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PLANT, SOIL AND MICROBIAL CONTROLS ON GRASSLAND DIVERSITY
RESTORATION: A LONG-TERM, MULTI-SITE MESOCOSM EXPERIMENT

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Running Title: Priority effects are drivers of diversity restoration.

SUMMARY

1. The success of grassland biodiversity restoration schemes is determined by many factors; as such their outcomes can be unpredictable. There is a need for improved understanding of the relative importance of belowground factors to restoration success, such as contrasting soil type and management intensities, as well as plant community composition and order of assembly.
2. We carried out an eight-year mesocosm experiment across three locations in the UK to explore the relative and interactive roles of various aboveground and belowground factors in the establishment of target species, to determine general constraints on grassland restoration. Each location had a series of mesocosms with contrasting soil types and management status, which were initially sown with six grasses typical of species-poor grasslands targeted for restoration.
3. Over five years, sets of plant species were added, to test how different vegetation treatments, including early-coloniser species and the hemiparasite *Rhinanthus minor*, and soil type and management, influenced the establishment of target plant species and community diversity.
4. The addition of early-coloniser species to model grasslands suppressed the establishment of target species, indicating a strong priority effect. Soil type was also

an important factor, but effects varied considerably across locations. In the absence of early-coloniser species, low soil nutrient availability improved establishment of target species across locations, although *R. minor* had no beneficial effect.

5. *Synthesis and applications.* Our long-term, multi-site study indicates that successful restoration of species rich grassland is dependent primarily on priority effects, especially in the form of early-coloniser species that suppress establishment of slow-growing target species. We also show that priority effects vary with soil conditions, being stronger in clay than sandy soils, and on soils of high nutrient availability. As such, our work emphasises the importance of considering priority effects and local soil conditions in developing management strategies for restoring plant species diversity in grassland.

Key-words: ecological restoration; grassland; nutrients; plant-soil interactions; plant species composition; priority effects; soil; soil microbial community

INTRODUCTION

The introduction of intensive farming practices across Europe has led to declines of once widespread traditionally managed, species-rich meadows (Smith *et al.* 2003; Bullock *et al.* 2011). This has resulted in the widespread implementation of agri-environment schemes, which offer incentives for farmers to manage their land to enhance botanical diversity and the delivery of ecosystem services (Whittingham 2011). While many different approaches have been proposed, they have had mixed success, largely because different factors constrain restoration success in different contexts (Török *et al.* 2011; Bucharova *et al.* 2016).

A key goal of ecological restoration is the introduction and establishment of late-colonising grassland species, henceforth ‘target species’ (Pywell *et al.* 2002; Kiehl *et al.* 2010). While grasslands can establish quickly upon areas of bare ground, many factors can constrain the restoration of target species, but their relative importance is poorly understood (Pywell *et al.* 2007; Kiehl *et al.* 2010). Indeed, there is much debate as to the relative importance of different abiotic and biotic factors, such soil type and fertility, land management, and the order of plant species arrival and their resultant impact on the soil environment (known as priority effects), in the establishment of target species (Fukami *et al.* 2005; Ejrnaes *et al.* 2006; von Gillhaussen *et al.* 2014). Priority effects have received much attention because the order of arrival of plant species or groups can impact establishment success of target species and ecosystem functioning. In grasslands, for example, von Gillhaussen *et al.* (2014) demonstrated that an initial legume dominated sward can have strong and lasting impacts on later community dynamics compared with grassy swards, and Wilsey *et al.* (2015) who found a similar effect when exotic grasses were added before natives.

A potential constraint on ecological restoration is intensive land management, which typically creates a legacy of nutrient rich soil with bacterial dominated microbial communities; conditions that promote the growth of fast-growing plant species that readily utilize available nutrients and competitively exclude target species (Maskell *et al.* 2009; De Vries *et al.* 2012). Suppressive effects of fast-growing species that typically establish in moderately fertile grasslands and during early stages of restoration are potentially key because of their effects on soil chemistry and microbes, as well as through competitively excluding target species. If suppression remains after a number of years of restoration, this indicates a priority effect (Fukami *et al.* 2005; Plückers *et al.* 2014). For instance, Kardol and colleagues (2006) showed that soils conditioned by early-coloniser species are less favourable for the establishment of target species, and Ejrnaes *et al.* (2006) found strong and consistent effects

of arrival order on species richness. Kardol observed that while early-coloniser species were unaffected by soil conditioning, target species exhibited significantly higher growth in soils conditioned by other target species compared with early-colonisers. Moreover, successful restoration might be influenced by soil microbial community composition; soils with fungal-dominated communities are associated with more conservative nutrient cycles and are more conducive to the establishment of target species than soils with bacterial dominated communities, which often have high rates of nutrient mineralisation (Donnison *et al.* 2000; Smith *et al.* 2003). It is unclear if observed shifts in microbial communities are a cause or effect of diversity restoration (Smith *et al.* 2003), although there is evidence that arbuscular mycorrhizal (AM) fungi can impede early-coloniser establishment with benefits for slow-growing plant species (Francis & Read 1995), and that fungi are effective in immobilizing soil nutrients, thereby reducing nutrient availability to fast-growing plants (Bardgett *et al.* 2003; De Vries *et al.* 2012). Further, early successional soils often have bacterial dominated microbial communities, which become increasingly fungal dominated as succession proceeds and soil organic matter increases (Bardgett *et al.* 2005; Cline & Zak 2015).

Attempts have also been made to reduce the competitive dominance of highly competitive species, and hence promote diversity restoration, through the introduction of the facultative root hemiparasite *Rhinanthus minor* (Bullock & Pywell 2005). *R. minor* is known to infect and reduce the competitive dominance of fast-growing grassland species, thereby allowing slower growing species to increase in abundance (Joshi *et al.* 2000; Hautier *et al.* 2010).

There is evidence that *R. minor* is associated with shifts in the composition of soil microbial communities, causing an increase in the abundance of bacteria relative to fungi, and accelerated N cycling (Bardgett *et al.* 2006), which is likely to offset its value for promoting slower growing species. Moreover, effects of *R. minor* on plant cover continue past the short life span of the plant, indicating that it may have long-lasting effects on plant species

diversity (Hartley *et al.* 2015). The importance of *R. minor* relative to other factors, such as soil type and management, remains to be tested.

