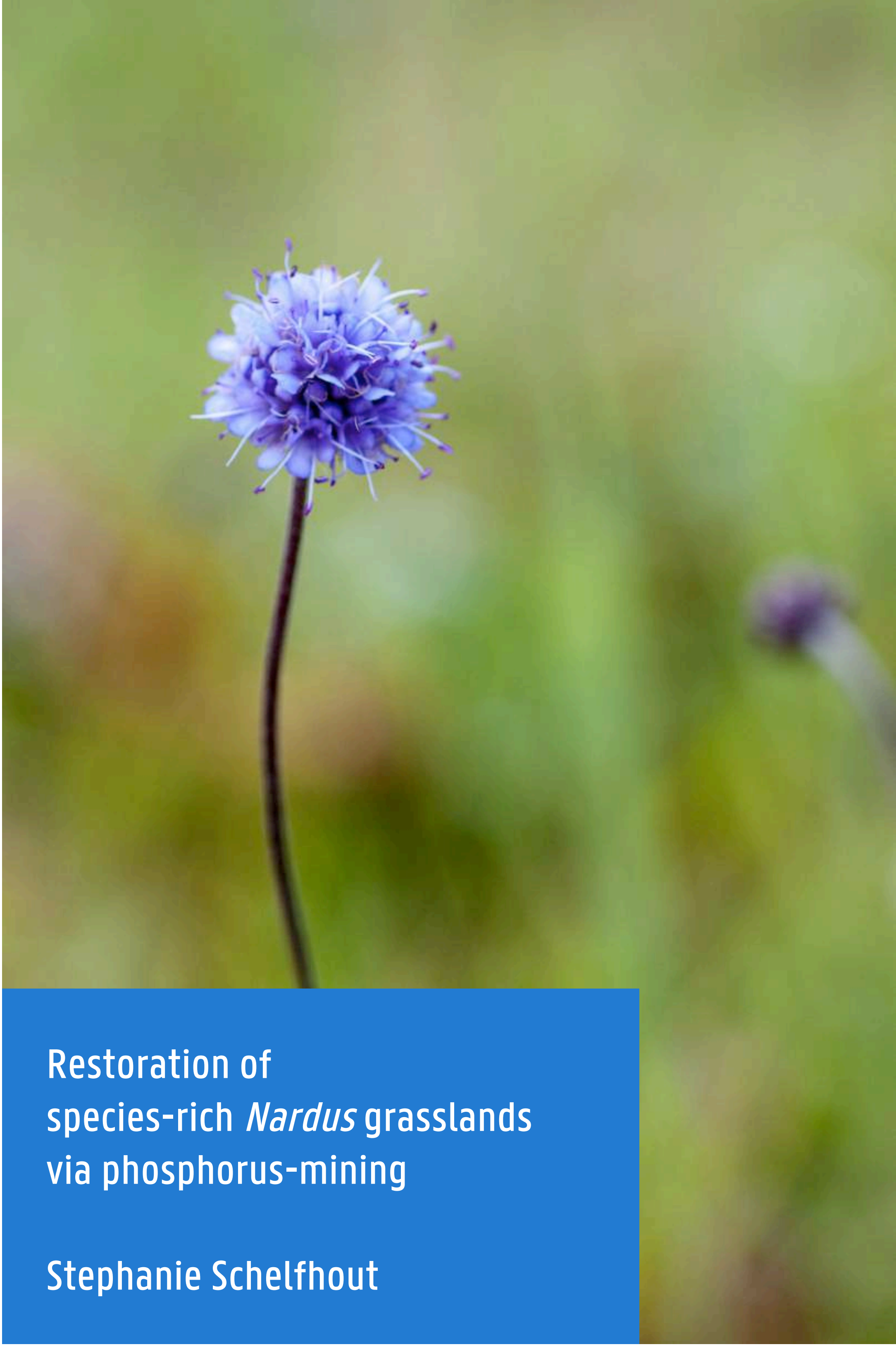

Restoration of species-rich *Nardus* grasslands via phosphorus-mining

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FACULTY OF BIOSCIENCE ENGINEERING

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Restoration of species-rich *Nardus* grasslands via phosphorus-mining

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Illustration on the cover: *Succisa pratensis* – Blauwe knoop (front); Mowing management in a post-fertilization field in Vrieselhof, Ranst, Belgium (back)

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E. O. Wilson in Letters to a young scientist (2013)

DANKWOORD

"Mag het iets meer zijn?" Dit onderzoek heeft iets langer mogen duren en gelukkig maar. Het speelt zich af op het spanningsveld tussen landbouw en natuur en deze complexiteit met al zijn stakeholders had ik niet op vier jaar tijd kunnen begrijpen (niet dat ik dit nu volledig begrijp maar toch een beetje). Dit onderzoek was een bijzondere uitdaging waar ik acht jaar lang erg graag aan werkte in het inspirerende Gontrode en het gezellige Schoonmeersen, samen met gepassioneerde promotoren en collega's, samen met de mensen die midden in de praktijk staan, nl. de natuurbeheerders en landbouwers die openstaan voor nieuwigheden, en samen met al die studentjes die ik mocht ontmoeten. Bedankt, het was leerrijk! Zoals je in dit doctoraat zal zien op de making-of-actie-foto's, kreeg ik erg veel hulp. Een woord van dank is hierbij op zijn plaats.

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18 Februari 2019

Stephanie

SUMMARY

Grassland ecosystems can be extremely species-rich and harbor a large proportion of European wildlife. Due to agricultural intensification in the 20th century, species-rich *Nardus* grasslands (a European priority habitat type) are nowadays largely confined to nature reserves, where they are further threatened by *e.g.* atmospheric nitrogen deposition. Typical plant species of *Nardus* grasslands are adapted to nutrient-poor soil conditions through their conservative resource use strategy. Land-use intensification causes the plant community to shift from the *slow-growth strategy* species in *Nardus* grasslands to *fast-growth strategy* species in eutrophic grasslands where nutrient cycling is fast and plant species richness is low. The soil biota community also changes, *i.e.* from fungi-dominated with omnivorous nematodes to bacteria-dominated microbial with herbivorous nematodes. Biodiversity conservation will benefit from better protection of (remnant, *i.e.* degraded) *Nardus* grasslands, reducing pressures on degraded grasslands and restoration of degraded grasslands.

The restoration of *Nardus* grasslands on former agricultural land is impeded by the phosphorus that has accumulated in the soil due to repeated fertilization. Bioavailable concentrations of phosphorus may be tenfold higher in former agricultural fields than in remnant *Nardus* grasslands. Nitrogen and potassium levels, in contrast, generally decrease again through mowing management when fertilization ceases. Hence, phosphorus is the key nutrient for evaluating the restoration potential of former agricultural grasslands.

In remnant *Nardus* grasslands and in a mesocosm experiment, we found a soil phosphorus threshold of 12 mg P_{Olsen} kg^{-1}. At higher bioavailable soil phosphorus concentrations, typical *Nardus* grassland species disappeared, probably due to being shaded out by fast-growing plant species (and inoculation with soil biota did not increase their performance). For ecological restoration, phosphorus can be extracted with plant biomass, *i.e.* through phytomining (by mowing or P-mining). We compared the potential and optimization opportunities of traditional mowing management, *i.e.* unfertilized cutting and removing hay two or three times a year, and P-mining, *i.e.* yield maximization by adding growth-limiting nutrients other than phosphorus (*i.e.* nitrogen and potassium). Mowing for 16-25 years in post-fertilization grasslands did not yield the floristic and phosphorus-poor targets of *Nardus* grassland. The biomass production was relatively low due to nitrogen and potassium limitation. Another 40 to 114 years of phosphorus removal would be needed to reach the soil phosphorus threshold of *Nardus* grasslands. With P-mining, the restoration time would be considerately shorter. The potential to phytomine phosphorus with P-mining depended on the soil phosphorus concentration: high phosphorus removal on phosphorus-rich soils, decreasing removal potential with decreasing soil phosphorus concentration. Adding biostimulants, *i.e.* phosphorus-solubilizing bacteria, humic substances or arbuscular mycorrhizal fungi, did not result in higher phosphorus removal with P-mining. Crop species such as buckwheat, sunflower and maize were considerably more effective at removing

phosphorus through P-mining than triticale or flax, at phosphorus-rich soils (all species performed similarly in soils with lower phosphorus concentrations).

Optimization opportunities for phytomining of phosphorus appear to be limited. Restoring phosphorus-poor soil conditions on former agricultural land will remain a challenge. Phytomining phosphorus will always be a long-term commitment, which can be made more efficient by lifting the limitation of nutrients other than phosphorus (P-mining) or selecting crop species that take up more phosphorus. Oligotrophic habitat types deserve high-priority conservation because it is nearly impossible to restore oligotrophic fields on eutrophic fields, unless a drastic technique is used such as topsoil removal. Ecological restoration of semi-natural grasslands on former agricultural land involves a large investment of time and/or money (*i.e.* decades of mowing or P-mining management or ten thousands of euros per hectare for topsoil removal). Therefore, it is necessary for practitioners in ecological restoration to focus their efforts and carefully select their goals, fields and techniques.

To aid practitioners, we developed a decision tree. We advise that the abiotic and biotic conditions be examined carefully and compared to the targeted habitat type, *i.e.* measuring the *distance to target.* We highlight the importance of restoring the abiotic conditions before starting biotic restoration and initiating suitable management. The abiotic *distance to target* for *Nardus* grassland restoration is the amount of phosphorus to be removed, which will determine the time needed for phytomining (mowing or P-mining). Evaluating the cost of the restoration technique, the time needed to reach the target and the potential value of the site in the landscape, can help in prioritizing restoration efforts. Restoring *Nardus* grassland on eutrophic soils requires topsoil removal, a costly measure, and is hence only likely with financial support of *e.g.* European Life funding or large national nature development projects. Low-budget projects can probably better focus on restoration on fields with a history of less intensive fertilization where phytomining by mowing or P-mining is attainable. It might be most efficient to invest some money (a small fraction when compared to the purchasing price) in the abiotic screening of parcels before purchasing them or before selecting a target habitat type, *i.e.* a habitat type with less stringent abiotic requirements on eutrophic fields (with high phosphorus levels). For instance, grasslands rich in common native herb species are easier to create, can be beneficial for *e.g.* pollinators and can be used strategically as corridors between oligotrophic habitats or as buffer zones to prevent inflow of fertilization from nearby agricultural fields. These *novel ecosystems*, however, can probably never fully replace highly specialized ecosystems such as *Nardus* grasslands.

SAMENVATTING

Graslanden kunnen enorm soortenrijk zijn. Een groot deel van de Europese plant- en diersoorten is gebonden aan deze soortenrijke graslanden. De intensivering van de landbouw tijdens de 20ste eeuw heeft ervoor gezorgd dat soortenrijke heischrale graslanden (een prioritair Europees habitattype) nu enkel nog voorkomen in natuurgebieden, waar ze verder bedreigd worden door o.a. atmosferische stikstofdepositie. Plantensoorten van heischrale graslanden zijn aangepast aan nutriëntenarme bodem; ze zijn gekenmerkt door een conservatief gebruik van nutriënten. Landgebruiksintensivering veroorzaakt een verschuiving in de soortensamenstelling van de plantengemeenschap, van de vele traaggroeiende soorten van heischrale graslanden naar een klein aantal snelgroeiende soorten in eutrofe graslanden. Ook de gemeenschap van bodembiota verandert, van een systeem gedomineerd door fungi en met omnivore nematoden naar een bacterie-gedomineerd systeem met herbivore nematoden. Biodiversiteitsbehoud is gebaat bij een betere bescherming van de resterende heischrale graslanden en bij het herstel van gedegradeerde graslanden.

Het herstel van heischrale graslanden op voormalige landbouwgrond is moeilijk. Jarenlange bemesting heeft gezorgd voor accumulatie van fosfor in de bodem. De concentratie van biobeschikbaar fosfor kan tot tien keer hoger zijn in voormalige landbouwgrond dan in heischrale graslanden. Ook stikstof en kalium zijn in hoge concentraties aanwezig na bemesting, maar deze elementen verdwijnen relatief snel uit de bodem door een verschralend maaibeheer zonder bemesting. Om de potentie voor herstel van heischraal grasland op voormalige landbouwgrond te evalueren, moeten we dus kijken naar fosfor.

In heischrale graslanden en in een mesocosm-experiment vonden we een grenswaarde voor bodemfosfor van 12 mg P_{Olsen} kg^{-1}. Bij hogere concentraties biobeschikbaar fosfor verdwenen de plantensoorten typisch voor heischraal grasland, waarschijnlijk door competitie voor licht met snelgroeiende plantensoorten, en inoculatie met bodembiota hielp hen niet om te overleven bij hoge fosforconcentraties. Bij natuurherstel kunnen we proberen om bodemfosfor te verwijderen via fytoextractie: fosfor wordt opgenomen in planten en afgevoerd met de plantenbiomassa bij de oogst. We vergeleken fytoextractie van fosfor bij twee hersteltechnieken: traditioneel maaibeheer (geen bemesting, twee tot drie keer per jaar maaien met afvoer van hooi) en uitmijnen (bemesting met stikstof en kalium om de biomassaproductie te maximaliseren). Na 16-25 jaar maaien in graslanden op voormalige landbouwgrond was er nog geen heischrale vegetatie en fosfor-arme bodem. De biomassaproductie van de bestudeerde graslanden was relatief laag, door stikstof- en kaliumlimitatie. Om de grenswaarde voor fosfor-arme heischrale graslanden te bereiken zou nog 40 tot 114 jaar maaibeheer nodig zijn. De duur van het beheer kan een flink stuk korter met uitmijnen. De potentie voor fosforafvoer via uitmijnen hangt weliswaar af van de concentratie fosfor in de bodem: hoge fosforafvoer uit fosforrijke bodems, dalende fosforafvoer met dalende concentratie bodemfosfor. Uitmijnen met toevoeging van biostimulanten (fosforsolubiliserende bacteriën, humuszuren of arbusculaire mycorrhiza)

zorgde niet voor een hogere fosforafvoer. Boekweit, zonnebloem en mais waren aanzienlijk effectiever in het afvoeren van fosfor via uitmijnbeheer dan triticale of vlas, in fosforrijke bodems; er was geen verschil in fosforafvoer tussen de gewassen in fosforarme bodems. Het blijkt moeilijk om fosforafvoer via uitmijnbeheer te optimaliseren. Het zal steeds een uitdaging blijven om fosfor-limiterende bodemomstandigheden te herstellen op voormalige landbouwgrond. Fytoextractie van fosfor is een langetermijnengagement, dat beperkt efficiënter gemaakt kan worden door nutriëntenlimitatie door andere elementen dan fosfor op te heffen via uitmijnen of door gewassen te gebruiken die meer fosfor opnemen. Het beschermen van bestaande oligotrofe habitats zou een hoge prioriteit moeten krijgen gezien het bijna onmogelijk is om van eutrofe bodems opnieuw oligotrofe bodems te maken, tenzij er ontgrondt wordt. Voor herstel van soortenrijk grasland op voormalige landbouwgrond is een grote investering nodig, van tijd én geld. Daarom is het belangrijk dat terreinbeheerders actief in natuurherstel kiezen voor een haalbaar doel gekoppeld aan een weloverwogen keuze van terreinen en hersteltechnieken.

Om terreinbeheerders te helpen bij het nemen van de nodige keuzes, ontwikkelden we een beslissingsboom. We adviseren om de abiotische en biotische terreincondities te meten en te vergelijken met de terreincondities van het doelhabitattype om de *afstand tot het doel* te bepalen. We benadrukken het belang van het herstellen van de abiotische terreincondities vóór er gewerkt wordt aan biotisch herstel. De afstand tot het doel voor heischrale graslanden is de hoeveelheid fosfor die verwijderd moet worden. Dit bepaalt de tijd nodig voor fytoextractie via maaien of uitmijnen. Door te evalueren hoeveel tijd nodig is om het doel te bereiken, wat de kost van het beheer zal zijn en hoe groot de potentiële waarde van het terrein in het landschap is, kunnen prioriteiten gesteld worden voor het natuurherstel. Om op eutrofe bodems soortenrijke heischrale graslanden te herstellen is ontgronden nodig, een dure maatregel die enkel mogelijk is met financiële steun van bijvoorbeeld Europese Life-projecten of nationale natuurherstelprojecten. Projecten met een klein budget kiezen beter voor natuurherstel op terreinen met een geschiedenis van minder intensief landgebruik waar via fytoextractie verschraald kan worden of voor een natuurdoeltype waarvoor geen fosfor-limiterende bodemomstandigheden nodig zijn zoals bijvoorbeeld kruidenrijke graslanden onder stikstof- of kalium-limitatie. Kruidenrijke graslanden met algemene inheemse kruidachtigen zijn ook waardevol voor o.a. pollinatoren. Ze kunnen bovendien corridors vormen voor soorten van oligotrofe habitats of als bufferzone het instromen van meststoffen uit nabijgelegen landbouwterreinen opvangen. Deze *nieuwe ecosystemen* zijn vermoedelijk geen volwaardige vervanging voor hoog-gespecialiseerde ecosystemen zoals heischrale graslanden, maar ze kunnen de lokale biodiversiteit wel helpen opkrikken.

LIST OF ABBREVIATIONS, SYMBOLS AND DEFINITIONS

oligotrophic soil	nutrient-poor soil; *e.g.* with *Nardus* grassland vegetation
mesotrophic soil	in between oligotrophic and eutrophic soil conditions; *e.g.* with grass-herb mixture
eutrophic soil	nutrient-rich soil; *e.g.* with fast-growing grass species
6230*	European Natura2000 priority habitat type of species-rich *Nardus* grasslands
6230_hn	subtype of dry *Nardus* grasslands specified for northern Belgium (De Saeger and Wouters, 2017; see Appendix 1.A for a species list)
Nardus grassland	intact *Nardus* grassland according to the criteria (see § 1.4)
remnant *Nardus* grassland	*Nardus* grassland that has been degraded somehow and lost species, for example by atmospheric nitrogen deposition or acidification
phytomining	phosphorus extraction by biomass production, through mowing or P-mining
mowing	cutting of grassland swards to remove nutrients with hay
P-mining	mowing + fertilization with growth-limiting nutrients (*e.g.* N and K); other crops are possible
CSR	plant strategies according to Grime 2001; Competitor, Stress-tolerator, Ruderal
SLA	Specific Leaf Area
P	Phosphorus
P_{CaCl2}	calcium chloride-extractable soil phosphorus; immediately available phosphorus
P_{Olsen}	sodium bicarbonate-extractable soil phosphorus; bioavailable phosphorus
P_{AL}	ammonium-lactate-extractable soil phosphorus; bioavailable phosphorus but > P_{Olsen}
$P_{Oxalate}$	ammonium-oxalate-oxalic acid-extractable soil phosphorus; slowly cycling phosphorus
P_{Total}	total soil P
ΔP_{Olsen}	change in P_{Olsen} over time
$\Delta P_{Oxalate}$	change in $P_{Oxalate}$ over time
$P_{Excessive}$	excess phosphorus stock in soil (versus target slowly cycling phosphorus stock)
PSD	Phosphate Saturation Degree
pH, pH_{H2O}, pH_{KCl}	measure of acidity, measurement of pH by extraction in H_2O or KCl
N	Nitrogen
K	Potassium
PNI	Phosphorus Nutrition Index
NNI	Nitrogen Nutrition Index
KNI	Potassium Nutrition Index
DM	Dry bioMass
P_{DM}, N_{DM}, K_{DM}	nutrient content in dry biomass of resp. phosphorus, nitrogen and potassium
AM	Arbuscular Mycorrhizal fungi
PSB	Phosphate-Solubilizing Bacteria
HS	Humic Substances; humic and fulvic acids
SD	Standard Deviation of the mean
SE	Standard Error of the mean
NMDS	Non-metric Multi-Dimensional Scaling analysis
LME	Linear Mixed Effects models
$(\Delta)AIC_c$	(change in) corrected Akaike Information Criteria; correction for small sample size
R^2	coefficient of determination
R^2_m	marginal R^2; proportion of variance explained by fixed factors alone
R^2_c	conditional R^2; proportion of variance explained by both fixed and random factors
ANOVA	ANalysis Of VAriance
p	significance of statistical test
F	statistical test; ratio between variation between sample means and variation within the samples
χ^2	statistical test; Pearson's chi-squared test
DF	Degrees of Freedom
TITAN	Threshold Indicator Taxa Analysis

"To our shame, we are careless with our language. We say that 'we've lost 97% of our flower rich meadows since the 1930s' or that 'we've lost 86% of the Corn Bunting population'. We speak of 'a loss of 97% of our Hedgehogs'. Loss, lost... as if this habitat and these species have mysteriously disappeared into the ether, as if they've accidentally vanished. But they haven't – they've been destroyed."

Chris Packham (2018) in A Peoples Manifesto for Wildlife

CHAPTER 1

Ecological restoration and the role of phosphorus

Past, present and future of European Nature © Jeroen Helmer, ARK Natuurontwikkeling

> *"Nature is not a 'nice to have' – it is our life-support system"*
>
> *Mike Barrett (Executive director of science and conservation at WWF)*

The safe operating space for human societies on earth has been concenptualized in nine planetary boundaries, *i.e.* scientifically based biophysical thresholds (Rockström et al. (2009) and updated by Steffen et al. (2015)). If crossed, they will inadvertently drive the Earth System into a much less hospitable state. Currently, four boundaries have been crossed as a result of human activity (Fig. 1.1). This PhD thesis, which aims to advance the knowledge on the restoration of species-rich grasslands, takes place in the context of two of the planetary boundaries: *biogeochemical flows* (more specifically: the phosphorus cycle) and *biosphere integrity* (more specifically: genetic diversity).

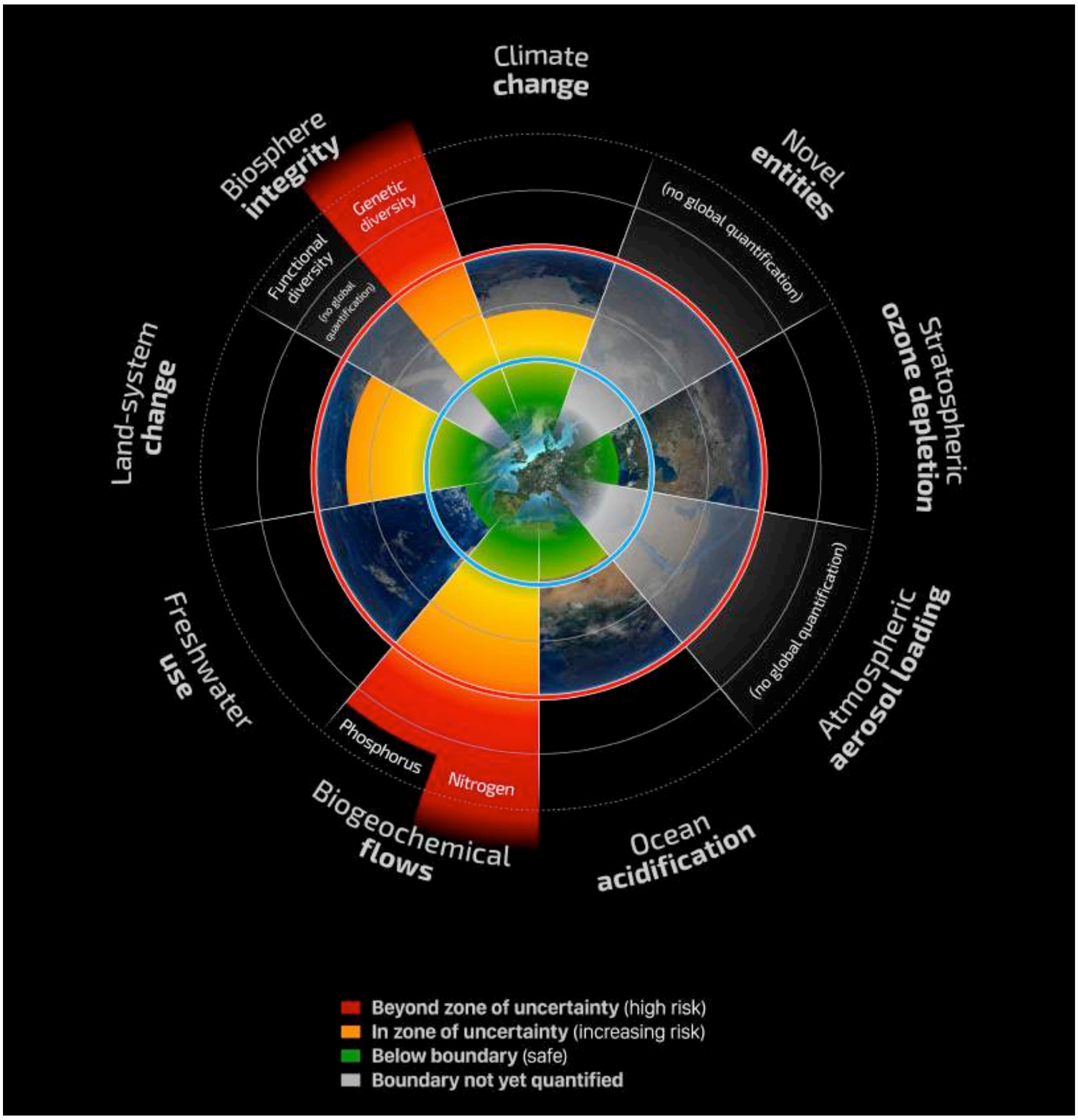


Figure 1.1 The current status of the control variables for the nine scientifically based biophysical thresholds, *i.e.* planetary boundaries. The green zone is the safe operating space (below the boundary), yellow represents the zone of uncertainty (increasing risk), and red is the high-risk zone. The inner blue circle depicts the planetary boundary itself; crossing the outer red circle means the high-risk zone is entered and could have disastrous consequences for humanity. The planetary boundaries are quantified into measurable control variables. The grey segments indicate there was no global quantification yet. Currently, four of the boundaries were crossed: the boundary of biogeochemical flows by the variables *nitrogen* and *phosphorus*, biosphere integrity by the variable *genetic diversity*, land-system change and, the boundary of climate change (Rockström et al. 2009; Steffen et al. 2015; Design by Globaïa)

1.1 The age of ecological restoration

Motivating people and policy makers to care for and restore natural habitats and their species has become a pressing matter (Barnosky et al. 2012; Ceballos et al. 2015) because the time window we have to halt further biodiversity loss is probably only two or three decades (Ceballos et al. 2017). We are thus in dire need for a concrete action plan to be able to reach the ambitious target of the Convention on Biological Diversity (CBD; Secretariat of the Convention on Biological Diversity 2014):

> *"…by 2050, biodiversity is valued, conserved, restored and wisely used, maintaining ecosystem services, sustaining a healthy planet and delivering benefits essential for all people"*

The global biodiversity commitment is one of the seventeen Sustainable Development Goals of the United Nations (United Nations 2015). Its aim is to conserve biodiversity worldwide and to restore degraded natural habitats and their biodiversity by 2030. We are not on track to reach this aim and need measurable goals and indicators to monitor our progress (Mace et al. 2018). The European Union incorporated the biodiversity commitment into policy, in the Habitats Directive (92/43/EEC) and Birds Directive (79/409/EEC - 2009/147/EC). Here as well, targets are far from reached. According to the 2007-2012 State of Nature Report, 60% of the non-bird species and more than three quarters of the European habitats are in an unfavourable or bad status, while only 4% of the non-bird species and habitats show an improving trend (European Commission 2015).

Preventing further habitat loss should be prioritized (Millenium Ecosystem Assessment 2005; Morris et al. 2006; Sutcliffe et al. 2014). It is far easier and cheaper to conserve natural systems than to restore them (Balmford et al. 2002). Involving all local stakeholders enables sustainable conservation that is also socially justifiable (Vucetich et al. 2018). Next to conservation as such, enlarging habitats of adequate environmental quality by restoring deteriorated habitats is necessary to halt biodiversity loss (Aronson and Alexander 2013). Perring et al. (2018) defined ecological restoration as:

> *"The process of assisting the recovery of damaged, degraded or destroyed socio-ecological systems in changing environments, for the benefit of people and nature across scales."*

This definition also takes account of the importance of the socio-ecological entanglement (restoration does not take place in a vacuum but are interconnectied with various social aspects), as well as of the various scales of needs (local to global) of restoration efforts and scales of whom to involve in restoration (from governments to local actors). Effective restoration requires identification of threats, understanding the underlying ecological mechanisms that can influence successful restoration, and recognition of appropriate interventions for a given context (Perring et al. 2015). Restoration efforts have to be carefully monitored, and success made measurable by defining restoration targets that are both ambitious and realistic. The *era of ecological restoration* (*sensu* E. O. Wilson) is around the corner, and evidence-based assessments are needed for verifying successful restoration techniques (Suding 2011).

1.2 Semi-natural grasslands: threatened hotspots of biodiversity

In 2013, semi-natural grasslands covered 28% of the European grassland area (197,400 km^2; data for EU28; Eurostat FSS). In contrast to primary grasslands, semi-natural grasslands are not climax vegetation and are man-made. They exist through continuous management, *i.e.* mowing (bi-)annually or grazing, to halt the succession towards forest (Hansson and Fogelfors 2000). Because of the link with traditional hay-making and pasturing, semi-natural grasslands are considered *Biological Cultural Heritage*, with high historical, cultural and esthetic values (Eriksson 2018). In their review on Palaearctic grasslands, Dengler et al. (2014) write:

> *"Semi-natural grasslands are an essential part of the cultural landscape of Europe and resulted from centuries or millennia of low-intensity land use since the beginning of the Neolithic period […] Such grasslands are mainly used for grazing by livestock (pastures) or hay-making (meadows), but might also be a successional stage of abandoned arable fields."*

Semi-natural grasslands can be extremely species-rich and break global records across biomes for vascular species density at survey areas below 100 m^2. For example, in a Transylvanian meadow in Romania, 98 plant species were found per 10 m^2 (Wilson et al. 2012). This high biodiversity is not limited to vascular plants but extends to other taxa as well, *e.g.* bryophytes (Virtanen et al. 2000), fungi (Griffith et al. 2012; Ozinga et al. 2013), butterflies (van Swaay et al. 2006; see Fig 1.3h), moths (Fox 2013), bumblebees (Goulson et al. 2005; Carvell et al. 2006) and oligolectic solitary bees (Wood et al. 2016; see Fig. 1.3i). For example, out of 436 well-studied butterfly species in Europe, 57% need semi-natural grasslands as their main habitat for feeding and completing their life-cycle (van Swaay et al. 2006).

European grasslands are recognized as hotspots of biodiversity, because they harbor a large proportion of the European biodiversity, but they are also threatened (Dengler et al. 2014; Habel et al. 2013) and among the most affected habitats in Europe and Central Asia (IPBES, 2018): about half of the grassland habitat types protected by the Natura 2000 framework (n=53) are threatened to some degree according to the European Red List of Habitats (Janssen et al. 2016). Among the deteriorating grassland habitat types are the species-rich *Nardus* grasslands, European habitat type 6230* and a priority habitat type in the Habitats Directive (Annex I), *i.e.* a habitat type in danger of disappearance with its natural range mainly within the territory of the European Union (95% inside the EU28 territory; Gigante et al. 2015). *Nardus* grasslands are listed on the European Red List of Habitats despite their large natural range (Fig. 1.2; Gigante et al. 2015). The conservation status "U2", *i.e.* "unfavourable – bad", in the Alpine, Atlantic, Boreal and Continental biogeographic regions is caused by the strong decrease in area as well as habitat quality during the second half of the 20th century. In the Atlantic biogeographic region (Belgium, the Netherlands, Northern Germany and Southern Denmark), *Nardus* grasslands were part of heathland systems with traditional pasturalism. Nowadays, they are almost completely converted into intensively used grasslands for forage production, arable land for intensive crop cultivation, forest, or urban land: in the Netherlands 95% of the heathland vegetation types were lost between 1850 and 2000 with similar declines in northern Belgium (Odé et al. 2001) and still continues to deteriorate: over the last 20 years, the quality of many of the *Nardus* grasslands in the Netherlands has decreased further; and, in 2016 the area of well-developed *Nardus* grassland was estimated at only 30-40 ha (van der Zee et al. 2017). Remnant *Nardus* grasslands are largely confined to nature reserves, often restricted to small areas (Gigante et al. 2015), and under pressure of acidification and eutrophication due to atmospheric nitrogen deposition (Stevens et al. 2011a), invasion by non-native species and altered precipitation due to climate change (Gigante et al. 2015).

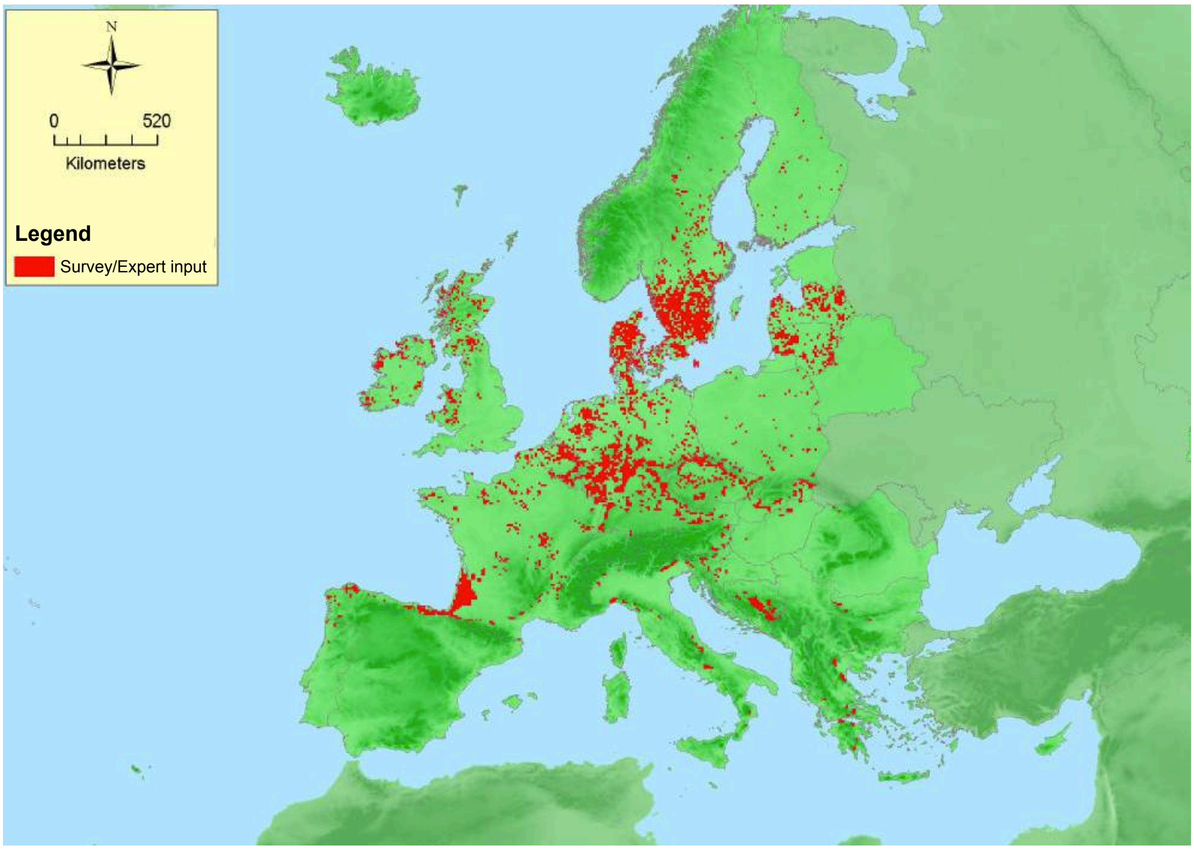


Figure 1.2 Distribution map of Habitat type E1.7 Lowland to submontane, dry to mesic *Nardus* grasslands (Gigante et al. 2015)

The 6230* habitat type includes a large variety of plant communities (*i.e.* associations), with varying ecological situations (hydrology, acidity, soil type), however, they are all typified by nutrient-poor (*i.e.* oligotrophic) soil conditions. In the Atlantic biogeographic region, a subtype of the 6230* habitat type according to the European Nature Information System EUNIS classification is categorized as "*E1.7 Lowland to submontane, dry to mesic Nardus grasslands*". In northern Belgium, *Nardus* grassland occurs on a wide variety of soil types: on sand, loam and peat soils but mostly on loamy sand or sandy loam soils (Zwaenepoel and Stieperaere, 2002). Three associations are distinguished by Zwaenepoel and Stieperaere (2002): (i) *Festuco rubrae-Genistelletum sagittalis*, the most calcareous type (pH_{H2O} 4.5-7.5), occurring on gravel depositis on top of calcareous substrate; (ii) *Galio hercynici-Festucetum ovinae*, occurring on sandy loam that has lost basic cations due to leaching (pH_{H2O} 4.3-6.0); and, (iii) *Gentiano pneumonanthes-Nardetum*, occurring on soils containing more organic material and typically acid to neutral (pH_{H2O} 4.0-6.4). More recently, De Saeger and Wouters (2017) defined four subtypes within the 6230-grasslands: (i) 6230_hn: dry *Nardus* grasslands; (ii) 6230_hmo: moist *Nardus* grasslands; (iii) 6230_hnk: dry, calcareous *Nardus* grassland; and, (iv) 6230_ha: species-rich grasslands of the *Agrostis capillaris* type (see Appendix 1.A for an identification key). This PhD thesis handles the restoration of the 6230_hn subtype, a species-rich grassland type (in Chapter 2, we report a species-richness of up to 49 species per 9 m^2) with typical species such as *Succisa pratensis*, *Nardus stricta*, *Potentilla erecta* (complete species list in Appendix-Table 1.A) and will be further simply called "*Nardus* grassland".

Figure 1.3 on the next page: Species of *Nardus* grasslands. From left to right, top to bottom: *Potentilla erecta*[a], *Succisa pratensis*[a], *Gentiana pneumonanthe*[b], *Briza media*[b], *Pedicularis sylvatica*[b] (Photo © Danny Laps), *Polygala serpyllifolia*[b], *Hygrocybe conica*[c], *Pyrgus malvae*[d] (Photo ©Vilda/Lars Soerink), *Andrena marginata*[e] (Photo © GBIF, Norway, 2017). The plant species are [a] near threatened or [b] vulnerable according to the Belgian Red List. [c] *H. conica* is among the most common waxcap species associated with *Nardus* grasslands, most other waxcap species are very rare (Ozinga et al. 2013). [d] *P. malvae* is associated with *P. erecta* (Maes and Van Dyck 1996) and is critically endangered in Flanders (Maes et al. 2012a). [e] *A. marginata* is associated with *S. pratensis*, but has gone extinct from the Netherlands since 1962 (Peeters et al. 2012; Reemer 2018) and probably from Flanders (personal communication Jens D'Haeseleer)


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

1.3 Land-use intensification: from oligotrophic to eutrophic ecosystems

1.3.1 Changes in historical and current land-use intensity

The intensification of grassland management is a global trend caused by economic-driven agricultural systems (Oenema et al. 2014). Farmers that aim to maximize their return on investment scale up their food and feed production and intensify their land-use; small, more traditional farmers generally quit farming. The environmental impacts of intensified farming practices, such as species-loss (Stoate et al. 2009), have indirect and direct effects on *Nardus* grasslands. Indirectly, neighbouring *Nardus* grasslands to intensively used farmland are mainly affected by atmospheric nitrogen deposition, an important indirect side-effect of intensification which leads to species-loss (Dupré et al. 2010, De Schrijver et al. 2011) and a shift to grass-dominated vegetation (Bobbink et al. 1998; Stevens et al. 2011a; see §1.3.3). Directly, *Nardus* grasslands are affected even more severely when they are converted into intensively used grasslands for forage production, such as has occurred to large proportions of *Nardus* grasslands in western Europe (Gigante et al. 2015). The level and period over which land-use has been intensified, results in a gradient of historical land-use intensification (*sensu* Wasof et al. 2019). We distinguish three types of grassland systems, according to historical and current management, soil chemistry, plant and soil biota communities (Fig. 1.5):

Oligotrophic grasslands such as *Nardus* grasslands on acid sandy soils that have been managed traditionally up to now: no fertilization and mowing once annually with occasional aftermath grazing. Net nutrient removal occurs annually through mowing combined with removing hay (Tilley 2014); autumn grazing leads to more patchyness in nutrient availability rather than to nutrient depletion (Mikola et al. 2009). The soil is nutrient-poor (see §1.3.2). Vegetation growth is limited by nutrients, limitation by phosphorus or co-limitation by nitrogen and phosphorus, which allows for species-rich vegetation with red-listed plant species (see §1.3.3).

Mesotrophic grasslands are grasslands for which land-use management has been intensified (see *Eutrophic grasslands*) and again turned into traditional management (without fertilization). If nutrient input by fertilization stops, after some years, biomass production will again be limited by nutrients, this time not by phosphorus but rather by nitrogen and/or potassium (Pegtel et al. 1996; see §1.3.2). The accumulated phosphorus is still present in the soil, though, and soil phosphorus levels are still high. The land-use history of these grasslands can differ widely. The species-richness varies from species-rich to species-poor (see §1.3.3).

Eutrophic grasslands are formerly oligotrophic grasslands transformed by land-use intensification: long-term intensive management, including practices to increase biomass production and feed quality such as fertilization, cultivation, tillage, altering hydrology, intensive grazing, herbicide use against dicotyledons and seeding with fast-growing plant species such as *Lolium perenne*, *Dactylis glomerata* and *Phleum pratense*. In Flanders, the recommendation for annual fertilization of intensively used grasslands on sandy soil, allowing four to six annual biomass harvests for forage production, is 354 kg nitrogen, 25 kg phosphorus and 292 kg potassium per hectare (Rombouts et al. 2015). The added nitrogen and potassium can be completely taken up and removed again in grasslands (Schellberg et al. 1999) when best practice principles for nutrient management are applied (Goulding et al. 2008). Nitrogen and potassium occur in highly mobile molecules prone to uptake by plant roots or leaching from the soil over time (Stevenson and Cole 1999). Phosphorus, on the other hand, rapidly becomes less available for plant uptake in the soil (Stevenson & Cole 1999), is oversupplied and accumulates in the soil through the years of high nutrient input (Box 1; see §1.3.2). For example, from 1970 to 2000, western European agricultural soils were fertilized annually with on average about 20 to 30 kg P ha^{-1} whilst only 10 kg P ha^{-1} was removed with the crops (Sattari et al. 2012). Legislation to regulate the maximal phosphorus input is very different between the European countries, with the highest maximum phosphorus applications standards in Flanders, Brittany and the Netherlands (Amery and Schoumans 2014), *n.b.* regions typified by intensive livestock production and consequently the largest phosphorus surpluses (Fig. 1.4; De Smet et al. 1996; Bomans et al. 2005); although, these legislations are becoming more strict, *e.g.* in Flanders the *Manure Action Plan 5* (MAP5) for the period 2015-2018 restricted for the first time the overuse of phosphorus. These regulations may have come too late for acid, sandy soils in Flanders where accumulation of phosphorus in the top soil layer (0-30 cm) has leached to deeper soil layers (30-90 cm) in a majority of fields (De Bolle et al. 2013a). The high nutrient availability due to fertilization causes the vegetation to shift from biomass production limited by nutrients to limitation by light as the vegetation is dominated by a few fast-growing plant species (Hautier et al. 2009; see §1.3.3).

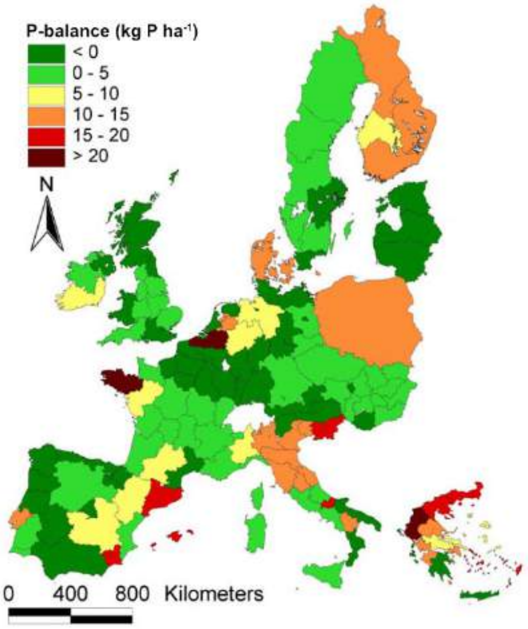


Figure 1.4 European map of the phosphorus balance (kg P ha^{-1}), *i.e.* the difference between input by fertilization and removal by crops derived from data between 1990 and 2003. This figure does not take into account manure transfers between regions or withdrawal of phosphorus from the phosphorus-cycle (Bomans et al. 2005)

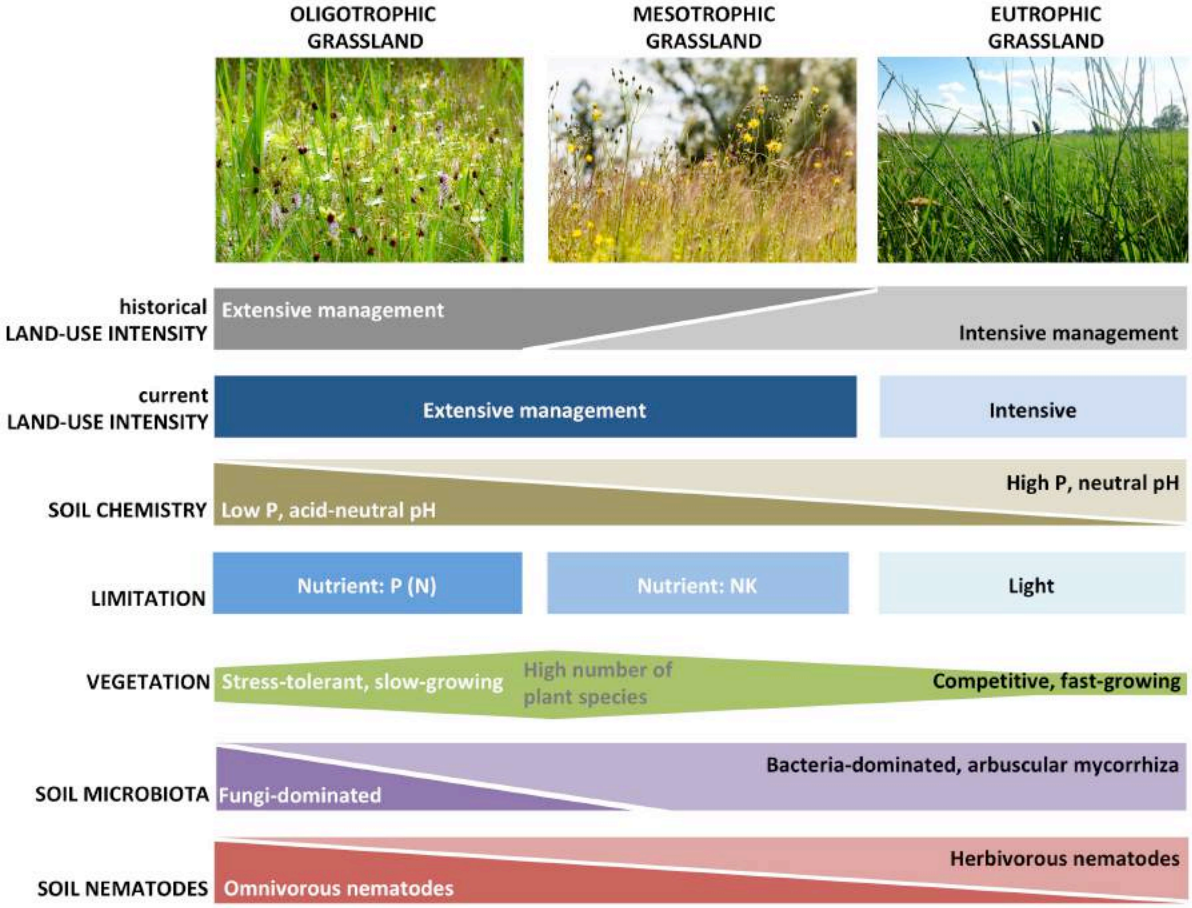


Figure 1.5 Remnant oligotrophic *Nardus* grasslands differ from mesotrophic and eutrophic grasslands that originated through increasing (historical) land-use intensity of former *Nardus* grasslands (see §1.3.1). The three grassland types occur along a gradient of (historical and current) land-use intensity (*sensu* Wasof et al. 2019). Soil chemistry variables, bioavailable phosphorus concentrations and pH, differ according to Wasof et al. (2019) and the other sources in §1.3.2. The type of nutrient limitation shifts from nutrient limitation in oligotrophic and mesotrophic grasslands to light limitation (Hautier et al. 2009). Oligotrophic grasslands are limited by phosphorus (see §1.3.3). Mesotrophic grasslands are, due to historical land-use intensification, not limited by phosphorus but by nitrogen and/or potassium (Pegtel et al. 1996). The vegetation differs according to Van Daele et al. (2017) and Wasof et al. (2019): from slow-growing stress-tolerant communities in oligotrophic grasslands to fast-growing competitive communities in eutrophic grasslands. Species-richness is high in oligotrophic grasslands, with a high number of rare species (see Chapter 2). Slight fertilization causes a turnover in the species assembly and species-richness may even increase (Bobbink et al. 1998; and other sources in §1.3.3); however, species-richness decreases with further nutrient-input (see §1.3.3). Soil microbiota and nematodes are represented according to the findings of Wasof et al. (2019)

Table 1.1 Characteristics of the management, soil and vegetation of oligo-, meso- and eutrophic grasslands (see Fig. 1.5)

	Oligotrophic grassland	Mesotrophic grassland	Eutrophic grassland	*p*-value
Floristic association [a]	Nardus	grass-herb mix	Lolium perenne	
Management				
Historical land-use intensity [b]	low	medium-high	high	
Current land-use intensity	low	low-medium	high	
Soil				
bioavailable phosphorus [c]	low	mid-high	high	
[min-max](mg P_{Olsen} kg^{-1})	2-14	27-128	73-170	
pH_{H2O} [min-max] [c]	4.2-6.8	4.7-5.8	4.7-5.8	
Limitation [d]	(nitrogen) phosphorus	nitrogen potassium	light	
Vegetation				
Productivity [e] **(t dry biomass ha^{-1} y^{-1})**	1-3	3-6	> 8	
No. plant species [f] **(No. per 9 m^2)**	22	8-13	8	
No. red-listed species [f] **(No. per 9 m^2)**	4	0	0	
Species characteristics [g]				
Ellenberg-N	1.4 ± 0.6^{A}	3.5 ± 1.3^{B}	4.8 ± 1.2^{C}	***
SLA (cm^2 g^{-1})	18.4 ± 8.9^{A}	27.4 ± 9.5^{B}	27.5 ± 9.8^{AB}	**
C	20 ± 21	27 ± 19	37 ± 13	NS
S	50 ± 30^{B}	27 ± 22^{A}	18 ± 18^{A}	***
R	30 ± 14^{A}	47 ± 19^{AB}	45 ± 21^{B}	**

[a] *Nardus* grassland = 6230* grasslands of the lowlands and submontaneous region (EUNIS E1.7; Gigante et al. 2015); grass-herb mix grasslands on acid, sandy soils in dry to moist conditions resemble either the basal community of *Anthoxantum odoratum* (syntaxon code: 16RG24 sensu Schaminée et al. 2015) or *Holcus lanatus-Silene flos-cuculi* (syntaxon code: 16RG7 sensu Schaminée et al. 2015); *Lolium perenne* grasslands on acid, sandy soils in dry to moist conditions resemble the basal community *Holcus lanatus-L. perenne* (syntaxon code: 16RG23 sensu Schaminée et al. 2015) [b] *sensu* Wasof et al. 2019 [c] Source of data: Oligotrophic grasslands: the remnant *Nardus* grasslands in chapter 2 (n=34); Meso- (n=17) and eutrophic (n=9) grasslands: Wasof et al. 2019 (pH_{H2O} converted from pH_{KCl} following Van Lierop 1981) [d] Assumption: see §1.3.3 [e] Assumption: *Nardus* grasslands according to (Bakker et al. 2002; Hejcman et al. 2010a; Bedia and Busqué 2013); grass-herb mix according to phase 3 grasslands in Schippers et al. (2012); *Lolium perenne* grasslands according to phase 0 and 1 grasslands in Schippers et al. (2012) [f] No. per 9 m^2; *Nardus* grasslands based on 34 Nardus grasslands (Chapter 2); grass-herb mix based on 7 post-fertilization grasslands (Chapter 2) and 16 experimental plots with mowing management for five years (Chapter 3); *Lolium perenne* grasslands based on 8 experimental plots with P-mining management, i.e. fertilized with nitrogen and potassium, on phosphorus-rich soil conditions (> 80 mg P_{Olsen} kg^{-1}) (Chapter 3) [g] Characteristics for the oligotrophic (n = 19), mesotrophic (n = 43) and eutrophic (n = 10) grassland species following the species selection of Van Daele et al. 2017). The species list is shown in Appendix-Table 1.B; Traits were derived from the LEDA Traitbase (Kleyer et al. 2008) with the function tr8 of the R package TR8 (Bocci 2015); CSR-values were derived from Pierce et al. (2017); Analyses of variance testing between the grassland groups with the functions lm and anova of the R package stats. Significant differences between grassland groups are indicated by the p-value: *** $p < 0.001$; ** $p < 0.01$; NS = not significant

1.3.2 Changes in soil chemistry

Repeated fertilization, particularly in areas with a surplus of animal manure, leads to nutrient accumulation in agricultural soils (Bouwman et al. 2012; Ringeval et al. 2017). The nitrogen and potassium levels generally decrease again through mowing management (up to > 200 kg N ha^{-1} y^{-1}, Jones et al. 2017) and leaching (on average 50 kg N ha^{-1} y^{-1} in intensively used arable land, Bouwman et al. 2012) after fertilization has ceased or atmospheric deposition has been reduced; phosphorus is more persistent in the soil (Storkey et al. 2015). Differences in historical land-use intensity cause different phosphorus loadings of grassland soils: oligotrophic < mesotrophic < eutrophic (Wasof et al. 2019; Fig. 1.6). In remnant, unfertilized *Nardus* grasslands, the bioavailable phosphorus concentration is generally lower than 12 mg P_{Olsen} kg^{-1} (Table 1.1; Chapter 2); fertilization leads to increased concentrations of bioavailable, slowly-cycling and total phosphorus (Box 1; Table 1.1).

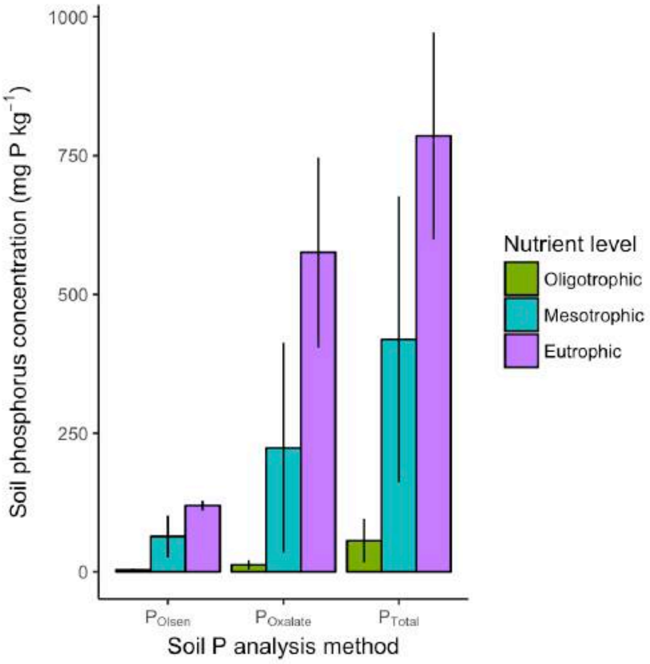


Figure 1.6 Soil phosphorus concentrations (mean with standard deviation) for the bioavailable (P_{Olsen}), slowly-cycling ($P_{Oxalate}$) and total phosphorus pool (P_{Total}) in soil samples of the top soil layer (0-10 cm) of oligo-, meso- and eutrophic grasslands (data was derived from the same set of grasslands shown in Table 1.1; different P pools are defined in Box 1)

Nardus grasslands are characterized by acid to neutral soil conditions (pH_{H2O} of 4.2-6.8; INBO). Intensified land-use in grasslands on acid soils is accompanied by increased soil pH by liming with calcium and magnesium-rich materials such as limestone till a level where phosphorus bioavailability is maximal (pH_{H2O} of 6; Goulding et al. 2008; Box-Fig. 1.3). The pH of mesotrophic and eutrophic grasslands was found to be increased (Wasof et al. 2019), which is not *per se* a negative aspect of intensification to oligotrophic grasslands under the condition liming is not accompanied by increased productivity (Hejcman et al. 2007; Holland et al. 2018). Acidification by atmospheric nitrogen (and sulfur) deposition is one of the threats to the vegetation of acid grasslands, such as *Nardus* grasslands, which are more sensitive to acidification than grasslands on well-buffered soils (Stevens et al. 2011a). Sensitive plant species may disappear due to acidification below a pH_{H2O} value of 4.6 (Merunková and Chytrý 2012) or 5 (Roem and Berendse 2000), due to toxic effects by increased bioavailability of aluminium and ammonia (Rout et al. 2001; Van Den Berg et al. 2005; Kleijn et al. 2008; De Graaf et al. 2009). The occurrence of some acid-tolerant species such as *Calluna vulgaris* and *Gentiana pneumonanthe* may be decreased by liming (Roem and Berendse 2000). However, in our study, the increased soil pH in the mesotrophic and eutrophic grasslands was accompanied by an increased availability of nutrients, which do pose a severe bottleneck for *Nardus* grassland species (see § 1.3.3).

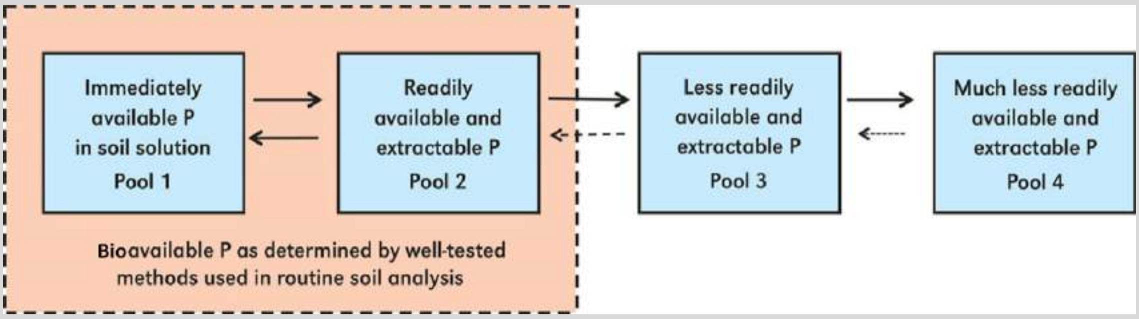
Box 1. Soil phosphorus

Understanding and predicting changes in soil phosphorus availability is complicated because of the various inorganic and organic types of phosphorus occurring in the soil (De Schrijver et al. 2012; Kruse et al. 2015) and the numerous chemical methods for phosphorus extraction (overview of current methods in Wuenscher et al. 2015). It is important to choose the appropriate extraction method for each research question. For instance, the total phosphorus concentration may give a quick view on the historical phosphorus fertilized to a field. Characterizing soil phosphorus concentrations into different pools or fractions may provide a more detailed insight, *e.g.* historical fertilization and depletion processes (Negassa and Leinweber 2009).

Various conceptual soil phosphorus models have been developed (Alvarez and Steinbach 2017), among which the *Phosphorus Pool Model* of Roberts and Johnston (2015), which considers inorganic soil phosphorus to reside in four inter-related pools that differ in their availability for plant-uptake (Box-Fig. 1.1).

The **bioavailable phosphorus pool** (first and second pools in Box-Fig. 1.1) contains phosphorus in the soil solution and phosphorus that is surface-adsorbed to soil components such as iron and aluminium (hydr)oxides, organic material and clay particles. This bioavailable phosphorus pool is immediately or within a growing season available to plants and can be determined through extraction in sodium bicarbonate ($NaHCO_3$; P_{Olsen}), a method developed for calcareous soils (Olsen et al. 1954) that gives satisfactory results also in acid soils (Renneson et al. 2016). In calcareous soils, the $NaHCO_3$ solution lowers the concentration of Ca^{2+} in solution as $CaCO_3$ precipitates, and therefore promotes calciumphosphate dissolution. In neutral or acid soils, the solubility of aluminum and iron phosphates increases as the OH^- ions in the Olsen extract lower the concentrations of Al^{3+} by aluminate complex formation and of Fe^{3+} by precipitation as iron oxide (Wuenscher et al. 2015). The concentration of P_{Olsen} in the soil correlates well with phosphorus uptake in plants in species-rich grasslands (Gilbert et al. 2009) and is used frequently for ecological research for quite a wide pH-range (Loeb et al. 2009; Ceulemans et al. 2014; Johnston et al. 2016).

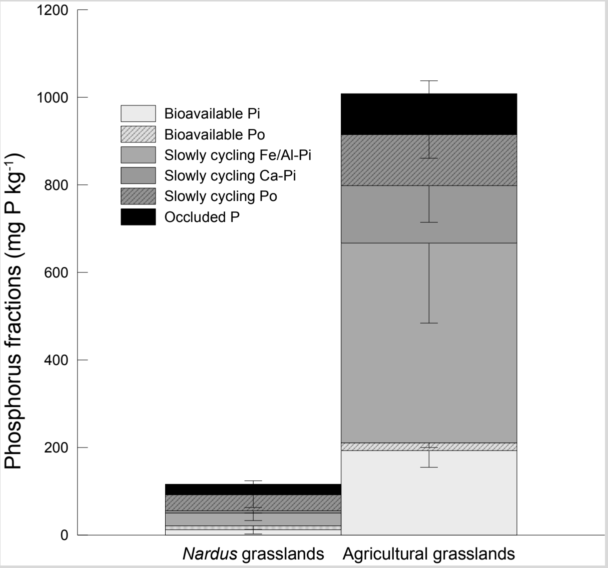
For the objective of re-establishing phosphorus-poor soil conditions, it is important to not only get insight into the bioavailable phosphorus pool, but also into the **slowly cycling phosphorus pool** that can feed the bioavailable pool on a timescale of many years (Syers et al. 2008; De Schrijver et al. 2012; Johnston et al. 2014). These third and fourth pools consist of phosphorus that is more strongly bound onto and contained in soil components. Extraction with ammonium oxalate ($P_{Oxalate}$; Schwertmann 1964) gives a measure of this slowly cycling phosphorus pool (van Rotterdam et al. 2012).



