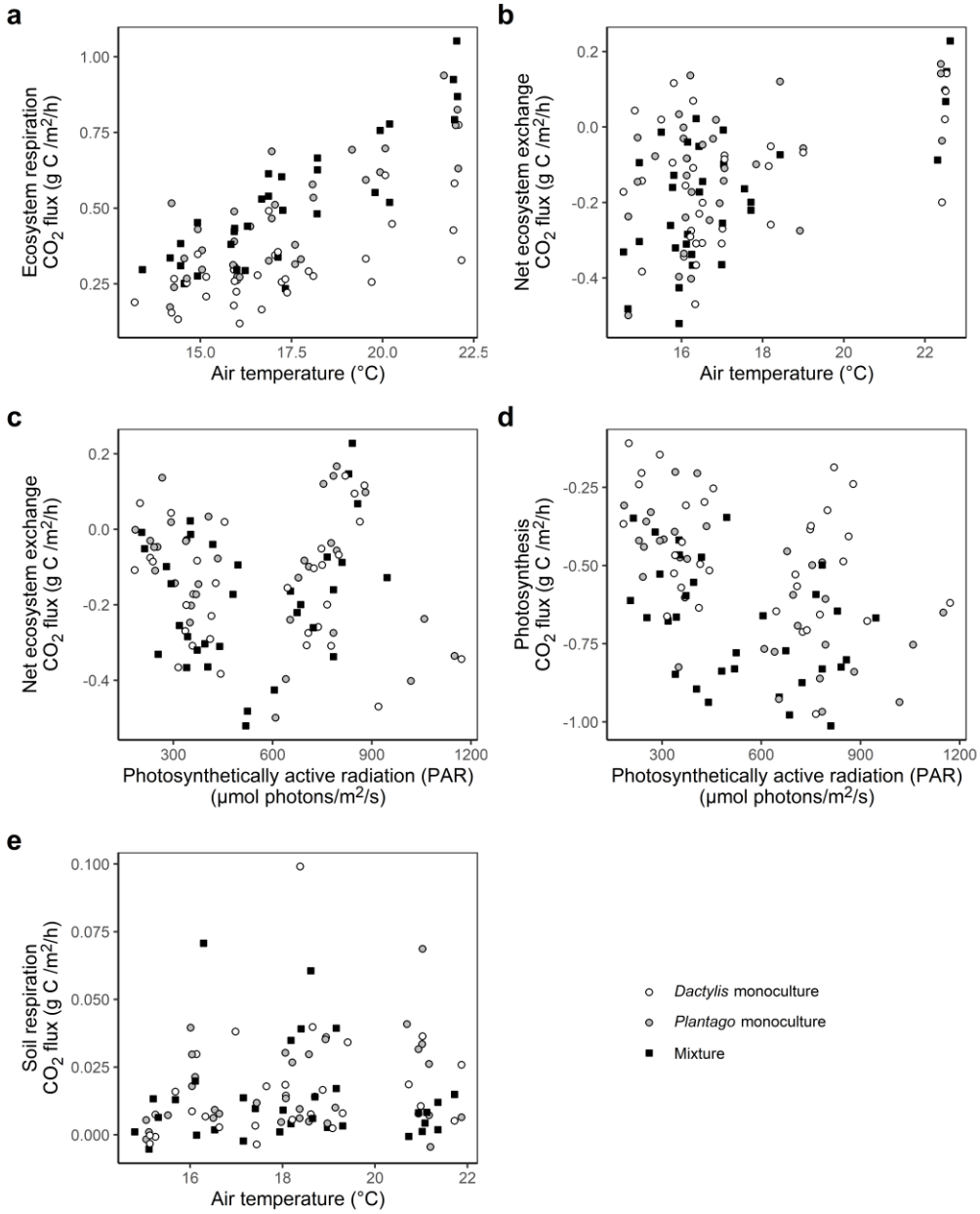

Figure 4.3: Ecosystem respiration (a), net ecosystem exchange (b, c), photosynthesis (d) and soil respiration (e) plotted against the model covariates air temperature (a, b, e) and photosynthetically active radiation (c, d).

4.3.3 Root, leaf and soil ^{13}C excess

^{13}C atom excess was positive in roots, shoots and soil starting from day 0 directly after labelling and also still 6 days afterwards, indicating that ^{13}C tracer was present in all compartments throughout the experiment.

Leaf ^{13}C excess was significantly affected by treatment, sampling timepoint, as well as their interaction ($p < 0.001$, Fig. 4.4 a, Table 4.2). It was generally highest on day 0, directly after labelling, and later gradually decreased. Tukey post hoc testing revealed that leaf ^{13}C excess was significantly higher on day 0 than on the other days ($p < 0.05$), with no statistically significant differences between the other days. Overall, ^{13}C excess was higher in *Plantago* leaves than in *Dactylis* leaves ($p < 0.05$), but there was no significant difference between mixtures and monocultures. On day 0, leaf ^{13}C excess was highest in *Dactylis* leaves growing in monoculture, significantly higher than in *Dactylis* growing in mixture and *Plantago* growing in monoculture ($p < 0.05$). From day 0 to day 1, ^{13}C excess in *Dactylis* declined by 77% in monoculture and by 75% in mixture. On day 1, leaf ^{13}C excess was highest in *Plantago* growing in monoculture, significantly higher than in all other treatments ($p < 0.05$). From day 0 to day 1, ^{13}C excess in *Plantago* declined by 36% in monoculture and by 60% in mixture. On day 3-5, leaf ^{13}C excess was higher in *Plantago* than in *Dactylis* ($p < 0.05$), with no significant difference between mixtures and monocultures. On day 6 there were no significant differences between any of the treatments ($p>0.05$).

Root ^{13}C excess was significantly affected by sampling timepoint ($p = 0.006$) and by the interaction between treatment sampling timepoint ($p < 0.001$, Fig. 4.4 b, Table 4.2). Overall, root ^{13}C excess was significantly lower on day 0 than on the other days ($p < 0.05$), with no statistically significant differences between the other days. The time

course of root ^{13}C excess differed between all treatments. In the *Dactylis* monocultures, there was a sharp increase of over 100% from day 0 to day 1 ($p < 0.01$), to twice the level found in the other treatments ($p > 0.05$). After day 1 it tended to decrease and on day 4-6 was significantly lower than on day 1 ($p < 0.05$). In the *Plantago* monocultures and in the mixtures, root ^{13}C excess tended to increase slowly from day 0 to day 6 with some variability throughout the time course. On day 4 and 6 it was significantly higher than on day 0 ($p < 0.05$) in the *Plantago* monocultures, and on day 3 and 6 in the mixtures ($p < 0.05$).

Soil ^{13}C excess was significantly affected by treatment, sampling timepoint and their interaction ($p < 0.01$, Fig. 4.4 c, Table 4.2). It was overall higher in the *Dactylis* monocultures than in the *Plantago* monocultures ($p < 0.05$) and overall higher on day 1 than on day 0 and day 6 ($p < 0.05$). The time course of soil ^{13}C excess differed between the three treatments. In *Dactylis* monocultures, as for root ^{13}C excess, there was a sharp increase in soil ^{13}C excess of over 100% from day 0 to day 1 ($p < 0.01$), when it was about twice as high as in the other treatments ($p < 0.01$). After that it decreased, with all other timepoints being significantly lower than day 1 ($p < 0.05$). In the *Plantago* monocultures there were no significant differences between timepoints ($p > 0.05$). In the mixtures, soil ^{13}C excess increased from day 0 up to a peak on day three and then decreased up to day 6 and on day 3 the soil ^{13}C excess was significantly higher than on day 0 and day 6 ($p < 0.05$).

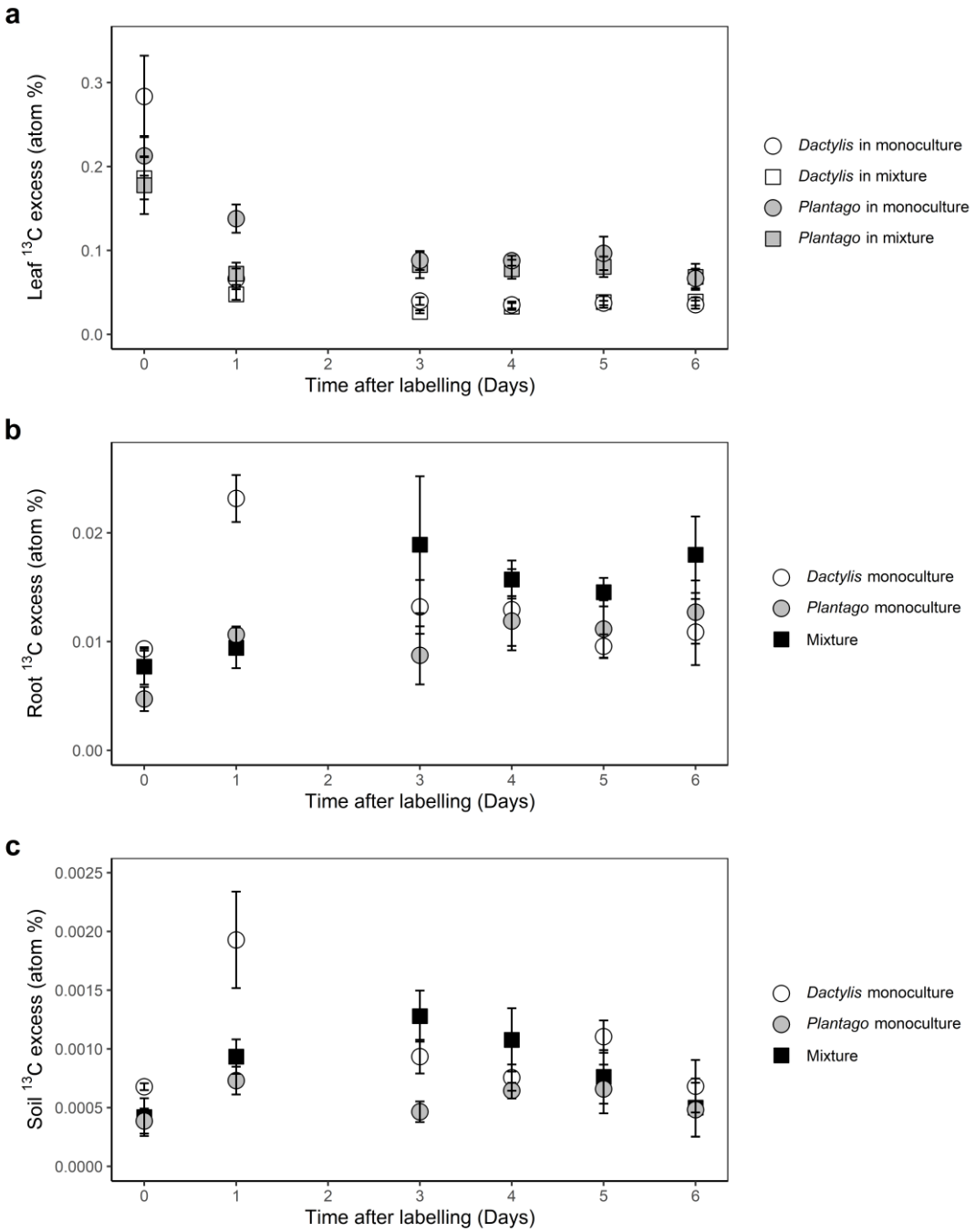

Figure 4.4: Time course of excess ^{13}C content in shoots (a), roots (b) and soil (c) in monocultures and mixture. Data represent mean +/- 1 standard error.

Table 4.2: Main and interactive effects of treatment (monocultures vs. mixture) and sampling timepoint on ^{13}C excess in shoots, roots, soil and ecosystem/soil respiration and on pulse-derived ^{13}C allocation in shoots, roots and their ratio per area, tested by likelihood ratio tests (LRT). df - degrees of freedom.

		Treatment			**Timepoint**			**Treatment x timepoint**		
		df	**LRT**	**p**	**df**	**LRT**	**p**	**df**	**LRT**	**p**
^{13}C excess	**Shoots**	**3**	**37.02**	**<0.001**	**5**	**125.2**	**<0.001**	**15**	**50.43**	**<0.001**
	Roots	2	4.817	0.089	**5**	**16.16**	**0.006**	**10**	**29.61**	**<0.001**
	Soil	**2**	**12.25**	**0.002**	**5**	**19.79**	**0.001**	**10**	**23.56**	**0.009**
	Ecosystem respiration	2	3.574	0.168	**5**	**216.8**	**<0.001**	**10**	**18.81**	**0.042**
	Soil respiration	2	5.379	0.068	**5**	**141.7**	**<0.001**	10	17.14	0.071
pulse-derived	**Shoots**	**2**	**23.03**	**<0.001**	**5**	**73.37**	**<0.001**	**10**	**44.53**	**<0.001**
^{13}C per area	**Roots**	**2**	**10.41**	**0.005**	**5**	**16.85**	**0.005**	**10**	**22.56**	**0.013**
	Root : shoot ratio	**2**	**29.60**	**<0.001**	**5**	**81.94**	**<0.001**	10	13.09	0.219

4.3.4 ^{13}C excess in respired CO_2 from ecosystem and soil

^{13}C excess in ecosystem respiration was significantly affected by sampling timepoint ($p < 0.001$, Fig. 4.5 a, Table 4.2) and by the interaction between treatment sampling timepoint ($p = 0.042$). In all treatments, ^{13}C excess was highest on day 0 and then decreased up to day 4, when it levelled off. Tukey post hoc testing revealed overall significant differences between all days from day 0 to 4 ($p < 0.01$), but no significant differences between day 4, 5 and 6 ($p > 0.05$). Treatments only differed significantly on day 6, when the ^{13}C excess was significantly higher in the mixtures than in the *Dactylis* monocultures.

^{13}C excess in soil respiration was significantly affected only by sampling timepoint ($p < 0.001$, Fig. 4.5 b, Table 4.2). In all treatments, ^{13}C excess was highest on day 0 and then decreased up to day 3, when it levelled off. Tukey post hoc testing revealed overall significant differences between all days from day 0 to 4 ($p < 0.01$), but no significant differences between day 4, 5 and 6 ($p > 0.05$). The effects of treatment and its interaction with timepoint were non-significant, but not by far ($p = 0.068$ for treatment and $p = 0.071$ for timepoint x treatment). *Dactylis* monocultures had on average slightly higher soil respiration than *Plantago* monocultures and mixtures, especially on day 0 and 1.

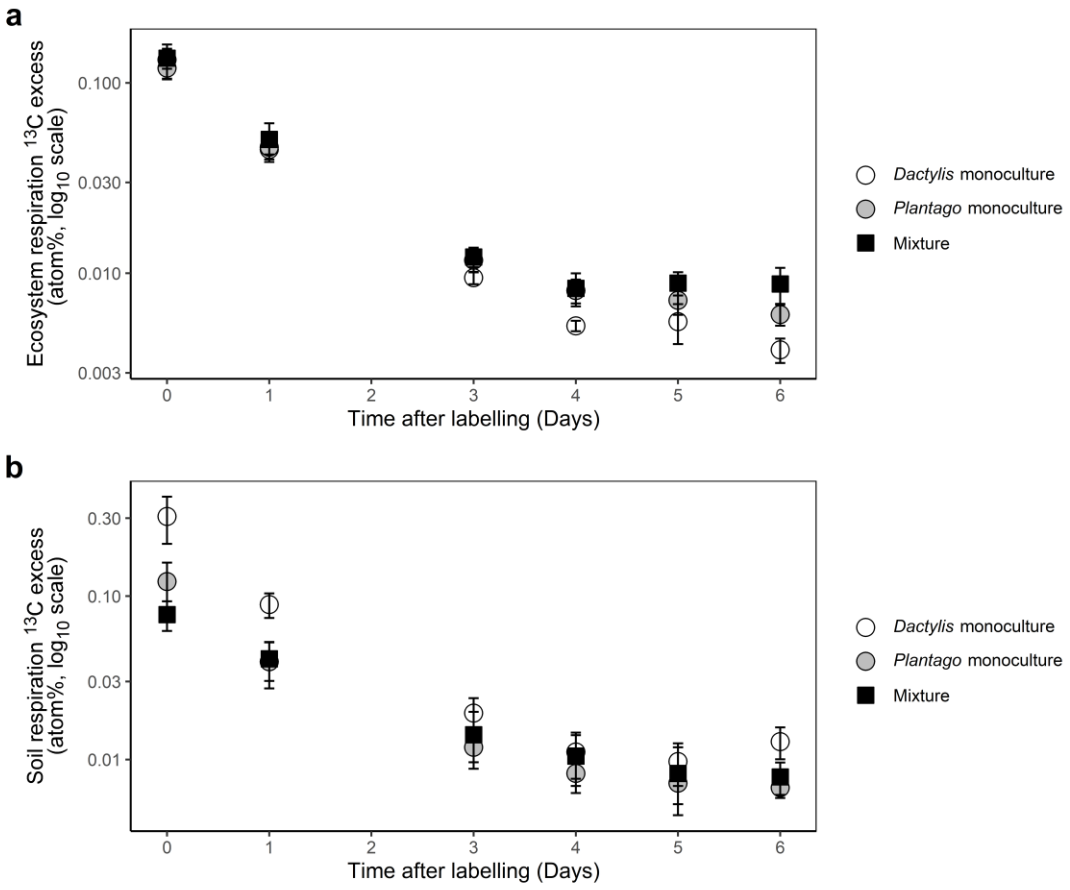

Figure 4.5: Time course of excess ^{13}C content in ecosystem- (a) and soil respiration (b) in monocultures and mixture. Data represent mean +/- 1 standard error. The y-axis is plotted logarithmically (log_{10}).