Our goal was to identify the dominant factors that promote, or impede, the establishment of target species, and determine the success of ecological restoration in mesotrophic grassland.

We tested the relative and interactive roles of different vegetation treatments, soil type, and historic management on the establishment of target species commonly used in diversity restoration. Our first hypothesis was that the primary factor impeding restoration success is the presence of competitive grass and forb species, which exhibit priority effects by excluding target species, and that this effect is especially pronounced in intensively managed soils. We further hypothesised that soil microbial community structure plays a secondary role, with a high abundance of fungi relative to bacteria and associated changes in soil nutrient availability offering a higher probability of restoration success, as does the presence of *R. minor* which suppresses fast-growing species. To investigate these hypotheses, we carried out an eight-year mesocosm experiment across three locations in England with contrasting soil conditions, with two soil types per site and an intensive and extensive management variant of each. We began with a depauperate mix of grass species typical of intensively managed agricultural grasslands targeted for diversity restoration (Pywell *et al.* 2002), which were allowed to establish for one year before adding a mix of early-coloniser species to half of the mesocosms and *R. minor* to half in a full factorial design. The early-coloniser species were chosen to represent a group of species which rapidly colonise moderately fertile agricultural grasslands (Pywell *et al.* 2003), and have marked effects on soil properties when grown in monoculture, including changes in soil microbial community structure (Innes *et al.* 2004; Harrison and Bardgett 2010). Following this, we added a set of target species to every mesocosm. The target species were a selection of slow-growing poorly competitive species typical of species rich grasslands of high nature conservation value,

including some species that were adapted to local conditions for each site. Their establishment success in light of prior establishment of more competitive species and associated alterations of soil characteristics was assessed after four years. The factorial design explicitly allowed us to test the relative importance of soil type, nutrient content and microbial community, relative to colonization order of grassland species, which has not been tested in such a long-term study across different sites. The effect of these species on establishment of target plant species and total community diversity were evaluated, as well as how these treatments influenced soil chemistry and microbial community composition.

MATERIALS AND METHODS

STUDY SITES AND EXPERIMENTAL DESIGN

The experiment was replicated at three locations across England: the University of Newcastle (54°59'N, 1°48'W), University of Reading (51°28'N, 0°54'W), and Rothamsted Research, North Wyke (50°46'N, 3°54'W), representing climatic conditions of the north-east, south-east and south-west of England, respectively (Table S1). At each location, 64 mesocosms were set up in April 2004 (80 cm diameter, 50 cm deep), which were buried into the ground to the top of the pot. The pots were placed on 15-10 cm of gravel to aid drainage. Mesocosms were then filled with two different soil types common to agricultural grasslands of each region: clay loam and sandy alluvial soils at Newcastle; chalk loam (brown rendzina) and neutral (clay loam) soils at Reading; and clay and silt-based alluvial soils at North Wyke (Table 1). For each soil type at each location, a history of intensive or extensive land management variant was included in the design, derived from paired grasslands on the same soil type that had been subject to either long term fertiliser use ($>100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ for >20 years), high grazing pressures and frequent cutting for silage, or extensive management with no known history of

fertiliser application, low grazing pressures and an annual hay cut (Ward *et al.* 2016). As shown previously, such historic management leads to differences in soil conditions, with soils of intensively managed grasslands having higher nutrient (N and P) content and availability of inorganic nutrients than extensively managed grasslands (Donnison *et al.* 2000; De Vries *et al.* 2012; hereafter, these soils are referred to as intensive and extensive management respectively).

A mixture of six common grassland species was hand sown into each mesocosm in September 2004 (Table S2; *Lolium perenne*, *Agrostis capillaris*, *Poa trivialis*, *Alopecurus pratensis*, *Holcus lanatus* and *Phleum pratense*, 1000 seeds per species per mesocosm; Emorsgate Seeds, Kings Lynn, UK), which were allowed to establish for one year. These species were chosen in order to simulate a baseline pre-restoration community of rank grassland. In September 2005, two more treatments were added to the full factorial design.

First, to half of the mesocosms we added a mix of the following species, 3g per species:

Lotus corniculatus, *Prunella vulgaris*, *Ranunculus acris*, *R. bulbosus*, *Anthoxanthum odoratum*, *Trifolium pratense*, and *Plantago lanceolata*. The species are termed early-coloniser due to their competitive abilities and rapidity of colonisation during restoration. The

hemiparasite *R. minor* was added to another half of the pots. This design was a full factorial, and yielded 64 mesocosms per location (2 soil x 2 management x 2(±) early-coloniser species

x 2(±) *R. minor* x 4 replicates). All mesocosms were subjected to simulated grazing and trampling in spring and autumn to simulate disturbances that occur in the field, using an

artificial hoof. Aboveground vegetation was harvested to 5 cm in July each year, with hay left on top of the mesocosms for one week with the cut herbage turned once to release seed; a

common management strategy. Farmyard manure (FYM) was added annually to all

mesocosms at Newcastle, and once in 2005 in Reading and North Wyke, to simulate farming practice.

In September 2007, two years after the early-coloniser species and *R. minor* treatments had been established, species typical of high nature value species rich grasslands (Rodwell 1992), were added to all mesocosms at all locations (Table S2). The two year “conditioning” period was considered sufficient time for the early-coloniser species and *R. minor* to establish and modify the soils through plant-soil feedback, thereby indirectly affecting restoration success. The target species were *Briza media*, *Centaurea nigra*, *Galium verum*, *Knautia arvensis*, *Leontodon hispidus*, *Pimpinella saxifraga*, *Primula veris*, *Succisa pratensis* and *Trisetum flavescens*. We also added a number of extra species at each location that were specific to grasslands of those locations. At Reading, *Sanguisorba. minor*, *Stachys officinalis*, *Filipendula vulgaris* and *Achillea ptarmica*; at Newcastle, *S. minor*, *Geranium sylvaticum* and *Achillea millefolium* and at North Wyke, *Serratula tinctoria*, *S. officinalis* and *A. ptarmica* were added.

