Soil and plant legacies affect restoration of species-rich grassland on clay soil

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Abstract

To maintain ecosystem functioning in a changing climate and with increasing anthropogenic effects, biodiversity should be restored and maintained. Challenges to restore natural systems include abiotic and biotic legacies from past land use, and non-native plant species migrating into these areas due to increased temperatures. To examine how these combined effects may influence species-rich grassland restoration, a plant-soil feedback pot experiment was performed. Three clay soil inocula (cropland, production grassland, conservation grassland) and a combination of agricultural, grassland, range expanding plant species were used to condition the soil for a subsequent experimental grassland community. The results showed that conditioning plant species with high productivity led to the highest community evenness, and that the high productive species were correlated to low nutrient availability for the subsequent community. Besides, community evenness showed to be more dependent on whether the soil was conditioned by a grass or forb, than showing clear species-specific effects of native or range expanding species. The difference between soil inocula led to a higher biomass of the community in cropland soils than production and conservation soils, but did not seem to affect the community evenness. To conclude, both abiotic and biotic plant legacies affect the grassland community, but the relative strength of these effects cannot be determined in this study. Besides, this study confirms that changing the relative proportion of plant species in a community can be challenging on clay soil, due to high nutrient availability. Further disentangling these abiotic and biotic legacies might help create effective management strategies that can contribute towards creating more resilient ecosystems.

Content

1. Introduction

Humans have changed ecosystems all over the world drastically. These anthropogenic changes affect the functioning of species, but also increase the speed at which the climate changes, resulting in altered biodiversity and ecosystem functioning (Zhu et al., 2012; Pecl et al., 2017). A major worry among people is that these ecosystem changes around the globe will reduce the quantity and quality of ecosystem services humans benefit from (Adhikari and Hartemink, 2016). As these ecosystem services are directly linked to ecosystem functions, loss of these functions should be prevented. This can be achieved my maintaining and restoring biodiversity around the globe. High biodiversity helps creating stable ecosystems, that are more resilient after environmental changes than low biodiverse systems (Mooney et al., 2009). Also, loss of biodiversity can lead to the loss of species with low-abundance but important roles, such as certain ecosystem engineers (Levin et al., 2012).

Even though goals are being made to protect ecosystems, the number of threatened species is still increasing (Díaz et al., 2019; IUCN, 2019a). To prevent further biodiversity loss, countries around the world implemented laws to protect nature and biodiversity (IUCN, 2019b). In Europe, protected areas were assigned to the Natura 2000 programme, in which certain species and their habitat are protected (Rijkswaterstaat, 2019). Within the Netherlands, previous nature related laws were replaced by the Law for Nature Conservation in 2017 to further prevent biodiversity loss and protect ecosystems. However, even before this new law, large scale projects were started to maintain and increase natural areas. For example, the Dutch National Ecological Network (NEN) was created to by protect species, prevent an excess of nitrogen in the soil, maintain or improve water quality, and practice nature-friendly agriculture (Rijksoverheid, 2019a; b). A successful project along the Meuse showed that biodiversity increased since 1990 and that rare species could increase in numbers (Peters and Kurstjens, 2008). This project included the protection of Natura 2000 areas, but also creating new nature areas and leaving room for natural development of the area.

1.1 Importance of soil in ecosystem restoration

To restore natural ecosystem, all components of the system should be considered. Over the last decades, researchers found that soil biodiversity affects both above- and belowground ecosystem functioning, and that the quality of the soil can affect ecosystem restoration efforts. Single soil manipulations of abiotic factors (e.g. pH, structure) can already increase the quality of degraded soils (Heneghan et al., 2008). However, Henegan et al. (2008) also state that applying soil ecological knowledge becomes more complex when complete ecosystem restoration is desired. For example, soil inoculation with mycorrhizal fungi may stimulate the plant community, but whether the mycorrhizal fungi will grow in the inoculated soil depends on abiotic factors such as nutrient availability. Besides, changing soil biota can also affect the competitive interactions between aboveground species. This concept is used in, for instance, containing the spread of invasive species (Heneghan et al., 2008).

As soil community changes during ecosystem restoration management, soil biota can be efficient bioindicators. For example, nematodes are often used because they are abundance in all soils and occur in multiple trophic levels (Ferris et al., 2001). Also, an increase in soil microbial biomass is often a consequence of grassland restoration, in which more perennial root systems develop during the restoration efforts. However, total microbial biomass does not automatically lead to higher soil biodiversity, as soil biodiversity depends on multiple variables, including the

present plant species, soil structure, and whether the soil is sandy or contains larger fractions or silt and clay (Bach et al., 2010).

1.2 Plant-soil feedback and legacy effects

Acknowledging the importance of soil and its biota during ecosystem restoration includes studying the interactions between the soil and the plants living in that soil. Soil biota, such as arbuscular mycorrhizal fungi (AMF) and nitrogen (N) fixing bacteria, can affect plants positively by supplying nutrients. On the other hand, damaging micro-organisms, such as herbivores, pathogens, and parasites, can hamper a plant's growth. In turn, plants also affect the soil and its biota with their root exudates and litter, which can then affect the plan again. This concept is also known as plant-soil feedback (PSF) (Van der Putten et al., 2013). PSF can occur interspecifically, where the soil changes induced by a plant species affect the same plant species, or intraspecifically, where other plant species are affected. Besides, PSF can be negative, neutral, or positive, depending on how a plant species performs with or without its soil biota, or compared to its performance on other species' soil biota.

Plant-soil feedback can drive natural succession, but also species coexistence. Negative PSF to dominant plant species can limit their growth, with increases the competitive advantage of less dominant species (Bonanomi et al., 2005). This was confirmed by a study that compared the relative abundance of species in a community, also referred to as community evenness, with and without soil biota. The results showed a higher community evenness in soils with fauna compared to without fauna (De Deyn et al., 2003). To conclude, more diverse ecosystems can exists when the right soil biota are present, as they have a key role in determining which plant species can grow where.

As PSF can operate on both spatial and temporal scales, certain plant species can affect subsequent plant communities. These long-lasting effects of a previous plant community are also called legacy effects, and can impact following communities for years (Van der Putten et al., 2013; Wubs et al., 2019). Additionally, legacy effects include all other ecosystem changes made by a species last after they cease their activity or leave the area. For example, nature restoration can be affected by agricultural activity, even if the area has not been in agricultural use for years. Human legacy effects after agriculture include high soil nutrient levels and altered soil. Legacy effects from the crops grown at the fields can include an accumulation of species specific soil biota, for example, specialist herbivores or pathogens. Burns et al. (2015) found that the fungal soil diversity is highly related to the plant species occurring in an area (Burns et al., 2015). Overall, agriculture reduces soil biodiversity (Tsiafouli et al., 2015). This becomes increasingly important in nature restoration projects, when often agricultural land was present before the natural plant community. Also, it is unclear how different agricultural practices affect later nature restoration efforts.

1.3 Plant range expansion

Predicting if restoration projects will be successful can be complicated due to climate change. Direct effects such as higher microbial activity due to higher temperatures alter ecosystem functioning. Also indirect effects occur, such as a changing plant community composition due to climate change, affecting soil biota and therefore soil processes (Kardol et al., 2010). Both effects can disrupt ecosystems, as not all species respond in the same way. For example, species can react to an increase in temperature by phenological shifts, in which their seasonal timing alters, or disperse to new areas where the climatic conditions better match their optimum (Mooney et al., 2009; Pecl et al., 2017). If species are unable to adapt to the changes they might go extinct

(Pecl et al., 2017). However, not only the individual response of species might determine what a future ecosystem looks like, but the changing interactions between species might be of greater importance (Berg et al., 2010). For example, a difference in dispersal speed between soil biota and plants can lead to dispersing plants being released from herbivores and pathogens, giving them an advantage over plants already settled in an area. Another mechanism is that the presence of local microbiota can have a positive feedback to an incoming plant species, also favouring the new species over locally adapted species (Wolfe and Klironomos, 2005).

Due to the increasing temperature, many species are increasing their northern range, or are expected to do so in the future (Huntley et al., 2006; Van Grunsven et al., 2010a). While the possible plant-soil feedback of range expanding plants is studied in detail, there is little attention for the effects of range expanding plants on natural areas or nature restoration projects. In the Meuse project, Kurstjens and Peters (2011) suggested that 10% of the studied plant species in the area increased their range due to a higher average temperature. However, the effects of these expansions are not mentioned (Kurstjens and Peters, 2011). Thus, more research will be needed to find out how range expanding species might affect future restoration projects.

1.4 Restoration of the Maasheggen area
An area in the Netherlands where currently nature conservation and restoration projects occur, is the Maasheggen area. The Maasheggen area consists of land separated by hedgerows, which were originally used to keep cattle on the pastures. Now, the area has a mix of arable land, pastures, and natural grasslands. National and regional organisations are aiming to expand and connect the natural grasslands to increase biodiversity. Moreover, the Maasheggen area was assigned to the UNESCO Man and Biosphere project, for being of historical, cultural, and economical importance (Bontenbal, 2017). This status also stimulates new and sustainable maintenance and development of the area. Together, new developments and connecting nature in the area should help make the Maasheggen more persistent against climate change (OverMorgen, 2016).

The land for new natural grasslands in the Maasheggen are former pastures and arable fields. The soils are expected to be relatively rich in nutrients, because the pastures and arable fields were fertilized. Also, clay depositions from the Meuse lead to a large clay fraction in the soils, increasing the soil capacity to bind nutrients (Peters and Kurstjens, 2008). This can affect the potential for natural grassland on clayey Maasheggen soils to become high in biodiversity, because high nutrient availability can stimulate the occurrence of dominant plant species (De Deyn et al., 2004; Peters and Kurstjens, 2008). Moreover, Peters and Kustjens (2008) suggested that plant species can settle less easily on former pasture soils compared to former arable fields, because of the high plant density of pastures. However, it is unknown if there is still a biotic effect once the former grass or crops of the pasture or arable land are removed.

