Restoration of nutrient-poor grasslands in Southern Limburg

Vegetation development and the role of soil processes

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Restoration of nutrient-poor grasslands in Southern Limburg

Vegetation development and the role of soil processes

Herstel van hellingschraallanden in Zuid-Limburg
Vegetatieontwikkeling en de rol van bodemprocessen
(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

Restoration ecology

The ongoing loss of biodiversity has led to an increasing concern for the conservation of threatened ecosystems and species all over the world (Delbaere 2004, 2006). Up to about twenty years ago, the focus was mainly on the conservation of the remaining species-rich areas by purchasing nature reserves and, if and where possible, by carrying out appropriate management measures. Since then, it has increasingly been recognized that the sustainable conservation of ecosystems and species needs a more offensive approach. Especially in the industrialized countries in Europe and North America, restoration activities are inevitable in order to maintain, restore or develop biodiversity that is lost by human interference. Experimental measures to restore degraded ecosystems are more and more being tested and applied. In The Netherlands, the first large-scale restoration project was carried out in 1950 in a moorland pool by restoring the water quality (Van Dijk & Westhoff 1960). As the knowledge about the underlying mechanisms still had to be developed, progress was made by trial and error. In the 1980s, this process has resulted in a new field of expertise within ecology, so called restoration ecology, to create a conceptual and scientific base for restoration (Nienhuis et al. 2002; Van Andel & Aronson 2006).

In the Netherlands, the main threats to biodiversity that were initially recognized – and led to national and regional policies for improvement – were acidification, eutrophication and ecosystem dehydration (so-called *ver-thema's: verzuring, vermesting, verdroging*). In 1990, landscape fragmentation (*versnippering*) was addressed for the first time in a national policy document, and added to the previous three themes. It was clear from the beginning that these problems could not only be solved by forcing back the causes, but that immediate action was needed to counterbalance the negative effects. Therefore, in 1989 the Ministry of Agriculture, Nature and Food quality started the EGM program (*Effect Gerichte Maatregelen*: 'Effect-oriented Measures'). This program involves subsidies for developing, testing and finally applying ecological restoration measures to counterbalance the impact of acidification, eutrophication and/or dehydration. The measures were not always as successful as hoped for, because of a clear lack of knowledge about the underlying ecological processes in several ecosystems. In 1995, the OBN (*Overlevingsplan Bos en Natuur*: 'Survivalplan Forest and Nature') was launched. This is a Dutch knowledge network on restoration ecology formed by researchers, management organizations and policy makers. In 2006, the scope of OBN was broadened to include all national policies regarding species and ecosystem conservation, as well as international policies relevant to the Dutch situation, including the EU Natura 2000 program, and the name changed into O+BN (*Ontwikkeling + Beheer Natuurkwaliteit:* 'Development and management of Nature quality') (Oosterlee & Smits 2009). This knowledge network is nowadays organized in seven expert teams for the major landscape types of the Netherlands.

One of the expert teams of O+BN has emphasized the lack of knowledge about the restoration of grasslands on slopes in Southern Limburg. In 2001, a research report on the current status of these grasslands clearly demonstrated the gaps of knowledge, especially concerning the status and restoration possibilities of the matgrass sward vegetation on these slopes and concerning the ecological coherence between the different vegetation zones (Bobbink & Willems 2001). This resulted in a four-year O+BN research project on the causes of the decline and the search for opportunities to restore these, originally very species-rich grassland ecosystems. Within this project, vegetation, soil and entomofauna of these grassland were studied, and the overall findings of the research were published in a Dutch research report in 2009 (Smits et al. 2009a). The current thesis, in which specific parts of the broader O+BN project are described in five scientific publications, is derived from this research project.

Grasslands on slopes in Southern Limburg

The grasslands that were studied in the current research are located in the most southern part of the Netherlands on slopes with a clear gradient in soil material. On top of the calcareous bedrock (especially in the western part of Limburg, near the river Maas), ancient sand and gravel deposits from the river occur (Figure 1.1). During the last glacial periods, parts of the slopes as well as the plateaus were covered with a loess layer of variable thickness. The current altitudinal topography of the Limburgian landscape was formed through erosion by the river Maas and its tributaries (Figure 1.2). Within the

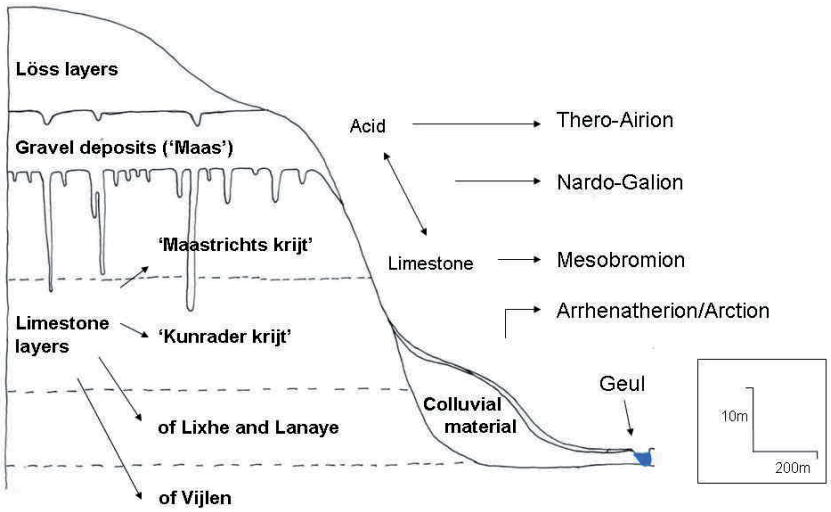


Figure 1.1. Schematic overview of the research gradient. Soil composition, geological substrate and location of the different grassland types (after Felder et al. 1981).

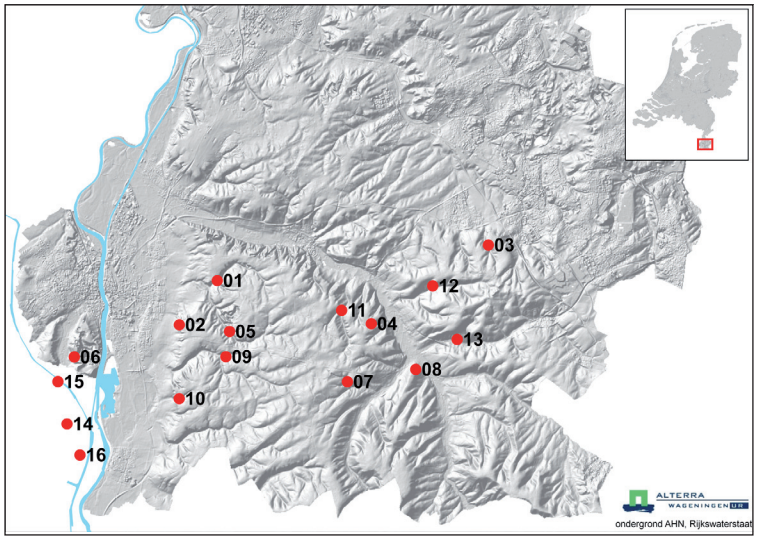


Figure 1.2. Heights (from around 30 to 322m above sea level) in Southern Limburg (AHN, 2009). Indicated are the different study sites: 1 = Bemelerberg (including Strooberg, Winkelberg, Hoefijzer and Verlengde Bemelerberg); 2 = Keerderberg; 3 = Kunderberg; 4 = Berghofweide; 5 = Schiepersberg (including Koeberg, Julianagroeve and Orchis simia-site); 6 = st. Pietersberg (Kannerhei and Popelmondedal); 7 = Vosgrubbe; 8 = Gulperberg; 9 = Wolfskop; 10 = Zure dries; 11 = Gerendal; 12 = Wrakelberg; 13 = Doeveberg and Eyserheide; 14 = Thier à la Tombe (B); 15 = Tiendeberg and Meerland (B); 16 = Thier de Lanaye (B).

USDA soil taxonomy the soils, belonging to the investigated grasslands, are classified as typic to rendollic eutrudept soils with (para)lithic contacts (Soil Survey Staff 1999).

The grasslands on these slopes often include a complex of vegetation types, depending on these soil characteristics (Figure 1.1). On the higher parts of the slopes, on the gravel deposits, acid grasslands occur, belonging to *Thero-Airion* communities. Calcareous grasslands (*Mesobromion erecti*) occur on calcareous outcrops, and matgrass swards (*Nardo-Galion saxatilis)* have developed in between (Schaminée et al. 1996). At the bottom of the slopes, where colluvial deposits have accumulated, more nutrient-rich communities belonging to *Arrhenatherion elatioris* and *Arction* can be found. Within the current study, we focus on the *Nardo-Galion* (*Betonico-Brachypodietum*) and *Mesobromion* communities (*Gentiano-Koelerietum*) on these grassland slopes (Table 1.1). In Northwestern Europe, these grasslands potentially are among the most species-rich vegetation types (Pärtel et al. 1996; Willems 2001; WallisdeVries et al. 2002). Nature reserves with these plant communities are integrated in the EU Natura 2000 network, because of their high plant diversity, which is also reflected by high faunal diversity. Matgrass swards and calcareous grasslands are included in the EU Habitat Directive as protected habitat types (Janssen & Schaminée 2003).

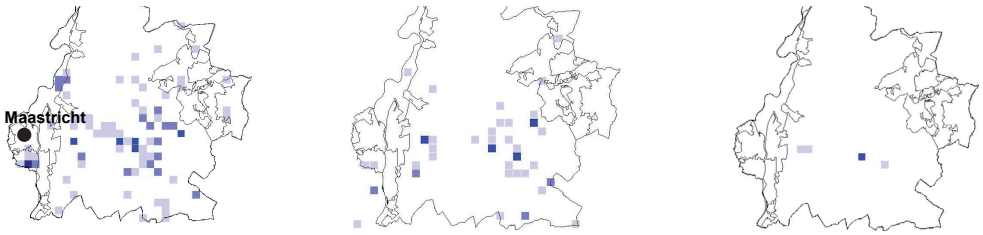
Table 1.1. Diagnostic vascular plant species of Betonico-Brachypodietum (BB) and Gentiano-Koelerietum (GK). c = character species, d = differential species, K = class, V = alliance, A = association (Schaminée et al. 1996).

Species	**BB**	**GK**		**BB**	**GK**
Achillea millefolium	dA	dK	*Knautia arvensis*	dA	
Agrostis capillaris	dK		*Koeleria macrantha*		dK
Antennaria dioica	cV		*Koeleria pyramidata*		cK
Anthoxanthum odoratum	dK		*Leontodon hispidus*	dA	dK
Anthyllis vulneraria		dK	*Linum catharticum*		dK
Arnica montana	cK		*Lotus corniculatus* var. *corniculatus*		dK
Botrychium lunaria	cV		*Luzula campestris*	dK	
Brachypodium pinnatum	dA	cV	*Nardus stricta*	cK	
Briza media	dA	dK	*Onobrychis viciifolia*		cV
Campanula rotundifolia	dA		*Ononis repens*		dK
Carex caryophyllea	dA	dK	*Parnassia palustris*	cA	
Carex ericetorum	cV		*Pimpinella saxifraga*	dA	dK
Carex flacca		dK	*Plantago lanceolata*		dK
Carlina vulgaris		dK	*Plantago media*		dK
Centaurea jacea	dA		*Platanthera bifolia*	cV	
Centaurea scabiosa		cK	*Poa pratensis*		dK
Cirsium acaule		dA	*Polygala comosa*		cV
Coeloglossum viride	cA		*Polygala vulgaris*	dA	
Danthonia decumbens	cK		*Potentilla erecta*	cK	
Euphrasia stricta	cV		*Ranunculus bulbosus*		dK
Festuca filiformis	dK		*Rumex acetosa*	dA	
Festuca rubra	dA	dK	*Rumex acetosella*	dK	
Galium pumilum		cA	*Sanguisorba minor*		dK
Gentianella ciliata		cA	*Scabiosa columbaria*		cK
Gentianella germanica		cA	*Schorzonera humilis*	cV	
Gymnadenia conopsea		cA	*Spiranthes spiralis*	dK	
Helianthemum nummularium		cK	*Stachys officinalis*	cA	
Helictotrichon pratense		cK	*Succisa pratensis*	dA	
Helictotrichon pubescens		dK	*Thymus pulegioides*		dK
Hieracium pilosella	dK		*Veronica officinalis*	cV	
Hypericum perforatum	dA		*Viola canina*	cV	
Hypochaeris radicata	dK				

The matgrass sward vegetation on the higher parts of the slope (*Nardo-Galion, Betonico-Brachypodietum*) was only recognized and published as a separate vegetation association for the Netherlands in 1982 (Willems 1982a). Therefore, only a limited amount of information is available about the historical species composition and distribution of the

matgrass sward vegetation, as most of the attention of botanists at the time was directed towards the calcareous parts of these grassland slopes. One of the characteristic species of the matgrass sward vegetation (*Parnassia palustris*) was known to have occurred on at least 50 locations in Southern Limburg, but at the moment only one of them remains (Kunderberg, Willems 1982b). Historical studies on calcareous grasslands in Southern Limburg (Diemont & Van de Ven 1953; Westhoff 1983b) already recognized elements of matgrass sward vegetation within the calcareous grasslands, but these were not described as separate vegetation units. This changed in 1975, when Willems & Blanckenborg studied the vegetation of the Sint-Pietersberg (Willems 1982a). The present name of the association and its position within the national vegetation classification of the Netherlands were given in 1996 (Schaminée et al. 1996).

To obtain a better insight in the (historical) distribution of the matgrass sward vegetation, a database was constructed with all available observations of characteristic matgrass sward species. This database, based on literature and herbarium material, contains over 500 observations. In Figure 1.3, the distribution of ten characteristic matgrass sward species is shown in three periods. The figure clearly shows the decrease of these species in the Southern Limburgian landscape (Smits et al. 2009a).



Before 1950 From 1950 onwards 2005-2008

Figure 1.3. Distribution of ten characteristic species of matgrass swards in Southern Limburg (Platanthera bifolia, Coeloglossum viride, Orchis morio, Spiranthes spiralis, Herminium monorchis, Antennaria dioica, Arnica montana, Parnassia palustris, Gentiana campestris and Botrychium lunaria) before 1950 (left), between 1950 and 2005 (middle), and recently (2005-2008; right). The intensity of the colours shows the number of species found in each square kilometre. Light grey = 1 species, middle grey = 2-3 species, and dark grey = 4-7 species. This figure is based on literature and herbarium material (Leiden, Maastricht, Utrecht) and only includes data that could be localized to a square kilometer grid cell. The data are not corrected for possible differences in observer densities.

Calcareous grasslands have been studied more intensively than matgrass swards as they always have been under the attention of botanists and ecologists, and therefore, there are more historical vegetation data available. Sites that have been managed more or less continuously, like the Laamhei (Gerendal), Wrakelberg and Kunderberg, still have calcareous grassland of a relatively good botanical quality. Sites that have been abandoned a long time ago and where restoration management was reintroduced around

1980, like the Bemelerberg, have shown an improvement of the botanical quality in the first years (Schaminée 1984; Willems et al. 1993; Bobbink & Willems 1993). On the long-term, however, this positive development stagnated (Bobbink & Willems 2001; Smits et al. 2007).

History and traditional land use

The geological formation of Southern Limburg has created the conditions for a characteristic, small-scale agricultural landscape. Originally, crop cultivation took place on the fertile loess soil on top of the plateaus. On the (steep) slopes, sheep herded by a shepherd, grazed during the day (Figure 1.4). The sheep were kept in overnight stables to collect the valuable dung. Subsequently, this manure was used on the arable fields, leading to a nutrient transport from the slopes to the plateaus. Within Southern Limburg, these sheep herds were small and occurred on the grassland slopes nearby the loess plateaus. Together with forest fringes, road verges, bare fields and orchards, the grassland slopes provided sufficient suitable foraging areas (Hillegers 1993).

Figure 1.4. Traditional management of grazing by herds of sheep.

The potential natural vegetation of the species-rich slope grasslands is deciduous forest (e.g. Westhoff 1983a). Therefore, they are called semi-natural, as regular management is necessary to maintain these grasslands and their species richness (e.g. Willems & Bik 1998). Without management, these open landscape units would quickly transform towards forest in natural succession (Figure 1.5).

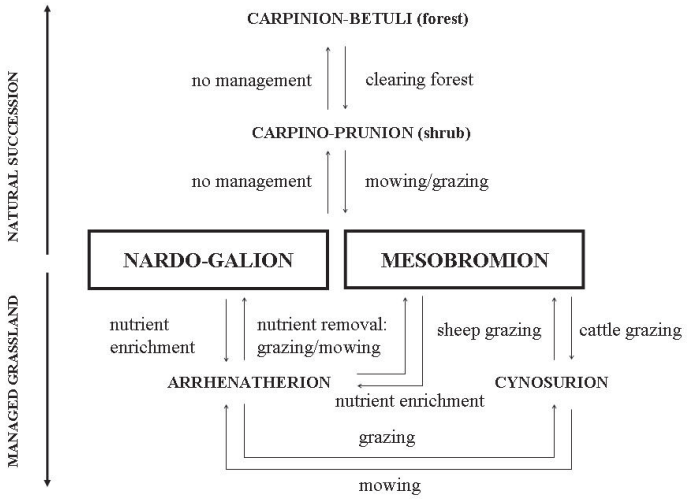


Figure 1.5. Schematic overview of the vegetation alliances on grassland slopes in Southern Limburg. Different management intensities (no management, grazing, mowing, clearing forest and nutrient enrichment) define the structure and vegetation composition of the slopes (after Schaminée et al. 2001).

Changes in land use and environmental quality

From the beginning of the twentieth century onwards, the traditional agricultural system collapsed (Poschlod et al. 2005) and intensification of agricultural practices led to a strong decline of the species richness in these grasslands. The introduction of artificial fertilizer is one of the major contributing factors (Willems 2001). The necessity to collect sheep manure ended and transporting the sheep every day was no longer feasible. At the same time, the application of barbed wire (from 1873) made it much easier to keep the sheep within fenced areas. Finally, when the Australian wool trade became more competitive than the European wool production, the traditional system of sheep grazing on these grasslands slopes largely finished around the 1940s. Many of the grassland slopes became abandoned and started their natural succession towards forest (Kohler et al. 2001, Figure 1.6). During the first decades of succession after abandonment, a strong increase of the grass *Brachypodium pinnatum* was recorded together with a decrease of species richness (Bobbink & Willems 1993). Around the same time, atmospheric N deposition was increasing due to intensification of agriculture and industrialization. Maxima of 40-80 kg N $ha^{-1}yr^{-1}$ in the late eighties and early nineties of the last century are estimated for the whole province of Limburg, whereas current levels are around 32 kg N $ha^{-1}yr^{-1}$ (Projectgroep Natuur- en Milieucompendium 2007). The latter still exceeds critical nitrogen loads that have been calculated. For matgrass swards the critical load ranges between 10-20 kg N $ha^{-1}yr^{-1}$ (Bobbink et al. 2003; Van Dobben & Van Hinsberg 2008). For calcareous grassland the range is between 15-25 kg N $ha^{-1}yr^{-1}$ (Bobbink et al. 2003; Van Dobben & Van Hinsberg 2008).

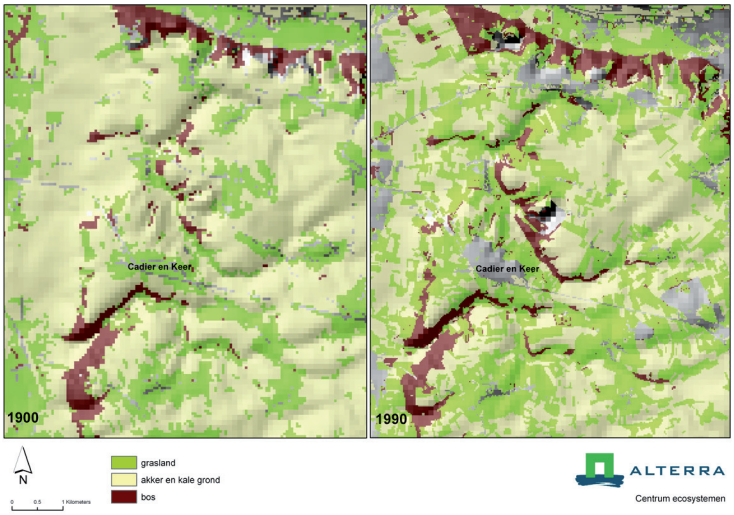


Figure 1.6. Land use around Cadier en Keer around 1900 (left) and 1990 (right). The shown map is based on the 'Actueel hoogte bestand' and the historical land use (Alterra 2009).

In the first part of the twentieth century, the increase of more intensive agriculture together with an increasing pressure on the land, led to fragmentation of the remaining grassland slopes (WallisdeVries et al. 2002), which is negatively correlated to colonization rates of most plant species (Joshi et al. 2006). In the last three to four decades, the isolation of the remaining reserves has particularly increased, due to a further land use intensification and renewed land allotment. Isolation of nature reserves is negatively correlated to their species richness (e.g. Reitalu et al. 2009). Re-colonization from nearby seed sources in such a fragmented landscape (with highly isolated and small habitats) is limited, because of the very restricted dispersal capacity of many chalk grassland species (Verkaar et al. 1983).

The studied species-rich grasslands occur on nutrient-poor soils, making them sensitive to nutrient enrichment. The intensification of agriculture on the plateaus has led to direct nutrient input, as fertilizer was running off from the agriculturally used loess plateaus on many of the grassland slopes. At the same time, abandonment of a large part of the remaining grasslands led to cessation of nutrient removal. This led, together with increased nitrogen deposition, to overall nutrient enrichment.

Nitrogen is one of the major limiting nutrients for plant growth, especially in nutrient-poor terrestrial habitats. Most of the characteristic plant species from the studied grassland slopes are adapted to nutrient-poor conditions, and can only survive or compete successfully on soils with low nutrient availability (e.g. Tamm 1991; Bobbink et al. 1998; Aerts & Chapin 2000). Therefore, the availability of nutrients in the soil is

expected to be one of the most important abiotic factors which determine plant species composition in these systems.

Preliminary measurements of nitrogen (nitrate and ammonium) in the soil revealed clear differences between the different vegetation zones (Table 1.2). Especially the high ammonium:nitrate ratio in the matgrass sward vegetation led to the question whether ammonium could have a negative effect on the target species of matgrass sward vegetation, thus being a bottleneck for restoration (Kleijn et al. 2008; De Graaf et al. 2009). Sensitivity to high soil ammonium concentrations, especially in combination with low pH, is known to exist for several endangered species of the matgrass sward vegetation (De Graaf et al. 1998a; Lucassen et al. 2002; Dorland et al. 2003; Van den Berg et al. 2005). The observed high ammonium:nitrate ratio in the matgras sward zone led us to hypothesize that nitrification might be hampered in this vegetation zone. Within this project, soil measurements of potential ammonia-oxidising activities (PAA) were carried out to provide insight in this process and in the influence of the vegetation.

Table 1.2. Measured soil characteristics of for the different vegetation types of the investigated grassland gradient (Summary of GLM, repeated measurements-results, including post-hoc Tukey-tests). Measurements are from week 6, 16, 27, 35, and 45 in 2005 from one transect at the Bemelerberg. Units of measurement for N-NH_4, N-NO_3, and total inorganic nitrogen are mg.kg^{-1} dry soil. Soil moisture content is calculated as (fresh soil-dried soil)/dried soil. N-inorganic is the sum of N-NH_4 and N-NO_3. Sphericity was assumed for pH, but not for N-NH_4, N-NO_3, Ammonium:nitrate ratio, and N-inorganic. The epsilon of Greenhouse-Geisser was therefore used for the latter factors. The standard deviation is between brackets, and significant differences between the vegetation types are indicated by different letters (P < 0.05) or with an asterisk for the factors (= 0.01 > P < 0.05; - = not significant).*

	Time	*Thero-Airion* (n= 5)	*Nardo-Galion* (n= 8)	*Mesobromion* (n = 7)	Time x vegetation type
pH_{demi}	F = 2.814, P = 0.032 *	5.05 (0.16), a	6.11 (0.12), b	7.98 (0.14), c	F = 2.547, P = 0.018 *
N in $NO_{3\ demi}$ (mg kg^{-1} dry soil)	F = 3.024, P = 0.057 -	10.07 (2.13), a	1.43 (1.68), b	3.23 (1.80), ab	F = 1.093, P = 0.377 -
N in $NH_{4\ KCl}$ (mg kg^{-1} dry soil)	F = 4.233, P = 0.033 *	11.16 (1.69), a	6.43 (1.33), ab	1.73 (1.42), b	F = 3.555, P = 0.025 *
Ammonium: nitrate ratio	F = 1.536, P = 0.234 -	2.86 (1.30), a	8.66 (1.30), b	1.04 (1.10), a	F = 1.415, P = 0.258 -
N-inorganic	F = 3.515, P = 0.045 *	21.23 (3.54), a	7.85 (2.80), b	4.96 (3.00), b	F = 2.238, P = 0.092 -

Restoration management

At the beginning of the 1970's, restoration of calcareous grasslands started to become a realistic option for nature conservation organizations (Hillegers 1985, Willems 2001, WallisdeVries et al. 2002). The characteristic sheep breed ("Mergellandschaap") was rediscovered and bred again (Figure 1.7). In some reserves the regenerated shrub and forest were cleared (Zure Dries, Schiepersberg). Around 1980, Mergelland sheep were reintroduced in calcareous grassland for restoration purposes (Hillegers 1993). Some experimentation took place with the grazing regime (high sheep density during a relatively short period vs. low density during a longer time), but the traditional management, in which the sheep were removed during the night, was not re-applied due to practical problems. Sheep grazing as a (restoration) management measure is a potential vector for seed dispersal. The traditional farming with associated sheep grazing implied the dispersal of large quantities of seed (Poschlod & Bonn 1998), but the current transport is limited, and plant species tend to be dispersed mostly from high- to low-productive landscape units (Mouissie et al. 2005, Kuiters & Huiskes 2009).
The effects of nutrient enrichment in calcareous grasslands (in relation to abandonment and the increased atmospheric nitrogen deposition) were investigated by Bobbink 1991, Bobbink & Willems 1987; 1988; 1991; 1993). The increase of the grass *Brachypodium pinnatum* was found to be strongly correlated to a decrease in species richness and diversity (Bobbink & Willems 1987; 1993, Willems 1990). On the short term, cutting the vegetation in mid-summer with removal of the mown material was found to be an

Figure 1.7. Mergellandschaap with two lambs (photo Peer de Win).

adequate measure to control *Brachypodium pinnatum* in continuously managed grassland reserves (Bobbink & Willems 1991), but on the longer term full restoration of these grasslands was not accomplished (Bobbink & Willems 1993; 2001; Smits et al. 2007).

Systematic analyses of the long-term development of the biodiversity in these grasslands were lacking (Bobbink & Willems 2001). Furthermore, it became increasingly clear that the area of these highly fragmented semi-natural habitats should be increased and connected to form a potentially stable ecological network (Opdam et al. 1993; Jongman & Pungette 2004; Ozinga et al. 2009). This led to the decision to re-create new species-rich grasslands to enlarge and connect the remaining grassland reserves. Dutch nature policy often considers agricultural (grass)lands with a history of intensive use to be the most available to enlarge the ecological network. Therefore, for the development of new grasslands, the focus is on highly enriched soil conditions.

Thesis objectives

The current study was initiated to contribute to a better understanding of 1) the causes of decline, and 2) opportunities for restoration of the nutrient-poor, but potentially species-rich grasslands on the slopes (matgrass sward vegetation and calcareous grassland) in Southern Limburg. The research can be divided into three parts. First of all, the past and present status of the grasslands is investigated to provide insight in long-term changes. The second part is dealing with possible constraints for restoration. In this part, the following questions have been investigated. Does eutrophication play a role in the observed long-term changes? Is the observed high ammonium:nitrate ratio in the matgrass sward zone caused by differences in potential nitrification activity along the vegetation gradient? Does the characteristic vegetation in the matgrass sward zone influence the potential nitrification activity? In this context, also topics related to fragmentation and isolation were addressed. The third, and last part of the research focuses on opportunities for restoration. Corresponding research questions deal with the long-term after-effects of fertilisation in calcareous grasslands, and with successful management measures to restore species-rich grasslands on slopes with a history of intensive agricultural use.

Thesis outline

In chapter 2, the **past and present status** of calcareous grasslands is investigated, using a large dataset of vegetation relevés from the Dutch Vegetation Database. These data range from 1939 to 2008 and provided the opportunity to investigate long-term changes in vegetation composition. Preliminary measurements on soil nitrogen revealed interesting differences along the investigated soil gradient. Therefore, soil nitrification processes,

being a possible **constraint for restoration**, were investigated in two chapters. In chapter 3 the potential nitrification activity along the investigated grassland gradient (acid grassland, matgrass sward vegetation and calcareous grassland) was investigated in a field survey. In chapter 4, the results from a greenhouse experiment are reported, in which different characteristic plant species of matgrass swards and calcareous grassland grew in a crosswise experiment on both matgrass sward soil and calcareous grassland soil. **Restoration opportunities** are reported in the last two chapters. In chapter 5, the long-term after-effects of fertilisation on the restoration of calcareous grassland are studied on the basis of time series of permanent plots in the Gerendal, starting in 1970. In chapter 6 the opportunities for accelerated restoration on agriculturally improved pastures are investigated. In two research sites, two experimental measures were tested with respect to the development of vegetation and soil conditions. The investigated measures included nutrient removal (by sod cutting and mowing) and seed addition (by transfer of mown material from a well-developed reference site). In the synthesis (chapter 7), the results are integrated, overall conclusions are drawn and results of additional, ongoing experiments are reported.

Chapter 2

Long-term vegetation change in Dutch calcareous grasslands: how effective are restoration measures?

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Abstract

The long-term effects of management as it was reintroduced around 1980 in Dutch calcareous grasslands are being investigated. Long-term vegetation changes in these grasslands have been analyzed using the large Dutch vegetation database with more than 500,000 relevés. From this database, 556 relevés were selected that could be classified as calcareous grassland (*Gentiano-Koelerietum*). Based on this selection, differences in floristic composition were analysed between four periods, using stratified resampling. We found clear changes in species composition. Species with a preference for open, dry habitats have significantly decreased, whereas species of more moist and nutrient-rich circumstances increased. This was confirmed by changes in mean Ellenberg indicator values. Restoration and conservation management in calcareous grasslands did not lead to full recovery of the former quality. The current species composition clearly differed from that recorded in the reference period (1931-1970). Moreover, species without adaptations for efficient long-distance dispersal have decreased. Apparently, the current restoration management has not been sufficient for full restoration of this nutrient-poor grassland ecosystem. Habitat fragmentation and isolation have led to a lower proportion of species without adaptations for long-distance dispersal, as they are no longer able to disperse among the isolated sites.

Key words

Calcareous grassland, *Gentiano-Koelerietum*, long-term changes, restoration, trend analysis, vegetation change.

Introduction

The calcareous grasslands of Northwest Europe belong to the most species-rich vegetation types of this part of the world (Pärtel et al. 1996; WalisdeVries et al. 2002). Their protection is a priority in the EU Habitat Directive, where they are featuring as habitat type 6120, semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*: 2002/83/EC Habitat Directive). In many European countries, this vegetation type has received much attention in initiatives for restoration and conservation management (Butaye et al. 2005; Köhler et al. 2005; Kiehl et al. 2006; Pärtel & Helm 2007; Dostálek & Frantík 2008; Fagan et al. 2008; Galvánek & Lepš 2008). In the Netherlands, these potentially species-rich grasslands are present in the most southern part of the Netherlands (Willems 2001; WallisdeVries et al. 2002) on slopes with calcareous bedrock at or near the surface, described as typic to rendollic eutrudept soils with (para)lithic contacts (Soil Survey Staff 1999).

Up to the 20th century, these grasslands (partly on steep slopes) were part of a traditional 'communal village' agricultural system, in which they were used as communal grazing lands for sheep, guarded by a shepherd. In the traditional system the sheep were brought inside at night to be able to collect their manure for the agricultural fields. This resulted in a nutrient transport from these grasslands to the agricultural fields, leading to a decrease of nutrient availability in the grassland slopes, together with an increase of nutrient availability in the agricultural fields. Around the turn of the 19th to the 20th century, important changes in land use started, e.g. due to the invention of artificial fertilizer and barbed wire. This resulted in a more intensified agricultural system on the plateaus and in the valleys, whereas many of the slopes became abandoned (Bobbink & Willems 1993; Hillegers 1993). In the second half of the 20th century, atmospheric N deposition increased, causing an extra nutrient input on these grassland slopes. Modelled and general atmospheric N deposition data for the province of Limburg reached maxima of 40-80 kg N $ha^{-1}.yr^{-1}$ in the late eighties and early nineties of the last century. Current levels are around 32 kg N $ha^{-1}.yr^{-1}$ (Projectgroep Natuur- en Milieucompendium 2007). Internationally used empirical critical loads for calcareous grassland are between 15-25 kg N $ha^{-1}.yr^{-1}$ (Bobbink et al. 2003) with the latest modelled calculations of 21.1 kg N $ha^{-1}.yr^{-1}$ for the Dutch calcareous grasslands (Van Dobben & Van Hinsberg 2008). The abandonment of these grasslands resulted in successional changes towards an increasing dominance of *Brachypodium pinnatum.* The increase of this species was also positively correlated to the enhanced N deposition in the Netherlands (Bobbink 1991; Bobbink et al. 1988). This resulted in a strong decrease of the number of species in the vegetation (Bobbink & Willems 1991, 1993; Willems 1990).

Around 1980, renewed attention for the conservation and restoration of the Dutch calcareous grasslands commenced, although some of these sites have remained

continuously managed. In abandoned sites with dominance of *Brachypodium pinnatum*, experiments with cutting regimes were carried out to investigate appropriate management to reduce the thick litter layer and to decrease the dominance of *Brachypodium pinnatum*. Results clearly showed that cutting the vegetation twice during three growing seasons was already adequate to reduce the negative effects of the thick litter layer and the abundant growth of *Brachypodium pinnatum* (Bobbink & Willems 1991, 1993), creating an appropriate starting point for a more long-term management plan. Renewed attention for breeding the local sheep breed (*Mergellander*) led to more intensified semi-traditional grazing management in many of these nutrient-poor grasslands. There are, though, clear differences compared to the traditional grazing management. The current management includes a 24-h stay at the grassland for several days, whereas the traditional herded, itinerant flock of sheep visited the grasslands during the day and spent the night in a stable elsewhere. Currently, almost 30 years after the first revive of restoration, it seems that the success of restoration efforts is stagnating, although little is known about the long-term overall changes in Dutch calcareous grasslands and the effectiveness of the restoration management.