SOIL AND VEGETATION ANALYSES

Each mesocosm was divided into a 10 cm x 10 cm sampling grid of cells. Five cells were randomly selected in September 2005 for soil sampling and a different random selection were sampled after the July harvest in 2011. A single soil core (1.5 cm diameter, 7.5 cm depth) was removed from each cell. The five samples from each mesocosm were passed through a 6 mm sieve and combined to produce a composite sample. The soil sampling in 2005 was carried out in September due to extremely dry soil conditions earlier in the season. The soils taken at the beginning and end of the study were air-dried and total carbon, nitrogen and phosphorus, Olsen extractable phosphorus, exchangeable potassium (K), calcium (Ca), magnesium (Mg), sodium (Na) and pH were measured using standard methodology (Allen 1989). In July 2011, aboveground vegetation was harvested from the same five cells as the

soil samples prior to the soil sampling, and combined to form a composite sample. Harvested vegetation was sorted to species level and material was oven dried at 80°C for 24 hours to measure dry weight of individual species.

To assess the biomass and structure of the soil microbial community, ester-linked phospholipid fatty acid (PLFA) composition was analysed in 2005 and 2011. Lipids were extracted from 1.5g fresh soil (Frostegård *et al.* 1991), and separated fatty acid methyl-esters were identified and quantified by chromatographic retention time and mass spectral comparison on a Hewlett Packard 5890 II gas chromatograph equipped with a 5972A mass selective detector (MSD II), using standard methyl ester mix ranging from C11 to C20 (Supelco UK, Poole, UK). The abundance of individual fatty acid methyl-esters was expressed as $\mu\text{g PLFA g}^{-1}$ dry soil and fatty acid nomenclature followed Frostegård *et al.* (1993). The PLFAs i15:0, a15:0, 15:0, i16:0, 17:0, cy17:0, 18:1 ω 7 and cy19:0 represented bacterial biomass (Federle, 1986; Frostegård *et al.*, 1993) and 18:2 ω 6 was used for fungal biomass (Federle, 1986), enabling calculation of fungal to bacterial PLFA ratios (Bardgett *et al.*, 1996). Actinomycetes were identified as fatty acids containing a methyl group, i.e. 10Me16:0, 10Me17:0 and 10Me18:0.

STATISTICAL ANALYSIS

The statistical analyses were split into two sets. First, we examined how soil type and management, early-coloniser species and *R. minor* treatments had affected biomass and species richness of both the target species group, and the total community of each mesocosm in 2011. Biomass and species richness of target plants and the total community were analysed using two-way Analysis of Variance (ANOVA) models in R3.1.0 (R Core Team 2013). Data were determined to fit the requirements of ANOVA using Box-Cox transformation and log

transformation. Each location was analysed independently, and models were not simplified to remove non-significant effects.

Subsequently we used two-way ANOVA to analyse the soil microbial community and chemistry data at each site in 2005 using soil type and management as the explanatory variables with an interaction term. We followed this with an analysis using all four treatments, using data from 2011 after the final harvest.

Finally, we used statistical modelling to identify the most important drivers of target species richness and biomass, and total community species richness and biomass at each site in 2011.

We began with a linear model that contained soil type and management, and two-way interactions and quadratic effects, before simplifying using likelihood ratio deletion tests and evaluating using Akaike's Information Criterion for small sample sizes (AICc; Hurvich & Tsai 1989). When the minimum adequate model was obtained, the next level included all soil chemistry variables, and all two-way interactions. The third step was to include total PLFA, bacterial, fungal and Actinomycete PLFA, and fungal to bacterial ratio. This was the final step for the total community biomass and species richness, but we added a further step for the target species to see if certain plant groups were directly affecting their establishment. It included biomass of the original six grass species, early-coloniser species, and *R. minor*. The final minimum adequate model offered the most parsimonious set of descriptors for successful establishment of target species.

RESULTS

STARTING CONDITIONS

In 2005, soil nutrient content varied with soil type and management, although patterns were not consistent across sites (Table 1). Generally, chalk soils at Reading were the most nutrient rich, while at the other two sites, management had left a legacy effect that was apparent as elevated nutrients in clay soils, but less frequently in alluvial soils where both management types had similar nutrient concentrations. At Reading, chalk soil was more alkaline than neutral soil ($F_{1,57}=128.23$, $p<0.001$), whereas at the other two sites sandy alluvial soil was more alkaline than clay soil. However, this effect varied with management: at Newcastle, extensively managed soils were more alkaline (interaction: $F_{1,57}=32.17$, $p<0.001$), while at North Wyke, soils with a history of intensive management were more alkaline (interaction: $F_{1,57}=12.99$, $p<0.001$). The C:N ratio was greater in chalk than neutral soil at Reading, especially in extensively managed soil (interaction: $F_{1,57}=43.00$, $p<0.001$). At Newcastle and North Wyke, the C:N ratio was higher in clay soils than sandy alluvial across management levels ($F_{1,57}=24.93$, $p<0.001$ and $F_{1,57}=60.17$, $p<0.001$ respectively).

At all sites, soil type explained variation in total microbial biomass, as measured by PLFA.

At Reading, total microbial biomass was greater in neutral than chalk soil (Table S3: $F_{1,57}=4.80$, $p=0.033$), whereas at Newcastle and North Wyke, this measure was greater in clay loam than sandy alluvial soil (Table S3: $F_{1,57}=54.09$, $p<0.001$; $F_{1,57}=40.87$, $p<0.001$). Total microbial biomass was not influenced by management at Reading or North Wyke, but at Newcastle total microbial biomass was higher in extensively managed than intensively managed clay loam soils, while in sandy alluvial soils, there was no effect (interaction: $F_{1,57}=12.25$, $p<0.001$).

Soil type, but not management, also affected the fungal to bacterial ratio at Reading, being greater in chalk than neutral soil ($F_{1,57}=4.92$, $p=0.031$), while at Newcastle and North Wyke no effect of soil type or management was observed.

TARGET SPECIES ESTABLISHMENT

In 2011, the biomass of added target species was affected by the presence of early-coloniser species and soil type, with no detectable effect of *R. minor* or management. As predicted, the primary driver of target species establishment was early-coloniser presence at all three sites, which had a highly suppressive effect (Reading: $F_{1,53}=12.90$, $p<0.001$). At Newcastle and North Wyke, this effect was stronger on clay than sandy alluvial soil (Figure 1; Newcastle: interaction: $F_{1,53}=18.05$, $p<0.001$, North Wyke: Soil type: $F_{1,53}=12.26$, $p<0.001$, early-coloniser species: $F_{1,53}=41.62$, $p<0.001$), while at Reading the effect was consistent across both soil types.