Box-Figure 1.1 The four pools of inorganic phosphorus in soils that can become bioavailable over different time scales, categorized in terms of bioavailability and extractability (Roberts and Johnston 2015). Pools 1 and 2 (*i.e.* bioavailable P) can be assessed by the Olsen-method (P_{Olsen}); pools 3 and 4 (*i.e.* slowly cycling P) can probably be measured by the oxalate-method ($P_{Oxalate}$), but it should be assessed whether the oxalate-method gives a complete picture of these pools

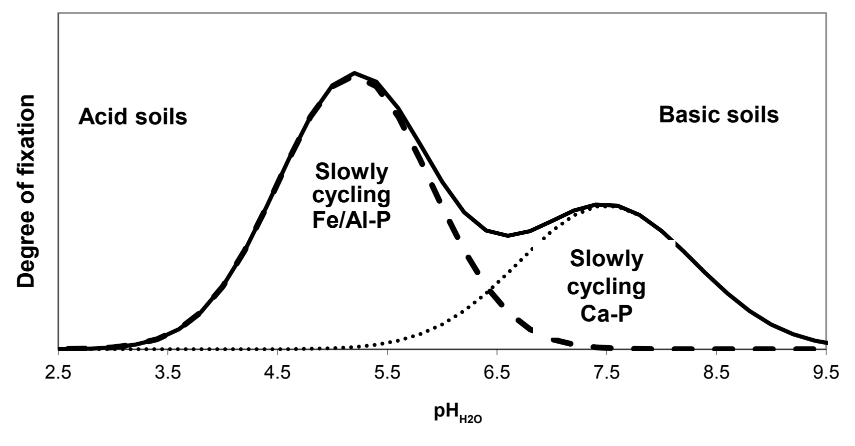
This model is a simplified, though understandable, interpretation of the various soil phosphorus fractions. Soil phosphorus can be characterized in greater detail by the sequential fractionation procedure of Hedley et al. (1982). Phosphorus is removed progressively from a soil sample by sequentially using stronger extractants in the chemical analysis. According to Richter et al. (2006), the nine fractions can be summarized into six inorganic and organic phosphorus fractions of variable bioavailability: bioavailable inorganic and organic phosphorus, inorganic and organic slowly cycling phosphorus associated to aluminium and iron oxides, slowcly cycling inorganic phosphorus associated to calcium and, the remaining occluded phosphorus, *i.e.* the residual phosphorus that could not be extracted by the previous steps. The occluded fraction is assumed to be unavailable for plant-uptake, even in the long-term, *i.e.* the non-biologically cycling phosphorus pool, and to have little impact on soil fertility (Stevenson and Cole 1999) and contains phosphorus that is associated to inorganic compounds that are very insoluble and to organic compounds that are considered resistant to mineralization. However, it was shown this pool may become available in the long-term (Richter et al. 2006).

Applying this analysis to oligotrophic and eutrophic grasslands on acid, sandy soils, reveals long-term fertilization affects all of the fractions, except the bioavailable organic phosphorus fraction (Box-Fig. 1.2). The occluded phosphorus pool takes a small proportion of the soil phosphorus fractions. In our further calculations of the time to phytomine phosphorus we do not consider the occluded pool, hence, we might (slightly) underestimate how long it takes for a soil to become depleted of phosphorus after fertilization. We further do not distinguish organic from inorganic fractions: the bioavailable phosphorus pool measured by extraction in sodium carbonate (P_{Olsen}) also contains organic phosphorus associated with soil organic surfaces and humic and fulvic acids (Blake et al. 2003; Alt et al. 2011); and, the slowly cycling phosphorus pool measured by extraction in ammonium oxalate contains a small amount of organic phosphorus (Koopmans et al. 2004).

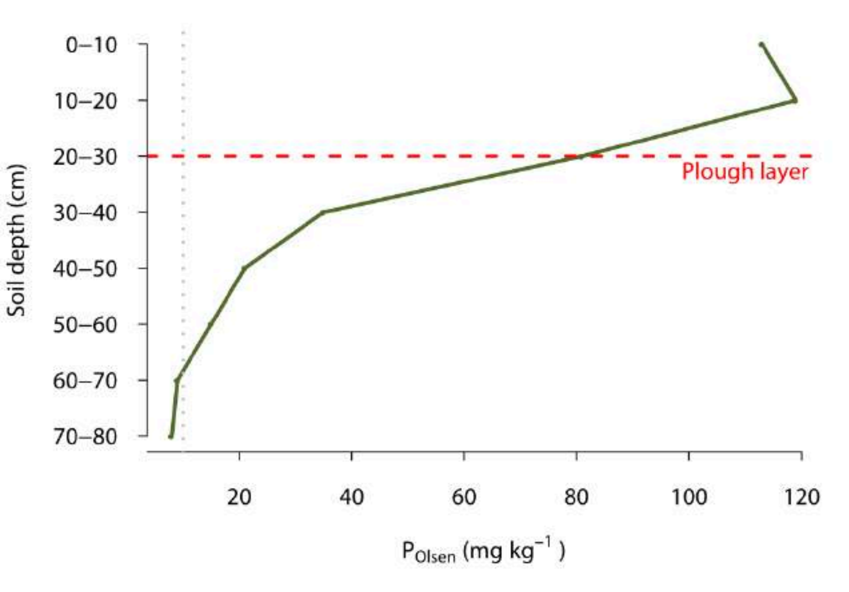


Box-Figure 1.2 Case study of the effects of fertilization on soil phosphorus fractions in a sandy soil: Inorganic (P_i) and organic (P_o) phosphorus fractions in *Nardus* grasslands (n=18) and intensively used eutrophic grasslands (n=12) in close vicinity to the *Nardus* grasslands in the 0-10 cm soil layer. These fractions are the result of Hedley fractionation (Hedley et al. 1982) and categorized according to (Richter et al. 2006). All fractions, except the bioavailable P_o-fraction were significantly higher in the eutrophic grasslands (De Schrijver et al. *unpublished data*)

The maximum sorption capacity of a soil increases with the proportion of small-size particles (sand < loam < clay), aluminium and iron oxide minerals and, is affected by the pH (McGechan and Lewis 2002). Sorption capacity is minimal and, hence, phosphorus bioavailability is maximal at pH_{H2O} around 6 (Goulding et al. 2008; Box-Fig. 1.3). Repeated excessive phosphorus fertilization increases the *phosphate saturation degree* ($PSD = [P_{Oxalate}/0.5\times(Al_{Oxalate}+Fe_{Oxalate})]\times 100(\%)$ with the concentrations of phosphorus, aluminium and iron in mmol kg^{-1}; (Van der Zee et al. 1990)). When a threshold is reached (25% for sandy soils), soils leach more than 0.1 mg orthophosphate per liter groundwater, a critical eutrophication limit for water bodies (Van der Zee et al. 1990; Rowe et al. 2016). These *phosphorus-saturated* soils are prone to leach phosphorus to deeper soil layers, as was found in the majority of intensively managed fields on acid, sandy soils and sandy loam soils in two large screenings (De Smet et al. 1996; De Bolle et al. 2013a). Phosphorus accumulation in the top soil layer (0-30 cm) was found to leach to deeper soil layers (30-90 cm) (De Bolle et al. 2013a) and was also observed in a eutrophic grassland by increasing the bioavailable phosphorus concentrations till the 50-60 cm soil layer (Box-Fig. 1.4).



Box-Figure 1.3 Degree of fixation of phosphorus in soils in relation to the soil acidity (pH_{H2O}). In acid soils with low soil pH, phosphorus is strongly adsorbed to iron (Fe) and aluminium (Al), while in soils with high pH values, phosphorus is absorbed to calcium (Ca). The maximal bioavailability of phosphorus lies around a pH of 6 (Stevenson and Cole 1999)



Box-Figure 1.4 Bioavailable phosphorus concentration (mg P_{Olsen} kg^{-1}) for each of the 10 cm soil layers until a depth of 80 cm in an intensively used eutrophic grassland on an acid, sandy soil in close vicinity to a remnant *Nardus* grassland (De Schrijver, *unpublished results*). Intensive management and fertilization caused soil phosphorus to leach from the 0-30 cm soil layer, which was tilled frequently, till deeper soil layers. The threshold value for the bioavailable phosphorus concentration in *Nardus* grasslands is indicated in a grey dashed line

1.3.3 Changes in vegetation

Oligotrophic *Nardus* grasslands have a low annual productivity, *i.e.* 1-3 t dry biomass ha^{-1} (Bakker et al. 2002; Hejcman et al. 2010a; Bedia and Busqué 2013). It is common knowledge that fertilization increases grassland productivity (Grime 1973; Parr 1986; Marrs 1993; Gough et al. 2000; Harpole and Tilman 2007). For example, in the field experiment in Rengen (Germany), several fertilizer treatments have been applied since 1941 to a *Nardus* grassland (Hejcman et al. 2010a) causing a range in annual productivity from on average 2.5 t dry biomass ha^{-1} in the unfertilized controls till 10 t dry biomass ha^{-1} in the plots annually receiving nitrogen, phosphorus, potassium and lime. Productivity in eutrophic grasslands may even reach more than 15 t dry biomass ha^{-1} (Liebisch et al. 2013).

The relation between species-richness and productivity has been studied for a long time (Grime 1973; Parr 1986; Gough and Marrs 1990; Marrs 1993; Grime 2001; Keddy 2005) and the quest for a general *law of nature* is still a hot-topic (*e.g.* Fraser et al. 2005; Adler et al. 2011; Pierce 2014; and the discussions that followed). Grassland vegetation shifts by fertilization from nutrient-limitation to light-limitation, which is accompanied by a loss in species-richness (Hautier et al. 2009). The study by Hautier et al. (2009) was performed with inorganic fertilizer containing nitrogen, phosphorus, potassium and some micronutrients at a medium rate of nitrogen and potassium input (2006: 40 kg N ha^{-1}, 35 kg P ha^{-1}, 60 kg K ha^{-1}; 2007: 200 kg N ha^{-1}, 50 kg P ha^{-1}; 80 kg K ha^{-1}), but was enough to favor the initially nutrient-limited fast-growing species to shade out the subordinate species. Adding light together with fertilizer to the grassland mesocosms resulted in productive *and* species-rich communities because light-limitation was lifted and did not hamper the subordinate species. Thus, when nutrient-limitation is removed from communities with numerous slow-growing species (*e.g. Nardus* grassland species), these communities are replaced by communities dominated by few fast-growing species that shade out the typical *Nardus* grassland species (Harpole and Tilman 2007; Hautier et al. 2009). The outcome of fertilization depends, however, on the quantity and type of nutrient or nutrient combinations added to the ecosystem. It is well-known that grasslands lose plant species due to fertilization with nitrogen (De Schrijver et al. 2011), phosphorus (Ceulemans et al. 2014; Hájek et al. 2017; van Dobben et al. 2017) or both (Schellberg and Hejcman 2007). It has been debated whether nitrogen or phosphorus fertilization was the most important driver for this biodiversity-loss with evidence in favor of nitrogen (Soons et al. 2017) and phosphorus (Schellberg and Hejcman 2007; Ceulemans et al. 2013; Ceulemans et al. 2014; van Dobben et al. 2017). Soons et al. (2017) and Roeling et al. (2018) argued that a shift from phosphorus limitation into nitrogen limitation does not necessarily lead to a loss of species, rather to a species turnover, *i.e.* species are being replaced by other species. Indeed, slight fertilization of oligotrophic grasslands was shown to result in a *status quo* in species-richness and in a species turnover (Bobbink et al. 1998; Kepfer-Rojas et al. 2018). However, endangered and rare species, such as the orchids *Dactylorhiza maculata, Platanthera bifolia* and *Listera ovata* (Hejcman et al. 2010b), persist in phosphorus or phosphorus-nitrogen-co-limited grasslands (Wassen et al. 2005; Fujita et al.

2014). The effect of potassium limitation on species-richness has been less-well researched and are less understood (Kayser and Isselstein 2005; Sardans and Peñuelas 2015); and, similarly co-limitation between nitrogen, phosphorus and/or potassium requires more investigation (Fay et al. 2015). There are, however strong indications that typical *Nardus* grassland species persist below a bioavailable phosphorus concentration of 12 mg P_{Olsen} kg^{-1} (see Chapters 2 and 6) and under phosphorus limitation (*e.g.* the Rengen experiment: Hejcman et al. 2010a; *e.g. Molinia caerulea*, *Potentilla erecta* and *Succisa pratensis* with high N:P ratio's: Roeling et al. 2018). But even below this bioavailable phosphorus threshold, a negative relation between bioavailable phosphorus and species-richness has been observed in *Nardus* grasslands (Riesch et al. 2018). We may assume the shift from phosphorus-limitation into nitrogen-limitation in our defined mesotrophic grasslands with an intensive fertilization history, will be accompanied by a loss of species (Table 1.1). As a hypothetical illustration, the "catastrophe model" by Parr (1986) may explain the shift from phosphorus-limitation to nitrogen-potassium co-limitation (shift from species-rich oligotrophic to species-rich mesotrophic community in Fig. 1.7), followed by a sudden drop to *e.g.* nitrogen-limitation (shift from species-rich mesotrophic to species-poor mesotrophic in Fig. 1.7), again followed by a shift to a system with light-limitation (shift from species-poor mesotrophic to species-poor eutrophic community in Fig. 1.7). According to Parr (1986), the introduction of a disturbance, *e.g.* increased mowing frequency, may lead to a jump back from the lower to the upper level of species-richness.

The characteristics of the plant species typical of oligotrophic, mesotrophic and eutrophic grassland vary considerably (Table 1.1). The plant community shifts from plant species with a *slow-growth strategy* in oligotrophic grasslands where nutrient cycling is slow, into a community with a *fast-growth strategy* in eutrophic grasslands where nutrient-cycling is fast (*sensu* the fast-slow plant economics spectrum in Reich 2014). For example, the Ellenberg nutrient indicator value, which correlates well with biomass production (Hill and Carey 1997; Chytrý et al. 2009), significantly distinguishes the three grassland types (Table 1.1). Further, the specific leaf area is lower and the Grime stress-score is higher in oligotrophic grasslands (Fig. 1.8; Table 1.1). Oligotrophic plant communities consists of slow-growing species, *e.g. Nardus stricta*, *Potentilla erecta, Danthonia decumbens, Veronica officinalis* and *Luzula multiflora* (De Saeger and Wouters, 2017; Appendix 1.A), which have a conservative and stress-tolerant resource use strategy (Fig. 1.8; Pierce et al. 2017). These traits typically include slow growth rate, low specific leaf area, high leaf longevity, high recalcitrance (*i.e.* with a low decomposition rate) and high resorption of nutrients from senescing leaves (Table 1.1; Wright et al. 2004). Mesotrophic grassland communities are typified by species with intermediate to fast-growing traits, *e.g. Anthoxanthum odoratum, Centaurea jacea* and *plantago lanceolata*. Fast-growing species are adapted to coping with light-limited growth conditions by *fast-growth traits*, such as high biomass production, low tissue lifespan, high rate of resource acquisition, low recalcitrance and high biogeochemical cycling (Wright et al. 2004). Eutrophic grassland communities are typified by such species are dominated by typically tall, long-lived and strongly competitive (high C-value on the CSR-scale; Fig. 1.8) species which require high nitrogen input (high Ellenberg nitrogen indicator value). Wesche et al. (2012) showed an increased average Ellenberg nitrogen indicator value is accompanied by a loss of nectar-producing grassland herbs, likely negatively affecting the abundance of pollinating insects.

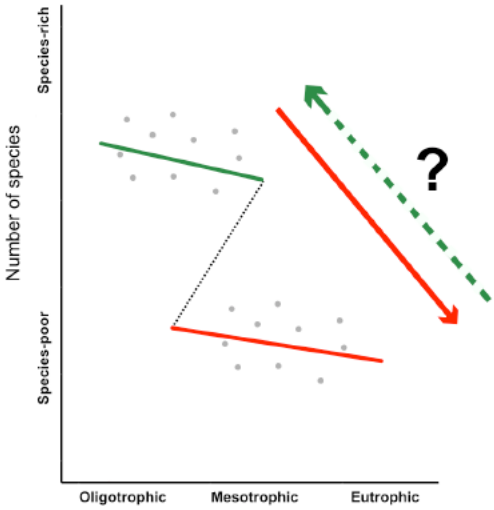


Figure 1.7 Theoretical effects of intensified land-use and nutrient enrichment on species-richness and the position of the three grassland categories defined in this thesis (oligotrophic, mesotrophic and eutrophic). Mesotrophic grasslands can be species-poor but also have the potential to harbor a high species-richness. Scheme of a discontinuous response in species-richness after the "catastrophe model" by Parr (1986). The red arrow shows changes in species-richness as it responds to small changes in nutrient status: starting with high species-richness in oligotrophic grasslands, species-composition alters when moving towards mesotrophic grasslands with initially no loss in species-richness. Suddenly, a jump occurs towards the species-poor mesotrophic grasslands (*i.e.* a catastrophe event) before continuing to become a species-poor eutrophic grassland. It is unclear whether the shift from species-poor eutrophic grasslands can be made back to species-rich oligotrophic grasslands (green arrow)

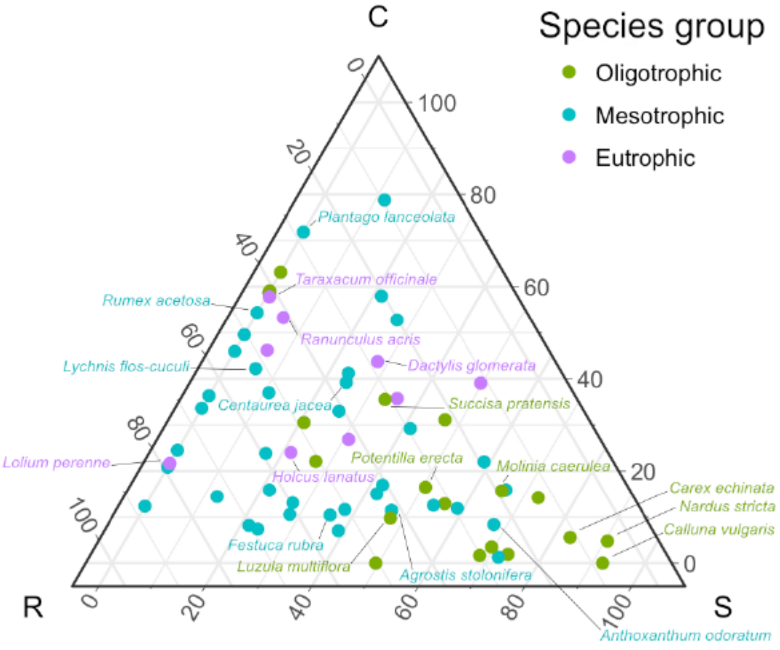


Figure 1.8 CSR-strategy of oligotrophic (green), mesotrophic (blue) and eutrophic (purple) grassland species (n = 72) in Van Daele et al. (2017). CSR-values as defined by Pierce et al. (2017); names shown for the nineteen species studied in Chapter 6; all 72 species listed in Appendix 1.B

1.3.4 Changes in soil biota communities

Below-ground biota are ecologically linked with above-ground biota (Wardle et al. 2004) and hence with the spectrum of slow to fast resource economics the plant community represents (*sensu* Reich 2014). In oligotrophic habitats, the slow-cycling nutrient turnover in the soil food web is fed by nutrient-poor plant material and typically consists of fungi, enchytraeid worms and macro- and micro-arthropods. In eutrophic conditions, the fast-cycling nutrient turnover in the soil food web is fed by nutrient-rich plant material and is made up of bacteria, earthworms and microfauna (*i.e.* nematodes). Through plant-soil feedbacks, soil biota communities can interact with and shape the plant community (Thakur and Wright 2017). Negative plant-soil feedbacks, *e.g.* through soil pathogens, were found to occur more in fast-growing plant species; positive plant-soil feedbacks, *e.g.* by mutualists such as mycorrhiza that help with nutrient acquisition, were more common in slow-growing plant species (Lekberg et al. 2018). Whether plant-soil feedbacks affect plant-plant competition depends on soil fertility: in eutrophic soils, negative plant-soil feedback effects are generally outweighed by plant-plant competition; and, in oligotrophic soils, mutualistic plant-soil feedbacks can prevent competitive exclusion and thus favour slow-growing plant species (Lekberg et al. 2018).

Land-use intensification can decrease the diversity and alter the community assembly of soil bacteria, fungi, collembola and nematodes (Wood et al. 2017). Fertilization, increased mowing frequency, liming, soil tillage and altered vegetation composition will each have either positive or negative effects on certain species groups. In a large grassland fertilization experiment, for instance, fertilization with nitrogen and phosphorus caused only slight changes to the diversity of the microbial community, but lead to a consistent shift in community composition (Leff et al. 2015). In the fertilized plots, microbial species with eutrophic traits were relatively more abundant while oligotrophic microbial species, such as arbuscular mycorrhiza, declined. Contrastingly, Wasof et al (2019) found a higher relative abundance of arbuscular mycorrhiza in eutrophic grasslands than in oligotrophic grasslands, which is probably explained by practice of liming in those intensively used grasslands. While these acid, sandy soils in unlimed conditions with possibly high bioavailability in aluminium can have negative effects on arbuscular mycorrhiza (Seguel et al. 2013), liming might have a positive effect. For arbuscular mycorrhiza, the abiotic soil conditions seem more important than the identity of the host plant species (Van Geel et al. 2017). Nematodes, on the other hand, do show a *plant species effect* (Viketoft et al. 2005). In Wasof et al (2019), fast-growing plant species (*Lolium perenne, Holcus lanatus* and *Taraxacum officinale*) were associated with herbivorous nematodes, which feed on the plant roots, and slow-growing plant species (*Molinia caerulea* and *Potentilla erecta*) were associated with fungivorous and bacterivorous nematodes. Much is still unknown about plant-soil feedback effects, but the field is rapidly developing through, *e.g.* more affordable molecular analysis techniques.

1.4 Restoration of oligotrophic grasslands

Ecological restoration involves decision making on many levels (local to whole landscape) and by multiple actors (restoration practitioner to legislators on international level) and begins with identifying the goal and assessing the starting situation by an ecosystem assessment to determine the bottlenecks to recovery (Fig 1.9; Hulvey et al. 2013). A restoration practitioner might not have the knowledge (*i.e.* scientific bottleneck) or means (*i.e.* financial bottleneck) to restore communities that went locally extinct (*i.e.* biotic bottleneck) on a field with altered soil conditions (*i.e.* abiotic bottleneck). In addition, not all management measures may be feasible at the particular field.

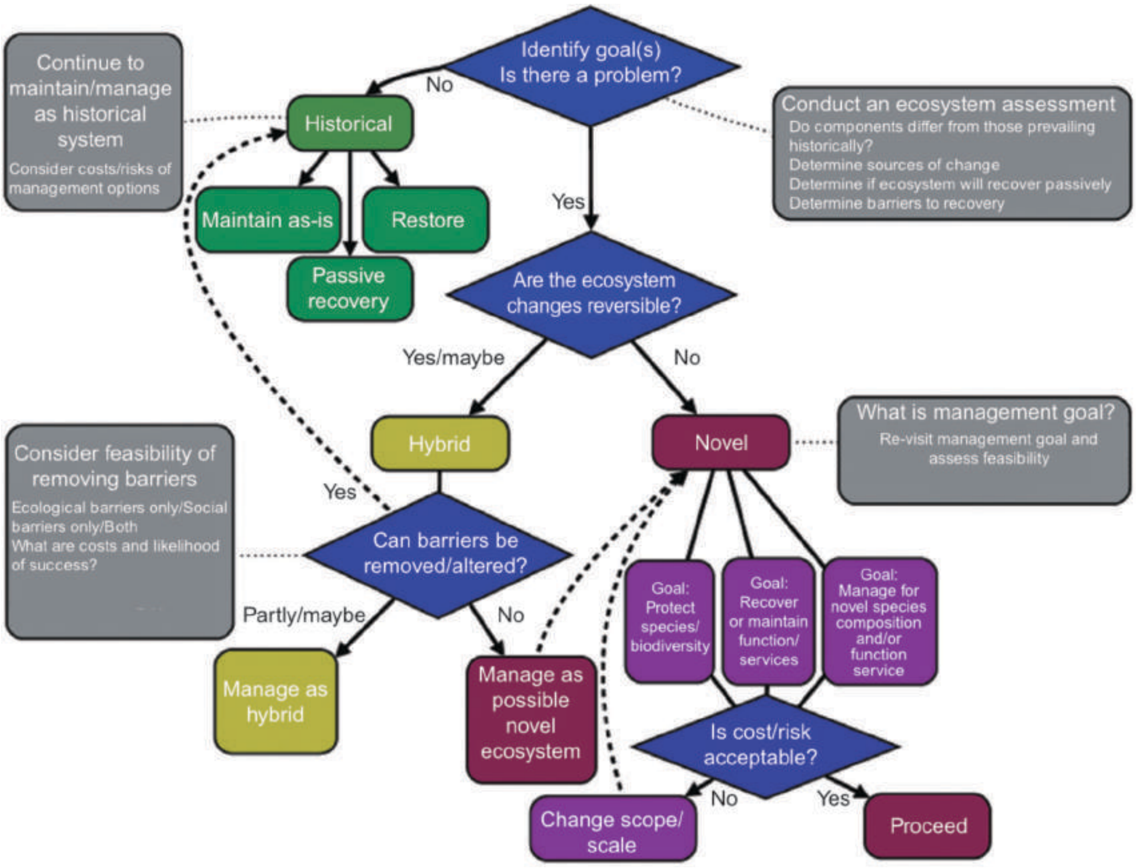


Figure 1.9 Framework to guide decisions for restoring ecosystems as an illustration for the multitude of options and constraints in restoration ecological projects (Hulvey et al. 2013)

An unambiguous and measurable goal should be put forward to be able to verify restoration success, adjust restoration efforts if necessary or even change the goal to an ecologically and economically more realistic target (Rohr et al. 2018). An example of an easily measurable goal is *area*, as in the 2014 decree of the Flemish government which aims to keep the 377 ha remnant *Nardus* grasslands in an optimal condition and restore an additional 637 ha *Nardus* grasslands (Appendix 1.C). The restoration success of species-rich *Nardus* grasslands can be also evaluated based on the following criteria:

- re-establishment of nutrient-limiting soil conditions (Marrs 1993; Roeling et al. 2018), **low phosphorus concentrations** in particular (cf. the 12 mg P_{Olsen} kg^{-1} threshold in Table 1.1, §1.3.2),
- **atmospheric nitrogen deposition** below the threshold of 12 kg N ha^{-1} y^{-1} (van Dobben et al. 2012),
- presence of minimally four **typical *Nardus* grassland species**, such as *Succisa pratensis*, *Nardus stricta*, *Potentilla erecta* (complete species list in Appendix 1.A), that together cover more than 10% (target for 6230_hn class of dry *Nardus* grasslands; De Saeger and Wouters 2017; key in Appendix 1.A),
- presence of **typical fauna species**, *e.g.* butterflies (*Pyrgus malvae*, *Maculinae alcon*), crickets (*Gryllus campestris*), reptiles (*Coronella austriaca*),

For evaluating restoration success, also a measure for the ‘benefit of people’ should be included, which leads to the disciplines of multifunctionality and ecosystem services. However, the various functions and ecosystem services of grasslands have been, apart from the relationship biodiversity-biomass production, hardly quantified and require further research (Bengtsson et al. 2019), *e.g.* Lindemann-Matthies et al. (2010), which quantified the aesthethic appreciation of grasslands along a gradient of varying species-richness.

In approved projects in Natura 2000 areas in Flanders, the target of restoring *Nardus* grasslands was almost always selected before assessing the (a)biotic field characteristics of the fields where restoration will take place (Appendix 1.C). It is hence unclear whether the proposed and budgeted management measures will enable to attain the ambitious goal of restoring 637 ha of additional *Nardus* grassland. In Flanders, a large part of planned habitat restoration will probably take place on former agricultural land (Gobin et al. 2009), where the soil conditions and vegetation are in generally eutrophic (see §1.3.2). Restoration of *Nardus* grasslands on such eutrophic fields in species-poor, fragmented and intensively used landscapes requires restoration of both the abiotic and biotic site conditions. Restoration of other species-rich grassland types such as lowland hay meadows (Natura2000 habitat type 6510) and calcareous grasslands (Natura2000 habitat type 6210) require similar attention to reinstalling abiotic and biotic site conditions (see §7.5).

Eutrophic soil conditions are an abiotic bottleneck with long-lasting negative effects on biodiversity (see Chapter 1.3). Elevated soil phosphorus concentrations in particular persist for a long time in the soil, *i.e.* decades to millennia (Dupouey et al. 2002; McLauchlan et al. 2006; Kepfer-Rojas et al. 2018). Hence, restoration efforts of this abiotic bottleneck are aimed at reducing the soil fertility, in particular soil phosphorus concentrations. Here, we introduce commonly used and novel techniques to reduce bioavailable soil phosphorus concentrations:

- The removal of the entire phosphorus-enriched soil layer, *i.e.* **topsoil removal**, is a quick and effective restoration technique (Verhagen et al. 2001; Smolders et al. 2008; Frouz et al. 2009; Török et al. 2011; Emsens et al. 2015). After intensive agriculture, phosphorus concentrations are high in the tillage layer (generally 0-30 cm), but phosphorus may have leached to deeper soil layers as well (Box-Fig. 1.4). It is therefore, important to beforehand accurately determine the depth of the soil layer that needs to be removed (Hölzel and Otte 2003). The large soil volumes to be removed and transported (*e.g.* 6000 m^3 per hectare for the top 0-60 cm) render topsoil removal expensive (Table 1.2). This high one-time cost could be reduced if the topsoil can be re-used, for construction of dikes, or to introduce topographic and plant compositional heterogeneity on site, as being demonstrated in Australian grasslands (Gibson-Roy and Mcdonald 2014). Near complete restoration of flora and fauna of *Nardus* grassland on formerly eutrophic soil has been reported only for restoration projects in which the nutrient-enriched soil layer is taken away by topsoil removal and target species have been re-introduced (*e.g.* Loeb et al. 2018). However, in some cases, *e.g.* if the water table is near the surface or due to project budget restrainments, topsoil removal is not possible.
- **Full-inversion tillage**, *i.e.* the *in situ* burial of the phosphorus-enriched topsoil layer (Glen et al. 2016), is likely a cheaper (Table 1.2) alternative to topsoil removal though the technique is not commonly used nor well-researched. Tillage may be performed until about one meter soil depth, but this technique requires the deeper soil layers (60-100 cm) to be phosphorus-poor (Milligan et al. 2017).
- ***In situ* fixation of phosphorus** by adding chemical amendments (*e.g.* iron chloride, aluminium sulphate, calcium carbonate, elemental sulphur and iron-rich materials) has shown variable success to decrease the bioavailable concentration of phosphorus (Ann et al. 2000; Grootjans et al. 2002; Gilbert et al. 2003; Stuckey et al. 2007; Geurts et al. 2011). To reduce the bioavailability of phosphorus significantly, large amounts are necessary (*i.e.* 4 – 63 t ha^{-1}), which may have negative side-effects such as toxicity to the target species (Marrs 1993; Ann et al. 2000).
- **Grazing** leads to patchiness in nutrient availability rather than nutrient depletion from the soil (Mikola et al. 2009). Marrs (1993) reported that sheep grazing annualy removed only 5 kg P ha^{-1}. Jewell et al. (2007) found cattle grazing locally removed less than 20 kg P $ha^{-1} y^{-1}$ and, elsewhere within the same field, added more than 50 kg P $ha^{-1} y^{-1}$, which renders this technique useless to restore phosphorus-poor soil conditions on eutrophic soils.
- Soil phosphorus concentrations may be reduced by phytoextraction or **phytomining**, *i.e.* phosphorus extraction by biomass production, through mowing or P-mining.

- **Mowing** is the practice of cutting grassland swards to remove nutrients with hay. No fertilizers are used. Grasslands that were intensively fertilized might annually produce more than 15 t dry biomass ha^{-1} (Pegtel et al. 1996). After several years of mowing without fertilization, biomass production significantly decreases due to limitation of nutrients other than phosphorus, namely nitrogen (Van Der Woude et al. 1994; Smits et al. 2008) and potassium (Oelmann et al. 2009). The annual biomass production generally falls back from 15 to 5 t dry biomass ha^{-1}, or even less (Berendse et al. 1992; Pegtel et al. 1996; Pavlu et al. 2011). With lower biomass production, phosphorus removal rates decline from about 45 to 6 kg P ha^{-1} (Pegtel et al. 1996). Therefore, this restoration method might take several decades and due to the cumulative cost over the years (Table 1.2), it may be more expensive than the one-time cost of topsoil removal. Mowing is an effective technique to reduce the bioavailability of nitrogen and potassium, but not of phosphorus.
- **P-mining** combines mowing management with fertilization of nitrogen and potassium (growth-limiting nutrients other than phosphorus) to maximize biomass production and thus phosphorus removal. The technique has been suggested by Marrs (1993) and Crawley et al. (2005) and can be used as an adjusted agricultural technique that may be used as a guided transition from agriculture to nature. P-mining can be done with the existing grassland vegetation or with arable crop that efficiently take up phosphorus and produce high quantities of biomass. Harvesting hay or other crops may deplete the soil of phosphorus. However, little long-term field evidence is available on how fast phosphorus can be removed by P-mining (MacDonald et al. 2012). Johnston et al. (2016) recently compiled several long-term phosphorus depletion experiments and found bioavailable phosphorus to decline exponentially, with the rate of decline depending on the soil's phosphorus-buffering capacity, actual phosphorus saturation and ability to restore the equilibrium between the different phosphorus pools. Modeling also suggested limited phosphorus removal over time with P-mining (Perring et al. 2009). In the first phase of P-mining, the bioavailable phosphorus pool is constantly and sufficiently replenished from the slowly cycling phosphorus pool (Vanden Nest et al. 2015). Later in the P-mining process, phosphorus usually becomes depleted in the rhizosphere, limiting plant-growth and, consequently, phosphorus removal (Koopmans et al. 2004). This complicates estimations of phosphorus removal along the restoration process. The cost of P-mining depends upon the quantity and quality of the biomass and its value for farmers; long-term P-mining may be expensive in case when the biomass has a low value (Table 1.2) or may be disadvantageous because during the period of P-mining little nature values are present due to intensive management.

Marrs (1993) stated that the success of reducing soil fertility depends upon how much the soil fertility was increased and how fast soil fertility can be reduced. Better predictions of the outcomes of measures to reduce soil phosphorus concentrations will lead to better management decisions (Brudvig 2017). In this PhD, we aim to provide additional data to better support decisions in the ecological restoration process.

Eutrophication and acidification caused by **atmospheric nitrogen deposition** are a second abiotic bottleneck, next to the eutrophic soil conditions resulting from former fertilization. A potential effect is increased grass cover at the expense of forb species (Stevens et al. 2006). A short-term solution is *in situ* mitigation by measures such as liming or increased mowing to overcome the acidifying and eutrophying effects (Stevens et al. 2011b; Jones et al. 2017). A more sustainable long-term strategy is to bring down atmospheric nitrogen depositions in Natura 2000 sites to non-detrimental levels (Soons et al. 2017), which cannot be done by local field managers but involves national and international legislation, *e.g.* the programmatic approach to nitrogen (Programmatische Aanpak Stikstof, PAS) in Flanders and the Netherlands. When long-term nitrogen input is ceased, mowing and removing biomass twice a year effectively leads to a decrease in soil nitrogen concentrations and plant diversity recovery in grasslands (Storkey et al. 2015).

Overcoming abiotic bottlenecks has rarely led to the targeted habitat recovery (Verhagen et al. 2001; Bischoff 2002; Poschlod and Biewer 2005). **Biotic bottlenecks** must be tackled as well. Seeds of target plant species (Ozinga et al. 2009) and the typical community of soil organisms (van der Heijden et al. 2008) may be absent or hindered by habitat fragmentation and low dispersal capacity of *Nardus* grassland species (Verhagen et al. 2001; Piessens et al. 2005). The soil seed bank of eutrophic grasslands does not contain species of semi-natural grassland types (Bekker et al. 1997). Spontaneous recolonization of target species may occur after the abiotic conditions are restored if the surrounding landscape is species-rich and landscape connectivity is high (Mitchley et al. 2012; Michalcová et al. 2013) and the source-communities are closeby (*e.g.* within a few meters, Berendse et al. 1992). In western Europe, however, oligotrophic grasslands are nutrient-poor habitat islands in a species-poor eutrophic landscape (Fibich et al. 2018). Hence, active re-introductions by *e.g.* transfer of fresh seed-containing hay or seeding of missing species are crucial for restoring the targeted species-rich communities in the short term (Kiehl et al. 2010). In the long term, connecting the oligotrophic habitats by strategic ecological restoration is a more sustainable solution (ecological intensification of the landscape *sensu* Kovács-Hostyánszki et al. (2017)).

Table 1.2 Costs, advantages and disadvantages of several ecological restoration techniques for restoring phosphorus-poor soil conditions on eutrophic soils. The underlined techniques are studied in this PhD

Technique	Cost (euro ha^{-1})	Advantages	Disadvantages
Top soil removal	15 500 – 20 200 – 25 000[a1] 75 000[a2]	One time intervention P-limitation in short-term	Expensive Deep excavation necessary if soil-P has leached to deeper soil layers Deep excavation not always possible Removes the seed bank, soil layer rich in organic matter and cations able to buffer against acidification
Full-inversion tillage	2170[b]	One time intervention P-limitation in short-term	Not suitable for soils with deep leaching of P
Grazing	284 – 600[c]	Close to traditional management of *Nardus* grassland Increased heterogeneity and patchiness	Limited P-removal
Mowing	580 – 1872[d]	Close to traditional management of *Nardus* grassland No nutrient or pesticide input NK-limitation within a few years	Limited P-removal Uncertaintly of time needed Hay quality decreases so cost increases High cumulative costs
P-mining	0 – 540[e]	Increased P-removal	Uncertaintly of time needed Uncertainty of productivity and economical attainability Requires fertilizers and intensive agricultural management High cumulative costs

[a1] removal of 0-50 cm including transportation cost for respectively 1 km, 2 km and 3 km (Oosterbaan et al. 2008);

[a2] average cost for removal of 0-50 cm including transportation cost (Anonymous 2012); costs may be lower if topsoil removal is combined with *e.g.* construction of dikes.

[b] conversion from Pound sterling to Euro (Luscombe et al. 2008)

[c] excluding placing of fences, including reparation costs to fences (Anonymous 2012)

[d] price difference depends upon difficulty to mow on terrain (Anonymous 2012) and upon the gain or loss to dispose of the hay, in Flanders and the Netherlands the processing of hay costs 50 euro per ton dry biomass (Compeer and Mattheij 2017)

[e] Cost may vary from break-even (or even profitable) till 540 euro per hectare (Oosterbaan et al. 2008)

1.5 Aim, research questions and thesis outline

In this PhD thesis, we aim to study how *Nardus* grassland communities can be restored after intensive land-use. Our three main research questions (RQ) are:

> RQ1: Can traditional management by mowing restore *Nardus* grassland on former agricultural land?
> RQ2: Is it possible to make phytomining of phosphorus more efficient and effective?
> RQ3: Do *Nardus* grassland species benefit from inoculation with soil biota along a soil phosphorus gradient?

In **Chapter 2**, we evaluate the effects of traditional mowing management on former agricultural land and compare the vegetation composition and soil phosphorus concentrations of formerly intensified grasslands with remnant *Nardus* grasslands (**RQ1**; Fig. 1.10). Based on field measurements of phosphorus removal by mowing, we estimate the time needed to reach the phosphorus threshold found in remnant *Nardus* grasslands. We further model the restoration time needed with P-mining and compare and discuss the attainability of the target with both management measures.

In **Chapters 3-5**, we use soil phosphorus gradients in one field and three pot experiments to study the optimization of phosphorus removal by P-mining (**RQ2**). We used the soil phosphorus gradient approach to simulate the chronosequence of soil phosphorus concentrations during restoration.

- **Chapter 3** handles a five-year field experiment situated in the nature reserves of Landschap de Liereman and Vloethemveld wherein the effects of mowing and P-mining management are compared with regard to phosphorus removal and soil phosphorus concentrations.
- **Chapter 4** reports the results of a pot experiment in which we aimed to speed up phosphorus removal by P-mining by adding either humic substances or arbuscular mycorrhiza.
- In **Chapter 5**, we compare phosphorus removal by phosphorus mining with five crop species in another pot experiment.

In **Chapter 6**, the first results of a multi-year mesocosm experiment are presented, in which we also use a soil phosphorus gradient to study the interactive effects of soil biota and soil phosphorus availability on target plant species abundance (**RQ3**). The slow-growing target species were planted in communities with fast-growing meso- and eutrophic species allowing for competition between the plant species.

In **Chapter 7**, we synthesize the new insights acquired during this PhD thesis to answer the three research questions and develop a decision scheme to guide practitioners in evaluating the attainability of their targets and in selecting the most appropriate restoration technique. Finally, we discuss the implications for practice and policy, recommend opportunities for further research and summarize the key findings.

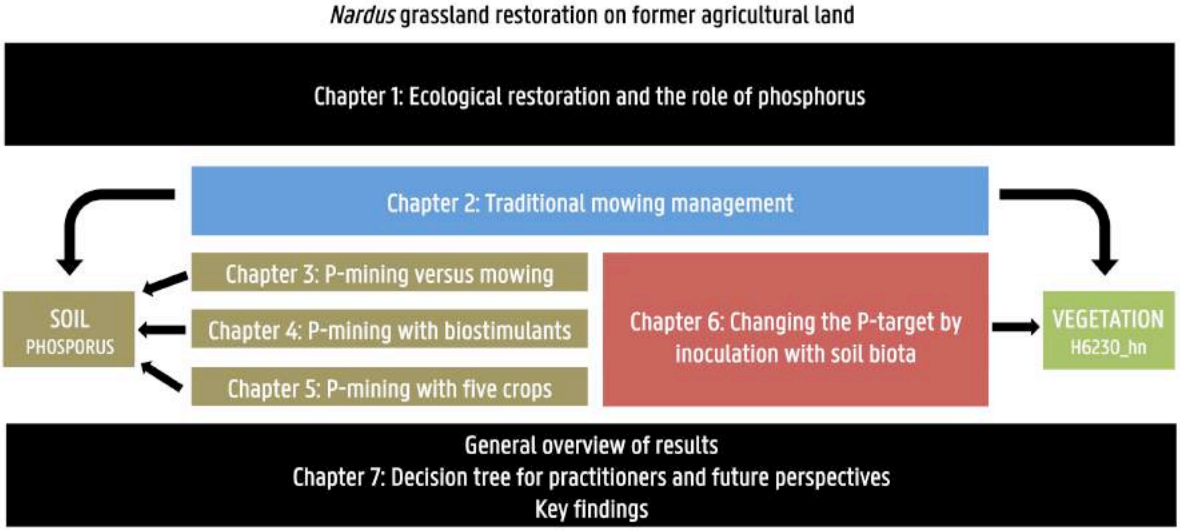


Figure 1.10 Thesis outline. In Chapter 2, we explored the effects of reinstating traditional mowing management after a period of intensive land-use and describe the *distance to target* for *Nardus* grasslands (Natura 2000 habitat type H6230*) in vegetation composition and soil phosphorus concentrations (Research Question 1). In Chapters 3-5, we experimentally explore possibilities to make phytomining of phosphorus (by mowing or P-mining) more efficient and effective (Research Question 2). Chapter 6 contains the first results of an experiment to allow *Nardus* grassland species to grow above their soil phosphorus threshold by adding soil biota (Research Question 3). In Chapter 7, the results of this thesis are discussed, a decision tree is presented to aid practitioners to select the most appropriate restoration technique; and, we explore the implications for practice, opportunities for further research and we give a concise answer to the research questions

CHAPTER 2

P-removal for restoration of *Nardus* grasslands on former agricultural land: cutting traditions

Mowing of mesotrophic grasslands on previously intensively managed fields in nature reserve *Vlijmens Ven* in ‘s-Hertogenbosch (NL) in June 2018

After: Schelfhout S, Mertens J, Perring MP, Raman M, Baeten L, Demey A, Reubens B, Oosterlynck S, Gibson-Roy P, Verheyen K, De Schrijver A. 2017. P-removal for restoration of Nardus grasslands on former agricultural land: cutting traditions. Restoration Ecology 25:S178–S187. Doi: 10.1111/rec.12531

Abstract

Past intensive land-use complicates the successful restoration of oligotrophic species-rich grassland types. One of the major bottlenecks are the elevated nutrient levels due to fertilization, especially residual phosphorus (P). Aiming to deplete nutrients, managers often reintroduce traditional haymaking management, sometimes combined with grazing. Here, we evaluate whether this technique restores the abiotic and biotic boundary conditions for restoration of *Nardus* grassland. Seven grasslands were selected in Flanders, Belgium, which had elevated nutrient levels after the cessation of intensive agriculture 16 to 24 years ago, and which have been mown and grazed since. We compared soil and vegetation data of these post-fertilization grasslands with 34 well-developed oligotrophic *Nardus* grasslands. Mowing and grazing did not cause community composition to resemble that of *Nardus* grassland. Furthermore, bioavailable P-concentrations were significantly higher in the post-fertilization grasslands and P-limitation was not obtained. Restoring P-poor soil conditions through continued mowing and grazing management would take at least decades. Phosphorus-mining can shorten the restoration time by increased P-removal.

2.1 Introduction

The soil phosphorus content can be decreased by removing phosphorus taken up by plants, *i.e.* phytomining which includes mowing and P-mining management (see §1.4). The phosphorus removal rate depends upon the amount of harvested biomass and upon its phosphorus concentration. In case of grassland restoration on fertilized land, the practice of mowing with hay removal after the cessation of fertilization is often used, sometimes combined with grazing. Mowing removes nitrogen effectively because nitrate is mobile and highly susceptible for leaching or plant-uptake (Storkey et al. 2015). However, mowing does not sufficiently decrease the soil phosphorus content on heavily fertilized agricultural land (Smits et al. 2008). The reason for low annual phosphorus removal with mowing is the declining biomass production due to limitation of other nutrients than phosphorus, namely nitrogen (Van Der Woude et al. 1994; Smits et al. 2008) and/or potassium (Oelmann et al. 2009). Restoring phosphorus-poor soil conditions through mowing may consequently take a long time and mowing can, therefore, fail –as a single measure– to restore biodiversity on heavily fertilized land (Smits et al. 2008).

Here, we study the restoration success of seven post-fertilization grasslands that have been mown followed by grazing for more than 15 years in order to restore *Nardus* grasslands. To get insight in the ‘distance to target’, of the post-fertilization grasslands, we compare their vegetation composition and soil phosphorus concentrations with well-developed remnant *Nardus* grasslands. We measured how much phosphorus has been removed by the practice of mowing and calculated the phosphorus stock that would have been removed by a management of P-mining. The effectiveness of both mowing and P-mining techniques was assessed by calculating the time needed for restoration.

2.2 Methods

2.2.1 Field measurements in Nardus grasslands and post-fertilization grasslands

To assess vegetation composition and soil data of remnant *Nardus* grasslands, we used a database containing data of 34 parcels spread over 11 locations in northern Belgium (INBO 2015). Most of these grasslands were degraded to some degree by (i) atmospheric nitrogen deposition in the past (critical load was exceeded in more than 70% of the area of species-rich grasslands in 2008 in northern Belgium) or present (critical load was exceeded in more than 47% of the area of species-rich grasslands in 2017; Vlaamse Milieumaatschappij 2018), and by (ii) habitat fragmentation. However, we can assume that the soil phosphorus concentrations were unchanged and can be regarded as reference values to compare with. The plant species cover was measured in a 9 m^2 quadrat per parcel in July - September 2012 – 2014. In each *Nardus* grassland, one representative quadrat was selected without using the presence or absence of target plant species as a selection criterion. Subsequently to these vegetation surveys, nine soil cores (0-10 cm) were collected in each quadrat and combined into one sample (0.5 l). These samples were dried (40°C for 48 h), sieved (2 mm sieve size) and chemically-analysed (see further chemical analyses).

We selected seven post-fertilization grasslands, all with comparable hydrology and soil texture as the *Nardus* grasslands. These grasslands were located on relatively dry, sandy soils (Podzol in WRB classification) in two neighbouring nature reserves in northern Belgium: Turnhouts Vennengebied and Landschap de Liereman (Appendix 2.B). The seven grasslands were in intensive agricultural use, and hence, fertilized until 16 – 24 years ago. Since then, although still exposed to atmospheric N-deposition (28-31 kg N ha^{-1} y^{-1}; Cools et al. 2015), active fertilization has ceased. The management consisted of mowing with hay removal once a year in July and grazing in late summer with ponies or cows.

Vegetation measurements were performed in July 2014 in 4 m^2 plots per parcel, the number of plots per parcel (two to six) depended on the size and the heterogeneity of the grassland. Four soil cores (0-10 cm) were collected from each quadrat and combined into one sample (0.3 l). The samples were dried, sieved and analysed for bioavailable and slowly cycling P (see further chemical analyses).

In the post-fertilization grasslands, P-removal by the current mowing practice was assessed by measuring biomass production and biomass P-concentration (P_{DM}) in one 0.25 m^2 subplot within each of the quadrats. The sward was cut two cm above the soil level on the same day as the vegetation survey. Vegetation samples were dried (70°C for 48h), weighed to obtain the total dry biomass (DM) and ground before chemical analysis (see further).

2.2.2 Chemical analyses

As a measure for the bioavailable phosphorus pool (Gilbert et al. 2009), soils were extracted in $NaHCO_3$ (P_{Olsen}) following ISO 11263:1994(E). In order to get insight in the slowly cycling phosphorus pool, we extracted soils in ammonium-oxalate-oxalic acid ($P_{Oxalate}$ according to

NEN 5776:2006; van Rotterdam et al. 2012). Extracted phosphorus was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999). $P_{Oxalate}$ concentrations were not available in the database of *Nardus* grasslands and were therefore calculated based on the P_{Olsen} concentrations. We assessed the relation between P_{Olsen} and $P_{Oxalate}$ in 120 soil samples taken in close vicinity to the post-fertilization grasslands. Linear regression analyses revealed a strong relation between P_{Olsen} and $P_{Oxalate}$: $P_{Oxalate} = 0.67 + 3.03 * P_{Olsen}$; $R^2 = 0.93$ and $p<0.001$ (*lm* function in the R package *stats*; Fig. 2.1). Additionally, soil-pH_{H2O} in the post-fertilization grasslands is presented in Appendix-Table 2.B.

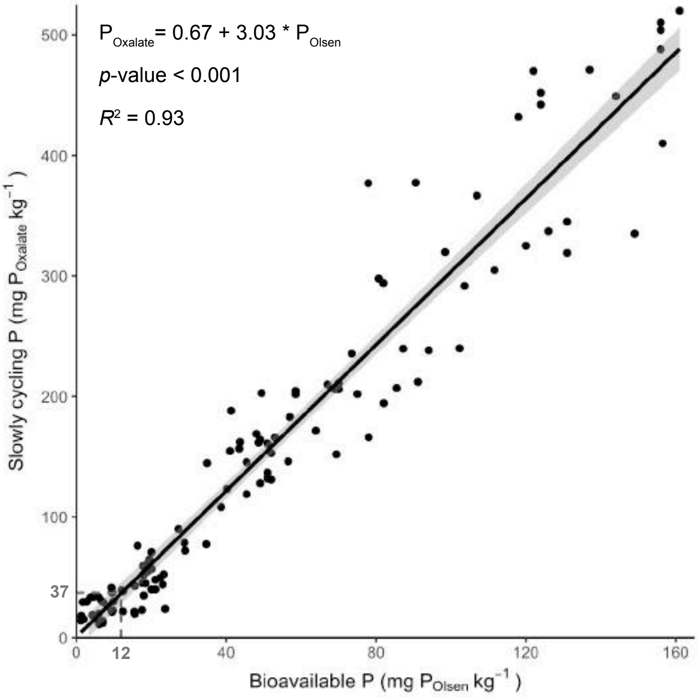


Figure 2.1 Linear regression between concentrations of bioavailable P (P_{Olsen}) and slowly cycling P ($P_{Oxalate}$) from 120 soil samples. The bioavailable P-threshold for *Nardus* grasslands, namely, 12 mg P_{Olsen} kg^{-1} (see Methods section), corresponds to a slowly cycling P-concentration of 37 mg $P_{Oxalate}$ kg^{-1} and is indicated with grey dashed lines. The black full line depicts the linear model; the shaded area illustrates the standard error; and, in the top left, the linear model and fit are shown

Plant biomass was analysed for total phosphorus concentration (P_{DM}) by digesting 100 mg of sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs for 4 h at 140°C. P was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999), and total potassium concentration (K_{DM}) by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS). Total nitrogen concentration (N_{DM}) was measured by high-temperature combustion at 1,150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Hanau, Germany).

2.2.3 Calculations and statistical analyses

We combined two datasets of vegetation surveys differing in plot size (*Nardus* grasslands of 9 m^2 and post-fertilization grasslands of 4 m^2 size). To be able to compare plant species richness and vegetation composition in *Nardus* and post-fertilization grasslands, we used rarefaction curves to convert the 4 m^2 quadrats in post-fertilization grasslands into one 9 m^2 quadrat per grassland, a procedure made possible by the multiple quadrats per post-fertilization grassland (Appendix 2.C, and see Gotelli and Colwell 2001).

We tested for differences in the number of plant species and typical species (Appendix 2.A) between remnant *Nardus* grasslands (n=34) and post-fertilization grasslands (n=7) with *t.test* in the R-package *stats* without equal variances ($p<0.05$; R Core Team 2015). To explore potential differences in plant community composition, we performed a non-metric multi-dimensional scaling analysis (NMDS) with *metaMDS* from R-package *vegan*. We used the Lennon dissimilarity index to quantify between-plot compositional differences with the original quadrat data. Since this index is derived from species turnover only, it excludes nestedness patterns derived from richness differences (Baselga 2010). Convex hulls were added with *ordihull* from R-package *vegan* (Fig. 2.2; Oksanen et al. 2017). We performed a permutational analysis of variance on the same dissimilarity matrix, with grassland type as predictor and a significance based on 999 permutations (Anderson 2001). Plant species significantly indicative for either *Nardus* or post-fertilization grasslands from the quadrats recorded here were obtained by indicator value analysis with *multipatt* in the R-package *indicspecies* (De Cáceres et al. 2010).

We tested for differences in soil P_{Olsen} and $P_{Oxalate}$ concentrations between remnant *Nardus* grasslands (n=34) and post-fertilization grasslands (n=7) by using *t.test* in the R-package stats without equal variances. To get insight in the abiotic 'distance to target' of the post-fertilization grasslands, we used a threshold value for bioavailable phosphorus of 12 mg P_{Olsen} kg^{-1} soil, which was calculated as the 95 percentile of the dataset gathered in Raman et al. (Instituut voor Natuur- en Bosonderzoek, Brussel, unpublished data). This value corresponds with a slowly cycling phosphorus pool of 37 mg $P_{Oxalate}$ kg^{-1} when converted with the linear regression as discussed previously.

$P_{Oxalate}$ stocks in the 0-10 cm soil layer were calculated by assuming soil bulk density of 1.4 g cm^{-3} for sandy soils. For Nardus grasslands, a threshold value of 51.8 kg $P_{Oxalate}$ ha^{-1} was herewith calculated. Subtracting this threshold value from the P-oxalate stocks of each post-fertilization grassland gave insight in the 'distance to target', *i.e.* the excess of slowly cycling phosphorus ($P_{Excessive}$).

The annual P-removal by mowing the post-fertilization quadrats was calculated by multiplying DM with P_{DM}. For each post-fertilization grassland, we estimated how many years it would take to reach the threshold of *Nardus* grassland by dividing $P_{Excessive}$ with its current annual P-removal.

How long it takes to reach the threshold value depends both on the phosphorus concentrations in the soil and the soil depth in which concentrations are elevated. When parcels are regularly ploughed, it is likely that P-concentrations are elevated in the complete furrow, or even deeper when soils were P-saturated and P-leaching occurred. If no regular ploughing has occurred, sometimes only the topsoil has elevated P-concentrations. We illustrated this issue by performing our calculations for two cases: i) case in which only the 0-10 cm topsoil and ii) case in which the furrow (0-30 cm soil) contains elevated P-concentrations. The P-concentration for the 0-30 cm soil was assumed to be the same as in the 0-10 cm soil. We here want to stress that these calculations are only estimations, as we here assume P-removal by mowing to stay constant in time. It might, however, be possible that annual P-removal would further decrease as a consequence of P-depletion in the soil (Chapter 4).

To verify whether the target of P-limitation or NP-co-limitation was obtained in the post-fertilization grasslands (see Introduction), we compared P_{DM} and N_{DM} to the ecological critical thresholds of P- and N-limitation in grasslands (P_{DM}<0.7 g P kg^{-1} and N_{DM}<14 g N kg^{-1} according to Wassen et al. 1995 and Güsewell 2004). To verify which nutrient(s) limited biomass production for P-mining purposes, we compared P-, N- and K-concentrations in plant biomass to agricultural standards (P_{DM}<2.6 g P kg^{-1}, N_{DM}<20 g N kg^{-1}, K_{DM}<20 g K kg^{-1}; Bailey et al. 1997).

For each post-fertilization grassland, we calculated potential P-extraction by P-mining. P-mining results in lifting N- and K-limitation through fertilization, and we assume biomass production will increase compared to the mowing management. Due to NK-fertilization, P-removal will increase with biomass production, while P_{DM} will probably not change, as it is mainly influenced by bioavailable soil-P-concentrations (Gilbert et al. 2009). We furthermore modelled how much time it will take to deplete $P_{Excessive}$ by P-mining. Also here we want to stress that our calculations are only rough estimations because little experimental knowledge is available on how effective P-mining is in the long-term (MacDonald et al. 2012). During P-mining management, annual P-removal declines over time with decreasing soil phosphorus bioavailability (Chapter 4). Therefore, we assume that initially, in a soil with high bioavailable phosphorus concentration (>65 mg P_{Olsen} kg^{-1}), annual P-removal is high (*i.e.* 45 kg P ha^{-1} according to unpublished results on P-mining fields in close vicinity to the post-fertilization grasslands). Further, we assumed P-mining to slow down until 20 mg P_{Olsen} kg^{-1} is reached in steps according to Chapter 4: 65-55 mg P_{Olsen} kg^{-1}, 33.5 kg P ha^{-1}; 55-36 mg P_{Olsen} kg^{-1}, 22 kg P ha^{-1}; 36-25 mg P_{Olsen} kg^{-1}, 14 kg P ha^{-1}; 25-20 mg P_{Olsen} kg^{-1}, 10 kg P ha^{-1}. When bioavailable phosphorus pools are depleted any further, P-removal by P-mining will likely approach phosphorus removal by mowing. Therefore, we suggest changing the management from P-mining to mowing without NK-fertilization when a bioavailable P-concentration of 20 mg P_{Olsen} kg^{-1} is achieved. In this last step, we use the measured P-removal by mowing from each post-fertilization quadrat until the target of 12 mg P_{Olsen} kg^{-1} is reached. Also these calculations were performed for two soil depths, 0-10 cm and 0-30 cm.

2.3 Results

The post-fertilization grasslands have a lower species-richness than the remnant *Nardus* grasslands: we found on average only eight vascular plant species per 9 m^2 in post-fertilization grasslands in contrast to on average 22 vascular plant species per 9 m^2 (Table 2.1) in the remnant *Nardus* grasslands. In the post-fertilization grasslands, no typical *Nardus* grassland species were found, whilst the remnant *Nardus* grasslands had on average four typical species. Furthermore, we found that plant communities of post-fertilization and remnant *Nardus* grasslands did not resemble each other (Fig. 2.2). The species-richness in 21 of the 34 remnant *Nardus* grasslands was lower than 20 species per 9 m^2; the botanical criterium (see §1.4 and Appendix-Table 1.A) for dry *Nardus* grasslands (habitat type 6230_hn) was met in 76% of the remnant grasslands and in neither of the post-fertilization quadrants.

Table 2.1 Vegetation and soil properties of the post-fertilization grasslands and remnant *Nardus* grasslands (mean ± SE)

	Post-fertilization grasslands with mowing and grazing for 15 – 24 years	**Remnant *Nardus* grasslands never fertilized**	***p*-value [a]**
Number of grasslands	7	34	
Number of vascular plant species per 9 m^2	8 ± 0.6 [c]	22 ± 1.6	***
Number of typical *Nardus* grassland species per 9 $m^{2,b}$	0 ± 0 [c]	4 ± 0.3	***
Bioavailable P (mg P_{Olsen} kg^{-1})	53 ± 11	3.9 ± 0.5	
Slowly cycling P (mg $P_{Oxalate}$ kg^{-1})	159 ± 32	13 ± 1.4 [c]	

[a] p-value of *** indicates *p* <0.001

[b] The list of typical Nardus grassland species is shown in Appendix Table 2.A

[c] Slowly cycling P-concentrations in remnant *Nardus* grasslands were calculated from bioavailable P-concentrations by linear regression (see Methods)

The bioavailable and slowly cycling phosphorus concentrations were significantly higher in the post-fertilization grasslands (Fig. 2.3; Table 2.1) compared to the *Nardus* grasslands. While the *Nardus* grasslands had very low bioavailable P-concentrations (1.5 – 14.1 mg P_{Olsen} kg^{-1}), concentrations in our post-fertilization grasslands ranged between 25 and 114 mg P_{Olsen} kg^{-1}; *i.e.* 1.8 to 13 times higher concentrations than the calculated threshold for *Nardus* grasslands (12 mg P_{Olsen} kg^{-1}, Raman et al., Instituut voor Natuur- en Bosonderzoek, Brussel, unpublished data). We estimated that on average 170 to 511 kg $P_{Oxalate}$ ha^{-1}, dependent on the soil depth with elevated phosphorus concentrations, should be removed from the post-fertilization grasslands to reach the bioavailable phosphorus threshold of 12 mg P_{Olsen} kg^{-1} (Table 2.2).