The aim of the current study is to investigate the long-term effects of management of calcareous grassland in the Netherlands, as it was reintroduced around 1980. To achieve this, general changes in Dutch calcareous grasslands are analysed, by using data from the Dutch Vegetation Database (Knollová et al. 2005; Schaminée et al. 2007; Haveman & Jansen 2008). Traditional habitat restoration measures aim at improving the local habitat quality, but as the current calcareous grasslands are very fragmented and isolated in the Southern Limburg landscape, dispersal processes probably also play an important role. To be able to distinguish between these different processes, Ellenberg indicator values were calculated for changes in the local habitat quality. To get insight into the importance of dispersal processes for restoration, vegetation datasets were combined with the LEDA database, containing quantitative information on plant traits (Knevel et al. 2003, 2005; Kleyer et al. 2008).

Material and Methods

Selection of plot data

The plot data with species composition in calcareous grasslands were obtained from the Dutch Vegetation Database, containing over 500.000 relevés (Schaminée et al. 2009). Using ASSOCIA (Van Tongeren et al. 2008), each relevé was assigned to one or several plant communities. ASSOCIA uses the synoptic tables as published in Schaminée et al (1995-1999) as a reference. The program calculates the likelihood that a given relevé belongs to a specific syntaxon by looking at constancy values and mean cover abundance of the species. It uses Eucledian distances to calculate the similarity between each

specific relevé and the syntaxon that it is compared to. Besides the likelihood, ASSOCIA also calculates the incompleteness and weirdness for each combination of relevé and plant community. The 'incompleteness' is a measure to what extent the typical species for a certain community are present in the relevé. The 'weirdness' is a measure to what extent weird (unexpected, non-characteristic) species are present in the relevé in relation to a certain community. Based on the output of ASSOCIA, relevés assigned to calcareous grassland were selected.

Several filters were applied to improve the quality of the dataset. First of all, only plots were included with a maximum index for incompleteness of 0.3 and a maximum index of weirdness of 0.4 (Van Tongeren et al. 2008). This led to a selection of relevés that are considered to represent well-developed calcareous grassland. Furthermore, only the relevés that had an indication of the date of collection and an accurate indication of the location were included.

To obtain a homogeneous dataset without a relationship between species numbers and plot size, the plots smaller than $1m^2$ and the plots bigger than $50m^2$ were excluded. For these plot sizes, the relationship between plot size and species numbers was tested with an Kruskall-Wallis test, using $1m^2$, $4m^2$ and $9m^2$ as cut-off points. Significant differences occur between the different groups ($X^2 = 10.765$, df = 3, P = 0.013) with slightly higher species numbers for the largest plot size (>$9m^2$; post-hoc Tukey B, Table 2.1). As the difference in average species numbers was only 2 species, they are considered to have a minor effect on the whole analysis. Therefore, $50m^2$ was kept as the upper size to be able to include as many data as possible. To check for outliers, the remaining dataset was classified with JUICE 6.5.41 (Tichy 2002) using TWINSPAN (Hill 1979).

Table 2.1. Relationship between plot size and average number of vascular plant species, with the standard deviation between brackets. Numbers followed by the same letter are not significantly different.

Plot Size	**N**	**Species number (± st.dev.)**
$\leq 1m^2$	174	31 (± 4) a
$1m^2 >$ and $\leq 4m^2$	294	31 (± 4) a
$4m^2 >$ and $\leq 9m^2$	36	33 (± 6) ab
$9m^2 >$ and $\leq 50m^2$	52	33 (± 6) b

Periods

To analyze the changes over the years, four research periods were chosen, partly based upon the availability of relevés. The choice for 1980 as a cut-off point was determined by the considerations that restoration management generally commenced around this time, and that atmospheric N deposition had its peak roughly around 1980.

The following four periods were chosen:
1931-1970: starting with the earliest available data. These data represent a reference for the intact, well-developed calcareous grasslands;
1971-1980: representing the period up to the start of restoration management around 1980;
1981-1990: the period just after restoration started;
1991-2008: representing the current period.

Stratified resampling
The following criteria have been applied for stratified resampling: (1) per decade and per 1 x 1 km grid cell, a maximum of 10 relevés are included to stratify for temporal and spatial aspects; (2) 100 iterations are carried out; and (3) the maximum number of relevés per period per iteration was set at 60 relevés to make sure that every iteration only contained a subset of the available data.

Species taxonomy
For some species with notorious identification problems, taxa were lumped and subspecies were removed to overcome taxonomical problems (Table 2.2).

Table 2.2. Renamed vascular plant species.

Old names	**New name**
Arrhenatherum elatius ssp. *elatius*	*Arrhenatherum elatius*
Bromus hordeaceus ssp. *hordeaceus*	*Bromus hordeaceus*
Cerastium fontanum	*Cerastium fontanum* ssp. *vulgare*
Festuca ovina, F. filiformis	*Festuca ovina* ag. *(*incl. *F. cinerea, F. filiformis)*
Festuca rubra	*Festuca rubra* ag.
Lotus corniculatus ssp. *corniculatus*	*Lotus corniculatus*
Medicago falcata	*Medicago falcata + M. sativa*
Ononis repens, O. repens ssp. *repens, O. repens* ssp. *spinosa*	*Ononis repens*
Platanthera bifolia, P. chlorantha, P. bifolia x chlorantha	*Platanthera bifolia + P. chlorantha*
Polygala comosa	*Polygala vulgaris*
Senecio jacobea ssp. *jacobea*	*Senecio jacobea*
Taraxacum officinale, T. tortilobum, T. sectie *Erythrosperma, T.* sectie *Tortilobum*	*Taraxacum species*
Thymus serpyllum, *T. pulegioides*, *T.* species	*Thymus pulegioides* ag.
Vicia sativa ssp. *nigra*	*Vicia sativa*

Explanatory variables for plant diversity changes
Based on presence/absence data of species, average Ellenberg indicator values for light, temperature, moisture and nutrients were calculated for each relevé (n= 556). Originally,

the 'nutrients' parameter was indicated as 'nitrogen', but it is probably best interpreted as a productivity or soil fertility parameter (Schaffers & Sýkora 2000), and therefore it is called 'nutrients' in the current publication. Secondly, we restricted the analysis to the subset of species that showed a significant trend in the stratified resampling procedure, for which again Ellenberg indicator values were calculated and compared.

Data on dispersal ability by various dispersal vectors were extracted from the LEDA database with life-history traits of the Northwest European flora (Knevel et al. 2003, 2005; Kleyer et al. 2008) and adapted to a binary classification (Ozinga et al. 2004, 2009). The following dispersal vectors, all capable of providing highly effective long-distance dispersal (> 100 m), were considered: wind, the fur of large mammals, the digestive tract of large mammals, and the digestive tract of frugivorous birds. It should be noted that many species have a high potential for more than one dispersal vector (Ozinga et al. 2004).

In order to include information on as many species as possible, the available data were aggregated into a binary classification. As a consequence the dispersal data are less precise as compared to the Ellenberg indicator values. We quantified the representation of dispersal syndromes in four periods, i.e. the proportion of species with a high potential for dispersal by the given long-distance dispersal vector (wind, fur, dung, birds) or with no adaptations for long-distance dispersal (No LDD). The proportions are weighted by the frequency of occurrence.

Statistical analysis

The species frequencies between period 1-2, 2-3, 3-4, and 1-4 were analysed with χ^2-statistics in JUICE (Tichy 2002) for each iteration. Only vascular plant species were included, as bryophytes had not been recorded on a regular basis. The number of permutations in which a species showed a significant difference between two research periods, was recorded for every plant species. Stratified resampling and statistical analysis were carried out with a threshold significance value of $P \leq 0.05$. If the number of permutations with a significant difference for a given species exceeds 75%, the significance is considered strong, if the number of permutations with a significant difference for a given species was between 51% and <75%, the significance is considered to be reasonable. Significant differences in Ellenberg indicator values between the two species groups were compared with a Student T-test ($p<0.05$). The significance of the effect of dispersal traits was analysed with logistic regression and we compared the subsets of species that showed a significant decrease with the subset with a significant increase (see Table 2.3).

Nomenclature follows Van der Meijden (2005) for vascular plant species and Schaminée et al. (1995-1999) for plant communities.

Table 2.3. Groups of species that showed significant trends over the total research period. Four species groups can be distinguished: decreasing trends, increasing trends,initial increasing followed by decreasing trend and the opposite: first decreasing, then increasing trends. In electronic appendix A, the complete results of the analyses are shown.

Decreasing species	**Increasing species**	**Increasing, then decreasing**
Anthyllis vulneraria	*Clematis vitalba*	*Hieracium umbellatum*
Arenaria serpyllifolia	*Clinopodium vulgare*	*Senecio erucifolius*
Bunium bulbocastanum	*Cornus sanguinea*	
Carlina vulgaris	*Crataegus monogyna*	**Decreasing, then increasing**
Cirsium acaule	*Crepis biennis*	*Carex caryophyllea*
Euphrasia stricta	*Dactylis glomerata*	*Campanula rotundifolia*
Festuca ovina ag.	*Dactylorhiza maculata* ssp. *fuchsii*	*Ranunculus bulbosus*
Galium verum	*Fraxinus excelsior*	*Plantago media*
Gentianella germanica	*Galium mollugo*	
Hieracium pilosella	*Gymnadenia conopsea*	
Koeleria macrantha	*Leucanthemum vulgare*	
Koeleria pyramidata	*Prunella vulgaris*	
Orchis militaris	*Prunus avium*	
Poa compressa	*Rhinanthus alectorolophus*	
Potentilla verna	*Rhinanthus minor*	
Thymus pulegioides ag.	*Rubus caesius*	

Results

The application of our selection criteria resulted in a set of 556 relevés and a total of 241 vascular plant species that were used for the analyses. Based on the outlier analysis in TWINSPAN, no further relevés were excluded. The different periods are represented by 86 (period 1931-1970), 133 (period 1971-1980), 189 (period 1981-1990), and 148 (period 1991-2008) relevés, respectively. The average number of vascular plant species per relevé slightly increased in time from resp. 30.1 (± 5.8), to 30.5 (± 4.3), to 32.1 (± 4.0), and 31.4 (± 4.4) for the period 1991-2008.

Stratified resembling and statistical analysis resulted in four groups of species that showed clear trends, while the majority of species (84%) showed random variation without significant trends. The following four groups of trends were distinguished: 16 species clearly increased over the selected periods, 16 species decreased, and 6 species showed two different, more complex changes.

The ecological characteristics of the increasing and decreasing species mentioned in Table 2.3 show clear general patterns. Twelve of the 16 species that significantly decreased are considered characteristic for calcareous grassland, whereas the remaining

four species (*Arenaria serpyllifolia*, *Festuca ovina*, *Galium verum* and *Poa compressa*) are typical for *Sedo-Scleranthetea* and/or *Koelerio-Corynephoretea* (Schaminée et al. 1995-1999; Schaminée et al. 2007). All species in this group are indicative of relatively dry, nutrient-poor and open habitats in the Netherlands. Five of the 16 species showing a significant increase over the research period were woody species (*Cornus sanguinea*, *Crataegus monogyna*, *Fraxinus excelsior*, *Prunus avium* and *Rubus caesius*), which clearly indicates that the current management allows woody species to invade these grasslands. *Gymnadenia conopsea* ,one of the few orchid species that has its optimal habitat in Dutch calcareous grassland, is the only characteristic plant species that has clearly benefited from the developments in these grasslands since 1990. The increase of this species may be explained by special measures to promote orchids in current management schemes: many grasslands are currently grazed or mown only after the period in which the orchids have set seeds. The remaining ten species with a significant increase, have various ecological characteristics: *Clinopodium vulgare, Clematis vitalba,Crepis biennis*, *Dactylis glomerata*, *Dactylorhiza maculata* ssp. *fuchsii*, *Galium mollugo*, *Rhinanthus minor, Leucanthemum vulgare*, and *Prunella vulgaris* occur in more nutrient-rich grasslands and fringes (*Molinio-Arrhenatheretea*, *Galio-Urticetea*, *Trifolio-Geranietea sanguinei*; Schaminée et al. 1995-1999; Schaminée et al. 2007). Finally, *Rhinanthus alectorolophus*, a Central European species, has been found spreading recently in Dutch calcareous grasslands. The two *Rhinanthus* species (*R. minor* and *R. alectorolophus*) are both annual, semi-parasites on grasses, that can disperse easily. Their boost can be explained by the increase of sites with relatively more nutrient-rich conditions and therefore more nutrient-rich grass species.

Six remaining species revealed significant bidirectional changes. *Hieracium umbellatum* and *Senecio erucifolius* increased up to 1980 and decreased afterwards, whereas *Campanula rotundifolia*, *Carex caryophyllea*, *Plantago media* and *Ranunculus bulbosus* decreased up to 1980 and increased afterwards (Table 2.3). The complete synoptic table, including the results from the stratified resembling and statistical analyses is included as an appendix (App. A).

Explanatory variables for plant diversity changes

The mean Ellenberg indicator values for the 556 relevés showed minor, but significant changes between the specific research periods (Table 2.4). The Ellenberg indicator values for light and temperature decreased over time, whereas the Ellenberg indicator values for moisture and nutrients increased. The same trends are visible in the two species groups showing a significant increase or decrease (Figure 2.1): the declining species had higher Ellenberg indicator values for light ($F = 1.328$, $df = 29$, $P = 0.006$) and temperature ($F = 0.143$, $df = 20$, $p = 0.028$), whereas they had lower average Ellenberg indicator values for moisture ($F = 0.458$, $df = 25$, $P < 0.001$) and nutrients ($F = 20.633$, $df = 15.217$, $p < 0.001$).

*Table 2.4. Average number of vascular plant species and average Ellenberg indicator values for light, temperature (temp.), moisture and nutrients for the different research periods with the standard deviation between brackets. The significance level is indicated with *** = P < 0.001.*

	1931-1970	**1971-1980**	**1981-1990**	**1991-2009**	**Statistics**
	n = 86	n = 133	n = 189	n = 148	
Nr.of species	30.1 (5.8) a	30.5 (4.3) a	32.1 (4.0) b	31.4 (4.4) ab	X^2 = 19.986, df = 3, P< 0.000 ***
Light	7.20 (0.10) a	7.13 (0.11) b	7.11 (0.11) b	7.10 (0.12) b	X^2 = 56.625, df = 3, P< 0.000 ***
Temp.	5.50 (0.10) a	5.46 (0.11) b	5.44 (0.10) b	5.43 (0.12) b	X^2 = 24.398, df = 3, P< 0.000 ***
Moisture	3.98 (0.18) a	4.03 (0.13) b	4.06 (0.13) b	4.20 (0.17) c	X^2 = 104.059, df = 3, P< 0.000 ***
Nutrients	3.05 (0.34) a	3.34 (0.27) b	3.40 (0.33) b	3.40 (0.43) b	X^2 = 57.926, df = 3, P< 0.000 ***

Spectra of dispersal syndromes across the four periods revealed only minor changes (Table 2.5). There was a decrease in the proportion of species with no adaptations for efficient long-distance dispersal and a small increase in the proportion of wind- and bird-dispersed species. The results of the logistic regression based on the comparison of dispersal traits of species that with a significant decline and those with a significant increase (see Table 2.3) revealed that species with no adaptations for efficient long-distance dispersal were significantly overrepresented among the declining species (Wald-5.0, Nagelkerke R^2=0.25, P = 0.025), while the effect of other traits was insignificant.

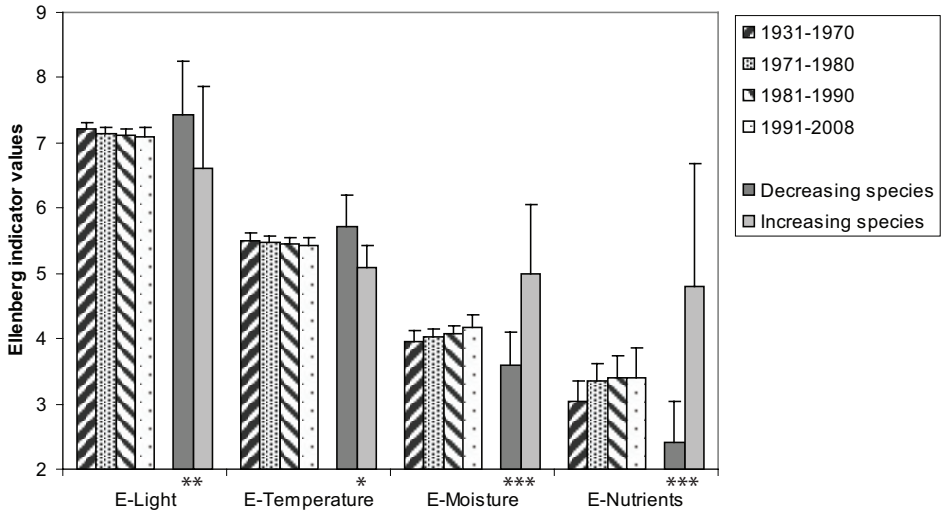

*Figure 2.1. Average Ellenberg indicator values for light, temperature, moisture and nutrients based on the relevés in the different research periods (n = 86, 133, 189, 148), and based on the species groups that showed significant trends. With asterisks the significance level between the two species groups is indicated: *** = P < 0.001; ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05, n.s. = not significant.*

Table 2.5. Representation of dispersal syndromes in the four periods. The data in the cells represent the proportion of species with a high potential for dispersal by the given long- distance dispersal vector (wind, fur, dung, birds) or with no adaptations for long-distance dispersal ('No LDD'). Indicated is the percentage of species in the vegetation with an adaptation for a specific dispersal vector. Note that species can have adaptation for more than one dispersal vector. The proportions are weighted by the frequency of occurrence.

Dispersal vector **Period**	**Wind** **(n = 42)**	**Mammals, fur** **(n = 50)**	**Mammals, dung** **(n = 90)**	**Birds** **(n = 16)**	**No LDD** **(n = 57)**
1900-1970	4.9	31.7	46.3	1.1	27.7
1970-1980	7.1	29.8	42.4	5.6	27.8
1980-1990	6.6	30.8	46.4	3.3	24.8
1990-2010	7.7	30.2	47.2	3.5	21.0

Discussion

Changes in species composition

In the present study we identified a clear and consistent shift in species composition in well developed Dutch calcareous grasslands during the second half of the 20th century. The observed shift towards less distinct calcareous grassland and more common mesotrophic grassland communities is similar to developments observed in other calcareous grassland areas in Europe. Bennie et al. have studied the long-term vegetation change in British chalk grassland and also concluded a shift towards a more nutrient-rich vegetation (Bennie et al. 2006). In the British research, also a decrease in total species numbers was found, in contradiction to the current study. In Belgium, the replacement of specialists by generalists in butterfly communities of calcareous grassland was linked to habitat loss and fragmentation in these habitats (Polus et al. 2007). In the Swiss alps, the resurvey of 151 relevés led to on average higher mean species numbers, but clear shift in community composition towards higher nutrient indicator values (Peter et al. 2009).

Explanatory variables

By using environmental indicators, such as Ellenberg indicator values, the observed changes in vegetation composition can expose the major drivers of vegetation change. As the selection of 556 relevés all represent well-developed calcareous grassland (due to the applied selection criteria), it is not surprisingly that the overall Ellenberg values do only show small differences between all the selected research periods. The only period that is significantly different from all others for all measured parameters is the reference period (1931-1970). Detailed analysis of the Ellenberg values of the species that did show a significant increase or decrease, however, clearly indicated various shifts in habitat conditions, i.e. lower availability of light, lower temperatures, higher availability of nutrients, and higher soil moisture content. These changes indicate a more dense vegetation structure. The results also confirm the trend that was derived from

the mentioned changes in phytosociological classes (4.1). Correlation of the Ellenberg indicator values with measured soil and vegetation parameters was studied by Schaffers & Sykora (2000) and did reveal the best correlation of the Ellenberg moisture parameter with the lowest moisture content in summer, whereas the Ellenberg nitrogen parameter correlated best with biomass production. Hill et al. (2000) published a study on the application of Ellenberg values to Great Britain, and found that except for continentality (which was therefore not used in the present study), in general Ellenberg indicator values correspond well to environmental gradients that can be observed in the many different habitats in Great Britain.

The evidence presented does not demonstrate the cause of the vegetation change, as several factors may have contributed to the found changes in species composition. However, it is known that three important processes (isolation, atmospheric N deposition and restoration management) took place during the investigated period, and the results are clearly in line with these processes. To disentangle the relative effects of these factors, detailed studies of the individual sites (e.g. effect of the surroundings, local atmospheric N deposition, and management history) are necessary, but they are unfortunately not available in a quantitative form to allow detailed comparisons.

In addition to the differences in management of calcareous grassland itself, changes in the direct surroundings of the calcareous grasslands probably have also played a major role. In the landscape of South Limburg, up to the first half of the nineteenth century, common grazing lands were much more widespread than nowadays and forests were restricted to small areas, due to much more intensive land-use (Hillegers 1993). For example, early data from the Bemelerberg (one of the hill slopes with calcareous grasslands) from around 1800 show that the area of nutrient-poor grasslands was twice as large as the current reserve and the vegetation on the slopes in this area consisted of 90% of grasslands and only 10% forest (Tranchot-maps from 1805). Nowadays, these percentages are more or less reversed.

Analyses of the dispersal syndromes revealed that species with no adaptations for efficient long-distance dispersal were significantly overrepresented among the declining species, while other dispersal traits showed no significant effect. This translates in a decrease in the proportion of species with no adaptations for efficient long-distance dispersal over the 20th century (Table 2.5). The decrease of the proportion of species without adaptations for efficient long-distance dispersal, is a clear indication of the fact that currently the nature reserves are rather small and isolated, without opportunities for these species to expand to other suitable habitats. This indicates that the long-term (restoration) management has failed to conserve the total pool of calcareous grassland species, probably due to the small size and isolated positions of the separate reserves. This is in line with Fischer & Stocklin (1997), Van Swaay (2002) and WallisdeVries et al. (2002), who concluded

that habitat loss and habitat fragmentation are the major reasons for species extinction in European semi-natural grasslands. On the other hand, the overall mechanisms for long-distance dispersal (> 100 m) that are considered in the current analysis do not seem to be the driving force for the changes in species composition that took place over the research period. This means that there is yet no significant evidence that the restoration of the sheep grazing in calcareous grasslands has lead to overall changes in the frequency of occurrence of species that are adapted to that specific long-distance dispersal (e.g. the fur of large mammals or the digestive tract of large mammals). Kuiters & Huiskes (2009) studied the endozoochorous seed dispersal in Dutch calcareous grasslands, and found that 43% of the local species pool germinated from sheep dung samples, although mostly in small numbers. They also found a positive correlation between seed dispersability of plant species and Ellenberg indicator value for nutrients, suggesting that species from nutrient-rich soils have a higher probability to disperse their seeds through sheep dung.

Conclusion

The aim of the present study was to quantify the long-term changes in vegetation in Dutch calcareous grasslands, and to relate the found changes to trends in environmental conditions and the implemented restoration management. Our findings are in line with other studies within Europe and clearly demonstrate a change in floristic composition towards more a more dense grassland structure, and a more nutrient-rich vegetation composition.

Since the start of restoration management, the vegetation has developed towards more moist and nutrient-rich circumstances, moving further away from the typical open, dry, nutrient-poor habitats. The current species composition is different from the situation in the reference period (up to 1970). Thus, the restoration management that was implemented around 1980 did not lead to the aimed recovery of the environmental conditions that prevailed during the reference period. Furthermore, species without adaptations for efficient long-distance dispersal decreased in the investigated period. Apparently, the current management was not sufficient for full restoration of the habitat, and species without adaptations for long-distance dispersal are less able to disperse among sites. Thus, the observed changes in species composition, and changes in the environmental parameters within calcareous grassland can be explained by three factors that have changed over the last six decades: isolation, atmospheric N deposition and restoration management

For the future, nitrogen emissions should be reduced further to stop the exceedance of the critical loads (Bobbink et al. 1998; Van Dobben & Hinsberg 2008), although it is not clear whether this will be sufficient to halt or reverse the observed changes in vegetation composition. The process of fragmentation of the calcareous grasslands already took place before the researched period, whereas the process of isolation of the remaining

grasslands strongly increased the last century. Connectivity of the remaining reserves, however, currently receives a lot of attention among scientists and practitioners. The development of biodiversity in these ecological corridors is still a difficult subject and more research is needed. Within the next years, a three year research project will start to investigate the opportunities to improve the management of the reserves themselves to improve conservation of these valuable habitats. In this study, the effects of a more intensively management, that is carried out in different compartments and phased in time will be followed.

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Chapter 3

Nitrification along a grassland gradient: Inhibition found in matgrass swards

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Abstract

Measurements along a hill-slope vegetation gradient in nutrient-poor grasslands from acid grasslands via matgrass swards to calcareous grasslands showed increased ammonium to nitrate ratios in the matgrass swards. These results generated the research question whether there might be a difference in nitrification activity or nitrifying community composition between the different zones in this hill-slope gradient. In each of the vegetation types along the gradient, soil samples were taken in five grassland nature reserves. Potential nitrification rates have been determined as an indication of the size of the active ammonia-oxidising microbial communities. Additionally, the dominant ammonia-oxidising sequences related to the b-Proteobacteria have been determined by a Polymerase Chain Reaction (PCR) based on the 16S rRNA gene in combination with Denaturing Gradient Gel Electrophoresis (DGGE) at one of the nature reserves. Compared to the top and lower zones of the vegetation gradient (i.e. acid grasslands and calcareous grasslands, respectively), potential nitrification rates were clearly repressed in the middle, matgrass swards zone. In contrast to the differences in potential nitrification activities observed in one of the nature reserves (Bemelerberg), no differences in dominant ammonia-oxidising sequences were observed at this location. One sequence belonging to cluster 3 of the Nitrosospira lineage appeared to be dominant among the sequences belonging to the ammonia-oxidising species of the b-Proteobacteria in all vegetation zones. Nitrification was apparently inhibited by the vegetation, whereas no shift in nitrifier populations could be shown. The possible role of repressed nitrification in the decline of this vegetation type is discussed.

Key words

Ammonia-oxidising activities, *Mesobromion*, *Nardo-Galion*, nitrification, nitrate production, Nitrosospira, *Thero-Airion*.

Introduction

The nutrient-poor grasslands on hill slopes in South Limburg are potentially one of the most species-rich ecosystems in the Netherlands (Willems 2001; WallisDeVries et al. 2002). Up to the beginning of the twentieth century, they were abundant in the form of common land, grazed by sheep led by shepherds. From the beginning of the previous century management practice changed and a strong decline in number of patches and area of these species-rich grasslands occurred. In the 1980s sheep grazing was reintroduced in most of them. Over the past 25 years, the floristic biodiversity of the calcareous grasslands increased in response to these restoration measures, although full restoration has not been accomplished yet. However, the botanical quality of the matgrass sward vegetation decreased even further (Bobbink & Willems 2001).

One factor that is thought to be important in causing biodiversity loss in European matgrass swards is nitrogen availability (e.g. Roelofs et al. 1996; Bobbink et al. 1998; Roem et al. 2002). Preliminary measurements of soil inorganic nitrogen concentrations in the above-mentioned grasslands showed increased ammonium to nitrate ratios in the matgrass sward vegetation, compared to the other vegetation types in this gradient. It is hypothesized that in this part of the vegetation gradient the process of nitrification is inhibited.

The process of nitrification, *i.e.* the oxidation of ammonium to nitrate, is performed by two physiologically different groups of bacteria. The group of ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) converts ammonium to nitrite, which is then transformed by the group of nitrite-oxidising bacteria (NOB) to nitrate. For the onset of nitrification the characteristics of the group of AOB/AOA is of most importance. Potential nitrification activities had been measured before in calcareous grasslands from the same area and turned out to be significantly ($p<0.01$) correlated with the Most Probable Numbers of ammonia-oxidising bacteria (Stienstra 2000). Both parameters were negatively correlated with the time period that passed since the fertilisation of the grasslands had been stopped. In addition, analysis of the AOB-related 16S rRNA genes from DNA isolated from the soils of these grasslands, showed a change in the community composition of β-proteobacterial ammonia oxidisers: A predominance of cluster 3 of the *Nitrosospira* lineage in early stages of succession after finishing fertilisation was replaced by cluster 4 of the same linage of β-AOB in later stages (Kowalchuk et al. 2000). As mentioned above, the success of the restoration measures taken in the nutrient-poor grasslands on hill slopes in South Limburg aimed at improving floristic biodiversity, varied between the vegetation types in the gradient. In line with the observations of Stienstra (2000), it is hypothesized that the restoration measures produced different communities of ammonia-oxidising betaproteobacteria giving rise to different ammonium to nitrate ratios along the vegetation gradient.

To verify both hypotheses, which relates to negatively affected nitrification and a different community composition of the β-AOB in the matgrass sward vegetation as compared to

both other vegetation types, the PAA was determined in nutrient-poor grasslands on slopes in five nature reserves. In addition, the dominant type of β-AOB was determined by molecular analysis for the different zones in the gradient of one of the nature reserves.

Materials and methods

Study site description

The studied grassland gradient only occurs in the most southern part of the Netherlands, in the province of Limburg. Between 100 and 150 meter above sea level, we find loess and acid gravel deposits from the river Meuse on top of the calcareous substrate, resulting in a unique 30 – 60 m long vegetation gradient with from the top downwards acid grasslands (*Thero-Airion,* Schaminée et al. 1996) on typic dystrudept soils, via matgrass sward vegetation (*Nardo-Galion,* Schaminée et al. 1996) on dystric eutrudept soils to calcareous grassland (*Mesobromion,* Schaminée et al. 1996) on typic to rendollic eutrudept soils (Soil Survey Staff 1999). Along the whole gradient (para)lithic contacts occurred. The acid grasslands are characterized by many annuals, e.g. *Rumex acetosella* and *Aira praecox*, next to different heathland moss and lichen species. The matgrass sward vegetation includes diagnostic species like *Danthonia decumbens*, *Stachys officinalis* and *Hieracium umbellatum,* and the calcareous grasslands contain typical species like *Sanguisorba minor*, *Briza media* and *Linum catharticum*. In the present study, data from five nature reserves are included: Bemelerberg (50º51’03’’N, 5º46’05’’E), Hoefijzer (50º50’58’’N, 5º46’29’’E), Zure dries (50º48’11’’N, 5º44’46’’E), Tiendeberg (50º48’34’’N, 5º40’03’’E) and Schiepersberg (50º49’55’’N, 5º46’42’’E). All of these five reserves contain a well developed grassland gradient or parts of it.

Soil sampling

For the chemical characteristics of the soil, mixed soil samples of the upper 10 cm (auger diameter 2 cm) were collected after removing the vegetation along the vegetation gradient at the Bemelerberg reserve in spring 2005, and at the Zure dries, Tiendeberg and Schiepersberg in spring 2007. The soil samples were stored at 4˚C and processed within 3 days. To determine pH and and NO_3-concentrations, we extracted soil (15 g) on a rotary shaker for 1 h (100 rpm) with 100 ml demineralised water, and for the measurement of NH_4-concentrations, 100 ml of a 0.2M KCl-solution was used. The soil suspensions were centrifuged for 5 min at 4000 rpm. Supernatants were filtered through a Whatman GF/C-filter and stored at -20˚C until further analysis. Soil moisture was determined after drying at 105˚C for 48 h. NH_4^+ and NO_3^--concentrations were determined colormetrically using a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). Total inorganic nitrogen is defined as the sum of NH_4^+ and NO_3-concentrations.

The size of the active ammonia-oxidising microbial community was estimated by measuring the potential ammonia-oxidising activities in soil samples. Therefore,

in each of the vegetation types along the gradient six soil samples were taken at the Bemelerberg and Hoefijzer (spring 2005). In spring 2007 an extra set of data was added, by sampling ten soil samples along the vegetation gradients at the Zure dries, Tiendeberg and Schiepersberg. The soil samples of the five nature reserves (n=66) were collected and processed according to the procedure described above for the determination of pH at the start of the potential measurements. To determine the total N, a destructive soil analysis was carried out. A dried, ground and sieved (sieve size 0.2 mm) soil sample is weighed into tin foil containers (around 3 mg). The containers are burnt with oxygen (dry combustion) in a stream of Helium gas, with Cr_2O_3 as a catalyst (1050 °C). Components are measured by a thermal conductivity detector TCD (Pella & Colombo 1973).

Potential ammonia-oxidising activities

Potential ammonia-oxidising activities (PAA) as estimation of numbers of actively ammonia-oxidising cells were determined in 250 ml Erlenmeyer flasks containing slurries of 15 g fresh, sieved (4 mm) soil in 100 ml buffered medium with 2mM $(NH_4)_2SO_4$. The buffer was composed of 2mM phosphate buffer (an equimolar mixture of KH_2PO_4 and K_2HPO_4, adapted to the prevailing soil pH), because pilot experiments had shown that the potential ammonia-oxidising activities in samples from the calcareous grassland and from the acid grassland were affected by the pH value of the slurries in which the potential activities were measured. For the calcareous grassland relatively high values were obtained at neutral pH, whereas for the acid grassland potential activities were repressed by pH 7 compared to pH 4 (data not shown). Hence, potential ammonia-oxidising activities were determined at pH values comparable with the original soil pH. During the activity measurements, the slurries were permanently shaken on a rotary shaker (RO 20, Gerhardt, Bonn, Germany; 100 rpm) in the dark at a temperature of 27ºC. Sub-samples of 3 ml were taken at t = 0, 2, 4, 6, 21, 27, 51, 74, 98, 122 and 146 hours, and centrifuged for 5 minutes at 13,000 rpm (Biofuge pico, Heraeus Instruments, South Plainfield, USA), decanted, and frozen (-18ºC) till analysed. At each sampling time, the pH of the incubation medium was checked, and restored to its original value with 0.1N NaOH or 0.1N HCl, if necessary. Concentrations of nitrate plus nitrite were measured on a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). Potential ammonia-oxidising activities were calculated from the changes in NO_2- plus NO_3-concentrations in time, using linear regressions. The slope of the regression lines were used as a measure for the potential ammonia-oxidising activity.

Data analysis

The measurements of the chemical characteristics of the soil samples were analysed with ANOVA (SPSS 15.01) for soil pH, and with non-parametric tests (Kruskal-Wallis) for soil moisture content, nitrate, ammonium, ammonium to nitrate ratio, total inorganic N, and total N. Post-hoc Tukey-tests were applied to distinguish between the three different vegetation types.

In the samples that were used to measure potential ammonia-oxidising activities, the interaction between the different vegetation types and the pH was tested with a one-way ANOVA procedure and a post-hoc Tukey test (SPSS 15.01). The interaction between the different vegetation types and the potential ammonia-oxidising activity was tested with a non-parametric test (Kruskal-Wallis, SPSS 15.01) and a post-hoc Tukey test.