Target species richness was also primarily dictated by presence of early-coloniser species, with some soil effects. Management and *R. minor* again had no effect on target species richness. The target species was the most species-rich group at both Reading and Newcastle, while at North Wyke the original six grasses sown in 2004 contributed more to species richness on average. Target species accounted for 73%, 36%, 32% of the total number of species within communities at Reading (8 spp.), Newcastle (5 spp.) and North Wyke (3 spp.) respectively (Figure 2). At Reading, chalk soils had a higher target species richness than neutral soils, and early-coloniser species suppressed species richness of target plants in both soil types (interaction: $F_{1,53}=5.66$, $p=0.021$). At both Newcastle and North Wyke, species richness of target plants was suppressed by early-coloniser species (Newcastle: $F_{1,53}=55.28$,

$p < 0.001$, North Wyke: $F_{1,53} = 26.15$, $p < 0.001$), and at North Wyke, species richness of target plants was greater on sandy alluvial soils than clay ($F_{1,53} = 16.47$, $p < 0.001$).

TOTAL COMMUNITY ESTABLISHMENT

At the final harvest in 2011, total aboveground biomass was affected by soil type and addition of early-coloniser species (Figure 1). Aboveground biomass was greatest at Newcastle and lowest at Reading. At Reading, soil type was the main driver of aboveground biomass, while early-colonisers had no effect. Total aboveground biomass was higher in chalk soils, especially when *R. minor* was present. On neutral soils, *R. minor* had no effect on overall biomass (Soil type: $F_{1,53} = 9.36$, $p = 0.004$, *R. minor*: $F_{1,53} = 20.68$, $p < 0.001$). At Newcastle and North Wyke, sandy alluvial soil had higher total biomass than clay soil, but there were contrasting effects of early-coloniser species presence; at Newcastle, total biomass was lower when early colonisers were present, while at North Wyke total biomass was higher (Newcastle: soil type x early-colonisers: $F_{1,53} = 8.61$, $p = 0.005$; North Wyke: Soil type: $F_{1,53} = 9.69$, $p = 0.003$, early-colonisers: $F_{1,53} = 22.22$, $p < 0.001$). There were no treatment effects of management on total aboveground biomass at any site.

At all sites, the strongest predictor of total species richness was soil type (Figure 2; Reading: $F_{1,53} = 8.50$, $p = 0.005$; Newcastle: $F_{1,53} = 9.31$, $p = 0.004$; North Wyke: $F_{1,53} = 9.69$, $p = 0.003$). Newcastle had the highest species richness and North Wyke the lowest, and at both sites, species richness was higher on alluvial soils, while at Reading the most diverse assemblages occurred on chalk soil. Addition of early-coloniser species had a site-specific effect on total species richness, having no net effect at Reading or Newcastle, but increasing total richness at North Wyke ($F_{1,53} = 14.96$, $p < 0.001$).

SOIL CHEMISTRY

At final harvest, soil type and management explained most variation in soil chemical properties (see Table S4a-c for all statistical output), although some effects of plant treatments were detected. At Reading, there were no effects of plant treatments (Table 4a), whereas at Newcastle, all soil nutrients except total C and N were impacted by early-colonisers (Table 4b), and extractable P and K were reduced by early colonisers, while Ca, Mg and Na were increased. At North Wyke, early-colonisers increased concentrations of every nutrient and also soil pH, but lowered extractable P (Table S4c).

Rhinanthus minor had variable effects on soil chemistry across sites. At Reading, *R. minor* had no impact on soil chemistry, whereas at Newcastle, K, Ca and Na were all higher with *R. minor*. Finally, the C:N ratio at North Wyke was significantly reduced by *R. minor*, but no other chemical properties were altered.

SOIL MICROBIAL COMMUNITIES

At Reading, total PLFA in 2011 was greater in the intensively managed than extensively managed grasslands (Figure 3a; $F_{1,53}=7.54$, $p=0.008$), whereas at Newcastle, there was an interaction between soil type and management: total PLFA was greater in clay soils of extensive grasslands than intensive, whereas there was no management effect in sandy alluvial soils (Figure 3b; $F_{1,53}=16.07$, $p<0.001$). At North Wyke, total PLFA was greater in the clay than the sandy alluvial soil (Figure 3c; $F_{1,53}=23.71$, $p<0.001$).

The ratio of fungal to bacterial PLFA was not affected by any treatments at Reading and (Figure 3d,f), whereas at Newcastle there was a highly significant interaction between soil type and management for this measure: sandy alluvial soils had higher fungal to bacterial

ratios than clay soils, and sandy soils of extensively managed grasslands had an even greater fungal to bacterial ratio (Figure 3e; $F_{1,53}=81.18$, $p<0.001$).

STATISTICAL MODELS

A priority effect caused by biomass of early-coloniser species was the most important variable determining target species biomass across sites, followed by soil chemistry. Soil type, management, and soil microbial community were not significant indicators of target species success and were removed from the models. At Reading, early-coloniser species biomass was the only variable that significantly affected target species biomass, displaying a strong suppressive effect (Figure 4a; $F_{1,59}=13.69$, $p<0.001$). At Newcastle, the minimum adequate model describing target species biomass showed that early-coloniser species biomass reduce target species biomass to near zero at a certain threshold (Figure 4b; EC mass: $F_{1,58}=67.47$, $p<0.001$, quadratic: $F_{1,58}=14.29$, $p<0.001$), and high soil Na also significantly reduced target species biomass (Figure 4c; $F_{1,58}=35.80$, $p<0.001$). At North Wyke, target species biomass was negatively related to soil C content (Figure 4d; $F_{1,58}=27.30$, $p<0.001$) and biomass of the original six grasses added (Figure 4e; $F_{1,58}=10.93$, $p=0.002$).