1.5 Aim and research questions
Although there has been extensive research into ecosystem restoration and biodiversity conservation, there a still a lot of unknowns, especially regarding the effects of previous land use on natural grassland restoration in the Netherlands. Besides, range expanding plant species are more likely to inhabit existing and future natural grasslands. Therefore, this study will investigate several factors that could affect the success of natural grassland restoration. The main question is how (biotic) soil and plant legacies may influence species-rich grassland restoration. As a model system, the soil of Maasheggen fields will be used. Here, different land uses are spread on a relatively small scale. Moreover, the Maasheggen has clay soil, while most

studies regarding PSF are done on sandy soils. This could validate whether PSF mechanisms explored in different studies also apply to clay soils.

In a plant-soil feedback pot experiment, I will investigate how soil characteristics and plant species of agricultural land (cropland), pastures (production grassland), and natural grassland (conservation grassland) affect the community evenness of an experimental natural grassland community with two grass and two forb species. The first hypothesis is that there will be a higher community evenness on soils from pastures compares to arable fields, due to the finding that grassland species have more difficulty growing on pasture soils compared to agricultural soils (Peters and Kurstjens, 2008). My second hypothesis is that a higher community evenness can be achieved by conditioning the soil with species of the natural grassland itself compared to pasture and agricultural plant species, due to an accumulation of species-specific soil biota that lead to a negative PSF. In the same experiment, the effect of several range expanding plant species on the experimental plant community will be assessed. Therefore, my third hypothesis is that soil conditioning with range expanding plant species will lead to a lower community evenness compared to native species, because of a difference in specific soil biota. Lastly, I will examine of any effect of the plant species conditioning is related to their functional group. Here, the hypothesis is that conditioning the soil with grasses will decrease the relative abundance of grasses in the experimental plant community, and vice versa for conditioning the soil with forbs.

2. Methods

To study the effects of soil and plant legacies on natural grassland restoration, a two-phase plant-soil feedback experiment was set-up. A full-factorial design was used with three soil inoculum types, nine plant species for the soil conditioning phase, and a mixture of natural grassland species for the feedback phase (Figure 1). As each treatment had six replicated, the experiment was performed with 186 pots in total (3 soil inoculums * 10 conditioning types * 6 replicates + 6 * sterile control).
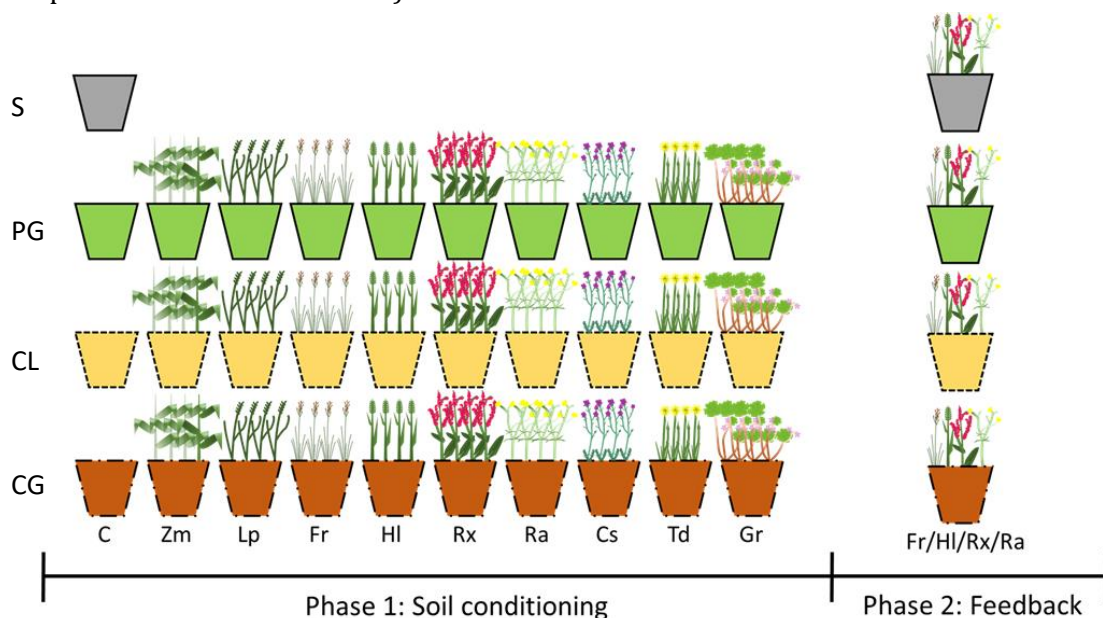

Figure 1. Experimental set-up of the soil conditioning (phase 1) and soil feedback (phase 2). Soil inoculums originate from PG = production grassland, CL = cropland, CG = conservation grassland, S = none (sterilized soil). The soil conditioning treatments are C = control, and conditioning with plant species Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*.

2.1 Soil sampling and plant species selection

Soil for the inoculums was collected across the Maasheggen area on 27 and 28 May 2019 (Figure 2). At the middle of each field, a sample was taken of 20x20x30 cm (12 dm³). After collection, the soil samples were stored at 4 °C until further use. Additionally, background soil was collected in the Maasheggen area (51°41'23.03", 5°57'13.79") on 28 May 2019 (Fig. 2). The soil was sieved (10mm), homogenized, and sterilized with 25 kGy γ-irradiation. The soil was stored at room temperature until further use.

For the conditioning phase, the plant species *Zea mays* L. var. Ronaldinio and *Lolium perenne* L. var. Tetra green were selected to represent the arable fields and pastures, respectively. The seeds used for these species originate from the test farm Vredepeel of Wageningen University and Research (WUR). The species of the natural grassland, *Holcus lanatus L., Festuca rubra L., Rumex acetosa L.,* and *Ranunculus acris L., w*ere selected after a vegetation inventory in the Maasheggen natural grasslands. Plant vegetation inventory was performed on four of the natural grassland fields in the Maasheggen area. Per field, two plots of 2x2m were marked and the inventory was done according to the Braun-Blanquet scale (Appendix A1). The seeds used for these species were ordered at the wild plant seed supplier Cruyd-Hoeck. The range expanding plant species *Centaurea stoebe L., Tragopogon dubius* Scop. and *Geranium rotundifolium L.* are species origination from the south of Europe, and are now expanding their range throughout the Netherlands. The seeds used for these species were collected in the Netherlands during previous NIOO research and stored locally.
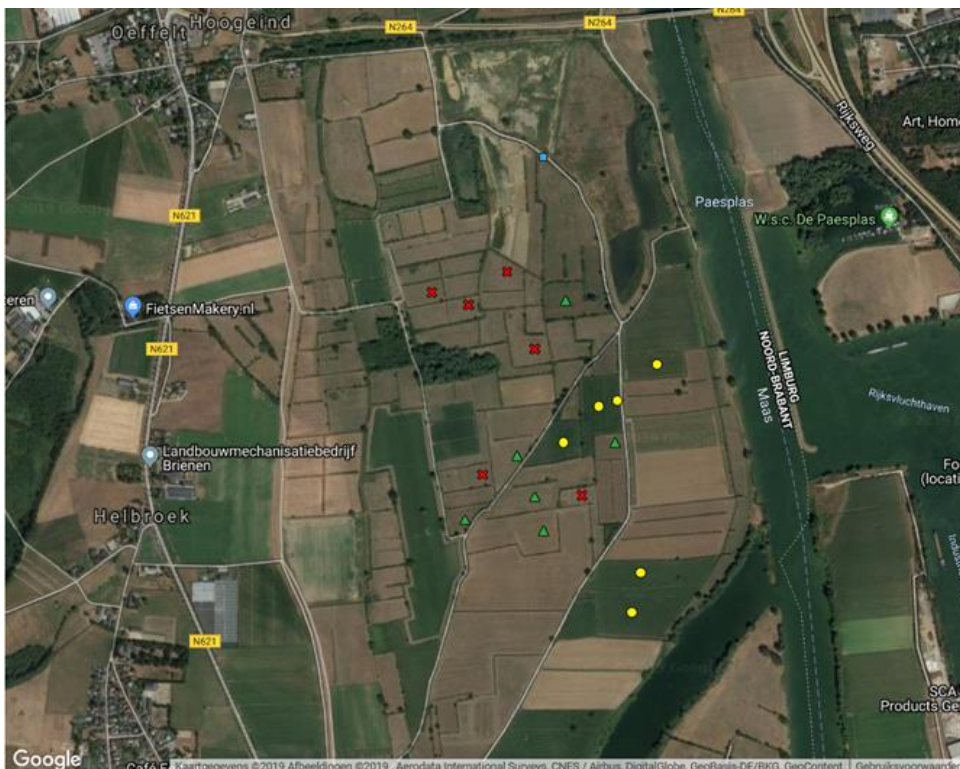

Figure 2. Soil sampling locations in the Maasheggen area for background soil (blue square), and at pastures (green triangles), arable fields (yellow circles), and natural grasslands (red crosses).

2.2 Seed germination

Before sowing, the seeds were sterilized in 10% bleach-water for 3 minutes. The seeds of *F. rubra* and *H. lanatus* were too small for sterilization, so these were sown without being sterilized. The seeds were sown on glass beads, moisturized with demineralized water, and grown in a growth chamber at 16/8 hours L/D. The temperature during the germination for the conditioning phase seedlings was 22/18 °C, and 20/10 °C for the feedback phase seedlings, to promote faster germination. Five days after germination, seedlings were stored at 4 °C with 16/8 hours L/D, until all species had germinated. Seedlings of *R. acris* did not germinate without a cold period, so these were stored up to two weeks on moisture glass beads 4 °C with 16/8 hours L/D. After that, the same germination procedure was followed as for the other species.