DNA extraction and purification

To obtain an indication of the possible role of the community composition of the ammonia-oxidising bacteria in the accumulation of ammonium in some of the vegetation zones and for comparison with former analyses (Kowalchuk et al. 2000), DNA was extracted from 0.5 g dried soil with the mechanical disruption protocol described by Henckel et al. (1999) from 18 samples of the Bemelerberg nature reserve. Subsequently, the extracted DNA was purified with a commercial purification kit (Wizard® DNA Clean-up system, Promega Corporation, USA) according to the manufacturer's recommendations. Purified DNA was resuspended in deionised water. Extraction and purification were verified by electrophoresis of 5 µl of the DNA solution in 1.2% agarose gel and 0.5X TBE buffer (5X Tris-Borate: 54 g Tris base, 27.5 boric acid, 20 ml 0.5 M EDTA) and then visualised by ethidium bromide fluorescence.

PCR amplification of β-proteobacterial 16S rRNA genes

PCR fragments of the 16S rRNA gene of the β-subclass of the ammonia-oxidising Proteobacteria were generated with the ßAMO161f-ßAMO1301r primer set (McCaig et al. 1994). These PCR fragments were used as a template for a nested PCR using the CTO primer set as described by Kowalchuk et al. (1997). This second primer set amplified an approximately 465 bp fragment. In this paper we refer to this fragment as the CTO fragment. The primers are degenerated with specificity and sensitivity clearly defined (Kowalchuk et al. 1997, 1999; Koops et al. 2006). PCR amplification was performed in a total volume of 25 µl containing 1X PCR Mg-free buffer, 200 µM of each deoxynucleotide, 1.75 mM $MgCl_2$, one units of *Taq* DNA Polymerase (Invitrogen, Tech-Line USA), 400 ng μl^{-1} BSA (purified bovine serum albumin, New England BioLabs, Beverly, MA, USA), 0.5 µM of each primer, and 2.5 µl of 1:100 diluted ßAMO161f-ßAMO1301r PCR fragment as template. Reactions were performed in a Multiblock Thermocycler System (Thermo Electron, USA) according to the following program: 3 min denaturation at 94 °C; 35 cycles of: 30 s denaturation at 92 °C, 60 s annealing at 57 °C and 45 s elongation at 72 °C; the last step was 5 min elongation at 72 °C. All the amplification reactions were verified loading 5 µl amplification products in 1.2% agarose gel, separated by electrophoresis in 0.5X TBE, stained in ethidium bromide solution and visualised with UV.

Denaturing Gradient Gel Electrophoresis (DGGE) analyses
Approximately 200–300 ng of PCR products were separated by DGGE with a Protean II system (Bio-Rad, USA) according to the protocol of Kowalchuk et al. (1997) for the study of ammonia-oxidising bacteria. The denaturant gradient to separate the nested CTO fragments was 35–55%, with a solution of 8% acrylamide, 7 M urea and 40% formamide defined as 100% denaturing. Gels were run for 17 h at 60 °C in 0.5X TAE buffer (50X Tris-acetate: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA per litre).

Sequencing and phylogenetic analyses
From the denaturing gradient gels, selected bands were cut out from the middle parts. In case the DGGE pattern showed only one band, the corresponding band from the original agarose gel that contained a longer fragment with base pairs was cut and used for further analysis. The acrylamide pieces were stored in water overnight at 4 °C for the elution of PCR fragments (Bollmann & Laanbroek 2002). Bands were used 1:10 diluted as template for a further 25 cycles PCR. Obtained PCR products were separated again with DGGE to confirm recovery of the desired single bands (Zwart et al. 1998). Sequencing reactions were performed with the BigDye Terminator V 3.1.Cycle Sequencing kit (Applied Biosystems, USA) and run on an Applied Biosystems 3130 capillary sequencer. Sequences obtained from DGGE bands were (http://www.ncbi.nml.nih.gov/BLAST) and subsequently aligned with published 16S rRNA gene sequences of cultured ammonia-oxidising bacteria, by means of the fast aligner tool of the ARB software and added to the related neighbour-joining trees by using the parsimony criterion and ad hoc created filters (Ludwig et al. 2004). Alignment of the sequences obtained from the DGGE bands have been done with another fragment of the 16S rRNA gene, i.e. with nucleotides from positions 210 to 506 of the 16S rRNA gene of *Escherichia coli*.

Results

Chemical characteristics of the soil
The soil pH (H_2O) of the different vegetation types was significantly different between all groups and was on average 5.08 for the acid grassland, 5.59 for the matgrass sward vegetation, and 7.69 for the calcareous grasslands (Table 3.1), whereas the soil moisture content did not show significant differences. The ammonium concentration was decreasing along the research gradient, with lowest values in samples from calcareous grassland, whereas the lowest amount of nitrate was present in the samples from matgrass sward vegetation. The ammonium:nitrate ratio was 4.44 in the middle (matgrass sward) zone and thus considerably higher than in the neighbouring vegetation types (2.75 & 1.0; Table 3.1). Total quantities of inorganic nitrogen or total nitrogen percentages did not reveal any differences along the vegetation gradient.

*Table 3.1. Summary of the soil characteristics from the different vegetation types collected in spring in the nature reserves Bemelerberg (2005), Hoefijzer (2005), Schiepersberg (2007), Tiendeberg (2007) and Zure dries (2007). Units of measurement for N-NH_4, N-NO_3, and total inorganic nitrogen are mg.kg^{-1} dry soil. Soil moisture content is calculated as (fresh soil-dried soil)/dried soil. Total N is measured as a percentage of the total soil. The standard deviation is between brackets, and significant differences between the vegetation types are indicated by different letters (P < 0.05). With asterisks the significance level is indicated: *** = P < 0.001; ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05; n.s. = not significant.*

	Acid grassland	**Matgrass sward**	**Calcareous grassland**	**Statistics**
pH_{demi}	5.08 (0.28) a	5.59 (0.49) b	7.69 (0.53) c	F = 213.50, P < 0.000 ***
Moisture content	0.26 (0.10) a	0.34 (0.12) a	0.32 (0.09) a	χ^2 = 4.00, P = 0.135 n.s.
N in $NO_{3\ demi}$	3.41 (3.84) ab	2.27 (1.95) a	6.26 (3.55) b	χ^2 = 7.72, P = 0.021 *
N in $NH_{4\ KCl}$	5.27 (4.35) a	4.47 (2.54) a	3.79 (2.82) b	χ^2 = 14.30, P = 0.001 **
Ammonium:nitrate ratio	2.75 (2.20) ab	4.44 (4.64) a	1.00 (1.41) b	χ^2 = 11.58, P = 0.003 **
Total inorganic N	8.69 (8.17) a	6.74 (3.01) a	8.02 (3.82) a	χ^2 = 1.00, P = 0.607 n.s.
Total N (%)	0.37 (0.06) a	0.40 (0.10) a	0.36 (0.11) a	χ^2 = 1.51, P = 0.471 n.s.

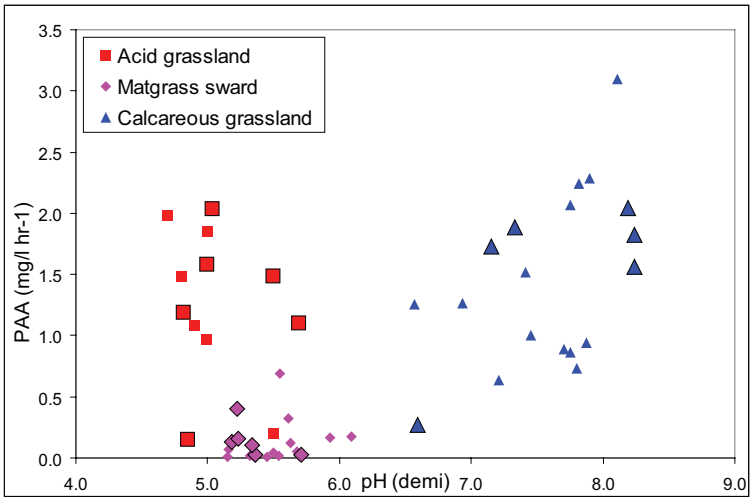

Figure 3.1. Potential Ammonia-oxidising Activities (mg $l^{-1}.h^{-1}$) measured in samples from the different vegetation types at the nature reserves of Bemelerberg, Hoefijzer, Schiepersberg, Tiendeberg and Zure dries. The pH (demi) represent the soil pH after extraction with demineralised water for 1 hour. The bigger symbols indicate samples from the Bemelerberg that were used in the DNA-analysis. The average R2 of the linear regressions used for the calculation of the potential activities was 0.98 for the acid grassland samples (s.d. 0.04), 0.91 for the matgrass sward samples (s.d. 0.12), and 0.99 for the calcareous grassland samples (s.d. 0.01).

Potential ammonia-oxidising activities

Within the five reserves, significant lower activities ($P < 0.001$) were encountered in the matgrass sward vegetation (on average 0.1 mg l^{-1} hr^{-1}, Figure 3.1), when compared to the relatively high potential ammonia-oxidising activities in the acid grassland and in the calcareous grassland (on average 1.3 and 1.5 mg l^{-1} hr^{-1}, respectively). The difference in potential ammonia-oxidising activities between the acid and the calcareous grassland was not significant at the 0.05-level. The same significant differences were found when analysing the data from the nature reserve Bemelerberg separately (data not shown). The soil pH (H_2O) of the different vegetation types of the Bemelerberg was again significantly different between all groups and was on average 5.07 for the acid grassland, 5.49 for the matgrass sward vegetation, and 7.61 for the calcareous grassland.

Diversity of ammonia-oxidising microbial communities

The diversity of ammonia-oxidising microbial communities was established by a nested PCR – DGGE approach for the vegetation zones from the nature reserve Bemelerberg. On the basis of the 16S rRNA gene, no major differences were observed between or within the vegetation types (Figure 3.2). All soil samples were dominated by one band, which on the basis of a BLAST analysis of its 553 nucleotides, was most closely related (98% identity) to *Nitrosospira* sp. Nsp2 (Bbband 9; Figure 3). In all samples, and irrespective

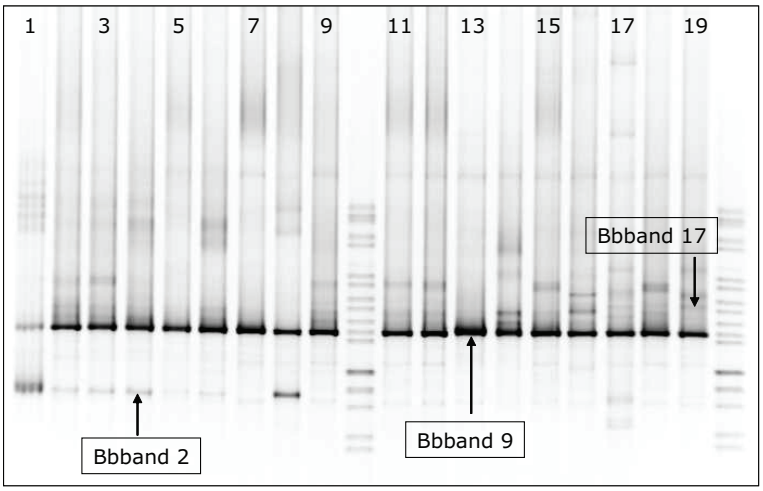

Figure 3.2. DGGE-banding pattern of ammonia-oxidising bacteria of the β-subclass of the Proteobacteria obtained by nested PCR from DNA isolated from soil samples from Bemelerberg nature reserve. Lanes 1-6 represent samples from the acid grassland; lanes 7-9 and 11-13 samples from the matgrass sward vegetation; lanes 14-19 samples from the calcareous grassland; lanes 10 and 20 are marker lanes. Indicated are the true bands belonging to the β-subclass of the ammonia-oxidising Proteobacteria. The two bands above Bbband 9 in lanes 14 and 16 migrated at the height of BBband 9 after cutting and reamplification. All other bands were not reamplifiable after cutting.

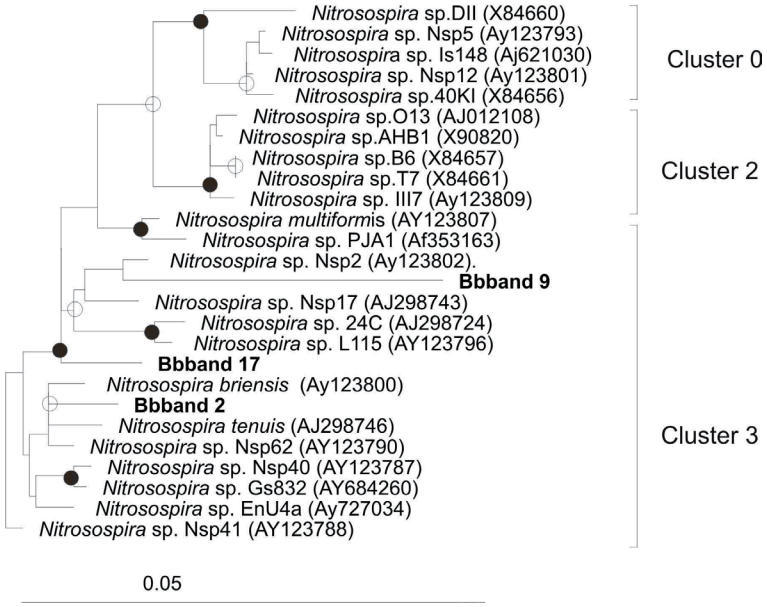

Figure 3.3. 16S rRNA-based neighbour-joining phylogenetic tree of the ammonia-oxidising Nitrosospira lineage of the β-subclass of the Proteobacteria (Purkhold et al. 2003). Bands retrieved in this study are marked in bold. Scale bar indicate 5% estimated sequence divergence. Boot trap values ≥90% or ≥70% are indicated in the tree by closed and open circles, respectively.

of the vegetation type, faint bands accompanied this dominant band. A selection of six faint bands from different locations in the gel was cut from the gel and reamplified. Only two of them turned out to belong to ammonia-oxidising bacteria of the β-subclass of the Proteobacteria. On the basis of a sequence of 402 nucleotides, one of these minor bands was mostly related (97% identity) to *Nitrosospira briensis* (Bbband 2; Figure 3.3). This band occurred faintly in most samples and as a subdominant band at one of the matgrass sward samples. The second faint band that belongs to the β-subclass of the ammonia-oxidising bacteria occurred only in a few samples, but apparently again irrespective of the type of vegetation. This latter band was mostly related (99% identity) to *Nitrosospira* sp. Nsp17 (Bbband 17; Figure 3.3) on the basis of a sequence of 455 base pairs. All other faint bands were either not reamplifiable after cutting or migrated to the same height of the dominant Bbband 9 in the case of the faint bands in lanes 14 and 16.

The dominant sequence of ammonia-oxidising bacteria encountered in all samples belongs to cluster 3 of the genus *Nitrosospira* (Purkhold et al. 2003). The two other subdominant sequences of this functional group (*Nitrosospira* sp. Nsp17 and *Nitrosospira briensis*) also belong to this cluster. The dominant *Nitrosospira* sp. Nsp2 and the subdominant

Nitrosospira sp. Nsp17 are even closely related to each other according to Purkhold et al. (2003). Originally these strains have been isolated from German and Icelandic soils, respectively (cf. Purkhold et al. 2003).

Discussion

We identified clear differences in the potential ammonia-oxidising activities of the soil samples from the different vegetation zones. Hence, the first hypothesis that increased ammonium to nitrate ratios in the matgrass sward vegetation were due to repressed nitrification was supported by the results obtained with the measurements of potential ammonia-oxidising activities. The values measured in calcareous grassland were of the same order as those determined by Stienstra (2000) for earlier stages of succession in calcareous grasslands from the region. The observed repressed activity of the ammonia-oxidising community in matgrass sward vegetation was not due to a lack of ammonium neither in the field nor during the potential rate measurements, as this was provided in excess during the activity measurements. The pH also does not seem to limit the activity of the ammonia-oxidising bacteria, because at the samples from acid grassland with an even lower pH, nitrification occurred. The findings of the present study are in contradiction with general assumptions that nitrification is rapid in soils with a pH ≥ 6 and slower in soils with a pH ≤ 5 (Subbarao et al. 2006a).This difference in potential activity, which points to differences in numbers of active, ammonia-oxidising cells, was not reflected by differences in the bacterial community composition. In all three vegetation types, species with 16S rRNA-based sequences that were mostly related to *Nitrosospira* sp. Nsp2 were the dominant ammonia-oxidising species. Hence, we have to reject the hypotheses that different ammonia to nitrate ratios observed in the matgrass sward vegetation were due to different communities of ammonia-oxidising β-Proteobacteria.

In an earlier study in calcareous grasslands in the Province of Limburg, the Netherlands, a shift from cluster 3 of the *Nitrosospira* lineage in early succession fields to cluster 4 of the same lineage of β-AOB in climax vegetation types has been observed (Kowalchuk et al. 2000). These authors proposed that ammonium availability, which was lower in the climax vegetation, may be a steering force affecting the ammonia-oxidising microbial community in grassland soils. As the current research sites have never been fertilised, except for atmospheric N deposition, and the vegetation in these grasslands has been relatively persistent at least since the beginning of the 20th century, our observations seem to correspond more with those of Horz et al. (2004), who found that increased nitrogen deposition altered the structure of the ammonia-oxidising bacterial community towards a community dominated by bacteria most closely related to *Nitrosospira* sp. Nsp2, which is also the dominant β-AOB at our locations and related to cluster 3 of the *Nitrosospira* lineage.

Changes in microbial diversity and community structure might have large-scale consequences for ecosystem functioning and underlying processes (Schimel 1995, Schimel et al. 2005). In our case, however, environmental conditions, *i.e.* the presence

of specific vegetation and associated habitat conditions, affected the activity of the ammonia-oxidising bacterial community, but not its composition. An explanation for the observed difference in behaviour between the potential ammonia-oxidising activities and the community composition of the ammonia-oxidising-proteobacteria in our soil samples as observed in the Bemelerberg nature reserve may be related to the presence and activity of ammonia-oxidising archaea. Copy numbers of the functional gene for ammonia oxidation in archaea, the so-called archaeal *amoA* gene, might be two orders of magnitude larger in pristine grasslands than the copy numbers of the bacterial *amoA* gene (Leininger et al. 2006). Furthermore, reverse transcription quantitative PCR studies and complementary DNA analysis using novel cloning-independent pyrosequencing technology applied by these authors demonstrated the activity of the archaea *in situ* and supported the numerical dominance of archaeal over bacterial ammonia oxidizers. In a series of agricultural soils in which pH had been maintained at distinct pH values of 4.5 – 7.5 for 35 years, Nicol et al. (2008) observed an increased number of copies of the archaeal *amoA* gene with decreasing pH, whereas the copy number of the bacterial *amoA* gene remained more or less the same. At pH 5.3 – 5.9, the authors observed a two orders of magnitude difference between the *amoA* gene copy numbers of archaea and bacteria, while the *amoA* gene transcript copies were almost three orders of magnitude larger for the archaea. In non-fertilized French grassland soils managed by sheep-grazing or mowing, copy numbers of the 16S rRNA gene were of the same order of magnitude for ammonia-oxidising bacteria and archaea (Le Roux et al. 2008). However, based on assumed lower transformation rates for ammonium into nitrite by AOA compared to AOB (Könneke et al. 2005), Le Roux et al. (2008) postulated that the major part of nitrification in the investigated grassland soils was related to AOB activity. Notwithstanding these considerations, we cannot not exclude that active AOA have been responsible for the observed disagreement between potential ammonia-oxidising rates and community compositions as determined for the different parts of our vegetation gradient.

A possible explanation for the observed repression of potential nitrification activity in the matgrass sward vegetation is the mechanism of repression by allelopathic compounds, which has been proposed by Rice and Pancholy (1972). The authors, however, did not provide adequate mineralization data to rule out ammonium shortage as an alternative explanation and also failed to provide in situ evidence for plant involvement in nitrification inhibition. Exudation of toxic compounds by plants, produced particularly by plant species from climax vegetation is not conclusively demonstrated in the field (*cf.* De Boer et al. 1990). Nevertheless, biological nitrification inhibition has recently been demonstrated by several authors. Exudates from the roots of the grass *Brachiaria humidicola* repressed nitrification (Subbarao et al. 2006b). Furthermore, it was demonstrated in *Brachiaria humidicola* that ammonium stimulates the synthesis and release of inhibiting compounds (Subbarao et al. 2007a, b). Grasslands dominated by *Andropogon* contained mostly ammonium, whereas nitrate nearly disappeared as the grassland matured (Lata et al. 1999). Lata et al. (2004) also demonstrated that nitrification can be suppressed or stimulated depending on the ecotype of *Hypparhenia diplandra*.

Finally, Zakir et al., (2008) found an increasing effect of plant age and ammonium on the release of nitrification inhibiting compounds by root exudates of *Sorgum bicolor*. As far as we know, such an effect involving natural vegetation has not been published.
Repression of nitrification by plant species of the matgrass sward vegetation could be an adaption to the nutrient-poor circumstances of these grasslands to conserve and use N efficiently, by maintaining inorganic nitrogen in the less mobile form of ammonium and thus preventing it from leaching as nitrate (Vitousek et al. 2002; Herrmann et al. 2005). However, the acid-sensitive species of the matgrass sward vegetation are sensitive to high soil ammonium concentrations, especially in combination with low soil pH (De Graaf et al. 1998a; Lucassen et al. 2002; Dorland et al. 2003; Van den Berg et al. 2005). Hence, if plants from the matgrass sward vegetation indeed inhibit ammonium oxidation by the release of allelopathic compounds, then a negative feedback could exist, leading to a decline of this vegetation type. Atmospheric deposition of reduced N (ammonium or ammonia) will increase the rate of this decline as ammonium would not be oxidised to nitrate by the ammonia-oxidising microorganisms and thus not leach to the groundwater. This will lead to a strong dominance of ammonium supply in this vegetation type. Studies are underway to evaluate plant species control of the nitrification process.

Conclusions

As hypothesized, the high ammonium to nitrate ratios observed in the matgrass sward part of the vegetation gradient along hill-slope grasslands could be ascribed to a repressed nitrification. However, the observed differences in potential ammonia oxidation rates were not related to differences in the composition of the community of ammonia-oxidising β-Proteobacteria. Members of the third cluster of the *Nitrosospira* lineage dominated in all soils independent on the prevailing ammonium to nitrate ratio.

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Chapter 4

Repression of potential nitrification activities by matgrass sward species

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Abstract
Soil nitrification is a key process in regulating the relative availability of the various inorganic N forms to plants. In the current study, we investigated the effect of different plant species on numbers of ammonia-oxidising microbial cells by measuring the potential nitrification activity (PAA). Soil from matgrass sward and from calcareous grassland was collected in the field and four characteristic plant species of each vegetation type were cultivated from seed. These plant species grew for four months in the two soil types in a cross-wise experiment. After those 4 months, PAA was significantly higher in calcareous soil compared to the matgrass sward soil and the presence of matgrass sward species had significantly decreased PAA in this soil. In soils from matgrass stands, PAA were much lower, and no effect of the different plant species could be detected. Plant biomass of the calcareous grassland species was overall positively correlated with PAA, whereas for matgrass plant species a negative trend was found. We conclude that matgrass sward plant species had a clear repressing effect on the potential ammonia-oxidising activity in calcareous grassland soil within this four-month growth experiment. The observed repression of PAA is in accordance with earlier field observations of PAA in the different vegetation zones, where repressed PAA and significant higher ammonium to nitrate ratios were observed in the matgrass sward vegetation compared to the other vegetation zones (Smits et al. 2010).

Keywords
Potential ammonia-oxidising activities, nitrification inhibition, ammonium toxicity, calcareous grassland, *Mesobromion*, *Nardo-Galion*, nitrate production.

Introduction

In the Netherlands, species-rich grasslands occur on hill slopes in South Limburg in a unique vegetation gradient where loess and acid gravel have been deposited by the river Meuse on top of calcareous substrate. This local geomorphology resulted in a vegetation gradient with acid grasslands on the acid gravel deposits, more buffered matgrass swards on thin layers of loess on top of acid gravel deposits in the middle reach, and calcareous grasslands on calcareous outcrops beneath. Potentially, these nutrient-poor grasslands on hill slopes are one of the most species-rich ecosystems in the Netherlands (Willems 2001; WallisDeVries et al. 2002), but from the beginning of twentieth century a strong decline in species richness was observed, due to changing land use practices (Willems 1990; Poschlod & WallisDeVries 2002) and the effects of atmospheric nitrogen deposition (Bobbink et al. 1998; Bakker & Berendse 1999; Stevens et al. 2004; Clark & Tilman 2008).

Increased inputs of nitrogen in these grasslands may have a direct eutrophication effect on the vegetation leading to changes in competitive interactions and shift in plant species composition (Bobbink 1991). In the acidic part of these grasslands, both soil acidification and shifts in the dominant form of inorganic nitrogen, e.g. nitrate or ammonium, are of main importance for the observed effects of N deposition (e.g. Bobbink & Lamers 2002; Kleijn et al. 2008; De Graaf et al. 2009; Dupré et al. 2009), whereas acidification and shifts in the dominant form of inorganic nitrogen do not play a major role in calcareous grasslands (Bobbink & Roelofs 1995). Increased ammonium concentrations in the soil solution can cause toxicity in plant species characteristic for acidic to slightly buffered habitats (De Graaf et al. 1998a; Britto & Kronzucker 2002; Dorland et al. 2003, Van den Berg et al. 2005). In contrast, no negative effect of ammonium is observed for grass species which are known to invade these vegetation types at high N deposition rates. Soil nitrification is a key process in regulating the relative availabilities of the differential N forms to plants, and therefore plays a crucial role in the effects of enhanced N inputs on the composition of the vegetation.

Recently, a clear spatial pattern has been observed in the dominant form of inorganic nitrogen in the soil of the above-mentioned South Limburg grassland gradient: nitrate concentrations were significantly lowest in the matgrass sward, whereas ammonium concentrations decreased along the gradient. Moreover, the ammonium to nitrate ratio was significantly higher in the matgrass swards zone (pH 6.1) in comparison to the acid grassland (pH 5.1) and the calcareous grassland (pH 8.0). The potential ammonia-oxidising activity, a measure for the number of actively ammonia-oxidising microbial cells (Verhagen et al. 1992), turned out to be significantly lower in the matgrass sward, when compared to the PAA in the acid grassland and the calcareous grassland (Smits et al. 2010). Molecular analysis of the ammonia-oxidising betaproteobacterial communities based on the 16S rRNA gene showed no major differences in the diversity between

these three vegetation zones (Smits et al. 2010). We are aware that, in general, lower pH correlates to lower PAA, but found that in this research gradient, nitrification was lowest at intermediate pH level. This gave reason for further research.

The observed repression of the potential nitrification activity in the matgrass sward may be explained by exudation of biological nitrification inhibitors (BNI) by plants. Up to now, several indications for exudation of BNI have been demonstrated in crop plant species (Subbarao et al. 2007c; 2007b, Zakir et al. 2008; Subbarao et al. 2009) and Hyperrhenia sp. dominated savanna (Lata et al. 1999; 2000; 2004). In the current article, we focus on the vegetation level. To investigate the extent to which the characteristic grassland vegetation affects potential nitrification, a cross-wise growth experiment was designed on soils of the two plant communities. The objective of this study was to examine if characteristic plant species of both matgrass sward and calcareous grasslands have an influence on the potential nitrification activities in the soil. We hypothesise that the repression of potential nitrification activity observed in the field (Smits et al. 2010) is due to biological nitrification inhibition by typical matgrass sward plant species.

Materials and methods

Description of the research site

The two vegetation types that are used in this study comprise matgrass sward vegetation (*Nardo-Galion saxatilis*, Swertz et al. 1996) on dystric eutrudept soils and calcareous grassland (*Mesobromion erecti,* Schaminée & Willems 1996) on a typic to rendollic eutrudept soils (Soil Survey Staff 1999). Along the whole gradient, (para)lithic contacts occur.

Experimental setup

A cross-wise pot experiment was set up in the greenhouse, in which eight selected plant species grew for four months from October 2007 to March 2008 on soil from the matgrass sward zone and soil from the calcareous grassland zone. Soil samples of the upper 10 cm were collected in the matgrass swards and in the calcareous grassland at the Bemelerberg (the Netherlands, 50°51'N, 5°46'O). The soil was stored at 4˚C and processed within two days after collection. Homogenized soil of each soil type was put into 1 litre pots. Seeds of four characteristic species of matgrass swards, and of four typical species of calcareous grassland were collected from different local nature reserves. Species selection took place based on seed availability and family similarity. For comparison across the two soil types, similar families (*i.e.* Asteraceae, Poaceae and Lamiaceae) were used for three out of four species. For matgrass swards, the selection resulted in *Hieracium umbellatum, Succisa pratensis, Danthonia decumbens* and *Stachys officinalis* as characteristic species, whereas *Leontodon hispidus, Sanguisorba minor, Briza media* and *Thymus pulegioides* were the selected species of calcareous grassland.

Seeds were germinated in the greenhouse in standard potting soil, and four-weeks-old seedlings were used in the experiment. The nomenclature was used according to Van der Meijden (2005) for the plant species, and Schaminée et al. (1996) for the vegetation.

Of every species, four seedlings were planted in matgrass sward soil (n=4) and in calcareous grassland soil (n=5). As there was not sufficient matgrass sward soil for five replicates, only 4 replicates were used with this soil type. Pots without seedlings were included as controls (n=5 for calcareous grassland soil and n=4 for matgrass sward soil). All 82 pots were randomly placed in the greenhouse with a 16-hour photoperiod, and watered four times a week during the whole experiment with rainwater. The used rainwater was analysed twice during the experiment for nitrate en ammonium concentrations to check on possible extra nitrogen input. In the first month, different plant species started to show interveinal chlorosis on both soil types. Spraying with 0.05M iron (Fe-EDDHA) and 0.02M manganese (Mn-DTPA; Brinkman Agro BV, NL) removed these effects. The treatment was repeated three times during the experiment.

Soil nutrient analysis

At the beginning of the experiment, soil of each grassland type was homogenized and extracted (15g, n=5) on a rotary shaker for 1 h (100 rpm) with 100 ml demineralised water for the determination of pH and nitrate concentrations. An extraction with 100 ml 0.2M KCl-solution was used for the measurement of ammonium concentrations. The soil suspensions were centrifuged for 5 min at 4000 rpm. Supernatants were filtered through a Whatman GF/C-filter and stored at -20˚C until further analysis. Soil moisture content was determined after drying at 105˚C for 48 h. Nitrate (nitrite plus nitrate) and ammonium concentrations in the soil extracts were determined colorimetrically using a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). The nitrate and ammonium concentrations in the pore water in the pots were measured bi-weekly during the growth experiment through rhizons (Eijkelkamp, the Netherlands) in 22 stratified randomly chosen pots. At each sampling moment, every combination of soil/plant species was included and three of the controls were also measured.

Potential ammonia-oxidising activities

Potential ammonia-oxidising activities (PAA) were determined at the start and at the end of the experiment in 250 ml Erlenmeyer flasks containing slurries of 15 g fresh, sieved (4 mm) soil in 100 ml buffered medium with 2mM $(NH_4)_2SO_4$. The buffer was composed of 2mM phosphate buffer (an equimolar mixture of KH_2PO_4 and K_2HPO_4, adapted to the prevailing soil pH), because preliminary experiments had shown that the PAA in samples were affected by the pH value of the slurries in which these activities were measured. Hence, PAA were determined at pH values comparable with the original soil pH (*i.e.* 5.4 for matgrass swards and 8.0 for calcareous grassland). During the PAA measurements, the slurries were permanently shaken on a rotary shaker (RO 20, Gerhardt, Bonn, Germany;

100 rpm) in the dark at a temperature of 27ºC. Sub-samples of 3 ml were taken at t = 0, 2, 4, 6, 21, 27, 51, 74, 98, 122 and 146 hours, and centrifuged for 5 minutes at 13,000 rpm (Biofuge pico, Heraeus instruments, South Plainfield, USA), decanted, and frozen (-20 ºC) till analysed. At each sampling time, the pH of the incubation medium was checked, and set to its original value with 0.1N NaOH or 0.1N HCl, if necessary. Concentrations of nitrite plus nitrate in the subsamples were measured on a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). PAA were calculated from the changes in nitrite plus nitrate concentrations in time, using linear regressions. The slope of the regression lines was used as a measure for the PAA. The average R^2 of the used linear regressions of the PAA at the end of the experiment was 0.99 for matgrass sward soils (s.d. 0.01), and 0.99 for calcareous grassland soils (s.d. 0.01).

Plant growth
To analyse systematic differences in plant growth between the two soil types, above- and belowground biomass of all plants was harvested after four months of growth, dried at 70° C and weighted.

Statistics
The measurements of the chemical characteristics of the soil and the PAA at the beginning of the experiment were analysed with a non-parametric Mann-Whitney U Test. The PAA at the end of the experiment was tested with a two-way ANOVA. As there was a clear interaction effect (soil * species), both soil types were further analysed with a one-way ANOVA, including a Tukey-b post-hoc test on the group level and on the species level, as both datasets were normally distributed and homogeneity of variance (Levene's Test) could be assumed. Nitrate and ammonium concentrations in the pore water samples of the experimental pots (rhizon samples) were analysed by repeated measurements analyses. For both parameters, the sphericity could not be assumed (Mauchly's Test of Sphericity). Therefore, the Greenhouse Geisser epsilon was used. Biomass production of the separate species between both soil types were tested with a non-parametric Mann-Whitney U Test, and the correlation of the biomass of the plants and the found PAA was tested with a non-parametric (Spearmans rho) correlation. All statistical analyses were carried out in PASW statistics 17.0.

Results

Chemical characteristics of the soil
At the start of the experiment, the pH values (demi) of the two soil types were significantly different: on average 5.4 in matgrass sward soil, and 8.0 in calcareous grassland soil (Table 4.1). Furthermore, nitrate concentrations were significantly higher and ammonium concentrations significantly lower in the pots with calcareous grassland

*Table 4.1. Soil characteristics at the beginning of the experiment (averages and between brackets the standard deviations). Significance level (Mann-Whitney U Test): ** = P<0.01; * = 0.01>P<0.05, n.s. = not significant. The R^2 of the calculated regression lines for Potential Ammonia-oxidising Activities at the start of the experiment (PAA_start) was on average 0.98 (st. dev. 0.03).*

	Calc. soil (n = 5) vs. Matgrass sward soil (n= 5)
pH_{demi}	8.0 (0.1) – 5.4 (0.4) **
N in $NO_{3\ demi}$ (mg kg^{-1} dry soil)	6.4 (1.1) – 2.5 (1.2) **
N in $NH_{4\ KCl}$ (mg kg^{-1} dry soil)	2.4 (2.3) – 12.9 (6.4) *
Ammonium:nitrate ratio	0.4 (0.4) – 7.7 (7.8) **
PAA_start experiment (mg l^{-1} hr^{-1})	0.42 (0.13) – 0.08 (0.04) **

soil, compared to those with matgrass sward soil (Table 4.1). Due to high variation, the ammonium:nitrate ratio was not significantly different at this sampling date in autumn (Table 4.1), in contrast to earlier field measurements in the same grassland zones (Smits et al. 2010).