DISCUSSION

Our overarching goal was to identify the dominant factors that promote, or impede, the establishment of target plant species, and hence diversity restoration in grassland. We found that the presence of early-coloniser species was the strongest, most consistent factor affecting the establishment of target species and grassland diversity restoration. Across three sites,

these species reduced the biomass of target species added to model grassland communities, particularly in the early stages of the experiment. Early-coloniser species also reduced species richness of target species at two of the three sites with more neutral soils (i.e. Newcastle and North Wyke), but had no consistent effect on total community species richness. We also found that soil type had a strong impact on grassland diversity restoration, with the biomass and species richness of target species, and overall community richness, being highest on sandy alluvial and chalk soils. Moreover, the strength of the negative effect of early-coloniser species on the biomass and richness of target species varied across soil types, suggesting that the success of diversity restoration is context dependent. We found some evidence that diversity restoration was related to fungal biomass, but soil nutrient availability was a consistently powerful determinant of target species biomass and richness, being lower with higher nutrient availability. In particular, high soil sodium and calcium concentrations are linked here with lower target biomass, and we showed here that both early colonisers and *R. minor* increased these nutrients. However, *R. minor* was not strongly linked with any measure of diversity restoration.

Our findings suggest that the key constraint for establishment of target species in grassland restoration was competition from common early-coloniser grassland species such as *Trifolium pratense* and *Ranunculus repens* (Kiehl *et al.* 2010). The strong priority effect of early-coloniser species indicates that competition is responsible for the negative effect on target species establishment, more than changes in soil chemistry or microbial community structure resulting from early-coloniser species presence. Competition remained strong several years after initial establishment of early-coloniser species, suggesting that priority effects are strong and consistent determinants of target species success that do not decline with time (Fukami *et al.* 2005). These effects have also been demonstrated using different

functional groups as starting communities, with the initial group remaining extremely dominant over later arrivals (von Gillhaussen *et al.* 2014).

When early-coloniser species were not added, target species were able to achieve higher biomass than when they were present, indicating that for restoration purposes, early-coloniser species should be suppressed prior to target species addition. Species richness overall was not strongly associated with either early-coloniser species or soil type by the end of the study, demonstrating that the target species were present in the sward, but their biomass was very low. Plant functional traits could be accountable for this result; trait values did not differ much between the early-colonisers and the target species (Table S2), so it possible that niche space was already filled when target species were added. The original six grasses had smaller seeds and higher specific leaf area than the subsequently added species groups; these are two characteristics identified by Fischer *et al.* (2013) as indicators of poor ability to establish in competition. Collectively, these results suggest that ecological restoration techniques should focus more on problematic forb and legume species than perennial grasses, as they are highly competitive and have strong impacts on soil fertility and microbial interactions (Fischer *et al.* 2013, von Gillhaussen *et al.* 2014).

Soil type also consistently emerged as a dominant factor affecting the establishment of target species, although the nature of these effects varied considerably across locations. Sandy alluvial soils were generally more productive and species rich than clay soils at both Newcastle and North Wyke, which was most likely due to differences in soil moisture and nutrient availability across soils. Surprisingly, differences in historic land management had limited effects on the establishment of late-colonising target species and overall community diversity. This could be due to the ability of soil to retain nutrients after fertilisation ceases; alluvial soils are less retentive than clay. However, as expected, soil nutrient availability was a key factor influencing plant production and target species establishment, with high soil

concentrations of Na and Ca being associated with lower target species biomass. It is well established that high soil fertility is detrimental to species diversity in grassland, especially the establishment of slow-growing target species (Dickson & Gross 2013), while early-coloniser species can maximise growth (Inouye *et al.* 1987; Paschke *et al.* 2000).

We found evidence that, on chalk soils, high fungal biomass is associated with lower establishment of target species. This is the reverse of findings on more acidic or mesotrophic grasslands. On chalk, such as at our Reading site, AM fungi have been shown to enhance the competitiveness of fast-growing competitive species, to the detriment of slower-growing more conservative species (Zobel & Moora 1995), suggesting that ecological restoration efforts must be targeted and appropriate for a sites soil conditions. Chalk soils also contrasted with those of mesotrophic grasslands, in that they had higher microbial biomass in intensively than extensively managed soils. This is likely to be due to higher soil N and P, which is a valuable substrate to microbes; also, microbial biomass is strongly correlated with organic matter, which tends to be low in chalk (Raiesi 2012).

In our study, the colonisation of *Rhinanthus minor* was poor and had limited and inconsistent effects on the biomass and richness of target species and total community species richness, contrary to our third hypothesis. As shown by Bardgett *et al.* (2006), *R. minor* suppresses grasses only when it is at high densities. Given the duration of this study and poor reseeded ability of this annual plant, it is possible that it only has minor and indirect benefits for increasing the diversity of a sward. For it to be truly effective, therefore, the restorer would need to add seed of *R. minor* every year to replenish the stock. The colonisation mechanism of *R. minor* appears to be largely opportunistic, and it is possible that the pots did not offer conditions conducive to successful establishment for this annual plant (Ameloot *et al.* 2005). However, despite the low establishment rates, *R. minor* did have some impact on soil micronutrients, increasing Ca and K and reducing Na at Newcastle, although these effects did

not feedback to the plant community. Calcium and Na are two nutrients that were shown to affect final biomass and species richness, although this effect was not consistent across sites.

While we did not observe many direct effects of *R. minor* on species richness and biomass, its early effects on soil chemistry could have indirect effects on community assembly. Hartley *et al.* (2015) observed soil effects that continued many months after the plant had senesced, which could mean that *R. minor* is an important inclusion in a restoration procedure.

CONCLUSION

The findings of this long-term, multi-site experiment indicate that successful restoration of species rich grasslands depends primarily on ensuring that there is sufficient empty niche space available for target species, which requires suppression of competing species with similar trait syndromes. Moreover, our results indicate that priority effects are a dominant driver of diversity restoration, although these effects vary with soil properties, being strongest in clay than sandy soils; as such, they need to be accounted for in restoration management.

Our results also demonstrate the general importance of soil properties for restoration success, with both soil type and nutrient status being key determinants of target species establishment, albeit in inconsistent ways. We also discovered that the influence of soil properties on establishment of target species varies depending on the presence of early-coloniser species, which again demonstrates the primary importance of priority effects in determining restoration success. Overall, our results suggest that species rich grassland restoration objectives cannot be met if early-coloniser species are present in abundance, especially in clay based soils that have been modified by agricultural management, and they point to the need for targeted restoration programmes informed by potential priority effect and local soil conditions.