2.3 Experimental set-up
Phase 1: soil conditioning

The soil conditioning phase was performed the greenhouse at the NIOO, with 16/8 hours L/D, 21/16 °C, and relative humidity of 50%. For soil conditioning with one plant species, four seedlings were be placed in 1.6L pots with background soil and inoculum at a ratio of 9:1. Until two weeks after planting, dead or lacking plants were be replaced or added. The plants were be watered 5 days a week, and once a week the pots were weighted to set the soil at 25% moisture level. For the control treatment, no seedlings were added. Additionally, soil for the sterilized control was stored in closed bags in the greenhouse. Seven weeks after initial planting, the aboveground biomass was clipped at ground level, and the biomass was determined by drying at 70 °C for at least 72 hours and weighing the samples.

To prepare the pots for phase 2 of the experiment, the roots from the conditioning phase plants were cut with a knife, the soil was mixed, and 1000g of soil was mixed with 250g of sterile soil to prevent plants in the feedback phase from being limited by too little space. The remaining soil from the pots was used for soil analyses, see below. Additionally, pots for the sterilized control were filled with 1250g of sterile soil.

Phase 2: soil feedback

Each of the pots from phase one received one seedling from each of the natural grassland species *(F. rubra, H. lanatus, R. acetosa,* and *R. acris*). The plants were be watered 3 days a week, and once a week the pots were weighted to set the soil at 25% moisture level. Watering occurred less regularly compared to the conditioning phase, to let the top soil dry and prevent growth of mosses.

After seven weeks all biomass was collected by clipping aboveground biomass at ground level and sorting per species, and washing all soil of the roots. Both shoots and roots were dried at 70 °C for at least 72 hours and weighted to measure dry weight.

2.4 Nematode analysis

To assess soil biodiversity of the different field types, nematodes were extracted out of ca. 200g of soil using Oostenbrink elutriators for each of the 18 soil samples collected for the inocula (Oostenbrink, 1960). Half of the extracted nematodes were fixated using 4% formalin, while the other half was suspended in 70% ethanol for possible future DNA analysis. The fixated nematodes were used to produce mass slides for visual identification. Due to low quality of some

mass slides, twelve samples were used for the analysis (four for each soil type). Per mass slide, the feeding type of 100 individuals was determined (Bongers, 1988).

2.5 Soil analyses
pH-H_2O of the fresh soil was measured using the InoLab pH7310 meter. The soil samples for nutrient analyses were dried for 144 hours at 40 °C, and sieved over a 4mm sieve. P-Olsen was measured in the SEAL QuAAtro Segmented Flow Analysis (SFA) system, using a 0.5 M $NaHCO_3$ extract. N and K were extracted using a 0.01M CaCl2 extract, and measured using the SFA system and ICP-OES, respectively.

2.6 Data analysis
All data was analysed using the statistical software R (R Core Team, 2017), and figures were made using the ggplot2 package (Wickham, 2016). Nematode counts were analysed using a chi-square test for independence.

Simpson's community evenness was calculated using the following equation: $SIEI = (\frac{1}{\sum P_i^2})(\frac{1}{S})$. In the equation, P_i represents the ith species proportional biomass, and S is the number of species in the community (De Deyn et al., 2003). SIEI or biomass across treatments were analysed with using one-way or two-way ANOVA. In case of significant results, a Tukey HSD post-hoc test was performed. Normality of the residuals and homogeneity of the variances were analysed visually using Q-Q plots and residuals vs fitted values plots, respectively. A linear regression model was used to determine the relationship between SIEI and the shoot biomass after the conditioning phase.

To relate the soil characteristics content to the biomass after both the conditioning and feedback phase, a principle component analysis was performed. The soil characteristics included in the analysis were the total N content, P-Olsen, and pH after the conditioning phase.

3. Results

3.1 Conditioning phase

As an indication for biotic differences between the soil inocula, nematode feeding types were determined per soil inoculum type (Figure 3). The results show that the proportion of feeding types is not distributed equally across the soil inocula from conservation grassland (CG), production grassland (PG) and cropland (CL) (X^2 = 78.334, p <0.001). Especially in the cropland soils, the proportion of bacterivores is larger than in production and conservation grassland soils, while the proportion of herbivores is lower.
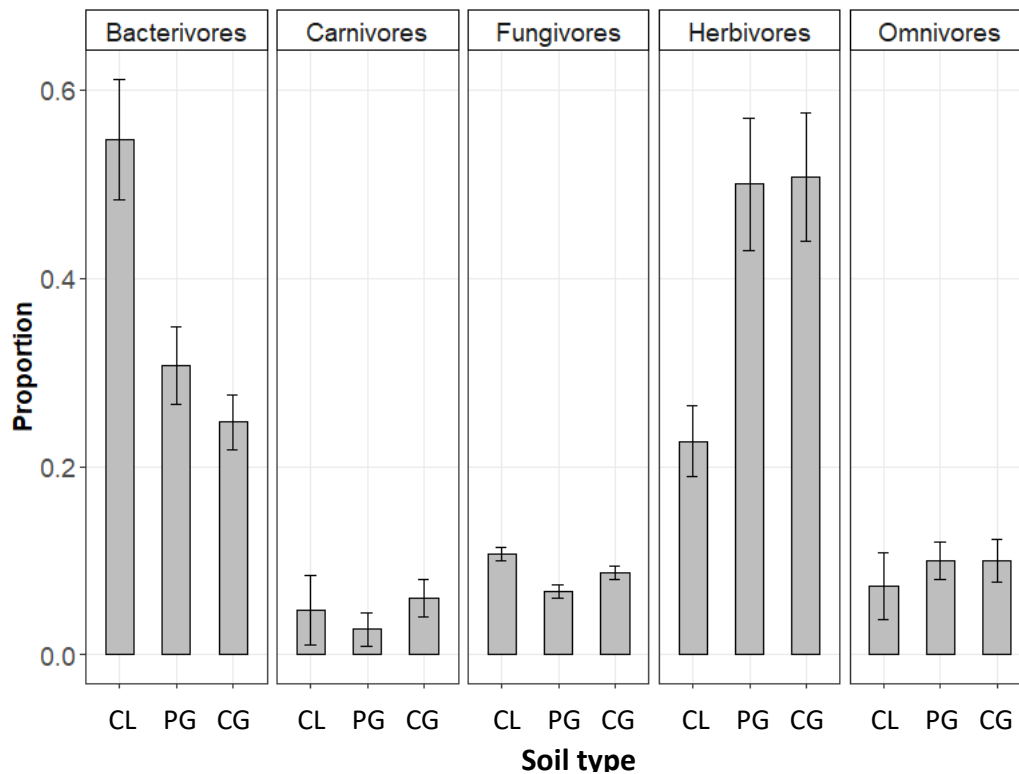

Figure 3. The proportion (±1 SE) of nematode feeding types in different soil types (CL = cropland, PG = production grassland, CG = conservation grassland).