4.3.5 Pulse-derived ^{13}C allocated to shoots and roots

The pulse-derived shoot ^{13}C per area was significantly affected by treatment, timepoint and their interaction ($p =< 0.001$, Fig. 4.6 a, Table 4.2). Tukey post hoc testing revealed that overall, the pulse-derived shoot ^{13}C was higher on day 0 than on all other days and higher on day 1 than on day 6 ($p < 0.01$). Overall, it was higher in the *Plantago* monoculture and the mixture than in the *Dactylis* monocultures ($p < 0.005$), with no significant difference between the *Plantago* monocultures and mixtures. These differences were also found on each individual day ($p < 0.005$) except on day 0, when there was no significant difference between any of the treatments ($p > 0.13$). The time course of pulse-derived shoot ^{13}C differed slightly between treatments. In the *Plantago* monocultures and the mixtures, it was significantly higher on day 0 than on the other days ($p < 0.05$), with no significant differences between the other days. Also, in the *Dactylis* monoculture, pulse-derived shoot ^{13}C was significantly higher on day 0 than on the other days ($p < 0.05$), but additionally it was significantly higher on day 1 than on day 4 and 6 ($p < 0.05$).

The pulse-derived root ^{13}C per area was significantly affected by treatment, timepoint and their interaction ($p =< 0.05$, Fig. 4.6 b, Table 4.2). Overall, it was significantly higher in the mixtures than in both types of monocultures ($p < 0.05$), with no significant difference between *Plantago* and *Dactylis* monocultures ($p = 0.99$). It was also overall significantly higher on day 1 and 6 than on day 0 ($p < 0.05$) with no significant differences between the other days ($p > 0.05$). Considering each day individually, the mixtures had a higher pulse-derived root ^{13}C than the monocultures only on day 4-6 ($p < 0.05$), but not before ($p > 0.05$). *Dactylis* monocultures had no significant variation between days ($p > 0.05$). In *Plantago* monocultures the pulse-derived root ^{13}C was

significantly higher on day 6 than on day 0 ($p < 0.05$) and in mixtures it was significantly higher on day 1 and 6 than on day 0 ($p < 0.05$).

The root : shoot ratio of pulse-derived ^{13}C was significantly affected by treatment and timepoint ($p < 0.001$, Fig. 4.6 c, Table 4.2), but not their interaction. All three treatments differed significantly ($p < 0.005$). The root : shoot ratio of pulse derived ^{13}C was highest in *Dactylis* monocultures, followed by mixtures and lowest in *Plantago* monocultures. It was also lower on day 0 than on all other days ($p < 0.001$) and lower on day 1 than on day 6 ($p < 0.05$).

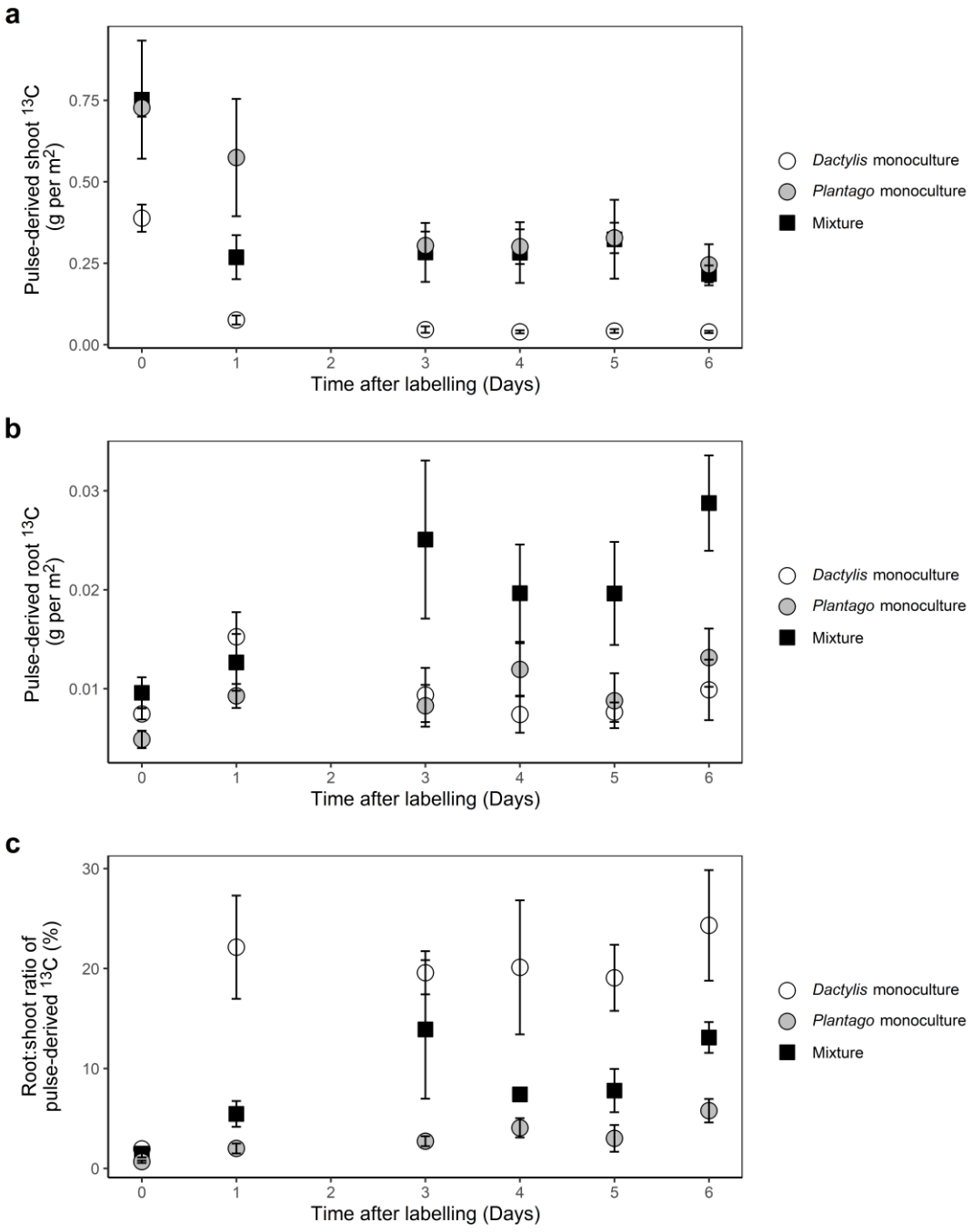

Figure 4.6: Time course of pulse-derived ^{13}C allocation per m^2 in shoots (a) and roots (b) and the root : shoot ratio of pulse-derived ^{13}C allocation (c) in monocultures and mixture. Data represent mean +/- 1 standard error

4.4 Discussion

The aim of this study was to investigate if the interaction between two grassland species has an effect on short-term C cycling. *Dactylis* and *Plantago* monocultures showed significant differences in C dynamics. *Plantago* assimilated more ^{13}C and allocated more to leaf biomass, while *Dactylis* allocated more ^{13}C to root biomass and rhizodeposition. In mixtures, almost twice as much ^{13}C was allocated to root biomass than in either monocultures. Additionally, the temporal pattern ^{13}C allocated to roots and soil differed between mixtures and monocultures. These findings suggests that the interaction between *Plantago* and *Dactylis* altered the fate of recently assimilated C compared to their monocultures with implications for the role of plant community composition on grassland C dynamics.

4.4.1 General patterns of short-term C dynamics

In all treatments, leaf ^{13}C excess was highest shortly after labelling, then rapidly declined within the first 24 hours, with much smaller or no declines in the following days (Fig. 4.4 a). This quick translocation is typical for grassland vegetation (De Deyn et al., 2011; de Deyn et al., 2012; Karlowsky et al., 2018). There was also a fast measurable allocation of ^{13}C into roots, soil and respired CO_2, clearly detectable in all compartments 4 hours after the start of pulse-labelling (Fig. 4.4 and 4.5). Root and soil ^{13}C excess increased with a time lag compared to the leaves, which is also consistent with other studies on grassland vegetation (De Deyn et al., 2011; Karlowsky et al., 2018). ^{13}C excess in respired CO_2 was also highest shortly after labelling, and then decreased first rapidly, then progressively more slowly over the measurement period (Fig. 4.5).

4.4.2 Comparison of short-term C dynamics in *Dactylis* and *Plantago* monocultures

Plantago retained more ^{13}C in leaves than *Dactylis*, both per unit of leaf biomass (Fig. 4.4 a) and in total above-ground biomass (Fig. 4.6 a). This is consistent with its faster growth rate and higher shoot biomass (Fig. 4.1 a). *Dactylis* had higher translocation below-ground and higher root : shoot ratio in allocation of ^{13}C (Fig, which is consistent with its much higher root : shoot ratio in biomass (Fig. 4.1 c). ^{13}C excess in roots and soil of *Dactylis* peaked one day after labelling. De Deyn et al. (2011) found in a grassland pulse chasing experiment that ^{13}C enrichment in arbuscular mycorrhizal fungi, as well as bacteria and saprophytic fungi peaked 24h after labelling. Consequently, the peak measured in *Dactylis* is likely due to ^{13}C being allocated to root exudates (which are then quickly consumed by microbes) and/or to mycorrhizal fungi associated to the roots, which is subsequently in part released back to the atmosphere through microbial respiration. In *Plantago* there was no visible peak in root or soil ^{13}C excess (Fig. 4.4 b and c). This is consistent with results from a study of Mediterranean grassland species, where grasses released more root exudates than forbs (Warembourg et al., 2003). However, it is possible that a peak occurred between the sampling timepoints, especially as there was no sampling on day 2 after labelling.

4.4.3 Comparison of short-term C dynamics between monocultures and mixture

In both species, the ^{13}C excess retained in leaves towards the end of the sampling period did not differ between monocultures and the mixture (Fig. 4.4 a). However, *Dactylis* leaves showed higher ^{13}C excess in monoculture than in mixture on day 0, directly after labelling. This is likely due to the lower above-ground biomass in *Dactylis* monoculture compared to the mixture (Fig. 4.1 a), which means there was less shading and less photosynthesis (Fig 4.2 c) reducing the levels of $^{13}CO_2$ in the system during labelling.

The difference did not persist beyond day 0, so this excess did not get incorporated in leaf tissue, but translocated quickly to other parts. In *Plantago*, the initial leaf ^{13}C excess did not differ between monoculture and mixture, but it declined more quickly in the mixture than in the monoculture (60% decline in mixture and 36% decline in monoculture from day 0 to day 1). Similarly, de Deyn et al. (2012) observed increased translocation from leaves in 6-species mixture compared to monoculture in some species of a grassland mesocosm experiment.

The most pronounced difference between monocultures and mixtures was that in mixtures about twice as much ^{13}C was retained in roots compared to both monocultures (Fig. 4.6 c), consistent with the higher root biomass found in mixtures compared to monocultures (Fig. 4.1 b). It has frequently been observed that diverse systems have higher root biomass than monocultures (e.g. Tilman et al., 2001). The increased below-ground allocation of recently assimilated C caused by species interactions found here could be a mechanism contributing this. However, it is not possible to tell if the increased below-ground allocation was caused by *Dactylis*, *Plantago*, or both species in the mixture. The faster translocation observed in *Plantago* leaves in the mixture compared to monoculture (Fig. 4.1 a) suggests that *Plantago* may have contributed. There are several possible reasons for the increased below-ground C allocation in mixture. One possibility is that there might have been more intense competition for water and nutrients in the mixture. However, the levels of soil moisture and plant available nitrogen were similar in the mixture and the *Plantago* monoculture (see Chapter 3, Fig. 3.8). Another possible mechanism is that the two species exhibited vertical niche differentiation, which could have led to a higher overall root biomass (Mommer et al., 2010). Additionally, there may have been reduced root biomass production in monocultures due to pathogens (Hendriks et al., 2013)

Root and soil ^{13}C peaked on day 1 in *Dactylis* monoculture, but on day 3 in the mixture. This could be due to the higher soil moisture in *Dactylis* monocultures (see Chapter 3, Fig. 3.8), as low soil moisture can decrease rates of root exudation (de Vries et al., 2019).

4.4.4 Conclusion

The results of this study show that interactions between species have the potential to alter grassland ecosystem C cycling and soil C storage. Here, mixtures showed an increased allocation of photosynthate C to roots, which likely leads to increased soil C storage in the longer term (Rasse et al., 2005). Also, species interactions altered temporal patterns of rhizodeposition. The underlying mechanisms for this and consequences for soil C storage require further investigation.

Importantly, only subtle effects of species interactions on short-term C cycling were measured above-ground, while much stronger effects could be observed below-ground. This highlights the need for detailed study of plant species and functional type effects on belowground processes to understand the effect of vegetation on soil C, rather than focussing only on above-ground vegetation properties.

5 Does drought-induced plasticity of root and shoot traits alter their decomposability?

Abstract

Drought has been shown to induce plastic changes in a range of plant root and shoot traits. These same traits have been shown to explain differences in root and shoot litter decomposability between species. However, it has not been studied whether drought-induced plasticity of root and shoot traits alters their decomposability accordingly. To investigate this, a grass, a forb and a legume common to European temperate grasslands were grown in the greenhouse and subjected to a 5-week moderate drought treatment. Root and shoot traits of the droughted plants were compared to well-watered controls to determine drought-induced trait plasticity. A decomposition assay of the senesced root and shoot material was conducted over 16 weeks, with mass loss measurements at 5 timepoints, to determine the effect of drought on litter decomposability. Drought had significant and sometimes strong effects on many morphological and chemical shoot and root traits of all three species, such as leaf and root dry matter content and specific leaf area, sometimes of similar magnitude as trait differences between species. Litter

decomposition was best described by an asymptotic exponential model including a labile litter fraction decomposing at a rate k and a residual litter fraction with a decomposition rate of zero. Drought had effects on litter decomposability in two species, accelerating decomposition of the labile fraction and either increasing or decreasing the residual litter fraction. This could have important implications for ecosystem carbon and nitrogen cycling. However, drought effects on litter decomposability were fewer and weaker than on plant traits, which suggests that these plant traits may not be indicative of drought-induced changes in decomposability within species.

Keywords: Plant functional traits, litter decomposition, intraspecific trait variation, plasticity, drought, plant functional groups

5.1 Introduction

Drought has become more frequent globally in recent years and model predictions show that the frequency and duration of drought are likely to increase as climate change proceeds (Dai, 2013). Ecosystem carbon (C) and nitrogen (N) cycles are affected by drought in a number ways, one of them is through its effect on vegetation. Plant traits have been increasingly used to better understand both the response of vegetation to environmental variation, such as droughts, and the effect of vegetation on ecosystem functions, such as for example litter decomposition and C and N cycling (Lavorel & Garnier, 2002; Funk et al., 2017). In many of these studies traits related to the 'resource economic spectrum' are of central importance, with trade-offs between a resource-acquisitive and a resource-conservative strategy (Reich, 2014).

Drought can alter plant community traits by affecting community composition and structure with some species being more susceptible to drought than others (Fry et al.,

2013). Additionally, drought can induce trait changes within species due to phenotypic plasticity, which is the ability of a single genotype to produce different forms and physiologies depending on environmental conditions (Sultan, 2000). Phenotypic plasticity can in turn have effects on C and N cycling (de Vries et al., 2016).

Plant species have evolved traits that allow them to cope with drought through avoidance and/or tolerance strategies (Lambers et al., 2008). Plant trait phenotypic plasticity as an adaptation to drought can amplify these strategies (de Vries et al., 2016). Plants with an avoidance strategy increase water uptake and/or reduce losses so that they remain hydrated, for example: (i) by growing more resource-acquisitive fine roots that improve water uptake, which may lead to an increase in specific root length (SRL) (Padilla et al., 2013), (ii) by growing more resource-conservative shoot and root tissues with thicker cell walls and higher lignin content (Fort et al., 2013); (iii) by accumulating non-structural carbohydrates (NSC) in shoots and roots lowering plant tissue osmotic potential (Zwicke et al., 2015), (iv) by increasing root to shoot ratio (Poorter et al., 2012); or (v) by adjusting water use efficiency and stomatal conductance (Klein, 2014). Both (ii) and (iii) could cause a reduction in specific leaf area (SLA), SRL and leaf and root N content, and an increase in leaf and root dry matter content (LDMC/ RDMC) and tissue C content. Plants with a tolerance strategy have the capacity to re-grow after drought, which can be achieved by accumulating osmoprotectant NSC in tissues that protect the plant against cell damage and facilitate re-growth (Zwicke *et al.*, 2015). On the other hand, intense water stress may inhibit plant growth to such a degree that plant trait plasticity is mostly related to reduced growth, rather than being an adaptation to drought. In this case, drought may lead to higher SLA, lower LDMC/RDMC and higher tissue N content (de Vries et al., 2016). In summary, drought can induce phenotypic

trait plasticity in different directions, depending on the plant's drought strategy and on the severity of the drought stress.