AUTHORS' CONTRIBUTIONS

RDB initiated and gained funding for the study, which was conceived and designed by RDB, JRBT, RSS and SRM. ESP, DAB, JS, SJH, RSS, HQ, KAH, CSL and PJH collected the data, and ELF analysed the data and wrote the manuscript in close consultation with RDB. All authors provided critical comment on the manuscript and contributed to its revision.

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DATA ACCESSIBILITY

- Species descriptions: uploaded as online supporting information
- Biomass, species richness, soil chemistry and microbial data: uploaded to Dryad Digital Repository <http://doi.org/10.5061/dryad.v7t4q> (Fry *et al.* 2017).

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SUPPORTING INFORMATION

Table S1: Regional 10 year weather averages for each location (www.metoffice.gov.uk)

Table S2: List of species in each group and seed weights added to pots.

Table S3: Baseline soil microbial community in 2005, as measured by PLFA.

Table S4: Treatment effects on soil chemistry in 2011.

TABLES

Table 1: Baseline soil chemistry from 2005 for each location \pm SEM. Letters denote differences at the $p < 0.05$ level. Sites were analysed individually.

Soil type	Management	pH	Total N g kg ⁻¹	Total P g kg ⁻¹	Ca g kg ⁻¹	Mg g kg ⁻¹	K g kg ⁻¹	C:N
Reading								
Chalk	Extensive	7.9 \pm 0.08a	4.3 \pm 0.10c	0.4 \pm 0.03c	307.7 \pm 3.34a	1.2 \pm 0.01a	0.2 \pm 0.01a	23.8 \pm 1.57a
	Intensive	7.8 \pm 0.02a	5.6 \pm 0.10b	1.4 \pm 0.04a	82.8 \pm 5.71b	0.3 \pm 0.02b	0.2 \pm 0.01b	11.9 \pm 0.36b
Neutral	Extensive	6.8 \pm 0.12b	4.6 \pm 0.20c	0.7 \pm 0.01b	3.0 \pm 0.11c	0.1 \pm 0.00c	0.1 \pm 0.00c	8.2 \pm 0.78c
	Intensive	7.0 \pm 0.09b	6.1 \pm 0.01a	1.3 \pm 0.06a	5.1 \pm 0.13d	0.1 \pm 0.00d	0.1 \pm 0.00d	8.4 \pm 0.11c
Newcastle								
Clay	Extensive	5.6 \pm 0.06c	5.6 \pm 0.90a	0.9 \pm 0.03a	2.3 \pm 0.08a	0.1 \pm 0.02b	0.1 \pm 0.00a	8.5 \pm 0.20a
	Intensive	5.6 \pm 0.03c	3.9 \pm 0.10ab	0.8 \pm 0.02b	1.8 \pm 0.05b	0.2 \pm 0.00a	0.1 \pm 0.00b	8.3 \pm 0.10a
Sandy alluvial	Extensive	6.4 \pm 0.02a	2.6 \pm 0.00c	0.5 \pm 0.02d	1.5 \pm 0.03c	0.1 \pm 0.00c	0.1 \pm 0.00c	7.6 \pm 0.27b
	Intensive	5.9 \pm 0.05b	3.3 \pm 0.30bc	0.6 \pm 0.02c	1.7 \pm 0.03c	0.1 \pm 0.00d	0.1 \pm 0.00c	7.3 \pm 0.09b
North Wyke								
Clay	Extensive	5.2 \pm 0.06c	4.4 \pm 0.10a	1.0 \pm 0.02ab	0.8 \pm 0.04c	0.1 \pm 0.00a	0.1 \pm 0.01b	8.0 \pm 0.14a
	Intensive	5.7 \pm 0.09b	3.9 \pm 0.10b	1.5 \pm 0.06a	1.3 \pm 0.16ab	0.1 \pm 0.00b	0.2 \pm 0.01a	7.9 \pm 0.10a
Sandy alluvial	Extensive	5.8 \pm 0.04ab	3.2 \pm 0.10c	0.9 \pm 0.05b	1.2 \pm 0.02b	0.1 \pm 0.00c	0.1 \pm 0.01b	6.8 \pm 0.12b
	Intensive	5.9 \pm 0.04a	3.2 \pm 0.10c	0.1 \pm 0.03c	1.4 \pm 0.03a	0.1 \pm 0.00c	0.1 \pm 0.01b	6.9 \pm 0.15b