After growing the conditioning plant species in soils with the various soil inocula, there was considerable variation in growth between and within species (Figure 4). The species with the lowest biomass per pot was *R. acris*, which had only one individual per pot, while the biomass of *Z. mays* exceeded that of all other species. These differences are reflected in the results, as the biomass per species differed significantly (F = 98.547, p < 0.001). However, there was no significant difference among soil inocula (F = 1.397, p = 0.251), and there was no significant interaction between species and soil inoculum (F = 0.464, p = 0.960; Figure 5).
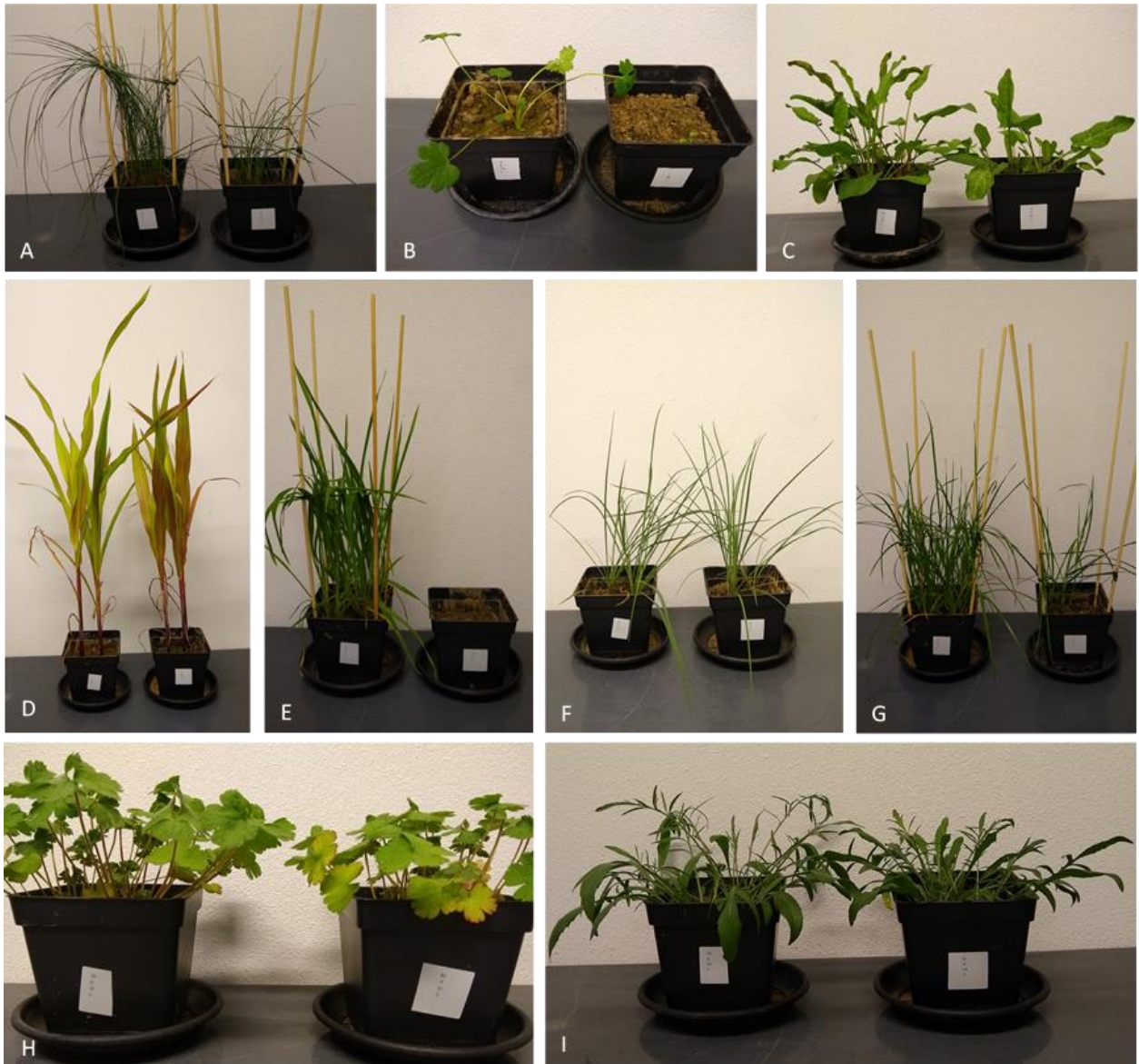
Figure 4. Plant biomass produced during the conditioning phase of 7 weeks. Plant species: *Festuca rubra* (A), *Ranunculus acris* (B), *Rumex acetosa* (C), *Zea mays* (D), *Holcus lanatus* (E), *Tragopogon dubius* (F), *Lolium perenne* (G), *Geranium rotundifolium* (H), *Centaurea stoebe* (I).
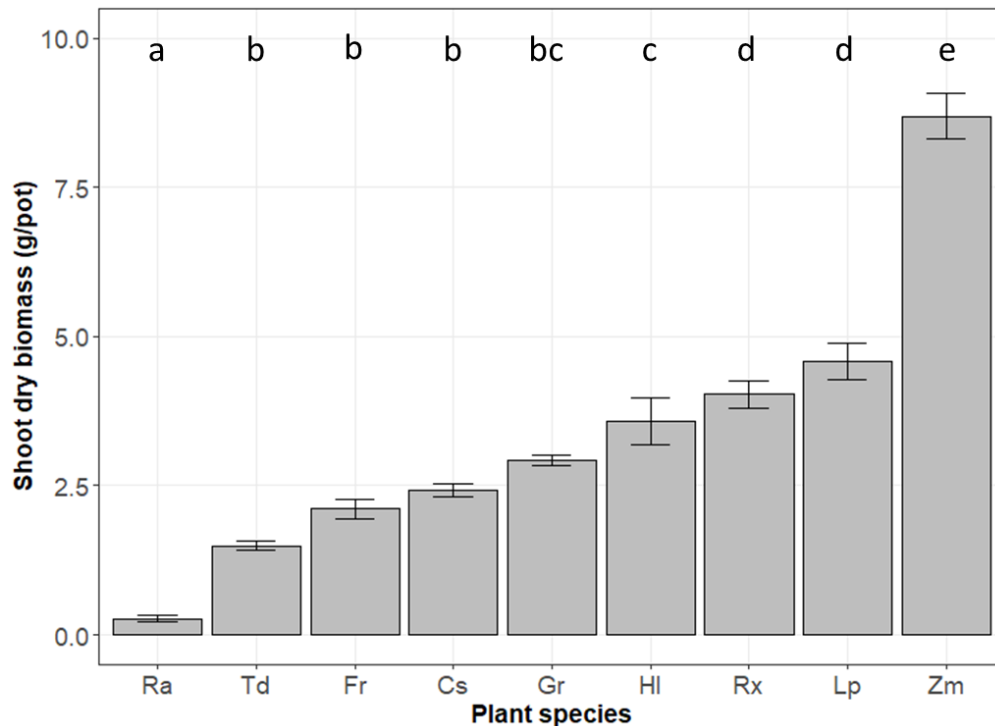
Figure 5. Shoot dry biomass of plant species after the conditioning phase. Plant species: Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*. Shoot biomass differed significantly per plant species (F = 98.547, p < 0.001). Different letters above plant species indicate significant differences.

3.2 Community evenness

At the end of the feedback phase, community evenness was determined using Simpson's evenness index. A two-way ANOVA showed that there was no significant interaction between soil inoculum type and plant species conditioning (F = 0.644, p = 0.860) or an effect of soil inoculum type (F = 2.370, p = 0.097; Figure 6A). However, plant species conditioning affected community evenness significantly (F = 3.360, p = 0.001; Figure 6B). Post-hoc analysis showed that soil conditioning with *Z. mays* led to a higher evenness than *H. lanatus*, *R. acris,* and the control. The range expanding plant species, *C. stoebe, T. dubius,* and *G. rotundifolium* did not lead to a significantly different evenness than the other conditioning plant species (Fig. 6B). Community evenness after conditioning with the different plant species and soil inocula was also compared to the sterilized control. The median of the sterile control was generally lower than those of the plant species treatments, although only the *L. perenne*, *R. acetosa*, and *Z. mays* treatments differed significantly from the sterilized control (Sterilized vs *L. perenne*: p = 0.014, sterilized vs *R. acetosa*: p = 0.048, sterilized vs *Z. mays*: p = 0.002). All soil inoculum types resulted in a significantly higher community evenness than the sterilized control (Sterilized vs cropland: p = 0.091, sterilized vs conservation grassland: p = 0.017, sterilized vs production grassland: p = 0.008).
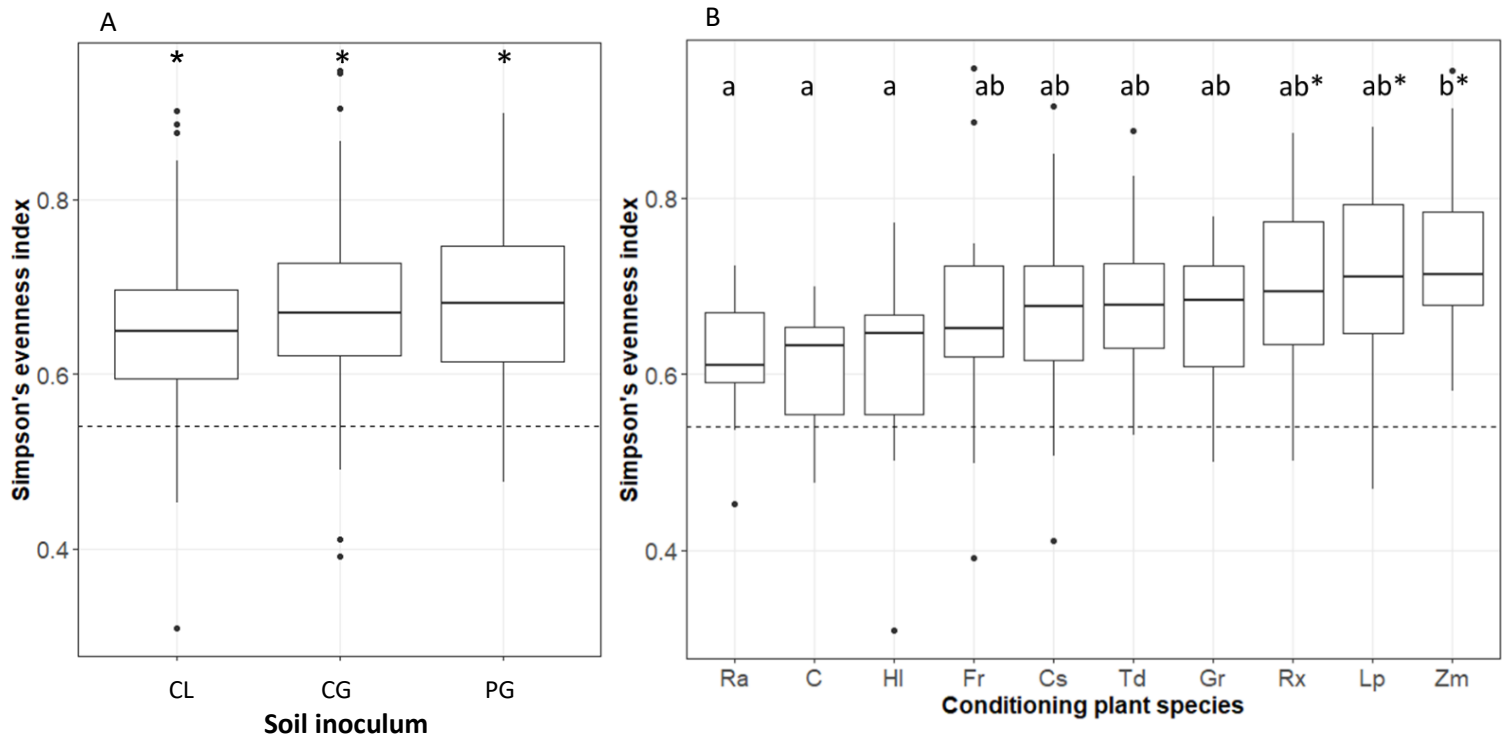
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Figure 6. Simpson's evenness index of the plant community at the end of the feedback phase. (A) Simpson's evenness index per soil inoculum (CL = cropland, CG = conservation grassland, PG = production grassland). Treatments did not differ significantly (F = 2.370, p = 0.097). (B) Simpson's evenness index per conditioning plant species. C = control, Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*. Treatments differed significantly across conditioning plant species (F = 3.360, p = 0.001). (A and B) Treatments with different letters are significantly different. Treatments with an asterisks differ significantly from the sterilized control (dashed line).
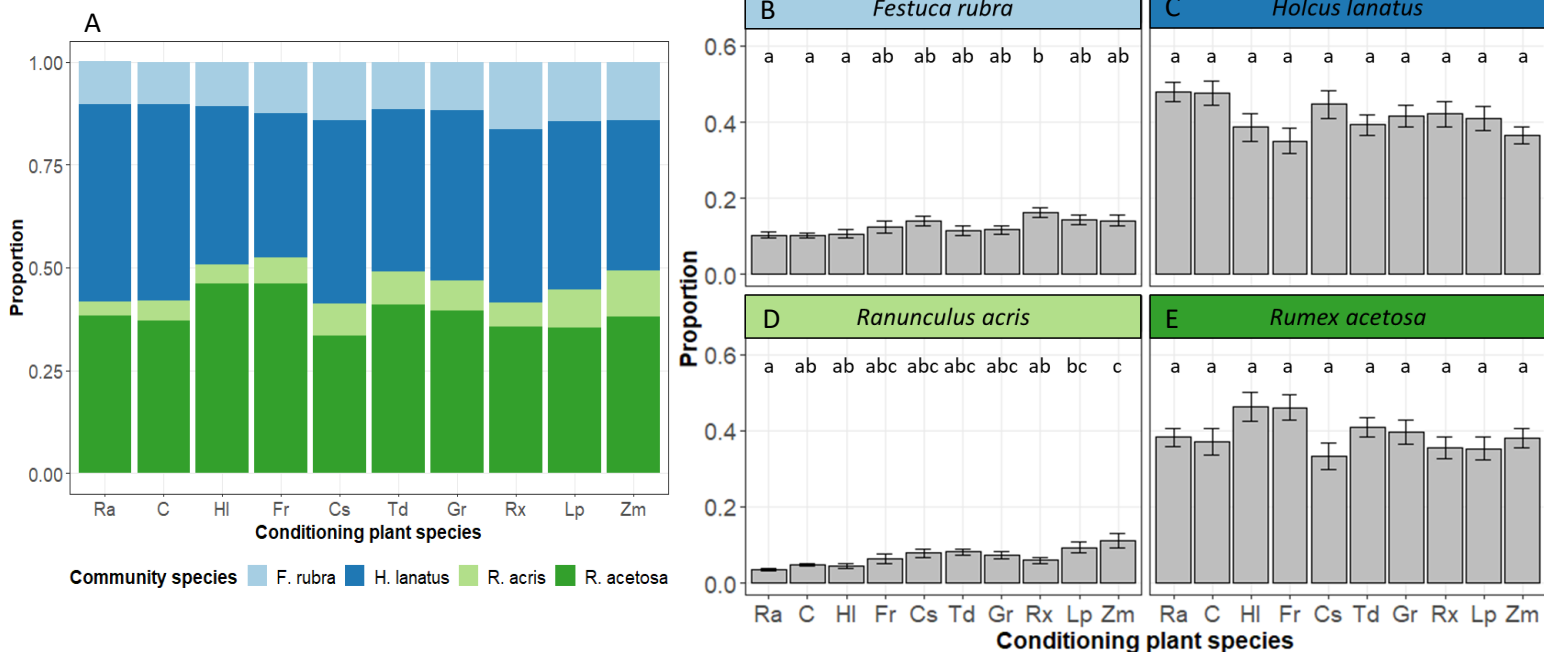
Figure 7. The proportion of each plant species in the experimental community. (A) Proportion of community plant species stacked per conditioning plant species (C = control, Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*). (B - E) The proportion (±1 SE) of *F. rubra* (B; F = 3.079, p = 0.002) *H. lanatus* (C; F = 2.02, p = 0.04), *R. acris*, (D; F = 4.78, p < 0.001), and *R. acetosa* (E; F = 1.986, p = 0.044). Different letters show significant differences between treatments.