Changes at the species level can affect several ecosystem processes, including litter decomposition. Litter decomposition is an important component of ecosystem C and N cycling, as it influences nutrient availability for plants and microbes and affects microbial community composition and C storage (De Deyn et al., 2008). Rates of litter decomposition depend on a number of interacting factors, including litter quality, climate, soil conditions and decomposer communities. However, results from a global meta-analysis of decomposition experiments show that the influence of litter quality is larger than the influence of climatic variation (Cornwell et al., 2008). Litter contains a large variety of chemical compounds and studies have shown that many of them play important roles in explaining differences in root and shoot decomposition rates between plant species. For example, a set of chemical traits including total N, cellulose, lignin and a range of NSC predicted differences in leaf litter decomposition between 10 grassland species (Gunnarsson et al., 2008). Also, plant secondary metabolites have been shown to explain differences in litter decomposability between species (Chomel et al., 2016). The trajectory of mass loss during the course of litter decomposition is often approximated by an exponential decay function (Olson, 1963), however this model does not always fit the data (Adair et al., 2010). Models including several phases throughout the time course of decomposition controlled by different chemical compounds can sometimes better explain trajectories of mass loss (Loranger et al., 2002). These models include an initial phase controlled by easily degradable, soluble compounds (such as NSC), followed by a mid-stage that is controlled by cellulose content and a late-stage that is controlled by recalcitrant compounds such as lignin.

Despite this complexity, a considerable part of the variation in shoot and root litter decomposability between species can often be linked to easily measurable, integrative traits of live plants. Even though litter chemical traits differ from fresh plant chemical traits due to nutrient resorption (Quested et al., 2003; Orwin et al., 2010), litter decomposability has been linked to live plant traits in many studies, as predicted by the afterlife hypothesis (Grime & Anderson, 1986). For example, plant traits related to the leaf economic spectrum, such as LDMC and leaf nitrogen content (LNC) have been found to correlate with decomposability across communities and species (Fortunel et al., 2009; Kazakou et al., 2009; Bumb et al., 2018). Root decomposability is also likely to be linked to traits, however fewer studies have been conducted with roots than with leaves. For example, in Mediterranean herbaceous species fine root decomposability was related to root chemical traits (phosphorus, NSC and hemicellulose), but not to morphological traits (Birouste et al., 2012). In contrast, tree root decomposition was correlated with root diameter, root hemicellulose and NSC, but not with root lignin (Hobbie et al., 2010).

These studies demonstrate that drought can induce phenotypic changes in the same traits that are correlated to differences in decomposition rates between species, such as content of lignin, cellulose and NSC, LDMC/RDMC and root diameter. However, it is unknown if plant phenotypic plasticity in response to drought affects litter decomposability in the way that these studies between species would suggest.

The aim of this study was to investigate whether drought-induced plasticity of root and shoot traits of grassland species alters their decomposability. Research has shown that in grasslands, functional group classification in grasses, forbs and legumes can help to understand ecosystem dynamics, as they differ in traits (Tjoelker et al., 2005), drought

response (Fry et al., 2013; Mackie et al., 2019) and effects on ecosystem functions (Fornara et al., 2009; Allan et al., 2013). To explore drought effects on traits and litter decomposability in all of these functional groups, a grass, a forb and a legume were grown in the greenhouse and subjected to a 5-week experimental drought. At the end of the drought, a range of shoot and root traits were measured and a decomposition assay of shoot and root senesced material was conducted. The following hypotheses were tested:

1. The effects of drought on shoot and root traits vary between grassland plant functional groups.
2. Drought affects root and shoot litter decomposability due to its effect on traits.

5.2 Methods

5.2.1 The drought experiment

Three species common to European temperate grasslands were selected for the experiment, which belong to different functional groups and are functionally distinct with different above-below ground growth forms: *Lolium perenne*, a fructan-accumulating grass, *Plantago lanceolata*, a rhizomatous forb and *Trifolium repens*, a shallow-rooted, stoloniferous N-fixing legume.

Plants were grown in the greenhouse at 16h light/8h dark. Seeds (Emorsgate Seeds, King's Lynn, Norfolk, UK) were germinated in plug trays using mesotrophic grassland soil collected at Hazelrigg field station (soil characterization see de Vries et al. (2018)) which had been sieved to 1 cm. After 2 weeks, seedlings were transplanted into monoculture pots with 7 individuals per pot. Each pot was built out of drain pipe with mesh at the bottom, 45 cm high and with 18 cm diameter. Each of the pots was filled

with a layer of chippings (1 kg) and 10 kg of field-moist (55% water holding capacity (WHC)) Hazelrigg soil.

The experiment was set up in a fully factorial block design. For each of the 3 species, 5 replicate pots were set up for the well-watered treatment and 5 pots for the drought treatment. This resulted in 30 pots in total. During the first five weeks all pots were watered evenly 3-4 times a week. During the following five weeks well-watered pots were kept at 60% WHC and droughted pots at 40% WHC adjusting gravimetrically 3-4 times a week. These WHC are comparable to previous drought experiments (de Vries et al., 2016; Lozano et al., 2020). The relatively mild drought at 40% WHC was chosen to allow the plants to adjust plastically while not wilting.

At the end of the growth period, when the plants were 12 weeks old, morphological root and shoot traits were measured (see section 3.2.2 below). Then, watering was stopped to allow plants to senesce for 2 weeks in the greenhouse. Senesced shoots from each pot were cut at the base, oven-dried at 40 °C for 48 hours, cut into pieces of max. 4 cm length and homogenized within the sample. Senesced roots were collected by removing the entire soil mass from the pot, working it gently with gloved hands and a rubber mallet and shaking it, no washing was necessary. Senesced roots were oven-dried at 40 °C for 48 hours, cut into pieces of max. 4 cm length and homogenized within the sample.

5.2.2 Trait measurements

Fresh plant traits were measured at the end of the growth period on one randomly selected individual in each pot following standard protocols (Pérez-Harguindeguy et al., 2013). Leaf area (LA), leaf length, SLA, LDMC were measured on five mature leaves per individual. For this, leaves were scanned using an EPSON flatbed scanner and leaf area was analysed using the software WinRhizo (Regent Instruments Inc., Sainte-Foy-

Sillery-Cap-Rouge, QC, Canada). Fresh leaves were weighed and leaf length was measured with a ruler. Leaf and shoot dry weight were determined after drying for 48 hours at 65 °C.

For root trait measurements, a soil core of 3 cm diameter and to full depth of the pot surrounding the harvested individual was taken in each pot. Roots were washed, scanned, weighed and dried at 65 °C for 48 hours to determine root dry biomass, SRL, root diameter, root tissue density (RTD) and RDMC. Roots were scanned using an EPSON flatbed scanner and scanned images were analysed using WinRhizo.

Senesced root and shoot materials were weighed and the number was divided by 6 to obtain senesced shoot and root dry weight, as there were 6 remaining individuals in each pot.

Chemical traits were measured on senesced material, as this may be more closely related to litter decomposability than chemical traits of fresh material. A dried sub-sample of litter from each shoot and root sample was ground in a ball mill and 3g (for shoots) or 4g (for roots) were used to analyse C and N content in an elementar analyser (EA 1108, Carlo Erba Instruments, Milan, Italy). Cellulose, hemicellulose, lignin and fibre content as well as dry matter digestibility were analysed using near infrared reflectance spectroscopy (NIRS), following the method outlined by Bumb et al. (2016). For this, duplicated sub-samples of root and shoot litter material were ground in a knife-mill and placed in ring cells equipped with quartz glass. Reflectance spectra were collected using a FOSS NIRSystem 6500 spectrometer (FOSS NIRSystems, Silver Spring, MD, USA) operating at 400–2500nm to produce an average spectrum with 870–1013 data points. Existing calibrations at CIRAD (French International Centre of Agricultural Research for Development) between the spectral properties and the measured chemical traits were

updated and adapted by conducting reference measurements of chemical traits on 12 samples. For this, cellulose, hemicellulose, lignin and fibre content were measured using the Van Soest method (Van Soest et al., 1991). Dry matter digestibility was measured by the pepsin-cellulase method (Aufrère et al., 2007). Calibration was performed using modified partial least square regression with the software WINISI (Version 4, Infrasoft International, Port Matilda, PA, USA).

5.2.3 Litter decomposition assay

The litter decomposition assay followed an approach developed by Wardle et al. (1998). For each assay, a petri dish was filled with 30 g field-moist Hazelrigg soil that had been sieved to 2 mm. The soil was covered with a circle of nylon mesh (1mm), that was cut to the same diameter as the petri dish and a 0.5 g sample of dried senesced plant material was spread out on top. To allow for destructive harvesting over time, 5 sub-samples of 0.5 g senesced shoot material were taken per plant pot and placed in individual petri dishes to be incubated for 2, 4, 8, 12 or 16 weeks. For *Lolium* and *Plantago*, 5 sub-samples of 0.5 g senesced root material were taken per plant pot and placed in petri dishes to be incubated for 2, 4, 8, 12 or 16 weeks. *Trifolium* had less root material, so only two sub-samples were taken to be incubated for 4 or 16 weeks.

This resulted in:

(5 timepoints x 5 replicate pots x 5 litter types (3 species for shoots and 2 species for roots)) + (2 timepoints x 5 replicate pots x 1 litter type (*Trifolium* roots)) = 270 petri dishes.

Petri dishes were sealed with electrical tape leaving a small gap to allow air circulation and incubated at 15 °C (the mean summer month temperature in Hazelrigg 2008-2018)

in the dark. Once a month, moisture was re-adjusted gravimetrically with sterile deionized water. At each destructive sampling (2, 4, 8, 12, 16 weeks) remaining litter was collected with tweezers, dried at 65°C for 48 hours and weighed.

5.2.4 Statistical analyses

Statistical analyses were conducted in R version 3.6.1 (R Core Team, 2019), and figures were produced using the *ggplot2* package (Wickham, 2016).

Plant traits

In order to test hypothesis 1, species-differences and drought-effects on all plant traits were determined using two-way ANOVA. Trait data was $\log_{10}$-transformed where necessary to fulfil model assumptions. Pairwise comparisons of significant effects were assessed using Tukey post hoc tests.

In order to compare magnitudes and directions of drought-effects on different plant traits, the log response ratios (LRR) were computed for each trait *x*:

$$LRR_x = \ln\left(\frac{\bar{x}_{drought}}{\bar{x}_{well-watered}}\right) \qquad Eq.\ 1$$

Where $\bar{x}$ is the mean of trait *x*. The LRR was chosen as a measure as it standardizes drought effects on different traits to the same unit, and also separates positive and negative drought effects on a comparable scale. For example, if $\bar{x}_{drought}$ is twice as high as $\bar{x}_{well-watered}$, the LRR is 0.69, while if $\bar{x}_{drought}$ is half as high as $\bar{x}_{well-watered}$, the LRR is -0.69.

The standard error $SE_{LRR\,x}$ of the LRR of each trait *x* was computed as:

$$SE_{LRR\,x} = \sqrt{\frac{(SE_{x\,drought})^2}{N_{drought}\,\bar{x}_{drought}} + \frac{(SE_{x\,well-watered})^2}{N_{well-watered}\,\bar{x}_{well-watered}}} \qquad Eq.\ 2$$

Where N is the number of samples in each treatment and SE_x is the standard error of the mean.

Litter decomposition

Models were fitted to the data of remaining litter mass after 2, 4, 8, 12 and 16 weeks using nonlinear least squares regression. This was done for shoot and root litter from each pot separately using a modified version of the code provided by Adair, Hobbie and Hobbie (2010). First, a simple exponential model (Olson, 1963) was fitted:

$$M(t) = M_0 e^{-kt} + \varepsilon \qquad Eq.\ 3$$

where $M(t)$ is remaining litter mass at time t, M_0 is initial litter mass and k is the decomposition rate.

As this model systematically underestimated initial mass loss and overestimated late-stage mass loss, an asymptotic exponential model was fitted, as described in Wieder and Lang (1982):

$$M(t) = A + (M_0 - A)e^{-kt} + \varepsilon \qquad Eq.\ 4$$

where $(1 - A)$ the labile litter fraction that decomposes at rate k and A is the residual litter fraction with a decomposition rate of zero.

Computation of AICc, a version of the Akaike information criterion corrected for small sample sizes, showed that the asymptotic exponential model provided a better fit than the simple exponential model (AICc difference > 2) for 44 cases, a similar fit for 5 cases

(AICc difference < 2 and > -2) and a worse fit (AICc difference < -2) for only one case, which contained an outlier. To ensure consistency, based on this, the asymptotic exponential model was fitted to all decomposition curves with the exception of *Trifolium* root litter, for which only two time points were available due to its smaller root biomass.

To test hypothesis 2, species differences and drought effects on the decomposition model parameters *A* and *k* were tested using two-way ANOVAs with log-transformation of variables where necessary to fulfil model assumptions. For *Trifolium* root litter the effect of species and drought on remaining mass % after 4 and 16 weeks were tested directly using two-way ANOVAs. Pairwise comparisons of significant effects were assessed using Tukey post hoc tests.

5.3 Results

5.3.1 Plant traits

Effects of species, drought and their interaction on plant traits (Hypothesis 1) were determined using two-way ANOVA and subsequent Tukey post hoc tests. There were significant differences between species means for all plant traits measured in fresh and senesced material ($p < 0.05$), except shoot individual weight where $p = 0.074$ (Fig. 5.1 and 5.2, Table 5.1).

A range of morphological and chemical traits measured on fresh or senesced material were affected significantly by drought across all species (Table 5.2). Drought increased LDMC, RDMC, fresh root dry weight and both fresh and senesced root : shoot ratio ($p < 0.05$). It also had a non-significant positive effect on root diameter ($p = 0.052$). Drought also decreased SLA, LA, leaf length, shoot and root cellulose and root lignin and fibre ($p < 0.05$) across all species. For some traits the drought effect varied between

species (species x drought, $p<0.05$). *Lolium* was the most affected species in terms of the number of additional traits affected including shoot fibre, shoot and root hemicellulose, shoot and root digestibility and root dry weight. *Trifolium* and *Plantago* had only two additional traits affected which were not consistent across species.

LRRs (see Eq. 1) were computed to compare the strength (magnitude of LRR) and direction (positive vs. negative LRR) of drought effects on traits (see Fig. 5.3). Generally, drought had the strongest negative effect on shoot morphological traits and a slightly weaker positive effect on root traits. Effects on shoot chemical traits were generally weakest with both positive and negative effects. Effects on root chemical traits were stronger than effects on shoot chemical traits, almost as strong as the effects on root morphological traits and also with both positive and negative effects.