The nitrate and ammonium concentrations in the pore water samples of the experimental pots (rhizon samples) were analysed in both soil types with repeated measurements analysis (GLM, Table 4.2). Three plant groups were distinguished: matgrass sward species, calcareous grassland species and no plant species (controls). Ammonium concentrations only turned out to be significantly different between soil types. Matgrass sward soil contained more ammonium than calcareous grassland soil (P = 0.006, Table 4.2), but there was no effect of time and measured ammonium concentrations were present during the whole experiment with at least 0.1 mg.kg^{-1} dry soil ammonium-nitrogen in the pore water. Therefore, ammonium was never limiting the nitrifiers in the soils during the growth experiment. On average, 2 mg.kg^{-1} dry soil ammonium-nitrogen

*Table 4.2. Summary of statistical analyses (GLM, repeated measurement analysis) for ammonium and nitrate concentrations in pore water samples during the experiment. Significance level: ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05; n.s. = not significant. At the end of the table, the average concentrations for both soil types are presented (N in ammonium and N in nitrate in mg.kg^{-1} dry soil) with the standard error between brackets.*

	Ammonium	**Nitrate**
Time (n = 6)	F = 0.770, P = 0.458 n.s.	F = 1.088, P = 0.360 n.s.
Plant	F = 1.092, P = 0.369 n.s.	F = 2.164, P = 0.161 n.s.
Soil	F = 11.833, P = 0.006 **	F = 0.646, P = 0.439 n.s.
Time x Plant	F = 1.539, P = 0.235 n.s.	F = 1.805, P = 0.151 n.s.
Time x Soil	F = 1.248, P = 0.304 n.s.	F = 3.935, P = 0.027 *
Plant x Soil	F = 1.397, P = 0.288 n.s.	F = 1.993, P = 0.183 n.s.
Time x Plant x Soil	F = 1.209, P = 0.337 n.s.	F = 0.421, P = 0.817 n.s.
Soil type	**Matgrass sward – Calcareous**	**Matgrass sward – Calcareous**
Av. Conc. (s.e.)	2.002 (0.338) – 0.324 (0.351)	215.666 (26.032) – 185.520 (27.015)

was available during the experiment in matgrass sward soil, whereas calcareous soil only contained 0.3 $mg.kg^{-1}$ dry soil of available ammonium-nitrogen (Table 4.2). Nitrate concentrations only showed significant differences for the interaction time * soil (Table 4.2) and on average 216 and 186 $mg.kg^{-1}$ dry soil nitrate-nitrogen was present during the experiment in the matgrass sward and calcareous grassland soil, respectively. Nitrate-nitrogen in the controls was increasing during the experiment in matgrass sward soil, indicating nitrification activity and confirming the higher PAA measured in the controls at the end of the experiment (data not shown). Nitrate concentrations in the pore water samples were on average over 500 times higher than the corresponding concentrations in the rainwater samples, whereas ammonium concentrations were more than 3 times higher (data not shown).

Potential ammonia-oxidising activities (PAAs)

The PAAs at the start of the experiment were significantly lower (Independent samples Test) in the matgrass swards soils compared to the calcareous grassland soils, with on average 0.08 vs. 0.42 mg N (nitrite plus nitrate) l^{-1} h^{-1}, respectively (Table 4.1). At the end of the experiment, the PAAs of the soils without plants were still significantly different between the two soil types with 0.16 (s.d. 0.03) and 0.34 (s.d. 0.03) mg N (nitrite plus nitrate) l^{-1} h^{-1} in the matgrass sward and the calcareous grassland soil, respectively. Two-way ANOVA revealed significant effects for soil type (lower PAA in matgrass sward) and species level, but also a strong interaction effect for soil * species, and therefore further analysis of the data was carried out for both soil types separately with a one-way ANOVA. For the matgrass sward soil, analysis of the three plant groups (matgrass sward species, calcareous grassland species and no species) did not show significant differences ($P = 0.068$) with slightly higher PAA in the pots without plant species. Analysis for the different species did also not show significant differences between the nine groups ($P = 0.052$). However, the PAA in calcareous soil data did show significant differences between the plant groups ($P<0.001$), with significant lower PAA for matgrass sward plants than for calcareous plants and controls. When testing the species separately, five homogeneous subsets could be distinguished (Figure 4.1, indicated with different letters). Thus, the PAA in calcareous soils did show distinct differences for the different species, with significant less nitrification in pots with matgrass sward species and with PAA comparable to the controls when calcareous grassland species were present (Figure 4.1).

Plant growth

In general, average above-ground, below-ground and total plant biomass were all significantly higher on calcareous soils. The matgrass sward species *H. umbellatum* showed a significantly higher ($p<0.05$) above- and below-ground and total biomass in pots with calcareous soil compared to the matgrass sward soil. The calcareous grassland species *T. pulegioides* and *S. minor* showed a significantly higher ($p <0.05$) above-ground biomass in the pots with calcareous soil compared to the matgrass sward soil

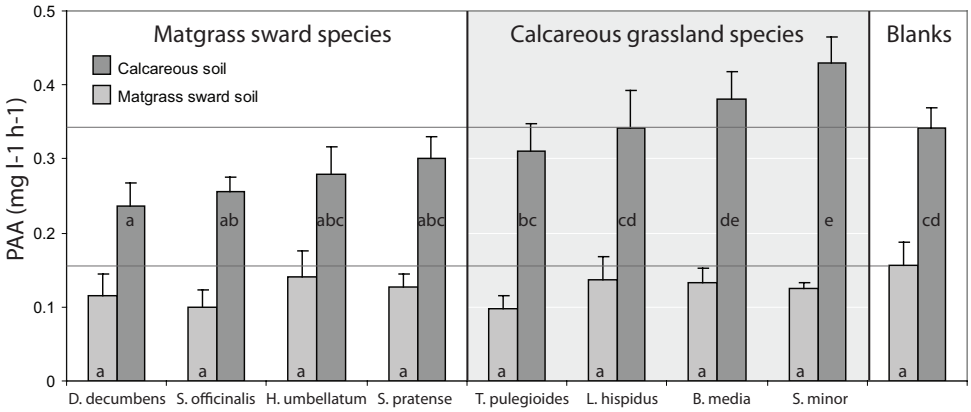

Figure 4.1. Potential Ammonia-oxidising Activity (PAA, in mg l-1 .h-1) at the end of the four-month growth experiment in both soils for all species and controls without plants. The letter codes indicate significant differences (at the P<0.05-level, ANOVA), and the horizontal lines represent the average value for the controls (soil without plant species).

(Table 4.3) and the calcareous grassland species *L. hispidus* and *T. pulegioides* showed a significantly higher below-ground in pots with calcareous grassland soil. *T. pulegioides* also showed a significantly higher total biomass in pots with calcareous grassland soil. The other species did not show any significant differences in dry weights for both soil types.

*Table 4.3. The above-ground, below-ground and total biomass (g) of the plants grown on calcareous grassland and on matgrass sward soils as determined at the end of the experiment. Shown are the averages (g) and standard deviations are between brackets. Significant differences (Mann-Whitney U Test) are shown with * for 0.01<P>0.05, and ** P<0.01, n.s. = not significant.*

	Above-ground biomass in	**Below-ground biomass in**	**Total biomass in**
Species	Calc. soil - Matgrass sward soil	Calc. soil - Matgrass sward soil	Calc. soil - Matgrass sward soil
Matgrass sward species			
H. umbellatum	0.92 (± 0.26) - 0.43 (± 0.17) *	0.78 (± 0.25) - 0.19 (± 0.53) *	1.70 (± 0.37) - 0.63 (± 0.17) *
D. decumbens	1.99 (± 0.64) - 1.98 (± 0.41) n.s.	0.80 (± 0.22) - 1.28 (± 0.57) n.s.	2.79 (± 0.85) - 3.25 (± 0.96) n.s.
S. officinalis	1.30 (± 0.19) - 1.32 (± 0.10) n.s.	0.82 (± 0.08) - 0.80 (± 0.32) n.s.	2.13 (± 0.25) - 2.12 (± 0.40) n.s.
S. pratensis	1.46 (± 0.43) - 1.22 (± 0.15) n.s.	0.39 (± 0.30) - 0.33 (± 0.06) n.s.	1.86 (± 0.71) - 1.55 (± 0.13) n.s.
Calcarous grassland species			
L. hispidus	1.50 (± 0.14) - 1.35 (± 0.29) n.s.	0.68 (± 0.19) 0.27 (± 0.04) *	2.18 (± 0.30) - 1.62 (± 0.32) n.s.
B. media	1.82 (± 0.35) - 1.31 (± 0.59) n.s.	0.60 (± 0.13) 0.66 (± 0.22) n.s.	2.42 (± 0.47) - 1.97 (± 0.80) n.s.
T. pulegioides	2.96 (± 0.89) - 1.14 (± 0.36) *	0.76 (± 0.24) 0.35 (± 0.15) *	3.72 (± 0.94) - 1.49 (± 0.49) *
S. minor	2.58 (± 0.34) - 1.61 (± 0.57) *	1.38 (± 0.25) 1.22 (± 0.34) n.s.	3.96 (± 0.47) - 2.83 (± 0.86) n.s.

Above-ground and total plant biomass were significantly positive correlated (Spearman's rho correlation) to PAA for all data together (Table 4.4). The biomass of the calcareous grassland species did not show a significant correlation with PAA when grown on matgrass sward or calcareous grassland soils separately, but the correlation between the biomass of these plant species and PAA was significantly positive when both soil types were taken together (Table 4.4, Figure 4.2, continuous lines). The biomass of the matgrass sward plants did only show a negative trend, but no significant correlation with PAA, except for the below-ground and total biomass of the plants grown on matgrass sward soil (Table 4.4, Figure 4.2, dotted lines).
The whole set of plants grown on calcareous grassland soil also was significantly positive correlated to above-ground and total plant biomass (Table 4.4), whereas no significant correlation was observed for the whole set of plants grown on the matgrass sward soil (Table 4.4) although a negative trend was visible (Figure 4.2, dotted lines).

*Table 4.4. Correlation coefficients of potential ammonia-oxidation activities (PAA) with plant biomass parameters (Spearman rho correlation). DW above = dry weight above-ground, DW below = dry weight below-ground, and DW total = Total dry weight. Significance level: ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05; n.s. = not significant.*

Soil type	Plant type	N	DW above	DW below	DW total
Both	Both	72	0.417 ***	0.203 n.s.	0.374 **
Both	Calcareous grassland	36	0.612 ***	0.461 **	0.597 ***
Both	Matgrass sward	36	-0.024 n.s.	-0.081 n.s.	-0.027 n.s.
Calcareous soil	Both	40	0.424 **	0.092 n.s.	0.372 *
Calcareous soil	Calcareous grassland	20	0.119 n.s.	0.382 n.s.	0.226 n.s.
Calcareous soil	Matgrass sward	20	-0.063 n.s.	-0.271 n.s.	-0.167 n.s.
Matgrass sward soil	Both	32	-0.138 n.s.	-0.227 n.s.	-0.222 n.s.
Matgrass sward soil	Calcareous grassland	16	0.291 n.s.	0.232 n.s.	0.179 n.s.
Matgrass sward soil	Matgrass sward	16	-0.438 n.s.	-0.594 *	-0.594 *

Discussion

The difference between plants from the matgrass swards and those from the calcareous grasslands with respect to potential ammonium oxidation activities was only observed on calcareous soil (Figure 4.1). On soil from the matgrass sward itself, no differences in PAA between plant species from both vegetation types were observed. This latter might be related to the significantly lower nitrification potential observed in the matgrass soil at the start of the growth experiment compared to the calcareous soil (Table 4.1). In the absence of plants, the PAA in the calcareous soil decreased on average with 19%, whereas the PAA in the controls of the matgrass sward soil increased with 93% on average, but was still well below the PAA measured in the calcareous soil. Although

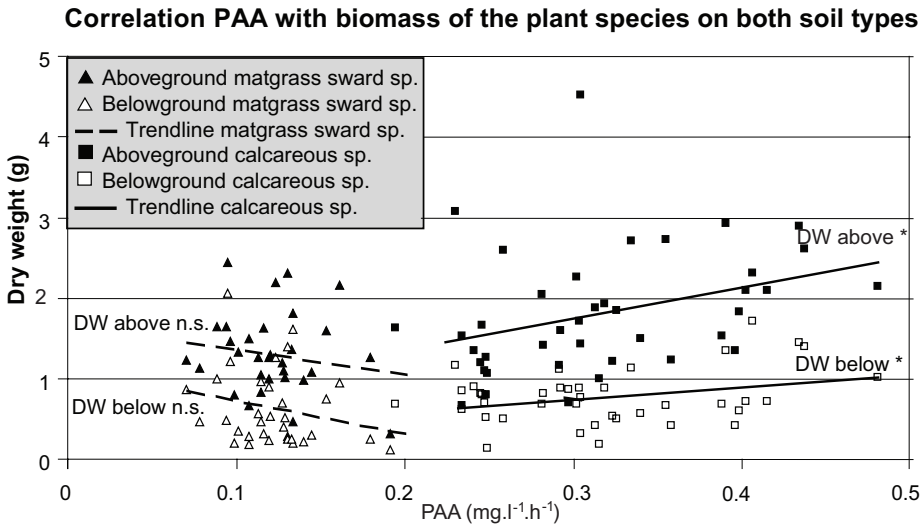

Figure 4.2. Scatter plot of the correlation of Potential Ammonia-oxidising Activity (PAA) and plant dry weight (above- and below-ground) for both species groups on both soil types. Trend lines are included to show the direction of the correlation, and the significance level is indicated (= P<0.05, n.s. = not significant).*

PAA had increased in the absence of plants during the growth period of four months in matgrass sward soil, the significant lower pH probably prevented a fast increase in PAA, which may have masked a possible effect of the plants on the development of the PAA. The time period of four months may not have been long enough to overcome the originally low population size of the ammonia-oxidising betaproteobacteria, although the PAA in matgrass sward soil was increasing during the experiment. An experimental period longer than four months might have shown the same differences between both plant groups in the matgrass sward soil as observed now for the calcareous soil. The restricted time period of four months might also have been responsible for the observed absence of the effects of plants on the ammonium and nitrate concentrations in the soils, as both soils contained sufficient nitrogen to sustain plant growth. Nitrate and ammonium availability was checked at regular intervals through pore-water sampling, and no effect of time was found. Sufficient ammonium remained present in the soil during the whole experiment for the AOB (Table 4.2). Hence, the observed repressed potential activity of the ammonia-oxidising community in the presence of plants from the matgrass swards was apparently not due to a lack of ammonium during the growth experiment. We cannot exclude that the significantly lower pH contributed to the low PAA found in the matgrass sward soil, although earlier field observations showed significant higher PAA at even lower soil pH values (<5.1, Smits et al. 2010).

Except for *H. umbellatum*, all species that grew better on calcareous grassland soil are typical for calcareous grassland. In the field *H. umbellatum* is characteristic for matgrass sward vegetation (SynBioSys, Schaminee et al. 2007). Moreover, biomass production of calcareous plant species was positively correlated to PAA. It is known that nitrate is the main nitrogen form in calcareous grasslands, and calcareous plant species prefer nitrate over ammonium for uptake (Gigon & Rorison 1972) and this is in line with the observed positive correlations. For matgrass plant species no overall correlation between the produced biomass and PAA was found, although on matgrass soil itself, there was a clear negative trend, with significant correlation for below-ground and total biomass. This is in agreement with our hypothesis that matgrass sward species negatively influence nitrification activity, leading to higher soil ammonium concentration and therefore more difficult growth conditions for the plant species themselves.

From correlation analyses of data derived from field samples, Stienstra et al. (1994) concluded that nitrification activities determined in grassland soils from a brook valley with different fertilisation histories were not related to plant species composition, but only to ammonium availability. These authors measured potential nitrification activities in root zones of *Holcus lanatus* and of one of the other locally dominant grass species (*Agrostis stolonifera*, *Agrostis capillaris* or *Anthoxanthum odoratum*). The species that they used are rather common species, and not sufficiently characteristic to be differentiating between different fertilisation histories in brook valley grasslands. In our experiment, on the other hand, characteristic plant species for climax grassland vegetation (i.e. matgrass swards and calcareous grassland) were used and the results lead to another conclusion than drawn by Stienstra et al. (1994). In the current experiment plant species composition does affect potential nitrification activity. Furthermore, the results from our experiment substantiate the results found by Groffman et al. (1996) who showed that plant species can create differences in microbial activity in soil.

The soils for this experiment were collected in autumn, when nutrient take-up by plants ceases and soil microbial processes rates, e.g. nitrification and mineralization are still high since temperatures are not decreasing sharply. Mineralization of recently senesced leave material and active nitrification could explain the observed high standard deviations in nitrate and ammonium concentrations observed in autumn soil samples as compared with analysis on other sampling dates. Potential nitrite-oxidising activities measured in a non-fertilized, semi-natural grassland, also showed increased nitrification activities at the end of the growing season (Both et al. 1992). A pattern with higher variation in autumn ammonium concentrations was also observed by Zhang et al. (2008) in grasslands in Inner Mongolia, China. Hence, the autumn setting may explain the relatively high variation in nitrate and ammonium concentrations. The general trend in these grassland types, however, is a higher ammonium:nitrate ratio in matgrass swards than in the adjacent vegetation zones (Smits et al. 2010).

The results of the current study confirm studies from Lata et al. (1999; 2000; 2004) on natural and semi-natural vegetation, and enlarge the view that not only crop plant species can exude biological nitrification inhibitors (BNI) (Subbarao et al. 2007c; 2007b, Zakir et al. 2008). As was shown by Subbarao et al. (2006, 2007a, 2009) exudates from the roots of the grass species Brachiaria humidicola and Leymus racemosus were able to influence the activity of nitrifying bacteria. For Brachiaria humidicola, it was shown by Subbarao et al. (2007b) that ammonium stimulates the synthesis and release of inhibition compounds. Zakir et al. (2008) found an increasing effect of ammonium and plant age on the release of nitrification-inhibiting compounds from the roots of Sorgum bicolor. Lata et al. (1999) discovered a strong correlation between the variation in potential nitrification rate and the characteristics of nitrogen nutrition of grasses in savannas. Mature grassland systems have the ability to inhibit nitrification or have a balanced inhibition-stimulation impact (Lata et al. 2000). Furthermore, Lata et al. (2004) demonstrated that nitrification can be suppressed or stimulated depending on the ecotype of Hypparhenia diplandra. Inhibition of nitrification can play a role in the functioning of these nutrient-poor grassland systems to control N losses (Lata et al. 2004; Fillery 2007; Phillippot et al. 2009).

In their discussion, Zakir et al. (2008) stated that the ecological significance of repressed nitrification is unknown. We hypothesize however, that repression of nitrification activities could be an adaptation of the nutrient-poor matgrass swards to conserve inorganic N and use it more efficiently by maintaining it in the less-soluble form of ammonium, thus preventing it from leaching in the form of nitrate (Vitousek et al. 2002; Lata et al. 2004; Herrmann et al. 2005). Atmospheric deposition of reduced N strongly increased over the last decades, and combined with reduced nitrification which prevented nitrate losses by leaching, this resulted in an ammonium excess for the nutrient-poor matgrass sward vegetation (Van den Berg et al. 2005, 2008). This present study shows that characteristic plant species from the matgrass sward vegetation might play a significant role in the inhibition of ammonium oxidation, at least in calcareous soils. If also present in the matgrass sward soil, a plant-soil feedback mechanism could have led to a further decline of this vegetation type. The findings of the current study fit well into the conceptual model of plant-microbe interactions concerning feedback dynamics (Bever et al. 1997; Reynolds & Haubensak 2008), as the plants turned out to influence the activity of the soil community. The observed inhibition of nitrification can be interpreted as a negative feedback on plant community diversity, under the current raised nitrogen levels due to atmospheric N deposition (Lee & Caporn 1998).

Acknowledgements

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Chapter 5

Long-term after-effects of fertilisation on the restoration of calcareous grasslands

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Abstract
Question: What are the long-term implications of former fertilisation for the ecological restoration of calcareous grasslands? Location: Gerendal, Limburg, The Netherlands. Methods: In 1970, ten permanent plots were established in just abandoned agricultural calcareous grassland under a regime of annual mowing in August. From 1971 to 1979, two different fertiliser treatments were applied twice a year to a subset of the plots (artificial fertiliser with different proportions of nitrogen and phosphorus). The vegetation of the plots was recorded yearly and vegetation biomass samples were taken for peak standing crop and total amounts of nitrogen, phosphorus and potassium. Species composition and floristic diversity were analysed over the research period (1970-2006) and between the treatments, including the use of multivariate techniques (Detrended Correspondence Analysis). Results: In terms of species number, there is a clear optimum 10 to 20 years after fertilisation has been terminated. Afterwards, there is a slow decrease; no new species appear and species of more nutrient-rich conditions gradually disappear. For the fertilised plots that received a relatively high proportion of N, effects are found only in the first years, whereas, for the plots that received a relatively high proportion of P, long-term after-effects are found in species composition, peak standing crop, total amounts of phosphorus in biomass, and in soil phosphorus data. Conclusions: The effect of artificial fertiliser with a large amount of nitrogen disappears in less than ten years when mown in August, including removal of the hay. This is a promising result for restoration of N-enriched calcareous grasslands, as the applied dose of nitrogen in this experiment largely exceeds the extra input of nitrogen via atmospheric deposition. Application of fertiliser with a large amount of phosphorus, however, has effects even more than 25 years after the last addition. There are no prospects that this effect will become reduced in the near future under the current mowing management.

Keywords
Chalk, fertilization, *Mesobromion erecti*, *Nardo-Galion saxatilis*, nitrogen, permanent plot, phosphorus, restoration.

Nomenclature
Van der Meijden (2005) for vascular plant species and Schaminée et al. (1996) for plant communities.

Introduction

The small, nutrient-poor grasslands on calcareous slopes in South Limburg are potentially one of the most species-rich ecosystems in The Netherlands (Willems 2001; WallisDeVries et al. 2002). Due to this high floristic diversity, which is also reflected in high faunal diversity, they are integrated in the EU Natura 2000 network, and two of their communities: calcareous grasslands of the *Mesobromion erecti* and matgrass swards of the *Nardo-Galion saxatilis*, are included in the appendix of the EU Habitat Directive (Romao 1996). These grasslands were once abundant as parts of the of common grazing land, but in the first half of the 20th century their number and area declined strongly due to changing land-use practices (Willems 1990; Poschlod & WallisDeVries 2002). Large areas were transformed either into arable fields and high production grasslands, or were abandoned and changed into forest (Bobbink & Willems 1987; Poschlod & WallisDeVries 2002).

To favour long-term restoration of calcareous grasslands in The Netherlands, these small remaining patches of habitat should be managed carefully. Furthermore, the area of these highly fragmented natural habitats should be increased and connected to form an ecological network (Opdam et al. 1993; Jongman & Pungette 2004). Therefore, restoration of adjacent heavily fertilised agricultural grasslands and fields is of major importance for the long-term maintenance of species richness in this calcareous region. As these originally nutrient-limited grasslands became highly productive because of their agricultural use (During & Willems 1984; Bobbink 1991; Willems et al. 1993), the removal of nutrients plays a central role in restoration of a species-rich vegetation. Removal of nutrients without removal of the upper nutrient-enriched soil layer thus conserving the present vegetation, can be accomplished by mowing or grazing, but these management measures are only successful when applied over a long period. Hence, long time series are of prime importance for understanding the long-term after-effects of fertilisation on restoration of these species-rich grassland types.

In 1970 ten permanent plots were established in a formerly fertilised calcareous grassland in South Limburg to study ecological restoration. The initial changes in vascular plant species composition in the permanent plots until 1993 had already been studied by Willems (1980, 1983), and Willems & van Nieuwstadt (1996), but one more report seemed to be justified, as the current publication includes vegetation data of 13 more years (until 2006); moreover, biomass analyses for all research years, not published previously, are now available. This paper investigates the development of vegetation, biomass and soil chemistry in relation to long-term after-effects of fertilisation in calcareous grassland. Specific research questions are: 1. Can long-term after-effects still be observed, even more than 25 years after cessation of fertilisation in (a) species composition, (b) production and (c) biomass composition? 2. Are these effects also reflected in soil chemistry?

Methods

Study area

The nature reserve Gerendal (50°50'13'' N, 5°50'54'' E) is located in the southernmost part of The Netherlands; it is a north-south running tributary valley of the river Geul, a tributary of the river Maas. The substrate consists of calcareous sediments (Upper Senonian Limestone), surfacing on the flanks of the valley, which was covered by layers of Pleistocene loss, and gravel deposits originating from the river Maas. The research site is located on a west-facing slope, with calcareous bedrock at a depth of ca. 40 cm (Willems 1980). Until 1967, this grassland was in agricultural use and heavily fertilised and heavily grazed. From 1967 to 1970, grazing and mowing took place, without the application of fertiliser. From 1970 onwards, the vegetation has been mown including removing the harvest annually in August (Willems 1980).

Experimental design

In 1970, three years after cessation of the intensive agricultural use, ten permanent plots (1.5 m x 1.5 m) have been established with a 1.5 m buffer zone between each plot. From 1971 to 1979, different treatments were applied:

1. Yearly fertiliser application of 170 kg-N.ha^{-1}, combined with 50 kg-P.ha^{-1} and 50 kg-K.ha^{-1}; this will be referred to as the Npk-serie (n = 2);
2. Yearly fertiliser application of 115 kg-N.ha^{-1}, combined with 355 kg-P.ha^{-1} and 115 kg-K.ha^{-1}; this will be referred to as the nPk-serie (n = 2);
3. No treatment; this will be referred to as the control series (n = 6). These six plots received their last fertilisation fertilisation in 1966 (under the former agricultural land use), and therefore show restoration since then. For more details, see Willems & van Nieuwstadt (1996).

The vegetation has been recorded almost annually from 1970 onwards mainly in August. The Braun-Blanquet scale was used (Barkman et al. 1964), providing data of more than 36 years in succession for analysis. The relevés were entered in the database managing system TURBOVEG (Hennekens & Schaminée 2001). As mosses have not been recorded every year, only vascular plant species were included in the analyses.

In addition, data on biomass were collected from harvested subsamples (50 cm x 50 cm) of the plots. The samples were dried (70 ºC, 48 h), weighed, and ground (Retsch centrifuge mill, sieve 0.5 mm). After grinding, 150 mg of the above-ground plant material was digested with an acid digestion containing 5 ml 30M sulphuric acid, together with salicylic acid. The nitrogen (N) and phosphorus (P) concentrations were determined calorimetrically and the concentration of potassium (K) flame-photometrically using a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, The Netherlands). Mixed soil samples of the upper 10 cm of each of the plots were collected (Auger diameter 2 cm) in August 2006. The soil samples were stored at 4 ºC and processed within one day. Soil (15 g) was extracted in a rotary shaker for 1 h (100 rpm) with 100 ml demin-

eralised water. The soil suspensions were centrifuged for 5 min at 4000 rpm (revolutions per minute). Supernatants were filtered through a Whatman GF/C-filter and stored at –20 ˚C until further analysis. Soil pH was determined in the remaining soil solutions. Soil moisture was determined after drying at 105˚C for 48 h. Ca, Mg, Al, K, NH4, NO3, SO4 and PO4-concentrations were determined colormetrically using a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, The Netherlands).

Data analysis

The development of the species composition under different treatments was analysed, using species richness (number of species per 1.5 m x 1.5 m). To analyse the effects of annual harvesting as a restoration measure in the control series (no fertiliser application after 1966), these data were divided in four periods, based on total species richness. Subsequently, the species composition between different periods was compared. To get an insight into the current difference in species composition of the nPk-series compared to all other plots, plots of the latest period (1996-2006) were used. Changes in species composition were analysed, using a $\chi 2$-test. If expected values < 5 appeared in the $\chi 2$-test, the results of the analysis are less reliable and therefore not presented. Ecological interpretation of the results is based on the sociological preferences of the individual species, according to SynBioSys (Schaminée & Hennekens 2001).

For trends in the changes in species richness and biomass as a result of treatments, data were tested by repeated measurement analyses in SPSS 11.5. To find the year where the effect of the Npk-treatment and nPk-treatment converged, one-way ANOVA (post-hoc: Tukey's b-test) was used for each year ($P < 0.05$). The soil data were also tested by one-way ANOVA. Development of the species composition over more than 35 years was investigated in a Detrended Correspondence Analysis (DCA). Indirect gradient analysis (DCA) was executed with percentage cover data in CANOCO 4.52 (ter Braak & Šmilauer 2002).

Results

Species richness

The general trend is a clear increase in number of plant species after the fertilisation ceased (at most after about 10-15 years), followed by a slow decrease (Figure 5.1). This is most clearly shown in the control treatment (n = 6), where the total number of species in the period 1980-1986 is largest with on average 39 species per plot. This amount decreased slowly until, on average, 31 species per plot in the period 2000-2004; a total decrease of 20%. The Npk-, and nPk-treatments show the same trend after the termination of the experimental additions in 1979, but the maximum number of species was lower (around 35). The current species richness of both sets of fertilised plots is about the same as the control series. During the period of fertiliser treatments (1971-

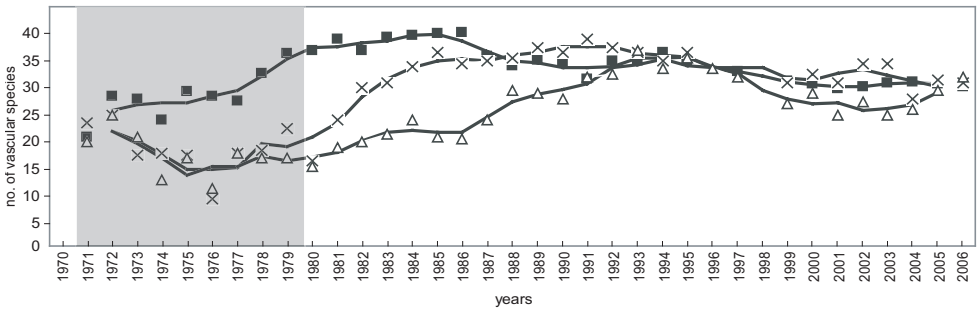

Figure 5.1. Average number of vascular plant species (1.5m x1.5m) of the control-series (n=6, filled squares), the Npk-series (n=2, crosses) and the nPk-series (n=2, triangles) in the Gerendal-plots (The Netherlands). From 1972 to 2005 the moving average (calculated by the average of three years) is shown for the three groups of plots. In grey, the years are indicated in which fertiliser was applied in the Npk- and nPk-series (1971 to 1979).

1979), the average number of species in the fertilised plots decreased, while it increased after the treatments were stopped until around 1990. Afterwards a slow decrease was found, comparable to the control series. Although the fertiliser treatment of the plots has been stopped already in 1979, it took until 1987 for the plots of the Npk-treatment and until 1991 for the plots of the nPk-treatment to reach the same number of vascular plant species as the other plots (ANOVA, $P < 0.05$).

Species composition

To get an insight into the major changes in species composition of the control series (which received no fertiliser application since 1967), $\chi 2$-tests were used (App. B). The total research period was divided into four periods, based on changes in species richness: (1) 1970-1979 (recovering period after fertilisation); (2) 1980-1986 (most species-rich period; on average 39 species per plot); (3) 1987-1995 (slow decrease; on average 35 species per plot); and (4) 1996-2006 (slow decrease; on average 31 species per plot).

Period 1-2

An increase in the number of species per plot took place from around 28 to 40 vascular species per plot in the first period. The second period is the most species-rich period. Of the 23 species that showed a significant trend between the first two periods, 22 increased in presence. Only *Bellis perennis* (indicative of nutrient-rich grasslands) decreased. Of the increasing species, four are characteristic of the *Mesobromion*, the other 18 species are rather common in dry grasslands in the area.

Period 2-3-4

A major loss of species took place during the last three periods from an average of 39 species per plot in period 2 to 35 in period 3 and 31 species per plot in period 4. 25 species

showed a significant trend between period 2 and 3: 15 of them decreased in presence, all rather common species in dry grasslands in the area, and ten species increased, of which *Brachypodium pinnatum* is the most indicative species of calcareous grasslands. In the period 3-4 23 species showed a significant trend: 15 rather common species decreased, and 8 rather common species increased.

Vegetation

Overall changes in species composition during the total research period show an increase in common dry grassland species in the first years after cessation of fertilisation, followed by a decrease in the same species group. The present characteristic calcareous species group remains quite stable over the years of the present research.

The DCA revealed strong differences in the development of the species composition under different treatments (Figure 5.2a-c). In 1971, all plots were still close together on the left side of the ordination space. By 2006, all plots had moved upwards, but are still clustered in the same part of the diagram, except for those of the nPk-treatment. All control plots follow more or less the same trend. The Npk-treated plots show a different development, but are currently (from about 1988 onwards) following the same development trend as the control-plots. The nPk-series show the same development as the Npk-series, but change around 1990 into another equilibrium (from the cloud of points in the right corner of the diagram into the cloud at the top of the diagram). So far, the species composition of the nPk-series has remained different from the species composition of all other plots. The same development is shown in the direct gradient analyses (CCA), although eigenvalues were lower (0.3 and 0.03 respectively; data not shown).

$\chi 2$-tests between the 18 plots of the nPk-series and 62 plots from all other plots in the latest research period (1996-2006) provided information on the difference in species composition between both groups. 14 species showed a significant change. Whereas more common species such as *Crepis biennis*, *Medicago lupulina*, *Hypericum perforatum*, and *Poa pratensis* are more present in the nPk-series, characteristic species of calcareous grassland such as *Carex caryophyllea*, *Campanula rotundifolia*, *Dactylorhiza fuchsii* and *Plantago media* are more present in all other plots (Figure 5.3).

Biomass and nutrient concentrations

The biomass production in the control series was on average 285 $g.m^{-2}$ (Figure 5.4). There was a significantly higher biomass-production in the fertilised plots than in the control plots until 1984 for the Npk-treatment and until 1987 for the nPk-treatment (ANOVA, $P < 0.05$). When tested with repeated measurements (Table 5.1), both treatments showed a significant raise in the first years (until 1985), while there is a significant increase of the nPk-treatment in the period 1986-2006.