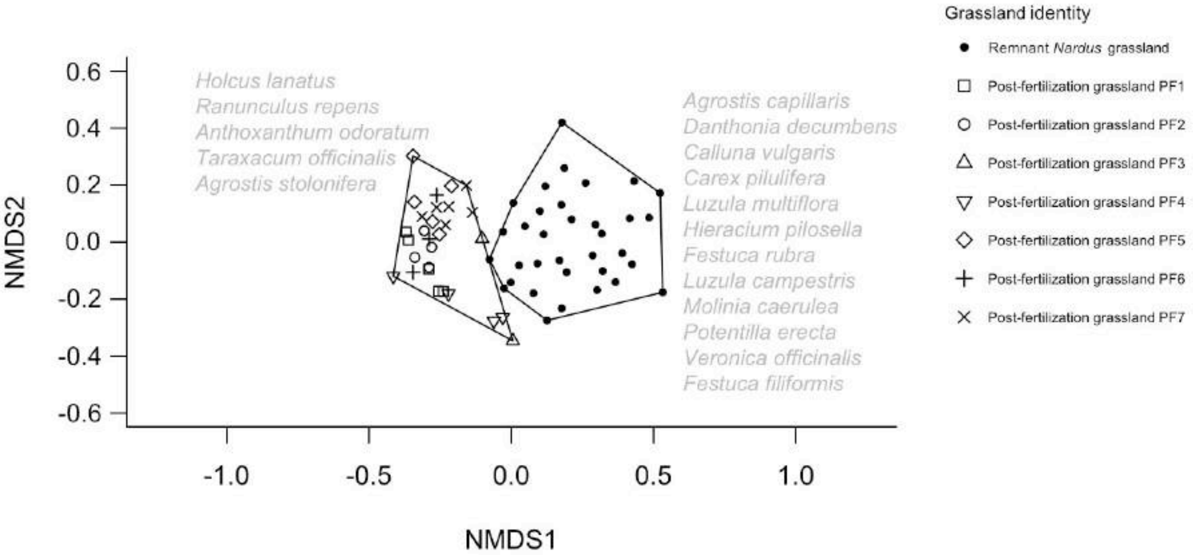


Figure 2.2 Results of a non-metric multi-dimentional scaling (NMDS) ordination of the plant communities in the 34 remnant *Nardus* grasslands and the seven post-fertilization grasslands (29 quadrats are shown). Distances between points represent differences in the composition of vegetation plots that are derived from turnover (Lennon dissimilarity; *F*=44.2; *p*<0.001) and are independent from variation in species richness. Indicative species significantly associated with our remnant *Nardus* grasslands and post-fertilization grasslands respectively, were shown respectively in the right and left corners in gray (*p*=0.001)

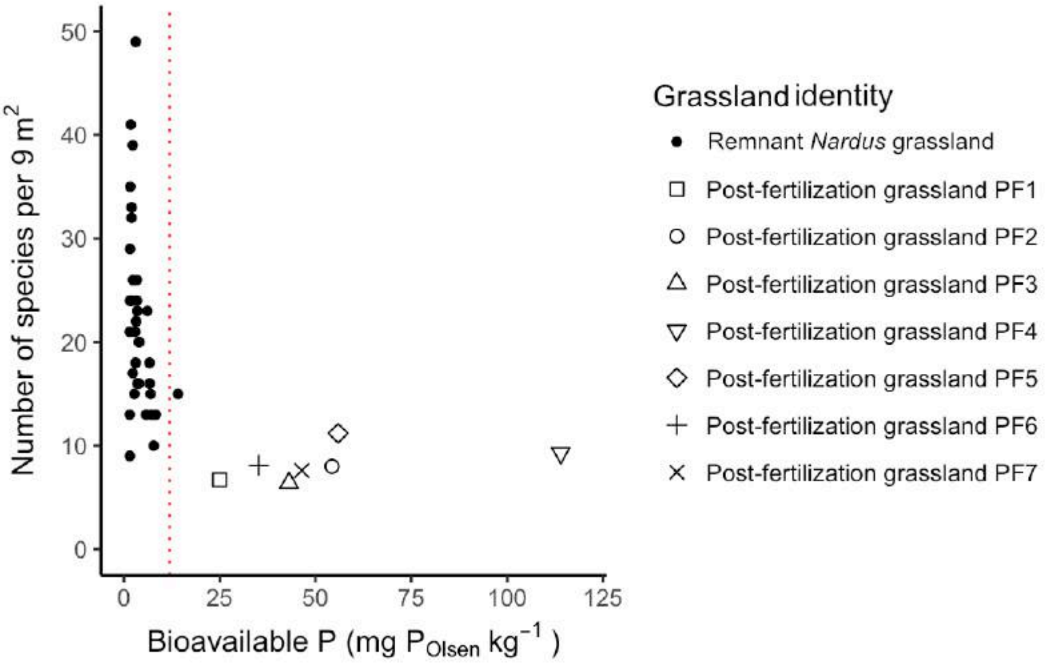


Figure 2.3 Number of vascular plant species found in 9 m^2 quadrats for remnant *Nardus* grasslands (n=34) and post-fertilization grasslands (n=7) versus bioavailable P-concentration (mg P_{Olsen} kg^{-1}). Number of plant species in post-fertilization grasslands was estimated from rarefaction curves (Appendix 2.C). The red dotted line indicates 12 mg P_{Olsen} kg^{-1}

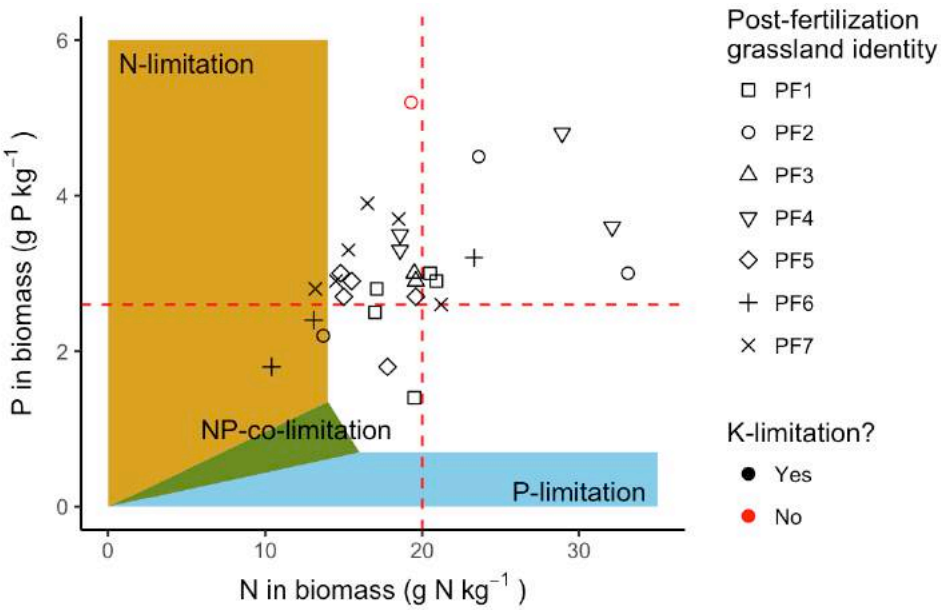


Figure 2.4 N_{DM}- and P_{DM}-concentration in the vegetation of post-fertilization quadrats (n=29) are shown with different shapes according to seven grasslands they were surveyed in. The colored polygons indicate N-, P- or NP-co-limitation in an ecological context (Wassen et al. 1995). The red dashed lines indicate N- and P-limitation in an agricultural context (Bailey et al. 1997). The red open circle shows the one quadrat where K was not limiting according to the agricultural standard of Bailey et al. (1997)

We compared N, K and P concentrations in the plant biomass of the post-fertilization grasslands to agricultural standard values (growth limitation when N_{DM}<20 g N kg^{-1}, K_{DM}<20 g K kg^{-1}, P_{DM}<2.6 g P kg^{-1}, Bailey et al. 1997). In five of the seven post-fertilization grasslands, we found a limitation of N and P, while K was limiting in all seven (see Fig. 2.4). A review on nutrient limitation by Güsewell (2004) revealed that an N:P ratio of less than 10 indicates that biomass production will be stimulated by N-fertilization. We found that the N:P ratio was less than 10 in all post-fertilization grasslands, and in none of them the ecological P-limitation was reached (P_{DM}<0.7 g P kg^{-1}, Wassen et al. 1995).

The current mowing management in the post-fertilization grasslands annually removes on average 1.9 t dry biomass ha^{-1} and 5.3 kg P ha^{-1}. We estimated that it would take about 40 to 118 years to reach the soil P threshold with this P-removal rate, the range dependent of the soil depth with elevated P-concentrations (Table 2.2). Our calculations show that with P-mining, the time needed to reach the restoration target is less than half of the time needed with mowing management (on average 14 to 46 years, depending on the depth of soil with elevated P-concentrations, see Table 2.2).

Table 2.2 Biomass production and nutrient concentrations in the first and only cut in post-fertilization grasslands (n=7; mean ± SE). $P_{Excessive}$, P-removal, P-removal time to reach the maximal threshold of 12 mg P_{Olsen} kg^{-1} in 0-10 cm and 0-30 cm were estimated for mowing and P-mining in the post-fertilization grasslands (n=7; mean ± SE)

	Post-fertilization grasslands with mowing and grazing for 15–24 years
Biomass production - first cut (t dry biomass ha^{-1})	1.9 ± 0.4
Nutrient concentrations in biomass	
P_{DM} (g P kg^{-1})	3.0 ± 0.2
N_{DM} (g N kg^{-1})	19 ± 1.3
K_{DM} (g K kg^{-1})	9.2 ± 1.1
Annual P-removal with biomass (kg P ha^{-1})	5.3 ± 1.0
Excessive soil-P-amount to remove	
$P_{Excessive}$ in 0 – 10 cm soil layer (kg P ha^{-1})	170 ± 45
$P_{Excessive}$ in 0 – 30 cm soil layer (kg P ha^{-1})	511 ± 135
Estimation of time to reach threshold	
0 – 10 cm mowing (years)	40 ± 11
0 – 10 cm P-mining (years)	14 ± 2
0 – 30 cm mowing (years)	118 ± 34
0 – 30 cm P-mining (years)	46 ± 5

2.4 Discussion

Mowing and grazing of post-fertilization grasslands for more than 15 years did not lead to plant communities that resembled the *Nardus* grasslands. Species-richness was significantly lower in the studied post-fertilization grasslands and typical *Nardus* species were absent. Further, P-limitation in plants, according to ecological thresholds, was not obtained, while it is an objective for restoring species-rich grasslands (Ceulemans et al. 2013). Also, compared to the threshold of 12 mg P_{Olsen} kg^{-1} for *Nardus* grasslands (Raman et al., Instituut voor Natuur- en Bosonderzoek, Brussel, unpublished data), the bioavailable soil P concentration in the post-fertilization grasslands was typically more than five times higher. Restoration efforts to a target of species-rich grasslands may thus be compromised by high residual soil fertility. Our small case study illustrates a gap between theory and practice: *i.e.* practitioners reinstating traditional grassland management on fields with an agricultural legacy without considering the abiotic and the biotic bottlenecks.

By the technique of mowing and hay removal, it would take many decades to reach the bioavailable P-threshold. The current P-removal rate was found to be much lower in our post-fertilization grasslands than in intensively-fertilized grasslands (on average 5.3 kg P ha^{-1} y^{-1} compared to more than 30 kg P ha^{-1} y^{-1} in optimal growing conditions (Liebisch et al. 2013). This low P-removal was due to the observed low biomass production. Intensively-fertilized grasslands (for example annually fertilized with 210 kg N ha^{-1} and 116 kg P ha^{-1}) can annually produce up to 15 t dry biomass ha^{-1} (Liebisch et al. 2013), in contrast to the post-fertilization grasslands where less than 3 t dry biomass ha^{-1} was removed by mowing once per year (at least in all quadrats but one, where 5 t dry biomass ha^{-1} was removed). This finding was similar to observations by Bakker et al. (2002) in grasslands mown for 25 years without fertilization. While we can assume that our post-fertilization grasslands were previously intensively-fertilized and were, hence, very productive, the low biomass production can be explained by nutrient limitation of N- and/or K, in some cases also of P, at least according to agricultural standard values.

P-mining aims at maximizing P-removal by NK-fertilization, and our calculations showed that this technique might halve the time needed to restore the necessary P-concentrations. Although some studies are investigating P-mining in the field (Dodd et al. 2012; Postma et al. 2015), more long-term field experiments are needed to get better insight in which crops are most optimal to reach restoration targets. Managers can choose to mine as quick as possible by means of an intensive agricultural practice, using crops that are not interesting for biodiversity (*e.g.* maize) and crop protection products, or undertake a more extensive way of mining, with crops being more interesting for biodiversity (*e.g.* grass-clover) and no crop protection (Carvell et al. 2006; Goulson et al. 2011).

Little field data is available on the costs/gains of P-mining. To make the technique of P-mining economically feasible for farmers, it is important that the crop or hay quality is guaranteed. In fields with low soil P-concentrations in an agricultural context, P in forage will also likely be suboptimal and this can be the cause of a lower nutritional value. For forage to serve as the only feed component of the diet of high yielding dairy cows, it should not contain less than 3 g P kg^{-1} of dry biomass (Valk et al. 1999). Possibly other less common used crops will be more optimal in the later stages of P-mining (*e.g.* buckwheat; Simpson et al. 2011).

2.5 Conclusion

The post-fertilization grasslands under study clearly show that the target of species-rich *Nardus* grasslands is far from reached after at least 15 years of traditional grassland management (mowing with hay removal and grazing). We estimated that restoring P-poor soil conditions through continued traditional mowing and grazing management may take many decades. P-mining might halve the time needed to restore the P-poor target concentration.

2.6 Acknowledgements

We thank the people from Natuurpunt Turnhouts Vennengebied and Landschap de Liereman. Also, we thank L. Willems and G. De bruyn for assisting with the chemical analysis of soil and biomass samples. We also thank coordinating editor K. Hulvey and the anonymous reviewers for their valuable comments on this manuscript. A.D.S. held a grant from the Research Foundation Flanders (FWO) and further support was provided by FWO project G050215N.

Illustrations from Chapter 3 on the next page (from left to right, top to bottom): Jelle Van den Berghe harvesting in Liereman-1; Danny Vereecke harvesting in Liereman-2; a mowing plot at Liereman-1 in June 2016; Kris and Filip Ceunen and Jelle Van den Berghe raking the cut grass in Liereman-2; the finger-bar; soil profile in Liereman-1; Robbe De Beelde in a packed car with the mower machine and fresh biomass samples


2.3 30-60
15-30
2.3 0-15cm

CHAPTER 3

Phytomining to re-establish phosphorus-poor soil conditions for nature restoration on former agricultural land

Mowing of the field experiment in Oud-Turnhout required a lot of (wo)man power

After: Schelfhout S, Schrijver A, Vanhellemont M, Vangansbeke P, Wasof S, Perring MP, Haesaert G, Verheyen K, Mertens J. Phytomining to re-establish phosphorus-poor soil conditions for nature restoration on former agricultural land. *Accepted pending minor revisions at Plant and Soil*

Abstract

To restore species-rich grasslands on former agricultural land, typically phosphorus-poor soil conditions need to be re-established. Here we assess the potential of phosphorus extraction by biomass production, *i.e.* phytomining. We compare two techniques: (i) *mowing*, *i.e.* cutting and removing hay two or three times a year, and (ii) *P-mining*, *i.e.* mowing with yield maximization by adding growth-limiting nutrients other than phosphorus (*i.e.* nitrogen and potassium).

In a five-year field experiment at three fields situated along a soil phosphorus gradient, we studied phosphorus removal through both biomass assessment and changes in two soil phosphorus pools: bioavailable phosphorus (P_{Olsen}) and slowly cycling phosphorus ($P_{Oxalate}$).

Phosphorus-mining doubled the phosphorus removal with biomass compared to mowing, and phosphorus removal with biomass was lower at fields with an initially lower concentration of P_{Olsen} in the soil. The P_{Olsen} concentrations decreased significantly during the experiment with the largest decreases in phosphorus-rich plots. Changes in the P_{Olsen} and $P_{Oxalate}$ stocks were correlated with the amount of phosphorus removed with biomass.

Phosphorus-mining effectively increases phosphorus removal compared to mowing, but becomes less efficient with decreasing soil phosphorus concentrations. Restoring phosphorus-poor soil conditions on formerly fertilized land remains a challenge: phytomining most often needs a long-term commitment.

3.1 Introduction

In a field-based experiment, we investigated the effectiveness of two restoration techniques for phosphorus removal and their effect on the concentrations and stocks of bioavailable and slowly cycling phosphorus in the soil. We compared (i) mowing, *i.e.* cutting grassland swards to remove nutrients with hay, and (ii) P-mining, *i.e.* mowing management combined with fertilization of nitrogen and potassium (growth-limiting nutrients other than phosphorus) to maximize biomass production and thus phosphorus removal (see §1.4). Mowing is widely used as a restoration technique on former agricultural land (*e.g.* Bakker et al. 2002; Török et al. 2011; Chapter 2). After several years of mowing, however, the biomass production generally decreases due to limitation by nitrogen and potassium (Van Der Woude et al. 1994; Oelmann et al. 2009), and therefore less phosphorus will be removed with hay. P-mining could be more effective at reducing soil phosphorus concentrations. Yet, only little field evidence on the effects of long-term P-mining is available, especially in comparison with mowing management. Recently, Johnston et al. (2016) compiled several long-term phosphorus depletion experiments and found bioavailable phosphorus to decline exponentially, with the rate of decline depending on the soil's phosphorus-buffering capacity, actual phosphorus saturation and ability to restore the equilibrium between the different phosphorus pools. We performed our experiment from 2011-2016 (*i.e.* during 5 years) in three fields differing in historical land-use intensity and thus representing a soil phosphorus gradient. For each studied field and restoration technique, we assessed the annual biomass yield and phosphorus removed with biomass. We expected (i) to remove more phosphorus by P-mining than by mowing; (ii) to deplete the stocks of bioavailable and slowly cycling phosphorus with a magnitude similar to the amount of phosphorus removed with biomass; and (iii) significant effects of the initial soil phosphorus concentration on (i) and (ii). We discuss the implication of our results in an ecological restoration context.

3.2 Methods

3.2.1 Study sites and experimental setup

We selected three fields with comparable soil conditions but different soil phosphorus concentrations to trade space for time, and hence allow for investigating phosphorus removal with biomass while soils become depleted of phosphorus. The study fields lay in Landschap de Liereman, a nature reserve in northern Belgium (Fig. 3.1). The fields were similar with regard to climate, hydrology and soil texture (Table 3.1), but differed in soil phosphorus concentrations because of differences in historical land-use intensity. The fields were previously managed as fertilized grasslands mown for hay production; fertilization ceased in 1990 for Liereman-1 and in 2005 for Liereman-2 and Liereman-3. The aim of the field owner (non-profit organization Natuurpunt) is to restore species-rich *Nardus* grasslands (European habitat type 6230*), which locally remain in small patches and road verges.

In April 2011, the experiment was set up by sowing the same species-mixture at all fields. First, the existing vegetation was removed by glyphosate application and ploughing the 0-30 cm soil layer. Second, the soils were limed with 1424 kg CCE (Calcium Carbonate Equivalent) ha^{-1} (2687 kg granular Dolokorn ha^{-1} containing: > 920 g [$CaCO_3$ + $MgCO_3$] kg^{-1}, from which at least 530 g CaO kg^{-1} and 300 g $MgCO_3$ kg^{-1}; following recommendations by the Belgian Soil Service) and harrowed. Third, we sowed the commercial Herbagreen plus mixture from Philip seeds at a seeding rate of 40 kg ha^{-1} with a seed drill: *Lolium perenne* L. cultivars Plenty (32%), Roy (20%), Milca (15%) and Alcancia (10%); *Phleum pratense* L. subsp. *pratense* cultivar Lirocco (10%); *Festuca pratensis* Huds. cultivar Preval (5%); *Poa pratensis* L. cultivar Balin (3%); and the herb species *Trifolium repens* L. (5%). This seed mixture contains species that can be used for forage production (*i.e.* the produced hay has an economical value to the farmer) on sandy soils. Liming was repeated every two years with 1200 kg CCE ha^{-1} (3000 kg granular Dolokorn ha^{-1} containing: > 600 g $CaCO_3$ kg^{-1} and > 300 g $MgCO_3$ kg^{-1}, from which at least 400 g CaO kg^{-1} and 150 g MgO; following recommendations by the Belgian Soil Service). In May 2012, we applied herbicides to favour the sown grass species (Bofix: 40 g [(4-Amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid L^{-1}, 20 g 3,6-Dichloropyridine-2-carboxylic acid L^{-1} and 200 g (4-Chloro-2-methylphenoxy)acetic acid L^{-1} at a rate of 5 L ha^{-1}). In 2013 and 2014, this was repeated in the parts of the fields managed by P-mining.

At each field, we marked eight plots of 4.5 m by 7 m, which we then further managed (from 2011-2016) by one of the two studied restoration techniques: mowing in four plots, P-mining in the other four plots, according to a randomized block design (Fig. 3.2). The total number of plots was 24 (8 plots x 3 fields). The mowing plots were managed without fertilization; the P-mining plots with annual fertilization of nitrogen (130 kg N ha^{-1} as NH_4NO_3) and potassium (225 kg K ha^{-1} as $K_2SO_4.MgSO_4$, *i.e.* following recommendations by the Belgian Soil Service). All plots were mown twice a year, except for 2011, *i.e.* the first year, when the vegetation was not fully developed and was mown only once, and 2013 when we mowed three times because of high standing crop biomass in August (see Appendix-Table 3.A). Each year, two thirds of the total annual amount of fertilizer were applied in March and one third after the first cut. We described the vegetation composition in June 2017 (see Appendix-Table 3.B).

3.2.2 Biomass sampling and chemical analyses

We sampled each plot two times per year, right before the fields were mown. All plots of a field were sampled (and mown) at the same time (see Appendix-Table 3.A for sampling dates). We harvested the biomass in the central part of each plot with a mower equipped with a finger-bar of 1.1 m width along a length of 2 to 4 m, which was measured immediately after cutting. We weighed the fresh biomass with hanging scales in the field, and took a sub-sample for measuring the dry matter content of the biomass (after drying the sample to constant weight at 65°C for at least 48 h).

The dry sub-samples were ground and digested with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs at 140°C for 4 h. In these extracts, the phosphorus concentration was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999) and the potassium concentration was measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS, Agilent, Santa Clara, United States of America). The nitrogen concentration was measured by high-temperature combustion at 1150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Hanau, Germany).

3.3.3 Soil sampling and chemical analyses

In April 2011 and January 2017, we collected soil samples with an auger of 3 cm diameter at two depths (0-15 and 15-30 cm) at three locations equally spread in the centre of each plot. We combined the three samples into a mixed sample per plot and per sampling depth, dried them at 40°C for 48 h and passed them through a 2 mm sieve. The bioavailable and slowly cycling phosphorus concentrations were measured by the following two methods: (i) extraction in sodium bicarbonate ($NaHCO_3$; according to ISO 11263:1994(E)) to measure bioavailable phosphorus (P_{Olsen}), which is available for plants within one growing season (Gilbert et al. 2009), and (ii) extraction in ammoniumoxalate-oxalic acid ($(NH_4)_2C_2O_4$ according to NEN 5776:2006) to measure slowly cycling phosphorus ($P_{Oxalate}$), which includes phosphorus that can become available on the longer term (van Rotterdam et al. 2012). In the 2011 samples, also the total phosphorus concentration (P_{Total}) was measured after complete destruction with perchloric acid ($HClO_4$; 65%), nitric acid (HNO_3; 70%) and sulphuric acid (H_2SO_4; 98%) in Teflon bombs at 150°C for 4 h. In the extracts, the phosphorus concentration was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999).

To measure soil bulk density at the two sampling depths, we collected undisturbed soil samples with standard sharpened steel 100 cm^3 Kopecky rings (diameter 5.1 cm, height 5 cm). We sampled at four locations in the centre of each field (5 sampling locations in Liereman-1 to cover the potential heterogeneity in this field). At each sampling location, we removed the topsoil containing most of the plant roots with a shovel and drove two rings in the 7-12 cm soil layer. We carefully exposed the 15-30 cm soil layer and collected another two rings from the 18-23 cm soil layer. The samples were dried until constant weight at 105°C for 48 h and weighed.

3.2.4 Calculations

We calculated the bulk density of the soil by dividing the dry weight of the soil sample by the volume of the Kopecky ring. Stocks of P_{Olsen} and $P_{Oxalate}$ were calculated by multiplying the bulk density with the phosphorus concentration and depth of the concerned soil layer. We calculated the changes in the P_{Olsen} and $P_{Oxalate}$ concentrations and stocks over time (ΔP_{Olsen}, $\Delta P_{Oxalate}$) by subtracting the 2011 from the 2017 values.

We calculated phosphorus removal with biomass by multiplying the biomass with its phosphorus concentration for each sampling time, and summed them per year to calculate annual biomass and phosphorus removal. We then calculated mean annual biomass and phosphorus removal for the years 2012 until 2016, leaving out 2011 because the vegetation was not yet fully developed then. The mean annual phosphorus concentration in dry biomass was calculated by dividing mean annual phosphorus removal by mean annual biomass.

We derived agronomic nutrient indices for phosphorus, nitrogen and potassium to describe the nutrient limitation for biomass growth according to Duru and Thélier-Huché (1995) and Duru and Ducrocq (1997). We calculated the phosphorus, nitrogen and potassium nutrition indices (PNI, NNI, KNI) as

$$PNI = 100 \times P\%/(0.065 \times N\% + 0.15),$$

$$NNI = 100 \times \; N\%/(4.8 \times biomass^{-0.32}),$$

$$KNI = 100 \times K\%/(1.6 + 0.525 \times N\%),$$

with *P%*, *N%* and *K%* the percentage of phosphorus, nitrogen and potassium in the *biomass* of the first cut (May-June). Values of 100% indicate there is no limitation by phosphorus, nitrogen or potassium; values above 100% indicate there is luxury consumption of this nutrient; and, values below 100% indicate deficiency in proportion to the index (Duru and Ducrocq 1997). For instance, values below 60% indicate severe limitation by this nutrient (Mládková et al. 2015).

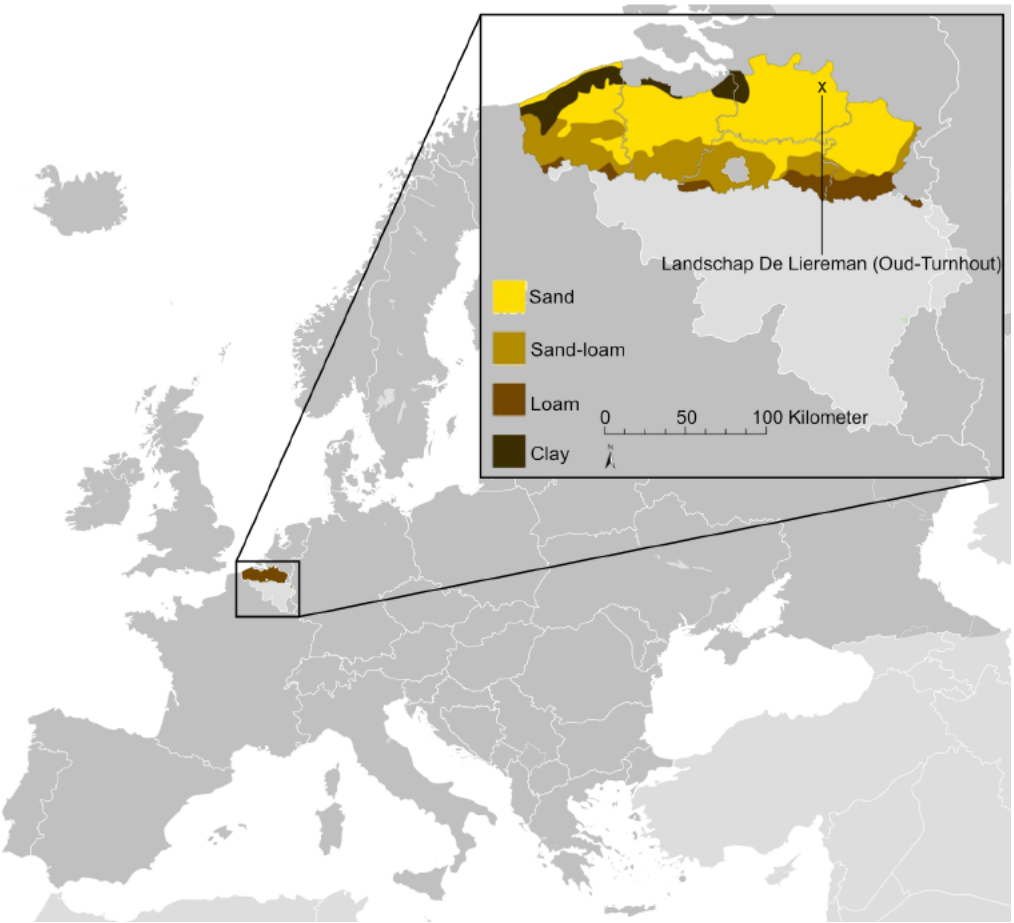


Figure 3.1 Map of Europe and northern Belgium (magnification in square) with the nature reserve (township between brackets) where the experiment took place. Soil classification according to the Belgian Soil Service

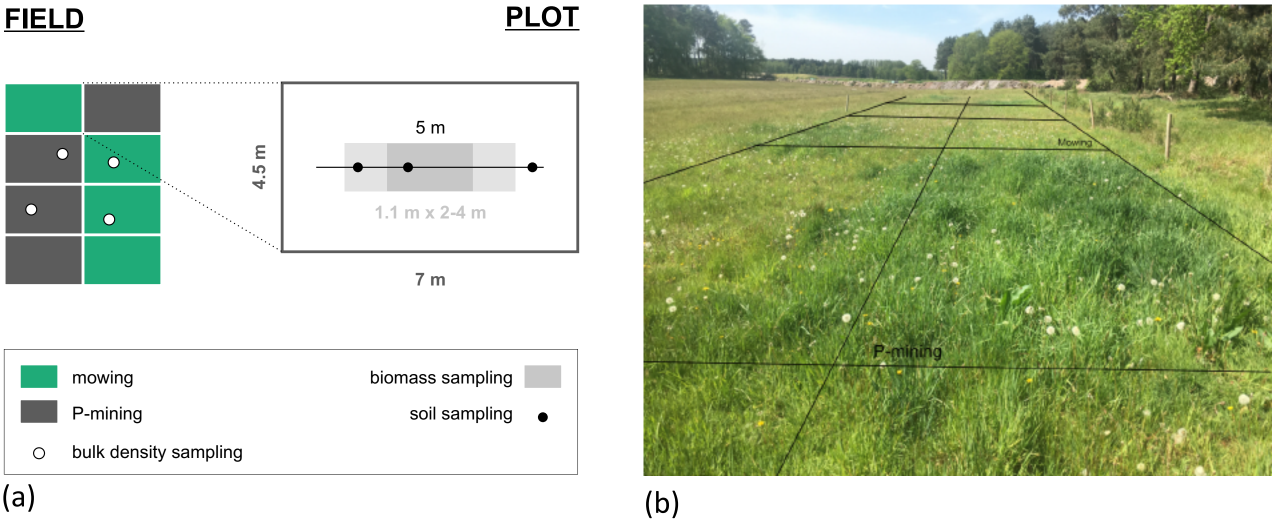


Figure 3.2 (a) Example of the experimental setup at each field. We sampled biomass and soil in the central zone within each of the mowing (green) and P-mining (grey) plots. Bulk density measurements were performed on four samples at each field. (b) Illustration of the effects of the restoration techniques mowing and P-mining in May 2016 in Liereman-1

Table 3.1 Initial soil properties of the three fields (2011). Samples from the 0-30 cm soil layer consisted of six soil cores bulked together to one sample per field. Samples from the 0-15 and 15-30 cm soil layers are shown by mean ± standard deviation with eight samples per field (*i.e.* one bulked sample for each plot); each of the samples consisted of three soil cores. Bulk density is shown by mean ± standard deviation with eight samples at four locations per field

	Depth (cm)	Liereman-1	Liereman-2	Liereman-3
Coordinates		51°20’0.1”N 5°1’1.7”E	51°19’58.4”N 5°0’57.4”E	51°20’50.5”N 5°1’11.2”E
Township		Oud-Turnhout	Oud-Turnhout	Oud-Turnhout
Nature reserve		Landschap De Liereman	Landschap De Liereman	Landschap De Liereman
Mean annnual precipitation[a] (mm)		870	870	870
Mean annual temperature[a] (°C)		10.5	10.5	10.5
BEMEX[b] classification	**0-30**	Coarse sand	Coarse sand	Coarse sand
WRB[c] classification	**0-30**	Gleyic Podzol (Arenic)	Albic Podzol (Arenic)	Gleyic Podzol (Arenic)
Organic C (weight %)[d]	**0-30**	4.3	1.5	1.4
pH_{KCl}[d]	**0-30**	4.1	4.8	4.6
P_{AL}(mg kg^{-1})[d]	**0-30**	30	130	300
K_{AL} (mg kg^{-1})[d]	**0-30**	30	<20	40
Ca_{AL} (mg kg^{-1})[d]	**0-30**	380	290	300
Mg_{AL} (mg kg^{-1})[d]	**0-30**	40	40	50
P_{Olsen} (mg kg^{-1})	**0-15**	29 ± 17	71 ± 16	112 ± 16
	15-30	27 ± 21	70 ± 15	108 ± 21
$P_{Oxalate}$ (mg kg^{-1})	**0-15**	66 ± 32	220 ± 84	422 ± 34[e]
	15-30	71 ± 47	225 ± 94	417 ± 52[e]
P_{Total} (mg kg^{-1})	**0-15**	196 ± 62	366 ± 118	605 ± 113
	15-30	245 ± 115	372 ± 116	565 ± 119
Bulk density (g cm^{-3})	**0-15**	1.5 ± 0.2	1.6 ± 0.1	1.5 ± 0.1
	15-30	1.5 ± 0.2	1.6 ± 0.1	1.5 ± 0.1

[a] Data between 1981 and 2010 by the Royal Meteorological Institute of Belgium for Oud-Turnhout;

[b] Soil classification according to Belgian Soil Service: “Coarse sand”: sandy fraction [50 µm - 2 mm] > 90% and median of sandy fraction > 200 µm and clay fraction [< 2 µm] < 8%;

[c] Soil classification according to the World Reference Base for Soil Resources classification (WRB);

[d] To characterize these fields, one bulked sample (regular grid, auger diameter = 3 cm, 6 soil cores per field) of the 0-30 cm soil layer per field was analysed by the lab of the Soil Service of Belgium: soil texture by manual characterization; organic carbon according to an SSB-adjusted Walkley and Black method (Walkley and Black 1934); P, K, Mg and Ca extracted in acid ammonium-lactate (resp. P_{AL}, K_{AL}, Mg_{AL} and Ca_{AL}; Egnér et al. 1960) measured with Inductively Coupled Plasma (ICP);

[e] Instead of eight samples, only six samples were analysed for $P_{Oxalate}$ in 2011

3.3.5 Statistical analyses

First, to investigate the effects of restoration technique (mowing vs. P-mining) and P_{Olsen} concentration in 2011 ($P_{Olsen-2011}$) on annual biomass properties for the period 2012-2016, we fitted linear mixed effects models with the function *lme* of the "nlme" package using the restricted estimates maximum likelihood method (REML) (Pinheiro et al. 2017). The different annual response variables we considered were: phosphorus removal with biomass, biomass yield (square root transformed), phosphorus concentration in biomass (log transformed), PNI (log transformed), NNI and KNI. The response variables were transformed if necessary to ensure normality of the residuals and homogeneity of variance following Zuur et al. (2009). The full models had the following form: response variable ~ restoration technique x $P_{Olsen-2011}$ + restoration technique x $P_{Olsen-2011}{}^2$. We used *plot* nested within *field* nested within *year* as a random factor to account for the nested structure of the data and the repeated measures (yearly observations) in a plot. We compared the null model including random effects only, the intermediate including all intermediate combinations of the model terms and full models based on the corrected Akaike Information Criteria (AICc) and Akaike weights (Zuur et al. 2009) with the *AICctab* function of the "bbmle" package (Bolker and Team 2016). In case of competitive models, *i.e.* multiple models with a difference in AIC_c (ΔAIC_c) of less than two (Goodenough et al. 2012), we used ANOVA tests to select the optimal model with the function *anova* of the "stats" package (R-Core-Team 2016). For the selected final models, we obtained chi-square (χ^2) values and significance of the fixed terms using Type II Wald chi-square tests with the *anova* function of the "stats" package. We assessed the goodness of fit of the optimal models by calculating the marginal and conditional R^2 (R^2_m and R^2_c) with the *r.squaredGLMM* function of the "MuMIn" package (Bartoń 2018), with R^2_m representing the variance explained by fixed factors and R^2_c the variance explained by both fixed and random factors.

Second, to investigate whether the soil phosphorus concentrations (P_{Olsen} and $P_{Oxalate}$) decreased over the course of the experiment, and whether this decrease was affected by the restoration technique, we fitted linear mixed effects models of the following form: P_{Olsen} or $P_{Oxalate}$ ~ restoration technique x Year, with *field* as random factor. We fitted linear models as we expected the decline in phosphorus concentrations to be linear during the relatively short time of our experiments (*i.e.* 5 years).

Third, we investigated the effects of restoration technique and $P_{Olsen-2011}$ on the changes in P_{Olsen} and $P_{Oxalate}$ concentrations (ΔP_{Olsen}, $\Delta P_{Oxalate}$) at 0-15 cm and 15-30 cm depth with linear mixed effects models of the form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ ~ restoration technique x $P_{Olsen-2011}$. We also evaluated the relation between the $\Delta P_{Oxalate}$ and ΔP_{Olsen} concentrations with a linear mixed effects model of the form: $\Delta P_{Oxalate}$ concentration ~ ΔP_{Olsen} concentration. Finally, the $\Delta P_{Oxalate}$ and ΔP_{Olsen} stocks were compared with the cumulative phosphorus removal with a model of the form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ stock ~ restoration technique x cumulative phosphorus removal, random = ~1|Field.

In the mixed effects models with heterogeneous variances among fields, we allowed for a different variance per field using weights *varIdent* (Pinheiro et al. 2017). Results were considered significant when the *p*-value was less than 0.05 unless indicated otherwise. All statistical analyses were performed with R (R-Core-Team 2016); graphs were made with the “ggplot” package (Wickham 2009).

3.3 Results

3.3.1 Phosphorus removal with biomass

The biomass yield, the phosphorus concentration in the biomass and consequently the annual phosphorus removal were all significantly affected by the applied restoration technique (Fig. 3.3; Fig. 3.4; Appendix-Tables 3.C.1 and 3.C.2). Between 2012 and 2016, we annually removed significantly more biomass and more phosphorus from the P-mining plots than from the mowing plots (biomass: 7 ± 2 vs. 3 ± 1 t dry biomass ha^{-1}; phosphorus: 18 ± 7 vs. 8 ± 5 kg P ha^{-1}; $p < 0.001$). The concentration of phosphorus in the biomass, however, was lower in the P-mining plots than in the mowing plots (2.5 ± 0.7 vs. 2.9 ± 0.7 g P kg^{-1}; $p < 0.001$ The biomass yield, the phosphorus concentration in the biomass and the annual phosphorus removal were significantly affected by the concentration of P_{Olsen} in the soil at the beginning of the experiment ($P_{Olsen-2011}$) and, except for the biomass yield, by the interactions between $P_{Olsen-2011}$ and the applied restoration technique (Appendix-Table 3.C.2). The applied restoration technique and $P_{Olsen-2011}$ explained 67% of the variation (R^2_m) in phosphorus removal with biomass. The interannual variation explained a large proportion of the remaining variation (51%): *e.g.* summer droughts, such as in 2014 and 2015, probably caused a drop in the biomass production during the dry months and consequently a drop in the phosphorus removal with biomass.

The agronomic phosphorus nutrient index (PNI) of the biomass in 2012-2016 differed significantly between the two restoration techniques and the interactions between $P_{Olsen-2011}$ and restoration technique ($p < 0.001$ for restoration technique; $p < 0.01$ for $P_{Olsen-2011}$ and the interactions; Appendix-Tables 3.C.3 and 3.C.4; Appendix-Fig. 3.C.1a). Phosphorus was not limiting in any of the mowing plots (mean PNI = 98 ± 21%), but appeared to be limiting in the mining plots of the field with the lowest soil phosphorus concentrations (mean PNI in Liereman-1 = 55 ± 13%). The biomass production in the mowing plots was limited by potassium (mean KNI 36 ± 15%; Appendix-Fig. 3.C.1c) and nitrogen (mean NNI 41 ± 13%; Appendix-Fig. 3.C.1b), with especially severe potassium limitation in the field Liereman-1 (mean KNI 24 ± 6%). The applied fertilizers in the mining plots resulted in only limited potassium limitation (mean KNI 82 ± 19%) but could not prevent nitrogen limitation (mean NNI 67 ± 19%), which was, however, less severe than in the mowing plots ($p < 0.001$).

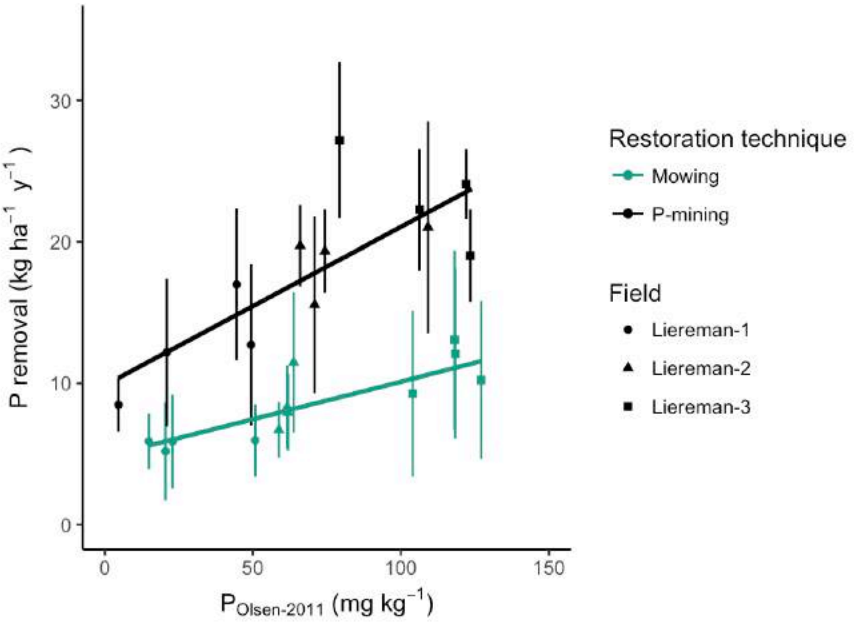


Figure 3.3 The mean and standard deviation of annual removal of phosphorus with biomass (kg ha^{-1} y^{-1}) plotted versus P$_{Olsen-2011}$ (mg kg^{-1}) for the 24 plots with mowing and P-mining treatment in the four study fields. The regression lines show the optimal linear mixed effect model fitted to the 120 data points for the 2012-2016 period

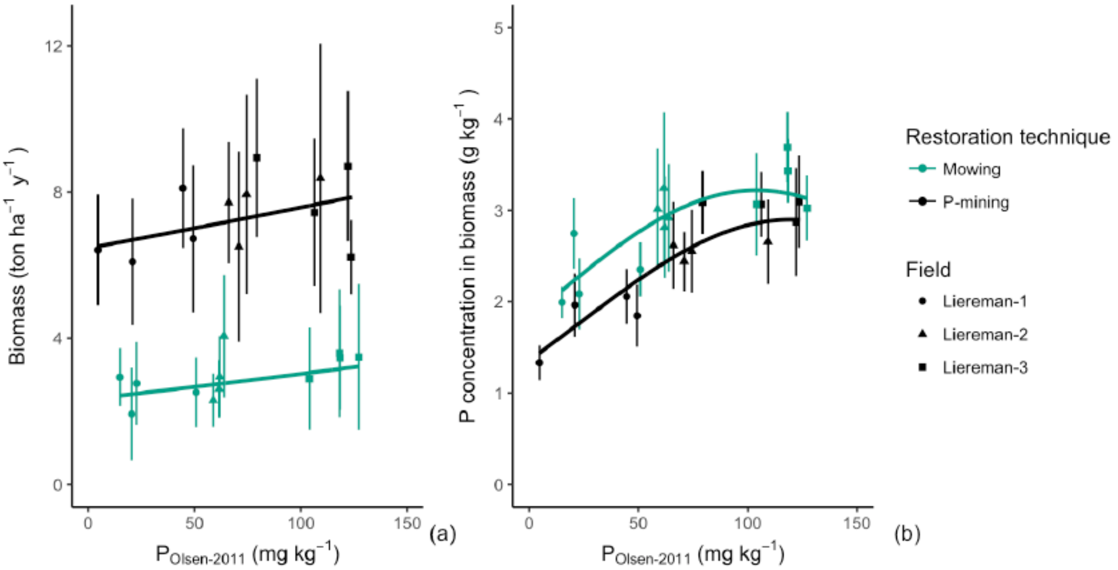


Figure 3.4 The mean and standard deviation of annual biomass (a; t dry biomass ha^{-1}) and annual phosphorus concentration in biomass (b; g P kg^{-1}) plotted versus P$_{Olsen-2011}$ (mg P$_{Olsen}$ kg^{-1}) for the 24 plots with mowing and P-mining treatment in the four study fields. The regression lines show the most optimal linear mixed effects model fitted to the 120 data points for the 2012-2016 period

3.3.2 Changes in soil phosphorus concentrations

The P_{Olsen} concentration in the 0-15 cm soil layer decreased significantly during the field experiment with on average 14 mg P_{Olsen} kg^{-1} ($p < 0.001$; Appendix-Fig. 3.C.2a). The 2011-2017 absolute decrease in P_{Olsen} concentration was larger in the soils with a high initial concentration of P_{Olsen} and larger in the P-mining plots of the Liereman-3 field (Appendix-Tables 3.C.5 and 3.C.6; Fig. 3.5a). The P_{Olsen} concentration in 2017 was on average 25% lower than in 2011 and this ratio was apparently lower for the highest initial P_{Olsen} concentrations, however, this effect was not significant (data not shown). We found no significant change in P_{Olsen} concentration in the 15-30 cm soil layer (Appendix-Fig. 3.C.2c). There was a strong positive correlation between the change in P_{Olsen} concentration and the change in $P_{Oxalate}$ concentration ($p < 0.001$; $R^2_m = 76\%$; Fig. 3.6; Appendix-Table 3.C.7). However, the change in the $P_{Oxalate}$ concentration varied widely in the 0-15 cm soil layer, and we found no significant change in $P_{Oxalate}$ concentrations for either soil layer (Appendix-Table 3.C.5; Appendix-Fig. 3.C.2b and 3.C.2d; Fig. 3.5b). Between 2011 and 2017, the stock in the P_{Olsen} decreased in the 0-15 and 0-30 cm soil layers, while the stock in $P_{Oxalate}$ remained the same (Fig. 3.7).

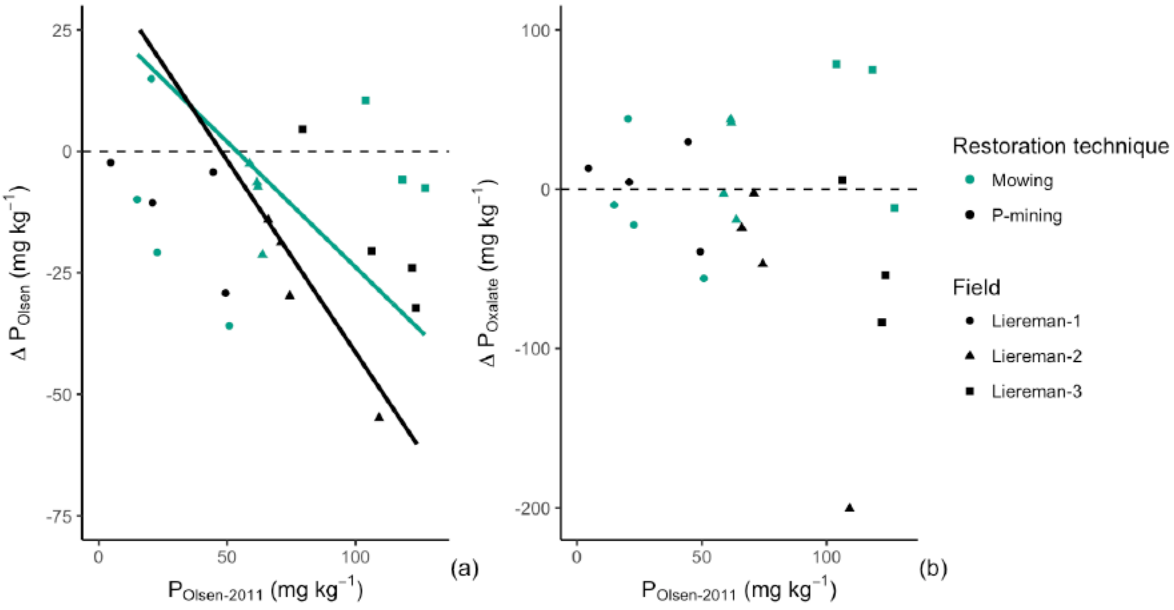


Figure 3.5 Changes in P_{Olsen} (a; mg kg^{-1}; n = 24) and $P_{Oxalate}$ (b; mg kg^{-1}; n = 22 because of two missing samples) in the 0-15 cm soil layer between 2011 and 2017 plotted versus $P_{Olsen-2011}$ (mg kg^{-1}). Negative values represent decreases in phosphorus concentrations; positive values show increases in phosphorus concentrations. The line was fitted according to the optimal linear mixed effects model for a transformed change in POlsen concentration as response variable

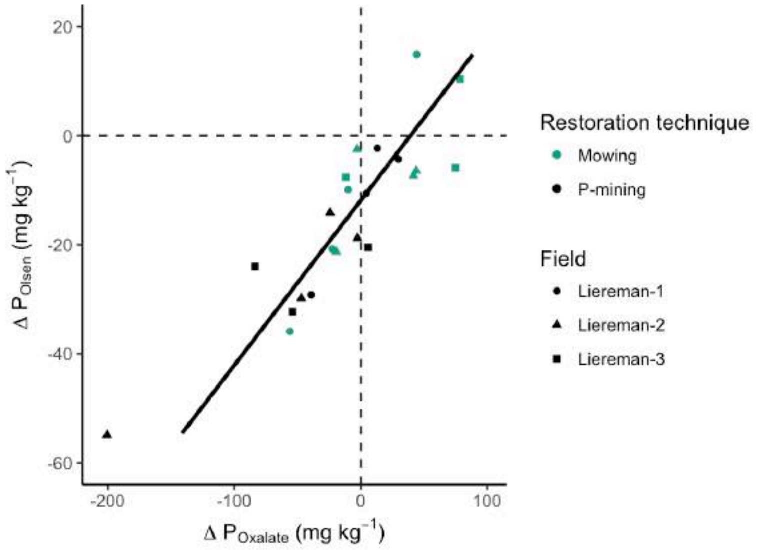


Figure 3.6 Changes in P_{Olsen} versus $P_{Oxalate}$ concentrations (mg kg^{-1}) in the 0-15 cm soil layer between 2011 and 2017 (n = 22). Negative values represent decreases in phosphorus concentrations; positive values show increases in phosphorus concentrations. The full black line was fitted according to the optimal linear mixed effects model for change in $P_{Oxalate}$ concentration as response variable

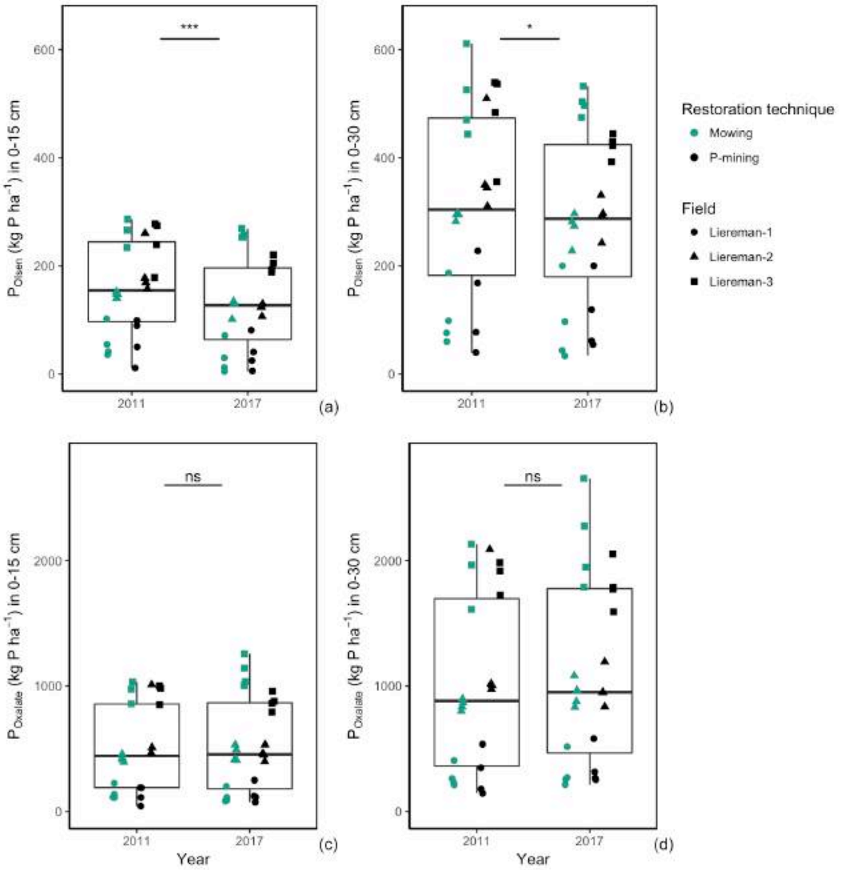


Figure 3.7 The stock of P_{Olsen} (a, b; in kg P ha^{-1}; n=24) and $P_{Oxalate}$ (c, d; in kg P ha^{-1}; n=22) in the 0-15 cm (a, c) and 0-30 cm (b, d) soil layers of the mowing and P-mining plots before and after the experiment (2011, 2017). Significance of effects is indicated by * *p* < 0.001; * *p* < 0.05; ns means the difference was not significant**

3.3.3 Phosphorus removal with biomass versus change in soil phosphorus stock

There was a weak negative relationship between the 2011-2017 change in the stock of P_{Olsen} in the 0-15 cm soil layer on the one hand and the removal of phosphorus with biomass on the other hand ($p < 0.1$, $R^2_m = 15\%$; Appendix-Tables 3.C.8 and 3.C.9; Fig. 3.8a). The relationship between the change in stock of P_{Olsen} in the 0-15 cm soil layer and the restoration technique was also weak ($p < 0.1$, $R^2_m = 13\%$; Supplementary Table S7). In general, the cumulative phosphorus removal with biomass was larger than the change in the stocks of P_{Olsen} in the soil (data points above the 1:1 line in Fig. 3.8a). The changes of $P_{Oxalate}$ stocks in the 0-15 cm soil layer varied widely and were significantly negatively correlated with the cumulative removal of phosphorus with biomass ($p < 0.05$; $R^2_m = 28\%$; Appendix-Tables 3.C.8 and 3.C.9; Fig. 3.8b).

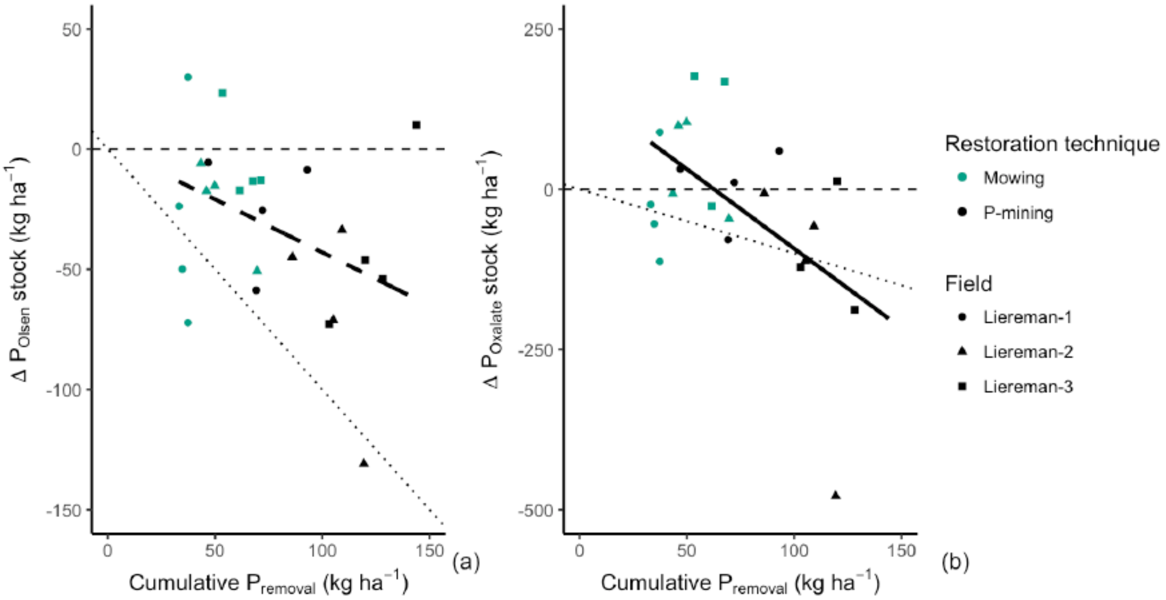


Figure 3.8 Change in P_{Olsen} stock (a; kg ha^{-1}; n = 24) and $P_{Oxalate}$ stock (b; kg ha^{-1}; n = 22) in the 0-15 cm soil layer between 2011 and 2017 plotted versus the cumulative P removal with biomass (kg ha^{-1}) between 2011 and 2016. Negative values represent decreases in phosphorus stocks; positive values show increases in phosphorus stocks. The dotted line is the 1:1 line. According to the linear mixed effects models, the dashed black line indicates a marginally significant relation ($p < 0.1$) and the full black line indicates a significant relation

3.4 Discussion

3.4.1 Phosphorus removal higher with P-mining and on phosphorus-rich soils

The removal of phosphorus with biomass in the P-mining plots was twice as large as in the mowing plots, and decreased with the initial concentration of P_{Olsen} in the soil. Johnston et al. (2016) also reported high phosphorus removal on phosphorus-rich soils (20-25 kg P ha^{-1}, similar to our results) and found phosphorus removal to be related to the soil phosphorus concentration on soils with less phosphorus. The lower phosphorus removal in the plots containing less P_{Olsen} in our space-for-time field experiment indicates that the extra phosphorus removal gained by P-mining will likely diminish over time during restoration. In our experiment, the phosphorus removal dropped linearly with decreasing P_{Olsen} concentration; in contrast, in the long-term Exhaustion Land experiment (Johnston et al. 2016), phosphorus removal was stable until the soil P_{Olsen} threshold decreased below 20 mg P_{Olsen} kg^{-1}, after which the relationship became linear as well. Hence, when estimating the time needed to restore phosphorus-poor soil conditions through mowing or P-mining, the decrease in annual phosphorus removal after a certain soil phosphorus level has been reached should be taken into account.

The difference in phosphorus removal between P-mining and mowing plots was mainly driven by the higher biomass yield in the P-mining plots, seeing that the phosphorus concentration in the biomass was lower in the P-mining plots. The swards in the P-mining plots yielded, on average, two times more biomass than in the mowing plots. Our mining swards annually yielded 7 ± 2 t dry biomass ha^{-1}, in line with other fertilized grasslands (on average 6 t dry biomass ha^{-1} y^{-1} in Duffková et al. 2015) but lower than the intensively fertilized grasslands that produced up to 14 t dry biomass ha^{-1} y^{-1} in Liebisch et al. (2013). The yields in our mowing plots, *i.e.* 3 ± 1 t dry biomass ha^{-1} y^{-1}, were similar to the yields in grasslands under nature management mown for 15 to 25 years in Belgium and the Netherlands (Bakker et al. 2002; Chapter 2). In the swards under mowing regime, the biomass production was severely limited by nitrogen and potassium from the second growing season onwards, in line with the well-described nitrogen and potassium depletion of soils through leaching or plant uptake (Van Der Woude et al. 1994; Smits et al. 2008; Oelmann et al. 2009; Storkey et al. 2015). The restraint on biomass production due to nitrogen and potassium limitation in the mowing plots probably explains that the biomass yield in these plots only weakly increased with soil phosphorus concentrations (Appendix-Table 3.C.1). Soil phosphorus appeared to be limiting only in the P-mining plots of the field with the lowest soil phosphorus concentration (Liereman-1; indicated by PNI < 60% *cf.* Mládková et al. 2015; Appendix-Fig. 3.C.1a).

The phosphorus concentration in the harvested biomass was affected by both the initial concentration of P_{Olsen} in the soil and the applied restoration technique. The plant phosphorus concentrations were positively correlated with the P_{Olsen} concentrations in the soil, *e.g.* in the P-mining plots we saw a twofold increase from on average 1.8 ± 0.3 mg P g^{-1} in the phosphorus-poor field (Liereman-1) to on average 3.0 ± 0.1 mg P g^{-1} in the phosphorus-rich field (Liereman-3; Fig. 3.4b). In the Liereman-3 mowing plots, the average PNI for June was equal to or greater than 100%, which indicates no limitation by phosphorus or even *luxury consumption* of this nutrient (Duru and Ducrocq 1997), *i.e.* an increase in phosphorus uptake with soil phosphorus concentration that does not result in additional biomass (Aerts and Chapin 1999). Interestingly, this luxury consumption of phosphorus also occurred in other P-mining studies: two pot experiments (Chapters 4 and 5) and a long-term field study (Bauke et al. 2018). The phosphorus concentration in the biomass was lower in the P-mining plots than in the mowing plots, which may be caused by: (i) *dilution*, *i.e.* concentrations of nutrients in plants tend to decrease - become diluted - as the plants produce more biomass (Hejcman et al. 2010), or (ii) *(temporary) phosphorus limitation* in the rhizosphere (Hinsinger 2001) as a result of a lag in the replenishment of the pools of bioavailable phosphorus in the soil, formerly described as *transactional phosphorus limitation* in Vitousek et al. (2010).

3.4.2 Decreasing P_{Olsen} over time

On average, the concentration of P_{Olsen} in the 0-15 cm soil layer was 25% lower at the end of the experiment than before the experiment started. In absolute numbers, the drop appeared to be larger in phosphorus-rich plots. We found a clear distinction in phosphorus removal with biomass between the two applied restoration techniques and the overall 2011-2016 phosphorus removal with biomass was related to the change in P_{Olsen} and $P_{Oxalate}$ stocks in the 0-15 cm soil layer. Changes in bioavailable soil phosphorus concentrations can be caused by plant phosphorus uptake through roots, translocation to shoots and subsequent removal with hay, but can also happen through phosphorus fixation in roots, uptake by microbial biomass or accumulation in soil organic matter. These organic pools of soil phosphorus can be a *sink* (*e.g.* incorporation of phosphorus into living microbial biomass), but can (later) also become a *source* of bioavailable phosphorus through mineralization and release of microbial phosphorus after cell death (von Lützow et al. 2006). Inorganic phosphorus can also be fixed by migration into organic phosphorus pools and occluded mineral pools (van der Salm et al. 2017), but phosphorus can also be released from organic or inorganic recalcitrant phosphorus pools. To gain full insight into the complex phosphorus cycle, a thorough fractionation procedure should be executed (cfr. De Schrijver et al. 2012), which was far beyond the scope of our study.

We want to note that our results are only valid for soils with a comparable sandy texture, soil acidity and organic matter content. The P_{Olsen} to $P_{Oxalate}$ ratio and the changes in this ratio after phytoextraction of phosphorus can differ across soil types (Cross and Schlesinger 1995; Johnston et al. 2016; Bauke et al. 2018)..