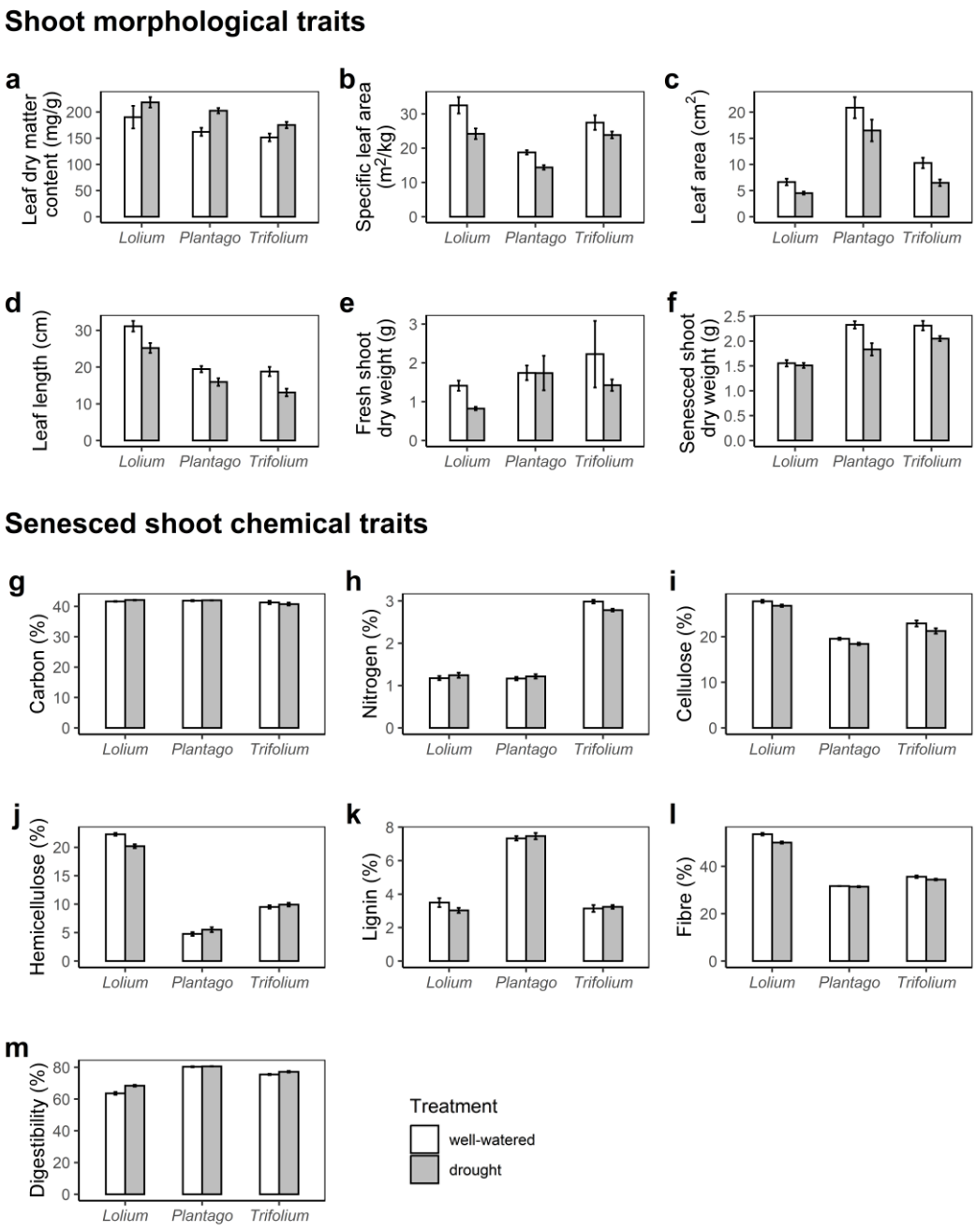

Figure 5.1: Effects of drought on shoot morphological (a-f) and senesced shoot chemical traits (g-m) of the three grassland species. Data represent mean +/- 1 standard error. Results from the ANOVA and subsequent Tukey post hoc testing for significant species differences and drought effects can be found in Tables 5.1 and 5.2.

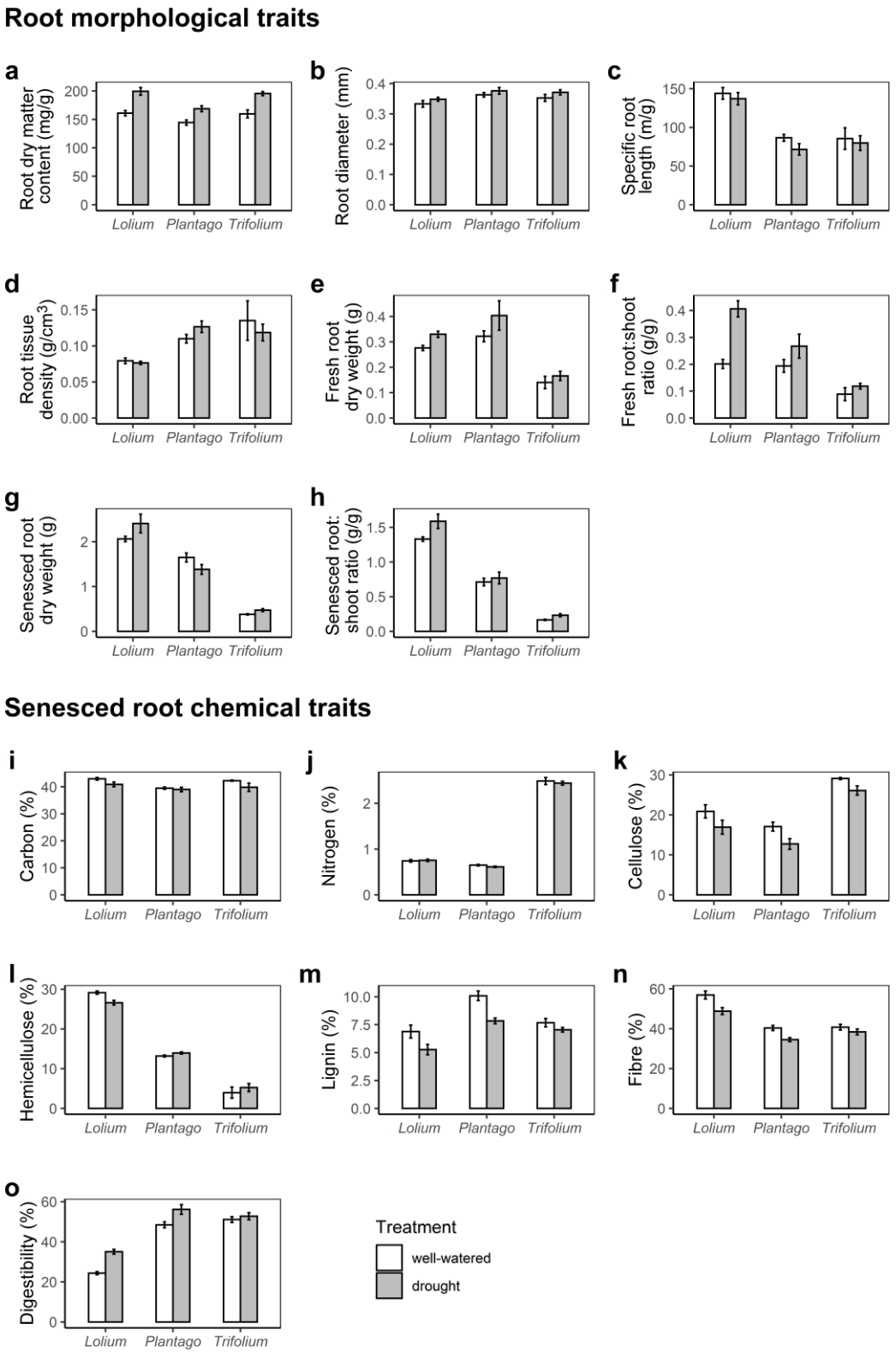

Figure 5.2: Effects of drought on root morphological (a-h) and senesced shoot chemical traits (i-o) of three grassland species. Data represent mean +/- 1 standard error. Results from the ANOVA and subsequent Tukey post hoc testing for significant species differences and drought effects can be found in Tables 5.1 and 5.2.

Table 5.1: Main and interactive effects of plant species and drought treatment on shoot and root plant traits, tested by two-way ANOVA.

Shoots

		Morphological traits						Chemical traits of senesced material						
Factor	**Statistic**	**Leaf dry matter content**	**Specific leaf area**	**Leaf area**	**Leaf length**	**Fresh shoot dry weight**	**Sensced shoot dry weight**	**Carbon**	**Nitrogen**	**Cellulose**	**Hemi-cellulose**	**Lignin**	**Fibre**	**Digesti-bility**
Species (df = 2)	**F**	**7.16**	**31.49**	**55.39**	**63.08**	2.92	**37.05**	**5.90**	**796.98**	**191.64**	**1267.60**	**344.11**	**1367.70**	**390.76**
	p	**0.003**	**<0.001**	**<0.001**	**<0.001**	0.074	**<0.001**	**0.009**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Drought treatment (df = 1)	**F**	**14.62**	**18.33**	**10.25**	**27.65**	3.47	**16.24**	0.01	0.27	**12.93**	1.35	0.28	**23.77**	**27.48**
	p	**<0.001**	**<0.001**	**0.004**	**<0.001**	0.075	**<0.001**	0.914	0.606	**0.001**	0.257	0.601	**<0.001**	**<0.001**
Species x drought treatment (df = 2)	**F**	0.28	1.31	0.40	0.64	0.65	**3.86**	1.31	**4.82**	0.34	**10.97**	1.68	**8.35**	**9.64**
	p	0.759	0.287	0.677	0.536	0.530	**0.035**	0.289	**0.018**	0.715	**<0.001**	0.207	**0.002**	**<0.001**

Roots

		Morphological traits							Chemical traits of senesced material						
Factor	**Statistic**	**Root dry matter content**	**Root diameter**	**Specific root length**	**Root tissue density**	**Fresh root dry weight**	**Fresh root : shoot ratio**	**Senesced root dry weight**	**Senesced root : shoot ratio**	**Carbon**	**Nitrogen**	**Cellulose**	**Hemi-cellulose**	**Lignin**	**Fibre**	**Digesti-bility**
Species (df = 2)	**F**	**11.38**	**5.04**	**30.00**	**8.23**	**28.41**	**26.90**	**141.74**	**403.44**	**5.39**	**1336.50**	**50.42**	**462.39**	**25.96**	**61.41**	**134.02**
	p	**<0.001**	**0.015**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.012**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Drought treatment (df = 1)	**F**	**55.15**	4.18	1.61	0.01	**5.31**	**14.07**	0.42	**10.33**	**6.17**	0.56	**12.80**	0.07	**20.95**	**19.49**	**26.85**
	p	**<0.001**	0.052	0.217	0.929	**0.030**	**<0.001**	0.524	**0.004**	**0.020**	0.461	**0.002**	0.799	**<0.001**	**<0.001**	**<0.001**
Species x drought treatment (df = 2)	**F**	0.96	0.04	0.16	0.84	0.48	0.85	**4.03**	1.73	0.78	0.30	0.14	**3.59**	2.07	1.84	**4.26**
	p	0.398	0.960	0.851	0.445	0.627	0.441	**0.031**	0.199	0.468	0.741	0.870	**0.043**	0.148	0.181	**0.026**

Table 5.2: Significant ($p < 0.05$) drought effects on traits of *Lolium*, *Plantago* and *Trifolium*, tested by 2-way ANOVA and subsequent Tukey post hoc tests. ↑ - positive drought effect, ↓ - negative drought effect.

	Trait	*Lolium*	*Plantago*	*Trifolium*
Shoot morphological traits	Leaf dry matter content	↑	↑	↑
	Specific leaf area	↓	↓	↓
	Leaf area	↓	↓	↓
	Leaf length	↓	↓	↓
	Fresh shoot dry weight	-	-	-
	Senesced shoot dry weight	-	↓	-
Senesced shoot chemical traits	Carbon	-	-	-
	Nitrogen	-	-	↓
	Cellulose	↓	↓	↓
	Hemicellulose	↓	-	-
	Lignin	-	-	-
	Fibre	↓	-	-
	Digestibility	↑	↑	-
Root morphological traits	Root dry matter content	↑	↑	↑
	Root diameter	-	-	-
	Specific root length	-	-	-
	Root tissue density	-	-	-
	Fresh root dry weight	↑	↑	↑
	Fresh root : shoot ratio	↑	↑	↑
	Senesced root dry weight	↑	-	-
	Senesced root : shoot ratio	↑	↑	↑
Senesced root chemical traits	Carbon	↓	↓	↓
	Nitrogen	-	-	-
	Cellulose	↓	↓	↓
	Hemicellulose	↓	-	-
	Lignin	↓	↓	↓
	Fibre	↓	↓	↓
	Digestibility	↑	↑	-

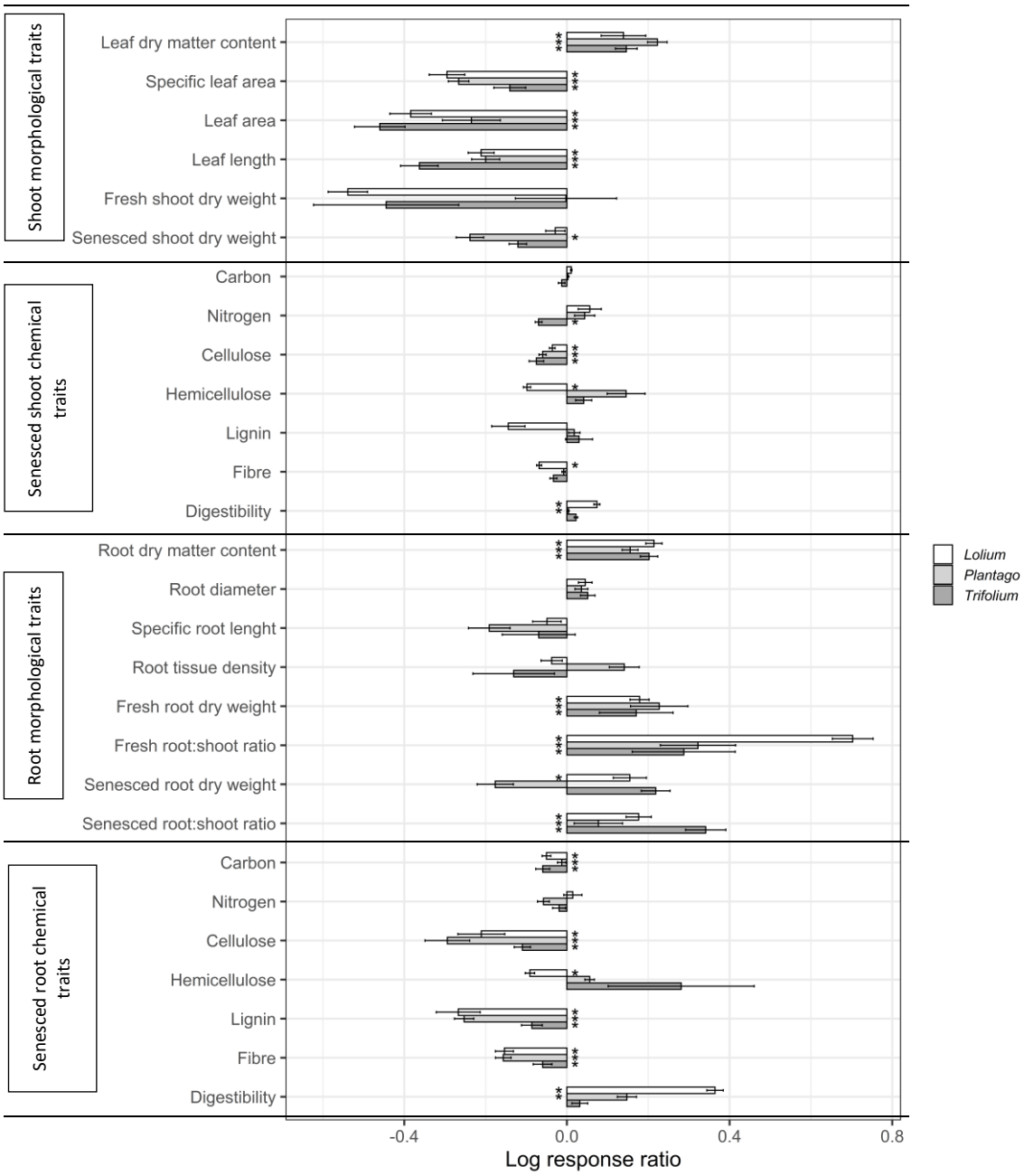

Figure 5.3: Log response ratios (LRR) for drought treatment effects on plant traits, see Eq. 1. If LRR > 0 there was a positive drought effect, if LRR < 0 there was a negative drought effect. Error bars represent +/- 1 standard error. * at the base of the bar plots indicate significant effects ($p < 0.05$) of drought treatment determined by 2-way ANOVA (see Table 5.1) subsequent Tukey post hoc tests.

5.3.2 Litter decomposition

To test whether drought affected the decomposability of shoots and roots following plant senescence (Hypothesis 2), an asymptotic exponential model (Eq. 4) was fitted to the mass loss data from the decomposition assay (Fig. S5.1). The model included a residual litter fraction *A* with a decomposition rate of zero and a fraction *(1-A)* that decomposes at rate *k*. Pearson correlation coefficients between measured and modelled values of remaining mass ranged between 0.974 and 0.999. Effects of species, drought and their interaction on *k* and *A* were determined using two-way ANOVA with Tukey post-hoc testing.

The decomposition rate of the labile litter fraction *k* was significantly different between species for shoots ($p < 0.001$), while the drought effect varied between species ($p < 0.05$, Fig. 5.4). Post-hoc testing revealed that drought increased *k* from 0.16 to 0.23 in *Plantago*, though not significantly ($p = 0.06$), but did not affect *k* in *Lolium* and *Trifolium*. For roots, *k* differed between species ($p < 0.001$) in droughted and well-watered treatments with a lower rate of decomposition in *Plantago* compared to *Lolium*.

The residual litter fraction *A* did not differ between species in shoots, but the drought effect varied between species (species x drought, $p < 0.05$, Fig. 5.4). Post-hoc testing revealed that drought increased the mean *A* from 0.316 to 0.430 in shoots of *Plantago* ($p < 0.05$), but did not affect *A* in *Lolium* and *Trifolium*. For roots, *A* differed between species ($p < 0.001$) and also the drought effect on *A* varied between species ($p < 0.001$). Post-hoc testing revealed that drought decreased mean *A* from 0.817 to 0.786 in *Lolium* ($p < 0.01$) but had no effect on *A* in *Plantago* roots.