The total N-content of the standing crop is in both fertiliser treatments significantly higher than the control treatment until 1981 (Figure 5.5). In 1998, the nPk treatment

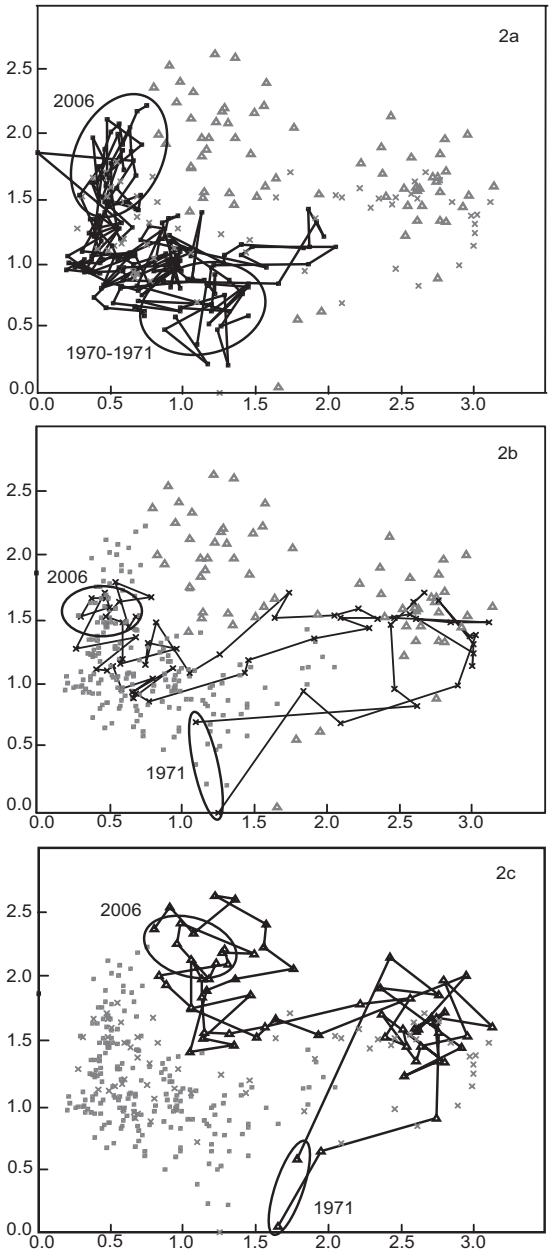

Figure 5.2. DCA ordination diagram of all 325 relevés of all treatments. In the different figures, the time series of each plot are connected with lines. A) control-series. All starting points are within the lowest circle (1970-1971), all data from 2006 are within the highest circle, B) Npk-series, and C) nPk-series. Eigenvalues of the first two axes are 0.4 and 0.2, respectively.

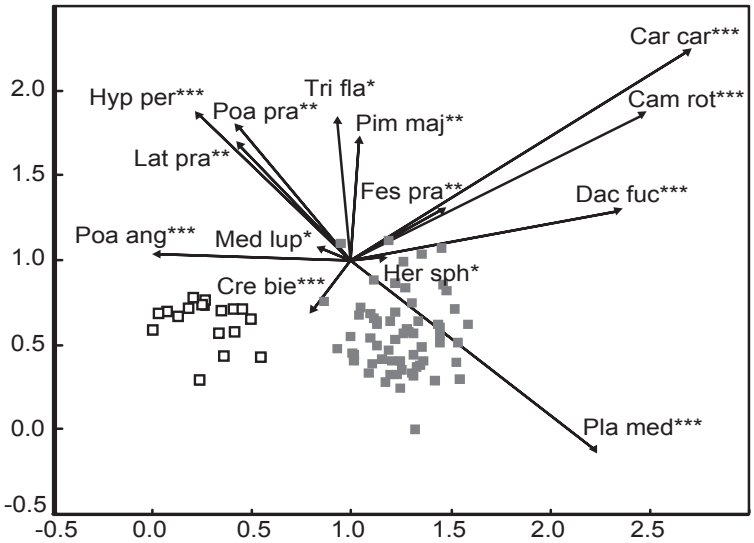

*Figure 5.3. DCA ordination diagram of 80 relevees of the latest period (1997-2006): 18 plots of the nPk-series (open squares), and 62 plots of the other series (both Npk-, and control-series: closed squares). With the arrows, the vascular plant species are indicated that show significant differences between the two groups. Significance level: P<0.001 ***, 0.001>P<0.01 **, 0.01>P<0.05 *. Eigenvalues of the first two axes are 0.2 and 0.1, respectively. Cam rot = Campanula rotundifolia, Car car = Carex caryophyllea, Cre bie = Crepis biennis, Dac fuc = Dactylorhiza fuchsii, Fes pra = Festuca pratensis, Her sph = Heracleum sphondylium, Hyp per = Hypericum perforatum, Lat pra = Lathyrus pratensis, Med lup = Medicago lupulina, Pim maj = Pimpinella major, Pla med = Plantago media, Poa ang = Poa angustifolia, Poa pra = Poa pratensis, Tri fla = Trisetum flavescens.*

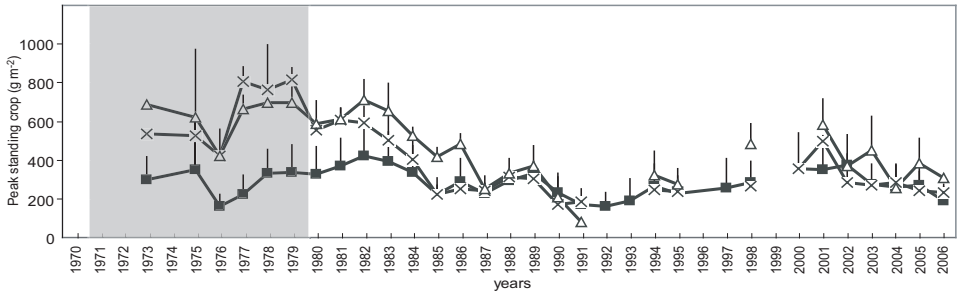

Figure 5.4. Peak standing crop (August) of the control-series (n=6, filled squares), the Npk-series (n=2, crosses) and the nPk-series (n=2, triangles), including the standard deviation in error bars. In grey, the years are indicated in which fertiliser was applied in the Npk- and nPk-series (1971 to 1979).

was significantly increased again. Testing data with repeated measurements, showed an effect for the Npk-fertilised plots, but only in the first years (Table 5.1). The total P-content of the standing crop (Figure 5.5) shows rather large standard deviations for

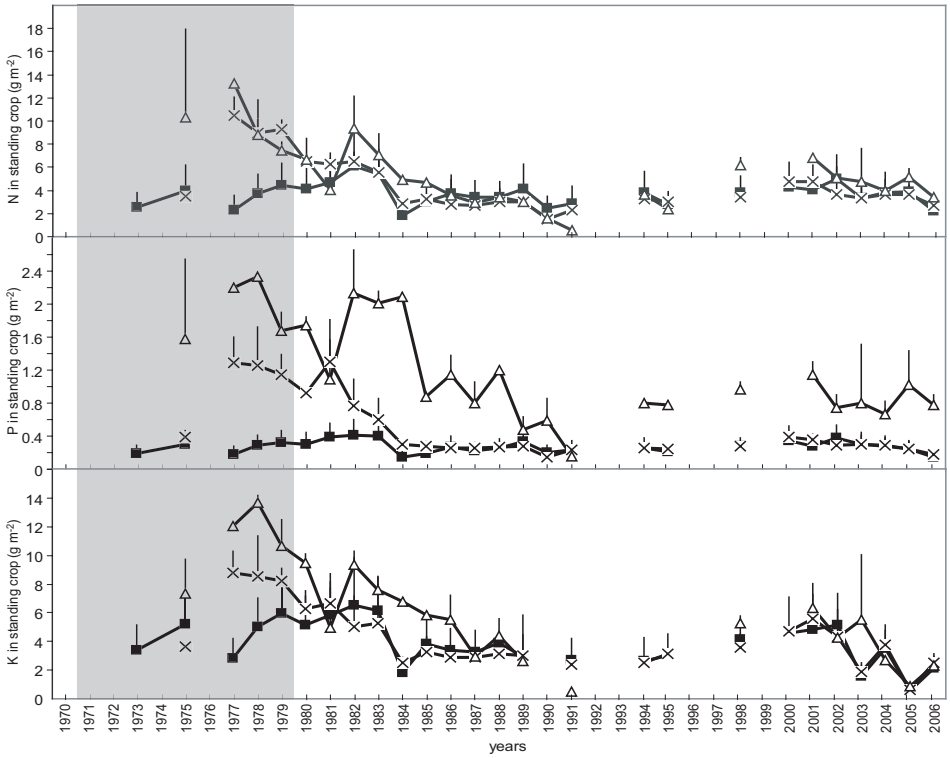

Figure 5.5. Total N, P, and K in standing crop of the control-series (n=6, filled squares), the Npk-series (n=2, crosses) and the nPk-series (n=2, triangles), including the standard deviation in error bars. In grey, the years are indicated in which fertiliser was applied in the Npk- and nPk-series (1971 to 1979).

the treatments, nevertheless a significant difference is found in almost all years for the nPk-series, whereas the results of the Npk-series converge to those of the control series from 1982. Testing data with repeated measurements reveals effects of treatments in both periods (Table 5.1). The total K-content of the standing crop (Figure 5.5) shows significant positive differences in 1978 for both treatments, whereas only the nPk-series show this difference in 1979 en 1980. Only in the first years (1978-1981), there was a positive effect of the P-treatment on total K-content of the standing crop.

Soil data

Soil data of 2006 showed a significantly (ANOVA, $P < 0.05$) higher concentration of only P-PO4-concentrations in the nPk-fertiliser treatment (control series: $9.57*10^{-3} \pm 2.34*10^{-2}$ mg.kg^{-1} dry soil; Npk-series: $4.91*10^{-3} \pm 6.95*10^{-3}$ mg.kg^{-1} dry soil; nPk-series: 1.43 ± 0.65 mg.kg^{-1} dry soil).

*Table 5.1. Summary of GLM, repeated measurements-results, including post-hoc Tukey-tests for the different treatments. As there were not sufficient data available for all years, selections were made, depending on the available data. A: 1975-'77-'79-'81-'83-'85 (No sphericity. Epsilon: Greenhouse-Geisser); B: 1986-'90-'94-'98-2002-'06 (Sphericity assumed); C: 1978-'79-'80-'81 (Sphericity assumed); D: 1986-'90-'94-'98-2002-'06 (Sphericity assumed); E: 1978-'79-'80-'81 (No sphericity. Epsilon: Greenhouse-Geisser); F: 1983-'86-'90-'94-'98-2002-'06 (No sphericity. Epsilon: Greenhouse-Geisser); G: 1978-'79-'80-'81 (Sphericity assumed); H: 1981-'83-'86-'89-'98-2002-'06 (Sphericity assumed). C = control treatment. Significance level: P<0.001 ***, 0.001>P≤0.01 **, 0.01>P<0.05 *, n.s. = -. For the post-hoc Tukey test, the group averages (g m^{-2}) are presented with letters indicating the significance level (P<0.05).*

Years	**N**	**Time**	**Treatment**			**Time * Treatment**
			C	**Npk**	**nPk**	
Peak standing crop						
A. 1975-1985	9	F = 13.276, *P* = 0.005 **	308 ^{a}	578 ^{b}	611 ^{b}	F = 4.932, *P* = 0.034 *
B. 1986-2006	10	F = 9.127, *P* = 0.000 ***	274 ^{a}	243 ^{a}	363 ^{b}	F = 2.650, *P* = 0.016 *
Total N in peak standing crop						
C: 1978-1981	8	F = 10.801, *P* = 0.000 ***	4.08 ^{a}	7.77 ^{b}	6.73 ^{a,b}	F = 6.176, *P* = 0.002 **
D: 1986-2006	10	F = 14.692, *P* = 0.000 ***	3.48 ^{a}	3.32 ^{a}	3.97 ^{a}	F = 3.417, *P* = 0.003 **
Total P in peak standing crop						
E: 1978-1981	8	F = 3.372, *P* = 0.112 -	0.32 ^{a}	1.16 ^{b}	1.72 ^{c}	F = 4.508, *P* = 0.058 -
F: 1983-2006	10	F = 24.050, *P* = 0.000 ***	0.27 ^{a}	0.33 ^{a}	1.06 ^{b}	F = 8.406, *P* = 0.001 **
Total K in peak standing crop						
G: 1978-1981	8	F = 14.715, *P* = 0.000 ***	5.39 ^{a}	7.42 ^{a,b}	9.71 ^{b}	F = 10.053, *P* = 0.000 ***
H: 1981-2006	10	F = 10.363, *P* = 0.000 ***	4.26 ^{a}	4.03 ^{a}	4.66 ^{a}	F = 1.012, *P* = 0.455 -

Discussion

Many studies have focused on the effects of fertiliser application on species composition and species richness in calcareous grasslands (Bobbink 1991; Willems et al. 1993; Morecroft et al. 1994; Carroll et al. 2003), but hardly any attention has been given to long-term after-effects of fertiliser application on plant diversity in restored grasslands. More than 25 years after the fertiliser application was finished, still significantly higher amounts of phosphorus are present. These effects are shown in species composition, as well as in biomass and soil chemistry. Effects of fertiliser application with a large amount of nitrogen, however, seem to have disappeared less than ten years after the application was finished. Furthermore, this research has made clear that under the current management (mowing once a year in August, including removal of the hay) after-effects of fertiliser applications with a high amount of phosphorus remain for periods much longer than 25 years, thus resulting in a different species composition with more common species, and less characteristic species of calcareous grasslands. These effects, however, are no longer detectable in general, functional vegetation characteristics, such as species richness.

All plots show an optimum in species richness 10-15 years after the fertiliser application stopped, followed by a slow decrease. This decrease might be explained by two mechanisms: firstly, by disappearance of the plant species that are characteristic of more nutrient-rich circumstances (the diminishing of nutrients, due to mowing management) and secondly, by the overall decline in species number in Dutch chalk grasslands as a result of soil eutrophication by atmospheric nitrogen input (Bobbink 1991; Bobbink & Willems 1987) and habitat fragmentation (Butaye et al. 2005).
The long duration of the after-effects of the highest P-applications in the experiment can also be explained by the relatively small amounts of P removed by the harvesting of the vegetation. A simple P mass balance, assuming fertilizer application as the main input and harvesting as the main output, shows that a total of about 320 $g.m^{-2}$ of P was added to the nPk plots during the 9-year nPk-fertiliser treatment. The amount of P that was removed with the summer harvest of the peak standing crop was at itshighest about 2.5 $g.m^{-2}$, and today is around 1 $g.m^{-2}$. In total about 37 $g.m^{-2}$ was removed in the nPk-series up to now (compared to 9 $g.m^{-2}$ in the control series and 16 $g.m^{-2}$ in the Npk-series, respectively), which is only 12% of the quantity applied. Due to the formation of poorly soluble calcium-phosphate minerals in calcareous grassland, management alone will not be sufficient to remove the extra inputs applied in the past. The P application in the nPk treatments was high, but still comparable to realistic fertiliser applications to these calcareous soils in the 1970s. However, in other fertiliser experiments, only 4.4 $g.m^{-2}.yr^{-1}$ phosphorus (during five years, see Willems et al. 1993) and 3 $g.m^{-2}.yr^{-1}$ (during three years, see Bobbink 1991) was added, much less than applied in the current experiment. The amounts applied in the current study are already three times the legally allowed amount in agricultural grasslands in 1987 (i.e. 11 $g.m^{-2}.yr^{-1}$; van Eerdt & Hijstraten 2005). However, it is known that the amount of phosphorus in artificial fertiliser has been declining since 1970. In 2006 the legally allowed amount of phosphorus was 5 $g.m^{-2}.yr^{-1}$ in grassland. The results of the current experiment are therefore only representative of severely phosphorus fertilised situations.
The amount of applied nitrogen in the Npk-series was 17 $g.m^{-2}.yr^{-1}$ (in total ca. 150 $g.m^{-2}$ was applied), and is therefore much higher than the measured atmospheric nitrogen input in these grasslands in The Netherlands (ca. 2-4 $g\text{-}N.m^{-2}.yr^{-1}$ in the 1990s: van Dam 1990). Nevertheless, the effects of this extra input disappeared under the current management within ten years. When compared to the legally allowed amount of nitrogen in agricultural grasslands in 1998 (i.e. 30 $g.m^{-2}.yr^{-1}$; van Eerdt & Hijstraten 2005) the application in the experiment was much lower. The amount of N that was removed with the summer harvest of the peak standing crop was at highest ca. 12 $g.m^{-2}$, and today is ca. 4 $g.m^{-2}$. These data are comparable to other studies on restoration of plant communities on nutrient-poor soils (Bakker et al. 2002). In total ca. 145 $g.m^{-2}$ was removed in the Npk-series up to now (compared to 121 $g.m^{-2}$ in the control series and 183 $g.m^{-2}$ in the nPk-series, respectively).
Furthermore, the results indicate that increased atmospheric nitrogen deposition has not

led to higher amounts of nitrogen in the calcareous grassland vegetation in the studied research period (1970-2006), as both peak standing crop and total N in peak standing crop (Figures 5.3 and 5.4) were stable over the whole research period. Therefore, mowing in August (including removal of the harvest) seems to be sufficient to remove the extra nitrogen supplied from the air in these grasslands. This is in accordance with the findings of Bobbink & Willems, who found that mowing in early August is an effective way to reduce Brachypodium pinnatum (Bobbink & Willems 1991). For restoration of other nutrient-poor communities, e.g. *Nardo-Galion* grasslands, annual mowing (including removal of the harvest) does not work (Bakker et al. 2002).

Restoration of degraded calcareous grasslands is a complicated long-term process which, in general, involves restoration of the abiotic as well as biotic conditions. Dispersal of target species can be a bottleneck in restoration, as many of these species have low dispersal possibilities (Verkaar et al. 1983; Poschlod et al. 1998) and in most cases have disappeared from the nearby surroundings. Furthermore, it is very unlikely that seeds of these species are still present in the soil seed bank, as most of the characteristic calcareous grassland species have a transient, rather than a persistent seed bank (Bekker et al. 1998; Willems & Bik 1998; Butaye et al. 2005). For example, many characteristic calcareous species such as Sanguisorba minor and Thymus pulegioides have not returned to the current research site, although they are present even within one kilometre. Therefore, restoration measures should preferably be carried out in areas, adjacent to more or less intact calcareous grasslands.

Although an ecological network provides the best properties for restoration of calcareous grasslands, long-term after-effects of fertilisation in former agricultural sites, in combination with restoration of biotic conditions (available species), are important factors when restoring calcareous grasslands from formerly fertilised systems (Bakker et al. 2002). Although long-term information on restoration is sparse (Smits et al. 2002), it proves to be indispensable to investigate long-term after-effects of fertilisation.

Acknowledgements

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TAKEUCHI

Chapter 6

Restoration of species-rich grasslands enhanced by sod cutting and hay transfer

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Abstract
In the Netherlands, the area of species-rich, nutrient-poor grasslands has been strongly reduced during the past hundred years, just like in other industrial and highly cultivated countries in northwest Europe. This loss is mainly due to changes in land use. Currently, species-rich grasslands in Southern Limburg only remain in small and isolated nature reserves, and almost all grassland reserves in this region are smaller than 10 ha. Apart from the conservation of the remaining sites, enhancement of landscape connectivity is necessary to achieve a sustainable ecological network. This paper studies measures to restore species-rich and nutrient-poor grasslands on pastures with a history of intensive agricultural use. Over a four-year period, a replicated factorial block design was used to test the effects of two methods of nutrient removal (sod cutting versus cutting with hay removal twice a year) and seed addition by hay transfer (spreading of autochthonous freshly mown plant material) on the development of vegetation and soil conditions. The best results were obtained with a combination of sod cutting and hay transfer, whereas up to now, mowing alone or in combination with hay transfer did not result in changes in soil and vegetation composition.

Keywords
Matgrass swards, *Nardo-Galion*, restoration, nutrient-poor grasslands, vegetation, species diversity, soil.

Introduction

During the past century, semi-natural species-rich grassland communities have become severely threatened due to large-scale changes in land use. In Southern Limburg (the Netherlands), these grasslands have been abundant on slopes as common land, grazed by herded sheep in the traditional land use system. From the beginning of the twentieth century onwards, there has been a strong decline in the number and area of the sites involved, due to changing land use practices (Willems 1990; Poschlod & WallisDeVries 2002). The grasslands often include a range of different vegetation types, depending on soil characteristics. On top of calcareous substrate, acid gravel deposits may occur, resulting in a unique vegetation gradient with acid grasslands (*Thero-Airion*) on the higher parts of the slopes, calcareous grasslands (*Mesobromion erecti*) on chalk outcrops, and matgrass swards (*Nardo-Galion saxatilis*) in between. At the bottom of the slope, on thicker soil layers, relatively more nutrient-rich grasslands, belonging to *Arrhenatherion elatioris* or *Arction* occur. Due to their (potentially) high diversity of plant and animal species, the grasslands on these slopes have a high conservation value, and two of the main vegetation types, e.g. the matgrass swards and the calcareous grasslands are therefore included in the appendix of the EU Habitat directive (habitat types 6230 and 6210; 2002/83/EC Habitat Directive). To favor long-term conservation of nutrient-poor grasslands, the small remaining patches of habitat should be managed carefully. Especially the restoration of calcareous grasslands has been the subject of many studies in NW Europe (Köhler et al. 2005; Kiehl & Pfadenhauer 2007; Fagan et al. 2008) as well as in the Netherlands (Willems 2001; WallisdeVries et al. 2002). The matgrass sward parts of these grasslands received much less attention than the calcareous grasslands, partly because this vegetation unit was only described as a separate plant community with its own characteristic soil conditions in 1982 (Willems 1982a; Swertz et al. 1996). Nevertheless, recent research on the historical distribution of the characteristic plant species made it clear that also in these more acidic grasslands much of the former botanical quality has become lost over the last century (Smits et al. 2009).
To form a potentially stable ecological network, the area of these highly fragmented semi-natural habitats should be increased and connected (Opdam et al. 1993; Jongman & Pungette 2004). Restoration of areas adjacent to nature reserves, which often have been heavily fertilized and intensively grazed, is of major importance for the long-term maintenance of the biodiversity in the sites. For this reason, the national policy for nature conservation in The Netherlands has resulted in a governmental fund for development of an 'Ecological main structure', a network of (large) nature reserves connected by landscape corridors in the Netherlands (Ministry of Agriculture, Nature and Food Quality 1990).

To restore or re-create new landscape corridors and thus facilitate habitats for the potentially species-rich vegetation types under discussion, both abiotic and biotic

constraints should be considered. The most important abiotic constraints in these dry grasslands are constituted by residual high soil fertility in grasslands with a history of intensive agricultural use and nitrogen input from atmospheric deposition (Bobbink et al. 1998;Walker et al. 2004). Sod cutting or topsoil removal is an often applied, but drastic and expensive way to reduce soil nutrients. Alternatively, hay making or grazing without fertilizer application can be applied, although the effect on soil fertility is more moderate and only visible on the longer term (Bakker et al. 2002; Smits et al. 2008). Moreover, in dry and wet degraded acid heathland areas, an unexpected extra restriction was discovered after sod cutting: soil ammonium concentrations can increase up to toxic levels for characteristic plant species under acidic soil conditions (De Graaf et al. 1995, 1998a, Dorland et al. 2003). Biotic constraints concern the absence of viable seeds and the limited opportunities for species to disperse to new, appropriate habitat. Seed bank development studies in comparable vegetation types demonstrated that their dynamics are mainly controlled by input from the established vegetation (Willems & Bik 1998; Bekker et al. 2000), whereas most of the characteristic species in the studied grasslands have a transient or short-term persistent seed bank (Knevel et al. 2003, 2005; Kleyer et al. 2008). Local seed sources of the target species have often become limited through habitat loss and fragmentation (Poschlod et al. 1998; Verhagen et al. 2001) and important potential vectors for seed dispersal have become reduced. Due to the changed agricultural land use, grazing practices and the accompanying widespread transport of grassland species have ceased (Fischer et al. 1996; Ozinga et al. 2009; Wallin et al. 2009). Seed addition experiments confirm that the absence of species in appropriate, abiotic conditions can be caused by dispersal-related limitation (Turnbull et al. 2000; Franzen & Eriksson 2003).

In this paper, we report the results of a four-year experiment evaluating the effects of measures to restore nutrient-poor, species-rich grasslands in pastures with a history of intensive agricultural use. The aim is to test the efficiency of two types of restoration mechanisms, e.g. nutrient removal (twice a year mowing, including removal of the hay, and sod cutting of the upper 10 cm soil once) with and without seed addition by hay transfer (using freshly mown material from a local, well-developed, species-rich grassland). We investigated which of the tested methods provides the best results for accelerated, adequate restoration. We hypothesized that the combination of sod cutting and hay transfer contributes most towards restoration on the short-term. For this reason, we compared soil parameters and plant community composition between harvested, sod cut and control (grazed) plots within two sites in the Southern part of the Netherlands, which had been highly nutrient enriched by formerly intensive agricultural grassland use.

Methods

Study sites

The experiments were carried out on two different locations in Southern Limburg, the Netherlands. The first is a site called 'Verlengde Bemelerberg' (VB; 50º51'12 N; 5º 46 22 E) and is located next to one of the best developed nutrient-poor grassland reserves in Southern Limburg, the 'Bemelerberg'. The grassland site had previously been in intensive agricultural use and was acquired by a regional management organization (Stichting Het Limburgs Landschap) in 2000. By that time, it had not been fertilized for a substantial period (about ten years; personal communication Stichting Het Limburgs Landschap). From 2000 onwards, the grassland has been grazed by a sheep of a local breed, the so-called Mergellandschaap. The grazing takes place in several short periods of some weeks during the year with a varying amount of sheep. The species-poor sward is dominated by crop grass species like *Dactylis glomerata* (Cocksfoot), *Lolium perenne* (Perennial ryegrass) and *Holcus lanatus* (Common velvetgrass).

The second research site is called "Keerderberg" (KB; 50º50'00 N; 5º 44 49 E) and consists of a grassland that used to belong to a boarding school (Huize st. Joseph). Descriptions of the flora of the estate and its surroundings around 1940 mention various matgrass sward species, like *Jasione montana* (Sheepbit), *Genista pilosa* (Hairy greenweed) and *Succisa pratensis* (Devil's-bit'scabious). None of these species were found again by the authors. Since 10-15 years, the grassland slope is managed by the State Forestry Service (Staatsbosbeheer). In the beginning it was grazed by cattle, and since 5-10 years by sheep (Mergellandschapen). The sheep stay in the grassland for a period of some weeks between mid August and mid April. Current dominant species in the grassland are *Festuca rubra* (Red fescue), *Agrostis stolonifera* (Creeping bent) and *Dactylis glomerata*.

Both sites were recommended to us by the local managers of the nature conservation agencies, because they themselves were not successful in restoring any of the potential biodiversity under the current management.

Experimental design

Experimental plots measured 3 x 3m, of which the inner 2 x 2m were used for analysis of vegetation and soil parameters. The two types of restoration measures (nutrient removal and hay transfer) are investigated in a full factorial design. The plots were fenced off from the current grazing management and the treatments were randomly ordered in the five series (Figure 6.1). This set-up was repeated five times (five blocks of treatments) in every site, and every block also included an unfenced control plot where the current management was continued. These were grazed according to the standard grazing regime (some weeks a year in spring and sometimes in autumn in varying numbers).

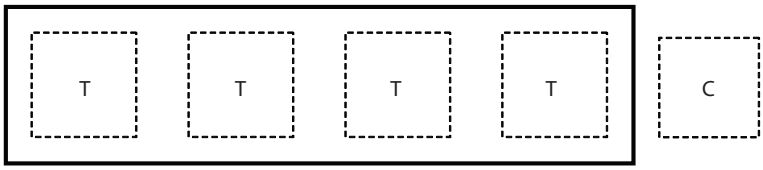

Figure 6.1. Lay out of the experiment (plot size 3x3 m, of which the inner 2x2 m is used for monitoring). The four treatments (T) are randomly assigned within the fenced off surface of 3x12 m (5 series; two sites). The four treatments were: (1) sod cutting; (2) sod cutting and hay transfer; (3) mowing twice a year; (4) mowing twice a year and hay transfer; (5) The control plot (C) was located outside the fence in the grazed area of both sites.

In spring 2005, soil samples were collected at different depths from both experimental sites and from the adjacent nature reserve to investigate the starting conditions. In the upper 10 cm, we found 1.5-3 times higher water-extractable nitrate concentrations and 4-6 times higher water-extractable phosphate concentrations in the experimental sites than in the nearby nature reserve, whereas from 10 cm onwards, the concentrations were comparable to the nature reserve (data not shown). Most phosphate and nitrate appeared to be stored in the upper 10 cm. To remove these extra nutrients, two experimental treatments were included. The first experimental treatment consisted of a regime of hay cutting twice a year, by mid June and late September/early October. The plots were mown mechanically and the harvested biomass was directly removed with a rake. The second treatment consisted of the removal of the upper, nutrient-rich 10 cm soil layer at the beginning of August 2005. From 2006 onwards, these plots were mown, including removal of the mown material once a year in autumn.

At the start of the experiment, both sites did not contain characteristic species of matgrass swards, whereas species of well-developed nutrient-poor grasslands were absent in the near surroundings. Therefore, the removal of nutrients was combined with a second experimental factor: the dispersal of plant diaspores, through a one-time application of freshly mown material. The material was harvested on 8 September 2005 on the adjacent Bemelerberg nature reserve, in a well-developed, species-rich matgrass sward, classified as *Betonico-Brachypodietum* (Swertz et al. 1996). Before harvesting, a species list, including the coverage and phenological status was compiled (Table 6.1).

Fresh plant material was spread directly on the soil in half of the experimental plots, resulting in a thin layer of biomass on the bare soil or on top of the present vegetation (roughly in the proportion ½:1). The first winter, the mown material was fastened on the plots with nets to reduce the risk of movement by wind and animals. In spring 2006, most of the mown material was decayed, and the nets were removed.

Data collection & analysis

Vegetation relevés (of the vascular plant species) were made in the inner 2x2 m of the plots using the adjusted Braun-Blanquet scale (Barkman et al. 1964) in early June, from 2005 to 2008. The relevés were imported in Turboveg (Hennekens & Schaminée 2001)

Table 6.1. Species composition and phenology of the vegetation (3x10 m) that was mown for the plant material transfer. The coverage (cov) is given as f = frequent; o = occasional; s = sparse, and the phenology (phen) is indicated with v = vegetative or g = generative (in flower and/or fruit). The vegetation was characterized by ASSOCIA (Van Tongeren 2008) as Betonico-Brachypodietum, with a weirdness of -0.1, and an incompleteness of 0.4.

Species	**Cov-Phen**	**Species**	**Cov-Phen**
Agrostis capillaris	f-g	*Hieracium pilosella*	o-g
Agrostis stolonifera	f-g	*Hieracium umbellatum*	f-g
Anthoxanthum odoratum	s-v	*Hypericum perforatum*	o-g
Brachypodium pinnatum	f-g	*Hypochaeris radicata*	o-g
Calluna vulgaris	f-g	*Jasione montana*	f-g
Campanula rotundifolia	f-g	*Leontodon hispidus*	s-g
Cerastium fontanum	s-g	*Plantago lanceolata*	f-g
Cytisus scoparius	o-v	*Potentilla erecta*	s-v
Danthonia decumbens	f-g	*Rosa* species	s-v
Festuca filiformis	f-g	*Rumex acetosella*	o-g
Festuca rubra	f-g	*Stachys officinalis*	f-g

and the vegetation composition was analysed with JUICE (Tichy 2002) and Associa (Van Tongeren 2000). Just before mowing the plots, 50x50 cm of the vegetation of each mown plot was harvested to determine the biomass removal.
In the summers of 2005 (just after sod cutting), 2006, 2007 and 2008 mixed soil samples were taken in all plots with a Gauge auger (Ø 2 cm). In the non sod-cut plots, samples at a depth of 0-10 cm and 10-20 cm were taken, whereas in the sod-cut plots, only the upper layer (0-10 cm) was sampled, as this is comparable to the 10-20cm of the original soil after removing the upper 10 cm. For the sod-cut plots and mown plots (both without hay transfer), an extra sampling was carried out in spring 2006 to test the effect of sod cutting on soil nitrogen processes.

Soil samples were kept at 4 ˚C and processed within a few days. 15 g of fresh soil was shaken during one hour (100 rpm) in a solution of 100 ml demi water (for the analysis of aluminium, phosphate and nitrate) or in 100 ml 0.2M KCl (for the analysis of ammonium). Afterwards, the slurries were centrifuged for 5 minutes (4,000 rpm) and the supernatant was filtered (Whatman GF/C-filter) and stored at -20˚C until further analysis was carried out. Soil pH was determined in the remaining slurries (WTW pH 540 GLP). For the soil moisture content, 20 g of soil was dried during 48 hours at 105 ˚C. NH_4, NO_3, and PO_4-concentrations were measured using an auto analyser (Skalar 40, Skalar Analytical BV, Breda, NL).

Statistical analysis
As the treatment 'sod cutting' took place in 2005, no vegetation was present in 2005 for this treatment. Therefore, complete sets of vegetation data for all treatments are only

available for 2006, 2007 and 2008. Species richness (the number of vascular plants per plot) was analyzed using a repeated measurements analysis for the years 2006, 2007, and 2008 with 'block' as a random factor and 'treatment' as a fixed factor (GLM, repeated measurements analysis, $P = 0.05$). Subsequently, the species composition between sod-cut plots (with and without mown material) and the other treatments was compared for 2008 data in PC-ORD (McCune & Mefford 1999) with an Indicator Species Analysis (Dufrene & Legendre 1997) to explore significant differences. The development of the species composition within the treatments over the four research years was investigated in a Detrended Correspondence Analysis (DCA). Indirect gradient analysis (DCA) was executed with percentage cover data in CANOCO 4.55 (Ter Braak & Šmilauer 1997-2006).

Soil variables (pH, aluminium, nitrate, ammonium, phosphate) were analysed using repeated measurements analyses for the years 2005, 2006, 2007, and 2008 with 'block' as a random factor and 'treatment' as a fixed factor (GLM, repeated measurements analysis, $P = 0.05$).

Vegetation data together with soil data of 2008 were analysed in a direct gradient analysis (CCA). Significant attributing environmental variables were selected by using the Monte Carlo permutation tests (automatic selection, 499 permutations, $P < 0.05$). Only significant variables are included in the final analysis.

Nomenclature follows Van der Meijden (2005) for vascular plant species and Schaminée et al. (1995-1999) for plant communities.

Results

Species number

A total of 127 species of vascular plants were found in the experimental plots. The hay transfer has resulted in the settlement of a range of plant species not previously present at the site, including characteristic *Nardo-Galion* species.

The number of vascular plant species per plot showed significant differences between the treatments in both sites (Figure 6.2, Table 6.2). On the Verlengde Bemelerberg, the average number of species in the sod-cut plots which had received mown material was 24, and therefore significantly higher than in the other treatments. Also on the Keerderberg, the largest number of vascular plants (average 29) was found in the sod-cut plots where mown material was added. The sod-cut plots without added mown material showed a higher species richness (average 24) than all remaining plots, whereas the unfenced control plot showed the smallest number of species (average 17).