The experimental plant community of *F. rubra, H. lanatus, R. acris*, and *R. acetosa* showed variation in the proportion per species across plant conditioning treatments (Figure 7A). However, conditioning the soil with of the conservation grassland plant species, did not lower the proportion of that species in the community (Figures 7B-E). The increased community evenness after conditioning with *Z. mays* was associated with a significantly increased proportion of *R. acris* ($F = 4.78$, $p < 0.001$; Figure 7D). However, the most dominant species, *H. lanatus* and *R. acetosa* did not decrease in proportion significantly after conditioning with *Z. mays*. For *H. lanatus*, the ANOVA showed a significant difference between treatments ($F = 2.02$, $p = 0.04$), but this was not reflected in the post-hoc analysis (Figure 7C). Similarly, a significant difference between the abundances of *R. acetosa* was observed ($F = 1.986$, $p = 0.044$), but this significance did not show up anymore under the conditions of the post-hoc analysis (Figure 7E). The proportion of *F. rubra* was higher after conditioning with *R. acetosa* than after conditioning with *R. acris, H. lanatus* and the control (Figure 7B). Thus, an increase in community evenness seems to be associated with relatively small changes in the community.
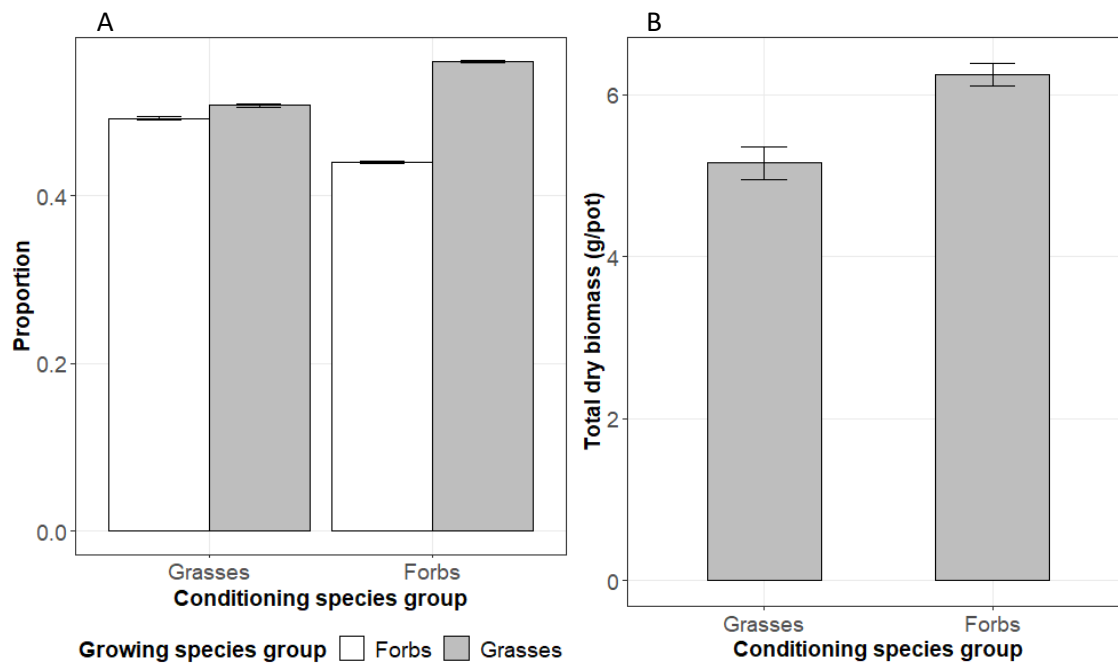

Figure 8. Growth of species functional groups after conditioning with grasses or forbs. (A) Proportion (±1 SE) of forbs and grasses per conditioning treatment with grasses or forbs. The conditioning treatments differed significantly (F = 6.163, p = 0.014). (B) Total dry biomass of the community after conditioning with grasses or forbs (F = 28.14, p < 0.001).

Figure 9. Example of a grass dominated community (left) versus a forb dominated community (right).

The changes in community evenness were more strongly related to whether the soil was conditioned by a grass or a forb. After conditioning with a grass, the overall relative proportion of grasses decreased significantly, and vice versa for forbs ($F = 6.163$, $p = 0.014$, Figure 8A). Visual observations showed clear differences between a community being dominated by a grass of a forb (Figure 9). Also, the total biomass of the community was lower after conditioning with grasses than with forbs ($F = 28.14$, $p < 0.001$; Figure 8B).

The species with the highest biomass during the conditioning phase seemed to have the highest community evenness after the feedback phase (Fig. 5 & 6B). This also shows from the

correlation between the biomass after the conditioning phase compared to the Simpson's evenness index (Adj. R^2 = 0.094, p < 0.001; Figure 10).
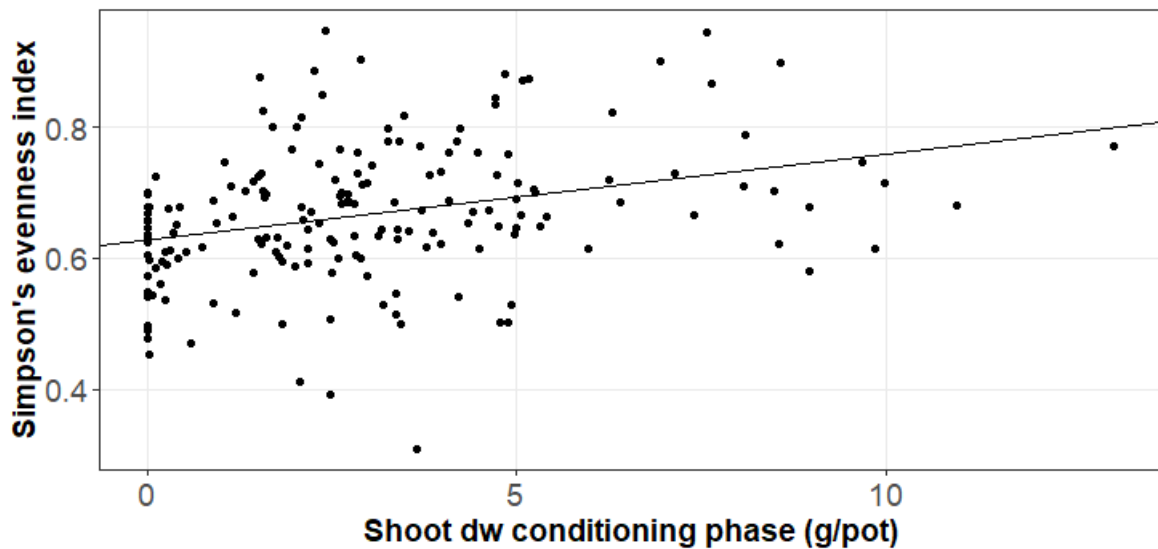

Figure 10. Correlation between the shoot dry weight during the conditioning phase and Simpson's evenness index (Adjusted R^2 = 0.094, p < 0.001).