FIGURES

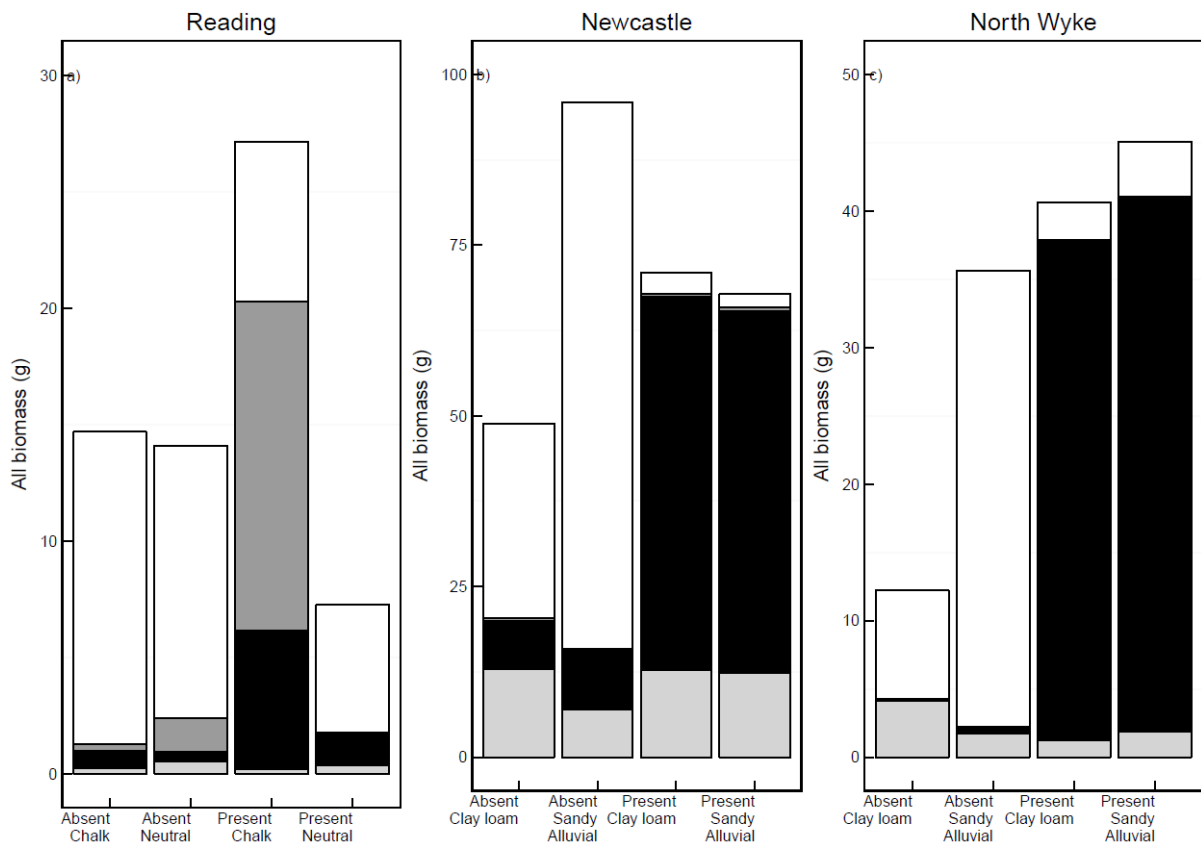


Figure 1: Effect of location, soil type and presence of early-coloniser species on all plant group biomass in 2011. Absent: early-coloniser species not added; Present: early-coloniser species added. Light grey: original six grass species, black: early-coloniser species, dark grey: *R. minor*, white: target species.

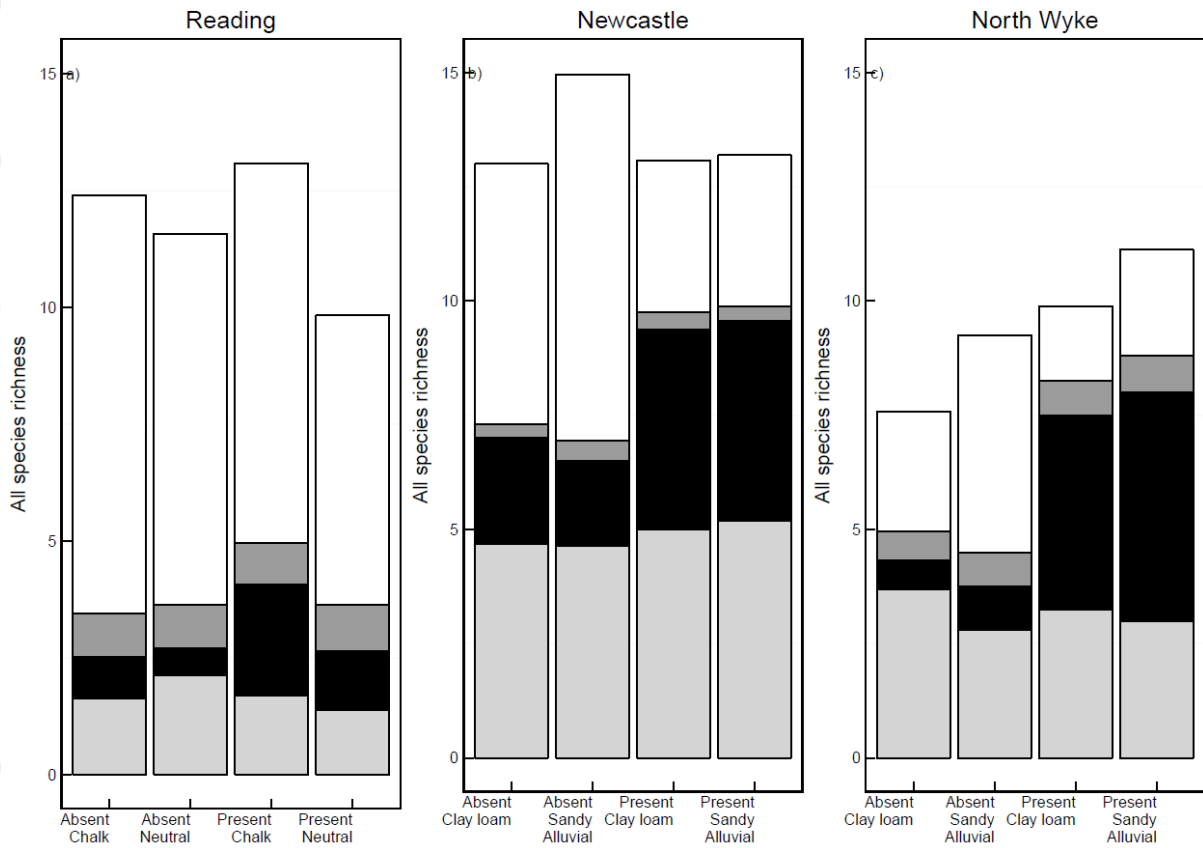


Figure 2: Effect of location, soil type and presence of early-coloniser species on all plant group species richness in 2011. Absent: early-coloniser species not added; Present: early-coloniser species added. Light grey: original six grass species, black: early-coloniser species, dark grey: *R. minor*, white: target species.