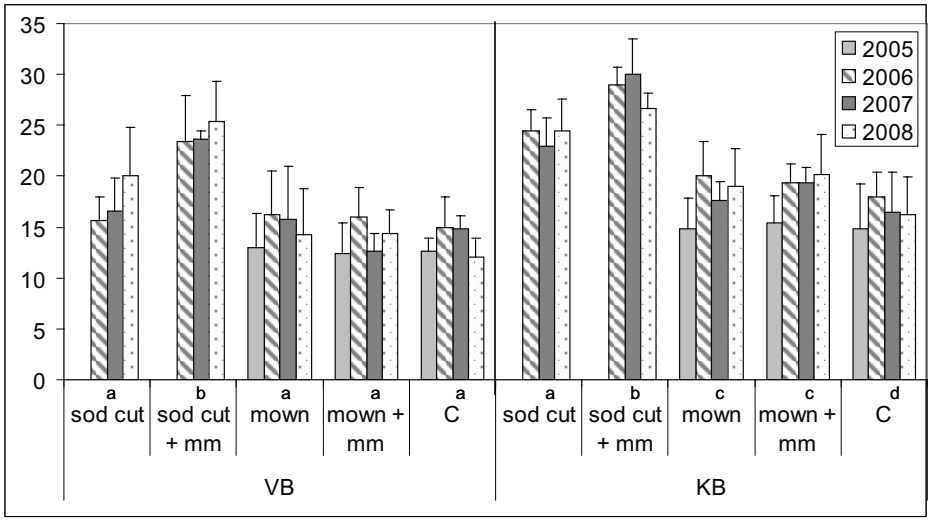

Figure 6.2. Average species richness (vascular plants) per plot (mean ± standard deviation) of the different treatments from 2005 to 2008 at both sites: Verlengde Bemelerberg (VB) and Keerderberg (KB). The four treatments were: (1) sod cut = sod cutting; (2) sod cut + mm = sod cutting and hay transfer; (3) mown = mowing twice a year; (4) mown + mm = mowing twice a year and hay transfer; (5) C = control. The different letters indicate significant different groups (GLM, repeated measurements analysis, P = 0.05).

Vegetation development

The species composition of the sod-cut plots showed a temporal increase of annual species characteristic for agricultural fields. Due to natural succession towards a stable grassland vegetation, only 50 % of these annuals was still present after three years (data not shown). In total, 27 of the species present in 2008 show significant differences in absence and presence between the sod cut plots and the other treatments (Table 6.3). Of these 27, eight have established from the mown material and these species can be considered to be characteristic for matgrass sward vegetation (Swertz et al. 1996; indicated with * in Table 6.3). Three years after the hay transfer, these eight species were still present and thus seem to have become established. The vegetation composition of the remaining plots (the control as well as both mown plots) remained rather constant over the whole research period (2005-2008).

The DCA revealed strong differences in the development of the species composition for the different treatments. In both sites, the species composition of the sod-cut plots (with and without mown material) was clearly different from that of the remaining treatments and controls. The species composition of the sod cut plots differed the most from that of the control plots in the first year after sod cutting (in 2006). At the Verlengde Bemelerberg,

*Table 6.2. Summary of statistical analyses (GLM, repeated measurement results) for species richness of the different treatments on de Verlengde Bemelerberg (VB) and Keerderberg (KB). All treatments were only analysed for 2006, 2007 and 2008, as the sod cut plots were only monitored from 2006 onwards. For the two sites, the covariance matrices were equal across the treatments, and therefore sphericity could be assumed. Sod cut = sod cutting; sod cut + mm = sod cutting and hay transfer; mown = mowing twice a year; mown + mm = mowing twice a year and hay transfer, and C = control. Significance level: *** = P ≤ 0.001; ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05; n.s. = not significant. Group averages are presented (mean & s.e.) with different letters indicating significant differences (post-hoc Tukey test, P < 0.05).*

	VB	**KB**
Years	2006-2007-2008	2006-2007-2008
N	25	25
Time	F = 0.236, P=0.791 n.s	F = 4.553, P = 0.017 *
Block	F = 4.785, P = 0.041 *	F = 0.825, P = 0.375 n.s.
Treatment	F = 12.680, P = 0.000 ***	F = 48.050, P = 0.000 ***
Time * Block	F = 0.048, P = 0.953 n.s.	F = 4.201, P = 0.022 *
Time * Treatment	F = 3.153, P = 0.008 **	F = 0.799, P = 0.607 n.s.
Treatment		
- Sod cut	17.4 ± 1.176 a	23.9 ± 0.673 a
- Sod cut + mm	24.1 ± 1.176 b	28.5 ± 0.673 b
- Mown	15.4 ± 1.176 a	18.7 ± 0.673 c
- Mown + mm	14.3 ± 1.176 a	19.7 ± 0.673 c
- Controll	13.9 ± 1.176 a	16.9 ± 0.673 d

the species composition between the sod-cut treatments with and without mown material started to diverge in the two following years (Figure 6.3, top picture). The plots that were only sod-cut, slowly started to move back in the direction of the control plots, whereas the sod-cut plots that had received mown material remained more comparable to the mown material sample itself. At the Keerderberg, the plots with and without the hay transfer showed the same developments, although the plots without mown material were slightly more similar to the other treatments (Figure 6.3, below picture). However, the species composition of the mown treatments (with and without mown material) remained similar to the grazed control plots during this 4-yrs experimental period.

Biomass

Annual maximum biomass did show high variation over the years. After four years of mowing no (decreasing) trend can be distinguished in the mown plots. The average biomass that was removed by mowing each year was 440 g m^{-2} (± 130) for the Bemelerberg and 472 g m^{-2} (± 84) from the Keerderberg. These amounts are (still) much higher than in well developed calcareous grassland vegetation (275 g.m^{-2}; Smits et al. 2008).

Soil nutrients

Local variation in soil composition led to differences in pH between the different blocks

Table 6.3. Species that show a significant difference between the sod cut plots and the other treatments in 2008 (Indicator species analysis, including 1000 permutations in a Monte Carlo test, P < 0.05). The data are shown for both sites together and for all treatments: S = sod cutting; Sm = sod cutting and hay transfer; M = mowing twice a year; Mm = mowing twice a year and hay transfer; C = control. For every species the presence (average of the ten replicates in percentage) and median coverage (in superscript, Braun-Blanquet codes) are shown. With an asterisk (*), species from the hay that are characteristic for nutrient-poor grasslands on slopes are indicated.

	Group No.	**S**		**Sm**		**M**		**Mm**		**C**	
	No. of relevees	**10**		**10**		**10**		**10**		**10**	
*	*Brachypodium pinnatum*	.		60	+	.		.		.	
	Bromus hordeaceus	80	+	80	+	40	+	60	+	40	+
*	*Campanula rotundifolia*	.		90	+	.		10	+	.	
	Cerastium fontanum	90	1	100	+	90	+	70	+	40	+
	Cerastium glomeratum	20	+	30	+	.		10	+	.	
	Conyza canadensis	20	+	30	+	10	+	.		.	
	Crepis capillaris	90	2	90	1	20	+	60	+	40	+
	Dactylis glomerata	80	+	50	1	80	1	90	+	100	2
	Daucus carota	80	+	80	+	40	+	10	1	20	r
	Epilobium obscurum	20	r	40	+	.		10	+	.	
*	*Festuca filiformis*	10	r	100	1	.		.		.	
	Fraxinus excelsior	70	+	70	+	20	+	40	+	20	r
*	*Hieracium pilosella*	.		50	1	.		.		.	
*	*Hieracium umbellatum*	.		50	+	.		.		.	
	Holcus lanatus	100	2	100	1	100	3	100	3	100	2
	Hypericum perforatum	20	+	80	+	.		30	+	.	
	Hypochaeris radicata	60	1	90	1	50	+	50	+	.	
*	*Jasione montana*	10	+	30	+	.		.		.	
*	*Luzula campestris*	20	+	80	+	.		10	r	.	
	Plantago lanceolata	40	+	90	+	30	1	50	+	20	+
	Poa pratensis	20	+	.		50	+	50	+	30	1
	Sonchus oleraceus	40	r	20	+	10	+	.		.	
*	*Stachys officinalis*	.		50	+	.		10	+	.	
	Taraxacum species	60	+	60	+	30	+	40	+	10	+
	Trifolium dubium	50	1	40	+	20	+	10	+	10	+
	Trifolium repens	80	2	80	2	50	1	50	1	70	1
	Veronica serpyllifolia	40	+	40	+	10	+	.		.	

at the Bemelerberg. Two of the blocks turned out to be located on more calcium-rich soil, leading to a significantly higher pH in these blocks (Table 6.4). The average pH values for the different blocks are shown for the 2005 data (Kruskall-Wallis, P<0.05). As pH is known to be strongly correlated to other soil variables, 'block' was indeed significantly correlated to the other measured soil variables phosphate, ammonium and aluminium

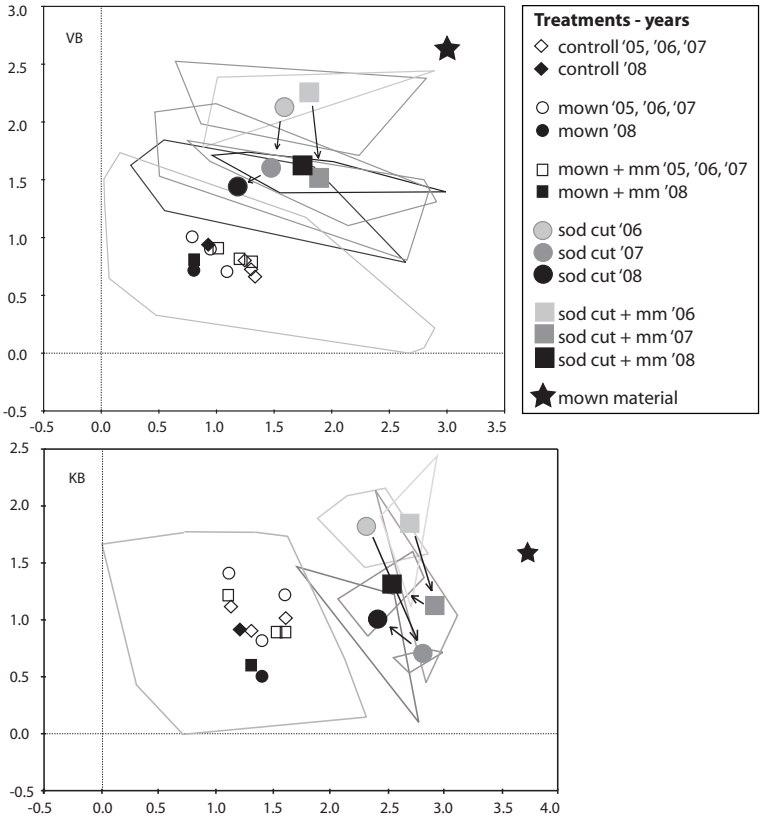

Figure 6.3. DCA of the Verlengde Bemelerberg (VB) data and Keerderberg data (KB) with the centroïds of all treatments for all years (2005-2008). The star symbol indicates the position of the mown material itself, and the lines surrounding the centroïds indicate the variation within the replicates. For the control and mowing treatments one line is drawn, as variation between these treatments and years was minimal. Eigenvalues are for the VB (top picture) 0.641 for the x-ax and 0.310 for the y-ax and for the KB (below picture) 0.463 for the x-ax and 0.250 for the y-ax.

(Pearson, $P < 0.05$). The treatments only had significant effects on nitrate and phosphate, and therefore only for these parameters averages per treatment are shown (Table 6.4). In the first soil monitoring after sod cutting (August 2005), nitrate concentrations were higher in the sod-cut treatments than in the other treatments, although this effect was only significant at the Keerderberg (ANOVA, $P = 0.001$). Nitrate concentrations in the sod-cut treatments were significantly higher just after sod cutting compared to the other treatments, but from half a year onwards significantly lower. Repeated measurements analysis of the data of the whole period (2005-2008; Figure 6.4, water-extractable soil N in nitrate) showed significant differences for the interaction effect (time * treatment). Therefore, only data from 2006 onwards were analysed to calculate group averages

*Table 6.4. Summary of statistical analyses (GLM, repeated measurements analyses) for pH, aluminium, nitrate, ammonium and phosphate concentrations (in mg kg-1 dry soil) of the different treatments for 2005-2006-2007-2008 on de Verlengde Bemelerberg (VB) and Keerderberg (KB). For pH and aluminium, the covariance matrices were equal across the treatments in both sites, and sphericity could be assumed. For nitrate, ammonium and phosphate at both sites, the covariance matrices were not equal across the treatments, and sphericity could not be assumed. In these cases, the greenhouse Geisser epsilon was used. Sod cut = sod cut plots, mm = mown material, Mown = mown plots, Controll = control treatment. Significance level: *** = P ≤ 0.001; ** = 0.001 > P ≤ 0.01; * = 0.01 > P < 0.05; n.s. = not significant. For the post-hoc Tukey test, the group averages (mean and s.e.) are presented with different letters indicating significant differences (P < 0.05).*

	VB	**KB**
pH (KCl data of 2005, 2006, 2007, 2008)		
N	25	25
Time	F = 6.616, P = 0.001 **	F = 0.509, P = 0.678 n.s.
Block	F = 25.753, P < 0.000 ***	F = 0.241, P = 0.629 n.s.
Treatment	F = 0.014, P = 1.00 n.s.	F = 0.490, P = 0.743 n.s.
Time * Block	F = 1.922, P = 0.136 n.s.	F = 0.196, P = 0.899 n.s.
Time * Treatment	F = 0.779, P = 0.669 n.s.	F = 0.876, P = 0.575 n.s.
pH averages (per block, KCl data of 2005)		
- 1	7.49 ± 0.05 a	5.17 ± 0.06 bc
- 2	5.35 ± 0.11 b	5.45 ± 0.05 cd
- 3	7.34 ± 0.18 a	4.95 ± 0.09 ab
- 4	4.27 ± 0.05 c	4.68 ± 0.14 a
- 5	4.03 ± 0.30 c	5.70 ± 0.20 d
Nitrate (water-extractable data of 2005, 2006, 2007, 2008)		
N	25	24
Time	F = 2.007, P = 0.151 n.s.	F = 2.950, P = 0.084 n.s.
Block	F = 0.024, P = 0.879 n.s.	F = 0.716, P = 0.408 n.s.
Treatment	F = 3.716, P = 0.021 *	F = 0.686, P = 0.611 n.s.
Time * Block	F = 2.946, P = 0.068 n.s.	F = 0.606, P = 0.502 n.s.
Time * Treatment	F = 3.398, P = 0.006 **	F = 12.809, P < 0.000 ***
Nitrate averages (N-NO3; waterextractable data of 2006-2007-2008)		
- Sod cut	0.573 ± 0.413 a	0.589 ± 0.412 a
- Sod cut + mm	0.387 ± 0.413 a	0.455 ± 0.368 a
- Mown	2.941 ± 0.413 b	1.952 ± 0.368 ab
- Mown + mm	4.114 ± 0.413 b	2.229 ± 0.368 b
- Control	2.652 ± 0.413 b	1.887 ± 0.368 ab
Phosphate (P-PO4; water-extractable data of 2005, 2006, 2007, 2008)		
N	25	25
Time	F = 2.143, P = 0.136 n.s.	F = 0.848, P = 0.428 n.s.
Block	F = 4.839, P = 0.040 *	F = 1.188, P = 0.289 n.s.
Treatment	F = 1.392, P = 0.274 n.s.	F = 3.958, P = 0.017 *
Time * Block	F = 1.473, P = 0.243 n.s.	F = 0.386, P = 0.664 n.s.
Time * Treatment	F = 1.270, P = 0.293 n.s.	F = 1.169, P = 0.345 n.s.
Phosphate averages (P-PO4, water-extractable data of 2005, 2006, 2007, 2008)		
- Sod cut	0.104 ± 0.264 a	0.193 ± 0.261 a
- Sod cut + mm	0.077 ± 0.264 a	0.169 ± 0.261 a
- Mown	0.355 ± 0.264 a	0.439 ± 0.261 ab
- Mown + mm	0.804 ± 0.264 a	1.267 ± 0.261 c
- Control	0.575 ± 0.264 a	1.112 ± 0.261 bc

Ammonium (N-NH4; KCl data of 2005, 2006, 2007, 2008)		
N	25	25
Time	F = 1.654, P = 0.202 n.s.	F = 1.448, P = 0.249 n.s.
Block	F = 32.717, P = 0.000 ***	F = 4.682, P = 0.043 *
Treatment	F = 2.575, P = 0.071 n.s.	F = 3.378, P = 0.030 *
Time * Block	F = 6.385, P = 0.003 **	F = 1.203, P = 0.307 n.s.
Time * Treatment	F = 1.729, P = 0.115 n.s.	F = 0.720, P = 0.650 n.s.
Aluminium (water-extractable data of 2005, 2006, 2007, 2008)		
N	25	25
Time	F = 1.665, P = 0.185 n.s.	F = 0.209, P = 0.890 n.s.
Block	F = 39.409, P = 0.000 ***	F = 0.036, P = 0.852 n.s.
Treatment	F = 0.180, P = 0.946 n.s.	F = 2.394, P = 0.087 n.s.
Time * Block	F = 2.620, P = 0.059 n.s.	F = 0.221, P = 0.881 n.s.
Time * Treatment	F = 1.512, P = 0.147 n.s.	F = 1.540, P = 0.137 n.s.

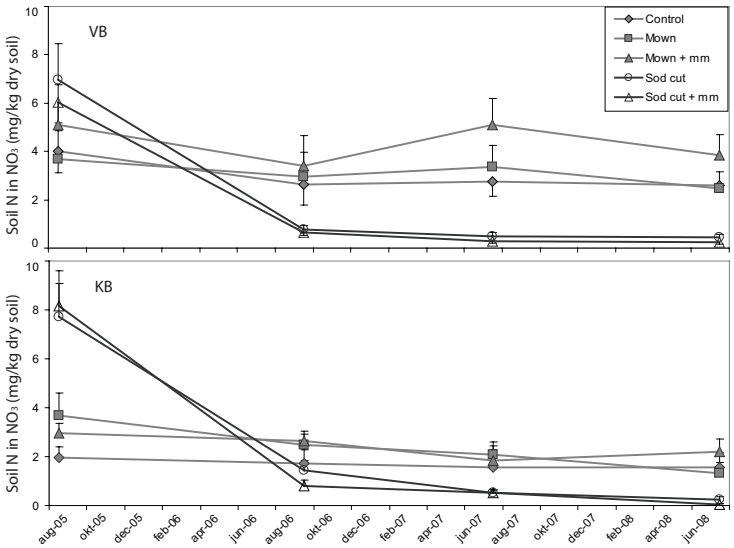

Figure 6.4. Water-extractable nitrate (mg N-NO_3 kg^{-1} dry soil) in the upper 0-10 cm soil in the Verlengde Bemelerberg (VB) and Keerderberg (KB) plots over the four research years (2005-2008).

(Table 6.4). Phosphate concentrations were least in the sod-cut plots. The differences can only be interpreted as a trend, because of the high standard errors (Table 6.4). Soil phosphate data were highest in the upper layer of the mown plots and control plots, and the concentrations measured in the sod-cut plots were comparable to those of the 10-20 cm layer in the mown and control plots. By removing the upper layer, most of the phosphate (over 80 %) was removed (Table 6.4). Ammonium concentrations did not show significant differences in time or treatment (Table 6.4), although significant differences were found between the different blocks at the Verlengde Bemelerberg

(block 1 and 3 contained overall less ammonium, associated with the higher pH). As expected, hay transfer did not attribute to the soil nutrient level, because only a thin layer of harvested material was applied.

Direct gradient analysis

Canonical correspondence analysis of the data of 2008 (both sites) together with the soil variables that contributed significantly (nitrate, ammonium, phosphate and pH) is shown in Figure 6.5. Although the two blocks with higher pH are clearly separated from the other eight blocks, the effects of sod cutting are visible in all blocks. Sod cutting turns out to be negatively correlated with nitrate- and phosphate concentrations, whereas pH and ammonium do not show correlation.

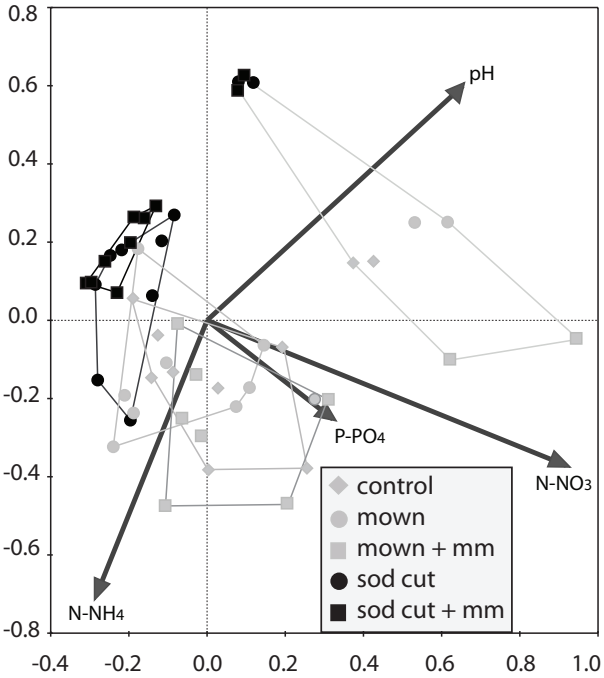

Figure 6.5. CCA of the total dataset of 2008 (both sites) with envelopes around the different treatments. The two blocks with a significant higher pH (block 1 and 3 of the Verlengde Bemelerberg) have been separated, and are located in the envelope in the upper right of the diagram. The significant soil parameters are indicated with arrows. Eigenvalues are 0.317 for the x-ax and 0.194 for the y-ax.

Discussion

Based on similar restoration studies in nutrient-poor fens (Rasran et al. 2007), wet grasslands (Manchester et al. 1999; Hölzel & Otte 2003) and dry calcareous grasslands (Kiehl et al. 2006, Kiehl & Pfadenhauer 2007), we hypothesized that sod cutting in combination with hay transfer (freshly mown material from local, species-rich grasslands) contributes most efficiently to short-term restoration of the investigated species-rich grasslands. Sod cutting did remove most soil nutrients in the current experiment, whereas twice a year mowing (including removal of the hay) did not lead to significant reduction of the soil nutrient concentrations after three years. Continuation of the experiment has to clarify if there is improvement in soil conditions on the long-term, as the effect of mowing with removal of the hay and, to a much lesser content, grazing on soil fertility can only be observed on the longer term (Bakker et al. 2002, Smits et al. 2008, Oelmann et al. 2009).

High local variation in the soil substrate (as indicated by the significant differences in soil pH) led to high variation in the measured soil parameters. Sod cutting did contribute to reduction of the soil nutrient level, although on average the soil remained more rich in phosphate than in nearby nature reserves. It is evident from survey data that soil P is very variable (Walker et al. 2004), and therefore the preliminary measurements, in which the upper 10 cm was found to have the most phosphate, could have been too coarse. More detailed soil P measurements could have been worthwhile to be sure that the layer which contains the most nutrients is removed. Fortunately, soil ammonium accumulation after sod cutting, as was demonstrated in dry heaths and wet heath areas (De Graaf et al. 1995, 1998b, Dorland et al. 2003) did not occur in the more buffered soils (pH 5-6.5) that are subject of the current study. Therefore, restoration of the soil pH is not important in these grassland slopes in South Limburg, as the buffering of the soil is much better than in less buffered and generally more acid soils, due to the clearly higher amount of exchangeable cations (Ca). Nitrate levels increased directly after sod cutting. Disturbance of the soil probably led to temporarily increased mineralization and nitrification, because after half a year nitrate concentrations were significantly lower than in the soils of the plots without topsoil removal. Therefore, sod cutting indeed led to a clear reduction of the nitrate availability. Studies on former arable lands have shown that sowing of later-successional plant species can be more effective on the short-term for grasslands restoration than measures aiming at nutrient removal (Kardol et al. 2008). The results in the current study, however, show that sod cutting was more successful than only adding hay. Topsoil removal comprises, next to nutrient-removal, a complete removal of the current vegetation and creates bare soil. This may be a suitable gap for low-competitive species, like weed species (often present in the soil seed bank due to long-living seed) and the characteristic species of matgrass sward vegetation from the added mown material.

The results of the treatments without hay transfer clearly indicate that biotic constraints also play an important role in the restoration of these degraded grasslands (Verhagen et al. 2001). Extinction effects are less frequent for species with a long-term persistent seedbank (Stöcklin & Fischer 1999), but the characteristic species of these grasslands on slopes (*Nardo-Galion*, *Mesobromion*) in general have short-lived seeds (Knevel et al. 2003, 2005; Kleyer et al. 2008). Therefore, seed addition with autochthonous material seems to be a logical step in the re-creation of these grasslands. Addition of the seed (by hay transfer) clearly had positive effects. Characteristic species became established within the relatively short research period of four years in the sod-cut plots, whereas in the established vegetation the hay transfer did not lead to establishment of the target species at all. Thus, seed dispersal limitations were partly overcome by adding the mown material. It is promising that several species established within a couple of years, but how the development/succession will take place on the longer term is still unknown.

Although seed dispersal by large herbivores can provide a sustainable mechanism for restoration by connecting the source communities with habitat restoration sites, many seeds from highly competitive species are dispersed from high productive to low productive parts of the sites (Mouissie et al. 2005; Kuiters & Huiskes 2009) by endozoochory. Therefore, restoration of the traditional management of these grasslands, including the possibility for dispersal of target species, only effectively works when the abiotic site conditions (nutrient-poor circumstances) are firstly fulfilled.

Local differences in habitat also play a role in the feasibility to achieve similar quality of the areas adjacent to the nature reserves, as the location of the remaining patches of species-rich grasslands are not random. In the current study, the location of the experimental plots are situated on slightly less steep slopes, and therefore more fertile soils, containing loess material. Chances to develop similar vegetation types next to the nearby nature reserves are therefore limited. In the current study, we have shown that the quality of corridors between the biodiversity hotspots can be strongly improved to contribute to a sustainable ecological network. It is, however, possible that the long-term development of the vegetation still will not be fully in the direction of the target communities (Woodcock et al. 2008). However, to enhance plant establishment using freshly mown material in combination with removal of the upper, nutrient-rich soil layer will raise the conservation value. Successful restoration of nutrient-poor grasslands will depend on the established biological diversity, but ultimately on a sustainable functioning ecosystem.

Conclusion

The current experiment clearly showed the opportunities for accelerated restoration of species-rich grasslands on slopes with formerly intensively used agricultural grassland. Removal of the upper, most nutrient-rich layer led to significantly less nutrient-rich (N + P) soil conditions, whereas the combination with of mown material from a well-developed site led to the establishment of characteristic species within a few years. Within the research period, mowing twice a year did not lead to an improvement of the situation.

Provided that source populations are at close range and care is taken that complete plant communities are restored, the re-assembly of entomological biodiversity is a much slower process (Kiehl & Wagner 2006; WallisDeVries & Ens 2008). A future challenge is to obtain more long-term insights in the effects of these restoration measures on the microbial, fungal and (entomo)faunal communities of these grasslands, because of their importance both as functional components of these grasslands and in their role in structuring vegetation composition (De Deyn et al. 2003; Walker et al. 2004; Woodcock et al. 2008). Furthermore, the effects at the landscape scale should be included, to be able to study for example the restoration of the structural heterogeneity (WallisdeVries et al. 2002) and erosion processes.
Thus, in degraded grasslands with a history of intensive agricultural use, sod cutting in combination with hay transfer has led to a significant increase of the establishment of characteristic species of nutrient-poor grasslands in less than five years.

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Chapter 7

Synthesis

The objective of the present study was to achieve a better understanding of the causes of decline and opportunities for restoration of the nutrient-poor, and potentially species-rich grasslands with matgrass sward and calcareous grassland vegetation (*Nardo-Galion* and *Mesobromion*) in Southern Limburg. This thesis focuses on the past and current composition of the grassland vegetation and on measures to restore the vegetation (species composition and species richness) towards well-known plant community references. An important aim was also to generate a better understanding of the causes of the deterioration of these vegetation types, with special attention to potential soil nitrification processes along the grassland gradient, especially in matgrass sward vegetation. Within the framework of the comprehensive O+BN research program, restoration of the (entomo)faunal aspects of these grasslands was studied alongside this project, and preliminary, integrated results have been published in Smits et al. (2009a).

The research on vegetation development and soil processes was centered around three main themes: (1) past and present status, (2) constraints for restoration, and (3) restoration opportunities. For each of these themes specific research questions were raised in chapter 1. The current chapter evaluates and integrates the results discussed in the previous chapters in the context of the main objectives of this thesis. Next to the research questions addressed in the five chapters in this thesis, some more subjects were explored in the four year O+BN project. Some of the results of these additional, and partly still ongoing experiments are also presented in the current chapter.

Past and present status

Up to the beginning of the twentieth century, the South Limburgian grasslands on slopes had an important function as common grazing lands, mainly for sheep. Nutrient removal was achieved by grazing the grasslands during the day and housing the sheep overnight to be able to collect the sheep dung to use it on the arable fields on the plateaus. Due to several changes in land use (Willems 2001; Poschlod et al. 2005), many of the grassland slopes became abandoned and their vegetation developed towards forest by natural succession. This, in combination with the increased atmospheric nitrogen deposition, led to severe habitat loss and degeneration of the floristic and faunal quality together with a decline in the spatial coherence of the remaining habitat patches. Even the (few) locations where a management of mowing and/or grazing was maintained, showed a declining species richness. Restoration opportunities for the calcareous grasslands were explored from the beginning of the 1970's (Schaminée & Hennekens 1982; Hillegers 1985, Willems 2001, WallisdeVries et al. 2002). Reintroduction of sheep grazing started around 1980 (Hillegers 1993) and the effects of nutrient enrichment in calcareous grasslands (in relation to the previous abandonment and the increased atmospheric nitrogen deposition) were investigated by Bobbink & Willems (Bobbink 1991, Bobbink & Willems 1987,

1988, 1991, 1993). Monitoring the long-term development of the calcareous grassland vegetation, however, shows that full restoration (recovery of the target vegetation type) has not been accomplished. Since the start of restoration management (around 1980) in calcareous grassland, changes in the species composition have been indicative of more nutrient-rich circumstances. The current species composition is different from the situation in the reference period (1931-1970): the 16 species that significantly decreased are all considered indicative of relatively dry, nutrient-poor and open habitats, whereas the 16 increasing species are all indicative of more common grasslands, and five of the 16 increasing species were even woody species (Chapter 2). Long-term monitoring of the experimental permanent plots indicates that long-term after-effects are found for fertilisation with phosphorus. Even 25 years after the fertilisation has been terminated, the current species composition is still different with less characteristic calcareous grassland species (Chapter 5).

Distribution data of matgrass sward species (Chapter 1) show a strong decline of the characteristic plant species in the twentieth century. As the availability of historical vegetation data for this grassland type is limited, Smits et al. (2009a) compared Dutch matgrass swards in Southern Limburg to well-developed sites in Belgium and Germany, including the type location of *Betonico-Brachypodietum* (Willems 1982a). The species richness of the Dutch sites was significantly lower than the reference sites, with on average 25 and 37 species, respectively. Comparison of the soil nutrient concentrations revealed on average more than 10 times higher nitrate concentrations in the Dutch sites than in the foreign sites. The results show that lower species richness of the Dutch matgrass sward vegetation is related to higher nitrogen availability (Smits et al. 2009a).

Additionally, a comparison was made of vegetation relevés and soil nutrient measurements collected along an altitudinal gradient in the Bemelerberg reserve (acid grassland, matgrass sward and calcareous grassland) at two moments in time. Data collected in 2005 were compared to data from 1977, which is just before the re-introduction of sheep grazing that had ceased around 1930-1940. Although the same vegetation types were present in both datasets, several changes were observed at the species level. No new species were found in 2005 compared to 1977, whereas some characteristic calcareous grassland species had disappeared from the transect. Soil nutrient measurements, as well as Ellenberg indicator values, demonstrated that nutrient enrichment had taken place from 1977 to 2005 (Smits et al. 2007, Smits et al. 2009a). Thus, the restoration management that was started in 1977, has not led to the aimed recovery of the environmental soil conditions and vegetation that had prevailed during the reference period.

When analyzing the differences in past and present status of the grassland slopes, one has to keep in mind that current species diversity can reflect historical landscape patterns to a certain extent, because changes in the vegetation often occur at a slow pace. (Eriksson et

al. 2002; Lindborg & Eriksson 2004; Ozinga et al. 2009). Therefore, it can be misleading to relate current species diversity to present day environmental conditions, landscape properties and management. It is important to be aware of a possible extinction debt when analyzing the consequences of fragmentation and landscape changes (Cousins et al. 2007, Ozinga et al. 2007, but see Adriaens et al. 2006). The investigated grasslands are part of a highly transformed landscape, and especially in highly transformed landscapes (<10% of the original left) Cousins (2009) found a correlation between plant species richness and the current landscape or habitat pattern.

Constraints for restoration

Eutrophication

In chapter 2, it has been demonstrated that long-term changes in calcareous grasslands demonstrate a clear shift in species composition. This shift was confirmed by changes in the Ellenberg indicator values as derived from the vegetation. We found indications that the microclimate turned colder, wetter and darker, indicating overall higher biomass (Chapter 2). The same indications for nutrient enrichment were found in the comparison of the Bemelerberg vegetation and soil data (1977-2005), and for the Dutch matgrass sward vegetation in relation to foreign sites. A similar development was found in British calcareous grasslands (Bennie et al. 2006) and Swiss alps (Peter et al. 2009). As nutrient-poor conditions are essential for these grassland types, the current nutrient enrichment (eutrophication) of these slope grasslands is an essential factor hampering restoration.

This nutrient enrichment originates from four possible, not mutually exclusive sources. First of all, the leaching from adjacent uphill intensive agricultural fields (Hillegers 1985); secondly, the use of fertilizer in the past on the grassland site itself (Chapter 6); thirdly, the failure of the current management to halt the natural succession, as indicated by the increase of woody species, leading to the accumulation of organic material (Chapter 2). There also seems to be an effect of different management regimes (time, intensity and overnight housing) nowadays and in the past. Finally, an important trigger for the decline of species diversity in nutrient-poor grasslands, like matgrass swards and calcareous grasslands, is considered to be atmospheric nitrogen deposition (Kleijn et al. 2008; De Graaf et al. 2009), which is still exceeding the critical loads. For the future, nitrogen emissions should be reduced further to stop the surpassing of critical loads (Bobbink et al. 1998; Van Dobben & Hinsberg 2008) and thus the continuous negative effect on our species-rich grasslands (Dupré et al. 2009).