For *Trifolium* roots remaining litter mass could only be obtained after 4 and 16 weeks due to its smaller root biomass, so it was not possible to fit Eq. 4. In this case, t-tests

were used to determine the effect of drought on mass loss after 4 and 16 months and showed that there was no significant effect of drought. Mean remaining mass after 16 weeks for *Trifolium* roots was 35.5 % for the well-watered treatment and 34.2 % for the drought treatment.

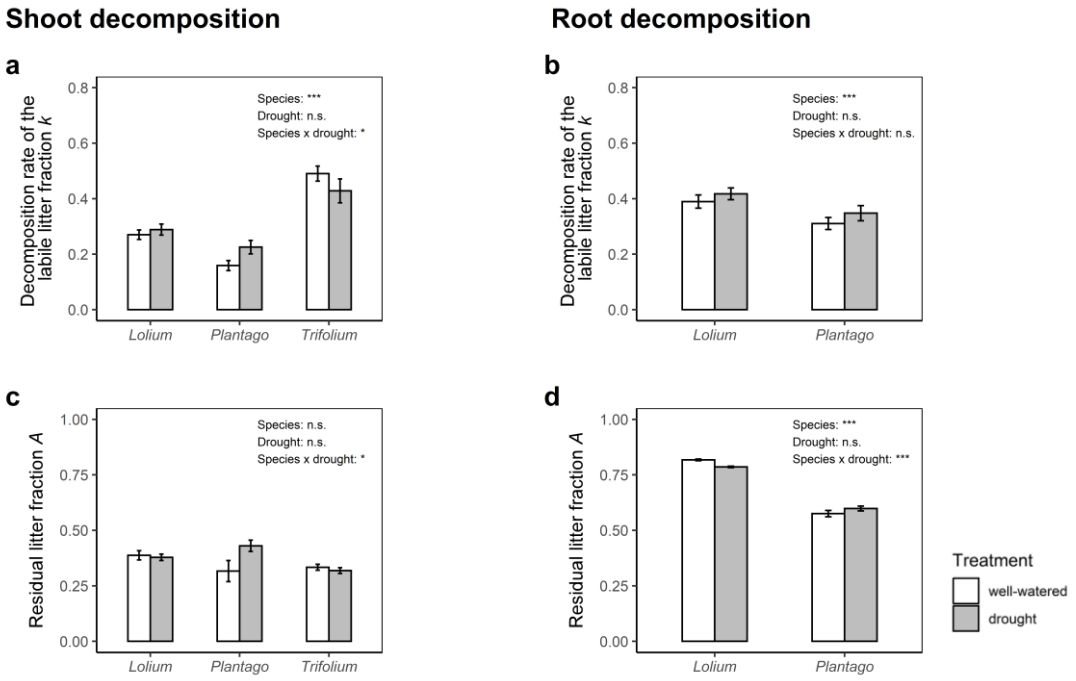

Figure 5.4: The effect of drought and plant species on the decomposition rate of the labile litter fraction *k* (a, b) and the residual litter fraction *A* (c, d). Data represent mean +/- 1 standard error. *k* and *A* were determined by fitting Eq. 2 to remaining litter mass after 2, 4, 8, 12 and 16 weeks using nonlinear least squares regression. Significance of main and interactive effects of species and drought treatment were assessed using ANOVA with significance indicated as: $p < 0.001$ ***, $p < 0.01$ **, $p =< 0.05$ *.

5.4 Discussion

The aim of this study was to investigate whether the drought-induced plasticity of root and shoot traits alters their decomposability. Drought had significant and sometimes strong effects on many morphological and chemical shoot and root traits of all three temperate grassland plant species. Morphological shoot traits were the most strongly affected by drought. For example, negative effects of drought on LDMC and SLA were of similar magnitude as differences between species. Drought also affected decomposition in two species, accelerating decomposition of the labile litter fraction and either increasing or decreasing the residual litter fraction. However, drought effects on litter decomposability were fewer and much weaker than drought effects on traits.

5.4.1 Drought effects on plant traits

In contrast to Hypothesis 1, drought had similar effects on most traits of the three species, irrespective of their functional group (Fig. 5.1 and 5.2, Table 5.1 and 5.2). Drought effects on morphological traits of both shoots and roots (increased LDMC, RDMC and root diameter and decreased SLA, LA and leaf length) were consistent with a shift towards a more resource conservative strategy in all three species. These results confirm findings from recent studies on the effect of droughts of varying duration on traits of temperate grassland species. Lozano et al. (2020) found similar responses in morphological shoot and root traits of the same three species to a more severe drought (2 months at 30% WHC), in that drought generally increased LDMC, RDMC and root diameter and decreased SLA. Also, de Vries et al. (2016) observed a shift to more conservative root traits in grassland species as a response to a two-week drought at 30% WHC.

However, fibre (cellulose, hemicellulose, lignin) and C content were either decreased or not affected by drought and digestibility was increased. This is inconsistent with conservative resource-use as suggested by the morphological traits. Even though it could not be measured in this study, an increase of NSC content in response to drought could explain this pattern. A drought-induced increase in NSC has been observed in other studies (e.g. Brunner *et al.*, 2015) and might also increase tissue dry matter content and density. It is surprising that despite a drought effect on RDMC there was no significant effect of drought on RTD, but effects might be explained by a higher density in the dry fraction of the root biomass due to NSC accumulation.

Other studies have investigated the effect of drought on plant chemical traits. For example, drought by withholding water for two weeks increased levels of NSC in leaves of *Lolium perenne*, but did not affect lignin content (AbdElgawad et al., 2014), which is consistent with the results of this study. Also, feedstock species generally showed increased NSC content and decreased lignin content in a year of drought compared to a non-drought year (Emerson et al., 2014). On the other hand, in contrast with this study, leaf lignin content was found to increase after 12 days of withholding water in *Trifolium repens* (Li et al., 2013). Also, in a meta-analysis, Dumont et al. (2015) found on average a small increase in lignin content of grassland shoots as a response to drought, but also a small increase in digestibility, and high variation between experiments. The contrasting results of these studies indicate that plant chemical responses to drought are variable, possibly depending on plant species and duration and intensity of drought.

Log response ratios revealed that, generally, shoots had a slightly stronger drought response in morphological traits than roots (Fig. 5.3). This might be due to the fact that root morphological traits are more physically constrained by the soil. On the other hand,

chemical traits of senesced plants showed a stronger drought-response in roots than in shoots. This might be due to the fact that roots are responsible for water acquisition and are directly in contact with the soil. Also, the increase in root : shoot ratio in all species implies that more C was allocated to roots under drought, which gives more opportunity for plastic adaptations.

5.4.2 Litter decomposition was best described by an asymptotic exponential model

Litter decomposition was best described by an asymptotic exponential model including a labile fraction *(1-A)* that decomposes at rate *k* and a recalcitrant fraction *A* with decomposition rate of zero. Even though a non-decomposable fraction of litter is unrealistic under field conditions, it is not unreasonable over the experimental timeframe and with the exclusion of larger decomposer organisms. Other studies have also reported an asymptotic model as the best fit (Howard & Howard, 1974), especially for roots (Hobbie et al., 2010). The good fit of the asymptotic model indicates that the plant material contained a labile fraction that decomposed distinctly faster than the remaining part, rather than containing a continuum from labile to more recalcitrant components. This is relevant for ecosystem C and N cycling as both labile and recalcitrant litter fractions can contribute to forming stable soil organic matter, but through different pathways (Cotrufo et al., 2015). Labile compounds are efficiently incorporated by microbes and their residues can form stable soil organic matter (Kallenbach et al., 2016). Recalcitrant compounds decompose slowly and small fragments of litter can remain in the soil due to their recalcitrance and/or through physical protection in soil aggregates (Cotrufo et al., 2015). The labile fraction might also contribute to fuelling the commonly observed peak of microbial activity after a drought ends (Birch, 1958). Additionally, labile and recalcitrant litter fractions are

likely related to different plant/litter traits (Loranger et al., 2002). Thus, a single parameter *k* can be insufficient to characterize litter decomposability.

5.4.3 Drought effects on litter decomposability

Hypothesis 2 was partially supported. Drought effects on litter decomposability were observed in *Plantago* shoots and *Lolium* roots and were not very large given the magnitude of trait changes, which could sometimes be as large as differences between species (Fig. 5.4). However, some of the observed drought effects on litter decomposability can be linked to the trait plastic responses to drought measured.

Drought increased both the decomposition rate of the labile litter fraction *k* and the residual litter fraction *A* for *Plantago* shoots. This means that the initial slope of the mass loss trajectory was higher, which could be due to an increased accumulation of NSC. Also, the fraction decomposing at rate zero was higher. No trait showed a higher drought-response in *Plantago* than in the other two species, so this pattern cannot be directly explained by the traits measured. An explanation could be that *Plantago* leaves were much larger than leaves of *Lolium* and *Trifolium*, so the decrease in SLA and LA could have led to a larger decrease in the total leaf surface area that can be accessed by microbes (Hanlon, 1981).

Drought decreased the residual litter fraction *A* for *Lolium* roots, which means that the initial slope of the mass loss trajectory was higher and the residual litter fraction was smaller. *Lolium* also had the highest mean increase in senesced root lignin content and digestibility with drought. The increase in digestibility was much higher than for the other two species. This might explain why drought affected decomposition in *Lolium*, but not the other two species, as digestibility has been shown to be a good predictor of decomposability (Bumb et al., 2018).

Even though to date no other studies have investigated the effect of drought-induced plant trait plasticity on litter decomposability, there have been studies of the effect of intraspecific trait variability on litter decomposability in other contexts. All of them found that integrative traits did not predict differences in litter decomposition within species well, even though integrative traits can be good predictors of differences between species. For example, LDMC, SLA and leaf/litter C, N and P could not explain the considerable within-species variation in litter decomposability in 16 temperate rain forest species from sites differing in soil nutrient status (Jackson et al., 2013), however more detailed chemical litter traits were not measured. In a French Mediterranean old-field succession, N-addition induced phenotypic trait-changes in herbaceous species, but they did not translate in changes in decomposition rates (Kazakou et al., 2009). Also, decomposition rates between genotypes of *Arabidopsis thaliana* were strongly correlated with secondary metabolites, but only weakly with morphophysiological traits (Kazakou et al., 2019).

5.4.4 Future work

There are some caveats to this experimental design. In the framing of this study the best solution to producing sufficient amounts of litter was to let plants die by imposing an additional fatal drought at the end of the experiment. Even though the fatal drought was much shorter that the main experimental drought, this may have introduced bias as all plants will have had an additional plastic response. Consequently, drought effects might be larger for decomposition in real-world ecosystems. On the other hand, roots often survive droughts and leaves will re-sprout using resources stored in the roots, so only part of the root system is decomposed. Drought can also affect litter decomposition through other mechanisms than changes in litter quality, such as a changes in litter quantity, root : shoot ratio (Poorter et al., 2012), the soil physical environment and in

microbial community and activity. Thus, further experiments are needed to better understand the effect of drought on decomposition under field conditions, and based on the results of this study they should include measurements of not only morphological traits, but also detailed chemical traits, especially of NSC.

5.4.5 Conclusion

The results of this study indicate that drought-induced plasticity of root and shoot traits can affect litter decomposability in some European temperate grassland species. It can lead to faster rates of initial litter decomposition, potentially due to drought-induced accumulation of easily decomposable non-structural carbohydrates. Drought can also either increase or decrease the recalcitrant litter fraction that decomposes at a slower rate. These changes could affect ecosystem C storage and also amplify the commonly observed flush of microbial activity after rewetting soils following drought. However, drought had much stronger effects on root and shoot traits than on litter decomposability. Especially in morphological traits, drought effects on plant traits could be as strong as differences between species, while drought effects on decomposability were much weaker than differences between species. This suggests that integrative morphological traits, which perform well at predicting differences in decomposability between species, may not be indicative of drought-induced changes in decomposability within species.

6 General Discussion

Plant traits can serve as easily measurable proxies for plant function, useful for predicting vegetation responses to environmental change and effects of vegetation on ecosystem function. However, many trait-based studies do not take into account intraspecific trait variability (ITV) and it is unclear how much uncertainty this introduces. The aim of this thesis was to improve understanding of the drivers that control ITV as well as the consequences of ITV for ecosystem functions related to C and N cycling in grassland ecosystems.

The main findings of the four experimental chapters are summarized in Fig. 6.1. Five overarching themes emerged, which will be discussed in the following sections:

1. Some of the potentially important drivers of ITV identified in the field (Chapter 2) induced substantial plasticity in similar traits in the mesocosm/greenhouse experiments, but others did not (Chapter 3-5).
2. Shoot biomass was overall the most variable trait. Leaf morphological traits were more variable than leaf chemical traits in response to drought (Chapter 4), but there was no clear difference in variability between leaf morphological and

chemical traits in response to neighbouring plants and N availability (Chapter 2 and 3).

3. Patterns of ITV were highly species-specific in the field and mesocosm experiment (Chapter 2-4), but less in the drought experiment (Chapter 5).
4. ITV affected ecosystem functions only sometimes and not very strongly (Chapter 3, 4 and 5).
5. Species interactions also affected ecosystem functions through mechanisms not related to ITV (Chapter 3 and 4).
6. Implications of the results of this thesis for the use of trait databases.

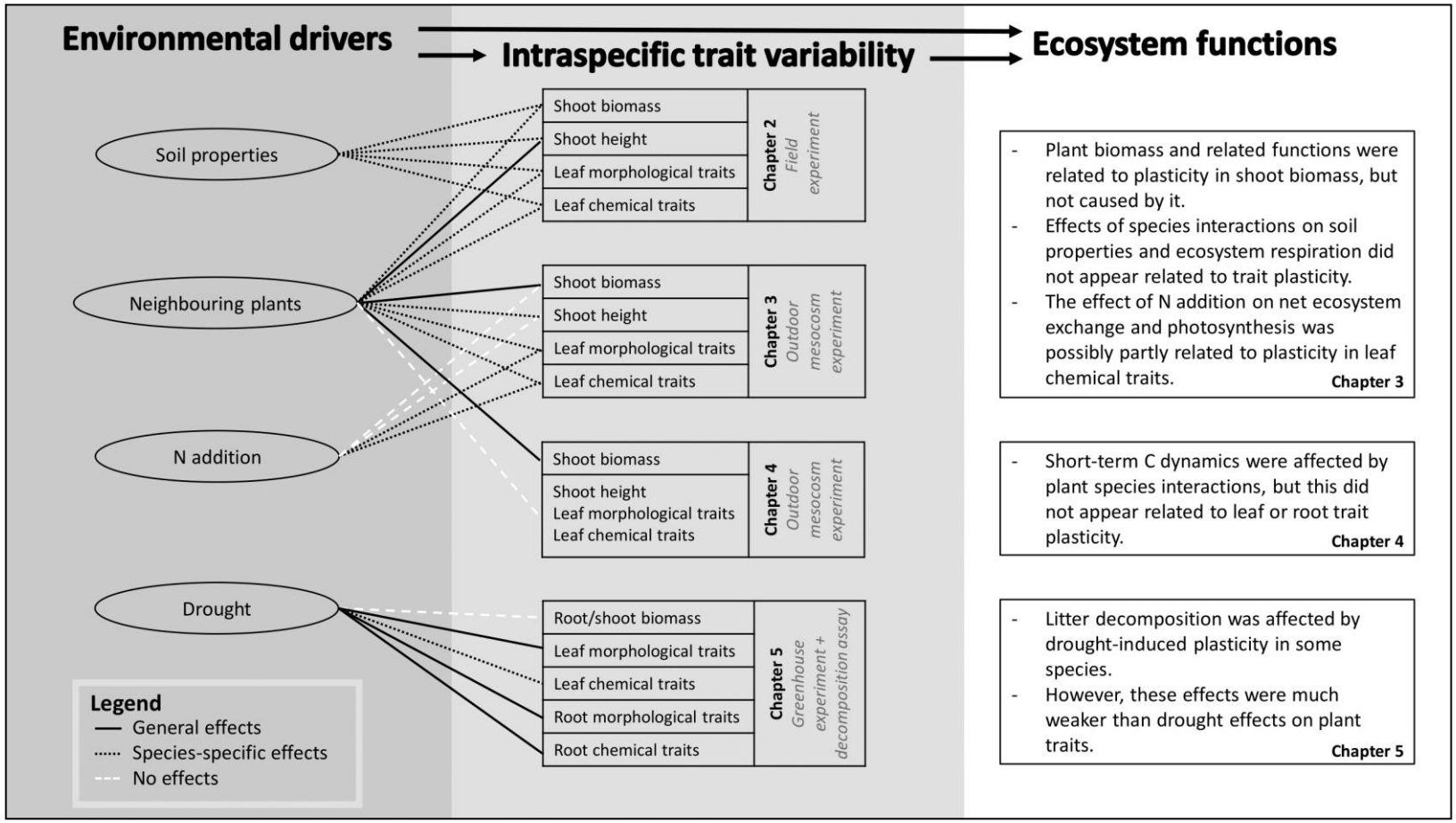

Figure 6.1: Conceptual diagram summarizing the main findings of this thesis, building from Fig. 1.1, showing the effects of environmental drivers on intraspecific trait variability, as well as effects of both on ecosystem functions. Black solid lines represent general effects, black dashed lines represent species-specific effects, and white dashed lines represent no significant effects.