3.4.3 Limitations of space-for-time substitution

A chronosequence, or a space-for-time experimental setup, is a substitution for when long-term time-series are not available. Our observations cover five years, which is shorter than the period over which we want to make predictions (several decades). According to Johnson and Mijanishi (2008), a space-for-time substitution is:

> *"… a time sequence of development from a series of plots differing in age since some initial condition, i.e. time since the site became available for occupation or colonization or the time since last disturbance. It makes the critical assumption that each site in the sequence differs only in age and that each site has traced the same history in both its abiotic and biotic components. If these assumptions are correct, then each site will have repeated the successional sequence of every other older site up to its present age."*

In our field experiment, despite similarities between the fields (soil texture, landscape, land-use as a grassland), each of the three fields were managed and fertilized in a different manner affecting not only soil phosphorus concentrations but also in *e.g.* pH (slightly more acidic in Liereman-1) and organic carbon content (higher in Liereman-1; Table 3.1). Another limitation of a space-for-time substitution is the environmental fluctuation caused by time, which could mask results (Foster and Tilman 2000). In our experiment atmospheric nitrogen deposition was higher in the 90's (on average 45 kg N ha^{-1} y^{-1} in 1990 in northern Belgium; Cools et al. 2015) than at the time of the experiment (on average between 26 and 24 kg N ha^{-1} y^{-1} between 2011 and 2016 in northern Belgium; Vlaamse Milieumaatschappij, 2018). The difference in biomass production and phosphorus removal between the mowing and P-mining treatments is currently probably larger than in the 90's because a higher atmospheric nitrogen input would have made the mowing plots more productive. Despite these limitations, such a space-for-time substitution remains valuable to get insight into the interactive effects of initial soil phosphorus concentrations and management on biomass production and phosphorus removal. However, supplementing the current time-for-space experiment with repeated measurements in a long-term experiment could help validating the results and getting insight into the effects of environmental fluctuations (Johnson and Miyanishi 2008; Racz et al 2013).

3.4.4 Implications for ecological restoration

Phytomining is an effective technique for removing phosphorus from soils: it has a large capability for phosphorus removal, *i.e.* up to 34 kg P ha^{-1} y^{-1} at phosphorus-rich soils. When considering phytomining to re-establish phosphorus-poor soil conditions, managers should however be aware of four drawbacks.

First, on phosphorus-rich fields, large amounts of phosphorus need to be removed to restore phosphorus-poor soil conditions, and this can take several decades (Chapter 2). For the depletion of bioavailable phosphorus pools, it is necessary to also reduce the slowly cycling phosphorus pools that replenish the bioavailable pools. To estimate the restoration potential of specific fields for re-establishing phosphorus-poor soil conditions, practitioners therefore need to assess both the bioavailable and slowly cycling phosphorus pools.

Secondly, the available time and financial budget should match with the choice for a restoration technique despite the law of diminishing returns (see Kubanek 2017 on optimal decision making). The law of diminishing returns impedes the use of phytomining for restoration (Marrs 1993): phosphorus removal with biomass declines with decreasing bioavailable phosphorus concentrations in the soil despite constant restoration efforts. This decrease in phosphorus removal efficiency has to be taken into account when estimating the restoration time needed with phytomining. As we showed here, the potential to remove phosphorus with biomass by either mowing or P-mining did differ depending on the initial soil phosphorus concentration. The advantage of P-mining over mowing (*i.e.* more phosphorus removal through mining) was smaller when the soils contained less bioavailable (< 50 mg P_{Olsen} kg^{-1}) and slowly cycling phosphorus. The choice for a restoration technique is also an economical question (Rohr et al. 2018) because during restoration via phytomining, hay yields will likely decrease, as well as the quality of the produced hay. Feed containing less than 3 g P kg^{-1} dry biomass is inadequate for high yielding dairy cows according to Valk et al. (1999). The produced hay may thus change over time from a valuable product (feed) into a waste product (but see the solutions proposed by the interregional GrasGoed project: www.grasgoed.eu). It is advisable to account for this potential cost at the start of the restoration project; governments could encourage the perseverance of restoration efforts to achieve phosphorus-poor soil conditions via phytomining by subsidizing.

A third drawback to consider is that neither the bioavailable nor the slowly cycling phosphorus concentrations in the deeper soil layer (15-30 cm) had been decreased after five years of phytomining (contrary to the significant decline in the bioavailable phosphorus concentrations in the upper 0-15 cm soil layer). Yet, the phosphorus pools in the 15-30 cm soil layer and even the subsoil layer (> 30 cm deep) are likely to be accessible by plant roots or mycorrhiza in symbiosis with these plants, especially with P-mining (Bauke et al 2018). The phosphorus pools in these deeper soil layers should thus be taken into consideration when estimating the restoration potential of a field.

The fourth drawback specifically for P-mining is that fertilization with nitrogen can have negative effects on the surrounding environment if the fertilizer is administered incorrectly. When the applied nitrogen doses are excessive, residual soil mineral nitrogen can leach to the ground water (D'Haene et al. 2014). The risks of using nitrogen fertilizers can be minimized by following fertilizer recommendations to limit off-site loss. Reducing the temporarily high soil nitrogen and potassium concentrations after P-mining is possible by mowing without fertilizer input for a few years (Smits et al. 2008).

Restoring phosphorus-poor soil conditions (*e.g.* <12 mg P_{Olsen} kg^{-1} for restoration of *Nardus* grasslands; Chapter 2) on phosphorus-rich soils, such as the Liereman-3 field in our study, via

P-mining or mowing is a time-consuming approach that will take many decades (see estimation of the duration in Chapter 2 and Box 2). For these parcels, more drastic measures such as mechanically removal (*i.e.* topsoil removal; cf. Török et al. 2011) or *in situ* burial (*i.e.* full-inversion tillage; cf. Milligan et al. 2016) of the phosphorus-enriched soil layer can be considered. When reducing the bioavailable and slowly cycling phosphorus pools is not feasible, it might be more appropriate to aim for floristically diverse mesotrophic plant communities that require less phosphorus-poor soil conditions, and certainly are interesting for diverse insect communities that benefit *e.g.* pollination and natural pest control in the surrounding (Woodcock et al. 2014). To work towards this aim, mowing to obtain nitrogen-, potassium- or co-limitation of these nutrients within a few years can be combined with the active introduction of common non-endangered legume and forb species following sward disturbance to enhance their germination (Klaus et al. 2018).

3.5 Conclusions

The potential to phytomine phosphorus (with either P-mining or mowing) depends on the phosphorus concentration in the soil, with high phosphorus removal possible on phosphorus-rich soils and the removal potential becoming lower on soils with lower phosphorus concentrations. Phosphorus mining led to larger phosphorus removal with biomass compared to conventional mowing management. It is clear that restoring phosphorus-poor soil conditions on formerly fertilized land will remain a challenge and that phytomining phosphorus is always a long-term commitment. For initially phosphorus-rich parcels, turning the nature restoration goal towards communities of mesotrophic habitats might be more realistic when drastic measures such as removal of the phosphorus-rich topsoil are not an option.

3.6 Acknowledgements

We are grateful for the admission to conduct our field experiment in Landschap De Liereman (Natuurpunt); the cooperation with the people of Natuurpunt, Agency of Forest and Nature (ANB) and local farmers was greatly appreciated. Special thanks to Kris Van der Steen, Jan Van den Berghe and Dieter Dijckmans. Further, we thank our lab technicians Luc Willems and Greet de Bruyn for processing and analysing our samples. Kris Ceunen, Filip Ceunen, Luc Willems, Robbe De Beelde, Jelle Van den Berghe, Danny Vereecke and Tomohiro Nagata are thanked for helping with fieldwork. Lander Baeten and Haben Blondeel are thanked for helping out with the statistics. We thank three anonymous reviewers for their helpful comments.

CHAPTER 4

Phosphorus-mining for ecological restoration on former agricultural land

Second harvest in the pot experiment

After: Schelfhout S, De Schrijver A, De Bolle S, De Gelder L, Demey A, Du Pré T, De Neve S, Haesaert G, Verheyen K, Mertens J. 2015. Phosphorus mining for ecological restoration on former agricultural land. Restoration Ecology. 23:842–851. Doi: 10.1111/rec.12264

Abstract

To restore species-rich terrestrial ecosystems on ex-agricultural land, establishing nutrient limitation for dominant plant growth is essential, because in nutrient-rich soils, fast-growing species often exclude target species. However N-limitation is easier to achieve than P-limitation (because of a difference in biogeochemical behavior), biodiversity is generally highest under P-limitation. Commonly-used restoration methods to achieve low soil P-concentrations are either very expensive or take a very long time. A promising restoration technique is P-mining, an adjusted agricultural technique that aims at depleting soil-P. High biomass production and hence high P-removal with biomass is obtained by fertilizing with nutrients other than P. A pot experiment was set up to study P-mining with *Lolium perenne* L. on sandy soils with varying P-concentrations: from an intensively-used agricultural soil to a soil near the soil P-target for *Nardus* grassland. All pots received N- and K-fertilization. The effects of biostimulants on P-uptake were also assessed by the addition of arbuscular mycorrhiza (*Glomus* spp.), humic substances or phosphate-solubilizing bacteria (*Bacillus* sp. and *Pseudomonas* spp.). In our P-rich soil (111 mg P_{Olsen} kg^{-1}), P-removal rate was high but bioavailable soil-P did not decrease. At lower soil P-concentrations (64 and 36 mg P_{Olsen} kg^{-1}), bioavailable soil-P had decreased but the P-removal rate had by then dropped 60% despite N- and K-fertilization and despite that the target (< 12 mg P_{Olsen} kg^{-1}) was still far away. None of the biostimulants altered this trajectory. Therefore, restoration will still take decades when starting with ex-agricultural soils unless P-fertilization history was much lower than average.

4.1 Introduction

Possibly, P-mining might be optimized by increasing the P-uptake through the use of plant biostimulants (Calvo et al. 2014): *e.g.* the addition of arbuscular mycorrhiza, humic substances or phosphate-solubilizing bacteria. Mycorrhiza can provide P to the plant by overcoming the P-depletion zone in the rhizosphere with an extensive hyphal network (Smith and Read 2008). Humic substances are humic and fulvic acids that can mobilize and solubilize P by (i) blocking P-adsorption-sites (ligand exchange), (ii) oxide dissolution by complexing aluminum (Al) or iron (Fe) held in minerals, (iii) mobilization of P held in metal-humic substances or (iv) via alteration of the soil pH (Gyaneshwar et al. 2002; Drouillon and Merckx 2003; Jones et al. 2003). Application of humic substances resulted in higher yield and consequently higher P-uptake for several crops (Verlinden et al. 2009; Daur and Bakhashwain 2013). Phosphate-solubilizing bacteria are free-living, plant-growth-promoting rhizobacteria mainly from the genera *Pseudomonas* and *Bacillus* that can solubilize P from calcium (Ca) or Al phosphate sources (Rodriguez and Fraga 1999; Rosas et al. 2006; Trivedi and Sa 2008). Recently, a pot experiment showed the positive effect of *Bacillus brevis*, *Pseudomonas putida* and *P. corrugata* on the available P conditions in acid, sandy soils with high total P-contents (De Bolle et al. 2013b).

Here we present the results of a pot experiment where P-mining with N- and K-fertilization, was tested in combination with several biostimulants at varying soil P-concentrations with *Lolium perenne* L. The different P-concentrations represent three phases in the development from agricultural soils towards soils with low bioavailable P-pools, a soil P-chronosequence. We hypothesized that P-removal through P-mining will become less effective with decreasing soil P-concentration despite N- and K-fertilization and that biostimulants enhance P-mining especially at the lowest soil P-concentrations.

4.2 Methods

A pot experiment was conducted to test the effect of (i) the soil P-concentration and (ii) additions of mycorrhiza, humic substances and P-solubilizing bacteria on biomass production, P-concentrations, P-removal and final plant available P-concentrations.

4.2.1 Set-up of the experiment

The soil originated from three sites located within a five hundred meter radius in Landschap De Liereman (Oud-Turnhout) nature reserve in the Belgian Campine region. On these sites, the ultimate target was the restoration of European Natura 2000 priority habitat 6230*, species-rich semi-natural *Nardus* grassland (Galvánek and Janák 2008). Non-degraded *Nardus* grasslands are closed dry or mesophilic, perennial grasslands on oligotrophic soils that have a bioavailable P-concentration of less than 12 mg P_{Olsen} kg^{-1} (Raman et al. *unpublished results*; Chapters 2 and 6), this will be considered as the target in this paper. The three sandy soils were selected based on their bioavailable P-concentrations: 111 mg P_{Olsen} kg^{-1} further referred to as *High-P*, 64 mg P_{Olsen} kg^{-1} further referred to as *Mid-P* and 36 mg P_{Olsen} kg^{-1} further referred to as *Low-P* (Table 4.1), and represented three phases in the development towards P-poor soil conditions (12 mg P_{Olsen} kg^{-1}), *i.e.* a soil P-chronosequence. The three sites are currently managed as hay meadows and cropped annually since they were taken out of agriculture. In the Low-P site, fertilization stopped about twenty years ago. The Mid-P and High-P sites were taken out of agriculture five years ago but P-concentrations differed due to different fertilization histories. When the three parcels were still in agricultural use, they were plowed regularly and therefore, the topsoil of at least 20 cm was quite homogenous in nutrient concentrations. In August 2011, soil of the three parcels was collected from the 5 – 15 cm soil layer to avoid the plant roots from the top 0 – 5 cm layer. We sieved (25 mm mesh), thoroughly homogenized, sampled and analyzed to assess the initial humidity and chemical soil conditions. Afterwards, each soil was hydrated up to 20% (V%). For each soil P-concentration and treatment with biostimulants or control, seven pots of 15 cm diameter and 12 cm depth were filled with 1.4 kg soil. Pots were lined with a polyethylene bag in order to avoid soil and P from leaching during watering. In each pot, 1 g ryegrass seed (*Lolium perenne* L., variety Plenty) was sown on the 18th of August 2011 (day 1). Pots were placed randomly in a growth chamber with a day/night regime of 16/8 h, temperature of 18°C and a relative humidity maintained at 75% for 123 days. The humidity of the soil was kept around 20% (V%) with purified water.

Table 4.1 Characterization of the initial soil properties for the 5-15 cm soil layer of the selected sites.

Soil properties	High-P	Mid-P	Low-P
Coordinates	51°20’50.5”N 5°1’11.2”E	51°19’58.4”N 5°0’57.4”E	51°20’0.1”N 5°1’1.7”E
P_{CaCl2} (mg kg^{-1})	7.00	0.93	0.23
P_{Olsen} (mg kg^{-1})	111	64	36
$P_{Oxalate}$ (mg kg^{-1})	414	196	59.4
$Al_{Oxalate}$ (mg kg^{-1})	619	543	380
$Fe_{Oxalate}$ (mg kg^{-1})	349	246	175
PSD (%)	46	26	11
P_{Total} (mg kg^{-1})	656	329	171
Agricultural value P-concentration[a]	Rather high	Rather low	Very low
pH (KCl)	4.64	4.39	3.94
C_{Total} (%)	1.60	1.00	0.99
N_{Total} (%)	0.11	0.06	0.05
C:N	14.5	16.7	19.8
K_{BaCl2} (mg kg^{-1})	16.2	10.2	14.7
Ca_{BaCl2} (mg kg^{-1})	741	355	128
Mg_{BaCl2} (mg kg^{-1})	62.4	30.2	9.85
Na_{BaCl2} (mg kg^{-1})	2.49	2.49	4.00
Al_{BaCl2} (mg kg^{-1})	30.0	52.0	136
CEC (meq kg^{-1})	4.60	2.63	2.29
BS (%)	93	78	34

[a] Rating of the P-concentration following advice by the Soil Service of Belgium (Bodemkundige dienst)

Based on recommendations by the Soil Service of Belgium and to avoid limitation effects by nutrients other than P during the experiment, we fertilized all pots three times with N as NH_4NO_3 and K as KNO_3 on day 0, 44 and 95 (for details see Appendix-Table 4.A). In total, 351 mg N and 468 mg K were added per pot, corresponding with 198 kg N ha^{-1} and 319 kg K_2O ha^{-1} in the field. Soils were limed according to their initial soil pH (the lime consisted of 60% $CaCO_3$ and 30% $MgCO_3$; High-P received 336 mg pot^{-1}, Mid-P 434 mg pot^{-1} and Low-P 602 mg pot^{-1}).

Next to the control that only received N- and K-fertilization, four treatment levels of biostimulants were applied on day 1 of the experiment: (i) the application of arbuscular mycorrhiza, (ii and iii) addition of humic substances at two levels (HS1 and HS2), and (iv) application of phosphate-solubilizing bacteria. For the mycorrhiza treatment, the commercially-available product *INOQ Agri* was used (from INOQ GmbH, Schnega, Germany). This contained three species of the genus *Glomus* coated on vermiculite as carrier material: *Glomus etunicatum* Becker and Gerd., *G. intraradices* Schenck and Sm. and *G. claroideum* Schenck and Sm.. After sowing, mycorrhiza were applied on top of the soil at a dose of 1.5 g per pot. The humic substances used in this experiment were a liquid mixture of humic (12% w/w) and fulvic acids (3% w/w) from the commercially-available product *Humifirst®*. Humic substances were applied in two concentration levels; HS1 with 0.05 ml Humifirst per pot (50 liter ha^{-1}, the recommended dose by the producer) and HS2 with 7 ml Humifirst per pot (7,000 liter ha^{-1}, a similar dose as used in experiments to fixate P with various chemical amendments (Ann et al. 2000; Diaz et al. 2008)). The addition of Phosphate-solubilizing bacteria was done as described by De Bolle et al. (2013) and consisted of a mixture of *B. brevis* (ATCC 8246), *P. putida* (ATCC 12633) and *P. corrugata* (ATCC 29736) obtained from DSMZ (Braunschweig, Germany). The bacterial inoculum consisted of 2.2×10^8 colony-forming units (CFU) g^{-1} of dry soil, based on the population size of phosphate-solubilizing bacteria in soils as found by Hu et al. (2009).

Germination rates of *L. perenne* were low in two pots of the Low-P soil with the phosphate-solubilizing bacteria treatment, therefore biomass production was much lower than in the other five pots. These two pots were removed from the dataset.

To verify mycorrhizal survival at the end of the experiment, roots of *L. perenne* were cleared and stained using to the ink and vinegar method (Vierheilig et al. 1998). Colonization by mycorrhiza was affirmed by microscopy in the treatments where mycorrhiza were added. The roots in pots without addition of mycorrhiza were not infected.

To determine the prevalence of the phosphate-solubilizing bacterial inoculations, we tried to assess the three species added to the pots in an extra experiment. The wild type strain was first made rifampicin (Rif) resistant, we only succeeded to do this with *P. corrugata*. This resistant strain was then inoculated and after 30 days monitored through plating on LBagar supplemented with rifampicin and kanamycin (for detailed information on the method, see Appendix 4.B). Overall, the amount of colony forming units per gram of soil decreased from circa $3x10^7$ to $2x10^5$ after 30 days (data not shown) indicating that though the bacteria were present at a lower concentration than initially, they could have an effect in the pot experiment (see also Canbolat et al. 2006 and Yu et al. 2011).

4.2.2 Sampling and chemical analysis

To get an insight in how the three soils differ in chemical composition, initial soil characteristics were assessed on sub-samples of the three collected soils after mixing the bulk samples thoroughly and before fertilization. Samples were dried at 40°C for 48 h and then passed through a 1-mm sieve. Soil pH (with KCl as extractant) was measured using a glass electrode (Orion, Orion Europe, Cambridge, England, model 920A) following the procedure described in ISO 10390:1994(E). Exchangeable K^+, Ca^{2+}, Mg^{2+}, Na^+ and Al^{3+} concentrations were measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS) after extraction in $BaCl_2$ (NEN 5738:1996). For calculation of effective cation exchange capacity (CEC) of the soils, all extracted exchangeable cations (K^+, Ca^{2+}, Mg^{2+}, Na^+ and Al^{3+} in meq kg^{-1}) were summed. Effective base saturation (BS) was calculated by the ratio of the sum of K^+, Ca^{2+}, Mg^{2+} and Na^+ over the sum of K^+, Ca^{2+}, Mg^{2+}, Na^+ and Al^{3+}. Total carbon (C) and total N content were measured by dry combustion at 850°C using an elemental analyzer (Vario MAX CNS, Elementar, Germany). Further, we quantified the original soil P-concentrations by analyzing bulk samples on:

i. Soluble and readily soluble P extracted in $CaCl_2$ (P_{CaCl2}; Simonis & Setatou 1996) and measured by ICP (CAP 6000 series, Thermo Fisher-scientific Inc., Waltham, Massachusetts, USA);

ii. Bioavailable P which is available for plants within one growing season (Gilbert et al. 2009) by extraction in $NaHCO_3$ (P_{Olsen}; according to ISO 11263:1994(E)) and colorimetric measurement according to the malachite green procedure (Lajtha et al. 1999);

iii. Slowly cycling P, which also includes P that can become available on the longer term and is adsorbed by Al and Fe. This P-fraction was extracted in ammoniumoxalate-oxalic acid ($P_{Oxalate}$, $Al_{Oxalate}$ and $Fe_{Oxalate}$; according to NEN 5776:2006). P-concentrations were measured according to the malachite green procedure. Al and Fe concentrations were measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS) and

iv. Total P measured after complete destruction with $HClO_4$ (65%), HNO_3 (70%) and H_2SO_4 (98%) in Teflon bombs for 4 h at 150°C (P_{Total}). P-concentrations were measured according to the malachite green procedure.

The soil P-concentrations at the end of the experiment (day 123) were also analyzed on bioavailable P (P_{Olsen}, as described above in ii). Grass was cut two cm above the soil level, at four times (day 29, 60, 95 and 123). For each grass cutting, grass samples were weighed (dry mass, DM) after drying to constant weight at 70°C for 48 h and P-concentration was obtained after digesting 100 mg of the sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs for 4 h at 140°C. Phosphorus was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999). Plant K and N were measured in a subset of the samples to get an estimation of how much K- and N-fertilizer was recovered with the biomass cuttings and whether these elements were limiting plant growth. The subset consisted of six samples: two biomass samples from each soil-P level in the control treatment at day 95. Plant K-concentrations were obtained also after digestion in Teflon bombs as described previously and measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS). Plant N-concentrations were measured by high temperature combustion at 1150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Germany). At day 95, none of the samples indicated to have K- or N-deficiencies based on optimal concentrations and the average P nutrition index (see Appendix 4.C).

4.2.3 Calculations

Per harvest time, P-removal was calculated by multiplying biomass production and P-concentration in biomass. Total biomass production and total P-removal are the sums over the four harvest times. The mean biomass P-concentration was calculated by dividing the total P-removal by the total biomass removal. The phosphate saturation degree (PSD) of the initial soil samples estimates P losses to the ground water and also the potential P to be desorbed and thus supplied to the bioavailable pool. We calculated PSD by $PSD = [P_{Oxalate}/0.5\times(Al_{Oxalate} + Fe_{Oxalate})]\times 100(\%)$ with $P_{Oxalate}$, $Al_{Oxalate}$ and $Fe_{Oxalate}$ representing the numbers of moles of P, Al and Fe per kg of soil extracted with ammoniumoxalate-oxalic acid (see Sampling and Chemical Analysis). The denominator is an estimation of the total P sorption capacity of the soil. Soils are ranked as ‘P-critical’ at a PSD of higher than 24%, which corresponds with more than 0.1 mg orthophosphate/liter groundwater. This means these soils can lead to eutrophication of surface waters. Soils with PSD-values higher than 35% are regarded as ‘P-saturated’ and result in more than 0.3 mg orthophosphate/liter groundwater (Van Meirvenne et al. 2007). We further calculated the difference in bioavailable P-stocks at the beginning and the end of the pot experiment. Bioavailable P-stock (in mg P pot^{-1}) at day 0 and day 123 were calculated by multiplying the bioavailable P-concentration (in mg P_{Olsen} kg^{-1}) at that time with the amount of soil per pot (1.4 kg).

4.2.4 Statistical analyses

Homogeneity of variances was tested using Bartlett tests, heterogenic response variables (P_{Olsen}) were transformed (square root) to achieve the homogeneity assumption. Comparisons of means (total biomass production, mean P-concentration, total P-removal and final bioavailable P-concentration) were done using two-way ANOVA tests with soil P-concentration and treatment and their interaction as factors. Residuals from these tests were examined for normal distribution with quantile-quantile plots. If the interaction between soil P-concentration and treatment was not significant, this interaction term was omitted from the model and the additive model was used for further comparison of the means. Multiple comparisons of treatments, soil P-concentrations and their interaction if significant were performed by means of Tukey using the HSD.test from R-package “agricolae”. A one-way student’s t-test was used to find differences between the bioavailable P-concentration at the end of the experiment and at the beginning. All statistical tests were computed with R 2.14.0 (R Core Team 2013) and statistical significance was set at *p* less than 0.05.

4.3 Results

4.3.1 Effects of soil P-concentrations on P-removal

The main determinant of the amount of P extracted in biomass was the soil P-concentration (Table 4.2). Both biomass production and biomass P-concentration decreased significantly when going from High-P to Mid-P (resp. 23% and 45% lower) and from High-P to Low-P (resp. 36% and 53% lower) (Tables 4.2 and 4.3). The further decrease in P-removal from Mid-P to Low-P was not significant (Fig. 4.1).

4.3.2 Changes in bioavailable P-concentration

Bioavailable P-concentrations (P_{Olsen}) seemed to increase 5% between day 0 and day 123 for High-P (student’s t-test, $p<0.05$), despite high P-removal with biomass (Fig. 4.1). In Mid-P, bioavailable soil P-stocks decreased 9% ($p<0.001$) and this seemed equal to the amount of P removed with biomass. In contrast, in Low-P, bioavailable soil P-stocks decreased 29% ($p<0.001$), more than the amount of P removed with biomass.

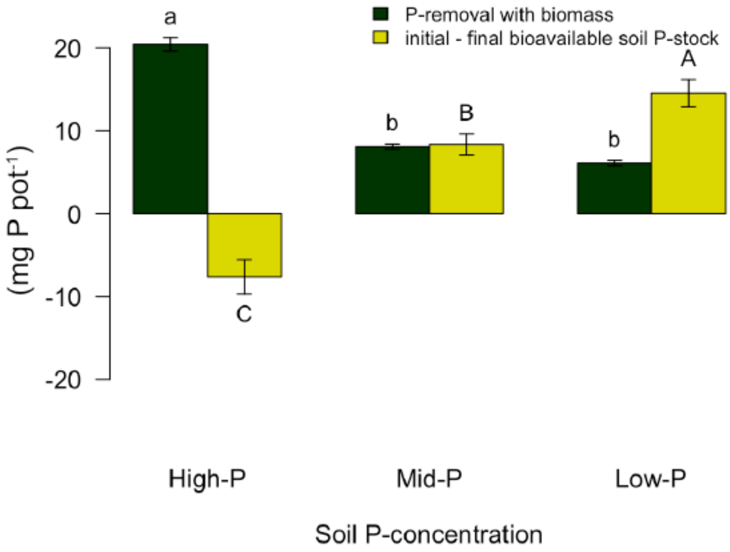


Figure 4.1 In High-P, P-removal did not result in a decline of bioavailable soil-P, while in Mid-P, P-removal was equal to the decrease in bioavailable soil-P. In Low-P, bioavailable soil P-stocks decreased more than the amount of P that was removed with biomass. Mean ± SE (n = 7) of change in bioavailable soil P-stocks (initial − final bioavailable soil P-stock) and total P-removal with L. perenne over 123 days (mg P pot^{-1}). Upper- and lowercase letters, respectively, indicate significant differences between mean changes in bioavailable soil-P stocks and mean P-removal with biomass (Table 4.2)

Table 4.2 Effects of soil P-concentration, the addition of biostimulants and their interaction on total biomass production, mean P-concentration, total P-removal, Initial – final bioavailable P-stock

	Soil P-concentration		Biostimulant addition		Interaction		
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	Error
Total biomass production	144.8	***	21.7	***	11.2	***	
Mean P-concentration	450.8	***	18.7	***	1.3	NS	
Total P-removal	1675.5	***	5.0	**	21.9	***	
Final bioavailable P-concentration	4284.9	***	5.4	***	1.5	NS	
Initial – final bioavailable P-stock	65.7	***	5.9	***	1.9	NS	
Degrees of freedom	2		4		8		88

Results of two-way ANOVA (f-values and significance levels) are shown (n = 7). Significance levels are *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS = not significant

Table 4.3 Mean ± SD (n=7, except for Low-P with P solubilizing bacteria where n=5) for each soil P-concentration and treatment with biostimulants of total biomass production, mean P-concentration in biomass and final bioavailable P-concentration

	Soil P-concentration		P-mining		P-mining + arbuscular mycorrhiza		P-mining + humic substances (50 liter ha^{-1})		P-mining + humic substances (7000 liter ha^{-1})		P-mining + phosphate-solubilizing bacteria	
Total biomass production (g dry biomass pot^{-1})			B		B		AB		A		C	
	High-P	A	4.80 ± 0.65		5.04 ± 0.49		5.37 ± 0.21		4.65 ± 0.66		5.00 ± 0.69	
	Mid-P	B	3.69 ± 0.66	a	3.89 ± 0.53	a	3.86 ± 0.53	a	4.16 ± 0.59	a	1.70 ± 0.43	b
	Low-P	C	3.09 ± 0.48	b	2.64 ± 0.44	bc	2.76 ± 0.30	bc	4.11 ± 0.37	a	1.88 ± 0.27	c
Mean biomass P-concentration (mg P g^{-1} dry biomass)			BC		BC		C		B		A	
	High-P	A	4.30 ± 0.44		4.09 ± 0.22		3.72 ± 0.24		4.05 ± 0.48		4.69 ± 0.46	
	Mid-P	B	2.22 ± 0.19		2.12 ± 0.19		2.10 ± 0.15		2.29 ± 0.23		2.92 ± 0.33	
	Low-P	C	2.01 ± 0.42		1.98 ± 0.23		1.97 ± 0.11		2.28 ± 0.33		2.54 ± 0.31	
Final bioavailable P-concentration (mg P_{Olsen} kg^{-1}) at 123 days			B		C		C		C		A	
	High-P	A	116.29 ± 3.9		108.46 ± 2.4		107.57 ± 7.5		107.40 ± 5.5		112.59 ± 4.4	
	Mid-P	B	57.90 ± 2.4		57.31 ± 4.1		55.84 ± 2.5		56.40 ± 2.5		58.74 ± 2.9	
	Low-P	C	25.46 ± 3.1		23.11 ± 3.6		23.64 ± 1.2		25.67 ± 2.4		28.20 ± 4.0	

All pots received N- and K-fertilization, more additions were as follows: arbuscular mycorrhiza; humic substances at 50 liter ha^{-1}; humic substances at 7,000 liter ha^{-1}; and phosphate-solubilizing bacteria.

Lowercase letters show the significant differences within one soil P-level if the interaction was significant (two-way ANOVA and Tukey HSD post hoc tests). Uppercase letters show the main effects of soil P-concentration and biostimulant addition. The target for species-rich Nardus grasslands is < 10 mg P_{Olsen} kg^{-1} (Raman et al. 2014)

4.3.3 Effects of biostimulants on P-removal

While biostimulants had little overall effects on P-removal in biomass (Fig. 4.2), there were some interesting effects of biostimulants at specific soil P-concentrations. In High-P, the addition of phosphate-solubilizing bacteria resulted in the highest P-removal compared to all other treatments. In Mid-P, however, this addition caused a significant decline in P removal (40%). In the Low-P soil, the humic substances treatment with high dosage (HS2) significantly improved the total P-removal by 52% because of a significant 33% increase in biomass production. Over all soil P-concentrations, the P-concentration in biomass was significantly increased with addition of phosphate-solubilizing bacteria.

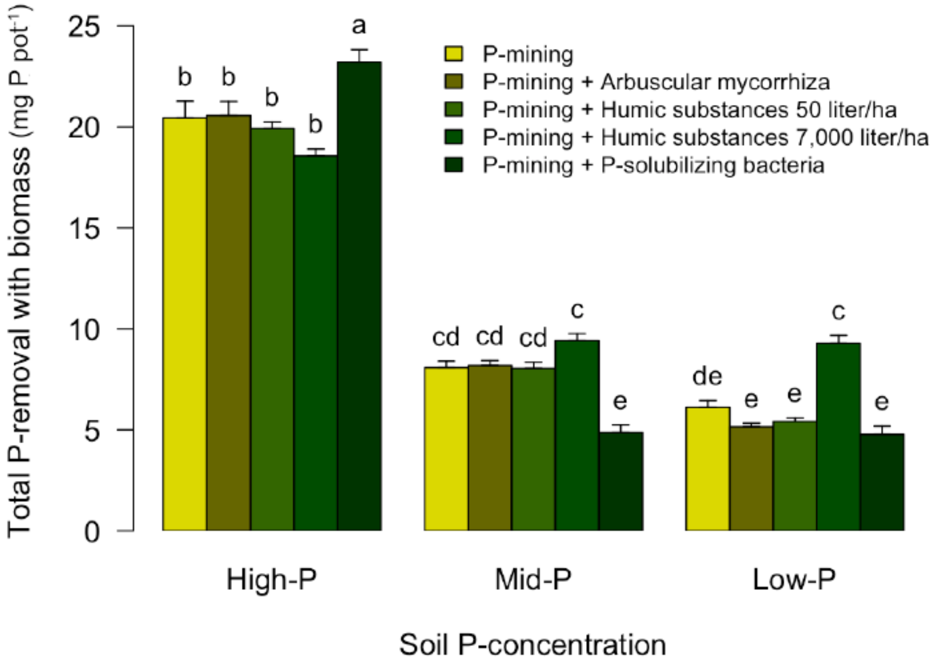


Figure 4.2 Mean + SE (n = 7) of the total P-removal with L. perenne over 123 days (mg P pot^{-1}). High-P contains 111 mg P_{Olsen} kg^{-1}, Mid-P 64 mg P_{Olsen} kg^{-1} and Low-P 36 mg P_{Olsen} kg^{-1}. Letters indicate significant differences between all means ($p < 0.05$) based on a Tukey HSD post hoc test following a two-way ANOVA (Table 4.2)

4.3.4 Effects of biostimulants on bioavailable P in soil

Final bioavailable P-concentrations on day 123 were significantly affected by the addition of biostimulants (Tables 4.2 and 4.3). Over all soil P-levels, addition with phosphate-solubilizing bacteria resulted in higher bioavailable P-concentrations than the control. With the other biostimulants, final bioavailable P-concentrations were lower.

4.4 Discussion

4.4.1 P-removal decreases over time due to P-limitation

Our results, across sandy soils with different initial concentrations of bioavailable P, showed reduced P-removal from those soils with lower bioavailable P despite N- and K-fertilization. This means that P-mining will slow down over time, as bioavailable soil P-concentrations decrease. P-mining will, therefore, take longer than would be assumed from initial P-uptake rates on recently-abandoned agricultural land. The main cause for the decline of P-uptake with decreasing soil P-bioavailability was the increasing P-limitation as indicated by the P nutrition index and plant P-concentrations (Appendix 4.C). N- and K-availability did not limit P-mining. We recovered in all pots 50 – 70% of the added N and 50 – 65% of the added K with the harvested grass. This indicates an overfertilization, more so in the Low-P pots. During restoration in practice, N- and K-fertilization should be adjusted to the needs of the crop and lowered if the production decreases over time. It might however take some time before P-removal will decline. In a long-term field experiment with several soil P-concentrations on a sandy texture, McCollum (1991) saw that in the beginning of the P-mining-process a plateau phase occurred, during which production would be constantly high. This was followed by a phase in which yields dropped significantly with further decreasing soil P-concentration. The plateau phase can last quite long: Oberson et al. (2010) showed in a pot experiment that P-removal with *L. multiflorum* was still at the same amount of our High-P soil, although the soil used was not fertilized with P for 22 years. Nevertheless, it seems like the decrease in P-uptake is not a steady decrease as uptake in Low-P was not significantly lower than Mid-P. P-removal remains more effective than would be expected. This is, however, difficult to explain.

4.4.2 Change of bioavailable soil P-stock is not only driven by removal of P with biomass

In our High-P, despite the high P-removal with biomass, we did not see a decline in bioavailable P. Bioavailable soil-P even increased. We can assume that in P-rich soils, the transfer of P from the slowly cycling pool to the bioavailable pool was as fast as or faster than P-removal with biomass. During the P-mining process, when the easily extractable P has been taken up by plants, P will become depleted in the rhizosphere and will start to limit plant growth and consequently P-removal. We could confirm this in our Mid-P soil where final bioavailable P-concentrations were 9% lower than at the beginning of the experiment. Here, the decrease in bioavailable P was roughly equivalent to the amount of P that was removed with biomass during the 123 days of the experiment. Availability decreased even more in our Low-P soil where bioavailable P decreased 29%, which is much more than the amount of P removed with biomass. This pattern may be due to the formation of an organic stable P-pool through roots and microbial immobilisation of P (De Schrijver et al. 2012). P taken up by plants can be removed with shoots when mown, be fixed in roots or accumulated in other organic soil pools such as microbial biomass and soil organic matter. The latter can become both a source of bioavailable P (remineralisation, release of microbial-P after cell death) or an important sink (immobilisation, incorporation of P into living microbial biomass) (von Lützow et al. 2006). Although the accumulation of an organic stable P-pool is happening in all three soil P-concentrations, it is probably relatively high in the Low-P soil.

4.4.3 Effects of biostimulants

The second aim of our experiment was to test whether additions of biostimulants could significantly increase the phytoextraction of P from the soil. At three soil P-levels we tested arbuscular mycorrhiza, humic substances at two concentrations and phosphate-solubilizing bacteria. The biostimulants significantly increased P-removal in only two cases: phosphate-solubilizing bacteria at High-P and a high concentration of humic substances at Low-P. Neither of the other treatments increased P-removal. And contrary to what we expected, P-removal decreased significantly by the phosphate-solubilizing bacteria addition in Low-P.

Overall, we did not find significant improvements by the addition of mycorrhiza despite affirmed colonization in the roots. We acknowledge that crop responses to added mycorrhiza have proven to be often unpredictable (Ryan and Graham 2002) and that inoculation is not always successful. Therefore, the experiment was repeated using a different application method for mycorrhiza and phosphate-solubilizing bacteria. They were mixed with the soil instead of applied on top of the soil. This did not have an effect on P-removal and does not have an impact on the results of the main pot experiment (see Appendix 4.D). In contrast to our results, a sterile Low-P soil (37 mg P_{Olsen} kg^{-1}) inoculated with *G. intraradices* and sown with *L. perenne* resulted in significantly positive effects from mycorrhiza (Lee et al. 2012). These positive effects on biomass production and P-concentration were similar to the effects of P fertilization. Lee et al. (2012) did not fertilize their soil with N while we did fertilize N and K in all of our pots according to agronomic standards. It is likely that N fertilization in our experiment reduced possible effects of the added mycorrhiza (Johnson et al. 2003). Also, Azcón et al. (2003) observed that the beneficial effects of *G. mosseae* in a soil very poor in P (4.5 mg P_{Olsen} kg^{-1}) were absent at high N- and P-fertilization regimes. P-mining will, due to N- and K-fertilization, be a technique that is incompatible with potentially beneficial arbuscular mycorrhiza.

We also tested the effects of humic substances in this experiment. These organic acids can, depending on soil properties, mobilize and solubilize P from the slowly cycling soil P-pool (Jones and Darrah 1994; Gang et al. 2012). In our treatment with a high dose (HS2), humic substances caused a significant increase in biomass production and P-removal in the Low-P concentration. This dose was however unreasonably large. HS2 was applied at 7,000 liter ha^{-1}, much higher than the standard dose (HS1: 50 liter ha^{-1}), and therefore, very expensive. This low dose did not improve biomass production nor P-removal in any of the soil P-concentrations. This is in accordance with the greenhouse studies of Jones et al. (2007) with wheat and of Pilanalı & Kaplan (2003) with strawberries, but in contrast with field experiments where positive effects of low HS dosage on (root) biomass production and P-uptake were observed (Sharif 2002; Verlinden et al. 2009; Verlinden et al. 2010; Daur and

Bakhashwain 2013). The effects of humic substances in concentrations in between our tested doses should be tested experimentally. It is possible that in a mid-range concentration, humic substances are still effective for enhancing P-uptake in P-poor soils.

Thirdly, we included phosphate-solubilizing bacteria in our experiment. Recently, a pot experiment gave insight in the positive effects of adding *B. brevis*, *P. putida* and *P. corrugata* on the available P conditions in acid, sandy soils with high total P-contents (De Bolle et al. 2013b). However, we should be cautious with concluding that phosphate-solubilizing bacteria addition can have beneficial effects on crop growth, since results from pot and field experiments are often conflicting. For instance, Malboobi et al. (2009) found that biomass production of potatoes in a field experiment was reduced by several PSB strains. While in the same paper, a positive influence on biomass production of potatoes in a pot experiment was reported. In our experiment, we have found for the High-P concentration that P-removal was enhanced with 14% (from 20.4 g P pot^{-1} to 23.2 g P pot^{-1}) by the addition of phosphate-solubilizing bacteria. In the Mid-P concentration, on the contrary, this treatment caused a significant drop in P-removal, which was mainly caused by low biomass production. Despite higher P-concentrations in biomass, P-removal in Low-P and Mid-P was not improved by the phosphate-solubilizing bacteria addition. But these organisms may be a useful tool at soils with high P-concentrations. However, the addition of phosphate-solubilizing bacteria to fields may still be unrealistic since the effects on crop growth were not consistent, and production of these organisms for soil applications on a large scale might not yet be economically attainable.

Calculations of the time needed to reach low soil P-concentrations with P-mining need to into account that P-removal will decrease in time (see Appendix 4.E). We do not know how long exactly how long it would take to restore low-P concentrations with P-mining, to get better insight in this, field data at different P-mining phases is needed. From our pot experiment, it also seems that in the later stages of the P-mining process, the decrease of bioavailable soil-P might actually go faster than assumed from the amount of P removed with biomass. We think a considerable fraction of bioavailable soil-P will be fixed temporarily in plant roots. Therefore, in the end of P-mining, it might be better to also use crops from which also roots are harvested or a transition from high-productive crops to crops better adjusted to low soil P-concentrations are worth considering (Delorme et al. 2000). Also, during restoration in practice, fertilization of N and K should be adjusted to the needs of the crop and lowered if the production decreases over time.

4.5 Conclusion

We have shown P-removal rate was 60% higher in our P-rich soil than in the P-poorest soil. This finding implies P-removal will likely slow down when soil P concentrations decrease, and hence, with time. None of the biostimulants altered this trajectory. Calculations of the restoration time needed to reach low soil P-concentrations with P-mining need to take this delay into account. Restoration of *Nardus* grasslands on ex-agricultural soils by P-mining will probably take decades unless P-fertilization history was much lower than average.

4.6 Acknowledgements

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Masterthesis student Tom Du Pré and internship student Predrag Miljkovic performing the second harvest and NK-fertilization in the pot experiment

Colorimetric determination of phosphorus in extractions from biomass or soil samples

Measurement of soil acidity with an electrode after shaking dry soil in demineralized water or KCl solution

CHAPTER 5

Phosphorus mining efficiency declines with decreasing soil P concentration and varies across crop species

The pot experiment at 88 days

After: Schelfhout S, De Schrijver A, Verheyen K, De Beelde R, Haesaert G, Mertens J. 2018. Phosphorus mining efficiency declines with decreasing soil P concentration and varies across crop species. International Journal of Phytoremediation. 20:9. 939-946. DOI: *10.1080/15226514.2018.1448363*

Abstract

High soil P concentrations hinder ecological restoration of biological communities typical for nutrient-poor soils. Phosphorus mining, *i.e.* growing crops with fertilization other than P, might reduce soil P concentrations more quickly. However, crop species have different P-uptake rates, which may also vary with soil P concentration. In a pot experiment with three soil-P-levels (High-P: 125-155 mg P_{Olsen} kg^{-1}; Mid-P: 51-70 mg P_{Olsen} kg^{-1}; Low-P: 6-21 mg P_{Olsen} kg^{-1}), we measured how much P was removed by five crop species: buckwheat (*Fagopyrum esculetum* Moench), maize (*Zea mays* L.), sunflower (*Helianthus annuus* L.), flax (*Linum usitatissimum* L.) and triticale (X *Triticosecale* Wittmack). Total P removal decreased with soil-P-level and depended upon crop identity. Buckwheat and maize removed most P from High-P and Mid-P soils and triticale removed less P than buckwheat, maize and sunflower at every soil-P-level. The difference in P removal between crops was, however, almost absent in Low-P soils. Absolute and relative P removal with seeds depended upon crop species and, for maize and triticale, also upon soil-P-level. None of the previously-grown crop species significantly affected P removal by the follow-up crop, perennial ryegrass (*Lolium perenne* L.). We can conclude that for maximizing P removal, buckwheat or maize could be grown.

5.1 Introduction

Currently, the sustainable use of P is also a hot topic in an agronomic context; consensus exists to keep soil P within an agronomic optimum where P concentrations are not limiting crop yields and there is no risk for P losses (Withers et al. 2017). This P optimum depends on various soil factors, rainfall and the crop species (Tang et al. 2009; Bai et al. 2013; Johnston et al. 2014; Sánchez-Alcalá et al. 2015), but can be generalized to range between 15 and 25 mg P_{Olsen} kg^{-1} (Syers et al. 2008; Poulton et al. 2013).

A potential solution for reducing soil P concentrations from P-saturated soils (> 85 mg P_{Olsen} kg^{-1}) may be to grow crops with zero-P fertilization, and, hence, using legacy soil P stocks (Jordan-Meille et al. 2012; Sharpley et al. 2013; Rowe et al. 2016). This practice of depleting soils of P by cultivating crops or grass whereby biomass production, and hence the removal rate of P, is optimized by fertilization with growth-limiting nutrients, other than P, is also named P-mining or phytoextraction of P. Initially, P depletion over time follows a linear curve which becomes curvilinear later on, and thus, slows down over time (Johnston et al. 2016). The trajectory of P-rich towards P-poor soils depends, among other things, upon the P buffering capacity of the soil and its ability to restore the equilibrium between different P pools (see Box 1). Inorganic P in soil can be considered in four pools of varying availability for plant uptake: when immediately accessible P, or also, P in the soil solution (pool 1), becomes depleted in the rhizosphere, further P uptake depends upon how fast readily extractable (pool 2) and low extractable P-pools (pools 3 and 4) can resupply the soil solution pool (Johnston et al. 2014).

The P-mining trajectory will further be affected by the growing crop species. Sharma et al. (2007) found large variations in P uptake among different crop species and specified that a potential candidate for P-mining should have high biomass production, high P-concentration and a relevant economic value. Some plant species are able to increase P uptake because of specific P acquisition strategies, and can even use organic P (Turner 2008), for example via root excretion of protons and organic anions to mobilize P from less bioavailable P fractions (Horst et al. 2001; Simpson et al. 2011). Phosphorus-mining might be optimized by using those crop species in crop rotation where specific P acquisition strategies can increase P uptake for themselves or for the follow-up crop and, thereby, improving P acquisition by the whole system (Horst et al. 2001; Rowe et al. 2016). Specific crop rotations have been developed for a multitude of purposes, *e.g.* using N-fixing crops for managing N-fertility (Crews and Peoples 2004) or using nematode antagonistic crop species (Barker and Koenning 1998). However, only a limited number of long-term field experiments for selecting crop rotations with the purpose of maximizing P extraction exist (described by Kratochvil et al. 2006 and Fiorellino et al. 2017). In these two studies, it was concluded that rotations for silage removed more P than grain rotations. These rotations were tested at different initial soil P concentrations, ranging from roughly 20 to 140 mg P_{Olsen} kg^{-1} (conversion from Mehlich-3 extractions based on Wolf and Baker 1985) and illustrated a P removal rate dependent on the initial soil P concentration. The investigated rotations for silage contained rye (*Secale cereale* L.), maize (*Zea mays* L.), alfalfa (*Medicago sativa* L.) and ryegrass (*Lolium perenne* L.) but this list could be further extended with, *e.g.* buckwheat (*Fagopyrum esculetum* Moench) able to mobilize P under zero-P-fertilization due to root excretion of organic acids (Possinger et al. 2013).

To aid with this crop selection we have set up a pot experiment containing five crop species and a follow-up crop along a series of soils with differing P concentrations. Our research questions are: (i) How large is the difference in P removal between the studied crop species? (ii) Do the initial soil P concentrations affect this difference in P removal? (iii) Is P removal of the follow-up crop affected by the previous crop species?

5.2 Methods

5.2.1 Soil collection

Based on an unpublished extensive screening, nine fields were selected within 1 km radius in "Landschap De Liereman" nature reserve (Oud-Turnhout, Belgium; Table 5.1). Fields differed in soil P concentrations, and had comparable sandy textures, hydrological conditions and acidity. We grouped the fields according to their bioavailable soil-P-concentration in three soil-P-levels: 125-155 mg P_{Olsen} kg^{-1} further referred to as *High-P*, 51-70 mg P_{Olsen} kg^{-1} further referred to as *Mid-P*, and, 6-21 mg P_{Olsen} kg^{-1} further referred to as *Low-P*. Per field, 150 kg soil was collected from the 5-20 cm soil layer between July and September 2012 (Fig. 5.1). The collected soil was sieved (1 cm mesh), homogenized with a cement mixer and consequently sampled to determine the soil moisture content gravimetrically. Soil moisture content was brought up to a volumetric level of 20% with deionized water.

Figure 5.1 Soil collection in nature reserve Landschap De Liereman in 2012

Table 5.1 Characterization of the initial soil properties for the soils used in the pot experiment (n=5)

Soil-ID	Soil-P-level	Coordinates	P_{Olsen} (mg kg^{-1})	P_{Total} (mg kg^{-1})	$P_{Oxalate}$ (mg kg^{-1})	$Fe_{Oxalate}$ (mg kg^{-1})	$Al_{Oxalate}$ (mg kg^{-1})	PSD (%)	pH_{KCl}
P-9	High-P	51°19'46.3"N 5°01'58.9"E	155 ± 6	693 ± 20	494 ± 28	419 ± 18	1284 ± 75	33 ± 1.0	4.2 ± 0.0
P-8	High-P	51°19'52.1"N 5°00'56.9"E	131 ± 11	517 ± 26	332 ± 10	466 ± 21	949 ± 33	28 ± 0.4	4.3 ± 0.1
P-7	High-P	51°19'58.1"N 5°00'58.0"E	125 ± 7	675 ± 45	453 ± 17	369 ± 12	925 ± 16	41 ± 1.0	4.6 ± 0.0
P-6	Mid-P	51°20'08.2"N 5°01'12.0"E	70 ± 3	364 ± 21	207 ± 4	265 ± 12	423 ± 14	37 ± 0.8	4.5 ± 0.0
P-5	Mid-P	51°20'00.1"N 5°01'01.3"E	51 ± 1	274 ± 7	132 ± 3	213 ± 5	357 ± 11	29 ± 0.4	4.5 ± 0.0
P-4	Mid-P	51°20'23.5"N 5°00'53.2"E	51 ± 2	388 ± 9	162 ± 6	567 ± 23	1353 ± 36	10 ± 0.2	4.5 ± 0.0
P-3	Low-P	51°20'50.4"N 5°01'11.6"E	21 ± 2	217 ± 95	42 ± 5	456 ± 57	563 ± 45	5 ± 0.2	4.3 ± 0.0
P-2	Low-P	51°20'21.5"N 5°01'14.6"E	19 ± 1	113 ± 10	61 ± 6	280 ± 23	430 ± 14	11 ± 1.0	4.3 ± 0.0
P-1	Low-P	51°20'03.6"N 5°01'12.9"E	6 ± 1	78 ± 5	12 ± 1	155 ± 18	878 ± 37	1 ± 0.1	3.8 ± 0.0

5.2.2 Chemical soil analyses

To verify initial chemical soil conditions, we sampled the bulk soil of each selected field by taking five samples after the homogenization procedure. Soil samples were dried at 40°C for 48 h and then passed through a 2 mm sieve. Soil pH (with 1 M KCl as extractant) was measured using a glass electrode (Orion, Orion Europe, Cambridge, England, model 920A) following the procedure described in ISO 10390:1994(E). Further, we analyzed soil P concentrations by following extraction methods:

i. Bioavailable P, which is available for plants within one growing season (Gilbert et al. 2009) by extraction in $NaHCO_3$ (P_{Olsen}; according to ISO 11263:1994(E)); and

ii. Slowly cycling P, which also includes P that can become available on the longer term and is adsorbed by Al and Fe. This P-fraction was extracted in ammonium oxalate-oxalic acid ($P_{Oxalate}$; according to NEN 5776:2006). Al and Fe concentrations were also measured in these extracts ($Al_{Oxalate}$ and $Fe_{Oxalate}$) by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS); and

iii. Total P measured after complete destruction with $HClO_4$ (65%), HNO_3 (70%) and H_2SO_4 (98%) in Teflon bombs for 4 h at 150°C (P_{Total}).

In the extracts, P was measured colorimetrically according to the malachite green procedure (Lajtha et al. 1999). We calculated the phosphate saturation degree (PSD), which estimates the potential P to be desorbed and thus supplied from the slowly cycling pool to the bioavailable pool by $PSD = [P_{Oxalate}/0.5\times(Al_{Oxalate} + Fe_{Oxalate})] \times 100(\%)$ with the P, Al and Fe contents (in mmol kg^{-1}) extracted with ammonium oxalate-oxalic acid. Soil PSD of 25% is seen as the critical limit above which the potential leaching of P to groundwater becomes larger than 0.1 mg orthophosphate $liter^{-1}$ groundwater (Van der Zee et al. 1990).

5.2.3 Setup of the pot experiment

For optimal P-availability, soil pH_{H2O} should be between 5.5 and 7.0 (Goulding et al. 2008; which relates to a pH-KCl range of 4.8 to 6.4; Van Lierop 1981). Hence, before filling pots, the soils were limed with chalk powder (Dolokorn: 60% $CaCO_3$ and 30% $MgCO_3$): according to their differing soil pH, 9.4 g Dolokorn pot^{-1} was added to soils P-1, P-2 and P-3 and 5.1 g Dolokorn pot^{-1} was added to soils P-4, P-5, P-6, P-7, P-8 and P-9. For each soil and crop combination, three pots of 26 cm diameter were filled with 7 kg soil. Five crop species were selected based on traits that could benefit P extraction along different soil P concentrations: high yield potential in maize (*Zea mays* L.; Ehlert et al. 2009) and sunflower (*Helianthus annuus* L.; Sharma et al. 2007); specific P acquiring strategy in buckwheat (*Fagopyrum esculetum* Moench; Possinger et al. 2013) and flax (*Linum usitatissimum* L.; Kranz and Jacob 1977 in Hakala et al. 2009); and, tolerance for low soil P concentrations in triticale (X *Triticosecale* Wittmack; van Dijk and van Geel 2012; see Appendix 5.A for varieties). Seeds were sown at depths and densities according to field recommendations on 24-26 October 2012 (Anonymous 2003; Pannecoucque et al. 2012; see also Appendix-Table 5.A.1). Next, pots were placed randomly in a greenhouse with day/night regime of 14/10h, temperature of 19°C, and a relative humidity maintained at 75% from 24 October 2012 until 15 Mai 2013. The pots were watered to keep the soil humidity around a volumetric level of 20%. All pots were fertilized with nutrients other than P to make sure limitation by these nutrients was minimal. All fertilizations were done with nutrient solutions containing NH_4NO_3 and KNO_3 following field recommendations (Debaeke et al. 1998; Björkman 2010; Maes et al. 2012b; van Dijk and van Geel 2012; see also Appendix-Table 5.A.2), except for day 30 when each pot was fertilized with macro- and micronutrients: 76.6 mg N as NH_4NO_3 and $Ca(NO_3)_2 \cdot H_2O$, 50 mg K as K_2SO_4, 100 mg Mg as $MgSO_4 \cdot 7H_2O$, 1.2 mg B as H_3BO_3, 2 mg Mn as $MnCl_2 \cdot 4H_2O$, 3.7 mg Zn as $ZnSO_4 \cdot 7H_2O$, 11.1 mg Cu as $CuCl_2 \cdot 2H_2O$ and 13.9 mg Mo as $Na_2MoO_4 \cdot 2H_2O$.

5.2.4 Plant sampling and chemical analyses

Depending on the crop species, crops were grown for 105-202 days, *i.e.* until seeds started to ripen but before leaves wilted (see Table S1). We cut crops 2 cm above the soil surface and kept the above-ground vegetative parts and seeds separately. These samples were weighed (dry mass, DM) and ground after drying to constant weight at 70°C for 48 h. Phosphorus concentration in biomass was obtained after digesting 100 mg of the sample with 0.4 ml $HClO_4$ (65%) and 2 ml HNO_3 (70%) in Teflon bombs for 4 h at 140°C and, then, colorimetrically measuring according to the malachite green procedure (Lajtha et al. 1999). Total above-ground biomass was calculated by summing seed and above-ground vegetative biomass. Phosphorus removal with seeds and vegetative parts was calculated by multiplying total above-ground biomass and P concentration. Total P removal was calculated as the sum of P removal with seeds and P removal with vegetative parts.

5.2.5 The follow-up crop: perennial ryegrass

To evaluate the effects of the different crops on P-removal with a next crop, we grew perennial ryegrass in each pot as a phytometer. After the final crop harvest, on 13 September 2013, the soil in all pots was harrowed superficially with a hand cultivator to create optimal seeding conditions and sown with 2 grams of *Lolium perenne* variety 'Plenty'. To exclude limitation by other nutrients than P, we fertilized all pots with 250 mg N and 350 mg K as NH_4NO_3 and KNO_3 on day 0. Ryegrass was grown for 52 days at the same greenhouse conditions, harvested and chemically-analyzed as described above.

5.2.6 Statistical analyses

All statistical analyses were performed with R (R Core Team 2016) and statistical significance was set at p less than 0.05. We clustered the nine soils into three soil-P-levels according to their soil P concentration to allow testing the effect of soil-P-level on the response variables with analysis of variance (ANOVA). We used two-way ANOVA (the aov function from R-package "stats") to test the effects of crop identity, soil-P-level and their interaction on (i) total above-ground biomass, mean P concentration and total P removal of the five crops and the follow-up crop and (ii) P removal with seeds and vegetative parts. To achieve the homogeneity assumption, the crop response variables total above-ground biomass, total P removal and P removal with seeds were square root-transformed; P concentration in biomass, P removal with vegetative parts, P concentration in the follow-up crop and P removal with the follow-up crop were $\log_{10}$-transformed. Residuals from the ANOVA tests were examined for normal distribution with quantile-quantile plots. Multiple comparisons of crop identity and soil-P-level were performed by means of Tukey using the HSD.test function from R-package “agricolae” (de Mendiburu 2016). Finally, for each crop species except for triticale, we performed second order linear regressions for total P removal versus Olsen P concentration using the lm function from the R-package “stats”. For triticale, a first order linear regression gave a better fit based on the adjusted R^2. According to ANOVA testing and the adjusted R^2, the second order linear regressions fitted the data significantly better, except for triticale where a first order linear regression was more appropriate.

5.3 Results

The removal of P with above-ground biomass was significantly influenced by the soil-P-level (High-P > Mid-P > Low-P; $F_{2,120} = 155.5$, $p < 0.001$) and crop identity (buckwheat > maize = sunflower > flax > triticale*;* $F_{4,120} = 97.6$, $p < 0.001$; see Tables 5.2 and 5.3). Interactions between soil-P-level and crop identity were also significant ($F_{8,120} = 7.3$, $p < 0.001$). Maximal P removal was obtained by buckwheat and maize at High-P (on average 232 mg P pot^{-1}) and minimal P removal by triticale at Low-P (on average 10 mg P pot^{-1}). P removal by triticale reached only 14 – 20 % of P removal with buckwheat across all soil-P-levels. We observed that the magnitude of the decline in P removal with soil-P-level depended upon crop identity with the steepest decline in buckwheat and (nearly) no decline in flax and triticale (Fig. 5.2 and Appendix-Table 5.B). For buckwheat, maize and sunflower, P removal decreased respectively 31 – 37 % when soil-P-level decreased from High-P to Mid-P and, 60 – 68 % when soil-P-level decreased from Mid-P to Low-P. And further, while P content in buckwheat, sunflower and flax seeds remained the same with increasing soil-P-level, P removal with seeds became relatively more important for maize and triticale (Figure 5.3).

Total above-ground biomass was mostly affected by crop identity ($F_{4,120} = 164.8$, $p < 0.001$; Table 5.2). For maize, total above-ground biomass was also significantly affected by the soil-P-level (High-P > Mid-P > Low-P). The effect of soil-P-level on total above-ground biomass was less clear in the other crops and completely absent in flax.

P removal with the follow-up crop, perennial ryegrass, was affected significantly only by the soil-P-level and not by the identity of the previous crop species (Tables 5.2 and 5.4). Meanwhile, biomass P concentration was affected by both soil-P-level (High-P > Mid-P > Low-P; $F_{2,120} = 123.5$, $p < 0.001$) and by the previous crop ($F_{4,120} = 4.9$, $p < 0.001$): in pots where flax was grown previously, P-concentration in the follow-up crop was significantly higher than where triticale, buckwheat or maize was grown (Tables 5.2 and 5.4).