Soil nitrification processes

Nitrogen availability is known to play an important role in causing the biodiversity losses in European matgrass swards (e.g. Roelofs et al. 1996; Bobbink et al. 1998; Roem &

Berendse 2002). Therefore, the phenomena of eutrophication, and the known strong effect of nutrient availability on plant growth and vegetation composition, led to measurements of nitrogen (nitrate and ammonium) in the soil over the research gradient. The observed high ammonium:nitrate ratio found in the matgrass sward vegetation (Chapter 1, Table 1) led to the hypothesis that ammonium could have a negative effect on the characteristic plant species (Britto & Kronzucker 2002; Kleijn et al. 2008), and therefore could be a bottleneck for restoration. This sensitivity to high soil ammonium concentrations of characteristic plant species, especially in combination with low soil pH, had already been demonstrated in several studies (De Graaf et al. 1998a; Lucassen et al. 2002; Dorland et al. 2003; Van den Berg et al. 2005). It was hypothesized that in the matgrass sward part of the vegetation gradient the process of nitrification is inhibited. Repression of nitrification by plant exudates from characteristic species of the matgrass sward vegetation could be an adaption to the former nutrient-poor circumstances of these grasslands to conserve and use N efficiently. By repression of the nitrification activity, the inorganic nitrogen remains in the less mobile form of ammonium which prevents it from leaching as nitrate (Vitousek et al. 2002; Herrmann et al. 2005).

This hypothesis was tested with measurements of potential ammonia-oxidising activities (PAA), first in soils directly taken from the field, and later also in soils from the field that were used in a growth experiment in the greenhouse. Significant lower potential nitrification activity was found in soils from the matgrass sward vegetation compared to acid grasslands and calcareous grassland (Chapter 3). In contrast to the differences in potential nitrification activities, no differences in dominant ammonia-oxidising microorganisms were observed based on 16SRNA sequence analysis. These results implied that nitrification processes are strongly hampered in the matgrass sward soil and that this inhibition has resulted in the high ammonium:nitrate ratio in these soils. The cause of the inhibition of nitrification was further investigated in a subsequent growth experiment in the greenhouse (Chapter 4). Matgrass sward plant species turned out to influence the nitrification activity, at least in calcareous soils. Up to now, the exudation of Biological Nitrification Inhibitors (BNI) have been demonstrated in several crop plant species (Subbarao et al. 2007a; 2007b, Zakir et al. 2008; Subbarao et al. 2009) and in *Hyparrhenia* species dominated savannah (Lata et al. 1999; 2000; 2004). The discovery of repression by a set of species of the same vegetation, indicating a mutual strategy for a specific vegetation, is new. More research is needed to confirm our hypothesis that a negative plant-soil feedback mechanism exists, leading to a further decline of this vegetation type. The found repressed nitrification in matgrass sward vegetation could lead to extra stress for this grassland community, as in the current era about 70% of the atmospheric nitrogen deposition exists of reduced nitrogen.

Fragmentation and isolation

The main process of fragmentation of calcareous grasslands in the Netherlands already took place before the period studied in Chapter 2, whereas the process of isolation of

the remaining grasslands strongly increased in the last century (Hillegers 1993). Due to mainly agricultural intensification of the surrounding landscape, characteristic species of these grasslands on slopes became more and more limited to the remaining reserves. Literature on fragmentation and isolation revealed a strong negative relationship with species richness caused by increased risk of extinctions (Fischer & Stocklin 1997) and decreased colonization opportunities (Joshi et al. 2006; Krauss et al. 2004). As a strong relationship between area and total species richness exists (theory of island biogeography, e.g. Stöcklin & Fischer 1999; Joshi et al. 2006), enlargement of the remaining grassland reserves and increase of their connectivity are of main importance. Ozinga et al. (2009) found that the magnitude of the effect of reducing connectivity is comparable to that of the eutrophication effect in explaining plant diversity losses in NW Europe. The development of local and regional biodiversity through connecting ecological corridors is still an important and actual topic in restoration ecology, for which more study is needed. Within the current project, no direct research was carried out about the negative effects of fragmentation and isolation on restoration success. However, it is clear that the opportunity for species to reach appropriate habitats is crucial, because most of the characteristic species in the studied grasslands (matgrass sward vegetation and calcareous grassland) have a transient or short-term persistent seed bank (Hutchings & Booth 1996; Knevel et al. 2003, 2005; Kleyer et al. 2008). Studies on calcareous grassland species by Stocklin and Fischer confirmed that species with higher habitat specificity tended to have shorter-lived seeds than species with lower habitat specificity. As most characteristic calcareous grassland species do not persist in the seed bank, the seed bank provides no buffer against local extinction (Stöcklin & Fischer 1999). Moreover, germination experiments with matgrass sward species have revealed that almost no germination and no establishment take place, indicating that the abiotic conditions are probably not suitable (yet) (Smits et al. 2009a).

Traditional farming practices resulted in massive seed dispersal through transports in sheep herds and mowing devices (Poschlod & Bonn 1998), whereas currently these important potential vectors for seed dispersal are strongly reduced. Grazing practices and the accompanying widespread transport of grassland species stopped, due to the changed agricultural land use (Fischer et al. 1996; Ozinga et al. 2009; Wallin et al. 2009). Local seed sources of the target species have often become limited through habitat loss and fragmentation (Poschlod et al. 1998; Verhagen et al. 2001), as confirmed in seed addition experiments (Turnbull et al. 2000; Franzen & Eriksson 2003). Studies in mesotrophic hay meadows revealed a strong relationship between the chances for species to be present and the frequency of distribution in the near (<3 km) surroundings (Ozinga et al. 2005), stressing the importance of dispersal limitations for species richness. Furthermore, grazing can have a mitigating effect on the genetic consequences of habitat fragmentation by seed exchange (e.g. Vergeer et al. 2003, Honnay et al. 2006). In Chapter 2, we found that species without adaptation to long-distance dispersal (e.g.

having plumes) decreased, indicating that these species are no longer able to reach or recolonize the remaining, isolated and fragmented habitats in the current landscape.

In the small, fragmented and isolated remaining sites, long-distance seed dispersal (LDD) by sheep might contribute to successful restoration of these grasslands. In our long-term dataset of calcareous grassland relevés (Chapter 2), however, we did not find an increase in species with LDD after restoration management (with grazing by sheep) was re-introduced. Hence, the current grazing management does not effectively contribute to restoration success by dispersal of seed. Endozochorous seed dispersal by sheep in these grasslands was studied by Kuiters & Huiskes (2009). They found high densities of *Urtica dioica*, although this species was not very common in the vegetation itself. Moreover, 43% of the species in the local species pool germinated from the dung sample, suggesting a high potential for sheep as a dispersal vector (Kuiters & Huiskes 2009). However, the available seeds of target species were only present in small quantities. They also found a positive correlation between seed dispersal capability of plant species and Ellenberg indicator value for nutrients, suggesting that species from nutrient-rich soils have a higher probability to disperse their seeds through sheep dung. Both Moussie et al. (2005) and Kuiters & Huiskes (2009) suggest that this often leads to the transport of nutrient-rich species to nutrient-poor sites.

Restoration opportunities

Restoration within existing nature reserves

On the short term, cutting the vegetation of relatively well-developed calcareous grasslands in mid-summer with removal of the mown material did result in a decrease of the grass *Brachypodium pinnatum* and an increase in species richness (Bobbink & Willems 1991). After abandonment, cutting twice a year proved to be successful to reduce the negative effects of a thick litter layer and dominance of *Brachypodium pinnatum* (Bobbink & Willems 1993). On the longer term, continuation or re-instalment of the traditional management (mowing/grazing), however, did not lead to full restoration (Bobbink & Willems 2001; Smits et al. 2007), i.e. the aimed nutrient-poor conditions were not established (Chapter 2). Abandonment has led to accumulation of organic material, and therefore nutrient enrichment. Moreover, the increased nutrient input by air causes mowing and especially grazing to be much less effective in nutrient removal than in the past (Bakker & Olff 1995). Therefore, more intensive management is necessary to counterbalance the effects of nutrient enrichment due to the past abandonment, agricultural fertilisation and/or atmospheric nitrogen deposition. The current management of relatively intensive late autumn grazing and too extensive (early) summer grazing turned out to be also bottlenecks for the life cycle of different entomofauna groups within the O+BN research (Smits et al. 2009a).

Due to the steepness of the slopes, grazing is often the most appropriate management measure, and it can also be preferred over mowing, because it produces a more heterogeneous structure across the whole terrain with mosaic patterns of tall and short stands. The success of grazing is highly dependent on the way it is implemented. As nutrient removal is the aim, effective removal of the eaten biomass should be accomplished, for instance by taking out the sheep during the night to remove nutrients. Management which leads to a heterogeneous, structure-rich grassland is important for the conservation of the entomofauna diversity. Some differentiation in management within one reserve is therefore necessary. This is an important option to consider while intensifying the management focussing on restoration of plant diversity (Smits et al. 2009a). Mowing cannot be considered everywhere as an appropriate management measure to remove nutrients, because of the situation of the grassland slopes. If mowing is applicable, in addition to the right timing of the cutting, quick and effective removal of the harvest is also essential, because nutrients from the mown material will leach and to return to the soil within one or two weeks (Schaffers et al. 1998).

In Chapter 5 the long-term after-effects of fertilisation were investigated in calcareous grasslands. Nitrogen fertilisation was effectively compensated for by a harvesting management in August within ten years. Phosphate fertilisation effects are still visible in the vegetation (a decreased number of characteristic species) plant composition and soil 25 years after the last fertilizer treatment took place. Restoration of degraded, previously fertilized grasslands is a complicated long-term process with much better prospects in the case of mainly nitrogen fertilizer use, compared to phosphorus. The effects of nutrient removal by mowing were also investigated in formerly abandoned grasslands on slopes. Due to natural succession, forest had developed. For restoration purposes, parts of the Schiepersberg complex were first clear-cut and then subjected to a harvesting regime. Four years of mowing twice a year, including removal of the mown material, did not result in an increase in species richness or a decrease in soil nutrient content, although vegetation structure did improve towards a more open, and less productive sward. Continuation of the experiment has to clarify if a decline in the nutrient status will be reached on the longer term (Smits et al. 2009a). After-effects of the past abandonment were also studied by Jeschke et al. (2008) who confirmed that five years of mowing was not sufficient to compensate for the effects of abandonment in terms of restored species richness and the number of characteristic plant species).

Restoration on former agricultural land

For restoration of nutrient-poor grassland on intensively used agricultural grasslands, the removal of the nutrient overdose is even more crucial to return to the necessary, more nutrient-poor soil conditions. Removal of the nutrient overdose is even more important when considering the creation of these nutrient-poor grassland systems on

former intensively used agricultural grasslands. Prolonged grazing by sheep without further fertilizer use in these previously heavily fertilized grasslands did not lead to any improvement (Chapter 6). Nutrient removal by sod cutting and subsequent transfer of freshly mown material from species-rich sites turned out to be the most successful combination of measures to restore *Nardo-Galion* communities (Chapter 6). The implementation of a twice-a-year mowing regime did not lead to any restoration within the four-year research period, and the combination of the mowing regime with the transfer of freshly mown material did not have any effect on species richness. A former intensively used grassland site on calcareous substrate was selected to investigate the re-creation of calcareous grassland. After four years, four species seem to profit from the experimental mowing regime (*Crepis biennis, Plantago media, Scabiosa columbaria, Carlina vulgaris*), of which the three last ones are characteristic calcareous grassland species. Twice a year mowing already had a significant decreasing effect on the maximum standing crop in 2008. Continuation of the experiment has to clarify whether there is more improvement in soil conditions on the long-term and how effective seed dispersal from the nearby source takes place (Smits et al. 2009a).

Effective dispersal of species

In order to get insight into the effectiveness of seed dispersal by sheep and transfer of mown material, we compared the seed contents of sheep dung and mown material from a well-developed reference site in Belgium (Tiendeberg). Eight samples of 0.50 x 0.50 m mown material were collected and put to germinate in the greenhouse for ten months. In total, about 15.500 seedlings were monitored, of which 3.000 were herbs. 35 species were identified, 18 of them are characteristic for species-rich grasslands. Comparison of these data with the endozoochorous seed dispersal data by sheep (Kuiters & Huiskes 2009) revealed that 500 g fresh dung samples from Tiendeberg contained about 100 viable seeds, whereas on average 50 viable seeds were found in comparable samples from Dutch nature reserves. Eleven species were identified, of which one is considered to be characteristic for these species-rich grasslands. Thus, endozoochorous seed dispersal by sheep is much less effective than dispersal by transfer of freshly mown material. In order to restore species-rich grasslands on former intensively used grassland, hay transfer of mown material can be a useful and relatively quick first step as more (characteristic) species can be dispersed (Chapter 6; Smits et al. 2009a).

Conclusion

The results of this thesis, and additionally the results of the O+BN research, clearly show that the current situation in these grasslands on slopes is far from optimal. Due to nutrient enrichment, common species have increased, whereas characteristic species have decreased. Full restoration was not accomplished in the calcareous grassland

zone, whereas the floristic quality of the matgrass sward has decreased even further. Furthermore, isolation between the remaining small size grassland reserves has led to negative effects on species richness and current management does not actively attribute to dispersal of characteristic species. Soil nitrification processes are repressed in matgrass swards, leading to a high ammonium:nitrate ratio. Characteristic plant species are known to be sensitive to high soil ammonium concentrations, indicating a negative plant-soil feedback, possibly leading to further decline of the matgrass sward vegetation. Additional research on the found repressed nitrification should reveal if this negative effect on the biodiversity in these grasslands exists and how this can be counterbalanced. Two approaches are needed to ensure optimal conservation and restoration. Firstly, the management in the reserves should be optimalized to ensure more nutrient removal. This should be combined with a more heterogeneous vegetation structure throughout the year that is required especially by the (entomo)fauna. Secondly, the dispersal opportunities for species should be increased by enlarging and connecting the remaining grasslands, therefore reducing fragmentation and isolation of these grasslands. To create suitable corridors on grasslands with a history of intensive agricultural use, sod-cutting, in combination with the transfer of freshly mown material is found to be a useful first step towards restoration of species-rich grassland.

Perspectives for the future

Restoring matgrass sward vegetation and calcareous grassland on slopes in Southern Limburg should focus on nutrient removal, as these grasslands depend on low nutrient availability. The (restoration) management of the last 25-30 years has not led to full recovery of the biodiversity in these grasslands. In order to improve the current situation, management in the reserves should be optimized to achieve more nutrient removal. Currently, a follow-up of the O+BN research is initiated, in which the exact design of such management will be studied. Except for nutrient removal, more insight in the soil nitrogen processes is needed, as the found high ammonium to nitrate ratio in the matgrass sward vegetation is associated with the decline of matgrass sward species. The results of this thesis indicate that matgrass sward vegetation itself represses nitrification, but exact knowledge how this process works and how the negative impact on the recovery of vegetation can be overcome should be subject of further research. In addition to nutrient removal, dispersal opportunities of (target) species is a key issue for restoration. To enhance the connectivity between the remaining grasslands, new corridors have to be created and restoration management is necessary to establish appropriate habitat in these areas that often have a history of intensive agricultural use. Although the first results of restoration management seem fairly successful for the establishment of the target species, further monitoring is needed to get an insight in the long-term development in these habitats.

Summary

The grasslands on slopes in Southern Limburg belong to the most species-rich grasslands in the Netherlands. They are located on slopes with a clear gradient in soil material with gravel deposits from the river Maas on top of the calcareous bedrock (especially in the western part of Limburg). The grasslands on these slopes often include a complex of vegetation types, with acid grasslands (*Thero-Airion*) on the acid gravel deposits, calcareous grasslands (*Mesobromion erecti*) on calcareous outcrops, and matgrass swards (*Nardo-Galion saxatilis)* in between. At the foot of slopes, on colluvial soil, more nutrient-rich grasslands or forbs (*Arrhenatherion elatioris/Arction)* may occur. Due to the high plant biodiversity, nature reserves with these vegetation types are integrated in the EU Natura 2000 network, based on the occurrence of matgrass swards and calcareous grasslands which are included in the EU Habitat Directive as protected habitat types.
In the 20th century, the number and area of these grasslands decreased due to changing land use and the small, remaining reserves became fragmented, isolated, and lost characteristic species. Around 1980 restoration management started. Sheep grazing was reintroduced and slopes with forest encroachment were cleared. Extra management measures included mowing, including the removal of the mown material and removing woody species. In the first years, restoration seemed successful with a clear increase in species richness, but on the long-term however, the floristical quality and species-richness have declined even further. To create a sustainable habitat with viable plant populations, it is necessary to enlarge the small remaining grassland reserves and increase the connectivity between them. Therefore, there is an increasing need for appropriate measures to re-create species-rich corridors between reserves.

Further insight into the causes of this decline and opportunities for restoration of these grassland slopes were the main aims of a four-year O+BN research project. In this project, vegetation, soil and faunal aspects of these grassland were studied and the findings of the research were published in a Dutch research report in 2009. The current thesis, in which specific parts of the broader O+BN project are described in five scientific publications, is derived from this research.

Analyses of the long-term vegetation development in calcareous grassland (Chapter 2) showed a consistent shift in species composition in well-developed Dutch calcareous grasslands during the second half of the 20th century. Distinct calcareous grassland species declined and more common mesotrophic/eutrophic grassland species increased. Generally, the vegetation has developed towards more nutrient-rich circumstances. Besides the vegetation composition, eutrophication was also observed in a comparison of soil data from 1977 and 2005 (Chapter 7). An analysis of the distribution of ten characteristic matgras sward species revealed a strong decrease in the last century (Chapter 1). Compared to foreign references, the matgrass sward vegetation in the Netherlands is less species-rich and more nutrient-rich (Chapter 7).

Constraints for restoration

Eutrophication is one of the main factors hampering restoration, as nutrient-poor conditions are required for the target grassland vegetation. There are four main causes for nutrient enrichment in these grassland slopes. First of all, there are after-effects of previous fertilisation (Chapter 5) in some of these grassland reserves. Secondly, due to the location of the grasslands on slopes, there is high run-off of fertilizer from the intensively agricultural used land on the plateaus. Thirdly, atmospheric nitrogen deposition has gradually enriched these sites. Although the atmospheric nitrogen deposition has been reduced since the 1990's, current levels still exceed the critical loads. Moreover, within the last ten years, no further reduction was accomplished. Finally, the current management is not sufficiently intensive: derived parameters indicated more nutrient-rich, and therefore a more moist, cooler and darker microclimate (Chapter 2). Moreover, woody species have increased, indicating that the current management fails to maintain an open grassland vegetation structure.

Nitrogen is one of the most important limiting nutrients for terrestrial plant growth, especially under nutrient-poor circumstances. Most of the characteristic species are adapted to these nutrient-poor soil conditions and can survive and compete successfully with other species. Measurements of available soil nitrogen (extractable nitrate and ammonium) showed high ammonium:nitrate ratios in the matgrass sward soil, an indication for lower nitrification activity in this part of the gradient. Experimental studies indeed confirmed repressed potential nitrification activity in the matgrass sward vegetation, compared to the two adjacent vegetation zones (Chapter 3) and a four-month growth experiment revealed a negative effect of characteristic matgrass sward species on potential nitrification activity (Chapter 4). High ammonium concentrations and high ammonium:nitrate ratio are known to have toxic effects on the characteristic plant species, and therefore this can have contributed to the decline of this species-rich grassland vegetation.

The grasslands studied in the current research are geographically limited, as specific site conditions (gravel deposits on top of calcareous soils) are required. Therefore, both fragmentation and isolation seems to other important bottlenecks for flora and (entomo) fauna. The exchange of species between the remaining, isolated grasslands is for most of the vascular plant species nearly impossible, whereas the soil seed bank of the characteristic plant species is predominantly short-lived. Therefore, species that have disappeared from a certain site are not able to return spontaneously. In the traditional land use (up to the beginning of the twentieth century), dispersal of plant species was widespread due to migrating sheep herds, hay transport and other small-scale transports connected to the land-use system of that time. The results from the long-term vegetation change in chapter 2 show that species without adaptation to long distance dispersal

(e.g. wind, mammals and birds) have decreased, indicating that those species are no longer able to disperse between the small and isolated reserves. Moreover, current (grazing) management has not led to an increase in species with adaptations for ecto- and endozoochorous seed dispesal, e.g. the restoration management with sheep grazing did not result in an effective increase of species with those adaptations. Germination experiments with matgrass sward species have revealed that almost no germination and no establishment take place, indicating that the abiotic conditions are probably not suitable (yet).

Restoration opportunities

Restoration management for the accelerated re-creation of species-rich grasslands was studied on agricultural grasslands with a history of intensive use in an experimental design (Chapter 6) and on grasslands that have been abandoned for a long time (Chapter 7). The combination of sod cutting with hay transfer (the transfer of freshly mown material from a nearby well-developed grassland) was the most successful measure. On the short term, mainly species from pioneer communities (e.g. arable fields) established after top soil removal of the most nutrient-rich upper 10 cm. Half of these species had disappeared after four years due to natural succession. At the same time, a number of characteristic species from the transferred hay were able to establish. The combination of hay transfer with twice a year mowing (without sod-cutting) did not result in raised species richness. Nutrient removal was neither accomplished on agricultural grasslands with a history of intensive use nor on previously abandoned grasslands in the treatment of twice a year mowing, including removal of the harvest. This is in agreement with earlier published studies, which showed that results of nutrient removal by mowing are only visible on the longer term (5-10 years). In calcareous grasslands that had been fertilized in the past, the development on the longer term (25 years after the fertilisation has stopped) showed that the effects of nitrogen application have disappeared after 10 years, whereas the effects of phosphate application are still visible (Chapter 5). Although the species richness of the P-fertilized plots is comparable to the control plots, the composition is still different with less characteristic calcareous grassland species.

To obtain an insight in the effectiveness of seed dispersal through sheep dung and mown material, a comparison was made between data of the same grassland slope (Chapter 7). Endozochorous seed dispersal by sheep works potentially for many characteristic plant species. Sheep dung collected at this slope (the Tiendeberg) contained the seeds of eleven species, of which only one was characteristic for species-rich grassland on slopes. Mown material contained 35 species, of which 18 were characteristic. On the short-term, transfer of mown material can be an effective first step towards restoration or re-creation of species-rich grasslands. Many seeds in the sheep dung belonged to more

common species, indicating more nutrient-rich habitats. Under the current, eutrophicated circumstances, it is important to prevent transport of common species towards the well-developed sites, and this should be taken into account in the grazing scheme.

Concluding

The results of this thesis and additional results of the O+BN research show that two approaches are needed to ensure optimal conservation and restoration. Firstly, the management in the reserves should be optimalized to ensure more nutrient removal together with a more heterogeneous vegetation structure throughout the year. This more heterogeneous vegetation structure is required especially by the (entomo)fauna. Secondly, the dispersal opportunities for species should be increased by enlarging and connecting the remaining grasslands, therefore reducing fragmentation and isolation of these grasslands. On the short term, sod-cutting in combination with the transfer of mown material seems to be a useful first step towards restoration of species-rich grasslands on former agriculturally used grasslands. Additional research on the found repressed nitrification should reveal if this effect has a negative effect on the biodiversity in these grasslands and how this can be counterbalanced.

Samenvatting

De hellingschraallanden in Zuid-Limburg behoren tot de meest soortenrijke graslandtypen van ons land. Deze graslanden liggen doorgaans op een gradiënt van verschillende moedermaterialen, met zure kiezelkopgraslanden (*Thero-Airion*) en heischrale graslanden (*Nardo-Galion*) in het bovenste deel van de helling. In het middendeel van de hellingen, op plekken waar kalkgesteente dagzoomt, komen kalkgraslanden (*Mesobromion erecti*) voor. Onderaan de helling, op colluviaal materiaal, bevinden zich matig voedselrijke graslanden of ruigten (*Arrhenatherion elatioris/Arction*). Vanwege de hoge biodiversiteit zijn zowel de heischrale graslanden als de kalkgraslanden opgenomen in de Europese natuurbescherming (Natura 2000). Het areaal van deze graslanden is in de loop van de 20^{e} eeuw sterk gekrompen en versnipperd geraakt door veranderd landgebruik. De overgebleven reservaten zijn sterk in kwaliteit achteruitgegaan: veel kenmerkende planten- en diersoorten zijn verdwenen. Omstreeks 1980 werd op veel plaatsen herstelbeheer in gang gezet. Hellingen die met bos waren dichtgegroeid, werden van opslag ontdaan en graslanden werden weer beheerd. Begrazing met schapen werd opnieuw ingesteld, waarbij gebruik werd gemaakt van het traditionele schapenras (Mergellander). Daarnaast werd door geregeld kappen van de houtige opslag en in een aantal reservaten door middel van hooien, de extra toevoer van voedingsstoffen vanuit de lucht (atmosferische stikstof depositie) en bemesting vanuit belendende landbouwpercelen teruggedrongen. In de eerste jaren leek dit herstelbeheer behoorlijk succesvol: de dominantie van het gras Gevinde kortsteel nam af en de soortenrijkdom toe. Twintig jaar later moest echter worden geconstateerd dat een volledig herstel van het kalkgrasland niet heeft plaatsgevonden en dat het areaal heischraal grasland in veel terreinen zelfs verder achteruit is gegaan.

De belangrijkste oorzaken voor de geconstateerde ecologische achteruitgang en de mogelijkheden voor herstel van dergelijke graslanden werden onderwerp van een vierjarig onderzoek in het kader van O+BN (kennisnetwerk Ontwikkeling + Beheer Natuurkwaliteit, van het ministerie van Landbouw, Natuur en Voedselkwaliteit). Dit proefschrift, waarin een vijftal onderwerpen als wetenschappelijk artikel zijn uitgewerkt, is afgeleid van het O+BN onderzoek.

Analyses van vegetatiegegevens van kalkgrasland over de tijd (Hoofdstuk 2) hebben duidelijk laten zien dat het instellen van de beheermaatregelen omstreeks 1980 vooralsnog niet heeft geleid tot een volledig herstel van de karakteristieke kalkgraslandvegetatie. Karakteristieke soorten zijn zelfs afgenomen, terwijl algemene (deels houtige) soorten zijn toegenomen. De Ellenberg indicatorwaarden geven aan dat de huidige vegetatie hoort bij meer voedselrijke omstandigheden. Dit wordt onderschreven door de resultaten van een gedetailleerde vergelijking van metingen uit 1977 en uit 2005 in het natuurreservaat de Bemelerberg. Ook hier werd aangetoond dat de voedselrijkdom van de bodem is toegenomen, terwijl een afname werd beoogd met het instellen van het traditionele beheer. Het voorkomen van een tiental karakteristieke heischrale soorten is

in detail onderzocht en deze blijken sterk te zijn afgenomen (Hoofdstuk 1). Ook blijkt het heischrale grasland ten opzichte van overeenkomstige gebieden in het buitenland minder soortenrijk, terwijl de bodem in Nederland meer stikstof bevat.

Beperkingen voor herstel

De voedselrijkdom van de bodem is tegenwoordig duidelijk hoger dan tijdens het instellen van herstelbeheer (omstreeks 1980). Hiervoor zijn diverse oorzaken aan te wijzen. Allereerst blijven de effecten van bemesting in het verleden nog lang in de vegetatie en bodem zichtbaar (Hoofdstuk 5, 6). Daarnaast leidt de ligging van de hellingschraallanden in het landschap tot verhoogde kans op inspoeling van voedingsstoffen vanuit de aangrenzende, hoger gelegen plateaus, die veelal in intensief agrarisch gebruik zijn. De atmosferische stikstofdepositie is weliswaar afgenomen, maar overschrijdt tegenwoordig nog steeds de kritische waarden voor heischraal grasland en kalkgrasland. De afgelopen tien jaar is geen verdere afname in stikstofdepositie meer gerealiseerd, waardoor de te hogere stikstofbelasting en overschrijding van de kritische waarden vooralsnog voortduren. Tenslotte draagt het huidige beheer onvoldoende bij aan afvoer van nutriënten. De afgeleide parameters duiden zelfs op een voedselrijker, koeler, vochtiger en donkerder microklimaat dan ten tijde van het ingezette herstel (Hoofdstuk 2). Daarnaast vindt successie plaats in de richting van bos: op de hellingen slaan houtige soorten op, wat betekent dat het beheer niet intensief genoeg is om de grasland structuur te fixeren.

Stikstof is een van de belangrijkste limiterende voedingsstoffen voor plantengroei, zeker onder voedselarme omstandigheden. De meeste karakteristieke soorten zijn dan ook aangepast aan voedselarme condities en kunnen juist hier overleven en succesvol concurreren met andere soorten. Metingen aan de hoeveelheid en vorm waarin stikstof in de bodem voorkomt, duidden op verschillen in nitrificatieactiviteit (het omzetten van ammonium naar nitraat) binnen de onderzochte gradiënt. Uit experimenten kwam achtereenvolgens naar voren dat in het heischrale grasland de potentiële nitrificatieactiviteit sterk geremd is (Hoofdstuk 3). Uit een vervolgexperiment (Hoofdstuk 4) bleken karakteristieke soorten uit de heischrale vegetatie een remmende invloed te hebben op de potentiële nitrificatie. De ammonium-nitraat ratio is door de remming van de nitrificatie verhoogd en een hoge ammonium-nitraat ratio is mogelijk toxisch voor heischrale soorten. Dit proces kan daarom hebben bijgedragen aan de achteruitgang van dit waardevolle grasland.

Zoals eerder opgemerkt zijn de onderzochte graslanden in het landschap geografisch beperkt door de aanwezigheid van hellingen en kalkgesteente in de ondergrond. Hierdoor lijkt voor zowel flora als fauna de hoge mate van versnippering en isolatie van de hellingschraallanden een belangrijk knelpunt te zijn. Uitwisseling tussen reservaten

is voor de meeste karakteristieke plantensoorten niet of nauwelijks mogelijk en van de karakteristieke soorten is bekend dat zij veelal een kortlevende zaadbank hebben. Hierdoor kunnen soorten die eenmaal uit een terrein zijn verdwenen in de huidige situatie niet op eigen kracht terugkomen. Verspreiding van soorten vond in het traditionele landgebruik veel meer plaats dan nu het geval is. Door middel van rondtrekkende, gescheperde schaapskudden (waarbij wegbermen, akkers en andere landschapselementen een cruciale rol in de begrazing speelden), maar ook door hooien en het verslepen van zaden via landbouwapparatuur werden zaden op grote schaal verspreid. Uit de resultaten van Hoofdstuk 2 bleken soorten die geen aanpassingen hebben aan zaden voor verspreiding over lange afstand (door wind, zoogdieren, vogels), te zijn afgenomen. Dit vormt een indicatie dat dergelijke soorten niet langer in staat zijn om de overgebleven, kleine en geïsoleerde habitats te bereiken. De huidige begrazing bleek bovendien niet te hebben geleid tot een toename van soorten die wèl aanpassingen aan zaden voor lange afstand verspreiding door zoogdieren (intern of extern) hebben. Herstel van de schapenbegrazing heeft tot op heden geen meetbaar positief effect gehad op de verspreiding van dergelijke soorten. Kiemingsexperimenten met karakteristieke heischrale soorten lieten zien dat de standplaats op dit moment waarschijnlijk (nog) niet geschikt is. Soorten die inmiddels uit de Nederlandse hellingschraallanden zijn verdwenen, kiemen en vestigen zich helemaal niet na herstelmaatregelen, terwijl de wat algemenere heischrale soorten zich na zaaien slechts mondjesmaat vestigen.

Mogelijkheden voor herstel

Experimenteel is onderzocht welke beheermaatregelen effectief zijn voor het herstel van hellingschraalland vanuit voormalig intensief gebruikt agrarisch grasland (Hoofdstuk 6) of vanuit een verruigde en/of verboste situatie (Hoofdstuk 7). Plaggen van de voedselrijke toplaag in combinatie met het uitleggen van maaisel, afkomstig van een goed ontwikkeld heischraal grasland, gaf de beste resultaten. Van het eenmalig plaggen profiteerden op de korte termijn vooral soorten van pionierbegroeiingen (o.a. akkersoorten), terwijl deze na vier jaar voor een groot deel weer waren verdwenen. Karakteristieke soorten van heischraal grasland, die mee waren gekomen met het maaisel, bleken in staat zich te vestigen in de geplagde proefvlakken. Daarentegen heeft het uitleggen van maaisel in combinatie met tweemaal maaien per jaar (dus zonder plaggen) niet geleid tot een toename van soorten. Tweemaal per jaar maaien, waarbij het maaisel wordt afgevoerd, heeft zowel in de experimenten op voormalig intensief gebruikt agrarisch grasland als op verruigde en/of verboste plekken binnen vier jaar niet tot verlaging van de voedingsstoffen in de biomassa of voedselrijkdom van de bodem geleid. Dit komt overeen met resultaten van overeenkomstig onderzoek elders, waaruit ook naar voren kwam dat effecten van maaien pas zichtbaar worden op de langere termijn. Toekomstig onderzoek zal moeten leren of dit op de Zuid-Limburgse hellingen ook het geval is. In kalkgraslanden die in

het verleden bemest zijn geweest, laat de ontwikkeling op de lange termijn (25 jaar na het stoppen van de bemesting) zien dat de effecten van stikstofbemesting weliswaar na ongeveer tien jaar zijn verdwenen, maar dat bemesting met fosfaat ook dan nog zichtbaar is in de soortensamenstelling (Hoofdstuk 5). Het soortenaantal is inmiddels gelijk aan het soortenaantal van de controle proefvlakken, maar de samenstelling van de soorten is anders met minder karakteristieke kalkgraslandsoorten.

Om inzicht te krijgen in de effectiviteit van verspreiding van zaden via schapenmest en maaisel zijn gegevens van dezelfde soortenrijke helling (Tiendeberg, België) met elkaar vergeleken (Hoofdstuk 7). Onderzoek naar het voorkomen van kiemkrachtige zaden in de mest van schapen heeft duidelijk gemaakt dat schapen zaden van veel karakteristieke soorten via de mest kunnen verspreiden. In verse mestmonsters van de Tiendeberg werden kiemkrachtige zaden van elf soorten aangetroffen, waarvan slechts één karakteristieke soort. In maaisel van dezelfde helling bevonden zich kiemkrachtige zaden van 35 soorten, waarvan 18 karakteristiek voor soortenrijke hellingschraallanden. Om op korte termijn karakteristiek soorten, en daarmee de karakteristieke vegetatie van de hellingschraallanden te herstellen, kan het uitleggen van maaisel uit een goed ontwikkeld grasland een effectieve, eerste stap zijn. Doordat de meeste zaden uit schapenmest bovendien afkomstig bleken van algemene soorten zoals de Grote brandnetel, Kruipend struisgras en Engels raaigras, is het dus erg belangrijk om bij de rotatieschema's rekening te houden dat de schapen voorafgaand aan beweiding van een natuurgrasland, niet worden geweid in grasland met een hoge dichtheid aan triviale en ruderale soorten.