6.1 Drivers of ITV

The field experiment (Chapter 2) pointed to a range of drivers controlling ITV, depending on the species and traits considered, such as the surrounding plant community, soil N availability, light availability, soil pH and soil microbial properties. Even though water availability was not directly measured, some results suggested that it might be important as well. In the mesocosm and greenhouse drought experiments (Chapter 3 and 5), neighbouring plants, N availability and water availability were experimentally manipulated. The patterns of ITV observed were to some degree similar to the ones found in Chapter 2, but there were also differences.

6.1.1 Neighbouring plants

In the field experiment, the presence of the most resource-acquisitive functional group increased the shoot height of two focal species and shoot dry weight of one focal species, likely related to higher N and lower light availability. In the mesocosm experiment an opposite pattern was found: the presences of a less resource-acquisitive species (*Rumex acetosa*) increased shoot biomass of all other species, likely due to higher N availability, while the presence of the most resource-acquisitive species (*Plantago lanceolata*) did not affect shoot dry weight. This difference could be due to the shorter duration of the mesocosm experiment, where faster-growing species probably took up soil nutrients faster compared to slower-growing species (de Vries & Bardgett, 2016), while in the field experiment the faster-growing species may have contributed to faster rates of nutrient cycling compared to slower-growing species (Orwin et al., 2010). In the mesocosm experiment, the height of the grasses was affected by neighbouring species, but this did not appear to be related to light availability as in the field experiment, but rather to particular interactions between species. Light

availability might not have been a limiting factor to the same degree in the pots as in the field, as light could enter from the sides and also the plant density was lower than in the field.

In both experiments, the effect of neighbouring plants on leaf traits was more limited compared to whole-plant traits. In the field experiment, the only leaf trait affected by neighbouring plants was leaf N in *Daucus carota*. In the mesocosm experiment, leaf traits were only sporadically affected by neighbouring plants, e.g. leaf C in *Plantago lanceolata* and some leaf morphological traits in *Anthoxanthum odoratum* and *Rumex acetosa*. In contrast, other studies have observed stronger and somewhat more consistent ITV of leaf chemical and morphological traits in response to biodiversity and legume presence (Gubsch et al., 2011; Lipowsky et al., 2015; Guiz et al., 2018). Reasons for this might be the high local heterogeneity of the rocky chalk soil in the field experiment, as well as the shorter duration and lack of strong light limitations in the mesocosm experiment.

6.1.2 N availability

In the field experiment, the height of *Daucus carota* was significantly correlated with K_2SO_4 extractable soil N and also leaf N, though not significantly. Additionally, leaf N, specific leaf area and leaf C : N ratio were correlated with total soil N in *Clinopodium vulgare*, which might reflect long-term N availability better than K_2SO_4-extractable N.

In the mesocosm experiment, N addition did not affect leaf N in any of the species, but it affected leaf C and C : N ratio in three of the species. The only morphological trait affected was leaf area in *Rumex acetosa*. In contrast, other studies have found considerable effects of N addition on leaf chemical and morphological traits (Kazakou et al., 2009; Siebenkäs et al., 2015). The weak effects observed here were likely due to

the low level of N addition, although it is worth noting that many experiments use unrealistically high levels of N addition and that grassland responses to N are non-linear with respect to the amount added (Niu et al., 2018). Additionally, the shoot biomass of all species was higher when growing with *Rumex acetosa*, which were also the treatments with the highest plant available N, but no leaf traits were strongly affected in these treatments.

6.1.3 Drought

Results of the field experiment suggested that low water availability might have decreased leaf dry matter content. This was confirmed in all three species in the drought experiment. Additionally, the experimental drought affected many other morphological and chemical root and shoot traits. The ITV observed in this experiment was generally larger than in the other two experiments, which is likely because here stress was imposed as a driver for ITV, rather than just a difference in growing conditions, and due to the severity of the treatment.

6.2 Which traits were the most variable?

In all experiments (Chapter 2, 3 and 5), individual shoot dry weight was the most variable trait. Individual shoot dry weight is not usually taken into account as a plant trait in studies (Pérez-Harguindeguy et al., 2013). This is firstly because it is unclear how the shoot dry weight of an individual should be defined in plants with clonal reproduction, for example in tussock-forming grasses, and in plants with indeterminate growth. Secondly, each species' contribution to total biomass (or to cover) is used as the weighting factor in the calculation of community-weighted mean traits or functional diversity indices. In this thesis, in the field experiment, individuals of the three chosen focal species could be clearly distinguished, as only one shoot could be found in each

of the turves sampled. In the mesocosm and greenhouse drought experiments, “shoot” was defined with respect to the initially planted seedlings, which could still be distinguished at the end of the experiments. The high variability observed in shoot dry weight in this thesis indicates the main response of plants to varying environmental conditions is an alteration of growth and that variation in other traits is less pronounced in comparison.

In all experiments, leaf and root C were the least variable traits. In the field and mesocosm experiments, leaf N and leaf morphological traits had similar overall degrees of ITV. In contrast, in the drought experiment, leaf morphological traits were overall more variable than shoot chemical traits, however this may also be related to the fact that shoot chemical traits were measured on senesced plant material. In an analysis of global trait data from woody and herbaceous species, the coefficients of variation did not differ strongly between leaf chemical and morphological traits, except for leaf C, which was less variable than all other traits (Kuppler et al., 2020).

Root morphological and chemical traits had similar degrees of variability in the drought experiment as leaf morphological traits. This might be because drought was the main driver of ITV here, which may affect roots more strongly, as they are directly involved in water acquisition and drought also shifted more biomass allocation belowground. Root chemical traits were also consistently more variable than leaf chemical traits in six understorey species across boreal and temperate Canadian forests (Kumordzi et al., 2019). The variability of leaf vs. root chemical traits is not often compared and it would be interesting to investigate this further in a wider range of contexts.

In addition to the absolute amount of ITV, expressed for example in coefficients of variation, studies including larger numbers of species have also considered the relative

magnitude of ITV in relation to variation between species (Kazakou et al., 2014; Siefert et al., 2015). In these studies, the relative magnitude of ITV tended to be higher in chemical traits than in morphological traits. This pattern included leaf C, as leaf C also shows little variability between species (Kattge et al., 2011). The relative magnitude of ITV is especially relevant for the question whether ITV should be taken into account in studies over gradients, while the absolute magnitude matters for evaluating ecosystem responses to environmental change.

6.3 Species specificity of ITV

In the field and mesocosm experiments (Chapter 2 and 3), some patterns of ITV were highly species-specific. For example, shoot biomass and height were correlated with microbial properties only in *Daucus carota*, suggesting competition for nutrients between plants and microbes. Only in *Leucanthemum vulgare* ITV was related to pH. In *Anthoxanthum odoratum*, ITV in several traits occurred specifically when this grass was grown in combination with another grass, which seemed related to tussock formation. However, the responses of three grassland species to drought (Chapter 5) were much more homogeneous.

Other studies have also observed species-specific idiosyncrasies in ITV of leaf and root traits along environmental gradients (e.g. Albert et al., 2010; Kumordzi et al., 2019; Weemstra et al., 2021). If species-specific idiosyncrasies in ITV were common and followed no recognizable underlying pattern, this would make generalizable predictions much more difficult (Shipley et al., 2016). However, common patterns of ITV among species have been observed in some contexts. For example, a global meta-analysis showed that ITV of leaf traits along elevation gradients followed common patterns (Midolo et al., 2019). Another meta-analysis found that drought-induced plasticity in

specific leaf area differed between temperate and sub-Mediterranean grassland species, and between grasses and forbs, but within these groups there were consistent patterns (Wellstein et al., 2017). In Chapter 5, drought responses were relatively consistent among functional groups, however only one species was studied for each functional group. Albert et al. (2010) proposed a conceptual model that could also help explain the species-specific idiosyncrasies observed in many studies. According to this model, ITV in response to an environmental gradient always follows a bell-shaped curve along the range that the species occupies. Depending on which part of the species range is covered in the gradient of a study, ITV in relation to the gradient can resemble a positive, a negative or a bell-shaped relationship. The findings of this thesis point towards a complicating factor: it can depend on the species which environmental factors are the most important in controlling ITV (e.g. pH, microbial properties, neighbouring species), and several of these factors might covary along environmental gradients.

6.4 ITV effects on ecosystem function

Through all the experiments, ITV affected ecosystem functions only sometimes and not very strongly. In the mesocosm experiment (Chapter 3 and 4), the effect of neighbouring plant species on shoot biomass was the only instance where patterns of ITV were consistent among species and this resulted in consistent effects of species interactions on total aboveground biomass. However, shoot biomass is not usually taken into account in trait-based studies (see section 6.2). As shoot biomass was defined here with respect to the initially planted seedling, it cannot be considered as a factor affecting total biomass, but it is essentially the same measure. Except for the relationship with shoot biomass, effects of species interactions on ecosystem properties and functions did not appear related to trait plasticity. Even though there were instances where ITV was affected by the neighbouring plant species (see section 6.1.1) community-weighted

mean traits in mixtures generally did not deviate significantly from what would be expected from their monocultures. The instances where ecosystem properties and functions deviated from what would be expected based on their monocultures (see section 6.5) did not appear related to ITV for any traits other than shoot biomass. N-addition affected only net ecosystem exchange and photosynthesis, which was possibly partly related to plasticity in leaf chemical traits, but these effects were not very strong. Litter decomposition was affected by drought-induced plasticity in some species (Chapter 5). However, these effects were much weaker than drought effects on plant traits.

6.5 Effects of species interactions on ecosystem functions

The interactions between plant species affected ecosystem properties and functions in idiosyncratic ways, depending on the particular ecosystem property or function and sometimes the species (Chapter 3 and 4). Compared to monocultures, the effects of the species in mixtures were sometimes additive (e.g. for soil moisture), sometimes synergistic (e.g. for aboveground biomass, photosynthesis and short-term C cycling) and sometimes one of the component species had a disproportional effect relative to its biomass (e.g. plant-available NO_3^-, ecosystem respiration and some soil enzyme activities). Disproportionate effects of one component species have commonly been reported for legumes (e.g. Lange et al., 2014), but also for *Lolium perenne* in the case of soil physical properties (Gould et al., 2016). If this phenomenon is more common, this might help to explain why the explanatory power of plant traits for predicting ecosystem functions is often low (van der Plas et al., 2020).

6.6 Implications of the results for the use of trait databases

These results of this thesis suggest that in cases where drivers of ITV are similar to the ones explored in this thesis it may be justified neglect ITV in studies examining the effects of plant traits on ecosystem functions. This gives some support to approaches using trait mean values from databases, which can allocate more resources to measuring other components of the study, such as more detailed environmental variables (e.g. soil properties) or ecosystem functions. However, Chapter 3-5 only considered phenotypic plasticity and not genetic variability, and only a relatively limited range of environmental drivers, so this may not be the case in other contexts. Indeed, other studies have observed stronger effects of ITV on ecosystem function (e.g. Lecerf & Chauvet, 2008; de Vries et al., 2016).

The considerable within-site ITV related to microenvironmental conditions found in Chapter 2, as well as in other studies (Albert et al., 2010b; Messier et al., 2010; Weemstra et al., 2021) also raises the question how ITV could be incorporated meaningfully in studies relating traits and ecosystem function. A possible approach following standard protocols (Pérez-Harguindeguy et al., 2013) would be to measure traits of at least 10 randomly chosen mature and healthy individuals of each species at each site of a gradient or in each experimental treatment, to average out differences in age and growth stage. However, this approach can only account for between-site/treatment ITV and not for within-site/treatment ITV. A sampling design accounting for within-site/treatment ITV would require even more sampling effort, which might be of more use being allocated to other aspects of the study.

6.7 Future work

The results of this thesis highlight several avenues for future research, for example:

- ITV in chemical root traits was larger than in chemical leaf traits, and generally there are much fewer studies on drivers of ITV in root traits, so this would benefit from further research.
- In this thesis, only the effect of plant trait plasticity on ecosystem functions was studied, but it would be interesting to also study the effect of genotypic variation and its interaction with phenotypic plasticity
- Generally, in this thesis the number of species (three to four in each experiment) was too small to model ecosystem function from traits. The next step would be to conduct a larger biodiversity experiment or a gradient study on plant trait effects on ecosystem functioning, to measure traits locally in each treatment, and to compare the predictive capacity of locally measured traits vs. mean traits taken from databases. In case of a gradient study, it would be especially important to include sufficiently detailed environmental variables as direct drivers of ecosystem function in the models.
- The results of this thesis suggested disproportional effects of some species on some ecosystem functions. It would be interesting to explore the underlying mechanisms of this.

6.8 Conclusion

This thesis has provided new insights into how plant traits affect ecosystem functions and the role of intraspecific trait variability in this. It has shown that plant species interactions, soil properties, nutrient availability and drought stress contribute to controlling intraspecific trait variability in grasslands, but that the exact patterns of intraspecific variability are often species-specific. Phenotypic plasticity in response to these environmental drivers had either weak or no effects on ecosystem functions related to C and N cycling. This suggests that in contexts similar to the ones examined

here it may be justified to ignore ITV in trait-based studies and focus on species means. In mixtures, one of the species sometimes had disproportionate effects on ecosystem functions relative to its contribution to biomass. This mechanism might limit the explanatory power of plant traits for predicting ecosystem functions.

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Supplemental material

Table S 2.1: Effects of FG combination on vegetation and soil properties. Means +- standard errors (SE) are shown for each FG combination. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 1). Significant effects ($p<0.05$) are shown in bold type and different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$).