3.3 Biomass

The total shoot biomass was not affected by interacting effects of soil inoculum type and conditioning plant species (F = 0.420, p = 0.982). However, effects on total shoot biomass of soil inoculum type (F = 4.432, p = 0.014) and conditioning plant species (F = 35.216, p < 0.001) were both significant. Post-hoc analysis showed that shoot biomass was significantly higher in cropland than in production grassland soils (p = 0.012, Figure 11A), and that several plant species conditioning treatments differed significantly. The control and conditioning with *R. acris* led to the highest shoot biomass, while conditioning with *L. perenne* and *Z. mays* led to the lowest shoot biomass (Figure 11B).

Similar to the shoot biomass, the total root biomass was significantly affected by conditioning plant species (F = 4.959, p < 0.001) and soil inoculum (F = 5.217, p = 0.006), but not by their interaction (F = 1.024, p = 0.436). However, post-hoc analysis showed that root biomass was significantly higher in soil conditioned with cropland soil inoculum than with conservation grassland inoculum (p = 0.005), but not compared to grassland inoculum (Figure 12A). Root biomass of several plant species differed significantly between conditioning treatments (Figure 12B), however, these differences did not necessarily not correspond with the differences found between the shoot biomasses.

To determine which factors may have contributed to the variation in shoot and root biomass, the soil properties (total nitrogen, P-Olsen, and pH) after the conditioning phase and biomass of both the conditioning and feedback phase were included in a principal component analysis (Figure 13). The results show that the included factors explain 77.4% of the variation on the first two axes. The shoot biomass after the feedback phase is most closely related to the N and P-Olsen content, and negatively related to the shoot biomass after the conditioning phase. The root biomass seems independent of these abiotic soil properties. Thus, the shoot and root biomass appeared to respond differently to nutrient availability.
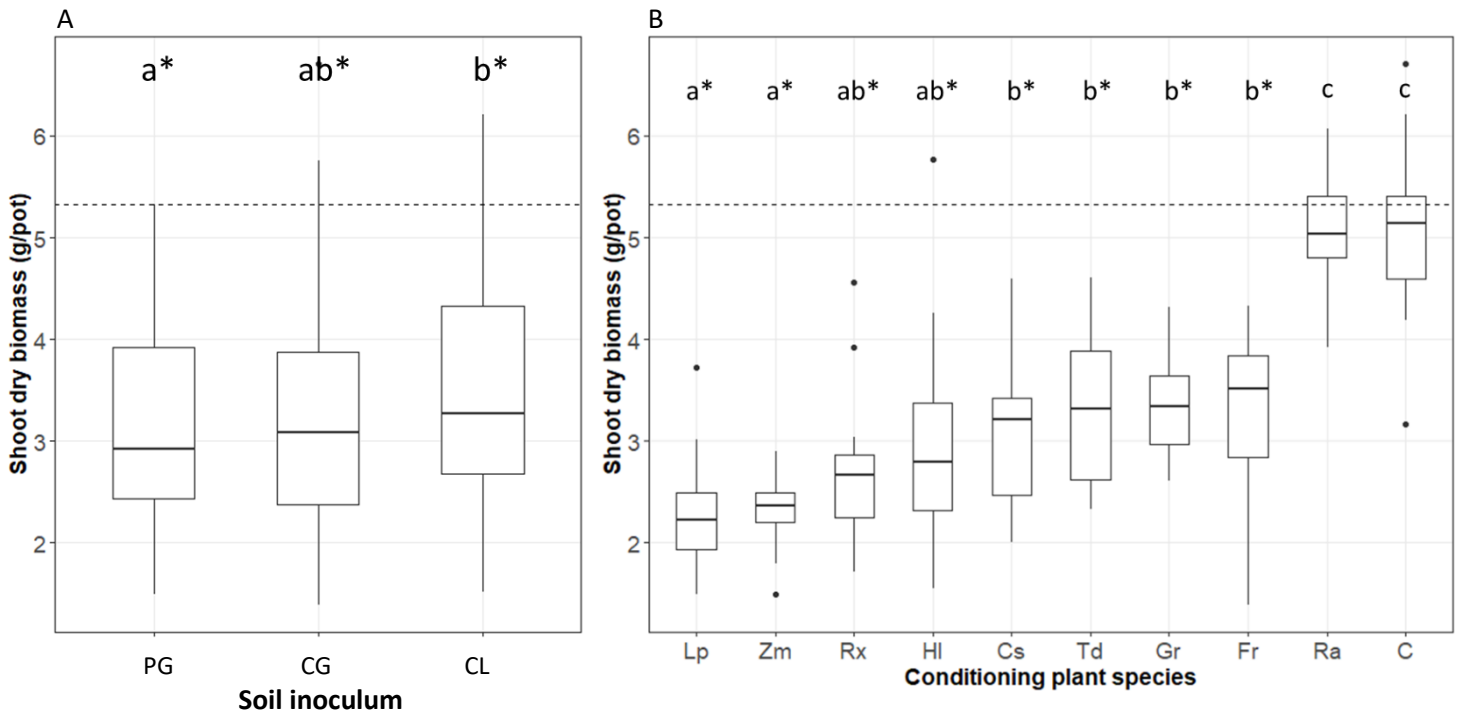
Figure 11. Total shoot biomass per soil inoculum type (A) and plant species conditioning treatment (B). Soil inoculum types: PG = production grassland, CG = conservation grassland, CL = cropland. Plant species conditioning treatments: C = control, Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*. Different letters above boxes indicate significant differences between treatments. Asterisks show significant differences from the sterilized control (dashed line).
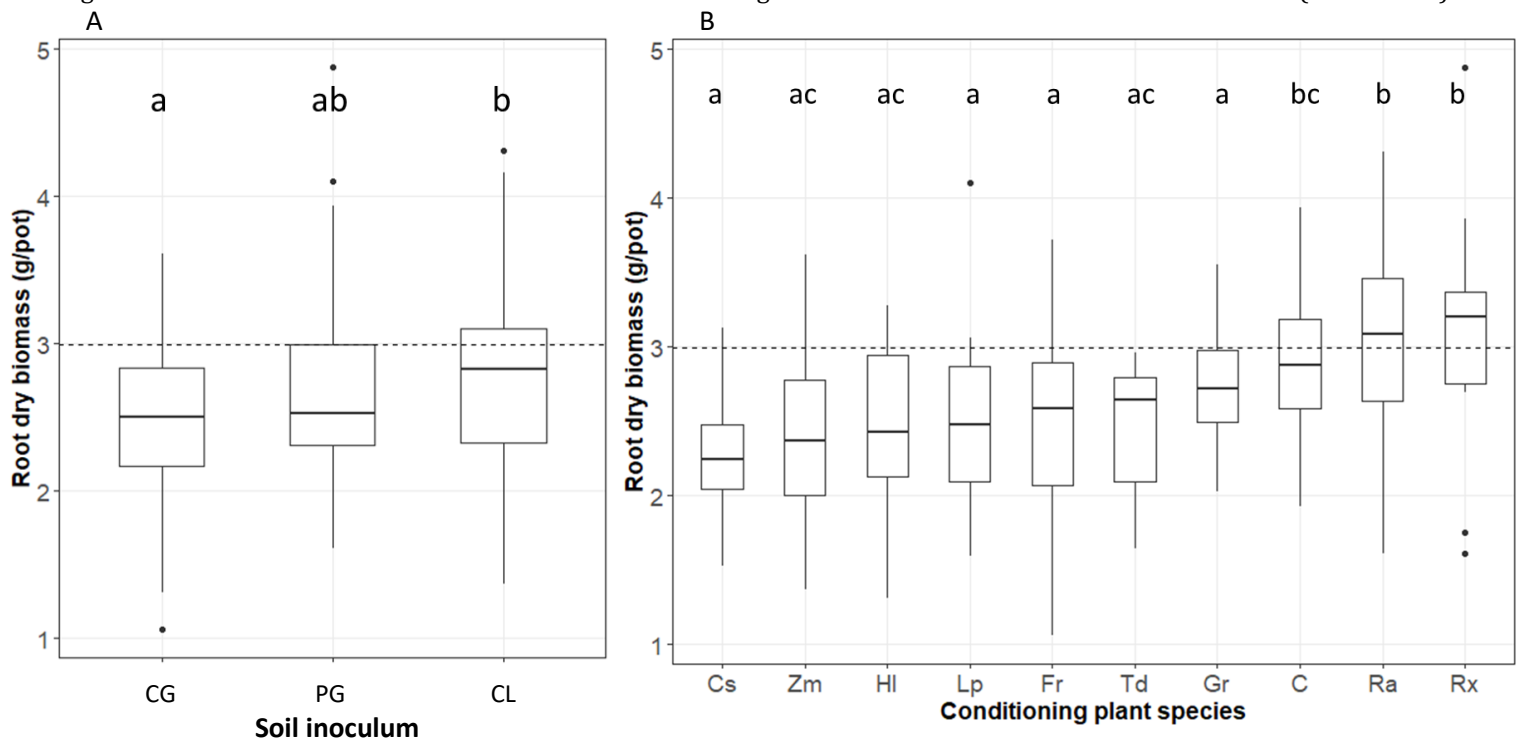
Figure 12. Total root biomass per soil inoculum type (A) and plant species conditioning treatment (B). Soil inoculum types: PG = production grassland, CG = conservation grassland, CL = cropland. Plant species conditioning treatments: C = control, Cs = *Centaurea stoebe*, Fr = *Festuca rubra*, Gr = *Geranium rotundifolium*, Hl = *Holcus lanatus*, Lp = *Lolium perenne*, Ra = *Ranunculus acris*, Rx = *Rumex acetosa*, Td = *Tragopogon dubius*, Zm = *Zea mays*. Different letters above boxes indicate significant differences between treatments. Asterisks show significant differences from the sterilized control (dashed line).
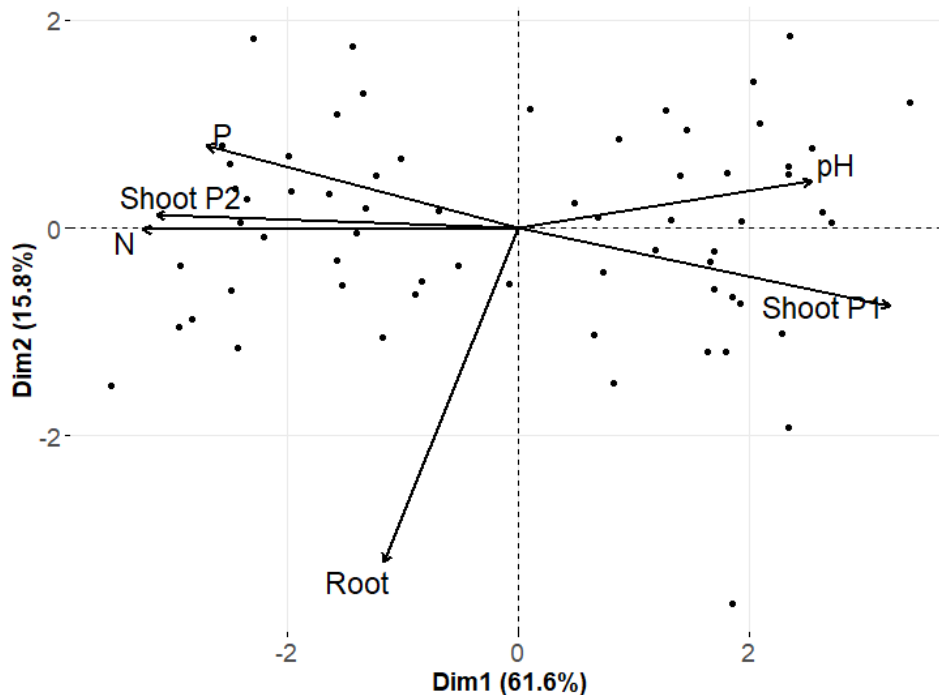
Figure 13. Principal component analysis containing the following variables: N = Total nitrogen content after the conditioning phase (mg/pot), P = P-Olsen after the conditioning phase (mg/pot), pH = soil pH after the conditioning phase, Root = root dry weight after the feedback phase, Shoot P1 = shoot dry weight after the conditioning phase, Shoot P2 = shoot dry weight after the feedback phase.