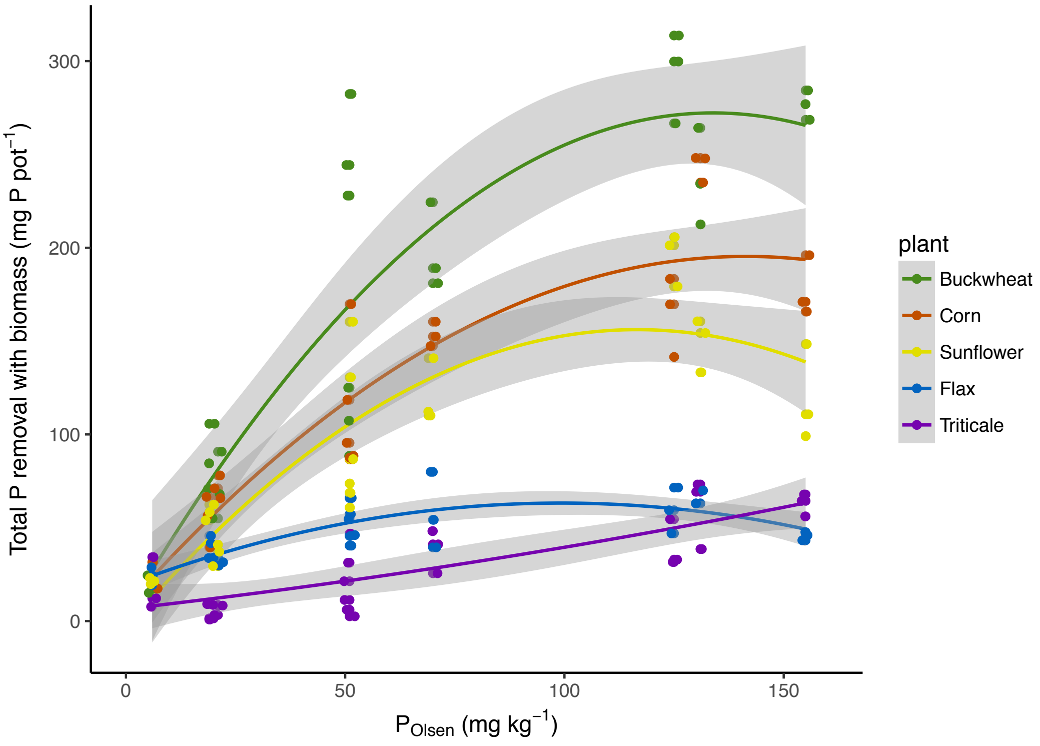

Figure 5.2 Total P removal with biomass versus bioavailable P concentration in the pot experiment (n=135). Colors indicate crop identity; curves represent first (triticale) and second order linear regressions (the four other crop species; for coefficients and statistics see Appendix-Table 5.B); shades show the standard error of the mean

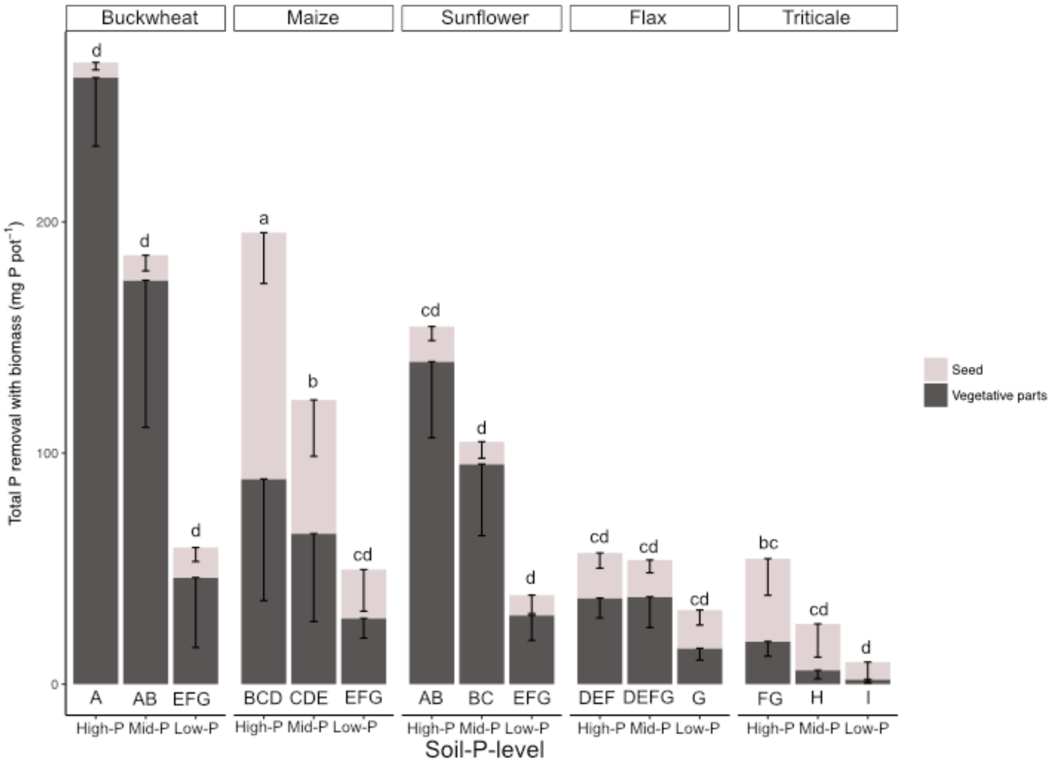

Figure 5.3 Effects of soil-P-level and crop identity on P removal with seeds and vegetative parts. Each bar represents the mean of three replicates minus one standard deviation of the mean. Letters indicate significant differences between all means of seed P removal (lowercase) and non-seed P removal (uppercase) based on a Tukey HSD post hoc test follow-up a two-way ANOVA ($p < 0.05$; Table 2)

Table 5.2 Effects of crop identity, soil-P-level and their interaction on total above-ground biomass, P-concentration in biomass and P-removal with biomass of the used crop and of the follow-up crop. The effect on P-removal with seeds and non-seeds is also shown

Experiment	Response variable	Crop ID		Soil-P-level		Crop ID x soil-P-level	
		F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Five crops	**Total above-ground biomass**	164.8	***	41.1	***	3.7	***
	P concentration in biomass	46.7	***	95.9	***	2.1	*
	P removal with biomass	97.6	***	155.5	***	7.3	***
	P removal with seeds	45.0	***	28.5	***	11.3	***
	P removal with vegetative parts	143.2	***	111.4	***	4.1	***
Follow-up crop	**Total above-ground biomass**	1.3	ns	17.5	***	0.3	ns
	P concentration in biomass	4.9	**	123.5	***	0.3	ns
	P removal with biomass	0.7	ns	76.9	***	0.3	ns
	Degrees of freedom	4		2		8	

Results of two-way ANOVA; Significance levels are *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, not significant

Table 5.3 Mean ± SD (n=9) for each cultivated crop species and soil P-concentration of total above-ground biomass, mean P-concentration and total P-removal

		Total above-ground biomass (g dry biomass pot^{-1})							**P concentration in biomass (mg P g^{-1} dry biomass)**							**Total P removal (mg P pot^{-1})**					
		High-P		**Mid-P**		**Low-P**			**High-P**		**Mid-P**		**Low-P**			**High-P**		**Mid-P**		**Low-P**	
		A		B		C			A		B		C			A		B		C	
Buckwheat	B	43 ± 10	cde	49 ± 18	cd	28 ± 8	efgh	A	6.5 ± 1.4	a	4.0 ± 1.6	b	2.0 ± 0.8	def	A	269 ± 31	a	186 ± 67	bc	59 ± 33	e
Maize	A	111 ± 15	a	84 ± 22	b	59 ± 16	c	D	1.8 ± 0.6	def	1.5 ± 0.5	ef	0.8 ± 0.2	g	B	195 ± 39	ab	123 ± 35	cd	50 ± 23	ef
Sunflower	C	40 ± 10	cdef	35 ± 12	defg	22 ± 5	ghi	B	3.9 ± 0.5	b	3.1 ± 0.8	bc	1.7 ± 0.5	ef	B	156 ± 37	bcd	105 ± 35	d	39 ± 17	ef
Flax	C	26 ± 3	fgh	28 ± 7	efgh	23 ± 3	gh	C	2.2 ± 0.5	cde	2.0 ± 0.7	cde	1.4 ± 0.4	ef	C	57 ± 11	e	54 ± 13	ef	32 ± 8	ef
Triticale	D	19 ± 5	hi	12 ± 7	ij	7 ± 5	j	C	2.8 ± 0.4	bcd	2.1 ± 0.5	cde	1.2 ± 0.4	fg	D	54 ± 16	ef	26 ± 17	fg	10 ± 10	g

Results from two-way ANOVA and Tukey post hoc tests are indicated with uppercase letters (main effects of crop ID and soil-P-level) and lowercase letters (interaction effect of crop ID x soil-P-level)

Table 5.4 Mean ± SD (n=9) for each cultivated pre-crop species and soil P-concentration of total above-ground biomass, mean P-concentration and total P-removal with the follow-up crop

	Above-ground biomass follow-up crop (g DM pot^{-1})				P-concentration follow-up crop (mg P g^{-1} dry biomass)			P-removal follow-up crop (mg P pot^{-1})		
	High-P	**Mid-P**	**Low-P**		**High-P**	**Mid-P**	**Low-P**	**High-P**	**Mid-P**	**Low-P**
	A	A	B		A	B	C	A	B	C
Buckwheat	11 ± 4	10 ± 3	7 ± 4	ABC	3.6 ± 0.6	2.7 ± 1.0	1.4 ± 0.3	41 ± 16	26 ± 11	9 ± 6
Maize	9 ± 4	9 ± 3	7 ± 4	BC	3.4 ± 1.1	2.4 ± 0.6	1.5 ± 0.5	32 ± 19	21 ± 5	10 ± 6
Sunflower	10 ± 3	11 ± 5	6 ± 3	AB	4.2 ± 0.8	3.1 ± 0.8	1.7 ± 0.5	42 ± 12	33 ± 18	10 ± 5
Flax	9 ± 4	8 ± 5	5 ± 3	A	4.5 ± 0.5	3.4 ± 0.9	1.6 ± 0.5	41 ± 19	29 ± 18	8 ± 4
Triticale	11 ± 3	9 ± 2	7 ± 2	C	3.2 ± 0.7	2.6 ± 0.6	1.3 ± 0.5	35 ± 12	23 ± 4	10 ± 5

Results from two-way ANOVA and Tukey post hoc tests are indicated with uppercase letters (main effects of crop ID and soil-P-level); The interaction of crop ID x soil-P-level was not significant

5.4 Discussion

5.4.1 P removal decreases with soil-P-level and varies across crop species

We investigated P removal with five crops across various soil-P-levels in a pot experiment during one growing season. Phosphorus removal was mainly affected by soil-P-level; across all crop species, it decreased with soil P concentration despite N- and K-fertilization. This finding is similar to the results of Chapter 4, a pot experiment on similar sandy soils with ryegrass, and also in accordance with long-term fertilization studies (Johnston et al. 2014; Fiorellino et al. 2017). In the context of ecological restoration, when estimating the time needed for P-mining towards P-poor soils (Chapter 2 and Box 2), it is relevant to account for the decline in P removal because this will likely cause a lengthening of the duration. We found that especially at High-P and Mid-P soils, the crop species used for P-mining mattered: buckwheat, maize and sunflower were crops with relatively large P removal compared to flax and triticale. Across all soil-P-levels, buckwheat and sunflower both had relatively high yields and P concentrations in biomass while maize only had relatively high yields. Similarly to Schiemenz and Eichler-Löbermann (2010), buckwheat stood out as a suitable crop for P-mining at High- and Mid-P levels. In contrast to Possinger et al. (2013), this was not the case at a Low-P level. In fact, in our Low-P soils the difference in P removal between crop species was almost absent, except for triticale removing significantly three to five times less P than the other crops. This corresponds to literature where generally, small grain cereals are not P-efficient in zero P systems, *e.g.* Colomb et al. (2007) showed with a long-term field study that wheat is more sensitive than maize and sunflower to the absence of P fertilization. Triticale, a hybrid between wheat and rye, seems to have inherited its P-efficiency traits from the lesser P-efficient wheat rather than from rye according to experiments with the triticale variety DT-46 (Pandey et al. 2005). The lesser capability of small grain cereals to extract P under zero-P fertilization was also confirmed in the long-term P-mining study reported by Kratochvil et al. (2006) and Fiorellino et al. (2017). Phosphorus removal potential of flax is inconsistent in literature: flax was reported in a P-fertilized pot experiment to accumulate P (Hakala et al. 2009), while it was also a poor P-mining crop when compared with buckwheat, sunflower and maize (Kalra 1971).

We further found that absolute and relative P removal with seeds depended upon the crop species (in accordance with Hakala et al. (2009)) and soil-P-level. In buckwheat and sunflower, P-removal with seeds varied from 2 to 23% of total P removal and the absolute P removal with seeds was not affected by soil-P-level. In contrast, maize seeds contained 43-55% of the above-ground P and this value significantly increased with soil-P-level. This observation may have implications for practice, for example, for maximizing P removal, buckwheat or sunflower could be grown as silage crops where the whole above-ground plant is harvested, *i.e.* no leaves are allowed to wilt. While for maize, both harvesting for silage and harvesting for grains are suitable options when maximizing P removal is the objective.

For buckwheat, sunflower and triticale, total above-ground biomass resided on a 'plateau phase' at High-P and Mid-P soils and, then, decreases suddenly in Low-P soils. According to literature, this is defined as the critical soil P concentration where yield responds to P fertilization and lies, depending on various soil factors and the crop species, between bioavailable P concentrations of 8 and 35 mg P_{Olsen} kg^{-1} (Tang et al. 2009; Bai et al. 2013; Poulton et al. 2013; Johnston et al. 2014; Sánchez-Alcalá et al. 2015).

Further, our results show a difference in luxury P consumption between the crop species. Luxury P consumption, defined as increasing plant P acquisition with soil P concentrations not resulting in additional biomass production (Aerts and Chapin 1999), was clearly shown for buckwheat and, slightly less apparent, for sunflower. The continued use of crop species with this trait in a zero P supply system would, with time, deplete soil P stocks. Crop species such as buckwheat, sunflower and also the high yielding maize, are preferably used when the objective is to reduce soil P concentrations from High-P to Mid-P concentrations. However, in the context of ecological restoration it is preferable to sustain the highest possible P-removal rates also towards or below Low-P soil levels. We have shown that at these levels, the crop species is less important; P-removal becomes very low in these soil conditions anyway. This implies that the last stretch of P-mining at Low-P soil-P-levels to restore P-poor reference conditions (< 12 mg P_{Olsen} kg^{-1}) will be time-consuming.

Contrastingly, for sustainable P use in an agricultural context, it is desirable to avoid both eutrophication and yield losses by staying within optimal bioavailable P concentrations (Syers et al. 2008). Temporarily P-mining management may be used in fields where the phosphate saturation degree (PSD) has exceeded 25%, *i.e.* the threshold for sandy soils from when eutrophication becomes critical for water bodies (Van der Zee et al. 1990; Rowe et al. 2016; see Box 1 in Chapter 1). The PSD may be decreased to safe levels by mining the soil-bound P-stock with crops taking up a lot of phosphorus such as buckwheat and maize. To continue crop production without yield losses for as long as possible in a zero-P fertilizing system, it seems advisable to use crop species that do not overly use soil P, *e.g.* small cereal grain crops such as triticale as this crop seemed to take up only small amounts P.

5.4.2 P-removal of the follow-up crop was not affected by the previous crop species

Despite the very different characteristics of each of the crops in our experiment, none of the crop species had a significant effect on P-removal by the follow-up crop, perennial ryegrass. Only the P concentration in the tissue of the follow-up crop was significantly affected by the identity of the previously-grown crop: it was significantly higher when it was grown after flax or sunflower than when it was grown after triticale. The positive effect of flax may be a reversed example of the 'dilution effect', when concentrations of nutrients in plants tend to decrease and hence become 'diluted' as the plant produces more biomass (Hejcman et al. 2010). In contrast to the effects of crop species, the follow-up crop was significantly affected by the soil-P-level.

5.5 Conclusion

Time estimations for ecological restoration with P mining should take into account the decrease of P removal and the identity of the used crop. When maximizing P-extraction is the objective, it is advisable to use crops such as buckwheat, maize and sunflower at high soil P concentrations. However, later in the P-mining process, crop identity was less important. Small grain cereals appear to be not efficient at P extraction. These crops can, however, be grown when sustainably using the legacy soil P for as long as possible is the objective. These results should be validated in a long-term field experiment, especially to see how P removal seems to depend upon the rate of resupplying readily extractable P pools.

5.6 Acknowledgements

We thank Natuurpunt Landschap De Liereman for the collaboration in this study. L Schelfhout is thanked for the transportation of the collected soil to Gontrode. Also, we thank L Willems and G De bruyn for the chemical analysis of soil and biomass samples. We also thank our students E Decadt, E Glorieux and H Vanheule for assisting with the lab work.

Illustrations from Chapter 6 on the next page (from left to right, top to bottom): Collection of background soil in nature reserve De Teut (Zonhoven); Robbe De Beelde and Luc Willems sieving the background soil; Safaa Wasof collecting soil inoculum in nature reserve Landschap De Liereman (Oud-Turnhout); Kris Ceunen preparing the greenhouse with an excavator; Andreas Demey filling the pots with soil freshly mixed with (or without) phosphorus and inoculum; ForNaLab team planting the tiny seedlings; Internship student planting tiny seedlings with a toothpick; ForNaLab team harvesting biomass; Iris Moeneclaey and Sumitra Dewan measuring plants


kubota
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CHAPTER 6

Effects of bioavailable phosphorus and soil biota on typical *Nardus* grassland species in competition with fast-growing plant species

The mesocosm experiment on 25/06/2018 with flowering *Centaurea jacea* visited by *Bombus lapidarius* (photo: Iris Moeneclaey)

After: Schelfhout S, Wasof S, Mertens, Vanhellemont M, Demey A, Haegeman A, Vangansbeke P, Viaene N, Baeyen S, De Sutter N, Maes M, van der Putten W, Verheyen K, De Schrijver A. Effects of bioavailable phosphorus and soil biota on typical Nardus grassland species in competition with fast-growing plant species. Submitted to Ecological Indicators

Abstract

The restoration of *Nardus* grasslands is often hampered by high bioavailability of soil phosphorus and disturbed biotic soil communities. In order to better understand these bottlenecks, we studied *Nardus* grassland species planted together with fast-growing species in 50-liter pots along a gradient of bioavailable phosphorus with or without inoculation of soil biota from oligo-, meso- or eutrophic grasslands.

In this mesocosm experiment, the threshold in bioavailable phosphorus for changes in the plant community was 11.5 mg P_{Olsen} kg^{-1} (Threshold Indicator Taxa Analysis). Above the threshold, a small increase in phosphorus resulted in a disproportionally large drop in abundance of the indicator species, among which four of the typical *Nardus* grassland species, whose low performance was independent of the soil biota treatment. Below the threshold, the typical *Nardus* grassland species flourished, most clearly so in the mesocosms in which no biota were added. The biomass of typical *Nardus* grassland species decreased with the total biomass of the plant community in each mesocosm.

Interestingly, the phosphorus threshold in our mesocosm experiment was close to the upper bioavailable phosphorus concentrations in remnant *Nardus* grasslands in northern Belgium. For the restoration of *Nardus* grasslands, phosphorus-poor soil conditions are essential. *Nardus* grassland species are able to handle nutrient limitation but not light limitation. We recommend further research to understand how soil biota inoculation alters the competitive ability of plant species in relation to soil phosphorus availability.

The mesocosm experiment on 20/05/2016

6.1 Introduction

Species-rich *Nardus* grasslands (European habitat type H6230*) are typified by nutrient-poor soils with very low bioavailable phosphorus concentrations (Chapter 2). Fertilization, *i.e.* lifting nutrient-limitation, causes the vegetation to shift from nutrient-limitation to light-limitation and a reduction in species-richness (Hautier et al. 2009; DeMalach et al. 2017; Hautier et al. 2018). In fertile soil conditions, species with a small initial advantage such as fast germination and growth rate to intercept light (*i.e.* species with fast early-growth: fast-growing species or grass-herb mix grassland species and *Lolium perenne* grassland species in Van Daele et al. 2017) asymmetrically outcompete species without this trait (*i.e.* species with slow early-growth: slow-growing species or *Nardus* grassland species in Van Daele et al. 2017; see §1.3.3). Reinstating phosphorus-limiting soil conditions in particular (Schellberg and Hejcman 2007; Ceulemans et al. 2014; van Dobben et al. 2017) allows rare plant species to thrive (Wassen et al. 2005). Bioavailable phosphorus concentrations in soils of remnant, unfertilized *Nardus* grasslands were found to be below a threshold of 12 mg P_{Olsen} kg^{-1} (the 95th percentile in the database of the Flemish Research Institute for Nature and Forest, reported in Chapter 2). Such a threshold value can, for instance, be used to direct restoration efforts towards candidate fields with a small abiotic 'distance to target' (cf. Chapter 2).

Plant communities are not only shaped by environmental filters, which affect plant-plant competition, but also by plant-soil feedback effects, *i.e.* interactions between plant species and their (micro)biotic environment (Thakur and Wright 2017). For ecological restoration, the development of a plant community may be directed towards a specific target by inoculation with soil biota sourced from reference fields (Wubs et al. 2016; van der Bij et al. 2018). Fast-growing plant species appear to be more susceptible to pathogens (negative plant-soil feedback); slow-growing plant species appear to benefit more from mutualists such as mycorrhiza that help with nutrient acquisition (positive plant-soil feedback; Lekberg et al. 2018). The abiotic context, *i.e.* resource availability, influences how plant-soil feedbacks affect plant-plant competition (Lekberg et al. 2018). In resource-rich conditions, plant-plant competition for light generally outweighs negative plant-soil feedback effects (*e.g.* root-feeding nematodes feeding on roots of fast-growing species); in resource-poor conditions, mutualistic plant-soil feedbacks can prevent competitive exclusion and favour slow-growing plant species (*e.g.* enhanced germination and growth of oligotrophic species by mycorhizzal associations). The restoration of *Nardus* grasslands may therefore benefit from tweaking the soil biota community in order to favour the growth and competitive ability of target plant species (Kardol et al. 2006; Brinkman et al. 2012; Torrez et al. 2016).

Soil biota inoculation, as a grassland restoration measure, may differentially affect slow-growing and fast-growing plant species in the community, with the outcome depending on resource availability, *e.g.* soil nutrient concentrations. Here, we study the interaction between soil biota and phosphorus availability on communities consisting of both fast- and slow-growing plant species in a mesocosm experiment allowing for the competition for light and nutrients between these species. Our first objective was to assess the changes in the plant community along the bioavailable phosphorus gradient. We expected to find a ‘community change point’ in bioavailable phosphorus concentration, or an environmental threshold, where the community composition shifts. Our second objective was to test the interactive effect of bioavailable phosphorus concentrations and soil biota inoculation on the typical *Nardus* grassland species. We expected that the addition of soil biota from *Nardus* grasslands has a positive effect on the plant species typical for *Nardus* grasslands, allowing them to grow at higher soil phosphorus concentrations.

6.2 Methods

In 110 mesocosms, *i.e.* medium-sized plant communities, we manipulated soil biota presence and bioavailable soil phosphorus concentrations and (unsuccessfully) simulated different levels of atmospheric nitrogen deposition (full design in Appendix 6.A; Fig. 6.1).

In March-April 2016, we filled 50-liter pots with 36 kg *γ*-irradiated (25 KGray) phosphorus-poor sandy soil mixed with an extra 4 kg of sandy soil for the soil biota treatment with four levels: “No biota” (using the same *γ*-irradiated soil), “Oligotrophic”, “Mesotrophic” and “Eutrophic” using freshly sampled soil from oligotrophic, mesotrophic and eutrophic grasslands (see Appendix 6.A.1 for information on the soil collection and processing; Appendix 6.A.2 and 6.A.3 for information on the abiotic characteristics of the background and inoculum soil).We repeated the soil biota inoculation treatment by collecting the three types of living soil in three regions in northern Belgium. Wasof et al. (2019) describes the vegetation, abiotic soil conditions, nematode and microbiotic communities of the donor grasslands.

To create a phosphorus gradient across the mesocosms with bioavailable phosphorus concentrations of <10, 15, 20, 25 and 60 mg P_{Olsen} kg^{-1}, we mixed either 0, 3, 5, 6 or 16 g NaH_2PO_4 in the 40 kg of soil, these quantities were derived from a small fertilization pot experiment preceding the mesocosm experiment. We did not succeed in creating the < 10 mg P_{Olsen} kg^{-1} x Eutrophic soil biota treatment combination in one of the replications (with inoculum from the Gulke Putten fields) due to the unavoidable addition of nutrients together with the 4 kg of soil inoculum (the N, P and K additions with inoculum are shown in Appendix-Table 6.A.5). In the soil measurement of June 2018, when comparing with the “No biota” treatment, the bioavailable phosphorus concentrations appeared to have increased slightly by adding eutrophic soil inoculum though not significantly (see Appendix-Fig. 6.A.5). The intended soil phosphorus levels (n=5) resulted in a smooth phosphorus gradient by adding the inoculums (see Appendix-Fig. 6.A.5). Therefore, the soil of each mesocosm was sampled every year to measure the concentration of bioavailable phosphorus at the time of the summer biomass harvest. In the analyses, we used the most recent measurements in bioavailable phosphorus concentrations (see Appendix-Fig. 6.A.5) as a continuous variable, instead of the categorical phosphorus levels.

The (failed) nitrogen treatment was aimed at simulating realistic atmospheric nitrogen deposition rates, *i.e.* <5, 20 or 60 kg N ha^{-1} y^{-1}. We spread 0, 90 or 270 mg NH_4NO_3 in a demineralized water solution evenly on each mesocosm, monthly from March until October. Despite these additions, in the plant tissue of mesocosms in the 20 or 60 kg N ha^{-1} y^{-1} treatments, we did not find increased nitrogen concentrations or increased nitrogen to phosphorus ratio; in the soil of these mesocosms, we did not find increased bioavailable NH_4^+ or NO_3^- concentrations. Probably, the high irrigation rate to compensate for the high greenhouse temperatures (summer mean of 20.4°C ± 6.4 SD and maximal temperature of 42.1°C in contrast to a summer mean of 17.5°C in Belgium according to the Royal Meteorologic Institute, KMI) caused the added nitrogen to leach from the mesocosms. We included the nitrogen treatment as an explanatory factor in the full GLM models. In none of our final models, the nitrogen treatment was retained as a significant explanatory factor (see Appendix C).

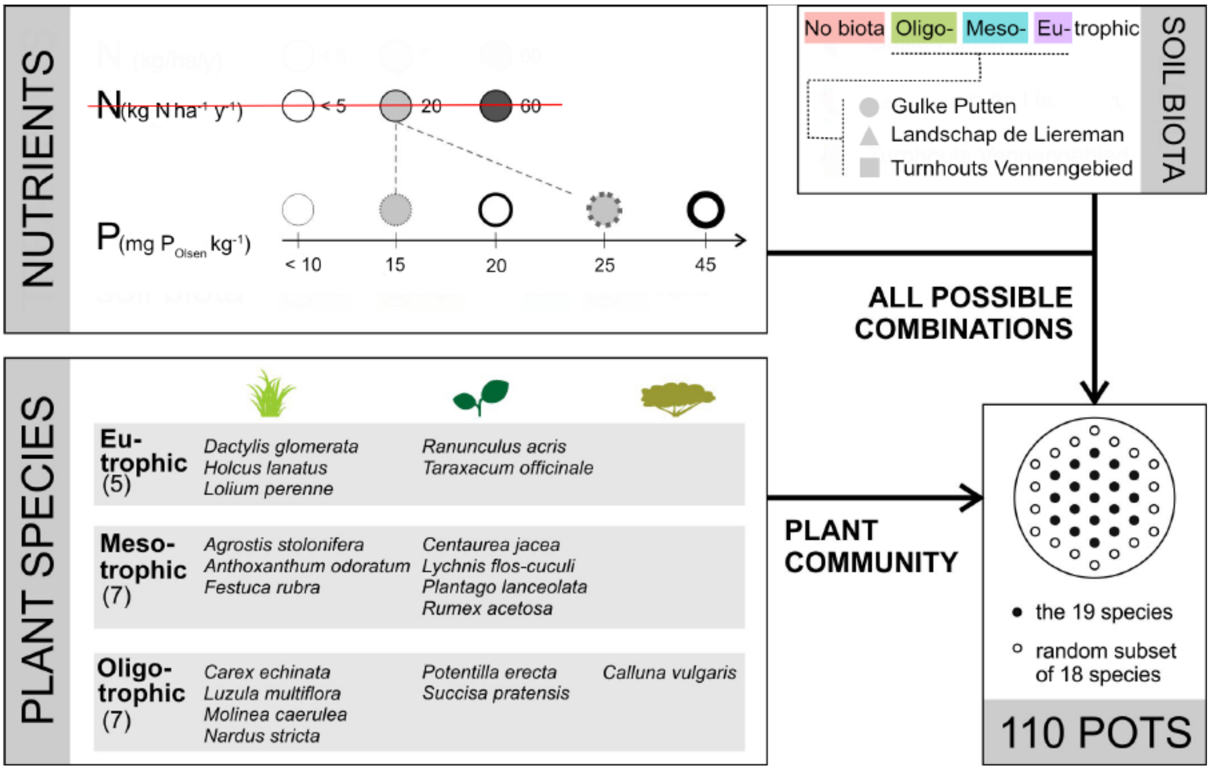


Figure 6.1 Design of the mesocosm experiment. Nineteen grass, herb or shrub species – typical of oligo-, meso-, eutrophic nutrient status – were planted together in pots. Soil nutrient availability was manipulated by adding nitrogen and phosphorus in different levels. The nitrogen treatment failed due to leaching effects following the high irrigation rate in the greenhouse to compensate for high summer temperatures. The soil biota community differed among pots as living soil from oligo-, meso-, and eutrophic grasslands (from each of three regions in northern Belgium) was added to part of the pots

In April 2016, we planted nineteen species in each mesocosm. We chose species typical of *Nardus* grasslands (oligotrophic species), grass-herb mixed grasslands (mesotrophic species) or grass-dominated *Lolium perenne* grasslands (eutrophic species) (cf. Van Daele et al. 2017; Fig. 6.2). The mesocosms were grown in a greenhouse, with an average temperature of 15°C

± 7 SD, a relative air humidity of 71% ± 14 SD and natural light conditions. We cut and removed all above-ground biomass three times per year (March, June, September).

In June 2018, in each mesocosm, we measured the total dry biomass and the separate dry biomasses of the three most abundant species of the oligo-, meso- and eutrophic species we planted (after drying for 48h at 70° C). For the other ten species, we estimated their cover in each mesocosm and used this cover and the total mesocosm biomass to estimate the species' biomass (Appendix 6.A.8). Per mesocosm, three samples of the 0-5 cm soil layer were taken with a 1 cm diameter auger, combined, dried (48h, 40°C) and analyzed for bioavailable phosphorus (extraction with sodium bicarbonate according to Olsen et al. (1954), colorimetric measurement with the malachite green procedure of Lajtha et al. (1999)).

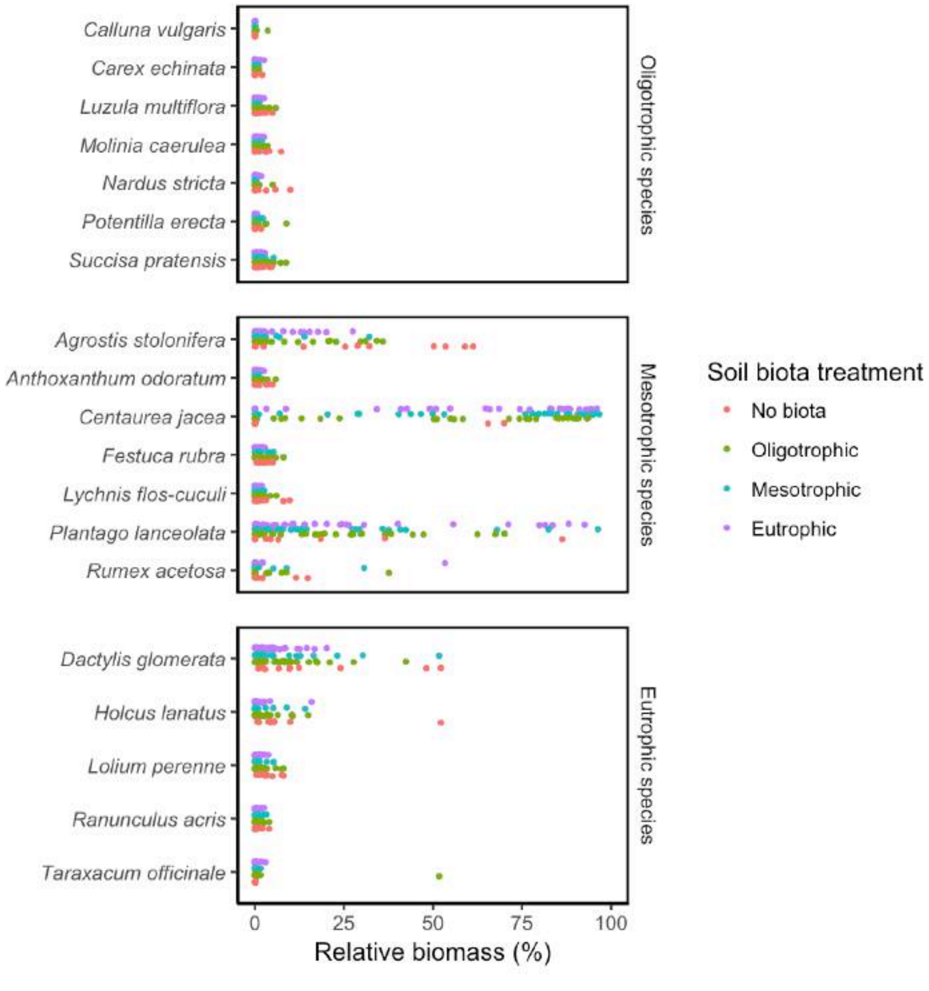


Figure 6.2 The biomass of the oligotrophic, mesotrophic and eutrophic plant species relative to the biomass of the entire community in the 110 mesocosms in the third year of the experiment (June 2018)

Using the species' biomass data, we looked for 'change points' along the phosphorus gradient using Threshold Indicator Taxa Analysis (TITAN, Baker and King 2010; function *titan*, *TITAN2* package, Baker et al. 2015). A change point, or ecological threshold, is a point at which a small increase in an environmental variable results in a disproportionally large change in community composition (Baker and King 2010). If along the phosphorus gradient, a synchronous change occurs in the abundance of a subset of species, this particular level of the phosphorus gradient is identified as a community change point. TITAN fits the abundance of individual species along the studied gradient and combines the abrupt changes of 'pure' and 'reliable' species (*i.e.* species that consistently show a significant change for > 95% of the 999 bootstrapped runs) into a positive and a negative community threshold (Appendix 6.B).

For the typical *Nardus* grassland species (oligotrophic species) identified as sensitive to soil phosphorus by TITAN (*i.e.* indicator species), we summed the dry biomass in each mesocosm ('absolute biomass') and divided it by the total dry biomass of the mesocosm ('relative biomass'). We fitted generalized linear models to study the effects of the bioavailable phosphorus concentration, the soil biota treatment, and their interaction on the absolute and relative biomass of the typical *Nardus* grassland species (function *glm*, *quasi* family with *identity* link and *constant* variance, *stats* package; model selection procedures in Appendix 6.C). To investigate the impact of plant-plant competition on the typical *Nardus* grassland species, we looked for a relationship between the biomass of these species and the total biomass of the mesocosms (function *glm*). Finally, we fitted generalized least squares models to study the effects of the experimental treatments on the total biomass (function *gls*, *weights = varIdent(form=~1|biota)* to allow for a different variance per biota level, *stats* package). We performed post-hoc pairwise comparisons to compare the differences between the soil biota levels (function *lsmeans* with *tukey* adjustment, package *lsmeans*, Lenth 2016). All analyses were performed in R (R-Core-Team, 2016); non-TITAN graphs were made with ggplot2 (Wickham, 2009).

6.3 Results

A negative environmental threshold of 11.5 mg P_{Olsen} kg^{-1} (*i.e.* the negative community change point; red line in Fig. 6.3) and a positive environmental threshold of 8.3 mg P_{Olsen} kg^{-1} (*i.e.* the positive community change point; grey line in Fig 6.3) were identified by the changes in the mesocosm communities. As the bioavailable phosphorus concentrations increase, the sum(*z*) scores will climb as the community-level response increases in magnitude: consequently, the negative threshold reveals a greater change in the community than the positive threshold. The peak in the negative sum(*z*) scores is sharp (filled points in Fig. 6.3), which provides strong evidence for a sharp, synchronous change in the community (King and Baker, 2014). In contrast, the peak in the positive sum(*z*) scores is broad (empty points in Fig. 6.3), which implies a more gradual change in the species' distributions along the phosphorus gradient. Around this threshold, six significant indicator species were observed with sharply declining responses to higher bioavailable phosphorus concentrations in the soil (Fig. 6.4; Table 6.1; $p < 0.05$). The narrower the uncertainty around the individual species' change point, the greater the support for a threshold-type response for a single species. The wider this uncertainty is, the weaker is the evidence for a sharp change in the distribution of a species along the gradient (King and Baker, 2014). Four of the significant indicator species were typical *Nardus* grassland species (oligotrophic species: *L. multiflora*, *N. stricta*, *Molinia caerulea* and *P. erecta*); two were typical for grass-herb mixed grasslands (mesotrophic species: *Anthoxanthum odoratum* and *Rumex acetosa*). The three other typical *Nardus* grassland species planted in the mesocosms were not considered 'reliable' species (*Succisa pratensis*, reliability score 0.94) or had too few occurrences in 2018 due to low survival (*Calluna vulgaris* and *Carex echinata*; Table 6.1), and were excluded from further statistical analyses. One eutrophic species, *Dactylis glomerata*, responded significantly positively towards increasing soil phosphorus concentrations (Table 6.1). The response of each of the nineteen species to the bioavailable phosphorus concentrations is shown in Appendix 6.B.

The relative cumulative biomass of the typical *Nardus* grassland species was significantly affected by the bioavailable phosphorus concentration ($p < 0.001$), the soil biota treatment ($p < 0.001$) and their interaction ($p < 0.05$; $D^2 = 28\%$; Fig. 6.5a; Appendix 6.C.1). The absolute cumulative biomass of these species was only affected significantly by the bioavailable phosphorus concentration ($p < 0.001$) and not by the soil biota treatment ($D^2 = 11\%$; Appendix 6.C.1). Below the negative environmental threshold of 11.5 mg P_{Olsen} kg^{-1}, the "No biota" mesocosms had a significantly higher relative biomass of the typical *Nardus* grassland species than the mesocosms in which soil biota had been added (average "No biota" 2.97% ± 1.10 SD, average mesocosms with biota 1.10% ± 0.92 SD; $p < 0.05$). In the "No biota" mesocosms, the relative biomass of the typical *Nardus* grassland species dropped steeply with increasing bioavailable phosphorus concentration.

Above a concentration of 17.1 mg P_{Olsen} kg^{-1}, there were no significant differences in relative biomass between the biota treatment levels (results post-hoc testing). The relative biomass was almost five times less than the average biomass of all "No biota" treatment mesocosms below the 11.5 mg P_{Olsen} kg^{-1} threshold (0.61% ± 0.65 SD). The typical *Nardus* grassland species were absent in 16 out of the 63 mesocosms with bioavailable phosphorus concentrations above the 11.5 mg P_{Olsen} kg^{-1} threshold and in 3 of the 47 mesocosms with phosphorus concentration below the threshold (2 mesocosms with oligotrophic biota added, 1 with eutrophic soil biota).

The relative biomass of the typical *Nardus* grassland species was significantly negatively correlated with the total plant biomass per mesocosm ($p < 0.001$; $D^2 = 42\%$; Fig. 6.5b). The total biomass was significantly affected by the bioavailable phosphorus concentration ($p < 0.01$), the soil biota treatment ($p < 0.001$), and their interaction ($p < 0.05$), but the final model explained only little variation ($R^2 = 12\%$; Fig. 6.5c). Below 22.9 mg P_{Olsen} kg^{-1}, the total biomass was significantly lower in the "No biota" mesocosms ($p < 0.05$; results post-hoc testing). The mesocosms inoculated with soil biota and below the 11.5 mg P_{Olsen} kg^{-1} threshold were dominated by *Centaurea jacea* and by *Plantago lanceolata* (below the threshold and with biota: on average 63% ± 28 SD compared to "No biota": 3% ± 5 SD; above the threshold: on average 67% ± 28 SD).

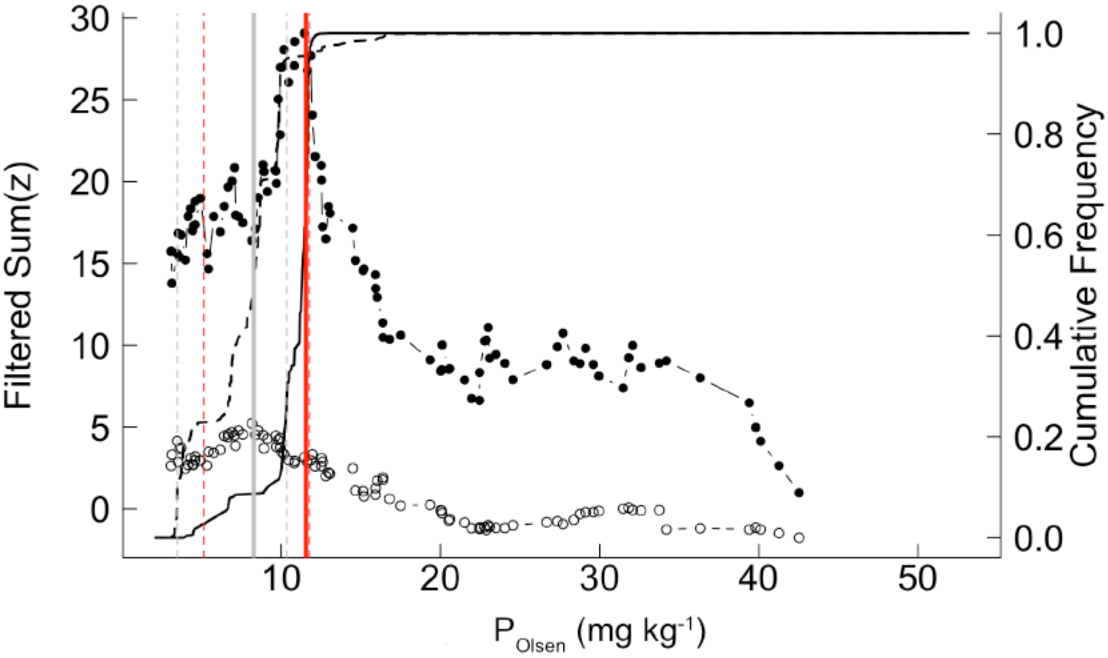


Figure 6.3 The negative (red line, 5th and 95th percentiles as dashed red lines) and the positive (grey line, 5th and 95th percentiles as dashed grey lines) change points of the community composition in the 110 mesocosms. Each point represents the sum of the negative (filled) and positive (empty) *z* scores at a candidate community-level change point of bioavailable phosphorus concentration; the greatest sum *z* point indicates the most abrupt change in community composition along the phosphorus gradient. The lines represent cumulative frequency distribution of change points among the 999 bootstrap replicates (full line: negative; dashed line: positive)

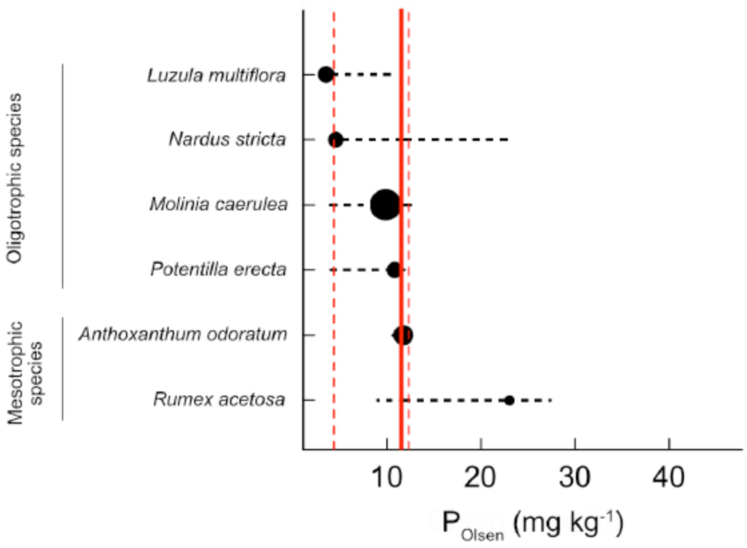


Figure 6.4 The negative community threshold (red line; 5th and 95th percentiles as dotted lines) and the negative species-specific thresholds for the significant indicator species along the studied gradient in bioavailable phosphorus (TITAN: purity ≥ 0.95; reliability ≥ 0.95. p < 0.05). Symbol size represents the magnitude of the response (*z* scores); black dotted lines represent the 5th and 95th percentiles among the bootstrap replicates. The positive community threshold and positive indicator species are not shown (see Table 6.1)

Table 6.1 The TITAN-derived thresholds in bioavailable phosphorus concentration in the soil (*threshold*, mg P_{Olsen} kg^{-1}, with confidence interval between brackets) for the nineteen species showing negative (-) or positive (+) responses in biomass (*direction*) along the studied gradient in bioavailable phosphorus in the 110 mesocosms. Significant indicator species sensitive to bioavailable phosphorus are marked by "x" (*significant* according to *purity* and *reliability*). Species are ordered by *z*-score and significance

Species		Occurrence [a]	Response		z		Indicator species according to		
name	type	June 2018	Direction	IndVal [b]	score	threshold [c]	purity [d]	reliability [e]	significant
Molinia caerulea	oligotrophic	44	-	58.5 **	11.0	9.9 [4.0 – 12.6]	1.00	1.00	x
Anthoxanthum odoratum	mesotrophic	47	-	48.4 **	6.6	11.8 [10.6 – 12.3]	1.00	1.00	x
Luzula multiflora	oligotrophic	52	-	71.3 **	5.4	3.6 [3.5 – 10.4]	0.99	1.00	x
Dactylis glomerata	eutrophic	105	+	69.7 **	5.2	8.3 [3.5 – 11.8]	0.99	1.00	x
Potentilla erecta	oligotrophic	64	-	57.6 **	5.1	10.8 [4.1 – 11.9]	1.00	1.00	x
Nardus stricta	oligotrophic	54	-	65.2 **	5.0	4.6 [4.4 – 22.9]	1.00	1.00	x
Rumex acetosa	mesotrophic	70	-	66.5 *	2.8	23.0 [9.0 – 27.4]	0.99	0.97	x
Ranunculus acris	eutrophic	71	+	50.3 **	3.8	20.1 [5.2 – 26.5]	0.95	0.94	
Succisa pratensis	oligotrophic	87	-	52.3 **	3.0	15.9 [4.2 – 44.2]	0.98	0.94	
Agrostis stolonifera	mesotrophic	91	+	69.6 *	2.8	4.1 [3.3 – 28.9]	0.76	0.95	
Calluna vulgaris [f]	oligotrophic	9	-	19.8 NS	2.4	3.1 [2.9 – 43.2]	0.60	0.66	
Lolium perenne	eutrophic	91	+	57.2 NS	2.0	4.8 [4.0 – 40.3]	0.80	0.87	
Plantago lanceolata	mesotrophic	101	+	62.9 NS	1.7	35.3 [4.3 – 42.0]	0.68	0.82	
Carex echinata [f]	oligotrophic	10	-	11.3 NS	1.6	18.4 [3.0 – 21.3]	0.92	0.71	
Holcus lanatus	eutrophic	91	-	64.5 NS	1.3	35.3 [3.6 – 35.3]	0.62	0.71	
Lychnis flos-cuculi	mesotrophic	56	+	33.7 NS	1.0	12.3 [3.0 – 41.7]	0.61	0.70	
Festuca rubra	mesotrophic	98	-	53.4 NS	1.0	29.8 [3.5 – 34.0]	0.55	0.66	
Taraxacum officinale	eutrophic	92	+	77.3 NS	1.0	43.2 [3.3 – 43.2]	0.70	0.53	
Centaurea jacea	mesotrophic	90	+	60.3 NS	0.9	3.1 [3.1 – 33.2]	0.58	0.71	

[a] number of mesocosms in which the species was present in June 2018 (out of the 110)

[b] significance of the response direction, ** $p < 0.01$, * $p < 0.05$, NS non-significant

[c] in mg P_{Olsen} kg^{-1}, with 5th-95th bootstrap confidence intervals of 999 simulation iterations

[d] mean proportion of correct response direction assignments

[e] mean proportion of *p*-values < 0.05 among 999 simulation iterations

[f] species excluded from analysis due to too few observations

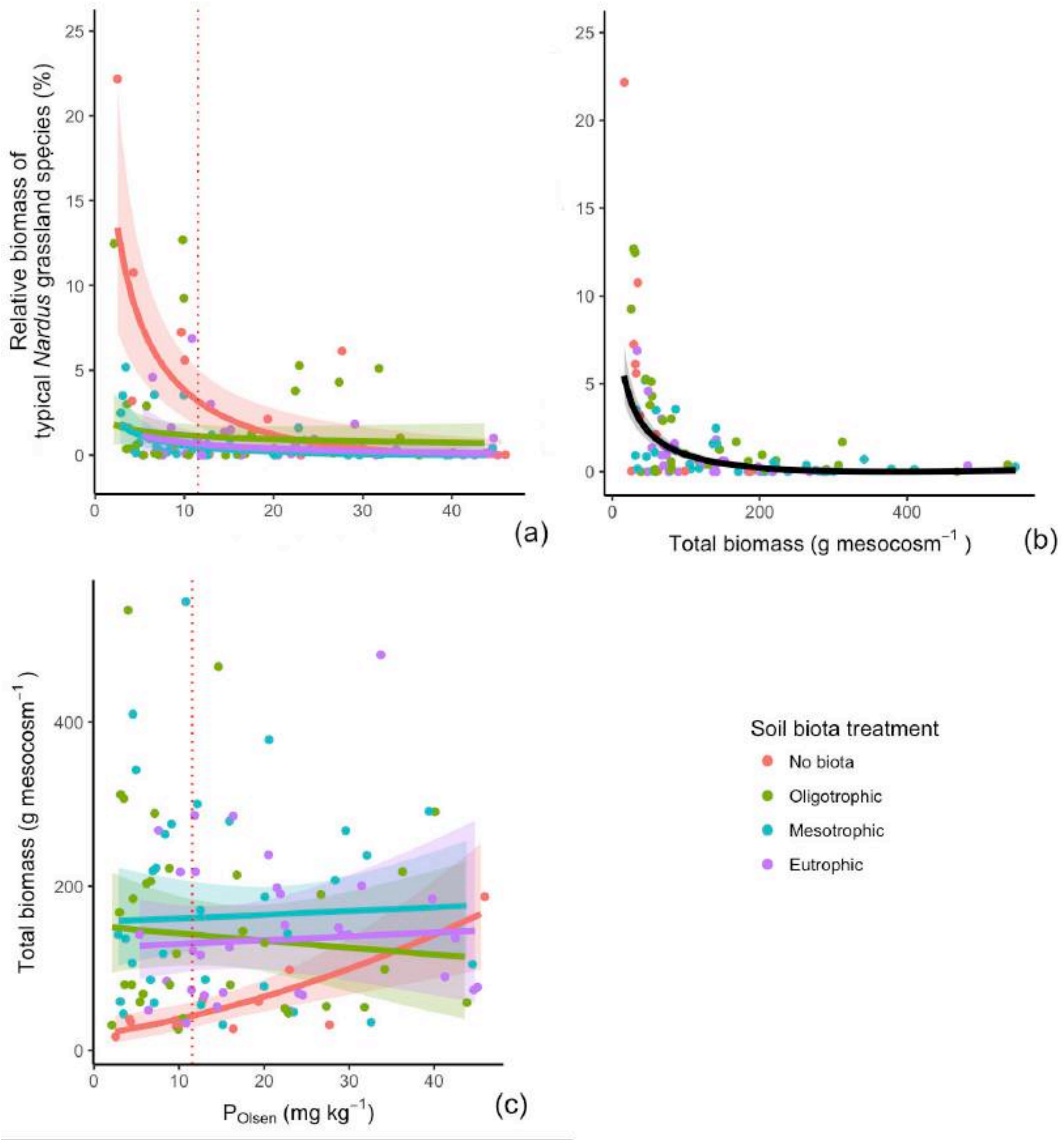


Figure 6.5 Cumulative biomass of the four identified oligotrophic indicator species typical for *Nardus* grasslands relative to the overall plant biomass of a mesocosm (%) versus the bioavailable phosphorus concentration (*a*, mg P_{Olsen} kg^{-1}) and the total biomass per mesocosm (*b*, g mesocosm^{-1}); and the total biomass per mesocosm versus the bioavailable phosphorus concentration (*c*). The lines were fitted according to the most optimal models (see Appendix 6.C); the shaded areas indicate 5th - 95th confidence intervals; the red dotted line indicates the negative community threshold; and the colors indicate the soil biota treatments.

6.4 Discussion

After three growing seasons in our mesocosm experiment, we evaluated the performance of typical *Nardus* grassland species planted together with fast-growing species (in total nineteen species typical of oligo- to eutrophic growing conditions) across a gradient in bioavailable phosphorus concentrations, and with or without inoculation of soil biota of different provenances (oligo- to eutrophic grasslands vs. a “No biota” treatment). The controlled nature of our greenhouse mesocosm experiment enabled us to investigate how the interaction of soil phosphorus concentrations and soil biota affect the competitive exclusion of typical *Nardus* grassland species, which are slow-growing oligotrophic plant species. Experiments rarely investigated such context dependency of plant communities to the inoculation of soil biota, *i.e.* dependency to bioavailable phosphorus concentration in the soil (Hoeksema et al. 2010; but see De Deyn et al. 2004).

We found a negative community threshold for bioavailable phosphorus of 11.5 mg P_{Olsen} kg^{-1}. This negative community threshold represents the bioavailable phosphorus concentration above which a small increase in phosphorus concentration results in a disproportionally large negative response of the four identified oligotrophic indicator species typical for *Nardus* grasslands. The biomass of these species (*L. multiflora*, *N. stricta, M. caerulea* and *P. erecta*) dropped sharply in mesocosms in which the soil phosphorus concentrations exceeded the threshold. The threshold in our artificial mesocosms was surprisingly similar to the threshold found in remnant *Nardus* grasslands (<12 mg P_{Olsen} kg^{-1}, Chapter 2). The negative species-specific thresholds for the typical *Nardus* grassland species in our mesocosms ranged from 3.6 and 4.6 mg P_{Olsen} kg^{-1} for *L. multiflora* and *N. stricta* to 9.9 and 10.8 mg P_{Olsen} kg^{-1} for *M. caerulea* and *P. erecta,* which suggests that a community-based ecological threshold (*e.g.* the threshold of 11.5 or 12 mg P_{Olsen} kg^{-1} here) may not be sufficient when managers aim to restore specific species (cf. 'ecological threshold' vs 'management threshold' in Jax 2016).

Apart from *A. odoratum* and *R. acetosa* (thresholds 11.8 and 23 mg P_{Olsen} kg^{-1}), neither of the other mesotrophic species nor any eutrophic species were identified as significant negative indicator species for increasing bioavailable phosphorus concentrations. Field experiments showed similar negative responses to phosphorus fertilization for *A. odoratum* (Hejcman et al. 2007; Hejcman et al. 2014; Duffková et al. 2015); a contrasting positive (Duffková et al., 2015; Hejcman et al., 2007) or neutral (Hejcman et al., 2014) response for *R. acetosa*; and a negative response for *P. lanceolata* (Duffková et al., 2015; Hejcman et al., 2007), for which we found no significant response in our mesocosms. Also in the field study of Hejcman et al. (2007), oligotrophic species (including our study species *S. pratensis*, *N. stricta*, *P. erecta* and *L.*

multiflora) responded negatively to phosphorus fertilization and mesotrophic plant species showed variable responses (including *P. lanceolata*, *R. acetosa* and *A. odoratum*). Eutrophic plant species responded positively (including *Holcus lanatus*, *Taraxacum officinale* and *D. glomerata*). In our experiment, *D. glomerata* was the only species with a significant positive response to increasing soil phosphorus concentrations.

In the mesocosms with soil phosphorus concentrations well above the community threshold, the typical *Nardus* grassland species were frequently absent or had low biomass, independent of the soil biota treatment. Lekberg et al. (2018) also showed that negative plant-soil feedback effects are outweighed by above-ground plant-plant competition, *i.e.* light-limitation, in resource-rich conditions. In the phosphorus-rich mesocosms, the fast-growing meso- and eutrophic species outcompeted and overgrew the slow-growing typical *Nardus* grassland species. This was indicated by a strong negative relationship between the relative biomass of the typical *Nardus* grassland species and the total biomass of the plant communities. In the mesocosms containing less phosphorus than the threshold, the relative biomass of the typical *Nardus* grassland species was twice (soil biota added) or even five times (no soil biota) as high as in the mescosms with high phosphorus availability. In the phosphorus-poor mesocosms, plant-plant competition was probably lower, which enabled the typical *Nardus* grassland species to survive and flourish, most clearly so in the mesocosms without soil biota in which the total biomass was lower than in the mesocosms with added soil biota. The higher total biomass in mesocosms with added soil biota may be the result of a positive plant-soil interaction that enabled certain plant species to acquire more nutrients in the phosphorus-poor soil conditions (cf. Lekberg et al., 2018). Two mesotrophic plant species, *C. jacea* and *P. lanceolata*, appeared to have benefited more from the soil biota than the typical *Nardus* grassland species, which is contrary to what we expected (Lekberg et al. 2018). These two fast-growing species produced clearly more biomass in the phosphorus-poor mesocosms with soil biota. Interestingly, according to the MaarjAM database, these two species appear more susceptible to colonization by arbuscular mycorrhiza than the typical *Nardus* grassland species in our experiment; 107 and 366 arbuscular mycorrhiza taxa are known to colonize *C. jacea* and *P. lanceolata*, whereas only 33 arbuscular mycorrhiza taxa are known to colonize *L. multiflora* and zero arbuscular mycorrhiza taxa are known to colonize *M. caerulea*, *N. stricta* and *P. erecta* (Öpik et al. 2010). Although not all mycorrhizal interactions are beneficial for plants (Hoeksema et al., 2010), this fact might be an explanation for our finding, however, requires further verification by, *e.g.* quantifying root colonization by arbuscular mycorrhiza species on the nineteen plant species used in the experiment. Alternatively, without the “No biota” treatment, it appears inoculation with soil biota ‘masks’ soil phosphorus effects on the typical

Nardus grassland species, similarly to the findings of De Deyn et al. (2004). However, a threshold analysis on the dataset whereas from the “No biota” treatment was excluded, confirmed the initial findings of significant sensitivity of the *Nardus* grassland species towards increasing soil phosphorus bioavailability (data not shown). We stress further investigation is needed by, *e.g.* comparing the soil biota communities in each mesocosm with the plant communities and by field experiments. For example, in a field and mesocosm experiment, Wubs et al. (2016) found the vegetation of an arable field, where the topsoil was removed and then inoculated with soil biota of *Nardus* grasslands, to shift towards the composition of the remnant *Nardus* grassland which was used as a donor site. Further, adding sods from heathlands shifted the plant community towards the composition of the heathland donor site. Similarly the below-ground soil community also shifted towards the donor sites. Also van der Bij et al. (2018) showed in a field experiment that adding soil biota, by spreading out sods containing both seeds and soil biota, enhanced the re-establishment of *Nardus* grassland and heathland species after topsoil removal. These field experiments, after five and three years, provide strong evidence that the inoculation with soil biota, *e.g.* by spreading out sods, may drive the plant community development in the direction of target habitat types.

The phosphorus-poor soil conditions typical of remnant *Nardus* grasslands were also essential for the survival of the typical *Nardus* grassland species amongst more competitive species in our mesocosms. However, restoring phosphorus-poor soil conditions and (re-)introducing target species will likely not warrant successful restoration of typical *Nardus* plant communities. In contrast to fast-growing species, slow-growing typical *Nardus* grassland species are known to be unable to switch from a growth strategy aimed at acquiring enough nutrients to a growth strategy of producing more biomass (Van Daele et al., 2017). Small changes in nutrient availability in nutrient-poor soils can, hence, lead to the loss of slow-growing species specialized at surviving under severe phosphorus-limitation (Roeling et al. 2018). Such changes may be induced by specific interactions with soil biota but still needs to be revealed by further research.

6.5 Conclusions

Phosphorus-poor soil conditions were essential for most typical *Nardus* grassland species in our mesocosm experiment. The negative community threshold of 11.5 mg P_{Olsen} kg^{-1} in the mesocosms corresponds to the upper limits of bioavailable soil phosphorus concentrations in remnant *Nardus* grasslands. Above the threshold, the soil biota treatment did not affect the biomass of the typical *Nardus* grassland species. Below the threshold, the typical *Nardus* grassland species could survive and flourish, most clearly so in the mesocosms of the “No biota” treatment. Increasing total biomass per mesocosm, due to fast-growing species, negatively affected the slow-growing *Nardus* grassland species indicating competition for light.

6.6 Acknowledgements

This project was funded by Research Foundation Flanders (project G050215N) and hosted at Flanders Research Institute for Agriculture, Fisheries and Food. We thank the nature reserve managers for permitting soil collection and helping with field selection, our ForNaLab colleagues and many students from the University College and Ghent University for their help in installing and managing the experiment and for sampling and sample processing.

The mesocosm experiment on 07/09/2016 (photo: Safaa Wasof)

Freshly cut meadow in a in *la vallée de la Meuse* in northern France

Discussing grassland restoration measures in the field with stakeholders in nature reserve Landschap De Liereman (Oud-Turnhout)

CHAPTER 7

General overview of results, decision tree for practitioners and future perspectives

Taking soil samples in a grassland in Landschap De Liereman to assess the depth of phosphorus leaching

> *"Not investing [in nature] is irreversible" (Fisher and Krutilla 1974)*

Decision tree after: Schelfhout S, Mertens J, Perring MP, Raman M, Baeten L, Demey A, Reubens B, Oosterlynck S, Gibson-Roy P, Verheyen K, De Schrijver A. 2017. P-removal for restoration of Nardus grasslands on former agricultural land: cutting traditions. Restoration Ecology. 25:S178–S187. Doi: 10.1111/rec.12531

Abstract

In this chapter, we discuss the three overarching research questions of this PhD thesis (cf. §1.5), using the results and data of our studies described in Chapters 2-6.

This thesis handles the restoration of *Nardus* grasslands, one of the European priority habitat types. Grassland ecosystems are important for a manifold of species from various taxa; however, large areas have been destroyed by land-use change or have deteriorated due to land-use intensification and atmospheric nitrogen deposition. Typical plant species of *Nardus* grasslands are adapted to nutrient-poor soil conditions by their conservative resource use strategy, *e.g.* slow-growth. In case of fertilization, they are likely to become shaded out by fast-growing plant species. Phosphorus is a key nutrient for evaluating the restoration potential of previously-fertilized grasslands, where bioavailable phosphorus concentrations are high.

We have shown that restoring *Nardus* grasslands on former agricultural land is not straightforward. Reinstating traditional management, such as mowing for 16-25 years (Chapter 2), did not restore plant communities of *Nardus* grasslands (cf. answer to RQ1 in §7.1; Fig. 7.1). The studied post-fertilization grasslands were mesotrophic to eutrophic, *i.e.* with fast-growing plant species and high concentrations of bioavailable phosphorus in the soil. The time needed to lower the soil phosphorus concentrations by traditional mowing management appeared to be very long (40 to 118 years).

In a five-year field experiment (Chapter 3), P-mining (*i.e.* lifting nutrient limitation by nitrogen and potassium) removed two times more phosphorus than mowing, whereby the period for abiotic restoration is halved. Optimization opportunities for phytomining phosphorus were limited (cf. answer to RQ2 in §7.2). Adding mycorrhiza or humic substances did not increase phosphorus removal with P-mining in a pot experiment (Chapter 4). However, certain crop species were more effective at removing phosphorus than others (pot experiment Chapter 5).