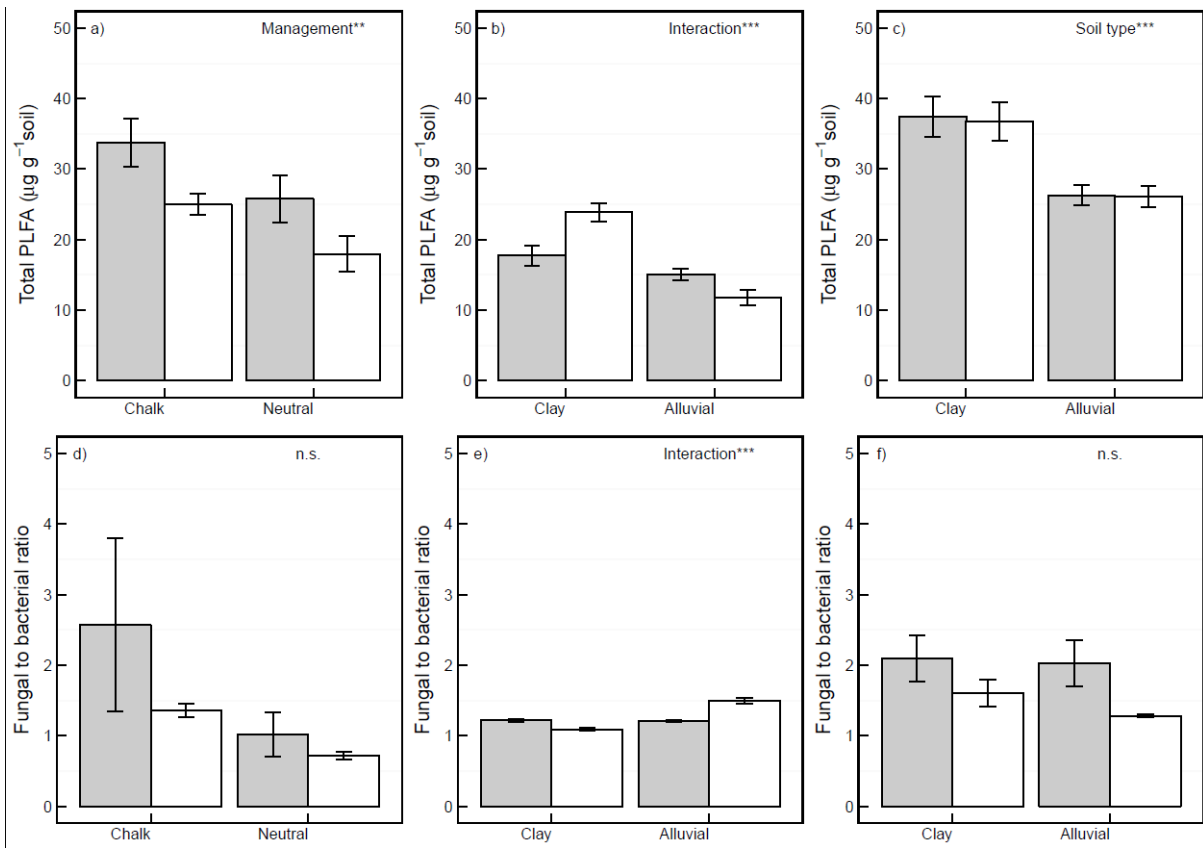


Figure 3:a-c) Effect of soil type and management on Total PLFA values in 2011. a) Reading, b) Newcastle, c) North Wyke. d-f) Effect of soil type and management on fungal to bacterial ratio in 2011. d) Reading, e) Newcastle, f) North Wyke. Grey bars refer to intensive management, while white bars denote extensive management.

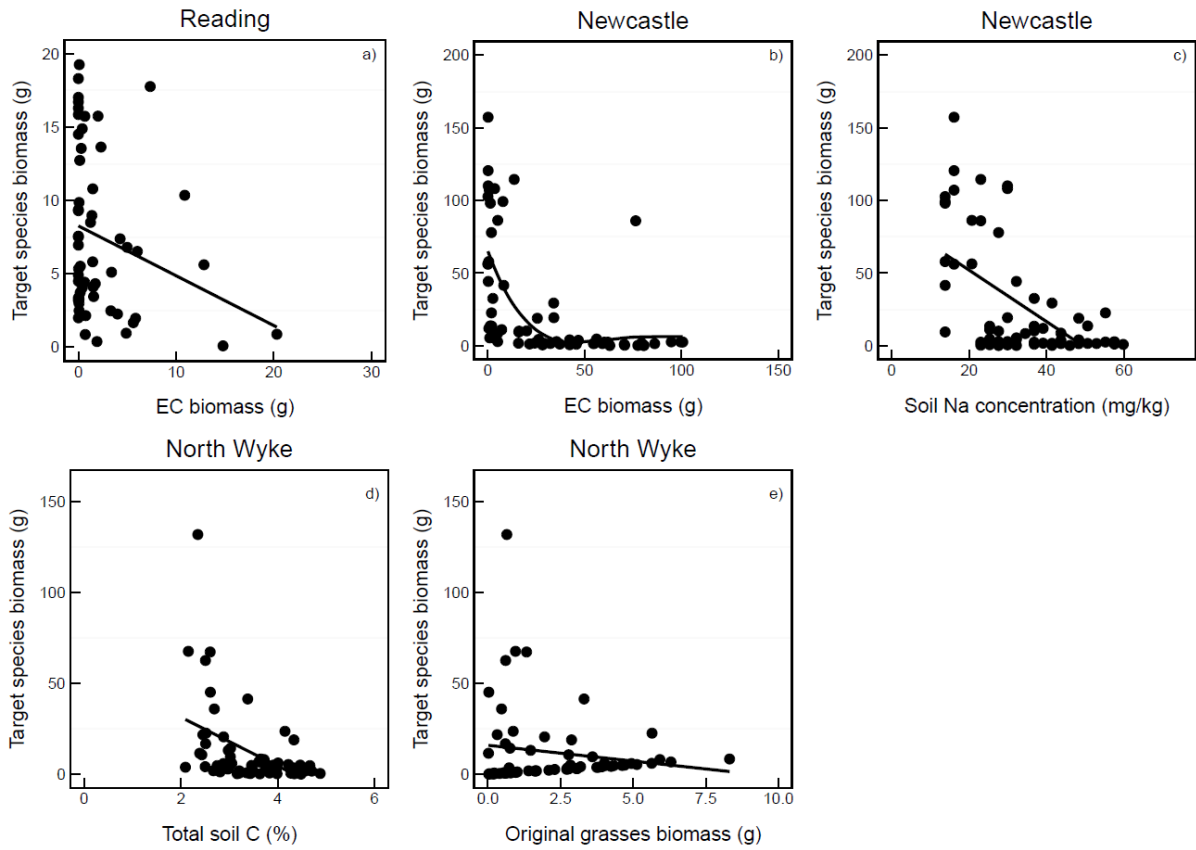


Figure 4: Effects of the strongest predictor of target biomass at three locations in 2011, derived from statistical models.