4. Discussion

The aim of the present study was to determine how (biotic) soil and plant legacies may influence grassland restoration. The question was raised what the effect is of the current plant species and belowground communities of various land types on community evenness of a subsequent natural grassland. Besides, I investigated the possible effect on community evenness of range expanding plant species migrating into an area. Lastly, the question was how plant-soil feedbacks may be species-specific or related to the plant functional group.

4.1 Community evenness and nutrient depletion
Community evenness was expected to be higher after conditioning with one of the community species (*Festuca rubra, Holcus lanatus, Ranunculus acris, Rumex acetosa*) than with *Zea mays* and *Lolium perenne*, due to accumulation of species-specific soil pathogens and herbivores, which could decrease the proportion of that species in the community. This hypothesis was not supported by the results, as the proportion of the plant species in the experimental community did not lower significantly after conditioning with one of those species.

Community evenness differed significantly between some of the conditioning plant species. Conditioning with *Z. mays* led to the highest community evenness, whereas *R. acris, H. lanatus* and the control showed a lower evenness. A more detailed view into the relative proportion of species shows that increased community evenness is mainly associated with the proportional increase of *R. acris*. The relative abundance of the dominant *H. lanatus* and *R. acetosa* did not lower significantly with higher evenness, and the abundance of *F. rubra* varied but its abundance

did not seem to relate to community evenness. These species-specific effects seem mostly related to the productivity of the conditioning plant species. *Z. mays* had a relatively high biomass after the conditioning phase, while *R. acris* biomass was limited. Moreover, the data showed community evenness to be significantly correlated to the shoot biomass during the conditioning phase, and shoot biomass of the conditioning phase shows to be negatively correlated to soil nitrogen and phosphorus levels. This suggests that higher productivity during the conditioning phase leads to nutrient limitation during the feedback phase. As a result, the slow growing plant species in the community, *F. rubra* and *R. acris,* could have more chance to compete against the more dominant *H. lanatus* and *R. acetosa*. These results are in line with the findings of Plantureux, Peeters and McCracken (2005). They suggested that only the fast growing species can compete in fields with high nutrient levels, and that an intermediate level of nutrient availability should be achieved for high biodiversity (Plantureux et al., 2005). Similarly, De Deyn et al. (2004) found a lower plant species diversity with increasing nutrient availability in a sandy loam soil with soil biota.

A limited amount of nutrients could also explain the overall lower shoot biomass of the communities that were conditioned by high productive plant species. However, these effects are less apparent for the root biomass, which is not as strongly correlated to the nutrient availability as shoot biomass. This can be explained by the finding that decreased nutrient levels are associated with increased root/shoot ratio, but not with an effect of root biomass itself (Cong et al., 2019).

A stronger effect of nutrient depletion than accumulation of soil biota on community evenness, might also explain why no effect was found after conditioning with the conservation grassland species. The conservation grassland species had a lower productivity during the conditioning phase than *Z. mays*. However, effects of soil biota cannot be ruled out, because the combined effect of nutrient depletion and soil biota cannot be separated in the current study.

4.2 Biotic soil legacies
The effect of soil biota from the different inocula seems limited. The results did not show an effect on community evenness, but did show an increased biomass in the cropland inoculated soils compared to the production and conservation grassland inoculated soils. It was expected that the community evenness would be higher with production grassland inoculum compared to cropland inoculum, because Peters and Kurstjens (2008) suggested that plant species could settle less easily on pastures compared to arable fields. The current results show that is probably not due to a difference in soil biota, but more likely due to high plant competition on pastures than agricultural fields.

The higher biomass in soils with cropland inoculum could be related to the lower proportion of herbivorous nematodes in the agricultural soils than in the production and conservation grassland soils. As agricultural fields are often associated with high abundances of herbivorous nematodes, this finding was unexpected (Yeates and Bongers, 1999). However, it has a few possible explanations. First, the agricultural fields had not been sown yet, while the pastures and natural grasslands both had vegetation at the time of soil sampling. Low food availability for the herbivorous nematodes during winter in the arable fields, where there was no cover crop sown, could have led to their relatively low abundance. This hypothesis is supported by Yeates and Bongers (1999), who stressed that the nematode community in agricultural fields show large seasonal variability. The community in spring usually shows a relatively high abundance of bacterial feeding nematodes, while at the end of the growing season the community is

dominated by plant feeding nematodes (Yeates and Bongers, 1999). Second, fertilisation on agricultural fields can have effects on the nematode community (Sarathchandra et al., 2001), but the effects are not consistent within feeding types. Sarathchandra et al. (2001) found both increasing and decreasing abundances of herbivorous nematode families, so the overall effect is not clear yet. Lastly, the current results show proportional abundances and not total abundance. A proportional change, for example by the increased number of bacterivores in the agricultural soils, could still mean that the total number of herbivorous nematodes is similar across all soil types. Therefore, a more detailed analysis of the nematode community and total counts would be useful.

4.3 Role of range expanding plant species
Opposite to my hypothesis, the range expanding plant species *T. dubius, G. rotundifolium* and *C. stoebe* did not show significant effects on community evenness. The results were comparable to the community evenness after conditioning with the other forbs in the study. Range expanding species that do show effects on plant communities or whole ecosystems are usually considered invasive (Weidenhamer & Callaway 2010, Vilà et al 2011). Although the currently studied range expanders are not considered invasive, they can still have altered biotic interactions than native species. For example, *T. dubius* had a higher productivity on soil from its expansion range than its natural range (Van Grunsven et al., 2010b), and range expanders generally experience less negative plant-soil feedback than related native plants (Engelkes et al., 2008).

Many studies regarding range expanding plant species focus on the possible geographical range that the species might have due to increased temperatures, or how biotic interactions of the range expanders differ from native plants (Bradley et al., 2009; Van Grunsven et al., 2010b; Corlett and Westcott, 2013; Engelkes et al., 2016). This might help predict what future plant communities might look like, and how ecosystems are affected by the non-native species. However, relatively little attention is given to range expanding plant species in nature restoration projects. The current study did not show effects of the range expanding species on the subsequent community, but it is possible that range expanders in newly formed natural areas affect the biotic interactions in that area. This might lead to ecosystem differences between the desired community and the actual community. Therefore, I would recommend to study the effect of range expanders on newly formed natural areas or future natural areas more closely.

4.4 Importance plant functional groups
The results showed a clear difference between soil conditioning with forbs or with grasses. Conditioning with forbs significantly lowered the proportion of forbs in the subsequent community, while conditioning with grasses led to a forb/grass ratio close to 50:50. This partly supports the hypothesis that the proportion of grasses in the community would decrease significantly after conditioning with grasses, and vice versa for forbs. These effects of plant functional groups are found more often. For example, one study showed that grasses performed better on soils conditioned with forbs, and forbs performed better on soils conditioned by grasses (Hendriks et al., 2013). Also, reduced plant growth has been found on soils conditioned with grass on sandy soil (Bezemer et al., 2006). The current study shows that these findings may also be relevant for clay soils.