The efficiency of removing phosphorus from the soil dropped steeply with decreasing soil phosphorus concentrations in the three experiments (Chapters 3-5). This decrease in annual phosphorus removal should be taken into account when estimating the time needed to restore phosphorus-poor soil conditions through mowing or P-mining.

Soil biota inoculation did not help *Nardus* grassland species to withstand soil phosphorus concentrations above the threshold of 12 mg P_{Olsen} kg^{-1} we observed in remnant *Nardus* grasslands (cf. Chapter 2) and our mesocosm experiment (Chapter 6, answer to RQ3 in §7.3). The slow-growing plant species typical of *Nardus* grasslands persisted in extremely phosphorus-poor environments and performed better if they were not shaded out by fast-growing species.

We developed a decision model to aid planners and managers in their choice of realistic restoration targets and interventions (§7.4). We highlight the importance of restoring abiotic conditions before starting biotic restoration and initiating traditional grassland management. Cost-effective efforts for restoration should be well-prepared and based on measurements of relevant initial soil characteristics. This allows for an evaluation of the distance to target and the selection of an effective restoration technique. These techniques may involve cutting with mowing and utilizing alternative techniques as P-mining or topsoil removal.

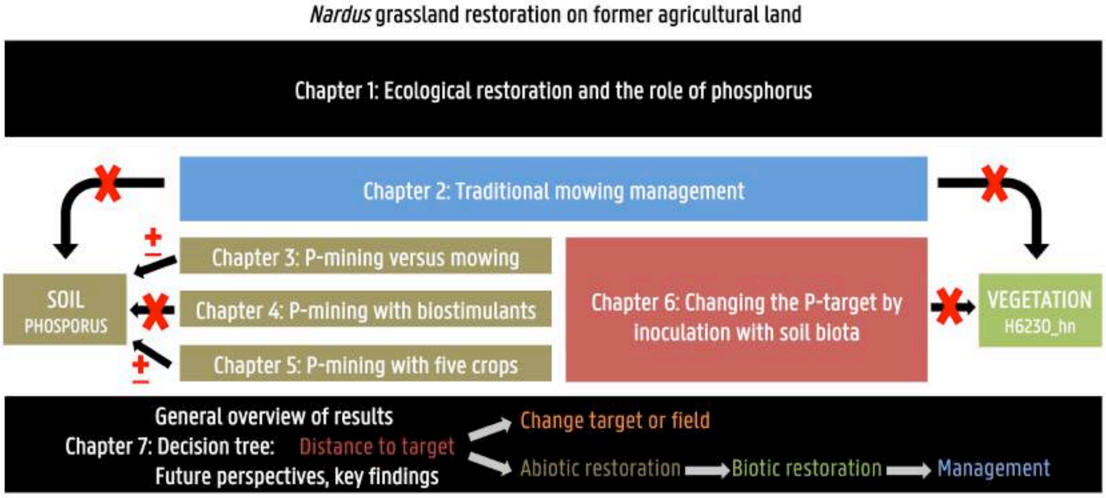


Figure 7.1 Thesis outline and summary of the key findings per chapter

7.1 RQ1: Can traditional management by mowing restore *Nardus* grassland on former agricultural land?

Traditional management, *i.e.* unfertilized mowing with hay removal and autumn grazing, of post-fertilization grasslands for at least 15 years did not yield the floristic and phosphorus-poor targets of *Nardus* grassland (Chapter 2). The remnant *Nardus* grasslands were typified by slow-growing species such as *Molinia caerulea*, *Potentilla erecta* and *Veronica officinalis*, of which some were Red List species such as *Polygala vulgaris*. The post-fertilization grasslands contained fewer species, only fast-growing common species and no Red List species.

Phosphorus-poor soil conditions are essential for the restoration of species-rich *Nardus* grasslands of the type 6230_hn (see Appendix 1.A). More specifically, a bioavailable phosphorus concentration of 12 mg P_{Olsen} kg^{-1} appears to be an upper limit for this habitat type. We observed this soil phosphorus threshold in remnant *Nardus* grasslands (95th percentile for 34 grasslands; Chapter 2) and in a mesocosm experiment in which we grew *Nardus* species together with fast-growing species along a gradient of bioavailable phosphorus (Threshold Indicator Taxa Analysis TITAN, Chapter 6). A small increase in phosphorus availability above the phosphorus threshold resulted in a disproportionally large drop in abundance of the typical *Nardus* species in the mesocosm communities (Chapter 6). However, in the post-fertilization grasslands, the bioavailable soil phosphorus concentrations were two to thirteen times higher than the phosphorus threshold of 12 mg P_{Olsen} kg^{-1}. Yet, the biomass production was relatively low (3 t dry biomass ha^{-1} y^{-1}) due to nitrogen and potassium limitation. With the current mowing management, another 40 years of phosphorus removal would be needed to reach the phosphorus threshold in the 0-10 cm soil layer, and another 118 years for the 0-30 cm soil layers. Enhancing the species-richness of grasslands with higher phosphorus concentrations than the threshold may be possible by choosing another target habitat type and (experimenting with) introducing the missing species (biotic restoration). For example, on acid, sandy soils where the threshold of 12 mg P_{Olsen} kg^{-1} is not realistic, the 6230_ha type (species-rich grasslands of the *Agrostis capillaris* group with some *Nardus* grassland species; Appendix 1.A; De Saeger and Wouters 2017) or the rbbha type (species-rich grasslands of the *Agrostis capillaris* group with less *Nardus* grassland species; Appendix 1.A; De Saeger and Wouters 2017) may be better targets.

Using traditional mowing management as a restoration technique is probably only effective in specific conditions, *i.e.* in fields where the abiotic conditions are close to the target and where the surrounding landscape matrix is species-rich and landscape connectivity is high (Winsa et al. 2015), *e.g.* in the White Carpathian Mountains of the Czech Republic (Michalcová et al. 2013). The grasslands we studied lay, however, in a species-poor, fragmented landscape. In addition, the remnant *Nardus* grasslands are small (<0.5 ha; larger areas usually contain more species (Keddy 2005)) and surrounded by eutrophic intensively used fields unhospitable for *Nardus* grassland species.

Restoration of *Nardus* grasslands on eutrophic fields in species-poor, fragmented and intensively used landscapes requires active restoration of both the abiotic and biotic site conditions.

The feasibility of restoring *Nardus* grasslands on previously-intensified grasslands depends on the (a) biotic starting point of the restoration trajectory, *i.e.* on the *distance to target*. The *distance to* this *target* is the amount of phosphorus to be removed and will determine the time needed for P-mining by phytomining (mowing or P-mining; Box 2); the extent of the phosphorus enrichment, *i.e.* the depth up to which soil phosphorus is elevated (Box-Fig. 1.4), will determine the cost of topsoil removal. The cost of the restoration technique and the time needed to reach the target may be weighed with the potential value of the site in the landscape that is being restored. Because of the high cost (*e.g.* €25 000 ha^{-1} in Table 1.2), topsoil removal is more likely to be used in large restoration projects, funded by *e.g.* European Life+ projects or within the framework of nature development projects of the Flemish Land Agency (VLM).

Biotic restoration should follow abiotic restoration and involves the active reintroduction of target plant species (Piessens et al. 2005; Helsen et al. 2013), *e.g.* via seed or hay transfer (Edwards et al. 2007; Hedberg and Kotowski 2010) or plug-planting (Walker et al. 2004) after soil disturbance (Kiehl et al. 2010). Biotic restoration also involves aiding the below- and above-ground fauna communities, *e.g.* by spreading out sods from donor *Nardus* grasslands to restore the below-ground micro- and mesofauna communities (Wubs et al. 2016; Loeb et al. 2018; van der Bij et al. 2018).

7.2 RQ2: Is it possible to phytomine phosphorus more efficiently?

Traditional mowing management removed only little phosphorus with biomass (*i.e.* 5.3 ± 1.0 kg P ha^{-1}, Chapter 2) due to limitation by nitrogen and potassium. The restoration time needed for reaching phosphorus-poor soil conditions would therefore be long (*i.e.* 40 to 114 years). With P-mining, *i.e.* mowing with yield maximization by adding the growth-limiting nutrients nitrogen and potassium, the restoration time would be considerately shorter (*i.e.* 14 to 46 years).

In our field experiment (Chapter 3), P-mining led to larger phosphorus removal with biomass compared to conventional mowing management, but the potential to phytomine phosphorus (with P-mining and mowing) depended on the soil phosphorus concentration: high phosphorus removal on phosphorus-rich soils, decreasing removal potential with decreasing soil phosphorus concentration. The results were similar in our pot experiments (Chapters 4-5). The phosphorus removal rate was 37 to 91% lower in the phosphorus-poor soil conditions than in the phosphorus-rich soil conditions (Table 7.1). The decrease in phosphorus removal with decreasing soil phosphorus concentrations implies a slowing down of phosphorus removal with biomass over time. A delay that should be taken into account in calculations of the restoration time needed to reach low soil phosphorus concentrations with P-mining. As such, restoration of *Nardus* grasslands on intensively fertilized soils by P-mining will probably take decades.

The use of specific crop species for P-mining may increase phosphorus removal and, thus, shorten the time needed for restoring phosphorus-poor soil conditions (Chapter 5). At high soil phosphorus concentrations, phosphorus removal with biomass was higher with buckwheat, maize and sunflower than with flax and triticale; at lower phosphorus concentrations, the difference in phosphorus removal was almost absent between crop species, except for triticale which consistently performed worst at removing phosphorus (Table 7.1). Before formulating recommendations on the possibilities of P-mining with crops, the results of our pot experiment need to be validated in a long-term field experiment. Adding biostimulants, *i.e.* phosphorus solubilizing bacteria (PSB), humic substances or arbuscular mycorrhizal fungi (AM) (Chapter 4), did not increase phosphorus removal with P-mining (Table 7.1).

Restoring phosphorus-poor soil conditions on post-agricultural land will remain a challenge. Phytomining phosphorus will always be a long-term commitment and can be made slightly more efficient by lifting the limitation of nutrients other than phosphorus (P-mining) or selecting crop species that take up more phosphorus.

Table 7.1 Mean phosphorus removal with biomass (standard deviation between brackets) in the experiments in this thesis (Chapter 3, Chapters 4-5) for different initial bioavailable soil phosphorus concentrations. Phosphorus removal declined consistently from phosphorus-rich soils (*High*) to phosphorus-poor soils (*Low*)

History P fertilization (mg P_{Olsen} kg^{-1})	**High (80-160)**	**Mid (40-80)**	**Low (20-40)**	**Low vs. High**
Field experiment 'mowing vs. P-mining': P removal (kg ha^{-1} y^{-1})				
mowing	10 (5)	8 (4)	6 (3)	- 42%
P-mining	20 (5)	19 (6)	12 (5)	- 37%
Pot experiment 'P-mining with biostimulants': P removal (mg pot^{-1})				
no addition	21 (2)	10 (4)	6 (1)	- 71%
+ HS	19 (1)	9 (1)	9 (1)	- 53%
+ HS2	20 (1)	8 (1)	5 (1)	- 75%
+ AMF	21 (2)	8 (1)	5 (1)	- 76%
+ PSB	23 (2)	5 (1)	5 (1)	- 78%
Pot experiment 'P-mining with crops': P removal (mg pot^{-1})				
buckwheat	269 (31)	186 (66)	79 (18)	- 71 %
maize	195 (39)	123 (35)	63 (14)	- 68 %
sunflower	155 (37)	105 (35)	47 (13)	- 70 %
flax	57 (11)	54 (13)	36 (6)	- 37 %
triticale	54 (16)	26 (17)	5 (4)	- 91 %

HS: Humic substances in a low concentration; HS: Humic substances in a high concentration
AMF: Arbuscular mycorrhizal fungi
PSB: Phosphorus solubilizing bacteria

Box 2. Estimating the time to phytomine phosphorus along a soil phosphorus gradient

We modelled the time needed to remove soil phosphorus by phytomining (*i.e.* by mowing or P-mining) until the target of *Nardus* grasslands (12 mg P_{Olsen} kg^{-1}) for soils with a bioavailable phosphorus concentration up to 150 mg P_{Olsen} kg^{-1} (Box-Fig. 2.1). This estimation is based on the results from Chapter 2 (bioavailable phosphorus versus slowly cycling phosphorus relationship) and Chapter 3 (phosphorus removal with biomass versus bioavailable phosphorus concentration and management type). Four considerations are needed when estimating the time needed to remove excess phosphorus from the soil with biomass:

First, the decline of bioavailable phosphorus concentrations depends upon many factors such as the soil type and annual phosphorus removal (Johnston et al. 2016). Johnston et al. (2016) suggests to predict the decline in bioavailable phosphorus concentrations by using data on phosphorus removal and on the decline of bioavailable phosphorus derived from a series of fields with the same soil type and different initial bioavailable phosphorus concentrations, *i.e.* **a soil phosphorus gradient**. The field experiment (Chapter 3) was performed along a soil phosphorus gradient. The soil type of the grasslands in our field study (Chapter 2) and experiment (Chapter 3) was sandy, acid and with low total iron (500-1500 mg Fe_{Total} kg^{-1}) and medium total aluminium concentrations (2200-4500 mg Al_{Total} kg^{-1}). This modeling exercise is valid only for such soils.

Second, **the yearly amount of phosphorus removed by biomass decreases with decreasing bioavailability of phosphorus in the soil** (see answer to RQ2) and has probably a large impact on the time needed to remove phosphorus (see Appendix 4.E). Here we assume grassland management, however, higher phosphorus removal may be possible by using arable crops such as maize or buckwheat (see Chapter 5). In our model we used the relation between phosphorus removal and bioavailable phosphorus for grassland management (P-mining or mowing) found in Chapter 3 (see Fig. 3.3; Equations 1 and 2). Below a bioavailable phosphorus concentration of 25 mg P_{Olsen} kg^{-1}, the advantage of P-mining over mowing (i.e. more phosphorus removal through mining) was small (Chapter 3). Therefore, at a bioavailable phosphorus concentration of 25 mg P_{Olsen} kg^{-1}, we assumed to **switch management from P-mining to mowing** in our time estimations. With phosphorus removal with biomass (in kg P ha^{-1} y^{-1}) as $P_{removal}$ and bioavailable phosphorus concentration as P_{Olsen} (in mg P_{Olsen} kg^{-1}), we assumed:

Annual phosphorus removal with mowing management:

$$P_{removal} = 4.8\ (\pm 3.2) + 0.05(\pm 0.03) \times P_{Olsen} \qquad \text{(Equation 1; Chapter 3)}$$

Annual phosphorus removal with P-mining management:

As long as bioavailable phosphorus concentration was higher than 25 mg P_{Olsen} kg^{-1}:

$$P_{removal} = 9.8(\pm 5.5) + 0.11(\pm 0.07) \times P_{Olsen} \qquad \text{(Equation 2; Chapter 3)}$$

For bioavailable phosphorus concentration lower than 25 mg P_{Olsen} kg^{-1}, we used (Equation 1).

Third, **it is necessary to take into account the slowly cycling phosphorus pools** that replenish the bioavailable pools (see Box 1 and Chapter 3). We, therefore, use the relation between bioavailable and slowly cycling phosphorus (Fig. 2.1; Equation 3) to predict the decline in bioavailable phosphorus concentrations when slowly cycling phosphorus stocks (in kg ha^{-1}) are depleted with phosphorus. With slowly cycling phosphorus concentration as $P_{Oxalate}$ (in mg $P_{Oxalate}$ kg^{-1}) and bioavailable phosphorus concentration as P_{Olsen} (in mg P_{Olsen} kg^{-1}), we assumed:

$$P_{Oxalate} = 0.67 + 3.03 \times P_{Olsen} \qquad \text{(Equation 3; Chapter 2)}$$

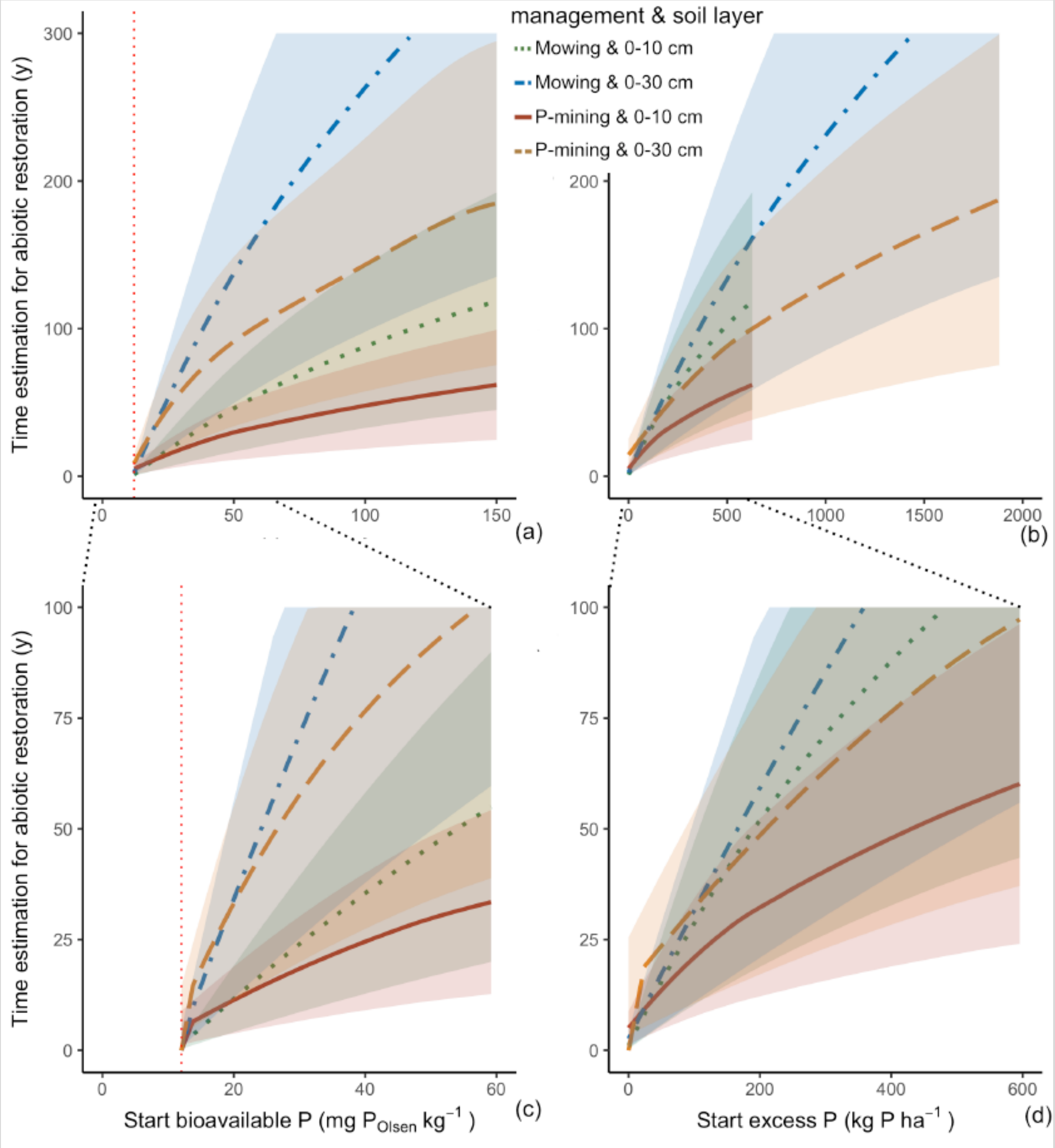
Fourth, in intensively used grasslands, it is very likely phosphorus concentrations are increased over the depth of 30 cm or even deeper by (the combination of) ploughing and leaching of phosphorus (Box-Fig. 1.4). In our field experiment, neither the bioavailable nor the slowly cycling phosphorus concentrations in the **deeper soil layer**

(15-30 cm) were decreased after five years of phytomining (contrary to the significant decline in the bioavailable phosphorus concentrations in the upper 0-15 cm soil layer; Chapter 3). Yet, the phosphorus pools in the 15-30 cm soil layer and even in the subsoil layer (> 30 cm deep) are likely to be accessible by plant roots or mycorrhiza in symbiosis with these plants, especially with P-mining (Bauke et al 2018). It is still unclear whether the phosphorus pools in these deeper soil layers should be taken into consideration when estimating the potential of a field for restoring an oligotrophic habitat type. We therefore performed this modelling exercise for both 0-10 cm and 0-30 cm soil layers.

The **time needed** to reach the phosphorus-poor threshold of *Nardus* grasslands (12 mg P_{Olsen} kg^{-1}) had **a wide range depending upon the soil depth and method to phytomine phosphorus** (Box-Fig. 2.1). Large excessive phosphorus stocks (> 500 kg P ha^{-1} in Box-Fig. 2.1b) are accompanied by a long time estimation of the abiotic restoration (> 50 years). The amount of excess phosphorus to remove is about three times higher for the 0-30 cm soil (up to 3000 kg P ha^{-1}) layer than for the 0-10 cm soil layer (up to 1000 kg P ha^{-1}). The time needed to remove phosphorus is markedly higher for mowing than for P-mining (Box-Fig. 2.1a). The longest time estimations were found for fields with high bioavailable phosphorus concentrations (Box-Fig. 2.1a).

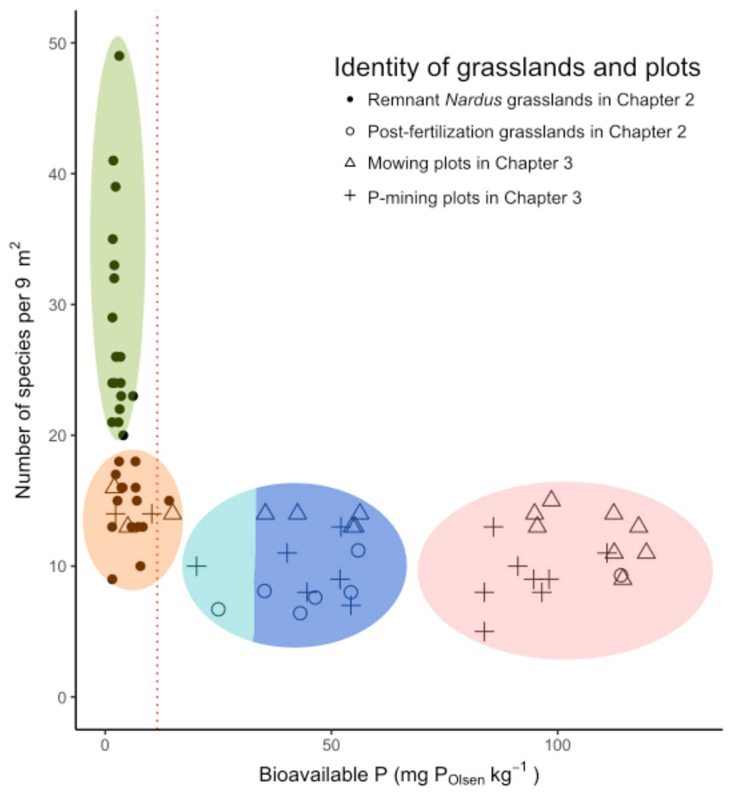
It appears **unrealistic to phytomine phosphorus (mowing or P-mining) from fields with very high soil phosphorus concentrations** (> 60 mg P_{Olsen} kg^{-1}) to reach the *Nardus* grassland threshold because this would take more than 50 years (Box-Fig. 2.1a). For this soil type, it may be more realistic to only consider fields with a bioavailable phosphorus concentration less than 60 mg P_{Olsen} kg^{-1} for restoration by phytomining (Box-Fig. 2.1c and d). If it suffices to phytomine the 0-10 cm soil layer, the time needed ranges up to 39 years for mowing management and up to 23 years for P-mining management depending upon the initial soil phosphorus bioavailability (Box-Fig. 2.1c). However, phytomining the 0-30 cm soil layer requires more time: up to 131 years for mowing management and up to 78 years for P-mining management depending upon the initial soil phosphorus bioavailability (Box-Fig. 2.1c).

In fields containing less than 25 mg P_{Olsen} kg^{-1}, it is better to use mowing as a restoration technique because there appears no gain in efficiency of P-mining over mowing management (see also Chapter 3).



Box-Figure 2.1 Estimation of time needed to phytomine phosphorus versus the initial bioavailable phosphorus concentration (mg P_{Olsen} kg^{-1}; a and c) and versus the amount of soil phosphorus in excess to the threshold of *Nardus* grasslands (kg P ha^{-1}; b and d). Panels c and d show a magnified part of panels a and b (0-60 mg P_{Olsen} kg^{-1} in c and the corresponding excess P stock in d). The time was estimated for phytomining by mowing (green dotted line and blue dot-dashed line) and P-mining (brown full line and yellow dashed line) management and of the 0-10 cm (green dotted line and brown full line) and the 0-30 cm (blue dot-dashed lineand yellow dashed line) soil layers. Estimations are based on the relation between bioavailable and slowly cycling phosphorus (Chapter 2) and on the model for phosphorus removal with biomass (Chapter 3). Shades show the 95% confidence interval derived from the phosphorus removal model (Chapter 3). Estimates are only valid for similar field conditions (sandy, acid, 500-1500 mg Fe_{Total} kg^{-1} and 2200-4500 mg Al_{Total} kg^{-1}) and management (bi-annual mowing without fertilization and P-mining by bi-annual mowing with N and K fertilization)

During the five-year field experiment of Chapter 3, four of the grassland plots reached the abiotic phosphorus-poor threshold of 12 mg P_{Olsen} kg^{-1} in the topsoil layer (Box-Fig. 2.2). In these plots, biotic restoration by introducing the missing target species by seeding or hay transfer may increase the species richness and shift the community composition towards the target of *Nardus* grasslands, though this remains to be tested. The other species-poor grasslands below the phosphorus threshold were remnant *Nardus* grasslands where species-richness may be increased as well by biotic restoration (Chapter 2). The acceptable time limit for abiotic restoration depends upon the project context. Grasslands with very high soil phosphorus concentrations (> 60 mg P_{Olsen} kg^{-1}) and low species numbers (10 species per 9 m^2) fall out of the scope for abiotic restoration by phytomining because it would probably take too long (up to 350 years). However, it seems reasonable to assume **phytomining is a realistic restoration technique in the grassland plots with intermediate bioavailable phosphorus concentrations (15 to 60 mg P_{Olsen} kg^{-1})**: phosphorus removal by mowing for fields below 25 mg P_{Olsen} kg^{-1} and by P-mining for fields between 25 and 60 mg P_{Olsen} kg^{-1}. P-mining immediately after intensive land-use does not imply a big change in the management and nature values (which are probably low). However, when using P-mining on mesotrophic grasslands that were managed by traditional mowing for a long time and species-rich, the nature values are decreased by the increased land-use intensity. It is worth also considering this loss



Box-Figure 2.2 The number of species per 9 m^2 versus bioavailable phosphorus concentrations (mg P$_{Olsen}$ kg^{-1}) for each of the grasslands in Chapters 2 and 3. The green oval shape indicates remnant *Nardus* grasslands in no apparant need of restoration measures. The orange oval shape indicates remnant grasslands and abiotically restored post-fertilization grasslands in need of biotic restoration. The blue oval shape indicates post-fertilization grasslands in need of abiotic and biotic restoration; light blue fields (below 25 mg P$_{Olsen}$ kg^{-1}) are probably suitable for restoration by mowing management; dark blue fields (between 25 and 60 mg P$_{Olsen}$ kg^{-1}) are probably suitable for restoration by P-mining. The red oval shape indicates grasslands for which phosphorus removal by phytomining would be too slow

7.3 RQ3: Do *Nardus* grassland species benefit from inoculation with soil biota?

In mesocosms with bioavailable soil phosphorus concentrations higher than 12 mg P_{Olsen} kg^{-1}, the biomass of typical *Nardus* grassland species was extremely low and not affected by the type of soil biota added (Chapter 6). In mesocosms with lower bioavailable soil phosphorus concentrations, the abundance of typical *Nardus* grassland species was impeded by the presence of soil biota from oligo-, meso- or eutrophic grasslands. Inoculation with soil biota did not enable typical *Nardus* grassland species to flourish at phosphorus concentrations higher than the threshold of 11.5 mg P_{Olsen} kg^{-1}. Yet, the mesocosm experiment is ongoing and more in-depth analysis of the plant-soil biota interactions might shed new light on this topic. In contrast to our preliminary results, other studies have shown inoculation of soil communities from target habitat types can help speed up the biotic restoration of oligotrophic grasslands and heathlands. Inoculation with soil biota, *e.g.* by spreading out sods, may drive the plant community development in the direction of target habitat types.

7.4 Decision tree

Based on our results, we present a decision tree to aid practitioners in selecting the appropriate restoration technique given the abiotic and biotic bottlenecks (Fig. 7.2). To obtain effective restoration management of *Nardus* grasslands after land-use intensification (cf. §1.3), we propose to focus on fields where phosphorus-poor soil conditions can be reached within a reasonable timespan and with a reasonable cost. What is *reasonable* may be different for projects. As an example, the outcome of the decision model is presented for a restoration project (Box 3).

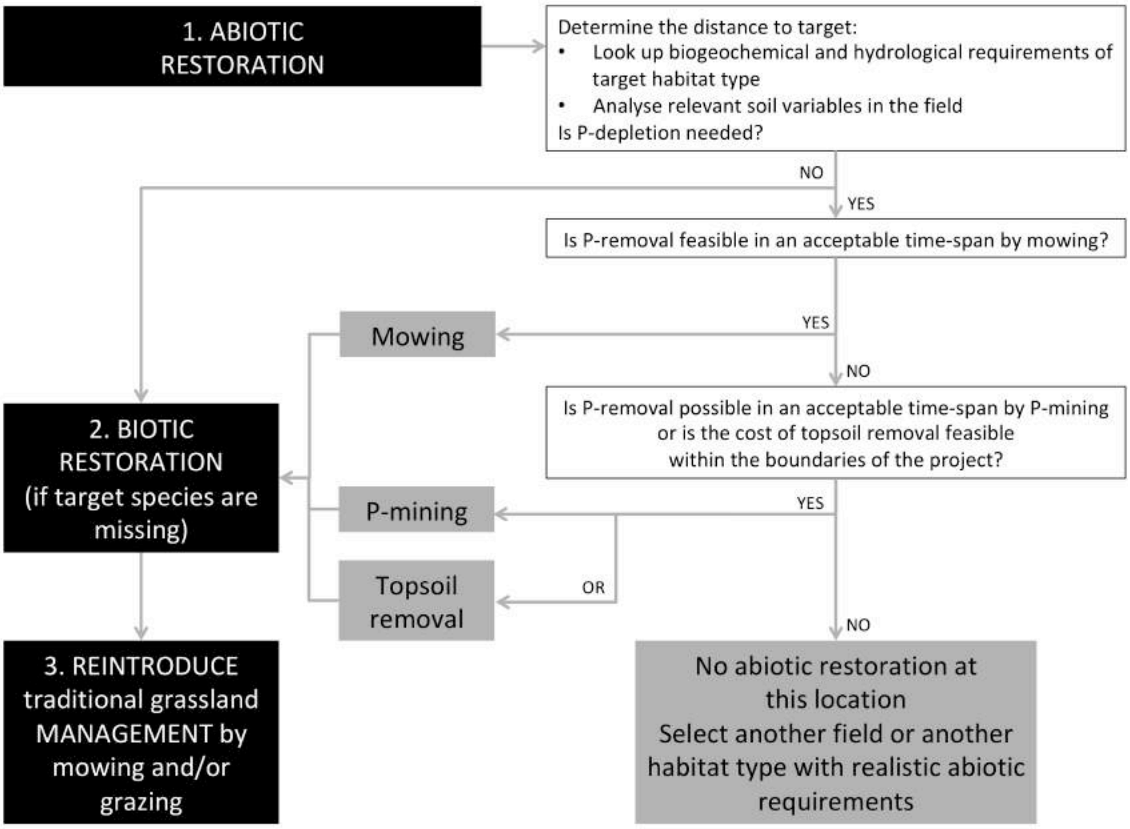


Figure 7.2 Decision tree for practitioners restoring species-rich semi-natural grasslands on fertilized land. This decision model was inspired by Kemmers and van Delft (2010) but with a particular focus on abiotic and biotic restoration. In cases where P-depletion is needed, the choice for an appropriate restoration technique is based upon the time and cost needed for restoration. The time needed with mowing or P-mining depends upon $P_{Excessive}$ while the cost for topsoil removal is dependent on the soil depth with elevated P-concentrations

The first step is to **assess the need for abiotic restoration** by determining the ***distance to target***, *i.e.* by measuring relevant abiotic variables (see §1.4) and comparing these values with their corresponding thresholds of the targeted habitat type (see Box 2). If the abiotic threshold is not met, **abiotic restoration is needed before biotic restoration** can happen. Which abiotic restoration technique is most appropriate depends on the *distance to target* and on the restoration project constraints such as budget and timing. One of these targets for the restoration of *Nardus* grasslands, is the phosphorus-poor target of 12 mg P_{Olsen} kg^{-1} (see §1.4, Chapters 2 and 6). The feasibility of restoring *Nardus* grassland on phosphorus-rich soils seems thus questionable without financial support of *e.g.* European Life+ funding or within the framework of nature development projects of the Flemish Land Agency (VLM). Topsoil removal is generally costly (see Table 1.2 in chapter 1). Low-budget projects can probably better focus on restoration on fields with a history of less intensive fertilization where phytomining by mowing or P-mining is attainable. It might be most efficient to invest some money in the abiotic screening of parcels before purchasing them or before selecting a target habitat type.

The second step is, if no substantial amount of phosphorus has to be removed or if abiotic restoration has been succesful, to **assess the need for biotic restoration** (see §1.4). We only found examples of successful restoration of *Nardus* grasslands on eutrophic land where the topsoil was removed followed by the reintroduction of target species (Berendse et al. 1992; Loeb et al. 2018); and, a similar finding for other grassland types: *Molinia* grassland (Natura2000 habitat type 6410; Tallowin and Smith 2001; Hölzel and Otte 2003; Walker et al. 2004; Klimkowska et al. 2007; Fig. 7.3), alluvial *Cnidion* grassland (Natura2000 habitat type 6440; Hölzel & Otte 2003), calcareous grasslands (Natura2000 habitat type 6210; Walker et al. 2004; Kiehl and Pfadenhauer 2007), lowland hay meadows (Natura2000 habitat type 6510; Walker et al. 2004), but also for various species-rich grasslands in Australia (Gibson-Roy et al. 2010).

Third, for **conserving these ecosystems**, the appropriate traditional grassland **management** (mowing and/or grazing) can be introduced and maintained (see §1.3.1).

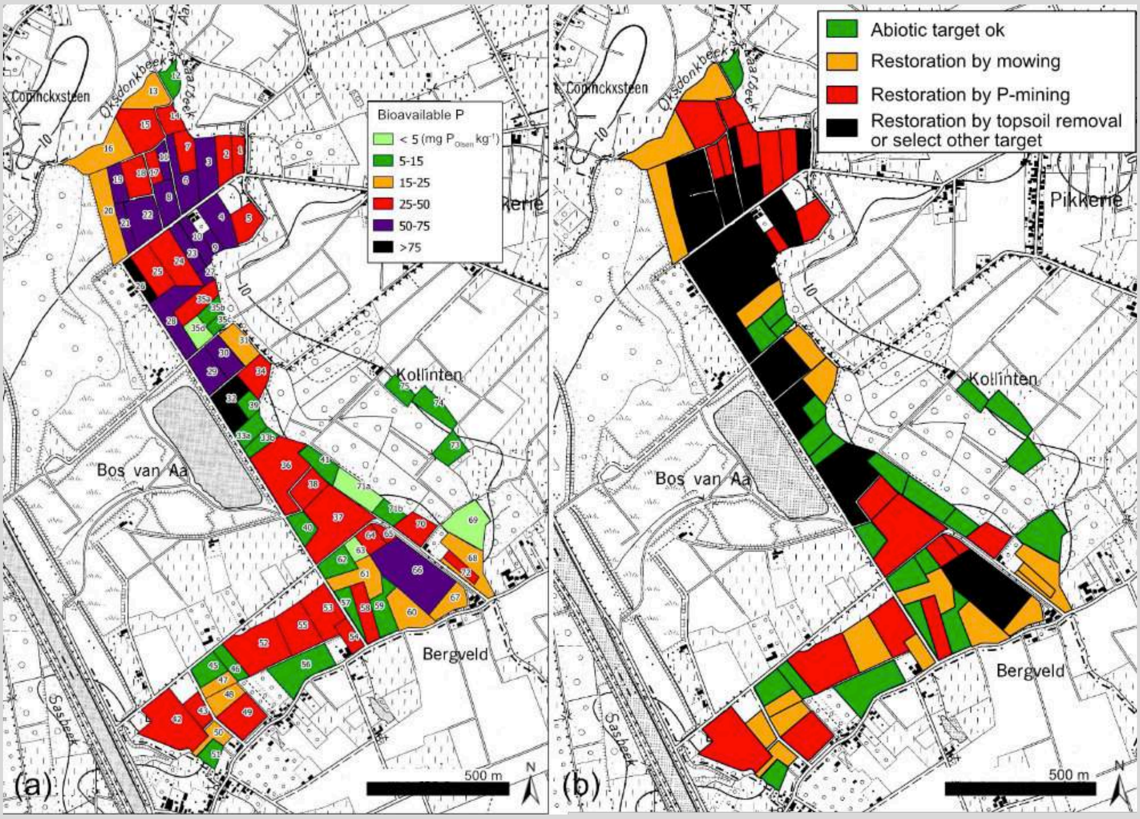
As a final step, **if the changes to the ecosystem are irreversible** and restoration of oligotrophic *Nardus* grasslands is unrealistic because of time or money constraints, one could decide to focus the restoration efforts on **other sites**, or one could **choose another habitat type** as a target for which the phosphorus concentrations may be higher; this may even be a *novel ecosystem* or a *hybrid ecosystem* between a novel and historical ecosystem (*sensu* Hobbs et al. 2006; Hobbs et al. 2009), which can result in certain ecological functions as well. In this case, species-rich habitat types that are less demanding with regard to soil phosphorus levels may be targeted, such as species-rich mesotrophic grass-herb mixture grasslands with common, flowering plant species such as *Leucanthemum vulgare*, *Centaurea jacea, Lotus corniculatus* and *Trifolium pratense*. Associations aimed at aiding pollinators, *e.g.* Bumblebee Conservation Trust (UK), are pioneers in this topic and have developed species lists of native pollinator-friendly plant species that tolerate growing in mesotrophic soil conditions (Appendix 7.A). Mesotrophic species-rich grasslands may be created by the combination of a few years of traditional mowing management to obtain nitrogen and/or potassium limitation and low biomass production (*cf.* 3 t DM ha^{-1} in Chapters 2 and 3) and the active introduction of common native legume and forb species on (patches of) naked soil via seeding or hay transfer (Klaus et al. 2017). Creating such species-rich grasslands on formerly intensively used land can *upgrade* biodiversity (Woodcock et al. 2014), *e.g.* by the provision of food and nesting places to a multitude of pollinators and other insects (Wratten et al. 2012; *e.g.* Fig. 7.4). Importantly, such *novel* grassland ecosystems can probably not replace highly specialized systems such as oligotrophic habitat types, which typically harbor a lot of Red List species. When located adjacent to sites of high ecological value, *e.g.* well-developed *Nardus* grasslands, such restored *novel* mesotrophic grasslands may be used as corridors by, *e.g.* butterflies, between oligotrophic habitats or as buffer zones to prevent inflow of fertilization from nearby agricultural fields. The continued (mowing) management of these mesotrophic grasslands may, however, become costly over time, especially if the produced hay is not valorized (see Table 1.2 in chapter 1). The management of arable land sown with (rare) annual wildflowers may be easier (superficial cultivation every three years; personal communication Mark Martens) and may also result in high diversity of flora (Albrecht et al. 2016; Lang et al. 2018) and fauna species, *i.e. pollination reservoirs* (*sensu* Venturini et al. 2017).

Figure 7.3 Successful floristic restoration of oligotrophic *Molinion* grasslands on eutrophic land after topsoil removal and hay transfer in 2012 in nature reserve Honderd Morgen in 's Hertogenbosch (the Netherlands) during Life project *Blues in the marshes.* Photo taken in June 2018

Figure 7.4 Result of introducing common herb species in the second year after introduction to a field with extremely high bioavailable phosphorus concentration (> 150 mg P_{Olsen} kg^{-1}) due to high historical land-use intensity (greenhouse with horticultural production of landscape plants) (a) in May 2018 with abundant *Leucanthemum vulgare* and and *Lychnis flos-cuculi*; and, (b) in June 2018 with abundant *Centaurea jacea, Achillea millefolium, Trifolium pratense* and *Lotus corniculatus* in Ruiter (Belgium). Pollinator sightings in 2018 include common species (*Aricia agestis*, *Lycaena phlaeas*, *Colias croceus*, *Smerinthus ocellata*, *Dasypoda hirtipes*) as well as rare species (*Argynnis paphia*, *Cupido argiades*)

Box 3. Screening fields for restoration of oligotrophic grasslands

In a consulting project commissioned by Waterwegen & Zeekanaal, a screening was performed to identify the difficulty of restoring species-rich oligotrophic grasslands (lowland hay meadow Natura 2000 type 6510) in fields close to nature reserve *Bos van Aa* in Zemst (Belgium; De Schrijver et al. 2013). The fields characterized by bioavailable phosphorus concentrations below the abiotic threshold (Box-Fig. 3.1a) had been traditionally managed for at least 15 years and were probably never fertilized intensively. The other fields were or had been used intensively, resulting in increased soil phosphorus concentrations: in fields with low bioavailable phosphorus concentrations, phosphorus-removal by mowing management was suggested (Box-Fig. 3.1b); in fields with slightly higher phosphorus concentrations, P-mining was advised until reaching a bioavailable phosphorus concentration of 25 mg P_{Olsen} kg^{-1} after which the restoration technique could be changed into mowing; in fields with very high phosphorus concentrations, topsoil removal or selecting another habitat type as a target was suggested.



Box-Figure 3.1 Output from consulting report for restoration of species-rich oligotrophic grasslands in nature reserve Bos van Aa in Zemst (Belgium). We found a wide variation in bioavailable phosphorus concentrations between fields (a; in mg P_{Olsen} kg^{-1} in the 0-10 cm). The outcome of the decision tree (Fig. 7.2) is shown in (b). The phosphorus-poor target was 15 mg P_{Olsen} kg^{-1} in the 0-30 cm soil layer; the distance to target was calculated by a similar calculation method to Box 2 based on bioavailable and slowly cycling phosphorus concentrations measured in the field; the acceptable time-limit for the project was to restore the abiotic targets by 2050 (De Schrijver et al. 2013)

7.5 Implications for practice and policy

To protect and restore European habitat types, cooperation between the various stakeholders of different levels, from local to regional, is required (cf. European Parliament resolution on 25/10/2018 for COP14; see examples in Table 7.2). Based on the insights acquired during this PhD, we formulate implications for practice and policy for some of the stakeholders.

In 2007, Flanders had 36.150 ha grasslands of (high) nature value (Biologische Waarderingskaart; Danckaert et al. 2008). However, only 16% (5806 ha, 0.4% of Flanders) were managed with a focus to conserve biodiversity; 19% (6988 ha) were legally unprotected as grassland; and, 62% were legally unprotected as grassland with ecological value and were allocated inadequately on the Flemish land-use map (translation: *zonevreemd*; Vlaamse Overheid 2000), *i.e.* as agricultural land which is probably intensively managed (*e.g.* with fertilizer and herbicide input). In contrast, in the semi-natural grasslands that cover only 0.4% of the land, 39% of the plant species of Flanders are found (over 600 species, of which 130 on the Red List, Dumortier et al. 2003) together with many other species (see §1.2). Hence**, upgrading the species richness of Flanders involves a better legal protection of the grasslands of high nature value and restoring biodiversity on degraded grasslands**.

Residual soil phosphorus, as a legacy effect of intensification, impedes ecological restoration of several terrestrial ecosystems: several species-rich grassland types (Janssens et al. 1998; Merunková and Chytrý 2012; Ceulemans et al. 2014), calcareous grasslands (Niinemets and Kull 2005), lowland hay meadows (Gilbert et al. 2009), heathlands (Aerts et al. 1995; Kooijman et al. 2016), forests (Verheyen and Hermy 2004; Baeten et al. 2010; Blondeel et al. 2018) and wetlands (Olde Venterink et al. 2001; Wassen et al. 2005). **We are obliged to treat oligotrophic habitat types with high-priority conservation** (cf. Moore et al. 1989 for oligotrophic wetlands) **because it is nearly impossible to turn eutrophic fields into oligotrophic fields without drastic and expensive measures such as topsoil removal.**

We advise to **carefully examine the abiotic and biotic conditions of a field before planning restoration**. This implies freeing up budget for chemical analyses and expert advice (see Box 3). The feasibility of particular targets can then be compared based on the *distance to target*, in combination with the attainable restoration techniques, the project budget available and the acceptable time limit within the project context. The distance to the target of *Nardus* grasslands is the amount of phosphorus to be removed, which will determine the time needed for phytomining (mowing or P-mining). For acid, sandy soils similar to our study, it seems reasonable to assume **fields with very high soil phosphorus concentrations** (> 60 mg P_{Olsen} kg^{-1}) **fall out of the scope for abiotic restoration by phytomining** because it would probably take too long (hundreds of years). Restoring high-level targets such as *Nardus* grasslands on eutrophic land may be limited to projects with large budgets, *i.e.* where it is possible to use **topsoil removal** as a restoration technique. **We advise projects and organizations with small budgets to focus their restoration efforts of oligotrophic habitat types on oligo- or mesotrophic fields or work towards habitat types with less stringent abiotic requirements on eutrophic fields** (*e.g.* nitrogen-limited flower-rich grasslands with common species) that may benefit the local biodiversity more than unsuccessful attempts at reaching high-level unattainable targets.

For acid, sandy soils similar to our study, we can further assume phytomining is a realistic restoration technique in the grassland plots with intermediate bioavailable phosphorus concentrations (15 to 60 mg P_{Olsen} kg^{-1}): phosphorus removal by P-mining for fields between 25 and 60 mg P_{Olsen} kg^{-1} and by mowing for fields below 25 mg P_{Olsen} kg^{-1}. P-mining immediately after intensive land-use does not imply a big change in the management and nature values (which are probably low). However, P-mining mesotrophic species-rich grasslands will cause a drop in biodiversity, hence it is worth considering this loss before selecting a target or restoration technique. Practitioners may use P-mining with existing grassland vegetation, with grass-clover (Timmermans and van Eekeren 2016) or with specific crops (see Chapter 5). Managers can choose to mine as quickly as possible by means of an intensive agricultural practice, using crops that are not interesting for biodiversity (*e.g.* maize) and crop protection products, or undertake a more extensive way of mining, with crops being more interesting for biodiversity (*e.g.* grass-clover, see Fig 7.5) and no crop protection (Carvell et al. 2006; Goulson et al. 2011). During the restoration trajectory towards phosphorus-poor soil conditions, it is probably better to switch P-mining into mowing management in fields containing less than 25 mg P_{Olsen} kg^{-1}, because there appears no gain in efficiency of P-mining over mowing management (see also Chapter 3) and it also takes a few years to decrease the nitrogen and potassium concentrations in the soil.

When the **abiotic target is (almost) reached**, restoration can be assisted by **reintroducing** the missing species. Counting on spontaneous recolonization will highly likely not suffice in fragmented species-poor landscapes as Flanders.

Perhaps most importantly, we urge practitioners to **try out (new) abiotic and biotic restoration techniques** in the field and to share the results with other practitioners and researchers, *e.g.* Conservation Evidence or the interregional GrasGoed project (see Fig. 7.6 and Table 7.2).

Figure 7.5 Grass-clover with P-mining management to phytomine phosphorus on former agricultal fields (in Life project *Blues in the Marshes* in ‘s Hertogenbosch, the Netherlands) is an attractive crop for bumblebees (Goulson et al. 2005)

Figure 7.6 Practitioners in ecological restoration of species-rich grasslands exchanging their experiences during the Interregional GrasGoed project by Inverde (see Table 7.2)

Table 7.2 Examples of actions in conservation and restoration of species-rich grasslands for stakeholders of different levels

Scale	Stakeholder	Action	Example
LOCAL	Practitioners	Conservation management on remnant oligotrophic habitats	Traditional management of remnant *Nardus* grasslands
LOCAL	Practitioners	Biotic restoration in sites that have lost species	Haytransfer from species-rich *Nardus* grasslands to species-poor oligotrophic grasslands
LOCAL	Practitioners	Use decision model for field selection for restoration of *Nardus* grasslands	Bos van Aa project (in Box 3)
	Practitioners, farmers, municipalities, Nature NGO	Create mesotrophic species-rich grasslands or *novel grassland ecosystems* on P-rich sites	Projects by entomological societies: Idylle projects by Vlinderstichting (NL)[a], Bijenlandschap (Groene Cirkels, EIS)[b], Bumblebee Conservation Trust grassland projects[c]; and, by floristic societies: Magnificent meadows[d]
LOCAL	Farmer cooperating in conservation management by *e.g.* mowing or grazing	Adjust intensity of management to the targeted plant and other species communities	KEMP vzw[e], a cooperation performing grazing on demand in remnant natural areas, adjusts the type of grazer (rare sheep breeds and goats), manages in function of target plant and other species (*e.g.* the snake *Coronella austriaca*) and valorizes the output by marketing lamb meat as a local specialty
LOCAL, REGIONAL INTERNATIONAL	Local companies owning oligotrophic sites	Identify, manage, restore and protect oligotrophic habitats	2B Connect[f] is an interregional project between Flanders and Netherlands to restore and manage biodiversity on company sites
LOCAL, REGIONAL	Local companies owning meso- or eutrophic sites	Create *novel ecosystem* and manage for biodiversity	Green4Grey[g] is Life+ project investing in green and blue infrastructure on company sites
LOCAL, REGIONAL, INTERNATIONAL	Municipal legislator	Protect and restore oligotrophic habitats Re-assign oligotrophic habitats in the land-use plan to get full protection	In 2018, the city of Lommel allowed and funded a multinational company to construct 110 ha of photovoltaic panels on an unprotected heathland (Kristal Solar Park)[h]
REGIONAL	Scientists, practitioners	Demonstrate successful and failing restoration techniques to practitioners	The field experiment of HerBioGras[i] aims at comparing and demonstrating several restoration techniques for mesotrophic species-rich grasslands
INTERNATIONAL	Scientists	Develop cheap and quick techniques to screen large areas for phosphorus-poor soil conditions	Use optical remote sensing techniques to map *sites of interest,* which are phosphorus-poor (pilot study to measure P_{Olsen} [l]). Cooperation with legislators to use these results to better protect oligotrophic areas.
INTERNATIONAL	Practitioners and scientists	Sharing failures and successes in grassland restoration	Conservation evidence[j]: summarizing evidence from conservation and restoration actions
INTERNATIONAL	Nature NGO's, scientists, advisors	Innovations to valorize hay produced in species-rich grasslands	GrasGoed[k] project, an interreg project to valorize hay of low feed quality

[a] https://www.vlinderstichting.nl/idylle/
[b] http://www.bestuivers.nl/Portals/5/Publicaties/Rapport_Wijk_en_Wouden_2018_klein_compleet.pdf?ver=2018-11-06-101249-947
[c] https://www.bumblebeeconservation.org/land-management-advice/
[d] http://www.magnificentmeadows.org.uk/advice-guidance/section/how-can-i-restore-or-recreate-a-meadow
[e] http://www.kempvzw.be/ [f] https://www.2b-connect.eu [g] https://green4grey.be [h] https://www.lommel.be/project/23/zonnepark-van-110-ha-op-kristalpark
[i] https://www.hogent.be/over-hogent/vakgroepen/natuur-en-voeding/herbiogras/ [j] https://www.conservationevidence.com/
[k] https://www.grasgoed.eu/ [l] (Kawamura et al. 2011)

7.6 Knowledge gaps and future research opportunities

Although other studies have investigated **P-mining in the field** (Dodd et al. 2012; Postma et al. 2015), more **long-term field experiments on various soil types** (see Johnston et al. 2016; Bauke et al. 2018) are needed to get better insight in: *e.g.* phosphorus removal rate, which crops are most optimal to reach restoration targets, which soil textures are more relevant to use P-mining as a restoration technique. Also, little field data is available on the **costs and gains of P-mining**. To make the technique of P-mining economically feasible for farmers, it is important that the crop or hay quality is guaranteed. In fields with low soil P-concentrations in an agricultural context, P in forage will also likely be suboptimal and this can be the cause of a lower nutritional value. For forage to serve as the only feed component of the diet of high yielding dairy cows, it should not contain less than 3 mg P g^{-1} of dry biomass (Valk et al. 1999).

Little information is found for the **last phase of phytomining** in particular, when the phosphorus removal rate can become very low despite soil phosphorus concentrations above the target of 12 mg P_{Olsen} kg^{-1} (see Chapter 3). Phosphorus can also be released from organic or inorganic recalcitrant phosphorus pools (Roberts and Johnston 2015). To get complete insight in the complexity of phosphorus cycling, a thorough fractionation procedure should be executed at different points in time during phytomining (cfr. De Schrijver et al. 2012).

A third gap in knowledge about phytomining for ecological restoration is the question of which soil depth to remove phosphorus from. We showed that neither the bioavailable nor the slowly cycling phosphorus concentrations in the deeper soil layer (15-30 cm) were decreased after five years of phytomining (contrary to the significant decline in the bioavailable phosphorus concentrations in the upper 0-15 cm soil layer). Yet, the phosphorus pools in the 15-30 cm soil layer and even in the subsoil layer (> 30 cm deep) are likely to be accessible by plant roots or mycorrhiza in symbiosis with these plants (Bauke et al 2018). **It is unclear whether the phosphorus pools in these deeper soil layers should be taken into consideration when estimating the restoration potential of a field**. Phosphorus uptake from deeper soil layers can be assessed by, *e.g.* tracer experiments with labelled phosphorus (^{33}P isotophic exchange; Kruse et al. 2015).

Testing and finetuning **novel restoration techniques** such as full-inversion tillage deserves more attention, as well as further research may focus on the ecological functions carried by novel grassland ecosystems.

The development of **cheap and quick techniques to screen large areas for phosphorus-poor soil conditions** can enhance the identification, restoration and conservation of oligotrophic habitats, but requires further research. It is quicker and may be cheaper to measure the canopy reflectance *in situ* to deduce bioavailable phosphorus concentrations (Kawamura et al 2011). Another option may be to use optical remote sensing to scan large areas (Kerr and Ostrovsky 2003).

We found the vegetation of the post-fertilization grasslands in Chapters 2 and 3 was (severely) limited by potassium. However, the **effect of potassium limitation on species-richness** has been less-well researched and are less understood than nitrogen and phosphorus limitation (Kayser and Isselstein 2005; Sardans and Peñuelas 2015); and, similarly co-limitation between nitrogen, phosphorus and/or potassium requires more investigation (Fay et al. 2015). Experimentation in the field or in more controlled environments (greenhouse) may be suited to investigate this further.

In the context of climate change, **summer droughts** are more likely to occur in Europe (Kovats et al. 2014). Target oligotrophic plant species may be able to withstand higher soil phosphorus concentrations in combination with a yearly drought period due to **limited biomass production**. Such complex interactions between soil fertility and (summer) drought periods may be researched by, *e.g.* adding a drought treatment to the mesocosm experiment presented in Chapter 6.

Based on the first results from the mesocosm, we **stress further investigation of the plant-soil interactions** is needed by, *e.g.* comparing the soil biota communities in each mesocosm with the plant communities. Small changes in nutrient availability in nutrient-poor soils appeared to lead to the loss of slow-growing species specialized in surviving under severe phosphorus-limitation (Roeling et al., 2018). Such changes may be induced by specific interactions with soil biota but still needs to be revealed by further research, such as a detailed investigation of the interactions in the mesocosm experiment.

Another **much-needed innovation is the economic valorization of the hay from species-rich grasslands** or grasslands in restoration management. Before the agricultural revolution, grasslands were much-needed for farmers and were traditionally managed for hay to overwinter cattle, horses and sheep. Nowadays, the hay quality does not suffice anymore nor do species-rich grasslands fit in the modern farming systems. The incentive to restore and continue management of these ecosystems needs to be found elsewhere because traditional mowing management is expensive (see Table 1.2) as well as the cost is high to dispose of the cut hay (50 euro t^{-1} dry biomass; Compeer and Mattheij 2017). Annually, about 40 000 t dry biomass is produced in Flanders (Compeer and Mattheij 2017). Examples of such innovations are using the fibres for production of paper or insulation material (*e.g.* the GrassGoed project).

APPENDICES

Appendices Chapter 1

Appendix 1.A Key for identifying 6230 Nardus grasslands (with subtypes)

The identification key by De Saeger and Wouters (2017) distinguishes four subtypes of the species-rich *Nardus* grasslands (Natura2000 6230*):

- 6230_hn: droog, heischraal grasland; translated as dry *Nardus* grassland
- 6230_hmo: vochtig,heischraal grasland; translated as moist *Nardus* grassland
- 6230_hnk: droog, kalkrijker heischraal grasland (*Betonico-Brachypodietum*); translated as dry, calcareous *Nardus* grassland
- 6230_ha: soortenrijke graslanden van het struisgrasverbond; translated as species-rich grasslands of the *Agrostis capillaris* type

In this PhD, we focus on 6230_hn (dry *Nardus* grasslands; see Appendix-Table 1.A for the identification of this habitat).

Appendix-Table 1.A Translation of the key for identifying *Nardus* grassland types by De Saeger and Wouters (2017)

Step [a]	Question	if YES	if NO
1.	Is it an open biotope with grassy vegetation (*Poaceae* or other) and herb species more than 50% of the cover? (it is a grassland)	2.	other type
2.	Is the cover by trees and shrubs (crown area) less than 10%	3.	other type
3.	Is the grassland is situated in the polder area (and at least 2 halophyte species are present) or in the coastal and dune area?	other type	4.
4.	Cover with large Carex species, Glyceria maxima, Iris pseudacorus, Equisetum fluviatile, Thypha sp., Schoenoplectus lacustris, Schoenoplectus tabernaemontani and/or Sparganium sp. is equal to or higher than 70% OR the cover with species from the wetland group[b] (excluding *Juncus effusus*, *Phragmites australis*, *Carex acuta* en *Carex acutiformis*) is higher than the cover with other species	other type	5.
5.	Was grassland management abandoned AND are peaty species[c] abundantly present?	other type	6.
6.	Are large sedge species[d] codominantly present?	other type	7.
7.	Is the vegetation part of the forest edge or part of the edge of a watercourse, and are typical species of the *Galio-Alliarion* vegetation type dominant?	other type	8.
8.	Are nitrophilous species[e] together (co-)dominant?	other type	9.
9.	Was grassland management by mowing or grazing abandoned AND at least two ruderal species[f] are abundantly present. OR it is a ruderal habitat with at least 30% of the cover occupied by ruderal species.	other type	10.
10.	Is the cover of species typical for wet ruderal site conditions[g] higher than 30%?	other type	11.