				mean +- SE						
	df	**Test statistic**	**p**	**FG 1**	**FG 2**	**FG 3**	**FG 1&2**	**FG 2&3**	**FG 1&3**	**FG 1,2&3**
Above-ground biomass	3, 9	LRT = 6.83	0.336	0.983 +- 0.054	0.801 +- 0.092	1.15 +- 0.11	0.792 +- 0.026	0.941 +- 0.0374	0.929 +- 0.043	1.12 +- 0.024
Sward height	**3, 9**	**LRT = 15.75**	**0.015**	**43.3 +- 1.7 ab**	**40.8 +- 3.1 ab**	**65 +- 5.9 b**	**38.3 +- 0.96 a**	**54.1 +- 1.73 ab**	**51.2 +- 1.5 ab**	**58.5 +- 0.86 b**
Above-ground biomass C	3, 9	LRT = 2.96	0.814	41.1 +- 0.12	42 +- 0.21	41.9 +- 0.19	41.7 +- 0.063	42.1 +- 0.151	41.9 +- 0.081	41.9 +- 0.077
Above-ground biomass N	3, 9	LRT = 12.13	0.059	1.37 +- 0.037	1.41 +- 0.043	1.3 +- 0.043	1.35 +- 0.018	1.48 +- 0.0118	1.25 +- 0.014	1.45 +- 0.012
Above-ground biomass C:N	3, 9	LRT = 11.68	0.069	30.8 +- 0.94	30.7 +- 0.94	33.3 +- 0.93	31.7 +- 0.42	28.8 +- 0.263	33.9 +- 0.39	29.4 +- 0.23
Total root dry weight	3, 9	LRT = 8.93	0.178	236 +- 32	334 +- 29	226 +- 22	504 +- 38	444 +- 22.2	315 +- 15	382 +- 20
Fine root dry weight	3, 9	LRT = 12.13	0.059	127 +- 6.3	306 +- 23	188 +- 16	326 +- 19	326 +- 11.8	212 +- 8	266 +- 9.3
Coarse root dry weight	6, 51	F = 0.9	0.499	109 +- 29	28.6 +- 6.8	37.6 +- 8.7	177 +- 21	119 +- 14	103 +- 8.4	116 +- 11
Root:shoot ratio	**3, 9**	**LRT = 17.39**	**0.008**	**1.73 +- 0.19 a**	**7.09 +- 0.54 ab**	**3.29 +- 0.43 ab**	**6.56 +- 0.38 b**	**4.44 +- 0.153 ab**	**3.9 +- 0.16 ab**	**3.64 +- 0.12 ab**
Mean root diameter	3, 9	LRT = 5.70	0.457	0.254 +- 0.011	0.198 +- 0.0016	0.192 +- 0.0039	0.204 +- 0.0021	0.192 +- 0.00341	0.198 +- 0.0026	0.22 +- 0.0047
SRL	6, 50	F = 0.80	0.573	115 +- 12	76.4 +- 7.1	124 +- 13	83 +- 5.5	93.3 +- 13	110 +- 7.3	85.3 +- 3.2
RTD	6, 50	F = 1.03	0.419	0.323 +- 0.026	0.481 +- 0.05	0.318 +- 0.031	0.66 +- 0.062	0.609 +- 0.035	0.399 +- 0.024	0.442 +- 0.019
RDMC	**6, 50**	**F = 3.20**	**0.010**	**0.245 +- 0.004 a**	**0.279 +- 0.0058 ab**	**0.284 +- 0.0071 b**	**0.25 +- 0.0018 ab**	**0.269 +- 0.002 ab**	**0.258 +- 0.0015 ab**	**0.262 +- 0.001 ab**
Total fine root length	4, 10	LRT = 1.11	0.981	131 +- 8.2	243 +- 29	222 +- 27	201 +- 8	261 +- 29.8	201 +- 7.1	196 +- 6.9
Root C	3, 9	LRT = 6.68	0.352	44.8 +- 0.085	45.1 +- 0.086	43.6 +- 0.16	44.9 +- 0.1	44.4 +- 0.128	44.4 +- 0.082	44.4 +- 0.058
Root N	3, 9	LRT = 3.62	0.728	1.51 +- 0.068	1.45 +- 0.014	1.45 +- 0.088	1.67 +- 0.034	1.67 +- 0.0408	1.56 +- 0.021	1.61 +- 0.023
Root C:N ratio	3, 9	LRT = 3.55	0.737	31.5 +- 1.5	31.1 +- 0.26	31.5 +- 2	28.2 +- 0.68	27.6 +- 0.726	28.9 +- 0.36	28.5 +- 0.38
Soil pH	3, 9	LRT = 1.59	0.953	7.64 +- 0.022	7.68 +- 0.058	7.64 +- 0.016	7.65 +- 0.011	7.62 +- 0.0169	7.6 +- 0.0098	7.63 +- 0.009
Soil C	6, 51	F = 0.53	0.781	11.4 +- 0.46	10.8 +- 0.66	11.1 +- 0.3	10.9 +- 0.16	11.7 +- 0.074	9.82 +- 0.28	10.5 +- 0.12
Soil N	3, 9	LRT = 8.99	0.174	0.767 +- 0.026	0.725 +- 0.031	0.664 +- 0.025	0.747 +- 0.0051	0.777 +- 0.0143	0.677 +- 0.014	0.745 +- 0.0058
Soil C:N ratio	3, 9	LRT = 8.04	0.236	14.7 +- 0.3	14.7 +- 0.57	16.9 +- 0.47	14.5 +- 0.15	15.4 +- 0.348	14.2 +- 0.19	14.1 +- 0.093
Olsen P	3, 9	LRT = 11.91	0.064	4.95 +- 0.57	4.56 +- 0.71	2.38 +- 0.92	1.81 +- 0.11	4.48 +- 0.283	2.7 +- 0.23	2.73 +- 0.2
K_2SO_4-extractable N	**4, 10**	**LRT = 16.41**	**0.012**	**31.3 +- 2.2 ab**	**20.3 +- 3.6 a**	**41.8 +- 4.4 ab**	**22.9 +- 0.64 a**	**44.5 +- 2.26 b**	**36 +- 2.5 ab**	**44.7 +- 2.2 ab**
microbial C	4, 10	LRT = 9.33	0.156	238 +- 11	189 +- 11	261 +- 6.5	217 +- 3.5	220 +- 6.03	190 +- 5.9	231 +- 4.3
microbial N	4, 10	LRT = 8.43	0.208	152 +- 8.2	110 +- 5.5	148 +- 3.7	123 +- 2.1	126 +- 2.03	119 +- 3.4	136 +- 2.1
microbial C:N ratio	3, 9	LRT = 6.96	0.325	1.6 +- 0.024	1.71 +- 0.015	1.76 +- 0.012	1.75 +- 0.016	1.74 +- 0.0196	1.61 +- 0.028	1.69 +- 0.017
Fungal PLFA	3, 9	LRT = 11.02	0.088	7.85 +- 0.4	6.27 +- 1.5	5.87 +- 0.57	6.09 +- 0.15	6.16 +- 0.0807	4.92 +- 0.076	6.14 +- 0.13
Bacterial PLFA	3, 9	LRT = 11.12	0.085	56.6 +- 2.7	42.9 +- 6.9	44.7 +- 0.77	44.3 +- 0.74	47.3 +- 0.77	41.3 +- 0.79	51.3 +- 0.99
Fungal:bacterial ratio	6, 47	F = 0.71	0.644	0.141 +- 0.005	0.137 +- 0.0093	0.13 +- 0.011	0.14 +- 0.004	0.132 +- 0.0026	0.122 +- 0.002	0.121 +- 0.0016
Gram negative PLFA	3, 9	LRT = 12.36	0.054	34 +- 1.6	25.7 +- 4.1	26.1 +- 0.5	26.1 +- 0.46	27.7 +- 0.503	24.1 +- 0.45	30.3 +- 0.58
Gram positive PLFA	3, 9	LRT = 9.36	0.155	21.8 +- 1.1	16.7 +- 2.7	18 +- 0.27	17.6 +- 0.28	19 +- 0.267	16.6 +- 0.33	20.3 +- 0.39
Gram positive:negative ratio	**6, 47**	**F = 3.11**	**0.012**	**0.64 +- 0.0029 a**	**0.646 +- 0.0037 ab**	**0.689 +- 0.0034 ab**	**0.674 +- 0.0026 ab**	**0.688 +- 0.0037 b**	**0.689 +- 0.0022 b**	**0.668 +- 0.0024 ab**
Total PLFA	3, 9	LRT = 11.68	0.07	127 +- 5.8	96 +- 16	99 +- 1.9	98 +- 1.5	105 +- 1.65	91.7 +- 1.7	113 +- 2.2

Table S 2.2: Effects of presence/absence of each FG on vegetation properties. Means +- standard errors (SE) are shown for presence and absence of each FG. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 1). Significant effects ($p<0.05$) are shown in bold type.

					mean +- SE	
	FG present/ absent	**df**	**Test statistic**	**p**	**FG present**	**FG absent**
Above-ground biomass	FG 1	3, 4	LRT = 0.34	0.561	0.972 +- 0.0090	0.959 +- 0.023
	FG 2	3, 4	LRT = 0.09	0.766	0.952 +- 0.0090	0.998 +- 0.022
	FG 3	3, 4	LRT = 2.74	0.098	1.03 +- 0.010	0.836 +- 0.016
Sward height	FG 1	3, 4	LRT = 0.59	0.444	49.8 +- 0.34	53.5 +- 1.1
	FG 2	3, 4	LRT = 0.30	0.584	50.1 +- 0.38	52.7 +- 0.94
	FG 3	**3, 4**	**LRT = 12.4**	**<0.001**	**56.4 +- 0.42**	**40.2 +- 0.54**
Above-ground biomass C	FG 1	3, 4	LRT = 0.04	0.848	41.7 +- 0.023	42 +- 0.06
	FG 2	3, 4	LRT = 0.14	0.707	41.9 +- 0.027	41.7 +- 0.041
	FG 3	3, 4	LRT = 2.50	0.114	41.9 +- 0.028	41.6 +- 0.038
Above-ground biomass N	FG 1	3, 4	LRT = 0.71	0.399	1.37 +- 0.0044	1.41 +- 0.0091
	FG 2	**3, 4**	**LRT = 7.25**	**0.007**	**1.43 +- 0.0043**	**1.29 +- 0.0084**
	FG 3	3, 4	LRT = 0.21	0.648	1.39 +- 0.0044	1.37 +- 0.0092
Above-ground biomass C:N	FG 1	3, 4	LRT = 0.45	0.502	31.3 +- 0.10	30.5 +- 0.2
	FG 2	**3, 4**	**LRT = 6.61**	**0.010**	**30 +- 0.093**	**33 +- 0.21**
	FG 3	3, 4	LRT = 0.08	0.772	30.9 +- 0.099	31.2 +- 0.21
Total root dry weight	FG 1	3, 4	LRT = 0.01	0.932	375 +- 7.1	362 +- 10
	FG 2	**3, 4**	**LRT = 5.33**	**0.021**	**426 +- 8.1**	**275 +- 7.6**
	FG 3	3, 4	LRT = 0.01	0.931	359 +- 5.9	395 +- 16
Fine root dry weight	FG 1	3, 4	LRT = 0.09	0.767	248 +- 3.6	286 +- 6.3
	FG 2	**3, 4**	**LRT = 7.30**	**0.007**	**301 +- 4.0**	**183 +- 3.8**
	FG 3	3, 4	LRT = 0.12	0.726	254 +- 3.0	265 +- 8.5
Coarse root dry weight	FG 1	1, 56	F = 1.53	0.221	128 +- 4.0	75.9 +- 5.8
	FG 2	1, 56	F = 0.56	0.456	125 +- 4.5	92.2 +- 5.4
	FG 3	1, 56	F = 0.11	0.744	104 +- 3.3	130 +- 9.2
Root:shoot ratio	FG 1	3, 4	LRT = 0.03	0.859	4.26 +- 0.072	4.82 +- 0.13
	FG 2	3, 4	LRT = 2.12	0.145	5.06 +- 0.08	3.24 +- 0.087
	FG 3	3, 4	LRT = 2.55	0.110	3.85 +- 0.043	5.46 +- 0.2
Mean root diameter	FG 1	3, 4	LRT = 0.42	0.517	0.215 +- 0.0012	0.194 +- 0.0013
	FG 2	3, 4	LRT = 0.03	0.858	0.207 +- 0.0012	0.213 +- 0.0023
	FG 3	3, 4	LRT = 0.16	0.692	0.204 +- 0.0012	0.217 +- 0.0021
SRL	FG 1	1, 55	F = 0.01	0.910	95.2 +- 1.5	96.7 +- 4.9
	FG 2	**1, 55**	**F = 4.63**	**0.036**	**85.4 +- 1.7**	**114 +- 3.3**
	FG 3	1, 55	F = 0.21	0.652	98.3 +- 1.9	91 +- 2.8
RTD	FG 1	1, 55	F = 0.79	0.378	0.473 +- 0.01	0.504 +- 0.016
	FG 2	**1, 55**	**F = 4.30**	**0.043**	**0.547 +- 0.012**	**0.36 +- 0.0097**
	FG 3	1, 55	F = 0.13	0.725	0.454 +- 0.0072	0.529 +- 0.025
RDMC	**FG 1**	**1, 55**	**F = 12.51**	**<0.001**	**0.256 +- 0.00044**	**0.275 +- 0.0013**
	FG 2	1, 55	F = 0.24	0.627	0.262 +- 0.00052	0.259 +- 0.0012
	FG 3	1, 55	F = 3.63	0.062	0.265 +- 0.00049	0.254 +- 0.0012
Total fine root length	FG 1	4, 5	LRT = 0.03	0.864	189 +- 2.0	247 +- 11
	FG 2	4, 5	LRT = 0.02	0.885	216 +- 3.7	184 +- 3.9
	FG 3	4, 5	LRT = 0.06	0.804	215 +- 3.7	189 +- 4.3
Root C	FG 1	3, 4	LRT = 1.07	0.302	44.6 +- 0.021	44.4 +- 0.059
	FG 2	3, 4	LRT = 0.04	0.851	44.6 +- 0.025	44.4 +- 0.039
	FG 3	**3, 4**	**LRT = 3.88**	**0.049**	**44.3 +- 0.024**	**44.9 +- 0.039**
Root N	FG 1	3, 4	LRT = 0.16	0.687	1.6 +- 0.0075	1.56 +- 0.018
	FG 2	3, 4	LRT = 1.30	0.253	1.62 +- 0.0085	1.53 +- 0.014
	FG 3	3, 4	LRT = 0.06	0.804	1.59 +- 0.0079	1.58 +- 0.017
Root C:N ratio	FG_1	3, 4	LRT = 0.07	0.798	29 +- 0.14	29.5 +- 0.35
	FG_2	3, 4	LRT = 1.07	0.301	28.5 +- 0.15	30.2 +- 0.3
	FG_3	3, 4	LRT = 0.45	0.504	28.8 +- 0.14	29.8 +- 0.34

Table S 2.3: Effects of presence/absence of each FG on soil properties. Means +- standard errors (SE) are shown for presence and absence of each FG. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 1). Significant effects ($p<0.05$) are shown in bold type.

					mean +- SE	
	FG present/ absent	**df**	**Test statistic**	**p**	**FG present**	**FG absent**
Soil pH	FG 1	3, 4	LRT = 0.05	0.832	7.63 +- 0.0028	7.64 +- 0.009
	FG 2	3, 4	LRT = 0.11	0.735	7.64 +- 0.0036	7.62 +- 0.0051
	FG 3	3, 4	LRT = 1.11	0.292	7.62 +- 0.0031	7.65 +- 0.0066
soil_C	FG_1	1, 56	F = 0.41	0.526	10.6 +- 0.054	11.3 +- 0.089
	FG_2	1, 56	F = 0.09	0.769	10.9 +- 0.044	10.5 +- 0.13
	FG_3	1, 56	F = 0.74	0.392	10.6 +- 0.056	11.0 +- 0.1
Soil N	FG 1	3, 4	LRT = 0.03	0.870	0.731 +- 0.0027	0.736 +- 0.0073
	FG 2	3, 4	LRT = 3.66	0.056	0.75 +- 0.0023	0.7 +- 0.007
	FG 3	3, 4	LRT = 0.81	0.369	0.723 +- 0.0032	0.749 +- 0.0048
Soil C:N ratio	**FG 1**	**3, 4**	**LRT = 4.82**	**0.028**	**14.3 +- 0.039**	**15.6 +- 0.15**
	FG 2	3, 4	LRT = 0.2	0.658	14.6 +- 0.051	14.9 +- 0.1
	FG 3	3, 4	LRT = 0.01	0.937	14.7 +- 0.057	14.6 +- 0.082
Olsen P	**FG 1**	**3, 4**	**LRT = 3.89**	**0.048**	**2.75 +- 0.061**	**4.08 +- 0.17**
	FG 2	3, 4	LRT = 0.04	0.832	3.03 +- 0.067	3.24 +- 0.14
	FG 3	3, 4	LRT = 0.18	0.675	3.08 +- 0.073	3.14 +- 0.12
K_2SO_4-extractable N	FG 1	4, 5	LRT = 0.36	0.546	35.1 +- 0.6	37.8 +- 1.20
	FG 2	4, 5	LRT = 0.12	0.724	35.9 +- 0.66	35.8 +- 1.00
	FG 3	**4, 5**	**LRT = 9.96**	**0.002**	**41.9 +- 0.69**	**24.9 +- 0.54**
Microbial C	FG 1	4, 5	LRT = 0.34	0.562	218 +- 1.4	223 +- 3.0
	FG 2	4, 5	LRT = 0.83	0.361	220 +- 1.4	218 +- 3.1
	FG 3	4, 5	LRT = 0.01	0.918	220 +- 1.6	218 +- 2.3
Microbial N	FG 1	4, 5	LRT = 0.37	0.542	131 +- 0.83	127 +- 1.3
	FG 2	4, 5	LRT = 0.21	0.646	127 +- 0.68	135 +- 1.9
	FG 3	4, 5	LRT = 0.01	0.908	130 +- 0.77	129 +- 1.7
Microbial C:N ratio	FG 1	3, 4	LRT = 1.32	0.250	1.67 +- 0.0055	1.74 +- 0.0072
	FG 2	3, 4	LRT = 3.78	0.052	1.72 +- 0.005	1.64 +- 0.011
	FG 3	3, 4	LRT = 0.47	0.491	1.69 +- 0.0061	1.69 +- 0.0075
Fungal PLFA	FG 1	3, 4	LRT = 0.24	0.622	6.01 +- 0.043	6.12 +- 0.14
	FG 2	3, 4	LRT = 0.59	0.443	6.14 +- 0.049	5.84 +- 0.095
	FG 3	3, 4	LRT = 1.77	0.183	5.74 +- 0.038	6.58 +- 0.12
Bacterial PLFA	FG 1	3, 4	LRT = 0.18	0.670	47.3 +- 0.29	45.8 +- 0.68
	FG 2	3, 4	LRT = 0.49	0.482	47.5 +- 0.32	45.8 +- 0.60
	FG 3	3, 4	LRT = 0.03	0.855	46.7 +- 0.30	47.3 +- 0.66
Fungal:bacterial ratio	FG 1	1, 52	F = 0.32	0.574	0.129 +- 0.00076	0.133 +- 0.0016
	FG 2	1, 52	F = 0.05	0.822	0.131 +- 0.00088	0.129 +- 0.0013
	FG 3	1, 52	F = 3.29	0.075	0.125 +- 0.00062	0.14 +- 0.0019
Gram negative PLFA	FG 1	3, 4	LRT = 0.23	0.633	27.9 +- 0.18	26.9 +- 0.41
	FG 2	3, 4	LRT = 0.48	0.486	28 +- 0.19	27 +- 0.36
	FG 3	3, 4	LRT = 0.15	0.702	27.4 +- 0.18	28.1 +- 0.4
Gram positive PLFA	FG 1	3, 4	LRT = 0.11	0.736	18.7 +- 0.11	18.3 +- 0.27
	FG 2	3, 4	LRT = 0.47	0.493	18.8 +- 0.12	18.2 +- 0.23
	FG 3	3, 4	LRT = 0.01	0.913	18.6 +- 0.12	18.5 +- 0.25
Gram positive:negative ratio	FG 1	1, 52	F = 0.59	0.444	0.672 +- 0.00076	0.679 +- 0.002
	FG 2	1, 52	F = 0.19	0.668	0.672 +- 0.00087	0.676 +- 0.0016
	FG 3	**1, 52**	**F = 6.35**	**0.015**	**0.681 +- 0.00082**	**0.66 +- 0.0015**
Total PLFA	FG 1	3, 4	LRT = 0.09	0.758	105 +- 0.63	102 +- 1.50
	FG 2	3, 4	LRT = 0.51	0.476	105 +- 0.68	102 +- 1.30
	FG 3	3, 4	LRT = 0.07	0.795	103 +- 0.64	105 +- 1.40