4.5 Species-rich grassland restoration on clay soil: practical implications
Creating a species-rich plant community can be more challenging on clay soil than on sandy soil, due to the high capacity of clay soils to bind nutrients (Peters and Kurstjens, 2008). This is also

reflected by the state of the current species-rich grasslands that were taken as a reference. Most fields were dominated by grasses and had a few dominant species (Appendix A1), including the species used in this study. Moreover, the species used in this experiment are considered to be the intermediate stage of natural grassland development (Ecopedia, 2019a). For farmers who want to create more species-rich grasslands, the Belgian nature organisation Ecopedia defined stages of natural grassland development. The least species-rich grassland is defined as phase 1, where *L. perenne* is dominating, while the most species-rich grassland is defined as phases 4 or 5. There, the community is dominated by forbs, has more than 30 species per 25 m², and preferably includes rare species that reflect the abiotic conditions. The Maasheggen natural grasslands could be defined as phase 2 grasslands, dominated by grasses such as *H. lanatus, Arrhenatherum elatius* and *Alopecurus pratensis*, and includes common forbs such as *R. acris, R. acetosa* and *Cerastium fontanum* (Ecopedia, 2019a; b)*.

In practice, little attention is given to natural grassland restoration on clay soils in the Netherlands. For example, *Natuurmonumenten* only has extensive management guides for species-rich grasslands on sandy soils and for increasing biodiversity in (clayey) arable fields. Besides, contradictory advices are being given by organisations. *Collectief Deltaplan Landschap*, which is active in the Maasheggen area, states that they advise farmers to deplete the nutrients in their fields to stimulate biodiversity, and possible use a rotary tiller to remove the previous vegetation and then sow the desired grasses and forbs (Deltaplan, 2018). *Vereniging Nederlands Cultuurlandschap*, who also operate in the area, indicate to not use any tillage methods as that would increase the risk of damaging the structure of clay soils (Groenontwikkelfonds, 2019).

This study shows that conditioning with a plant species before developing a species-rich grassland area on clay soils can affect the subsequent community, both by depletion of nutrients, and by selecting the desired plant functional group. However, depletion of nutrients by having vegetation on a field can take longer on clay soils compared to sandy soils, and frequent mowing and removing plant residue would be essential (Deltaplan, 2019). The results of this study show that high nutrient availability could indeed affect the ability for species-rich grasslands to develop, and large scale field trials are recommended to find how to manage clay soil in the most effective way to stimulate biodiversity.

4.6 Conclusion
The aim of the present study was to determine how (biotic) soil and plant legacies may influence grassland restoration. The highest community evenness was related to the plant species with the highest biomass during the conditioning phase, and correlated with the lowest nutrient availability, suggesting that evenness is related to nutrient availability. Besides, community evenness showed to be more dependent on whether the soil was conditioned by a grass or forb, than showing clear species-specific effects of native or range expanding species. The difference between soil inocula led to a higher biomass of the community in cropland soils than production and conservation, but did not seem to affect the community evenness. These results indicate that both nutrient depletion and soil biota influenced the plant community. From these findings, it cannot be concluded whether abiotic or biotic legacies are more important. To study their relative importance, an experiment could be performed in which abiotic and biotic factors should be manipulated separately.

Specific for the Maasheggen clay fields, the current natural grasslands need continuous management to become more diverse, and possible future fields can benefit from nutrient depletion by grasses to reduce nutrient availability and stimulate forb diversity. In the face of

climate change, including increasing temperatures, the species we find in natural grasslands might not be the same in the future. As long as these species are non-invasive, they might contribute to the biodiversity of future grasslands without affecting native species. To conclude, restoring natural grasslands on clay soils can be challenging due to high nutrient availability interfering with biotic interactions. Further disentangling these abiotic and biotic legacies might help create effective management strategies. Hopefully, this will also lead to more species-rich grasslands on clay soils, contributing towards more resilient ecosystems in the future.

5. Acknowledgements

6. References

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7. Appendix

A1: Plant inventory of the Maasheggen natural grassfields

Plant inventory data of the Maasheggen natural grasslands. Observed plant species and their coverage according to the Braun-Blanquet scale are included for each 2x2m plot.

Field:	N1		
Replicate:	1		
Plant family	**Species**	**Dutch species name**	**Coverage**
Poaceae	*Festuca rubra*	Rood zwenkgras	3
Polygonaceae	*Rumex acetosa*	Veldzuring	1
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	1
Fabaceae	*Trifolium dubium*	Kleine klaver	1
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	1
Poaceae	*Holcus lanatus*	Gestreepte witbol	1
Poaceae	*Poa pratensis*	Veldbeemdgras	2
Asteraceae	unknown	-	+
Field:	N1		
Replicate:	2		
Plant family	**Species**	**Dutch species name**	**Coverage**
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	3
Polygonaceae	*Rumex acetosa*	Veldzuring	+
Caryophyllaceae	*Cerastium fontanum*	Gewone hoornbloem	+
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	+
Poaceae	*Holcus lanatus*	Gestreepte witbol	2
Poaceae	*Festuca rubra*	Rood zwenkgras	3
Poaceae	*Poa pratensis*	Veldbeemdgras	3
Poaceae	*Alopecurus pratensis*	Grote vossenstaart	+
Field:	N2		
Replicate:	1		
Plant family	**Species**	**Dutch species name**	**Coverage**
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	3
Polygonaceae	*Rumex acetosa*	Veldzuring	+
Fabaceae	*Trifolium dubium*	Kleine klaver	2
Fabaceae	*Trifolium repens*	Witte klaver	+
Fabaceae	*Trifolium pratense*	Rode klaver	+
Asteraceae	unknown	-	r
Poaceae	*Alopecurus pratensis*	Grote vossenstaart	1
Poaceae	*Bromus hordeaceus*	Zachte dravik	2
Poaceae	*Holcus lanatus*	Gestreepte witbol	3

Field:	N2		
Replicate:	2		
Plant family	**Species**	**Dutch species name**	**Coverage**
Polygonaceae	*Rumex acetosa*	Veldzuring	3
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	2
Asteraceae	unknown	-	3
unknown	unknown	Small flowering plant	r
Fabaceae	*Trifolium dubium*	Kleine klaver	3
Rosaceae	*Crataegus monogyna*	Eenstijlige meidoorn	r
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	4
Fabaceae	*Trifolium pratense*	Rode klaver	r
Asteraceae	*Cirsium arvense*	Akkerdistel	r
Poaceae	*Poa pratensis*	Veldbeemdgras	2
Poaceae	*Dactylis glomerata*	Kropaar	+
Caryophyllaceae	*Cerastium fontanum*	Gewone hoornbloem	r
Poaceae	*Bromus hordeaceus*	Zachte dravik	+
Field:	N3		
Replicate:	1		
Plant family	**Species**	**Dutch species name**	**Coverage**
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	1
Polygonaceae	*Rumex acetosa*	Veldzuring	1
Fabaceae	*Trifolium dubium*	Kleine klaver	r
Fabaceae	*Ranunculus repens*	Kruipende boterbloem	2
Fabaceae	*Trifolium pratense*	Rode klaver	+
Caryophyllaceae	*Cerastium fontanum*	Gewone hoornbloem	+
Poaceae	*Bromus hordeaceus*	Zachte dravik	r
Poaceae	*Holcus lanatus*	Gestreepte witbol	3
Poaceae	*Poa pratensis*	Veldbeemgras	+
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	+
Poaceae	*Lolium perenne*	Engels raaigras	r
Poaceae	*Festuca rubra*	Rood zwenkgras	+
Field:	N3		
Replicate:	2		
Plant family	**Species**	**Dutch species name**	**Coverage**
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	2
Fabaceae	*Trifolium repens*	Witte klaver	3
Fabaceae	*Trifolium dubium*	Kleine klaver	3
Fabaceae	*Vicia*	Wikke	+
Asteraceae	unknown	-	1
Asteraceae	*Cirsium arvense*	Akkerdistel	r
Poaceae	*Holcus lanatus*	Gestreepte witbol	1
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	1
Poaceae	*Bromus hordeaceus*	Zachte dravik	+

Poaceae	*Dactylis glomerata*	Kropaar	r
Poaceae	*Festuca rubra*	Rood zwenkgras	1
Poaceae	*Poa pratensis*	Veldbeemdgras	2
Field:	N5		
Replicate:	1		
Plant family	**Species**	**Dutch species name**	**Coverage**
Plantaginaceae	*Plantago lanceolata*	Smalle weegbree	r
Poaceae	*Bromus hordeaceus*	Zachte dravik	2
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	2
Poaceae	*Dactylis glomerata*	Kropaar	1
Asteraceae	unknown	-	2
Poaceae	*Alopecurus geniculatus*	Geknikte vossenstaart	3
Poaceae	*Alopecurus pratensis*	Grote vossenstaart	r
Poaceae	*Lolium perenne*	Engels raaigras	3
Field:	N5		
Replicate:	2		
Plant family	**Species**	**Dutch species name**	**Coverage**
Poaceae	*Festuca rubra*	Rood zwenkgras	4
Ranunculaceae	*Ranunculus acris*	Scherpe boterbloem	2
Polygonaceae	*Rumex acetosa*	Veldzuring	2
Poaceae	*Poa pratensis*	Veldbeemdgras	1
Poaceae	*Alopecurus pratensis*	Grote vossenstaart	1
Convolvulaceae	Convolvulus arvensis	Akkerwinde	2
Poaceae	*Dactylis glomerata*	Kropaar	+
Asteraceae	unknown	-	2
Poaceae	*Lolium perenne*	Engels raaigras	2