Tot slot

Uit de resultaten van dit proefschrift, aangevuld met gegevens uit het O+BN onderzoek, komt naar voren dat een tweeledige aanpak nodig is voor herstel van de karakteristieke flora én fauna van de hellingschraallanden. Enerzijds dient het beheer binnen de reservaten verbeterd te worden, zodat meer afvoer van nutriënten plaatsvindt. Hier komt bij dat in het bijzonder voor de entomofauna een meer heterogene vegetatiestructuur moet worden nagestreefd gedurende het hele jaar. Anderzijds dienen de mogelijkheden van verspreiding voor soorten te worden vergroot door het uitbreiden en onderling verbinden van de huidige reservaten om de sterke mate van versnippering en isolatie van de hellingschraallanden terug te dringen. Vooralsnog lijkt plaggen in combinatie met het uitleggen van maaisel uit een goed ontwikkeld grasland een goede eerste stap om tot herstel of ontwikkeling van soortenrijke graslanden te komen om voormalig agrarische graslanden. Verder onderzoek aan de stikstofhuishouding in heischraal grasland moet uitwijzen of de gevonden remming van nitrificatie daadwerkelijk een negatief effect op de heischrale vegetatie heeft en zo ja, hoe dit kan worden opgeheven.

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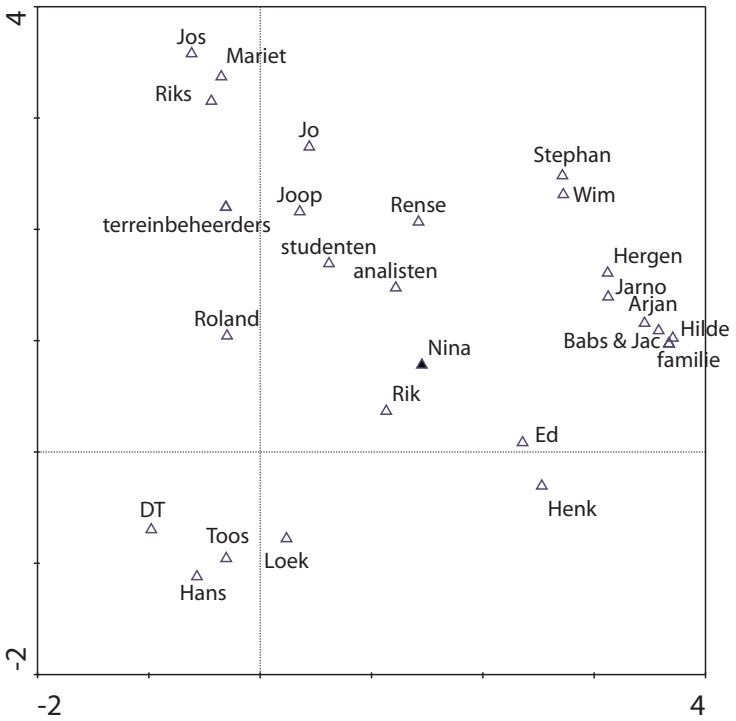
Dankwoord

Tabel 1. Twinspan tabel met activiteiten en medewerkers.

	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1
	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1
Tabelnummer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Nicole Cordewener (& team)			x	x													
Patrick Kloet (& team)			x	x													
Dennis van Schijndel				x		x	x										
Esther Boeter				x		x	x										
Aline Paalman				x	x	x	x				x						
Fred Siesling				x				x									
Sandra Robat						x		x						x			
Paul van de Ven						x		x									
Gerrit Rouwenhorst						x		x									
Loek Kuiters	x	x	x						x	x							
Toos van Noordwijk			x		x				x	x							
Hans Esselink (†)		x	x		x					x							
DT Heuvelland*			x						x								
Riks Laanbroek			x								x						
Joop Schaminee	x	x	x	x					x	x	x	x					
Jo Willems	x		x	x						x	x	x					
Roland Bobbink		x	x	x	x		x		x	x	x	x					
Jos Verhoeven			x								x	x					
Mariet Hefting			x	x							x	x					
Leescommissie**												x					
Henk Siepel	x	x								x			x				
Stephan Hennekens								x			x		x	x			
Rense Haveman			x	x				x						x			
Jarno Veldkamp	x			x									x	x			
Hergen Erhardt				x									x	x			
Ed Hazebroek		x		x	x	x							x	x			
Rik Huiskes				x	x	x			x	x	x		x	x		x	
Wim Ozinga											x		x	x		x	
Arjan Stroo													x	x	x		x
Babs Jasper													x	x	x		x
Jacomine van Wijk													x	x	x		x
Hilde Kauffman	x			x									x	x			x
Carla Coenders	x												x	x			x
Ton van Dril	x												x	x			x
Jet & Loes van Dril	x												x				x
Familie Smits	x																x

*Tabelnummer (activiteiten): 1 = Voorbereiding (tevens Karlè Sýkora); 2 = Vooronderzoek; 3 = Opzet onderzoek; 4 = Veldwerk (tevens Henk Hillegers, Victor Beumer, Susan Sollie, Edu Dorland); 5 = Referentie systemen (tevens Martine Lejeune, Willey Verbeke, Gabriel Erens, Remar Erens); 6 = Laboratorium; 7 = Analyse; 8 = Technische ondersteuning (tevens André Schaffers); 9 = Schrijven O+BN rapportages; 10 = Nederlandstalige artikelen; 11 = Schrijven proefschrift (en artikelen); 12 = Beoordeling proefschrift; 13 = Ontspanning; 14 = Stoom afblazen (tevens Eric Arets); 15 = Voorbereiden feest; 16 = Verdediging; 17 = After party. * DT Heuvelland: Bart van Tooren, Roland Bobbink, Friso van der Zee; Dries Boxman, Tim van der Broek, Harry van Buggenum, Jan Hermans, Hans de Mars, Toos van Noordwijk, Arjan Ovaa, Joop Schaminée, Michiel Wallis de Vries, Hans Weinreich, Freek van Westreenen & Jo Willems. ** Leescommissie: Kathrin Kiehl, Jan Bakker, Dennis Whigham, Annemiek Kooijman & Rudy van Diggelen.*



Figuur 1. DCA ordinatie diagram van de Dankwoord-tabel. Eigenwaarden van de eerste twee assen zijn respectievelijk 0.65 en 0.30.

Curriculum vitae

Op 30 december 1974 ben ik geboren in Tegelen. Na het behalen van het VWO diploma aan het Stedelijk Gymnasium in Nijmegen in 1993 ben ik in hetzelfde jaar begonnen aan de studie biologie in Wageningen. Gefascineerd geraakt door flora en vegetatie werd als eerste afstudeervak in 1996 de samenstelling van akkeronkruiden in de Cerdagne en Capcir (Pyreneeën, Frankrijk) en hun kiemingseigenschappen bestudeerd onder leiding van André Baudière, Peter Hilhorst en Ronald van den Berg. Vervolgens heb ik de successie op oude, verlaten akkers in Transkei (Zuid-Afrika) onderzocht in 1997, ter plekke begeleid door George Bredenkamp, Ladislav Mucina en vanuit Wageningen door Karlé Sykora die ook het aansluitende afstudeervak over de vegetatie langs de Grensmaas begeleidde. Als afsluiting volgde in 1998 een stage bij het IBN-DLO (thans Alterra) naar vegetatiestrategieën onder leiding van Joop Schaminée en wederom Karlé Sykora. Direct na mijn afstuderen ben ik in dienst gekomen bij het IBN-DLO (thans Alterra) en heb hier van 1998 tot 2004 gewerkt aan uiteenlopende projecten op het gebied van vegetatiekunde. Als opmaat naar het hier gepresenteerde onderzoek werd in 2003 een voorstudie verricht naar de schrale graslanden in Zuid-Limburg. De vier daaropvolgende jaren heb ik vanuit de universiteit Utrecht gewerkt aan vierjarig OBN-onderzoek (Overlevingsplan Bos en Natuur, thans Ontwikkeling en Beheer Natuurkwaliteit) naar de oorzaken van achteruitgang en mogelijkheden voor herstel van de Zuidlimburgse hellingschraallanden. Na afloop van deze periode werd er vanuit Alterra nog een half jaar langer aan het onderzoek doorgewerkt en in 2009 is het Nederlandstalige eindrapport verschenen, waarin ook de resultaten van het fauna-onderzoek zijn opgenomen. Aansluitend hebben de Universiteit Utrecht en Alterra de vertaling van de resultaten van dit eindrapport naar het voorliggende proefschrift mogelijk gemaakt. Sinds 2009 ben ik weer in dienst van Alterra als onderzoeker bij het team Vegetatie en landschapsecologie.

Overige activiteiten

2007-nu: Lid Council International Association for Vegetation Science (IAVS)
2007-nu: Secretaris sectie vegetatieonderzoek van de KNBV
2006-nu: Lid O+BN Deskundigenteam Heuvelland
2001-nu: Administrator International Association for Vegetation Science (IAVS)
2000-2005: Redactie Plantensociologische Kring Nederland (PKN)

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Appendix A

Synoptic table of the total species list (556 releves, stratified resampling) belonging to chapter 2. The frequency values after resampling are shown in columns 2-5. In the columns 6-9, the percentage of permutations with a significant difference between two periods is shown. No value is given when zero out of 100 runs showed significant results. For the different periods, stratified resampling led to the use of resp. 6000, 3927, 4374, and 6000 relevés. Species indicated with an asterisk (*) were excluded from the species lists that show trends, because of uncertainties with identification of these species.

		1900-	**1971-**	**1981-**	**1991-**	**% sign.**			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
↓	*Anthyllis vulneraria*	19	36.6	14.4	8.2	31	54	2	27
↓	*Arenaria serpyllifolia*	27.5	< 0.1	2.3	3.9	100			100
↓	*Bunium bulbocastanum*	10.1	2	5.8	< 0.1				78
↓	*Carlina vulgaris*	66.2	51.5	62.1	27.1			96	99
↓	*Cirsium acaule*	48.4	7.6	28	23.3	100	77	1	81
↓	*Euphrasia stricta*	28.4	46.2	35	11	15		89	68
↓	*Festuca ovina ag.*	51.4	21.2	12.6	4.3	85	2	13	100
↓	*Galium verum*	31.6	6.5	6.9	13.6	99			57
↓	*Gentianella germanica*	38.2	55.8	53.5	27.5	3		55	2
↓	*Hieracium pilosella*	69.1	65.6	39.9	21.3		22	31	100
↓	*Koeleria macrantha*	67.8	16.6	28.8	20.4	100	3	1	100
↓	*Koeleria pyramidata*	15.9	< 0.1	13	6.1	96	26	10	26
↓	*Orchis militaris*	16.2	2.5	1.2	2.9	63			79
↓	*Poa compressa*	12.7	17.4	14.2	< 0.1			24	98
↓	*Potentilla verna*	33.2	26.9	15.1	10.8		8	2	95
↓	*Thymus pulegioides ag.*	82.1	73.8	60.8	50.9			1	68
↓↑	*Campanula rotundifolia*	47.6	20.4	46.4	47.7	71	57		
↓↑	*Carex caryophyllea*	43	5.6	37.3	47.9	100	100		
↓↑	*Plantago media*	68.6	20	48.9	52.7	100	84		
↓↑	*Ranunculus bulbosus*	46.9	7.2	28.8	35	100	87		5
↑	*Clematis vitalba*	< 0.1	6.2	7.2	10.8				73
↑	*Clinopodium vulgare*	1.4	5.8	9.2	15.2		1	6	92
↑	*Cornus sanguinea*	< 0.1	19.6	11.9	7.2	57	2		20
↑	*Crataegus monogyna*	5.8	56.5	48.5	41.3	100			100
↑	*Crepis biennis*	< 0.1	< 0.1	2.7	12.2			26	85
↑	*Dactylis glomerata*	14.9	34.8	41.9	35.2	57		1	75
↑	*Dactylorhiza maculata s. fuchsii*	< 0.1	< 0.1	0.6	9.7			13	52
↑	*Fraxinus excelsior*	< 0.1	17.2	24	23.5	25			100
↑	*Galium mollugo*	< 0.1	0.4	4.8	11.1			6	74
↑	*Gymnadenia conopsea*	13.8	3.1	14.1	47	22	14	97	98
↑	*Leucanthemum vulgare*	56.5	92	71.4	67.1	75			
↑	*Prunella vulgaris*	3.9	13.3	22.7	29	27	1		100
↑	*Prunus avium*	1.5	21.6	11.7	5.2	88	6	8	
↑	*Rhinanthus alectorolophus*	< 0.1	< 0.1	6	29.2			96	100
↑	*Rhinanthus minor*	4.5	3.9	33.6	63.4		99	61	100

		1900-	1971-	1981-	1991-	% sign.			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
↑	*Rubus caesius*	5.6	31.9	18.6	23.9	100	8		94
↑↓	*Hieracium umbellatum*	2.9	16.5	22.1	1.2	63	3	98	
↑↓	*Senecio erucifolius*	14.3	36.4	14.9	19.8	73	44	2	
	Other species								
	Acer campestre	< 0.1	< 0.1	< 0.1	4.8				4
	Acer pseudoplatanus	< 0.1	2.5	< 0.1	5.6				7
	Acer species	< 0.1	< 0.1	< 0.1	1.1				
	Aceras anthropophorum	5.9	< 0.1	< 0.1	< 0.1				
	Achillea millefolium	59.4	70.6	69.2	53.9				
	Agrimonia eupatoria	54.3	53.9	53.2	49.8				
	Agrostis canina	3	< 0.1	< 0.1	< 0.1				
	Agrostis capillaris	9.2	2.5	2.3	4.8				1
	Agrostis gigantea	< 0.1	2.5	5.8	0.8				
*	*Agrostis stolonifera*	12.7	11.5	22.3	6.7		8	67	3
	Aira caryophyllea	1.5	< 0.1	< 0.1	< 0.1				
	Allium oleraceum	1.4	< 0.1	< 0.1	< 0.1				
	Allium vineale	< 0.1	1	0.5	2.3				
	Anisantha sterilis	< 0.1	< 0.1	2.3	< 0.1				
	Anthericum liliago	< 0.1	< 0.1	< 0.1	1.3				
	Anthoxanthum odoratum	4.7	< 0.1	2.3	6.4				
	Anthriscus caucalis	< 0.1	2.1	< 0.1	< 0.1				
	Arabis hirsuta	2.8	2.5	2.3	< 0.1				
	Arrhenatherum elatius	10	11.7	13.1	20.5		1	2	19
	Artemisia vulgaris	< 0.1	< 0.1	2.3	< 0.1				
	Bellis perennis	1.5	< 0.1	2.3	0.8				
	Betula pendula	< 0.1	0.7	1.2	0.9				
	Betula species	< 0.1	< 0.1	9.6	1.1		5	11	
	Botrychium lunaria	< 0.1	2.5	2.3	< 0.1				
	Brachypodium pinnatum	97.3	100	100	100				
	Briza media	93.3	87.6	82.8	89				
	Bromopsis erecta	4.3	< 0.1	< 0.1	4.1				
	Bromus hordeaceus	< 0.1	< 0.1	2.3	1.2				
	Calamagrostis epigejos	< 0.1	4.9	4.5	7.6			2	33
	Calluna vulgaris	1.9	< 0.1	< 0.1	< 0.1				
	Calystegia sepium	< 0.1	< 0.1	< 0.1	0.9				
	Campanula rapunculoides	1.5	< 0.1	< 0.1	2				
	Capsella bursa-pastoris	< 0.1	< 0.1	2.3	< 0.1				
	Carex digitata	1.5	0.6	< 0.1	< 0.1				
	Carex divulsa s. leersii	1.3	< 0.1	< 0.1	< 0.1				
	Carex flacca	81.3	72.2	83.7	92.8				
	Carex hirta	< 0.1	< 0.1	< 0.1	2.2				
	Centaurea jacea	71	58.1	77.6	81.3		1		
	Centaurea scabiosa	67.7	87.9	84.5	75.4				
	Centaurium erythraea	2.9	< 0.1	0.8	3.5				

		1900-	1971-	1981-	1991-	% sign.			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
	Cerastium arvense	0.4	< 0.1	< 0.1	1				
	Cerastium fontanum s. vulgare	5.9	2.1	< 0.1	6.5				
	Cerastium semidecandrum	3	< 0.1	2.3	< 0.1				
	Cichorium intybus	< 0.1	< 0.1	2.3	< 0.1				
	Cirsium arvense	< 0.1	0.8	2.3	7.1				19
	Cirsium palustre	1.5	< 0.1	< 0.1	< 0.1				
	Clinopodium acinos	5.8	< 0.1	< 0.1	< 0.1				
	Coeloglossum viride	1.5	< 0.1	< 0.1	< 0.1				
	Convolvulus arvensis	10.7	9	8.2	2.5		1	2	28
	Corylus avellana	2.8	1.6	4.7	< 0.1				
	Crataegus species	3	< 0.1	< 0.1	< 0.1				
	Cuscuta epithymum	1.4	< 0.1	< 0.1	2.1				
	Cymbalaria muralis	< 0.1	< 0.1	< 0.1	0.6				
	Cynosurus cristatus	< 0.1	< 0.1	2.3	< 0.1				
	Cytisus scoparius	< 0.1	< 0.1	2.3	1				
	Danthonia decumbens	6.2	< 0.1	2.3	5.3				
	Daucus carota	62	80.3	61.1	66.6				
	Echium vulgare	7.2	4.1	6.9	1				
	Elytrigia repens	1.4	< 0.1	0.7	1.3				
	Epipactis atrorubens	1.4	< 0.1	< 0.1	< 0.1				
	Erigeron acer	2.9	1.6	< 0.1	< 0.1				
	Erophila verna	1.5	< 0.1	< 0.1	< 0.1				
	Euphorbia cyparissias	1.4	< 0.1	< 0.1	< 0.1				
	Euphorbia helioscopia	1.4	< 0.1	< 0.1	< 0.1				
	Euphorbia stricta	1.4	< 0.1	< 0.1	< 0.1				
	Euphrasia rostkoviana	1.5	< 0.1	< 0.1	< 0.1				
	Euphrasia species	1.4	< 0.1	< 0.1	< 0.1				
	Fagus sylvatica	< 0.1	< 0.1	< 0.1	0.6				
	Festuca arundinacea	< 0.1	< 0.1	4.6	6.7				16
	Festuca pratensis	2.9	< 0.1	2.9	6.8			3	2
	Festuca rubra ag. (incl. F. arenaria)	57.9	59.2	75.9	79.9				5
	Fragaria vesca	5.7	15.4	4.1	4	15	22		
	Galeopsis species	2.9	< 0.1	< 0.1	< 0.1				
	Galium aparine	< 0.1	< 0.1	< 0.1	2.5				
	Galium pumilum	30.7	25.4	26.3	15.4			8	33
	Galium species	1.5	< 0.1	< 0.1	< 0.1				
	Genista tinctoria	8.7	13.1	4.6	13.9		16	21	3
	Gentianella campestris	1.5	< 0.1	< 0.1	< 0.1				
	Gentianella ciliata	3.4	< 0.1	4.6	< 0.1				
	Geranium columbinum	< 0.1	7.9	< 0.1	< 0.1				
	Geranium dissectum	< 0.1	< 0.1	< 0.1	0.6				
	Hedera helix	< 0.1	< 0.1	0.6	< 0.1				
	Helianthemum nummularium	2.9	< 0.1	2.3	1				

		1900-	1971-	1981-	1991-	% sign.			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
	Helictotrichon pratense	27	17.8	26.8	25.3	2	4		
	Helictotrichon pubescens	47.5	62.2	71.7	68.4				13
	Heracleum sphondylium	3.2	1.4	4.6	11.3			11	19
	Herminium monorchis	4.4	< 0.1	< 0.1	< 0.1				
*	*Hieracium laevigatum*	< 0.1	19.7	6.9	1.2	64	3		
	Hieracium sabaudum	< 0.1	< 0.1	0.8	3.6				
	Hieracium sect. hieracium	1.5	< 0.1	< 0.1	< 0.1				
	Hieracium sect. vulgata	< 0.1	< 0.1	< 0.1	0.9				
	Hieracium species	< 0.1	2.5	2.3	2.2				
	Holcus lanatus	4.3	< 0.1	< 0.1	7.7			7	1
	Hypericum dubium	< 0.1	< 0.1	< 0.1	1.8				
	Hypericum maculatum	< 0.1	< 0.1	< 0.1	0.4				
	Hypericum perforatum	28.8	47.6	34.6	17.4	15	2	34	7
	Hypericum pulchrum	1.5	< 0.1	< 0.1	< 0.1				
	Hypochaeris radicata	1.5	1.2	< 0.1	< 0.1				
	Inula conyzae	1.4	6.9	5.1	1				
	Juncus compressus	0.5	< 0.1	< 0.1	< 0.1				
	Knautia arvensis	54.5	64.3	65.5	66.1				1
	Koeleria species	1.4	< 0.1	< 0.1	< 0.1				
	Lathyrus pratensis	< 0.1	6	3.4	5.5				6
	Leontodon autumnalis	1.4	< 0.1	< 0.1	< 0.1				
	Leontodon hispidus	79.6	87.4	89.6	92.9				
	Leontodon saxatilis	2.9	< 0.1	< 0.1	< 0.1				
	Linaria vulgaris	< 0.1	< 0.1	< 0.1	4.1				
	Linum catharticum	83.7	93.2	95.1	90				
	Listera ovata	5.9	4.8	1.2	7.3			2	
	Lithospermum officinale	2.9	< 0.1	< 0.1	< 0.1				
	Lolium perenne	1.5	< 0.1	< 0.1	< 0.1				
	Lotus corniculatus ag. (incl. L. glaber)	95.1	96.5	98.5	94.9				
	Luzula campestris	1.5	1.9	< 0.1	2.1				
	Medicago falcata + M. sativa	0.5	< 0.1	2.3	2.9				
	Medicago lupulina	60.3	65.3	60.2	51.7				
	Medicago x varia	< 0.1	< 0.1	2.3	< 0.1				
	Melampyrum arvense	0.5	< 0.1	< 0.1	< 0.1				
	Melampyrum pratense	< 0.1	0.7	0.6	< 0.1				
	Melilotus altissimus	< 0.1	0.9	1.2	< 0.1				
	Minuartia hybrida	1.5	< 0.1	< 0.1	< 0.1				
	Ononis repens	48	43.8	47.1	70.1			7	13
	Ophrys apifera	2	3.5	< 0.1	5.6				
	Ophrys insectifera	3	< 0.1	< 0.1	< 0.1				
	Orchis simia	< 0.1	< 0.1	4.6	< 0.1				
	Orchis species	< 0.1	< 0.1	0.8	< 0.1				
	Origanum vulgare	59.3	82	78.7	55.1	1		5	

		1900-	1971-	1981-	1991-	% sign.			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
	Parnassia palustris	1.5	< 0.1	< 0.1	1				
	Picris hieracioides	26.5	29.1	18.4	18.7				5
	Pimpinella major	< 0.1	< 0.1	0.6	8.4			9	36
	Pimpinella saxifraga	75.5	65.9	83.5	82.7				
	Plantago lanceolata	68.6	60.2	66.9	86.4				
	Plantago major	< 0.1	< 0.1	2.3	0.6				
	Platanthera bifolia + P. chlorantha	2.8	13.7	12.8	8.4	33		1	8
*	*Poa angustifolia*	1.5	2.5	13.1	26.3		21	21	100
*	*Poa pratensis*	47.5	10.6	27.3	8.3	99	31	81	100
	Poa pratensis + P. angustifolia	1.4	< 0.1	< 0.1	< 0.1				
	Poa trivialis	< 0.1	3.8	< 0.1	< 0.1				
	Polygala vulgaris	66.1	73	73.1	57.4			1	
	Polygonatum species	< 0.1	< 0.1	< 0.1	0.3				
	Potentilla anserina	2.9	0.5	2.4	3				
	Potentilla erecta	4.7	< 0.1	2.3	< 0.1				
	Potentilla species	< 0.1	< 0.1	< 0.1	1				
	Primula veris	5.9	< 0.1	5.3	8.2				
	Prunus serotina	< 0.1	< 0.1	< 0.1	0.6				
	Prunus species	5.9	< 0.1	< 0.1	2.8				
	Prunus spinosa	4.2	2.2	< 0.1	6.5			1	
	Quercus robur	4.4	12.7	6.1	13	5		2	20
	Quercus species	< 0.1	< 0.1	< 0.1	0.6				
	Ranunculus acris	< 0.1	0.8	0.7	3.6				
	Ranunculus repens	1.5	2	3.2	1				
	Ranunculus species	< 0.1	< 0.1	< 0.1	2.5				
	Reseda lutea	< 0.1	< 0.1	2.3	< 0.1				
	Reseda luteola	1.5	< 0.1	< 0.1	< 0.1				
	Rhamnus frangula	< 0.1	2.5	0.6	< 0.1				
	Rhamnus species	< 0.1	< 0.1	< 0.1	1				
*	*Rhinanthus species*	< 0.1	< 0.1	1.2	10.3			15	67
	Rosa canina	< 0.1	1	4.6	2.4				
	Rosa rubiginosa	4.5	2.2	2.3	3.2				
	Rosa species	1.4	13.9	2.4	9.6	17	25	8	37
	Rubus species	1.4	2.5	0.9	8.5			7	27
	Rumex acetosa	4.9	< 0.1	6.9	3.7				
	Rumex acetosella	< 0.1	< 0.1	< 0.1	2.1				
	Salvia pratensis	< 0.1	< 0.1	< 0.1	2				
	Salvia verticillata	1.4	2.2	4.1	7.4				10
	Sanguisorba minor	92.9	84.8	90.7	82.5				
	Scabiosa columbaria	80.5	84.2	80	55.3			13	27
	Sedum acre	1.5	2.5	< 0.1	< 0.1				
	Sedum sexangulare	< 0.1	2.5	< 0.1	< 0.1				
	Senecio jacobaea	18.6	14.7	12.5	11.5	1			4

		1900-	1971-	1981-	1991-	% sign.			
	Species names	**1970**	**1980**	**1990**	**2010**	**1->2**	**2->3**	**3->4**	**1->4**
	N (strat. resampling) =	**6000**	**3957**	**4344**	**6000**				
	Senecio species	< 0.1	2.5	< 0.1	< 0.1				
	Silene vulgaris	4.5	8.5	12.4	7.5			1	
	Sonchus species	< 0.1	< 0.1	4.7	0.4				
	Stachys officinalis	5.9	< 0.1	2.3	3.6				
	Stachys sylvatica	1.4	< 0.1	< 0.1	< 0.1				
	Succisa pratensis	5.8	3.8	15.3	14.9		33		20
*	*Taraxacum species*	10.1	18.4	32.6	32.4	1	11		96
	Tetragonia tetragonioides	< 0.1	< 0.1	2.3	< 0.1				
	Tetragonolobus maritimus	0.4	< 0.1	< 0.1	5.9			1	5
	Teucrium botrys	< 0.1	< 0.1	2.3	< 0.1				
	Teucrium chamaedrys	3.4	< 0.1	2.3	< 0.1				
	Teucrium scorodonia	< 0.1	< 0.1	2.3	< 0.1				
	Thlaspi perfoliatum	< 0.1	< 0.1	4.6	1				
	Thymus praecox	< 0.1	< 0.1	2.3	< 0.1				
	Tragopogon pratensis	4.3	13.9	11.3	10.5	26		1	7
	Trifolium dubium	2.8	< 0.1	< 0.1	2.8				
	Trifolium pratense	14.1	8.5	22.8	27.5	3	26		30
	Trifolium repens	3	5.7	4.6	5.1				
	Trifolium species	< 0.1	< 0.1	< 0.1	3.2				
	Trisetum flavescens	47.1	27.4	34.6	32.7	16			5
	Triticum aestivum	< 0.1	< 0.1	< 0.1	1.1				
	Valeriana officinalis	1.5	4.3	0.9	< 0.1				
	Verbascum species	< 0.1	< 0.1	4.6	< 0.1				
	Verbascum thapsus	1.5	< 0.1	< 0.1	< 0.1				
	Viburnum opulus	< 0.1	< 0.1	< 0.1	0.6				
	Vicia cracca	27.1	50.7	46.8	32.9	47		2	
	Vicia hirsuta	< 0.1	3.4	< 0.1	4.4				
	Vicia sativa s. nigra	10.2	4	2.3	8				
	Vicia sepium	< 0.1	1.8	< 0.1	< 0.1				
*	*Vicia species*	< 0.1	< 0.1	0.6	10.5			25	72
	Vicia tetrasperma	< 0.1	5.6	< 0.1	< 0.1				
	Viola canina	< 0.1	< 0.1	< 0.1	1.1				
	Viola hirta	7.2	3.9	11.6	15.3		7	2	11
	Viola species	< 0.1	< 0.1	< 0.1	0.4				

Appendix B

Synoptic table of the control-plots (n=6) in four different periods belonging to chapter 5. Shown is the presence (in %) of each species per period with in superscript the characteristic coverage. The results of the chi-square test are presented in terms of significant changes between the periods (by comparing period 1 and 2, period 2, 3 and period 3 and 4) in the last two columns (++/ --: p<0.01, +/-: p< 0.05). Individuals that, due to taxonomic problems, could not be assigned to a specific species, are indicated only with the genus name + 'species'. Species that occur in only one period with a presence less than 10 % (presence-characteristic coverage): group 1: *Arrhenatherum elatius* (6-2), *Cerastium arvense* (2-1), *Cirsium vulgare* (8-2), *Elytrigia repens* (8-5), *Euphorbia helioscopia* (6-1), *Festuca cinerea* (4-2), *Glechoma hederacea* (2-1), *Hypochaeris radicata* (4-1) *Scabiosa columbaria* (4-1); group 2: *Hedera helix* (5-1), *Clinopodium vulgare* (2-1), *Sorbus aucuparia* (2-1), *Anagallis arvensis* (2-1), *Centaurium erythrea* (2-1); group 3: *Ranunculus* species (4-1); group 4: *Allium vineale* (2-1), *Gymnadenia conopsea* (2-1), *Plantago major* (2-1) *Prunus spinosa* (2-1).

Years (19../20..)	**71-79**		**80-86**		**87-95**		**96-06**		**Sign. trends**		
Period	**1**		2		3		4		**1-2**	**2-3**	**3-4**
Nr. of relevés	52		42		54		47				
Bellis perennis	28	2	5	1					--		
Veronica chamaedrys	9	2	24	1					+		
Festuca pratensis	77	5	93	3	48	2	60	2	+	--	
Lathyrus pratensis	49	2	86	2	63	1	55	2	++	-	
Luzula campestris	8	1	41	1			2	1	++	--	
Poa angustifolia	17	2	81	2	33	2	15	1	++	--	-
Carex caryophyllea	9	1	55	1	41	2	34	3	++		
Plantago lanceolata	77	4	100	2	98	2	96	2	++		
Ranunculus bulbosus	43	2	88	1	93	2	87	1	++		
Crataegus monogyna	15	2	50	1	33	1	23	1	++		
Senecio jacobaea	17	2	45	1	65	1	45	1	++		-
Hypericum perforatum	8	1	43	2	39	1	6	1	++		--
Origanum vulgare	8	1	29	1	33	1	4	1	++		--
Heracleum sphondylium	8	1	100	3	98	2	57	2	++		--
Clematis vitalba	6	1	81	2	91	2	21	1	++		--
Taraxacum species	32	2	79	1	94	1	81	2	++	+	-
Pimpinella major	21	2	67	2	85	1	55	1	++	+	--
Brachypodium pinnatum	47	5	79	5	93	6	100	7	++	+	
Campanula rotundifolia	4	1	33	1	63	1	70	2	++	++	
Rhinanthus species			62	2	100	3	83	2	++	++	
Vicia species			7	1	24	1	4	1		+	--
Trifolium species					33	2				++	--
Dactylorhiza maculata s. fuchsii			24	1	83	3	75	1		++	
Tragopogon pratensis			2	1	28	1	13	1		++	
Fraxinus excelsior	13	1	71	1	76	1	98	1	++		++
Festuca arundinacea	9	2	33	2	15	2	40	2	++	-	++
Vicia cracca	21	4	45	1	19	1	45	1	+	--	++
Crepis biennis					28	1	53	1		++	++
Quercus robur	8	1	21	1	4	1	45	1			++

Years (19../20..)	71-79		80-86		87-95		96-06		Sign. trends		
Period	**1**		2		3		4		**1-2**	**2-3**	**3-4**
Nr. of relevés	52		42		54		47				
Poa pratensis	36	2	21	2	6	1	21	1		-	+
Trifolium pratense	94	2	83	2	39	2	60	2			+
Dactylis glomerata	94	2	100	5	91	2	75	2			+
Gentianella germanica					4	1	17	1			
Cynosurus cristatus	83	2	79	2	2	1				--	
Trifolium repens	36	2	41	1	2	1				--	
Holcus lanatus	89	4	100	4	65	2	75	2		--	
Trisetum flavescens	87	4	100	2	61	2	60	2		--	
Medicago lupulina	94	3	88	1	52	1	38	2		--	
Agrostis stolonifera	77	3	88	2	22	1	30	1		--	
Ranunculus acris	93	2	98	2	63	1	23	1		--	--
Daucus carota	94	3	81	1	43	1	2	1		--	--
Prunella vulgaris	66	3	79	3	72	2	32	2			--
Agrimonia eupatoria			24	1	20	2	4	1			-
Listera ovata	15	1	29	1	32	1	13	1			-
Other species											
Linum catharticum	72	2	79	2	100	3	100	2			
Briza media	85	6	100	6	100	6	100	6			
Knautia arvensis	85	3	98	2	100	2	94	5			
Carex flacca	100	5	100	5	100	6	98	6			
Festuca rubra ag.	96	5	100	5	98	3	98	5			
Leontodon hispidus	100	7	100	7	100	8	98	7			
Leucanthemum vulgare	96	3	93	3	100	4	98	3			
Lotus corniculatus ag.	79	4	100	5	100	3	96	5			
Helictotrich pubescens	89	4	100	5	94	3	94	4			
Achillea millefolium	89	2	100	2	98	1	89	1			
Plantago media	100	5	100	3	100	2	89	4			
Centaurea jacea	100	3	98	3	96	2	87	2			
Pimpinella saxifraga	83	2	98	2	96	1	87	2			
Ononis species	79	5	83	5	96	6	96	6			
Vicia sativa			10	1			13	2			
Succisa pratensis					15	1	6	1			
Cerastium fontanum s. vulgare	9	2			2	1	2	1			
Prunus avium	9	1	24	1			2	1			
Vicia hirsuta			5	1	17	1	2	1			
Rosa canina	2	1	2	1	6	1					
Vicia sepium	4	1	7	1							
Cirsium arvense	8	1	7	1							
Lolium perenne	8	2	2	1							
Anthoxanthum odoratum	4	1	14	1	4	1					