11.	Are at least 3 calcareous species[h] occasionally present? OR is 1 calcareous species[h] frequently present?	Calcareous types including 6230_hnk	12.
12.	Is the cover with *Nardus stricta* equal to or higher than 30%?	6230, move to 14.	13.
13.	Is the soil oligotrophic and mostly acid AND are at least three species present from the *Nardus* group[i] AND are heathland species[j] present?	6230, move to 14.	other type
14.	Are multiple species indicating moist soil conditions[k] present in high frequency?	6230_hmo, see hmo-group[l]	6230_hn, see 15.
15.	Are at least four species from the *Nardus* or ha* group[m] present AND together cover more than 10%?	6230_hn	6230_hn[o]
NA	At least one species from the ha* group[m] frequently present OR at least three species from the (ha* and *Nardus*)-groups[m,i] each occasionally present	6230_ha	rbbha
NA	At least four species, each frequently present, from the ha-group[n] and or heathland species[j]	rbbha	other type

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[a] We filtered out the relevant steps for the identification of *Nardus* grasslands, consequently, the step numbers are different than in De Saeger and Wouters (2017)

[b] Marsh species: *Cardamine amara, Carex vesicaria, Juncus filiformis, Carex lasiocarpa, Cladium mariscus, Iris pseudacorus, Rorippa amphibia, Calamagrostis canescens, Carex pseudocyperus, Equisetum fluviatile, Peucedanum palustre, Epilobium palustre, Agrostis canina, Viola palustris, Galium palustre, Metha aquatica, Carex nigra, Carex curta, Eriophorum angustifolium…*

[c] Peat species: *Achillea ptarmica, Lysimachia vulgaris, Peucedanum palustre, Calamagrostis canescens, Galium uliginosum, Comarum palustre, Eriophorum angustifolium, Sphagnum sp.* and/or *Carex nigra*

[d] Large sedge species: *Juncus effusus, Juncus conglomeratus, Juncus acutiflorus, Juncus subnodulosus, Juncus inflexus*

[e] Nitrophilous species: *Urtica diocia, Galium aparine, Rumex obtusifolius, Cirsium arvense, Cirsium vulgare, Rubus sp., Arctium sp., Jacobea vulgaris subsp. vulgaris, Dactylis glomerata*

[f] Ruderal species: *Cirsium arvense, Convolvulus arvensis, Artemisia vulgaris, Tanacetum vulgare, Solidago canadensis, Calamagrostis epigeos, Conyza sp., Dipsacus fullonum, Equisetum arvensis, …*

[g] Species of wet ruderal soil conditions: *Persicaria bistorta, Valeriana officinalis, Angelica sylvestris, Lythrum salicaria …*

[h] Calcareous species: *Lathyrus tuberosus, Briza media, Cirsium acaule, Ophrys apifera, Silene vulgaris, Himantoglossum hircinum, Cinopodium vulgare, Helianthemum nummularium, Tragopogon pratensis subsp. pratensis, Brachypodium pinnatum, Agrimonia eupatoria, Trisetum flavescens, Centaurea scabiosa, Gymnadenia conopsea, Sanguisorba officinalis, Primula veris, Anacamptis morio, Pulicaria dysenterica, Origanum vulgare…*

[i] *Nardus* species: *Succisa pratensis, Carex panicea, Viola riviniana, Carex pallescens, Nardus stricta, Hieracium vulgatum, Solidago virgaurea, Festuca filiformis, Hypericum pulchrum, Ulex europaeus, Botrychium lunaria, Dactylorhiza maculata subsp. maculata, Pedicularis sylvatica, Viola canina, Thymus serpyllum, Gentiana pneumonanthe, Lathyrus linifolius, Potentilla anglica, Galium saxatile, Polygala serpyllifolia, Veronica officinalis, Hieracium lactucella, Genista anglica, Hieracium laevigatum, Euphrasia stricta, Danthonia decumbens, Potentilla erecta, Juncus squarrosus, Carex binervis, Luzula multiflora, Serratula tinctoria, Platanthera bifolia*

[j] Heathland species: *Calluna vulgaris, Erica cinerea, Erica tetralix, Juncus squarrosus, Carex pilulifera, Carex arenaria, Agrostis vinealis, Genista anglica, Genista pilosa, Cytisus scoparius, Vaccinium vitis-idaea, Vaccinium myrtillus, Trichophorum cespitosum subsp. germanicum, Deschampsia flexuosa, Ulex europaeus*

[k] Species of moist soil conditions: *Erica tetralix, Pedicularis sylvatica, Dactylorhiza maculata subsp. maculata, Gentiana pneumonanthe, Carex nigra, Carex panicea, Carex oederi subsp. oedocarpa, Agrostis canina, Hydrocotyle vulgaris, Juncus acutiflorus,…*

[l] hmo species: *Succisa pratensis, Carex panicea, Carex pallescens, Nardus stricta, Carex oederi subsp. oedocarpa, Dactylorhiza maculata subsp. maculata, Pedicularis sylvatica, Gentiana pneumonanthe, Danthonia decumbens, Juncus squarrosus, Carex binervis, Luzula multiflora, Platanthera bifolia, Serratula tinctoria*

[m] ha* species: *Gnaphalium sylvaticum, Hieracium vulgatum, Filago minima, Vulpia bromoides, Galium verum, Campanula rotundifolia, Thymus serpyllum, Dianthus deltoides, Potentilla argentea, Jasoine Montana*

[n] ha species: *Cerastium arvense, Achillea millefolium, Luzula campestris, Hypochaeris radicata, Agrostis capillaris, Trifolium arvense, Ornithopus perpusillus, Trifolium dubium, Leontodon saxatilis, Ranunculus bulbosus, Hieracium pilosella, Rumex acetosella, Plantago lanceolata, Aira praecox, Aira caryphyllea*

[o] *indicates the habitat has deteriorated or is weakly developed*

Appendix 1.B Species list oligotrophic, mesotrophic and eutrophic grasslands

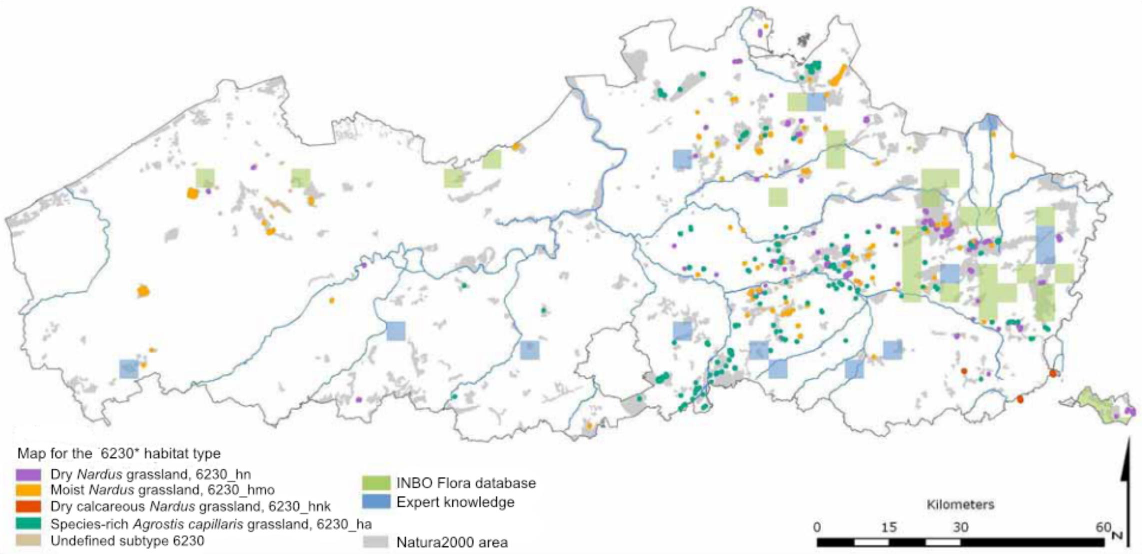
Appendix-Table 1.B Selected plant species (see Van Daele et al. 2017). Species selected for the mesocosm experiment in Chapter 6 are underlined

Oligotrophic species	**Mesotrophic species**		**Eutrophic species**
Arnica montana	*Achillea millefolium*	*Leontodon autumnalis*	*Artemisia vulgaris*
Briza media	*Achillea ptarmica*	*Leontodon hispidus*	*Bellis perennis*
Calluna vulgaris	*Agrostis capillaris*	*Leucanthemum vulgare*	*Dactylis glomerata*
Carex echinata	*Agrostis stolonifera*	*Linaria vulgaris*	*Holcus lanatus*
Carlina vulgaris	*Ajuga reptans*	*Lychnis flos-cuculi*	*Lolium perenne*
Deschampsia flexuosa	*Alopecurus geniculatus*	*Lysimachia vulgaris*	*Phalaris arundinacea*
Festuca ovina	*Alopecurus pratensis*	*Lythrum salicaria*	*Phleum pratense*
Hypochaeris radicata	*Angelica sylvestris*	*Myosotis scorpioides*	*Ranunculus acris*
Jasione montana	*Anthoxanthum odoratum*	*Plantago lanceolata*	*Ranunculus repens*
Koeleria macrantha	*Campanula rotundifolia*	*Poa pratensis*	*Taraxacum officinale*
Luzula campestris	*Cardamine pratensis*	*Prunella vulgaris*	
Molinia caerulea	*Centaurea jacea*	*Ranunculus bulbosus*	
Nardus stricta	*Cirsium palustre*	*Rumex acetosa*	
Pimpinella saxifraga	*Crepis biennis*	*Rumex acetosella*	
Potentilla erecta	*Crepis capillaris*	*Saxifraga granulata*	
Solidago virgaurea	*Cynosurus cristatus*	*Scirpus sylvaticus*	
Stachys officinalis	*Daucus carota*	*Silene dioica*	
Succisa pratensis	*Festuca rubra*	*Stellaria graminea*	
Veronica officinalis	*Galium mollugo*	*Tanacetum vulgare*	
	Galium verum	*Thalictrum flavum*	
	Hypericum perforatum	*Veronica chamaedrys*	
	Hypericum tetrapterum		

Appendix 1.C List of specific Nature targets for Nardus grasslands in Flanders

In 2007, 250 – 350 ha of Flanders were reported as *Nardus* grasslands (Natura 2000 type 6230*) and it was targeted to restore another 600 ha of *Nardus* grasslands (Appendix-Fig. 1.C; Paelinckx et al. 2009). Within the European Natura 2000 framework, the aim was formulated to keep the protected habitats (including their typical species) in an optimal state and to restore them if necessary.

In 2014, the Flemish government approved a decree with two goals: (i) to keep 377 ha of remnant *Nardus* grasslands in an optimal condition by *in situ* improving the quality of these grasslands, and (ii) to restore an additional 637 ha of *Nardus* grasslands (Appendix-Table 1.C). However, only vague descriptions were given of how this ambitious goal should be reached. The restoration of probably only a minority of these *additional grasslands to restore* will take place on phosphorus-poor soils: on abandoned *Nardus* grassland that became forest (1 ha) and on forests on non-agricultural land (121 ha). More than a quarter (174 ha) is planned to take place on sites with a fertilization history: on arable land and post-fertilization grasslands. For half of the *additional grasslands to restore* (334 ha), there is no clear indication of where this restoration will take place. Only for five of the 43 projects it is mentioned to work towards abiotic soil targets; mowing and grazing is mentioned in three of these projects; and, topsoil removal is mentioned in one project. Several projects (7) state to work on the biotic bottleneck. And for some projects (4), other target species than plant species were listed, *e.g.* butterflies, reptiles, crickets, birds, bats.



Appendix-Figure 1.C Distribution of remnant *Nardus* grasslands (Natura2000 habitat type 6230*) based on the occurrence of typical species in Flanders (Paelinckx et al. 2009)

Appendix-Table 1.C Specific nature targets for species-rich *Nardus* grasslands (Natura2000 6230*) in Flanders (Vlaamse Overheid, *n.d.*)

Region	Code	Area remnant *Nardus* grasslands (ha)	Quality target remnant	Area *Nardus* grasslands to restore (ha)	Previous landuse or habitat type not specified (ha)	From abandoned *Nardus* grassland (ha)	From forest (ha)	From heathland (ha)	From arable field (ha)	From post-fertilization grassland (ha)
Bos en Hei (Malle)	638	22	equal	15	0		8	0	8	0
Caestert	406	2	enhance	10	0		0	0	0	10
De Maten	908	5	enhance	23	0		12	0	0	12
Demervallei	745	8	enhance	82	82		0	0	0	0
Dijlevallei	724	3	enhance	10	10		0	0	0	0
Grote Nete	657	8	enhance	23	0		23	0	0	0
Hallerbos Brabantse heide	901	6	enhance	18	18		0	0	0	0
Haspengouw	416	2	enhance	7	7		3	0	0	0
Haspengouw (Vinne, Zoutleeuw)	417	5	enhance	6	6		0	0	0	0
Jekervallei	452	<0.5	enhance	1	1		0	0	0	0
Jekervallei 2	453	<0.5	enhance	1	1		0	0	0	0
Kleine Nete	828	1	enhance	6	6		0	0	0	0
Kleine Nete	851	2	enhance	6	0		3	3	0	0
Mangelbeek	687	2	enhance	5	5		0	0	0	0
Mangelbeek	688	20	enhance	7	0	7	0	0	0	7
Mechelse Heide	952	1	enhance	1	1		0	0	0	0
Mechelse Heide	953	10	enhance	6	0		3	0	0	3
Noord-Oost Limburg	672	9	enhance	16	0		0	0	0	16
Overgang Kempen Haspengouw	478	<0.5	enhance	2	0		2	0	0	0
Overgang Kempen Haspengouw	479	0	enhance	4	0		0	0	0	4
Overgang Kempen Haspengouw	480	<0.5	enhance	0	0		0	0	0	0
Overgang Kempen Haspengouw	481	<0.5	enhance	1	1		0	0	0	0

Schietvelden	624	13	enhance	9	0	0	0	0	9
Turnhouts Vennengebied	803	53	enhance	99	0	33	0	33	33
Valleigebied Kampenhout	531	<0.5	enhance	0	0	0	0	0	0
Vijvergebied Midden-Limburg	708	<0.5	enhance	13	13	0	0	0	0
Vijvergebied Midden-Limburg	709	1	enhance	15	0	0	0	0	15
Vlaamse Ardennen	545	<0.5	enhance	3	3	0	0	0	0
Voerstreek	439	2	enhance	15	15	0	0	0	0
Wingevallei	499-500	11	enhance	54	0	27	0	0	27
Zandig Vlaandere Oost	603	8	enhance	70	70	0	0	0	0
Zandig Vlaandere West	588	55	enhance	40	40	0	0	0	0
Zandleemstreek	560	1	enhance	0	0	0	0	0	0
Zeeschelde (sigma)	938	1	equal	0	0	0	0	0	0
Zonienwoud	512	8	enhance	29	29	0	0	0	0
Zonienwoud	519	1	enhance	9	0	9	0	0	0
Zwarte beek	855	25	enhance	15	15	0	0	0	0
Zwarte beek	856	24	enhance	0	0	0	0	0	0
Zwarte beek	871	1	enhance	5	5	0	0	0	0
Zwarte beek	874	66	enhance	6	0	0	0	0	0
Zwarte beek	888	0	enhance	7	7	0	0	0	0
TOTAL (ha)		377		637	334	121	3	40	135

Appendices Chapter 2

Appendix 2.A: Typical Nardus grassland species

Appendix-Table 2.A List with typical species in dry acidophilous lowland Atlantic *Nardus* grasslands within European habitat type 6230 (T'Jollyn et al. 2009)

Scientific name
Agrostis vinealis
Botrychium lunaria
Carex pilulifera
Danthonia decumbens
Euphrasia stricta
Festuca ovina
Galium saxatile
Genista anglica
Gnaphalium sylvaticum
Hieracium lactucella
Hieracium laevigatum
Lathyrus linifolius
Luzula multiflora
Nardus stricta
Polygala vulgaris
Potentilla erecta
Veronica officinalis
Viola canina

Appendix 2.B: Summary of the seven post-agricultural grasslands

Appendix-Table 2.B Location and soil characteristics of the seven post-fertilization grasslands (mean (minimum – maximum))

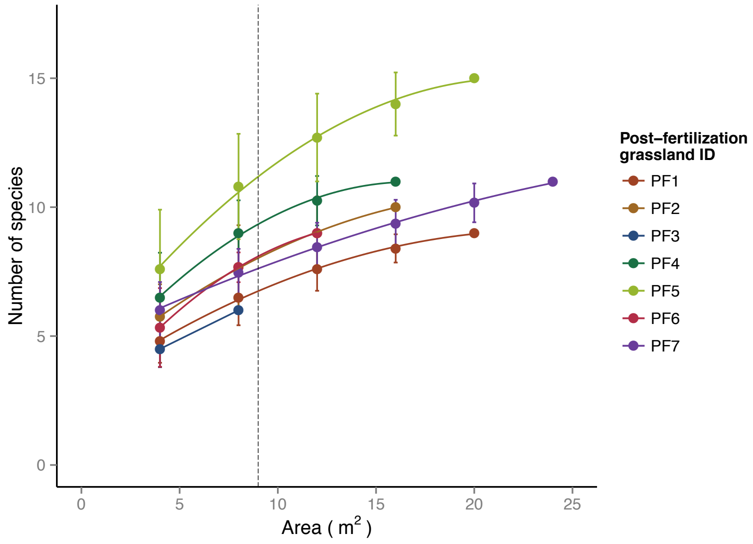
Grassland identity	Nature reserve	Coordinates	Time since fertilization (year)	Number of plots per grassland	Bioavailable P (mg P_{Olsen} kg^{-1})	Slowly cycling P (mg $P_{Oxalate}$ kg^{-1})	pH_{H2O}
PF1	Landschap de Liereman	51°20'24.1''N 5°1'14.8''E	21	5	25*	70*	5.5 (5.5 – 5.5)
PF2	Landschap de Liereman	51°20'7.6''N 5°1'2.3''E	24	4	54 (41 - 82)	166 (119 - 194)	5.5 (5.3 – 5.9)
PF3	Landschap de Liereman	51°20'4.9''N 5°1'13.0''E	25	2	43 (22 - 64)	111 (50 - 172)	4.8 (4.6 – 5.0)
PF4	Landschap de Liereman	51°19'46.2''N 5°2'0.2''E	16	4	114 (94 - 157)	334 (238 - 410)	5.6 (5.2 – 5.8)
PF5	Turnhouts Vennengebied	51°21'48.1''N 4°57'1.4''E	23	5	56 (41 - 87)	178 (146 - 240)	5.8 (5.5 – 6.1)
PF6	Turnhouts Vennengebied	51°21'39.7''N 4°55'25.4''E	17	3	35 (27 - 44)	132 (90 - 162)	5.9 (5.8 – 6.0)
PF7	Turnhouts Vennengebied	51°22'3.6'' N 4°57'44.9''E	23	6	46 (32 - 58)	120 (78 - 180)	5.7 (5.2 – 6.4)

*In one grassland (PF1), the soil sampling strategy was different: a composited sample was taken over the whole grassland (n=4) instead of one sample in each plot

Appendix 2.C: Rarefaction of the number of vascular plant species in post-fertilization grasslands

We are combining two datasets, one including the remnant *Nardus* grasslands and one including the post-fertilization grasslands. The methodology in these vegetation surveys was different: the size of the remnant *Nardus* grassland quadrats was 9 m^2 (3 m x 3 m) and one quadrat was positioned in each of 34 grasslands; while, the size of the quadrats in the post-fertilization grasslands was 4 m^2 (2 m x 2 m) but for each of seven grasslands, two to six quadrats were inventoried, depending on the size of the grassland.

Constructing sample-based rarefaction curves allows for comparing the number of species between the remnant grasslands and post-fertilization grasslands despite the difference in quadrat sizes (Gotelli and Colwell 2001). Therefore, we constructed these curves for the post-fertilization grasslands, plotted as function of sample size (Appendix-Fig. 2.C). All possible combinations between quadrats, within each of the seven grasslands, were made to calculate the mean species number for 8 m^2, 12 m^2, 16 m^2, 20 m^2 and 24 m^{2}plots. Then, we fitted second order linear models and calculated the number of species in 9 m^2 quadrats from these curves (Appendix-Table 2.C).



Appendix-Figure 2.C Rarefaction curves for the seven post-fertilization grasslands. The filled circles show the mean number of species and the flags show the standard error obtained by combining the vegetation surveys from two to six quadrats in each of the grasslands. The curves were fitted by second order linear models (see Appendix-Table 2.C)

Appendix-Table 2.C Fitted rarefaction curves, and their adjusted R-squared and *p*-values, for the seven post-fertilization grasslands according to second order linear models ($y = a + bx + cx^2$)

Grassland identity	Fitted value for *a*	Fitted value for *b*	Fitted value for *c*	Adjusted R^2	*p*-value	Estimated number of species for 9 m^2
PF1	2.920	0.525	-0.011	0.997	0.001	6.7
PF2	3.438	0.639	-0.014	0.999	0.018	8.0
PF3	3.000	0.375	0	*	*	6.4
PF4	3.313	0.916	-0.027	0.993	0.049	9.3
PF5	4.120	0.986	-0.022	0.994	0.003	11.2
PF6	2.000	0.958	-0.031	*	*	8.1
PF7	4.696	0.364	-0.004	0.996	0.0001	7.6

In the last column, the estimates for the number of vascular plant species are given for an area of 9 m^2

*The R^2 and *p*-values for PF3 and PF6 were not calculated because there were too few quadrats in these grasslands

Appendices Chapter 3

Appendix 3.A: Biomass sampling dates

Appendix-Table 3.A Biomass sampling dates for the three fields in Landschap De Liereman and the field in Vloethemveld

Year	Harvest	Liereman
2011	1	02/08
2012	1	15/05
	2	21/09
2013	1	25/05
	2	13/08
	3	23/09
2014	1	12/06
	2	10/10
2015	1	21/05
	2	22/10
2016	1	29/06
	2	21/10

Appendix 3.B: Species composition in 2011 and 2017

Appendix-Table 3.B Vegetation composition in July 2017 in mowing and P-mining plots: mean cover in %; minimal and maximal values are shown between brackets. Cover by grass species is indicated by "G"; cover by forb species by "F" and cover by moss species "M"

	Species	Type	Occurrence in Seed mix (%)	Mowing (%)	P-mining (%)
Sown species-mix in 2011	*Festuca pratensis*	G	5	0.4 (0-5)	0.4 (0-5)
	Lolium perenne	G	77	0.2 (0-1)	9 (0-80)
	Phleum pratensis	G	10	16 (0-40)	51 (5-90)
	Poa pratensis	G	3	0	0
	Trifolium repens	F	5	2 (0-10)	4 (0-25)
Spontaneous species found in 2017	*Achillea millefolium*	F		2 (0-25)	0
	Agrostis capillaris	G		36 (0-50)	18 (0-55)
	Agrostis stolonifera	G		9 (0-50)	9 (0-35)
	Anthoxanthum odoratum	G		10 (0-40)	3 (0-25)
	Arrhenatherum elatius	G		0	1 (0-15)
	Betula pendula	F		0.2 (0-1)	0
	Bromus hordeaceus	G		0.1 (0-1)	0
	Cardamine pratensis	F		0.1 (0-1)	0.4 (0-5)
	Carex ovalis	G		0.9 (0-5)	0.7 (0-7)
	Cerastium fontanum	F		7 (1-25)	3 (0-20)
	Cirsium arvense	F		0.5 (0-5)	11 (0-55)
	Cirsium palustre	F		0.7 (0-7)	1 (0-10)
	Conyza canadensis	F		0	0.1 (0-1)
	Crepis capillaris	F		2 (0-7)	0.5 (0-5)
	Dactylis glomerata	G		0	4 (0-30)
	Holcus lanatus	G		28 (10-60)	35 (10-60)
	Jacobaea vulgaris	F		5 (0-40)	3 (0-20)
	Juncus bufonius	G		0.8 (0-10)	0
	Juncus effusus	G		2 (0-10)	0.1 (0-1)
	Leontodon autumnalis	F		7 (0-20)	2 (0-15)
	Lotus pedunculatus	F		4 (0-25)	1 (0-7)
	Luzula campestris	F		3 (0-25)	0.1 (0-1)
	Pinus sylvestris	F		0.1 (0-1)	0
	Plantago lanceolata	F		0	0.1 (0-1)
	Plantago major	F		0.1 (0-1)	0
	Quercus robur	F		0.1 (0-1)	0
	Ranunculus repens	F		21 (0-50)	8 (0-20)
	Rhytidiadelphus squarrosus	M		19 (0-90)	0
	Rumex acetosa	F		0	0.3 (0-1)
	Rumex acetosella	F		7 (0-30)	0.2 (0-1)
	Rumex obtusifolius	F		0	1 (0-7)
	Salix caprea	F		0.2 (0-1)	0
	Sonchus oleraceus	F		1 (0-7)	0
	Taraxacum officinale	F		5 (0-25)	7 (0-20)
	Trifolium dubium	F		0.1 (0-1)	0
	Urtica dioica	F		0.1 (0-1)	2 (0-25)
	Veronica arvensis	F		0.1 (0-1)	0.2 (0-1)

Appendix 3.C: Model selection and optimal models

Appendix-Table 3.C.1 Model selection of the relationships between annual biomass properties in 2012-2016 and the restoration technique and soil phosphorus concentration at the start of the experiment ($P_{Olsen\text{-}2011}$)[a]. Only competitive models with ΔAIC_c of less than two are shown (Burnham and Anderson, 2002). In case of multiple suitable models according to ΔAIC_c, models were compared with ANOVA tests. The selected optimal models are shown in bold

Response Variable	Predictor Variables in Model	df	AIC_c	ΔAIC_c	*w*[b]	*p*-value[c]
Phosphorus removal with biomass	Restoration technique + $P_{Olsen\text{-}2011}$ + Restoration technique : $P_{Olsen\text{-}2011}$ + $P_{Olsen\text{-}2011}^2$ + Restoration technique : $P_{Olsen\text{-}2011}^2$	12	687	0	0.40	*ns*
	Restoration technique + $P_{Olsen\text{-}2011}$ + Restoration technique : $P_{Olsen\text{-}2011}$ + $P_{Olsen\text{-}2011}^2$	11	688	0.3	0.34	
	Restoration technique + $P_{Olsen\text{-}2011}$ + Restoration technique : $P_{Olsen\text{-}2011}$	**10**	**688**	**1.0**	**0.24**	
Biomass[d]	**Restoration technique + $P_{Olsen\text{-}2011}$**	**9**	**96**	**0**	**0.48**	*
	Restoration technique	8	98	1.7	0.21	
Phosphorus concentration in biomass[e]	**Restoration technique + $P_{Olsen\text{-}2011}$ + Restoration technique : $P_{Olsen\text{-}2011}$ + $P_{Olsen\text{-}2011}^2$**	**11**	**-94**	**0**	**0.59**	*ns*
	Restoration technique + $P_{Olsen\text{-}2011}$ + Restoration technique : $P_{Olsen\text{-}2011}$ + $P_{Olsen\text{-}2011}^2$ + Restoration technique : $P_{Olsen\text{-}2011}^2$	12	-93	1.4	0.29	

[a] The full models had the following form: response variable ~ restoration technique x $P_{Olsen\text{-}2011}$ + restoration technique x $P_{Olsen\text{-}2011}^2$. We used *plot* nested within *field* nested within *year* as random factors

[b] *w*: the Akaike weight indicates the probability that the model is the best model of the set of models tested

[c] * indicates a significant difference with $p < 0.05$; *ns* indicates no significant difference between the models

[d] Biomass was square root transformed

[e] Phosphorus concentration in biomass was log transformed

Appendix-Table 3.C.2 The optimal models for annual biomass yield (t ha^{-1}), mean phosphorus concentration in the biomass (mg kg^{-1}) and phosphorus removal with biomass (kg ha^{-1}), for 2012-2016, including the restoration technique treatment and the soil phosphorus concentration at the start of the experiment ($P_{Olsen-2011}$)[a]

Model	Estimat	Standard	χ^2	df	*p*-value[b]	R^2_m [c]	R^2_c
Biomass[d]				1,10		67%	97
Intercept	1.5	0.1	40		***		
Restoration technique	1.0	0.06	34		***		
$P_{Olsen-2011}$	0.002	0.001	5		*		
P concentration in biomass[e]				1,10		46%	80
Intercept	0.6	0.09	29		***		
Restoration technique	-0.3	0.06	33		***		
$P_{Olsen-2011}$	0.01	0.002	80		***		
$P_{Olsen-2011}^2$	-	0.00001	23		***		
Restoration technique : $P_{Olsen-2011}$	0.002	0.0007	6		*		
Phosphorus removal with				1,10		66%	96
Intercept	4.8	1.6	81		***		
Restoration technique	5.0	1.2	19		***		
$P_{Olsen-2011}$	0.05	0.01	61		***		
Restoration technique : $P_{Olsen-2011}$	0.06	0.02	11		**		

[a] The full models had the following form: response variable ~ restoration technique x $P_{Olsen-2011}$ + restoration technique x $P_{Olsen-2011}^2$. We used *plot* nested within *field* nested within *year* as random factors (see Supplementary Table S3 and Fig. S4)

[b] Significance of effects is indicated by *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

[c] The percentage of variance explained by the fixed effects (R^2_m) and the full model, including the random effects year, field and plot (R^2_c) are shown for each model

[d] Biomass was square root transformed [e] Phosphorus concentration in biomass was log transformed

Appendix-Table 3.C.3 Model selection of the relationships between the nutrient indices for phosphorus (PNI), nitrogen (NNI) and potassium (KNI), including the restoration technique treatment and the soil phosphorus concentration at the start of the experiment ($P_{Olsen-2011}$)[a]. Only competitive models with ΔAIC_c of less than two are shown (Burnham and Anderson, 2002). In case of multiple suitable models according to ΔAIC_c, models were compared with ANOVA tests. The selected optimal models are shown in bold

Response Variable	Predictor Variables in Model	df	AIC$_c$	ΔAIC$_c$	*w*[b]	*p*-value[c]
PNI[d]						
	Restoration technique + $P_{Olsen-2011}$ + Restoration technique : $P_{Olsen-2011}$ + $P_{Olsen-2011}^2$ + Restoration technique : $P_{Olsen-2011}^2$	**12**	**-123**	**0**	**0.87**	
NNI						
	Restoration technique + $P_{Olsen-2011}$	9	842	0	0.35	*ns*
	Restoration technique	**8**	**842**	**0.7**	**0.26**	
KNI						
	Restoration technique + $P_{Olsen-2011}$ + Restoration technique : $P_{Olsen-2011}$ + $P_{Olsen-2011}^2$	**11**	**884**	**0**	**0.74**	

[a] The full models had the following form: response variable ~ restoration technique x $P_{Olsen-2011}$ + restoration technique x $P_{Olsen-2011}^2$. We used *plot* nested within *field* nested within *year* as random factors

[b] *w*: the Akaike weight indicates the probability that the model is the best model of the set of models tested

[c] *ns* indicates no significant difference between the models

[d] PNI was log transformed

Appendix-Table 3.C.4 The optimal models for nutrient indices for phosphorus (PNI), nitrogen (NNI) and potassium (KNI) in May-June for 2012-2016, including the restoration technique treatment and the soil phosphorus concentration at the start of the experiment ($P_{Olsen\text{-}2011}$)[a]

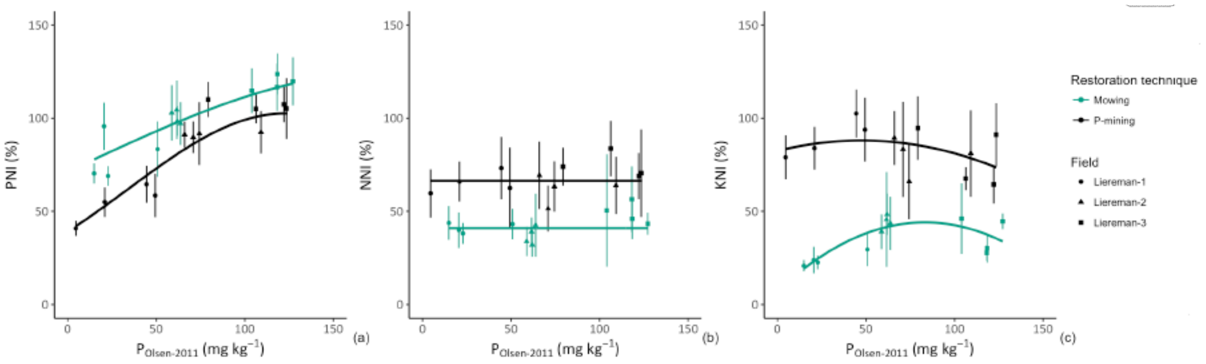
Model		Estimate	Standard error	χ^2	df	p-value[b]	R^2_m [c]	R^2_c [c]
PNI[d]					1,87		63%	75%
	Intercept	4	0.09	16809		***		
	Restoration technique	-0.6	0.1	62		***		
	$P_{Olsen\text{-}2011}$	0.006	0.002	105		***		
	$P_{Olsen\text{-}2011}^2$	-0.00002	0.00001	16		***		
	Restoration technique : $P_{Olsen\text{-}2011}$	0.009	0.003	16		***		
	Restoration technique : $P_{Olsen\text{-}2011}^2$	-0.00005	0.00002	7		**		
NNI					1,91		47%	96%
	Intercept	41	5	131		***		
	Restoration technique	25	2	171		***		
KNI					1,88		83%	99%
	Intercept	9	5	258		***		
	Restoration technique	71	4	600		***		
	$P_{Olsen\text{-}2011}$	0.7	0.1	3		.		
	$P_{Olsen\text{-}2011}^2$	-0.004	0.0009	17		***		
	Restoration technique : $P_{Olsen\text{-}2011}$	-0.3	0.06	24		***		

[a] The full models had the following form: response variable ~ restoration technique x $P_{Olsen\text{-}2011}$ + restoration technique x $P_{Olsen\text{-}2011}^2$. We used *plot* nested within *field* nested within *year* as random factors

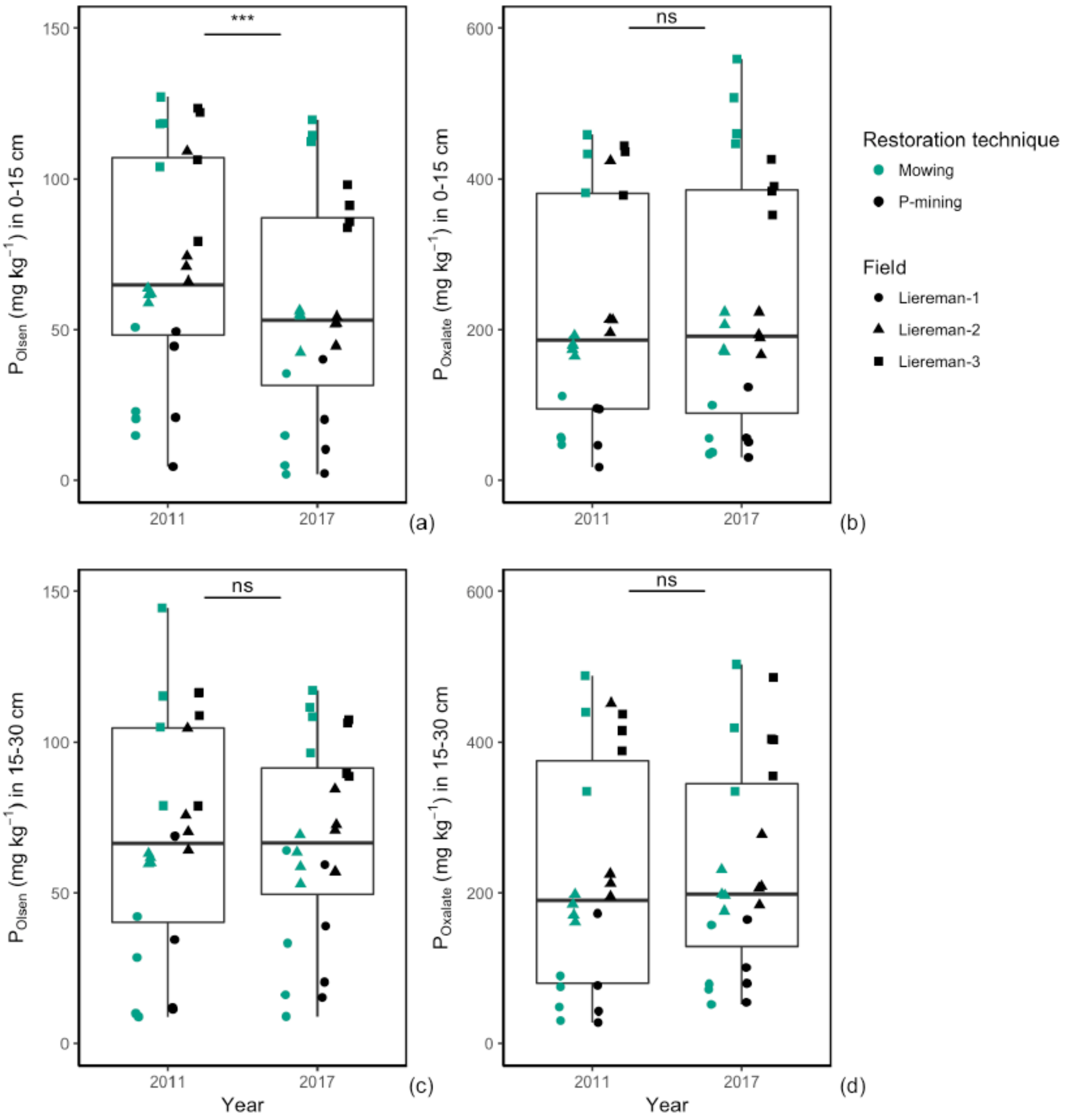
[b] Significance of effects is indicated by *** $p < 0.001$; ** $p < 0.01$; . $p < 0.1$.

[c] The percentage of variance explained by the fixed effects (R^2_m) and the full model, including the random effects year, field and plot (R^2_c) are shown for each model

[d] PNI was log transformed



Appendix-Figure 3.C.1 The nutrient induces for phosphorus (PNI; a), nitrogen (NNI; b) and potassium (KNI; c) in biomass harvested in May or June plotted versus $P_{Olsen\text{-}2011}$ (mg kg^{-1}) for the 24 plots. Mean and standard deviation for 2012-2016 are shown. The regression lines show the optimal linear mixed effects model fitted to the 120 data points



Appendix-Fig. 3.C.2 The concentration of P_{Olsen} (a, c; n=24) and $P_{Oxalate}$ (b, d; n=22) in the 0-15 cm (a, b) and 15-30 cm (c, d) soil layers of the mowing and P-mining plots before and after the experiment (2011, 2017). Significance of effects is indicated by *** p < 0.001; ns means the difference was not significant

Appendix-Table 3.C.5 Model selection of the relationships between the change in P_{Olsen} and $P_{Oxalate}$ concentrations between 2011 and 2017 in the 0-15 cm and 15-30 cm soil layers, including the restoration technique treatment and the soil phosphorus concentration at the start of the experiment ($P_{Olsen-2011}$)[a]. Only competitive models with ΔAIC_c of less than two are shown (Burnham and Anderson, 2002). In case of multiple suitable models according to ΔAIC_c, models were compared with ANOVA tests. The selected optimal models are shown in bold

Response Variable	Predictor Variables in Model	df	AIC_c	ΔAIC_c	w[b]
Change in 0-15 cm P_{Olsen} concentration	Restoration technique + $P_{Olsen-2011}$ + Restoration technique : $P_{Olsen-2011}$	8	198	0	0.83
Change in 0-15 cm $P_{Oxalate}$ concentration	null model	5	250	0	0.51
Change in 15-30 cm P_{Olsen} concentration	$P_{Olsen-2011}$	6	203	0	0.75
Change in 15-30 cm $P_{Oxalate}$ concentration	$P_{Olsen-2011}$	6	243	0	0.69

[a] The models had the following form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ ~ restoration technique x $P_{Olsen-2011}$. We used *field* as a random factor

[b] *w*: the Akaike weight indicates the probability that the model is the best model of the set of models tested

Appendix-Table 3.C.6 The optimal models for the change in P_{Olsen} and $P_{Oxalate}$ concentrations (mg kg^{-1}) between 2011 and 2017 in the 0-15 cm soil layer, including the restoration technique treatment and the soil phosphorus concentration at the start of the experiment ($P_{Olsen-2011}$)[a]

Model	Estimate	Standard	χ^2	df	*p*-value[b]	R^2_m[c]	R^2_c[c]
Change in P_{Olsen} concentration				1,18		40%	88%
Intercept	**-28**	18	1		ns		
Restoration technique	**0.5**	0.09	68		***		
$P_{Olsen-2011}$	**-10**	8	44		***		
Restoration technique : $P_{Olsen-2011}$	**0.3**	0.08	10		**		
Change in $P_{Oxalate}$ concentration				1,19		0%	0%
Intercept	**7**	10	0.4		ns		

[a] The models had the following form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ ~ restoration technique x $P_{Olsen-2011}$. We used *field* as a random factor (see Supplementary Table S6)

[b] Significance of effects is indicated by *** $p < 0.001$; ** $p < 0.01$; *ns*, not significant

[c] The percentage of variance explained by the fixed effects (R^2_m) and the full model, including the random effects field and plot (R^2_c) are shown for each model

Appendix-Table 3.C.7 The model for the change in P_{Olsen} versus P_{Ox} concentration (mg kg^{-1}) in the 0-15 cm soil layer between 2011 and 2017 (n = 22)[a]

Model	Estimate	Standard error	χ^2	df	*p*-value[b]	R^2_m[c]	R^2_c[c]
Change in $P_{Oxalate}$ concentration				1,18		76%	76%
Intercept	-39	9	3		ns		
Change in P_{Olsen} concentration	3	0.4	66		***		

[a] The model had the following form: $\Delta P_{Oxalate}$ ~ ΔP_{Olsen}. We used *field* as a random factor

[b] Significant effects are indicated by *** $p < 0.001$. *ns* indicates no significant difference between the models

[c] The percentage of variance explained by the fixed effects (R^2_m) and the full model, including the random effects field and plot (R^2_c) are shown for each model

Appendix-Table 3.C.8 Model selection of the relationships between the change in P_{Olsen} and $P_{Oxalate}$ concentrations between 2011 and 2017 in the 0-15 cm soil layer, including the restoration technique treatment and the cumulative phosphorus removal with biomass[a]. Only competitive models with ΔAIC_c of less than two are shown (Burnham and Anderson 2002). In case of multiple suitable models according to ΔAIC_c, models were compared with ANOVA tests. The selected optimal models are shown in bold

Response Variable	Predictor Variables in Model	df	AIC_c	ΔAIC_c	*w*[b]	*p*-value[c]
Change in P_{Olsen} stock	Restoration technique	4	245	0	0.37	*ns* .
	Cumulative P removal	4	245	0.4	0.29	.
	null model	3	246	0.8	0.24	
Change in $P_{Oxalate}$ stock	**Cumulative P removal**	4	282	0	0.54	

[a] The models had the following form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ ~ restoration technique x cumulative P removal. We used *field* as a random factor

[b] *w*: the Akaike weight indicates the probability that the model is the best model of the set of models tested

[c] Significant effects are indicated by . $p < 0.1$. *ns* indicates no significant difference between the models

Appendix-Table 3.C.9 The optimal models for the change in P_{Olsen} and $P_{Oxalate}$ stocks (kg ha^{-1}) between 2011 and 2017 in the 0-15 cm soil layer, including the restoration technique treatment and cumulative phosphorus removal with biomass[a]

Model	Estimate	Standard error	χ^2	df	*p*-value[b]	R^2_m [c]	R^2_c [c]
Change in P_{Olsen} stock				1,20		15%	24%
Intercept	-1.2	19	12		**		
cumulative $P_{removal}$	0.44	0.2	4		.		
Change in P_{Olsen} stock				1,20		13%	15%
Intercept	19	10	20		***		
Restoration technique	26	14	4		.		
Change in $P_{Oxalate}$ stock				1,18		28%	33%
Intercept	-155	71	0.6		ns		
cumulative $P_{removal}$	2.5	0.9	8		*		

[a] The models had the following form: ΔP_{Olsen} or $\Delta P_{Oxalate}$ ~ restoration technique x cumulative P removal. We used *field* as a random factor

[b] Significant effects are indicated by ** $p < 0.01$; * $p < 0.05$; $p < 0.1$. *ns* indicates no significant difference between the models

[c] The percentage of variance explained by the fixed effects (R^2_m) and the full model, including the random effects field and plot (R^2_c) are shown for each model

Appendices Chapter 4

Appendix 4.A Nitrogen and potassium fertilization

To avoid limitation effects by nutrients other than P on plant growth, soils were fertilized three times during the pot experiment (Appendix-Table 4.A). We followed the recommendations by the Soil Service of Belgium (Bodemkundige Dienst). During the pot experiment we recovered about 50 – 70% of the added N and 50 – 65% of the added K with four grass clippings.

Appendix-Table 4.A Fertilization rate during the main pot experiment (total duration 123 days)

	Dose per pot (1.4 kg soil, 15 cm diameter)	**Converted to dose per ha**
First fertilization: prior to filling the pots, N as NH_4NO_3 and K as K_2O	76 mg N pot^{-1} 55 mg K pot^{-1}	43 kg N ha^{-1} 37 kg K_2O ha^{-1}
Second fertilization: day 44, N and K as NH_4NO_3 and KNO_3	210 mg N pot^{-1} 315 mg K pot^{-1}	119 kg N ha^{-1} 215 kg K_2O ha^{-1}
Third fertilization: day 95, N and K as NH_4NO_3 and KNO_3	65 mg N pot^{-1} 98 mg K pot^{-1}	37 kg N ha^{-1} 67 kg K_2O ha^{-1}
TOTAL fertilization (in three doses)	351 mg N pot^{-1} 468 mg K pot^{-1}	198 kg N ha^{-1} 319 kg K_2O ha^{-1}

Appendix 4.B Additional pot experiment for testing the survival of added phosphate-solubilizing bacteria (PSB) in the three soil P-concentrations

To determine the prevalence of the phosphate-solubilizing bacterial inoculations, we tried to assess the three species (*Bacillus brevis, Pseudomonas putida and P. corrugata*) added to the pots in an extra experiment. The wild type strain was first made rifampicin (Rif) resistant, we only succeeded to do this with *P. corrugata*: a spontaneous mutant resistant of *P. corrugata* to rifampicin (Rif) was obtained by transferring an aliquot of a LBbroth culture into LBbroth with 100 mg $liter^{-1}$ Rif, incubating the culture at 30°C and isolating a Rif^R clone after growth. Subsequently, this clone was marked with the *rfp*-gene. The insertion of the mini-Tn5- PA1-04/03::rfp-cassette into pB10 was performed through a triparental mating, in which the helper plasmid pRK600 (Kessler et al. 1992), present in *Escherichia coli* HB101 (Boyer and Roulland-Dussoix 1969), mobilized the delivery plasmid pSM1833 (Haagensen et al. 2002) from the donor *E. coli* MV1190 (Herrero et al. 1990) into the recipient *P. corrugata* (ATCC 29736). Selection in LBbroth with rifampicin (50 mg/ml) and kanamycin (100 mg/ml) resulted in *P. corrugata* (ATCC 29736) derivative with the PA1-04/03::rfp-cassette inserted in the chromosome. The survival of this strain *P. corrugate rfp* in the three different soil types was monitored through plating on LBagar supplemented with rifampicin and kanamycin (both with a concentration of 100 mg/ml). Soil samples from bulk soil and rhizosphere combined were taken on day 4, day 14 and day 30. In extra pots, we also inoculated the wild type to see the effect on the biomass production of *L. perenne*.

Overall, the amount of CFU's per gram of soil decreased from circa $3x10^7$ to $2x10^5$ after 30 days (data not shown) indicating that though the bacteria were present at a lower concentration than initially, they could have an effect in the pot experiment. The production of grass biomass grown on soil supplemented with *P. corrugate rfp* was found not to differ significantly from the control with the wild type *P. corrugate*, such that the insertion of the rfp-cassette did not seem to have hampered the effects of the strain.

Appendix 4.C Nutrient limitations in the main pot experiment

P-mining is an adjusted agricultural technique that aims at depleting soil-P. High biomass production and hence high P-removal with biomass are obtained by fertilizing with nutrients other than P. It is important to know, whether the N- and K-fertilization performed in our experiment was sufficient to relieve the plants from N- or K-limitation. Therefore, we need to compare our plant N- and K-concentrations to agronomic thresholds from literature created for evaluating nutrients limiting optimal crop production. Table 4.C shows that, at day 95 of the main experiment, none of the samples indicated to have N- (45 to 67 mg N g^{-1} DM) or K-deficiencies (47 to 87 mg K g^{-1} DM) for optimal biomass production following agronomic concentration thresholds. In agricultural crop production, optimal concentrations are 20 mg N g^{-1} DM and 20.3 mg K g^{-1} DM (Bailey et al. 1997).

According to Liebisch et al. (2013), P-limitation in an agricultural context is indicated by P-concentrations less than 2.1 mg P g^{-1} DM. Bailey et al. (1997) is more strict and describes limitation for crop growth at concentrations less than 2.6 mg P g^{-1} DM. It is also possible to describe P-limitation for crop growth by the calculation of the P nutrition index (PNI): $PNI = (100 \times P_{biomass})/(0.065 \times N_{biomass} + 0.15)$ in which $P_{biomass}$ and $N_{biomass}$ are the measured P and N concentrations in %weight (Duru and Thélier-Huché 1997). With PNI-values of less than 80, crops are limited by P (Liebisch et al. 2013). According to the PNI, our plants were limited by P in the Mid-P and Low-P soils.

However, in an ecological context, nutrient limitation should be more severe to limit fast-growing species from outcompeting sub-ordinate target species. For example, at plant concentrations of less than 1 mg P g^{-1} DM, P can still not be the limiting nutrient for plant growth (Koerselman and Meuleman 1996). In this case, biomass production will still be too high for establishing species-rich grasslands.

Appendix-Table 4.C Mean ± SD of nutrient concentrations (n=7 for P, n=2 for N and K) and P nutrition index (PNI) in above-ground biomass for three soil P-concentrations (High-P, Mid-P and Low-P) in the control pots. P-limitation in an agricultural context is indicated by PNI < 80 (Liebisch et al. 2013), P-concentration < 2.1 mg P g^{-1} (Liebisch et al. 2013) or P-concentration < 2.6 g P kg^{-1}(Bailey et al. 1997)

Soil P-concentration	**P-concentration in biomass (mg P g^{-1} DM)**	**N-concentration in biomass (mg N g^{-1} DM)**	**K-concentration in biomass (mg K g^{-1} DM)**	**PNI**
High-P	4.30 ± 0.44	49.70 ± 3.39	62.51 ± 7.35	90.9
Mid-P	2.22 ± 0.19	51.82 ± 2.55	71.01 ± 5.09	45.6
Low-P	2.01 ± 0.42	55.92 ± 7.32	78.58 ± 5.39	39.1

Appendix 4.D Additional pot experiment designed for inoculation testing

A second pot experiment was executed to test whether the inoculation method of arbuscular mycorrhiza (AM) or phosphorus-solubilizing bacteria (PSB) affected the proportions between the treatments. The experiment was a repetition of the main pot experiment without the two humic substances treatments, except for the duration (60 d instead of 123 d), the number of replicates (five instead of seven), the number of grass cuttings (two instead of four) and the way AM and PSB were applied to the soil. The AM were not applied on top of the soil but mixed with the grass seeds before sowing. The modification in the PSB application was that the PSB solution was equally mixed in the soil by repeatedly layering one cm of soil and 5 ml of the PSB in solution. This experiment took place after the main pot experiment at a similar growth environment. The pots were fertilized with 94.25 mg N and 97.5 mg K each, corresponding with 53 kg N ha^{-1} and 66 kg K_2O ha^{-1}. The grass was cut two times at day 29 and day 60 and soil was collected at day 60. Initial and final soil characteristics were assessed according to the same methods as used in the main pot experiment.

Similarly to the main pot experiment, the responses in the AM treatment did not significantly differ from the control (Appendix-Tables 4.D.1 and 4.D.2). Also similarly, a significantly lower total biomass production was found on the Low and Mid-P level when NK+PSB were added. Mean P-concentration was significantly higher in all NK+PSB treatments than the other treatments in Exp2. In contrast to the main pot experiment, we did measure a significantly lower P_{Olsen} concentration of High-P after 60 days in the NK+PSB treatment when compared to the NK treatment.

Appendix-Table 4.D.1 Effects of soil P-level, treatment with biostimulants and their interaction on total biomass production, mean P-concentration, total P-removal and final bioavailable soil-P in the pot experiment of Appendix 2

	Soil P-concentration		**Biostimulant addition**		**Interaction**		
	***F*-value**	***p*-value**	***F*-value**	***p*-value**	***F*-value**	***p*-value**	**Error**
Total biomass production	7.1	***	23.8	***	4.7	**	
Mean P-concentration	22.8	***	22.8	***	0.5	NS	
Total P-removal	55.6	***	4.9	*	5.4	**	
Final bioavailable P-concentration	2063.0	***	12.0	***	14.2	***	
DF	2		2		4		36

Results of two-way ANOVA (f-values and significance levels) are shown (n = 5). Significance levels are *** *p* < 0.001; ** *p* < 0.01; * *p* < 0.05; NS = not significant

Appendix-Table 4.D.2 Mean ± SD (n=5) for each soil P-concentration and treatment with biostimulants of total biomass production, mean P-concentration, total P-removal and bioavailable P-concentration by the end of 60 days the pot experiment in Appendix 2. All pots received N- and K-fertilization, more additions were as follows: arbuscular mycorrhiza and phosphate-solubilizing bacteria

	Soil P-concentration		**P-mining**		**P-mining + arbuscular mycorrhiza**		**P-mining + phosphate-solubilizing bacteria**	
			A		A		B	
Total biomass production (g DM pot^{-1})	**High-P**	A	2.97 ± 0.18		3.31 ± 0.13		2.97 ± 0.75	
	Mid-P	B	2.65 ± 0.13	a	3.28 ± 0.36	a	1.70 ± 0.49	b
	Low-P	B	3.11 ± 0.12	a	2.98 ± 0.22	a	1.79 ± 0.75	b
			B		B		A	
Mean P-concentration (g P kg^{-1} DM)	**High-P**	A	3.10 ± 0.23		2.94 ± 0.13		3.61 ± 0.32	
	Mid-P	B	2.19 ± 0.10		2.26 ± 0.12		3.05 ± 0.67	
	Low-P	B	2.40 ± 0.22		2.43 ± 0.17		3.01 ± 0.39	
			AB		A		B	
Total P-removal (mg P pot^{-1})	**High-P**	A	9.22 ± 1.10		9.73 ± 0.67		10.55 ± 1.71	
	Mid-P	B	5.79 ± 0.30	ab	7.39 ± 0.73	a	4.95 ± 1.04	b
		B				a		
	Low-P		7.47 ± 0.87	a	7.22 ± 0.62	b	5.22 ± 1.63	b
			A		A		B	
Final bioavailable P-concentration (mg P_{Olsen} kg^{-1}) at 60 days	**High-P**	A	96.98 ± 3.44	a	99.29 ± 3.15	a	84.29 ± 2.85	b
	Mid-P	B	56.97 ± 2.85		53.89 ± 4.69		54.88 ± 1.98	
	Low-P	C	25.59 ± 2.18		24.94 ± 1.45		26.25 ± 2.27	

Lowercase letters show the significant differences within one soil P-level if the interaction was significant (two-way ANOVA and Tukey HSD post hoc tests). Uppercase letters show the main effects of soil P-concentration and biostimulant addition

Appendix 4.E Estimation of time needed to restore the P-poor conditions on a P-rich ex-agricultural field

To restore species-rich grasslands such as *Nardus* grassland on ex-agricultural fields, severe P-depletion is essential because due to fertilization history soil P-concentrations are enriched significantly. We give here a rough estimation of the time needed for this restoration (Appendix-Table 4.E.1). The bioavailable and slowly cycling P-concentrations come from field data (nature reserve Landschap De Liereman and surroundings in Oud-turnhout, Belgium).

Assuming the original P-poor conditions for *Nardus* grassland (<10 mg P_{Olsen} g^{-1}) should be reached during restoration, for example on a P-rich soil, 1500 kg P ha^{-1} should be removed from the 0-30 cm soil layer. When mowing without any fertilization as a nutrient depleting technique is used, 150 years are needed if we estimate an annual P-removal of 10 kg P ha^{-1} (Oelmann et al. 2009). In contrast, with P-mining without taking into account that P-removal slows down over time and remain as high as when still fertilized with P (P-removal of 45 kg P ha^{-1} y^{-1}, Gallet et al. 2003) it would require only 33 years. However, from our results it is clear that the actual P-removal time will be much longer (>50 years) because, initially, bioavailability of soil-P can remain high for a long time despite significant amounts of P removed with biomass and, because, P-removal slows down with decreasing soil P-concentration.

Appendix-Table 4.E.1 Estimation of time needed to restore abiotic conditions on a previously intensively-used agricultural field.

	Hypothetical P-rich soil	**Hypothetical target**
Bioavailable P-concentration (mg P_{Olsen} kg^{-1}): available for plants within one growing season	125	10
Slowly cycling P-concentration (mg $P_{Oxalate}$ kg^{-1}): can become available for plants in the long term, in equilibrium with bioavailable P-pool	400	50
Slowly cycling P-stock in top 0 – 30 cm (kg $P_{Oxalate}$ ha^{-1})	1700	200
P-stock to remove from 0 – 30 cm (kg P ha^{-1})	1500	
	Time to reach target (year)	
Mowing without any fertilization: annually removing 10[a] kg P ha^{-1}	150	
P-mining without slowing down: annually removing 45[b] kg P ha^{-1}	33	
P-mining with slowing down according to the results of our pot experiment: annually removing 45[b] kg P ha^{-1} until 65 mg P_{Olsen} kg^{-1} is reached, then annually removing 22[c] kg P ha^{-1} until 36 mg P_{Olsen} kg^{-1} is reached and then annually removing 14[d] kg P ha^{-1}	60	

[a] (Oelmann et al. 2009)

[b] (Gallet et al. 2003)

[c] decreasing the initial 45 kg P ha^{-1} with 61%, following the results from the pot experiment in Chapter 4

[d] decreasing the initial 45 kg P ha^{-1} with 70%, following the results from the pot experiment in Chapter 4

Appendices Chapter 5

Appendix 5.A Additional information for the pot experiment

Appendix-Table 5.A.1 Information on the used crop varieties, sowing density and number of growing days

Crop species	Scientific name and variety	Sowing density (# seeds pot^{-1})	Harvest at day nr
Buckwheat[a, c]	*Fagopyrym esculentum* MOENCH variety unknown	20	117
Maize[b]	*Zea mays* subsp. *mays* L. variety 'P8000'	8	139
Flax[a,c]	*Linum usitatissimum* L. variety unknown	20	202
Sunflower[b,c]	*Helianthus annuus* L. variety 'Uniflorus giganteus'	8	105
Triticale[b]	*x Triticosecale* Wittmack variety 'Orval'	20	202

[a] (Anonymous 2003)

[b] (Pannecoucque et al. 2012)

[c] seeds were obtained at "Het Vlaams Zaadhuis"

On the vernalization of triticale:

We used a winter variety, so when 4 to 5 leafs were unfolded, these pots were moved outdoors for vernalization from day 41 until 97 at about -5°C to 5°C following recommendations by (FAO 2004).

Appendix-Table 5.A.2 Information on the fertilization of the crops in the pot experiment

Crop species	Recommendations of fertilization in the field	Totally added N and K per pot as NH_4NO_3 and KNO_3	Totally added N and K per pot converted to dose per ha with pot surface	Times fertilized; at day nr
Buckwheat	30-35 kg N ha^{-1} 28-33 kg K ha^{-1} [a]	204 mg N pot^{-1} 104 mg K pot^{-1}	51 kg N ha^{-1} 26 kg K ha^{-1}	5 dosages: on days 13, 30, 55, 67 and 88
Maize	110-190 kg N ha^{-1} 74-173 kg K ha^{-1} [b]	421 mg N pot^{-1} 312 mg K pot^{-1}	106 kg N ha^{-1} 79 kg K ha^{-1}	8 dosages: on days 13, 30, 55, 67, 78, 85, 88 and 105
Flax	70 kg N ha^{-1} 42-100 kg K ha^{-1} [c]	279 mg N pot^{-1} 179 mg K pot^{-1}	70 kg N ha^{-1} 45 kg K ha^{-1}	6 dosages: on days 13, 30, 55, 67, 78 and 88
Sunflower	30-80 kg N ha^{-1} 50-100 kg K ha^{-1} [d]	346 mg N pot^{-1} 237 mg K pot^{-1}	87 kg N ha^{-1} 60 kg K ha^{-1}	7 dosages: on days 13, 30, 55, 67, 78, 85 and 88
Triticale	50-127 kg N ha^{-1} 24-90 kg K ha^{-1} [b]	354 mg N pot^{-1} 295 mg K pot^{-1}	89 kg N ha^{-1} 74 kg K ha^{-1}	5 dosages: on days 13, 30, 99, 124 and 139
Follow-up crop:	89-139 kg N ha^{-1} 59-141 kg K ha^{-1} [b]	250 mg N pot^{-1} 350 mg K pot^{-1}	63 kg N ha^{-1} 88 kg K ha^{-1}	1 dosage on day 0

[a] (Björkman 2010)

[b] (Maes et al. 2012)

[c] (van Dijk and van Geel 2012)

[d] (Debaeke et al. 1998)

Appendix 5.B Linear regressions

Appendix-Table 5.B Coefficients with standard error, adjusted R^2, f-values and p-values for linear regressions between soil-P-levels and P-removal for each of the five crop species

	a	b	c	adjusted R^2	*F*-value	*p*-value[a]
Buckwheat	3.6 ± 22	4.0 ± 0.74	-0.015 ± 0.004	0.80	54	***
Maize	7.9 ± 14	2.7 ± 0.48	-0.009 ± 0.003	0.83	64	***
Sunflower	-3.4 ± 14	2.7 ± 0.47	-0.012 ± 0.003	0.76	42	***
Flax	19.1 ± 4.6	0.89 ± 0.16	-0.004 ± 0.001	0.63	23	***
Triticale	4.1 ± 4.5	0.36 ± 0.05	-	0.66	52	***

[a] Significance: *** $p < 0.001$

Appendices Chapter 6

Appendix 6.A Set-up mesocosm experiment

In March-April 2016, we set up a mesocosm experiment to unravel the effects of nitrogen and phosphorus availability in interaction with soil biota on plant communities (Fig. 6.1) in the context of ecological restoration of species-rich *Nardus* grasslands on former agricultural land. The experiment consists of 110 mesocosms and is supposed to run for multiple years.

6.A.1. Soil collection and processing

In each pot (Fig. 6.1), we combined three different substrates:

1. **sterilized nutrient-poor mineral sand**, acquired at a horticultural center
2. **sterilized nutrient-poor background soil**, collected with a small excavator from the top 5 to 15 cm from grasslands in nature reserve De Teut (Zonhoven) in the sandy region of northern Belgium in early 2016 (Appendix-Table 6.A.1). The source sites had phosphorus-poor sandy soil with pH_{KCl} above 3.8 to avoid toxicity of aluminium (pH_{KCl} value converted from pH_{H2O} in the screening of De Graaf et al. 2009; conversion factor by Van Lierop, 1981; see Appendix 6.A.2). We sieved the collected soil over a 1 cm mesh and homogenized it.
3. **freshly sampled soil inoculum** from three types of sites (Fig. 6.1) - oligotrophic, mesotrophic and eutrophic (based on differences in vegetation, abiotic soil conditions, nematode and microbiotic communities; screening of sites described in Wasof et al. 2019). We collected soil from at least four locations per site (one or two fields; Appendix-Table 6.A.1) in a pooled soil sample to incorporate within-site variation. We sieved the soil over a 1 cm mesh and applied it fresh to the mesocosms within two weeks after sampling.

The nutrient-poor mineral sand and the background soil were wrapped in 50 kg packages and sterilized by γ-irradiation of 25 KGray (Synergy Health Ede B.V., Etten Leur, the Netherlands) to eliminate the majority of the microbial and mesofauna communities (Zhang et al. 2016).

Appendix-Table 6.A.1 The source sites where we collected the background soil and soil inoculum for the mesocosm experiment; classification into "nutrient status" based on Wasof et al. (2019)

	Soil	No. fields	Nutrient status	Region	Nature reserve	Coordinates
	Background	1	Oligotrophic	Zonhoven	De Teut	51°00'37.4"N 05°23'59.9"E
Soil inoculum	**Oligo-GP**	1	Oligotrophic	Wingene	Gulke Putten	51°04'42.1"N 03°20'18.2"E
	Meso-GP	1	Mesotrophic	Wingene	Gulke Putten	51°04'39.4"N 03°20'05.1"E
	Eutro-GP	2	Eutrophic	Wingene	Gulke Putten	51°04'37.9"N 03°19'44.6"E 51°04'41.9"N 03°19'39.7"E
	Oligo-L	2	Oligotrophic	Oud-Turnhout	Landschap de Liereman	51°20'23.1"N 05°00'05.0"E 51°20'01.8"N 05°00'39.7"E
	Meso-L	2	Mesotrophic	Oud-Turnhout	Landschap de Liereman	51°20'00.1"N 05°01'01.7"E 51°20'24.1"N 05°01'15.0"E
	Eutro-L	2	Eutrophic	Oud-Turnhout	Landschap de Liereman	51°20'20.9"N 05°00'12.8"E 51°19'53.2"N 05°00'59.7"E
	Oligo-TV	1	Oligotrophic	Turnhout	Hooiput	51°18'53.0"N 05°07'34.0"E
	Meso-TV	2	Mesotrophic	Turnhout	Turnhouts Vennengebied	51°21'42.7"N 04°56'48.6"E 51°22'17.7"N 04°55'40.8"E
	Eutro-TV	2	Eutrophic	Turnhout	Turnhouts Vennengebied	51°22'23.0"N 04°55'31.8"E 51°21'40.0"N 04°55'25.4"E

6.A.2 Abiotic characteristics of background and soil inoculum

To characterize the initial abiotic soil conditions, we took samples of the background and soil inocula. The samples were dried at 40°C for 48 h and passed through a 2 mm sieve. **Soil pH_{KCl}** (with KCl as extractant) was measured using a glass electrode (Orion, Orion Europe, Cambridge, England, model 920A) following the procedure described in ISO 10390:1994(E). The bioavailable **phosphorus concentration** was measured by extraction in sodium bicarbonate ($NaHCO_3$; Olsen et al. (1954); according to ISO 11263:1994(E)) to measure bioavailable phosphorus (**P_{Olsen}**), which is available for plants within one growing season (Gilbert et al., 2009).