Table S 2.4: Effects of FG combination (a) and for presence and absence of each FG (b) on traits of focal plant individuals of *Daucus*. Means +- standard errors (SE) are shown for each FG combination and for presence and absence of each FG. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 2). Significant effects ($p<0.05$) are shown in bold type and different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$).

a)				**mean +- SE**			
	df	**Test statistic**	**p**	**FG_1**	**FG_1_2**	**FG_1_3**	**FG_1_2_3**
Height	**3, 6**	**LRT = 11.06**	**0.011**	**52.7 +- 1.2 a**	**56.8 +- 3 ab**	**64.2 +- 1.3 b**	**64.8 +- 1.7 b**
Shoot dry weight	3, 17	F = 1.19	0.344	2.06 +- 0.18	2.38 +- 0.21	4.31 +- 0.56	3.71 +- 0.22
Height/ shoot dry weight	3, 17	F = 0.64	0.602	32 +- 2.5	24.8 +- 1	29.2 +- 4.3	18.8 +- 1.2
SLA	3, 6	LRT = 3.72	0.293	12.1 +- 0.34	10.3 +- 0.16	10.9 +- 0.31	12.3 +- 0.5
LDMC	3, 17	F = 0.65	0.593	0.335 +- 0.011	0.385 +- 0.0042	0.357 +- 0.011	0.37 +- 0.011
Leaf C	3, 6	LRT = 3.39	0.336	39.5 +- 0.22	40.2 +- 0.23	39.4 +- 0.11	40.1 +- 0.17
Leaf N	3, 6	LRT = 7.15	0.067	1.27 +- 0.033	1.19 +- 0.067	1.42 +- 0.04	1.57 +- 0.088
Leaf C:N ratio	3, 6	LRT = 7.42	0.06	31.9 +- 0.88	34.9 +- 1.6	28.3 +- 0.84	26.9 +- 1.4

b)					**mean +- SE**	
	FG present/absent	**df**	**Test statistic**	**p**	**FG present**	**FG absent**
Height	FG 2	3, 4	LRT = 0.84	0.359	61.2 +- 0.98	58.2 +- 0.87
	FG 3	**3, 4**	**LRT = 9.77**	**0.002**	**64.5 +- 0.7**	**54.3 +- 0.89**
Shoot dry weight	FG 2	1, 19	F = 0.1	0.759	3.54 +- 0.28	2.81 +- 0.12
	FG 3	1, 19	F = 3.38	0.082	4.04 +- 0.23	2.19 +- 0.096
Height/ shoot dry weight	FG 2	1, 19	F = 0.03	0.876	27.4 +- 1.9	26 +- 1.2
	FG 3	1, 19	F = 1.71	0.207	24.4 +- 1.8	29.1 +- 1.2
SLA	FG 2	3, 4	LRT = 3.7	0.055	10.7 +- 0.15	12.2 +- 0.2
	FG 3	3, 4	LRT = 0.03	0.852	11.6 +- 0.2	11.4 +- 0.18
LDMC	FG 2	1, 19	F = 0.46	0.505	0.368 +- 0.0051	0.351 +- 0.0057
	FG 3	1, 19	F = 0.10	0.758	0.363 +- 0.0054	0.355 +- 0.0057
Leaf C	FG 2	3, 4	LRT = 0.01	0.938	39.7 +- 0.083	39.8 +- 0.099
	FG 3	3, 4	LRT = 0.05	0.827	39.7 +- 0.071	39.8 +- 0.12
Leaf N	FG 2	3, 4	LRT = 0.34	0.561	1.33 +- 0.027	1.41 +- 0.032
	FG 3	**3, 4**	**LRT = 5.72**	**0.017**	**1.49 +- 0.03**	**1.24 +- 0.022**
Leaf C:N ratio	FG 2	3, 4	LRT = 0.3	0.581	31 +- 0.63	29.6 +- 0.57
	FG 3	**3, 4**	**LRT = 5.84**	**0.016**	**27.7 +- 0.51**	**33.1 +- 0.57**

Table S 2.5: Effects of FG combination (a) and for presence and absence of each FG (b) on traits of focal plant individuals of *Clinopodium*. Means +- standard errors (SE) are shown for each FG combination and for presence and absence of each FG. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 2). Significant effects ($p<0.05$) are shown in bold type and different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$).

a)				**mean +- SE**			
	df	**Test statistic**	**p**	**FG 2**	**FG 1&2**	**FG 2&3**	**FG 1,2&3**
Height	3, 18	F = 1.8	0.183	45.7 +- 2	42.4 +- 1.8	52.8 +- 2.1	54.2 +- 1.2
Shoot dry weight	3, 18	F = 1.14	0.361	0.782 +- 0.04	0.553 +- 0.038	0.702 +- 0.038	0.705 +- 0.033
Height/ shoot dry weight	3, 18	F = 1.56	0.234	60.4 +- 2.1	82.3 +- 4.9	80 +- 5.9	80.4 +- 2.9
SLA	3, 6	LRT = 1.51	0.679	18.1 +- 0.36	16.4 +- 0.5	19.6 +- 1.4	17.7 +- 0.55
LDMC	**3, 6**	**LRT = 9.40**	**0.024**	**0.336 +- 0.0047 a**	**0.457 +- 0.024 b**	**0.35 +- 0.013 ab**	**0.346 +- 0.0086 ab**
Leaf C	3, 18	F = 1.65	0.213	44.7 +- 0.073	44.5 +- 0.1	44.7 +- 0.079	43.8 +- 0.21
Leaf N	3, 6	LRT = 2.29	0.515	1.51 +- 0.045	1.33 +- 0.053	1.45 +- 0.065	1.36 +- 0.024
Leaf C:N ratio	3, 6	LRT = 1.98	0.577	30.3 +- 0.86	34.6 +- 1.6	32.4 +- 1.8	32.5 +- 0.66

b)					**mean +- SE**	
	FG present/absent	**df**	**Test statistic**	**p**	**FG present**	**FG absent**
Height	FG 1	1, 20	F = 0	0.984	48.8 +- 0.88	48.9 +- 1
	FG 3	**1, 20**	**F = 5.5**	**0.029**	**53.5 +- 0.76**	**44.2 +- 0.93**
Shoot dry weight	FG 1	1, 20	F = 1.55	0.228	0.636 +- 0.018	0.746 +- 0.019
	FG 3	1, 20	F = 0.08	0.782	0.704 +- 0.017	0.678 +- 0.022
Height/ shoot dry weight	FG 1	1, 20	F = 2.21	0.152	81.2 +- 1.8	69.3 +- 2.1
	FG 3	1, 20	F = 1.34	0.260	80.2 +- 2	70.4 +- 1.9
SLA	FG 1	3, 4	LRT = 0.98	0.322	17.1 +- 0.27	18.8 +- 0.42
	FG 3	3, 4	LRT = 0.3	0.585	18.6 +- 0.45	17.3 +- 0.22
LDMC	FG 1	3, 4	LRT = 2.61	0.106	0.397 +- 0.0093	0.342 +- 0.0041
	FG 3	3, 4	LRT = 1.62	0.203	0.348 +- 0.0049	0.391 +- 0.0092
Leaf C	FG 1	1, 20	F = 2.86	0.106	44.1 +- 0.093	44.7 +- 0.037
	FG 3	1, 20	F = 1.01	0.328	44.3 +- 0.096	44.6 +- 0.041
Leaf N	FG 1	3, 4	LRT = 1.61	0.204	1.35 +- 0.018	1.49 +- 0.026
	FG 3	3, 4	LRT = 0.08	0.776	1.4 +- 0.021	1.43 +- 0.025
Leaf C:N ratio	FG 1	3, 4	LRT = 1.14	0.285	33.5 +- 0.52	31.2 +- 0.62
	FG 3	3, 4	LRT = 0.03	0.873	32.5 +- 0.57	32.3 +- 0.59

Table S 2.6: Effects of FG combination (a) and for presence and absence of each FG (b) on traits of focal plant individuals of *Leucanthemum*. Means +- standard errors (SE) are shown for each FG combination and for presence and absence of each FG. Significant differences were tested using either ANOVA without random effects, in which case F-values are reported, or, when there was a pattern in the model residuals, using likelihood ratio testing (LRT) with focal species and/or row as random effect (random effect structure see table 2). Significant effects ($p<0.05$) are shown in bold type and different letters indicate significant differences identified by Tukey post hoc test ($p < 0.05$).

a)				**mean +- SE**			
	df	**Test statistic**	**p**	**FG 3**	**FG 1&3**	**FG 2&3**	**FG 1,2&3**
Height	3, 6	LRT = 1.18	0.757	59 +- 2	57.2 +- 0.93	55.7 +- 1.4	56 +- 1.6
Shoot dry weight	3, 6	LRT = 2.69	0.441	0.806 +- 0.06	0.958 +- 0.075	1.48 +- 0.18	0.796 +- 0.11
Height/ shoot dry weight	3, 6	LRT = 3.58	0.31	83.3 +- 4.8	71.5 +- 5.8	66.4 +- 8.2	90.1 +- 8.5
SLA	3, 6	LRT = 1.73	0.63	18.6 +- 0.36	19.3 +- 0.75	17.7 +- 0.98	20.3 +- 0.74
LDMC	3, 6	LRT = 3.95	0.267	0.232 +- 0.0064	0.21 +- 0.012	0.253 +- 0.013	0.239 +- 0.011
Leaf C	**3, 19**	**F = 3.6**	**0.033**	**42.1 +- 0.17**	**40.6 +- 0.18**	**42.1 +- 0.16**	**42.2 +- 0.12**
Leaf N	3, 6	LRT = 3.74	0.29	1.62 +- 0.088	1.5 +- 0.046	1.82 +- 0.051	1.74 +- 0.038
Leaf C:N ratio	3, 19	F = 1.05	0.394	27.9 +- 1.2	27.8 +- 0.90	23.7 +- 0.64	24.4 +- 0.55

b)					**mean +- SE**	
	FG present/absent	**df**	**Test statistic**	**p**	**FG present**	**FG absent**
Height	FG 1	3, 4	LRT = 0.13	0.72	56.6 +- 0.59	57.3 +- 0.83
	FG 2	3, 4	LRT = 0.13	0.72	56.4 +- 0.56	57.6 +- 0.91
Shoot dry weight	FG 1	3, 4	LRT = 0.39	0.531	0.884 +- 0.043	1.14 +- 0.071
	FG 2	3, 4	LRT = 2.03	0.154	1.22 +- 0.07	0.801 +- 0.039
Height/ shoot dry weight	FG 1	3, 4	LRT = 0.42	0.516	80 +- 3.4	74.9 +- 3.3
	FG 2	3, 4	LRT = 2.63	0.105	68.9 +- 3.4	86.4 +- 3.1
SLA	FG 1	3, 4	LRT = 0.98	0.323	19.7 +- 0.36	18.2 +- 0.35
	FG 2	3, 4	LRT = 0.61	0.434	18.5 +- 0.42	19.4 +- 0.27
LDMC	FG 1	3, 4	LRT = 2.11	0.147	0.223 +- 0.0056	0.243 +- 0.0049
	FG 2	3, 4	LRT = 0.00	0.983	0.231 +- 0.0061	0.236 +- 0.004
Leaf C	FG 1	1, 21	F = 2.76	0.112	41.3 +- 0.11	42.1 +- 0.08
	FG 2	1, 21	F = 3.09	0.093	41.3 +- 0.1	42.1 +- 0.075
Leaf N	FG 1	3, 4	LRT = 0.33	0.566	1.61 +- 0.024	1.72 +- 0.035
	FG 2	3, 4	LRT = 0	0.972	1.66 +- 0.027	1.68 +- 0.036
Leaf C:N ratio	FG 1	1, 21	F = 0.05	0.832	26.3 +- 0.41	25.8 +- 0.5
	FG 2	1, 21	F = 0.06	0.804	25.7 +- 0.41	26.3 +- 0.53

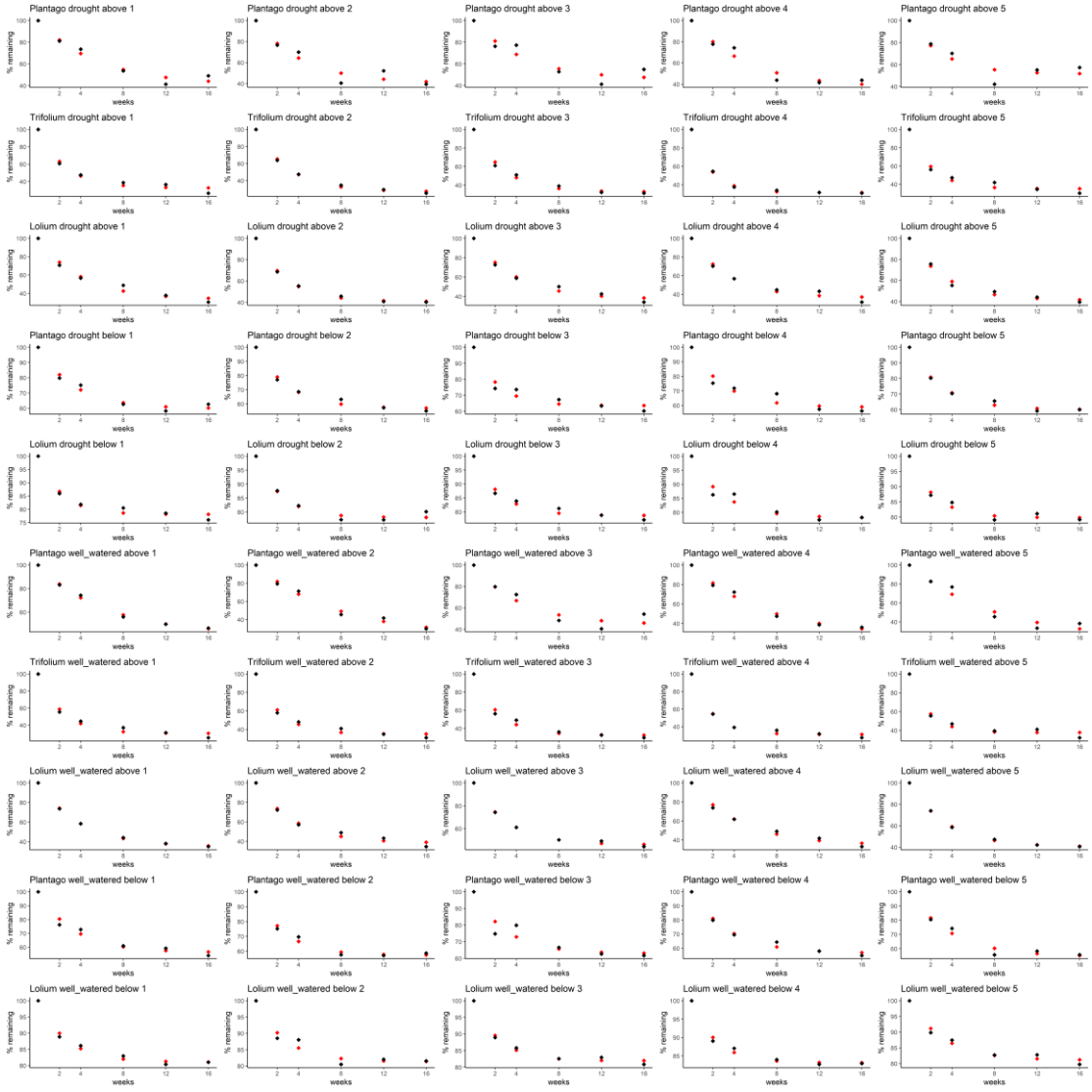

Figure S 5.1: Measured (black dots) and modelled (red dots) % litter mass remaining after 0, 2, 4, 8, 12 and 16 weeks. The model was an asymptotic exponential model (Eq.4) fit to the data using nonlinear least squares regression.