The total concentration of phosphorus, potassium, calcium, magnesium, sodium and aluminum was assessed after complete destruction of the samples with perchloric acid ($HClO_4$; 65%), nitric acid (HNO_3; 70%) and sulphuric acid (H_2SO_4; 98%) in Teflon bombs at 150°C for 4 h. The phosphorus concentration was measured colorimetrically according to the malachite green procedure (**P_{Total}**; Lajtha et al., 1999). The **concentration of potassium (K_{Total}), calcium (Ca_{Total}), magnesium (Mg_{Total}), sodium (Na_{Total}) and aluminum (Al_{Total})** in the acid-destructed samples were measured by atomic absorption spectrophotometry (AA240FS, Fast Sequential AAS). **Total carbon and nitrogen** content (C_{Total}, N_{Total}) were measured by dry combustion at 850°C using an elemental analyzer (Vario MAX CNS, Elementar, Germany).

The chemical soil characteristics of the background soil resembled the characteristics of the oligotrophic soil inoculum (Appendix-Table 6.A.2). The bioavailable phosphorus concentration in the oligotrophic soil samples was below the threshold of 12 mg P_{Olsen} kg^{-1} found in soils of remnant, unfertilized *Nardus* grasslands (the 95th percentile in the database of the Flemish Research Institute for Nature and Forest, reported in Schelfhout et al. (2017)). The bioavailable phosphorus concentrations of the Mesotrophic and Eutrophc soil inocula could be clustered as another two groups of nutrient status: Mesotrophic (13 – 28 mg P_{Olsen} kg^{-1}) and Eutrophic (67 – 107 mg P_{Olsen} kg^{-1}) soil inocula. According to the classification system of the Belgian Soil Service (BEMEX), the soil type of all soil except the Gulke Putten soil was *Course Sand*, which is comparable to the World Reference Base (WRB) soil type *Gleyic Podzol (Arenic)*. The soil type of the Gulke Putten soil was *Loamy Sand*, which is comparable to the WRB soil type *Gleyic Cambisol (Loamic).*

Appendix-Table 6.A.2 Characteristics of the collected background and inoculum soils in 2016

Soil		pH_{KCl}	P_{Olsen} (mg kg^{-1})	P_{Total} (mg kg^{-1})	N_{Total} (g kg^{-1})	K_{Total} (mg kg^{-1})	Ca_{Total} (mg kg^{-1})	Mg_{Total} (mg kg^{-1})	Na_{Total} (mg kg^{-1})	Al_{Total} (mg kg^{-1})	Fe_{Total} (mg kg^{-1})	C_{Total} (g kg^{-1})
Background		3.7	7.2	48	780	398	409	184	<	1628	2838	10
Inoculum	**Oligo-GP**	3.6	4.6	67	1470	1315	254	396	18	4445	6187	20
	Meso-GP	4.2	28.3	316	2270	1199	1050	494	19	5828	5959	26
	Eutro-GP	4.5	106.7	1067	3530	2129	1578	977	33	8159	12413	37
	Oligo-L	3.7	5.8	95	1220	328	349	139	<	3941	1476	15
	Meso-L	4.0	13.2	287	2080	574	758	177	16	5791	1757	24
	Eutro-L	4.1	67.2	419	1600	179	730	134	<	2186	1454	21
	Oligo-TV	3.5	5.6	71	1590	754	423	194	35	4084	1848	21
	Meso-TV	4.2	12.2	197	1950	583	670	202	4	4482	1541	22
	Eutro-TV	4.5	67.2	661	2340	539	1178	211	11	4623	2313	31

6.A.3. Biotic characteristics of the soil inocula

We took samples of the fresh soil inocula to assess the nematode communities by microscopic observation and the microbial communities by Next Generation Sequencing (NGS) followed by metabarcoding.

6.A.3.1. Microscopy and counting of nematodes

We measured the abundance and diversity of nematodes by extracting all nematodes from 100 cm^3 of soil by zonal centrifugation (Hendrickx 1995) as described in Wasof et al. (2019). The first 200 nematodes encountered in each sample were identified to genus level following the identification keys of Bongers (1988) and Brzeski (1998). The total number of nematodes per genus in each sample was then extrapolated based on this sub-sample of 200 nematodes.

6.A.3.2. Molecular characterization of bacteria and fungi

We extracted DNA from 250 mg soil using the PowerSoil® kit (MO BIO Laboratories Inc., QIAGEN, Venlo, the Netherlands) and ran two Polymerase Chain Reactions (PCRs) including purifications to amplify the molecular barcode and to add Illumina adapters to the fragments. For bacteria, the V3-V4 region of the 16S rDNA, which varies in length between 250-400 base pairs, was used with an adapted version of primer fITS7(GTGAATCATCRAATYTTTG) and for fungi, the primer ITS4NGSr (Ihrmark et al. 2012; Tedersoo et al. 2014); both primers had Illumina-specific adapter extensions. The first PCR consisted of an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation (95°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 30 min), and a final extension step at 72°C for 5 min. For the ITS2 fragment, 30 cycles were used instead of 25. A second PCR of 8 cycles was done to attach indices and sequencing adaptors to the amplicons using the Nextera XT index kit (Illumina, San Diego, CA, USA). The PCR mixes were prepared using the Kapa HiFi Hotstart ReadyMix (Kapabiosystems, Wilmington, MA, USA) according to the manufacturer's instructions. PCR products were cleaned after each step using the HighPrep PCR reagent kit (MAGBIO, Gaithersburg, MD). The libraries were quantified using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA), diluted to 10 nM and pooled in equal amounts. Paired-end sequencing (2x300bp) was done using the Illumina MiSeq v3 platform (Illumina, San Diego, California, USA) at Macrogen (South Korea). The demultiplexed data were further processed by removing the primers using Trimmomatic v0.32 (Bolger et al. 2014) and merging the forward and reverse reads using PEAR v.0.9.8 (Zhang et al. 2014). Length cut-off values for the merged sequences were set between 400 and 450 bp for the V3-V4 and between 200 and 480 bp for the ITS2 region and a minimum overlap size of 120 bp and quality score threshold of 30 were used. ITSx v.1.0.11 was used to extract the ITS2 fragments from the amplicons (Bengtsson-Palme et al. 2013). Next, sequences were quality filtered (maxEE=3), deduplicated and clustered into Operational Taxonomical Units (OTUs; clustering at 97% for bacteria and 98.5% for fungi) using Uparse software (Edgar 2013). For the V3-V4 sequences, chimeras were removed with the RDP Gold database as a reference (Edgar et al. 2011). On average, 84,384 sequences per sample were retained for the V3-V4 fragment, and 148,081 for the ITS2 fragment. Rarefaction analyses confirmed that we covered the sample's microbial diversity by running enough sequences for both bacterial OTUs (Appendix-Fig. 6.A.3.2a) and fungal OTUs (Appendix-Fig. 6.A.3.2b).

For each soil sample, the number of occurrences of each OTU was counted. The OTUs were taxonomically assigned with the *uclust* method, considering maximum three database hits with the QIIME software (version 1.9.0 standard settings; Caporaso et al. 2010) against the SILVA database for bacteria (version 119; Yilmaz et al. 2013) and the Unite database for fungi (version 7.0; Kõljalg et al. 2013) and using a minimum identity match of 90%. For each OTU, the highest taxonomic level present in more than 50% of the database hits was retained. The set of identified OTUs was then used to construct a bacterial and a fungal abundance matrix of OTUs across the soil inocula.

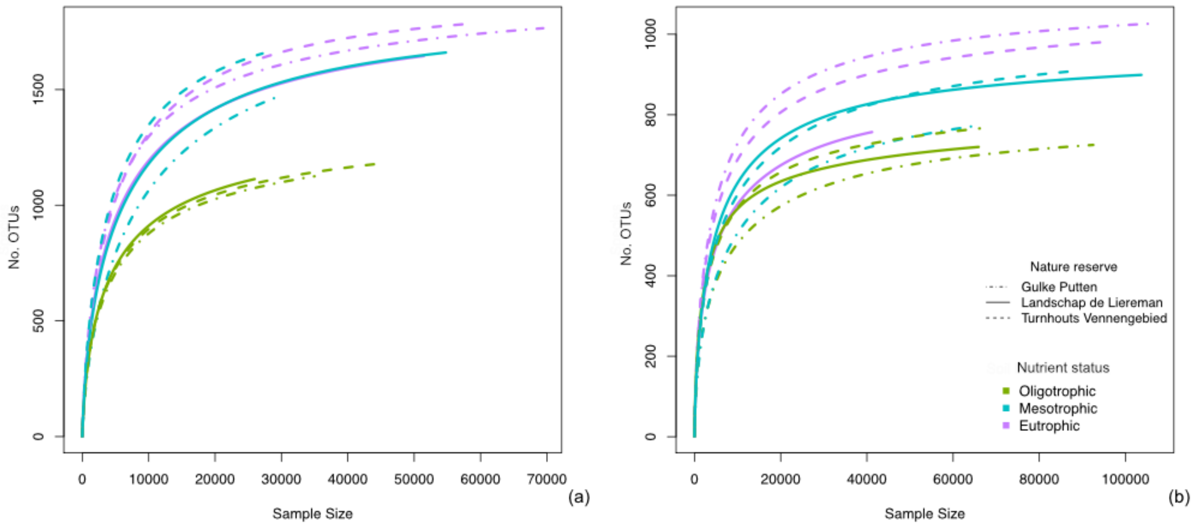


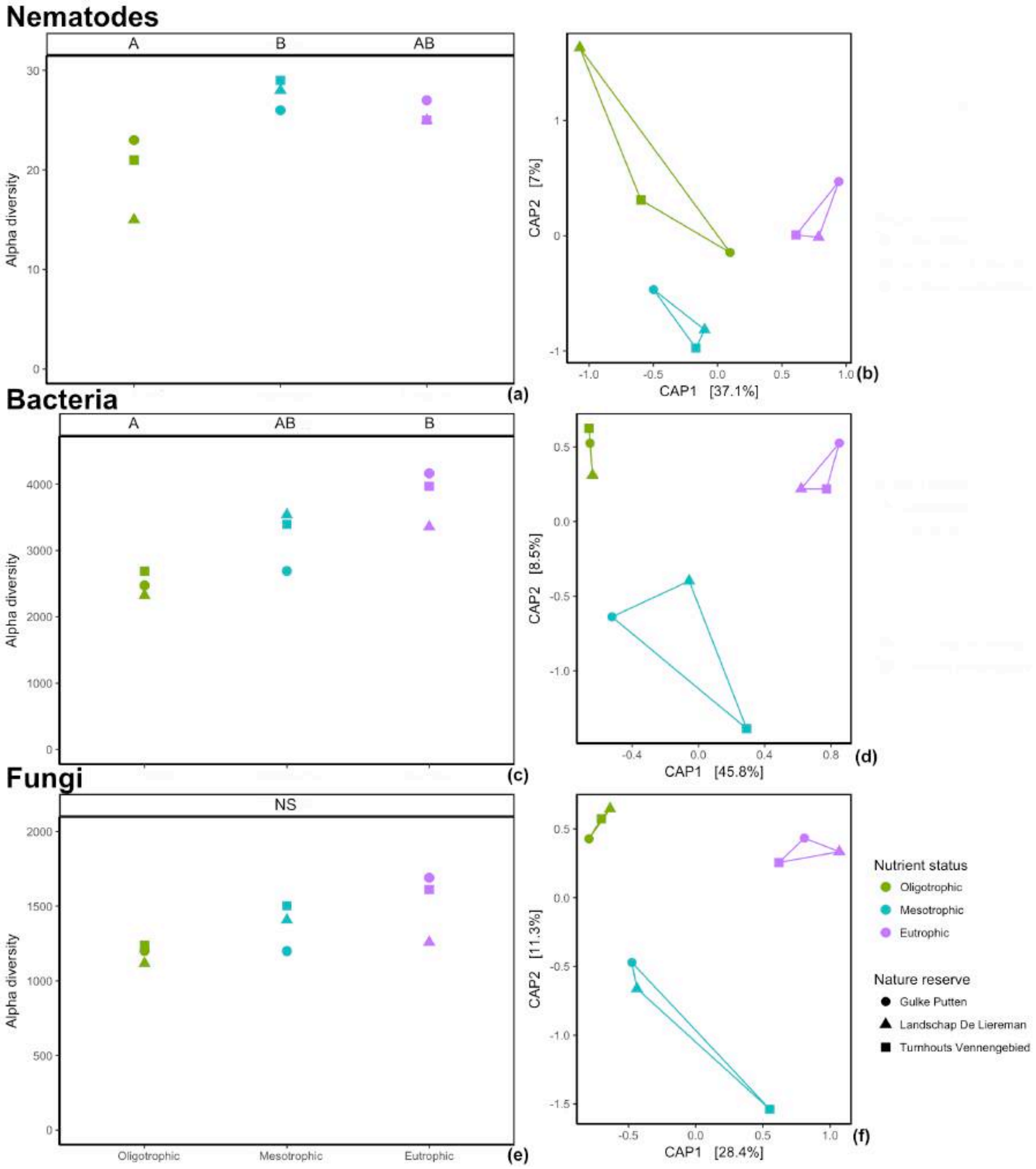
Figure 6.A.3.2 The rarefaction curves of the number of bacterial (a) and fungal (b) OTUs versus the number of sequences in the samples reach a plateau, which indicates that the number of sequences was high enough. Figures made with the function *rarecurve* of the *vegan* package (Oksanen et al., 2017)

6.A.3.3 The nematode, bacteria and fungi communities

The soil biota communities originating from the oligotrophic, mesotrophic and eutrophic soil inocula, *i.e.* the nutrient status levels, showed clear differences (Appendix-Fig. 6.A.3.3, Appendix-Table 6.A.3.3). Further in-depth analyses of the realized soil biota communities in the mesocosm experiment will be done in a future paper (Wasof et al., *unpublished*).

We plotted **alpha diversity** using the original, unfiltered nematode and OTU matrices (function *plot_richness* with measure *Observed* of the package *phyloseq*; McMurdie and Holmes 2013) and compared alpha diversity among the nutrient status levels by analysis of variance with the function *aov* of the package *stats* (R-Core-Team, 2016). In case of significant differences between the nutrient status levels ($p < 0.05$), we performed post-hoc pairwise comparisons of least-squares means by the function *lsmeans* with *tukey* adjustment of the package *lsmeans* (Lenth, 2016). In total, the soil samples contained 46 nematode genera, 6122 OTUs for the bacterial 16S primers and 4602 OTUs for the fungal ITS primers. The oligotrophic soils contained a significantly lower number of nematode genera than the mesotrophic soils (Appendix-Fig. 6.A.3.3a) and a significantly smaller number of bacterial OTUs than the eutrophic soils (Appendix-Fig. 6.A.3.3c). Fungal diversity did not differ significantly between the nutrient status levels (Appendix-Fig. 6.A.3.3e).

We studied differences in **community assembly** among the nutrient status levels using Constrained Analysis of Principal Coordinates (CAP) ordinations, constraining for the nutrient status, for the full, unfiltered nematode matrix and the filtered bacterial and fungal OTU matrices, *i.e.* for the subsets of OTUs that occurred 20 times or more across the samples (function *ordinate* with *Bray-Curtis* distance with the *phyloseq* package in R; McMurdie and Holmes (2013)). We tested for differences between the nutrient status levels using the function *anova* of the *stats* package. Ordinations were plotted with the function *plot_ordination* of the *phyloseq* package. The community assembly differed significantly among the nutrient status levels for nematodes (Appendix-Fig. 6.A.3.3b, constrained proportion 44%), bacteria (Appendix-Fig. 6.A.3.3d, constrained proportion 54%, 1952 OTUs after filtering) and fungi (Appendix-Fig. 6.A.3.3f, constrained proportion 39%, 1719 OTUs after filtering).



Appendix-Figure 6.A.3.3 Alpha diversity and CAP ordination (with constrained grouping for nutrient status) for nematode, bacteria and fungi communities in the soil inocula. Significant differences in alpha diversity between the oligotrophic, mesotrophic and eutrophic nutrient status are indicated by the capital letters A, B and AB; NS indicates no significant difference

Appendix-Table 6.A.3.3 Comparison of the species richness and community composition of nematodes, bacteria and fungi in the soil inocula between oligotrophic, mesotrophic or eutrophic nutrient status groups (ANOVA)

	Alpha diversity [a]			Community composition [b]		
soil biota	**df**	***F*-value**	***p*-value [c]**	**df**	***F*-value**	***p*-value [c]**
Nematodes	2,6	7.4	*	2,6	2.4	*
Bacteria	2,6	9.6	*	2,6	3.6	**
Fungi	2,6	3.1	NS	2,6	2.0	*

[a] number of genera (nematodes) or OTUs (bacteria, fungi)

[b] Multivariate analysis on the abundance matrices by CAP constraining for "nutrient status"

[c] The *p*-value indicates a significant difference between the three nutrient status groups: ** $p < 0.01$; * $p < 0.05$; NS not significant.

6.A.4 Filling the pots

We placed the empty 50-liter pots (with perforated bottom to allow drainage) on top of 10 cm of mineral sand, to allow drainage, in three trenches dug into the soil of the greenhouse (Appendix-Fig. 6.A.4). We filled the pots with the three substrates (see Appendix 6.A.2) in different layers. First, we added 8 kg γ-irradiated mineral sand on the bottom of each pot, covered it with root cloth, and added another 8 kg γ-irradiated mineral sand on top. Next, we used a cement mixer to homogenize 36 kg γ-irradiated background soil with 4 kg fresh soil inoculum. In the mesocosms without soil biota, we used 40 kg γ-irradiated background soil, which was also processed in the cement mixer. During mixing, we also added the different phosphorus levels to the soils (see Appendix 6.A.5). We added these soil-phosphorus mixtures on top of the mineral sand in the pots.

Appendix-Figure 6.A.4 The pots were placed in the greenhouse in three blocks following a randomized block design

6.A.5 Nutrient treatments

The nutrient treatments consisted of a monthly nitrogen addition (3 levels) during the experiment and a one-time phosphorus addition (3 or 5 levels) at the beginning of the experiment.

1. The **nitrogen** treatment was aimed at simulating realistic atmospheric nitrogen deposition rates, *i.e.* <5, 20 or 60 kg N ha^{-1} y^{-1}. We spread 0, 90 or 270 mg NH_4NO_3 in a demineralized water solution evenly on each mesocosm, monthly from March until October. We did not test the effect of nitrogen in our study, but we included the nitrogen treatment as an explanatory factor in our analyses for a complete representation of the experimental design. In none of our final models, the nitrogen treatment was retained as a significant explanatory factor (see Appendix C).
2. The **phosphorus** addition consisted of five dosage levels for constructing mesocosms with bioavailable soil phosphorus just below, just above and well above the threshold of 12 mg P_{Olsen} kg^{-1} described for remnant *Nardus* grasslands (Schelfhout et al. 2017). The targeted phosphorus concentrations were <10, 20 and 60 mg P_{Olsen} kg^{-1}. For the middle nitrogen level, we added two more phosphorus levels (targets 15 and 25 mg P_{Olsen} kg^{-1}) to investigate in detail the possible change point in plant community composition in the phosphorus concentration range of 15-25 mg P_{Olsen} kg^{-1}. We added 0, 3, 5, 6 or 16 g NaH_2PO_4 in a demineralized water solution to the 40 kg of soil used to fill each pot. With the addition of 4 kg of soil inoculum to the 36 kg sterilized background soil for each mesocosm (see section A4), extra nutrients were imported in the mesocosms (Appendix-Table 6.A.5), an undesired but unavoidable side effect in our experiment. Therefore, we sampled the soil of each pot every year to measure the concentration of bioavailable phosphorus and used the realized gradient in phosphorus concentration (see Appendix-Fig. 6.A.5) measured at the time of the biomass harvest as a continuous variable, instead of the categorical phosphorus levels, as an explanatory variable in our models. In the measurement of June 2018, when comparing with the "No biota" treatment, the bioavailable phosphorus concentrations appeared to have increased slightly by adding soil inoculum though not significantly (see Appendix-Fig. 6.A.5), most clearly in the pots in which eutrophic soil inoculum was added.

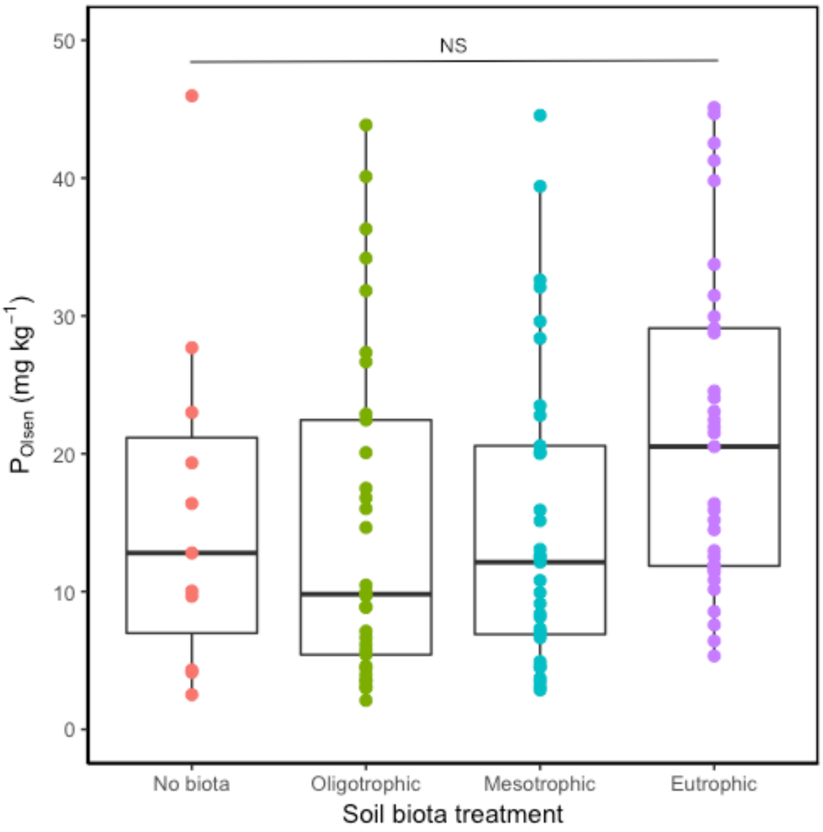
Appendix-Table 6.A.5 Stocks of the macronutrients phosphorus, nitrogen and potassium in the soils used in this experiment

	Name	Phosphorus ($g\ P\ mesocosm^{-1}$)	Nitrogen ($g\ N\ mesocosm^{-1}$)	Potassium ($g\ K\ mesocosm^{-1}$)
	Background (36 kg)[a]	1.7	28.1	14.3
inoculum	**No biota (4 kg)**[b]	0.2	3.1	1.6
	Oligotrophic (4 kg)[c]	0.3 (0.06)	5.7 (0.8)	3.2 (2.0)
	Mesotrophic (4 kg)[c]	1.1 (0.3)	8.4 (0.6)	3.1 (1.4)
	Eutrophic (4 kg)[c]	2.9 (1.3)	10.0 (3.9)	3.8 (4.2)

[a] 36 kg of background soil was used in each pot

[b] 4 kg of background soil was added to pots with the "No biota" treatment

[c] 4 kg of inoculum soil was added to pots with the oligotrophic, mesotrophic or eutrophic treatment; mean value with standard error of the mean between brackets (n=3, as in three sites per inoculum)

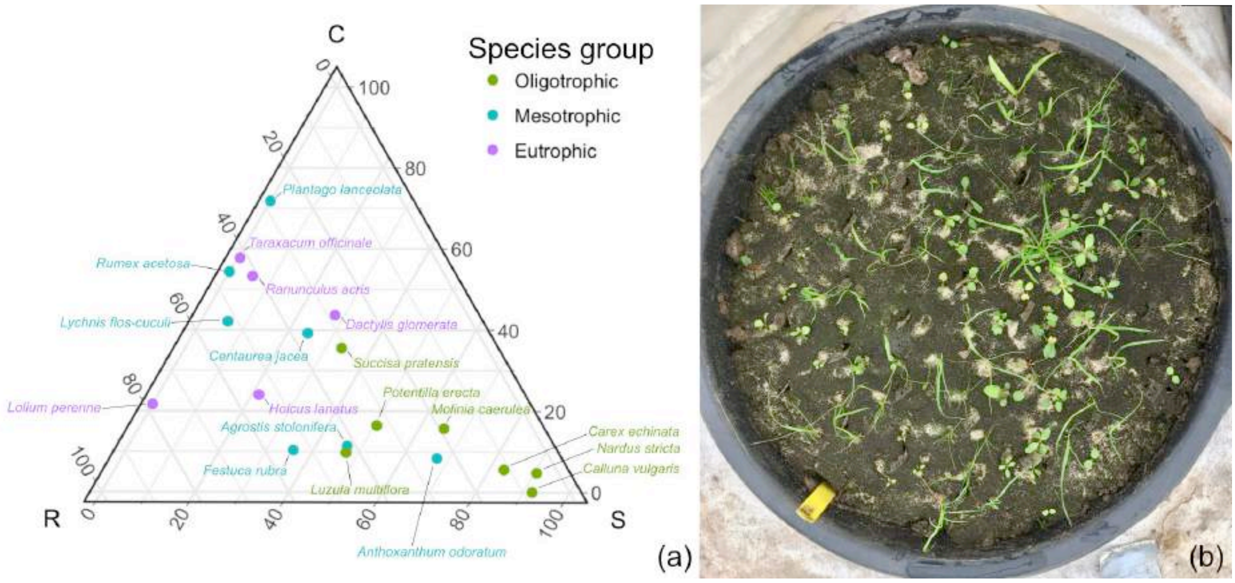


Appendix-Figure 6.A.5 The bioavailable phosphorus concentration (mg P_{Olsen} kg^{-1}) in the mesocosms (n = 110) did not differ significantly (NS) between the four levels of the soil biota treatment, according to an ANOVA test (*anova* function of the *stats* package with $p < 0.05$) followed by post-hoc testing (function *lsmeans* of the *lsmeans* package, Lenth 2016)

6.A.6 Plant species

We chose nineteen plant species typical of grasslands (see Fig. 6.1) based on the screening by Van Daele et al. (2017), excluding Leguminosae, hemiparasitic and annual plant species, and species typical of calcareous, wet or very dry soil conditions. The nineteen species' CSR-strategies are evenly spread along the CSR-triangle (Fig A6a) and they fall into three groups that can be linked to three different grassland communities along a historical land-use gradient (cf. Wasof et al., 2019). The five eutrophic plant species are typically found in grass-dominated *Lolium perenne* grasslands, the seven mesotrophic species in grass-herb mixed grasslands, the seven oligotrophic species in *Nardus* grasslands.

We obtained seeds of the nineteen species at Rieger-Hofmann® GmbH (Germany), a commercial nursery of plant seeds sourced in nature reserves. We surface-sterilized the seeds by soaking in 0.5% household bleach for 30 to 60 seconds to prevent fungal infection and rinsed them with demineralized water. Then, after stratification if necessary (following Van Daele et al., 2017), we put the seeds on heat-sterilized glass beads moistened with demineralized water in a germination cabinet (light regime 16h light, 8h dark; temperature 22°C when light, 18°C when dark) until a sufficient number of seedlings was obtained. After germination, which took one to three weeks, we transferred the seedlings to an acclimatized room (light regime 16h light, 8h dark; temperature 4°C) to keep all seedlings in a similar post-germination stage until planting. In April 2016, we carefully planted seedlings in the pots in the greenhouse using toothpicks. Each pot (45 cm diameter) received 114 seedlings, planted in 38 groups of three (Fig. 6.1; Appendix-Fig. 6.A.6b). The nineteen center positions were taken by each of the nineteen selected species at random positions. Due to the size of the pots, there were eighteen edge positions, which were planted at random with each of the oligotrophic and eutrophic species and with six of the seven mesotrophic species (the missing mesotrophic species was randomly assigned per pot). Two months after the transplantation, we recorded and replaced all dead seedlings; plants that died afterwards were not replaced. Around each mesocosm, we installed wire fencing to make sure that the above-ground plant biomass did not fall open in the open space between the mesocosms and to allow for light limitation within the plant communities (Cover-Fig. Chapter 6).



Appendix-Figure 6.A.6 (a) Overview of CSR-scores of the nineteen species after Pierce et al. (2017); (b) A planted pot with the nineteen species in April 2016

6.A.7 Growing conditions in the greenhouse

The mesocosms were grown in a non-heated greenhouse at Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) in Merelbeke. The average air temperature was 17.4°C ± 7.3 SD, the relative air humidity was 71% ± 14 SD, and there were natural light conditions. Irrigation was performed with an automated sprinkle watering system using tap water (Appendix-Table 6.A.7), up to two times per day depending on the climatic conditions in the greenhouse (hot weather required a higher irrigation frequency and dosage). The irrigation caused an estimated annual input of 3 kg nitrogen ha^{-1} (below the threshold for atmospheric nitrogen deposition of 12 kg nitrogen ha^{-1} y^{-1}; (van Dobben et al. 2012) and 282 kg calcium ha^{-1} (well below liming recommendations to increase the pH according to the Belgian Soil Service).

Appendix-Table 6.A.7 Chemical characteristics of the tap water used for irrigation in the greenhouse

	Concentration (mg l^{-1})							
pH	**NH_4 [a]**	**NO_3 [a]**	**PO_4 [a]**	**K [b]**	**SO_4 [a]**	**Na [b]**	**Ca [b]**	**Cl [c]**
6.9	0.5	< 0.04 [d]	< 0.04 [d]	1.4	23.7	3.1	35.2	10.1

[a] measured with an UV photometric method at k = 210 nm (NO_3), 640 nm (SO_4), 660 nm (NH_4), 830 nm (PO_4)

[b] measured by atomic absorption spectrophotometer AA240FS, Fast Sequential AAS

[c] measured with an ionspecific electrode

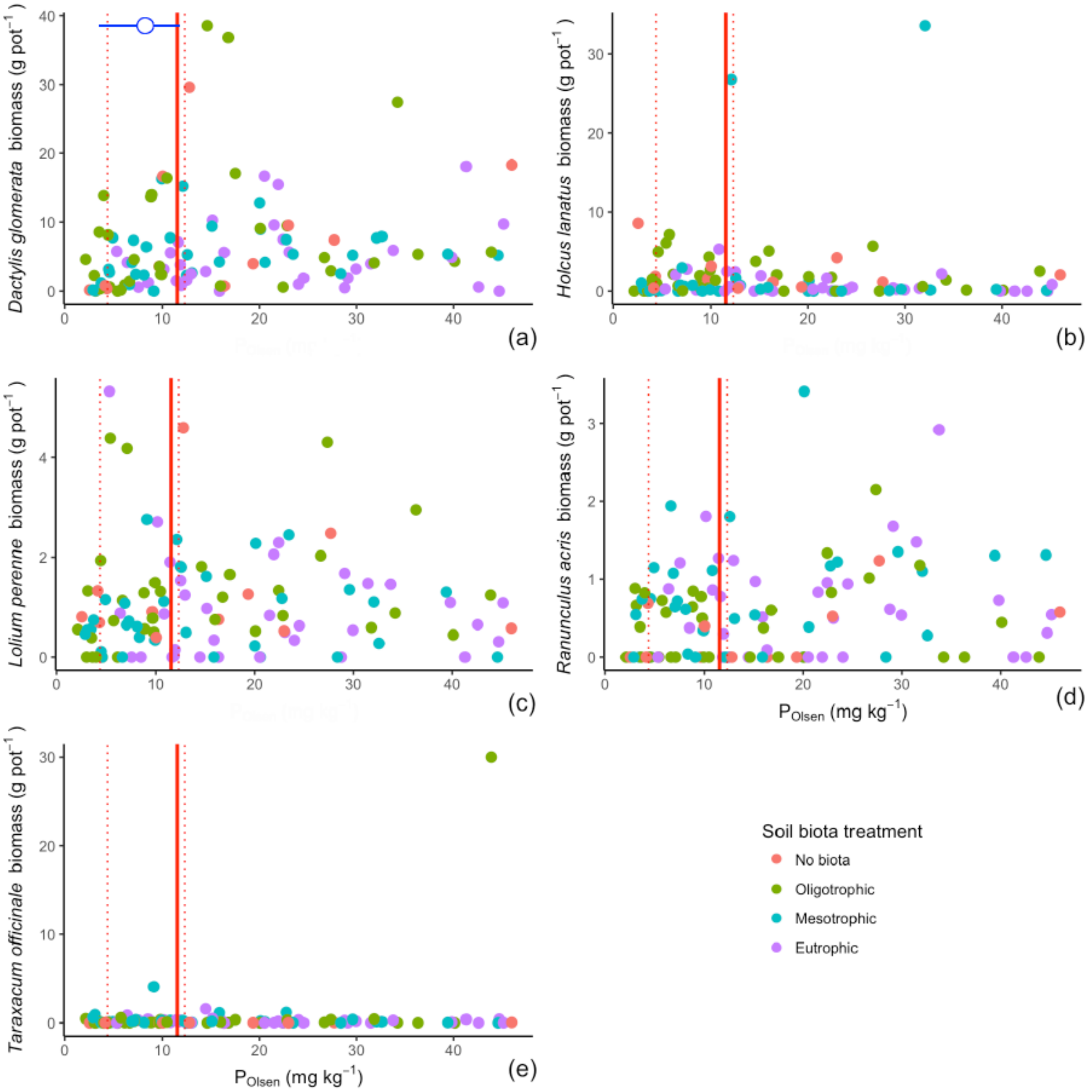
[d] below the detection limit

6.A.8 Biomass harvest June 2018

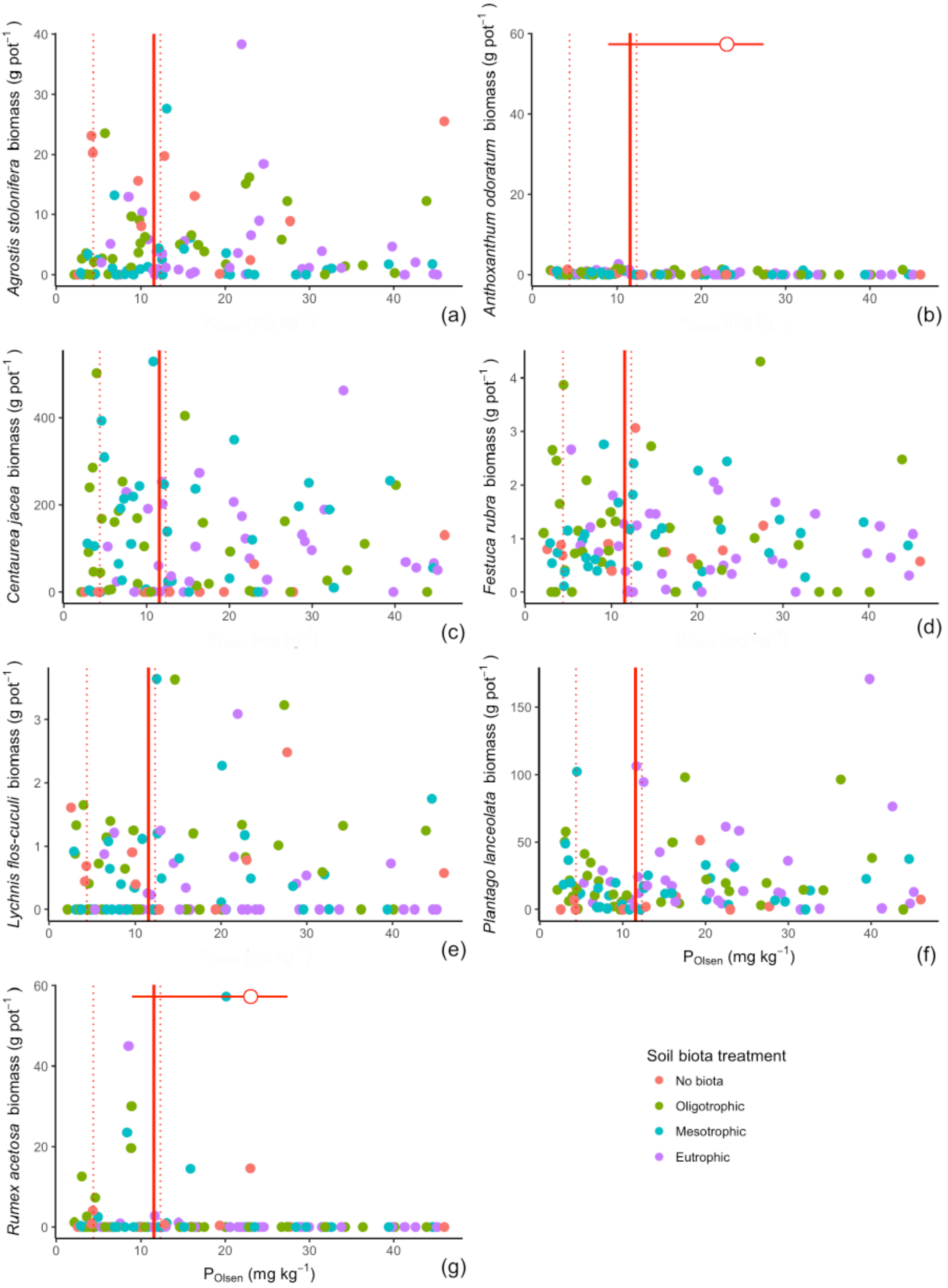
We harvested the above-ground biomass in each mesocosm by cutting the vegetation with grass scissors 4 cm above soil level. First, we separately harvested each of the nine most abundant species (*Agrostis stolonifera, Centaurea jacea, Dactylis glomerata, Holcus lanatus, Nardus stricta, Plantago lanceolata, Potentilla erecta, Rumex acetosa, Taraxacum officinale*). Second, we estimated the percentage cover for each of the other ten species (*Anthoxanthum odoratum, Calluna vulgaris, Carex echinata, Festuca rubra, Lolium perenne, Luzula multiflora, Lychnis flos-cuculi, Molinia caerulea, Ranunculus acris, Succisa pratensis*). Third, we jointly harvested the remaining above-ground biomass (of the ten species together) in each mesocosm. All biomass samples were dried for 48h at 70° C and weighed. We then used the dry weights to calculate the total dry biomass per mesocosm and estimate the dry biomass for the ten non-abundant species based on their species-specific cover and the total dry biomass in each mesocosm.

Appendix 6.B Supplementary information about the TITAN analysis

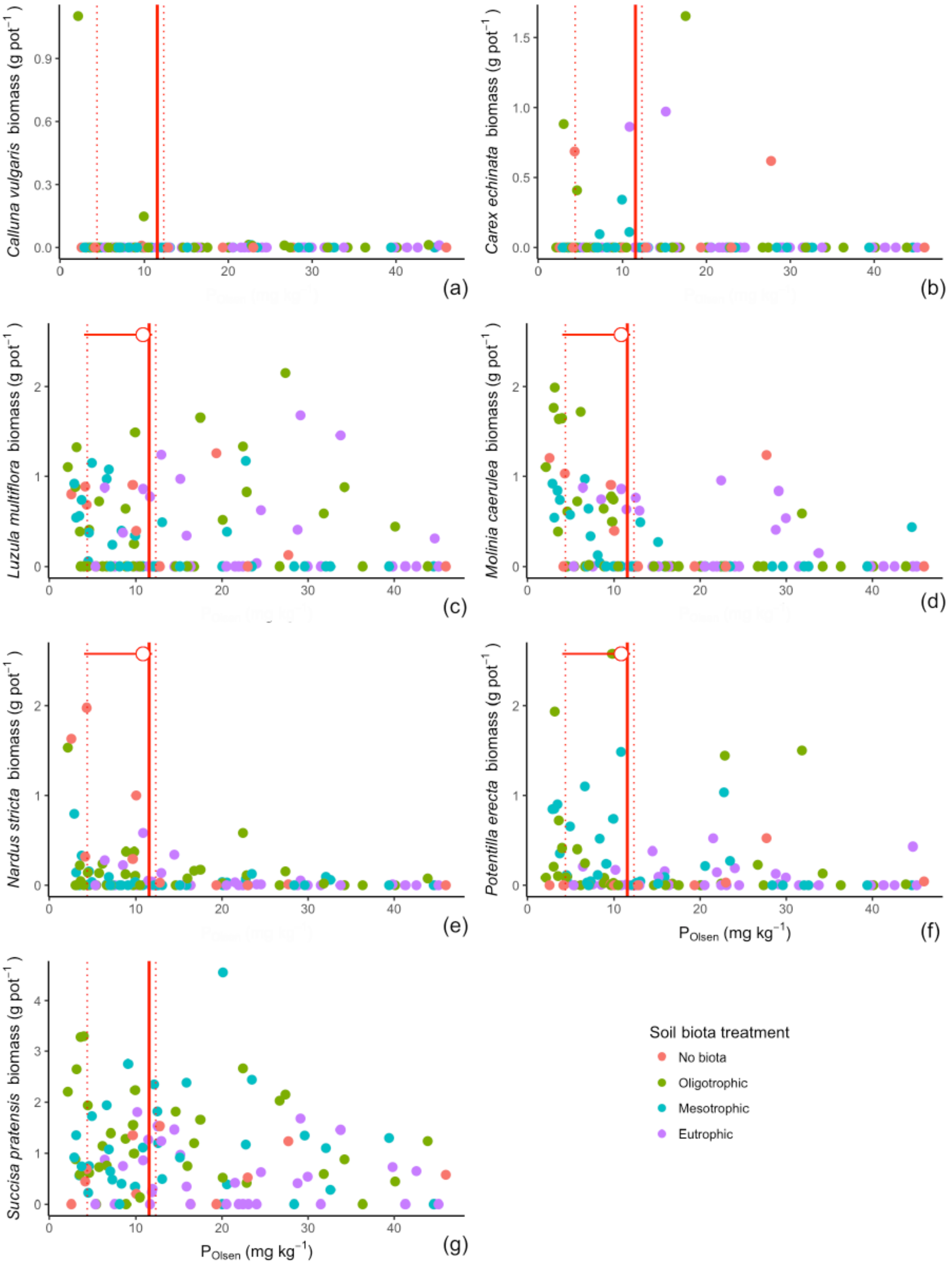
Of the five eutrophic species (Appendix-Fig. 6.B.1), one species, *i.e. Dactylis glomerata*, showed a positive response towards increasing bioavailable phosphorus concentrations, *i.e.* a positive change point. Of the seven mesotrophic species (Appendix-Fig. 6.B.2), the two indicator species, *i.e. Anthoxanthum odoratum* and *Rumex acetosa*, showed a negative change point. Of the seven oligotrophic species (Appendix-Fig. 6.B.3), four were significant indicator species showing a negative change point, *i.e. Luzula multiflora, Molinia caerulea, Nardus stricta* and *Potentilla erecta*.



Appendix-Figure 6.B.1 Absolute biomass per mesocosm (g mesocosm^{-1}) of the eutrophic indicator (a) and non-indicator (b-e) species. The blue circle indicates the positive change point of the indicator species (5-95th percentiles as blue line). The red full vertical line indicates the community sum *z*- threshold; the dotted red lines indicate the 5-95th percentiles for the community. Colors indicate the soil biota treatment



Appendix-Figure 6.B.2 Absolute biomass per mesocosm (g mesocosm^{-1}) of the mesotrophic indicator (*b* and *g*) and non-indicator (*a*, *c-f*) species. The red circles indicate the negative change point of the indicator species (with 5-95th percentiles as a red horizontal line). The red full vertical line indicates the community sum *z*- threshold; the dotted red lines indicate the 5-95th percentiles for the community. Colors indicate the soil biota treatment



Appendix-Figure 6.B.3 Absolute biomass per mesocosm (g mesocosm^{-1}) of the indicator oligotrophic species (*c-f*) and non-indicator oligotrophic species *(a*, *b* and *g)* according to TITAN. The red circles indicate the negative change point of the indicator species (with 5-95th percentiles as a red horizontal line). The red full vertical line indicates the community sum *z*- threshold; the dotted red lines indicate the 5-95th percentiles for the community. Colors indicate the soil biota treatment

Appendix 6.C Model selection and optimal models

6.C.1 Biomass of typical *Nardus* grassland species ~ experimental treatments

For the typical *Nardus* grassland species identified as sensitive to increasing soil phosphorus concentrations by TITAN (see Fig. 6.2; *Luzula multiflora, Molinia caerulea, Nardus stricta* and *Potentilla erecta*), we investigated the absolute biomass (sum of the biomasses of the four species per mesocosm) and the relative biomass (*i.e.* summed biomass of the four species relative to the total biomass of the mesocosm). The absolute biomass response variable was not transformed. The relative biomass response variable was square root transformed. Both variables contained a significant proportion of zeros (17%), which were true zero observations. We visually inspected the residuals as described in Zuur et al. (2009); generalized linear models with the *quasi* family with *identity* link and *constant* variance were suitable for the two response variables. We used the function *glm* of the *stats* package (R-Core-Team 2016). The full models contained the concentration of bioavailable soil phosphorus (log transformed), the soil biota treatment (4 levels) and the nitrogen treatment (3 levels) as explanatory variables.

We systematically deleted model terms from the full models until minimum adequate models were reached (Manning et al., 2004) by using the *drop1* function of the *stats* package as recommended by Zuur et al. (2009) with the likelihood ratio test *LRT*. The aim of our model selection was to minimize the residual deviance with the constraint of all explanatory variables being statistically significant (Table C1). We assessed the goodness of fit of the models by calculating the deviance reduction (D^2; Guisan and Zimmermann 2000), which is a goodness-of-fit statistic similar to the R^2 measure, by

$$D^2 = (Null\ deviance - Residual\ deviance)/Null\ deviance$$

For the final, selected models (Appendix-Table 6.C.1.2, Appendix-Fig. 6.C.1.2, Fig. 3a), we obtained likelihood ratio chi-square values and significance of the fixed terms using Type II Wald likelihood tests with the *Anova* function of the *car* package (test = *LR*; Fox and Weisberg, 2011). The nitrogen treatment was never retained as a significant explanatory factor.

Appendix-Table 6.C.1.1 Model selection by single term deletion starting with full models for the absolute and relative biomass of the typical *Nardus* grassland species in the mesocosms (n = 110). The objective was to maximize the deviance explained by the model with all explanatory variables being significant. A term was deleted when omitting the term from the model caused the smallest change in deviance

	Absolute biomass (g mesocosm^{-1})		**Relative biomass [b] (% mesocosm^{-1})**	
Explanatory variables in the model [a]	**Explained deviance**	**All terms significant?[c]**	**Explained deviance**	**All terms significant? [c]**
log(Olsen-P) * Soil biota * N	29%	no	43%	no
log(Olsen-P) * Soil biota + N	19%	no	29%	no
log(Olsen-P) + Soil biota * N	23%	no	29%	no
log(Olsen-P) * N level + Soil	18%	no	26%	no
log(Olsen-P) * Soil biota	20%	no	28%	yes
log(Olsen-P) + Soil biota	17%	no		
log(Olsen-P)	11%	yes		

[a] Generalized linear models with *quasi* family error distribution. The fit of the models is characterized by *explained deviance* and deletion of single terms is tested by likelihood ratio tests

[b] square root transformed

[c] *p* <0.05 is considered significant

Appendix-Table 6.C.1.2 Optimal models of the absolute and relative biomass of the typical *Nardus* grassland species in the mesocosms (n = 110). The fit of the models is characterized by the deviance (D^2)

Model[a]	Parameter estimate[b]	Likelihood ratio Chisquare	*p*-value[c]	df	Null deviance[d]	Residual deviance[d]	*D*^{2}
Absolute biomass (g mesocosm^{-1})					137 (109)	104 (108)	11%
Intercept	2.8 (0.3)						
log P_{Olsen}	-0.7 (0.1)	34.2	***	1			
Relative biomass (% mesocosm^{-1})[e]					94.7 (109)	63.5 (102)	28%
Intercept	22.1 (7.0)						
log P_{Olsen}	-1.5 (0.7)	18.3	***	1			
$SB_{Oligotrophic}$	-11.1 (5.6)						
$SB_{Mesotrophic}$	-8.9 (5.1)	16.4	***	3			
$SB_{Eutrophic}$	-9.9 (6.4)						
log P_{Olsen} : $SB_{Oligotrophic}$	1.1 (0.7)						
log P_{Olsen} : $SB_{Mesotrophic}$	0.6 (0.5)	11.1	*	3			
log P_{Olsen} : $SB_{Eutrophic}$	0.8 (0.7)						

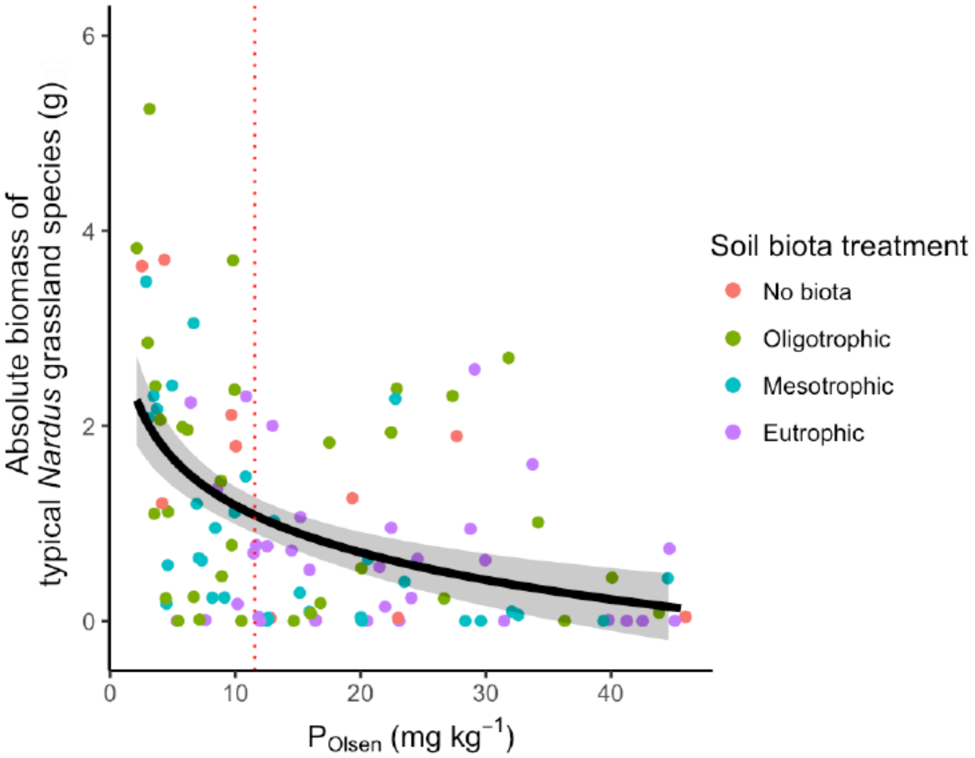
[a] Generalized linear models with quasi family error distribution. SB stands for 'Soil Biota treatment'.

[b] with standard error between brackets

[c] *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

[d] with degrees of freedom (*df*) between brackets

[e] The response variable was square root transformed, the coefficients and standard errors were back transformed in this table



Appendix-Figure 6.C.1 Absolute biomass of the typical *Nardus* grassland species in function of the bioavailable phosphorus concentration. The black line illustrates the significant relationship according to the *glm* fitted model. The shaded area indicates the 5th-95th confidence intervals. The dotted red line indicates the negative community change point identified by TITAN (see Appendix 6.B)

6.C.2 Biomass of typical *Nardus* grassland species ~ total biomass of the mesocosms

We modelled the absolute and relative biomass of the typical *Nardus* grassland species as a function of the total biomass per mesocosm (Appendix-Table 6.C.2, Appendix-Fig. 6.C.2, Fig. 3b), with *glm* models, as described in section C1. The full model contained only the total biomass per mesocosm.

Appendix-Table 6.C.2 Optimal models of the absolute and relative biomass of the typical *Nardus* grassland species in the mesocosms (n=110). The fit of the models is characterized by the deviance (D^2).

Model[a]		Parameter estimate[b]	Likelihood ratio Chisquare	*p*-value[c]	df	Null deviance[d]	Residual deviance[d]	*D*^{2}
Absolute biomass (g mesocosm^{-1})						137.3 (109)	125.8 (108)	42%
	Intercept	2.9 (0.6)						
	Total biomass	-0.4 (0.1)	9.9	**	1			
Relative biomass (% mesocosm^{-1})[e]						94.7 (109)	55.7 (108)	42%
	Intercept	19.2 (3.6)						
	Total biomass	-0.5 (0.1)	75.7	***	1			

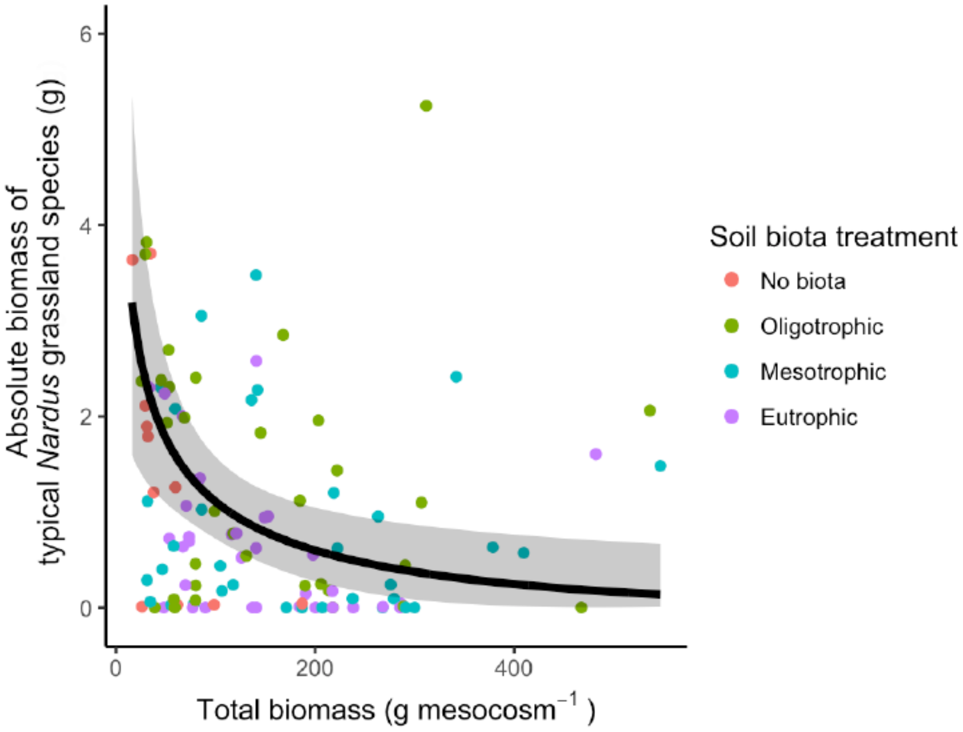
[a] Generalized linear models with quasi family error distribution

[b] with standard error between brackets

[c] *** p <0.001, ** $p < 0.01$, * $p < 0.05$

[d] with degrees of freedom (*df*) between brackets

[e] The response variable was square root transformed, the coefficients and standard errors were back transformed in this table



Appendix-Figure 6.C.2 Absolute biomass of the typical *Nardus* grassland species in function of the total biomass per mesocosm. The black line illustrates the significant relationship according to the *glm* fitted model. The shaded area indicates the 5th-95th confidence intervals

6.C.3 Total mesocosm biomass ~ experimental treatments

We investigated the effects of the experimental treatments on the total above-ground biomass harvested in each mesocosm (Appendix-Table 6.C.3, Fig. 3c). The total biomass response variable was square root transformed. We visually inspected the residuals as described in Zuur et al. (2009) and used generalized least-square models with the function *gls*, allowing for different variance per Biota level using *varIdent(form=~1|Biota)* as *weights*, with restricted estimates maximum likelihood (*REML*) as method from the *stats* package (R-Core-Team 2016). The full models contained the concentration of bioavailable soil phosphorus (log transformed), the soil biota treatment (4 levels) and the nitrogen treatment (3 levels) as explanatory variables.

We compared the null model, intermediate models and the full model based on the corrected Akaike Information Criteria (AIC$_c$) and Akaike weights (Zuur et al., 2009) with the function *AICctab* of the *bblme* package (Bolker and R-Core-Team 2016). We aimed to select 'competitive' models, *i.e.* models with a difference in AIC$_c$ (ΔAIC$_c$) of less than two (Goodenough et al., 2012). We retained only one model: the $\mathit{Soil\ biota}{*}P_{Olsen}$ model (*df* 12, *AICc* 636, Δ*AIC* 0, *weight* 0.67). We obtained chisquare values and significances of the explanatory terms using Type II Wald chisquare tests with the *Anova* function of the *car* package (Table C4; Fox and Weisberg 2011).

Appendix-Table 6.C.3 Optimal model describing the relationship between the total above-ground biomass in each mesocosm (n = 110) and the experimental treatments. Soil biota is abbreviated as 'SB'

Model[a]	**Parameter estimate**[b]	**Likelihood ratio Chisquare**	***p*-value**[c]	**df**	R^2
Total biomass (g mesocosm^{-1})[d]					15
Intercept	17.8 (6.7)				
P_{Olsen}	0.03 (0.01)	8.9	**	1	
$SB_{Oligotrophic}$	64.3 (25.0)				
$SB_{Mesotrophic}$	67.3 (26.8)	54.2	***	3	
$SB_{Eutrophic}$	49.6 (22.0)				
$P_{Olsen} : SB_{Oligotrophic}$	-0.05 (0.04)				
$P_{Olsen} : SB_{Mesotrophic}$	-0.03 (0.03)	11.0	*	3	
$P_{Olsen} : SB_{Eutrophic}$	-0.03 (0.02)				

[a] Generalized leased square models with quasi family error distribution. SB stands for 'Soil Biota treatment'.
[b] with standard error between brackets
[c] *** p <0.001, ** p < 0.01, * p < 0.05
[d] The response variable was square root transformed, the coefficients and standard errors were back transformed in this table

Appendices Chapter 7

Appendix 7.A Species-list of common herb species that tolerate mesotrophic soil conditions

Appendix-Table 7.A Plant species tolerant of growing in mesotrophic soil conditions

Species mix by Magnificent Meadows[a]	Species mix by bumblebee conservation trust[b]
Achillea millefolium	*Agrimonia eupatoria*
Ajuga reptans	*Scorzoneroides autumnalis*
Cardamine pratensis	*Stachys officinalis*
Centaurea nigra	*Lotus corniculatus*
Knautia arvensis	*Pimpinella saxifraga*
Lathyrus pratensis	*Hypochaeris radicata*
Leontodon autumnalis	*Centaurea nigra*
Leucanthemum vulgare	*Rumex acetosa*
Silene flos-cuculi	*Vicia sativa*
Prunella vulgaris	*Primula veris*
Ranunculus acris	*Succisa pratensis*
Ranunculus bulbosus	*Knautia arvensis*
Rhinanthus minor	*Galium verum*
Trifolium pretense	*Ranunculus acris*
Vicia cracca	*Lathyrus pratensis*
Vicia sativa	*Leucanthemum vulgare*
	Plantago lanceolata
	Leontodon hispidus
	Prunella vulgaris
	Trifolium pratense
	Achillea millefolium
	Rhinanthus minor

[a] Source: http://www.magnificentmeadows.org.uk/assets/pdfs/Wildflowers_tolerant_of_soil_fertility.pdf

b bbct: https://www.bumblebeeconservation.org/wp-content/uploads/2017/08/Bumblebee_seed_mix_for_neutral_soils.pdf

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Professional experience

2012-Present	PhD researcher at ForNaLab, Ghent University
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Education

2012-2018	PhD and Doctoral Schools programme Ghent University Title PhD Thesis: Role of phosphorus in the restoration of species-rich Nardus grasslands via phosphorus-mining (promotors: Jan Mertens, An De Schrijver, Kris Verheyen, Geert Haesaert)
2008-2010	MSc. in Bioscience Engineering, Forest and Nature Management, Ghent University. MSc. Thesis: *Tree species effect on earthworm communities in Danish and Flemish forests* (promotor: An De Schrijver)
2nd semester 2009	Erasmus exchange in at the Universität für Bodenkultur (BOKU) in Vienna
2004-2008	BSc. in Bioscience Engineering: Land and Forest Management, Ghent University BSc. Thesis: Bestrijding van Amerikaanse vogelkers in het Vlaamse bos: waar staan we anno 2007? (promotor: Margot Vanhellemont)
1998-2004	Secondary School, Moderne Talen-Wiskunde, Campus Lyceum, Wachtebeke

Scientific publications

Peer reviewed publications included in the Web of Science

Wasof S, De Schrijver A, **Schelfhout S**, Perring M P, Remy E, Mertens J, de la Peña E, De Sutter N, Viaene N, Verheyen K. 2019 Linkages between aboveground and belowground community compositions in grasslands along a historical land-use intensity gradient. Plant and Soil. 434:289. (IF 2017: 3.306) DOI: https://doi.org/10.1007/s11104-018-3855-7

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Setiawan N N, Vanhellemont M, De Schrijver A, **Schelfhout S**, Baeten L, Verheyen K. 2016. Mixing Effects on Litter Decomposition Rates in a Young Tree Diversity Experiment. Acta Oecologica 70: 79–86. (IF 2017: 1.615) DOI: https://doi.org/10.1016/j.actao.2015.12.003

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De Schrijver A, De Frenne P, Staelens J, Verstraeten G, Muys B, Vesterdal L, Wuyts K, Van Nevel L, **Schelfhout S**, De Neve S, Verheyen K. 2012. Tree Species Traits Cause Divergence in Soil Acidification During Four Decades of Postagricultural Forest Development. Global Change Biology 18 (3): 1127–1140. (IF 2017: 8.997) DOI: http://doi.wiley.com/10.1111/j.1365-2486.2011.02572.x

Under review

Schelfhout S, Wasof S, Mertens J, Vanhellemont M, Demey A, Haegeman A, Vangansbeke P, Baeyen S, De Sutter N, Maes M, Viaene N, van der Putten W H, Verheyen K, De Schrijver A. Effects of bioavailable phosphorus and soil biota on typical Nardus grassland species in competition with fast-growing plant species. Under review in Ecological Indicators (IF 2017: 3.983)

Schelfhout S, De Schrijver A, Vanhellemont M, Vangansbeke P, Wasof S, Perring M P, Haesaert G, Verheyen K, Mertens J. Phytomining to re-establish phosphorus-poor soil conditions for nature restoration on former agricultural land. Accepted pending minor revisions in Plant and Soil (IF 2017: 3.306)

Articles in other journals

Vanhellemont M, **Schelfhout S**, Mertens J, Verheyen K. 2014. Bos in De Vallei Van De Gondebeek: 1775-2013. Bosrevue (50): 1–4

Schelfhout S, De Schrijver A, Mertens J, De Block M, Herr C, De Smedt P, Verheyen K. 2014. Natuurontwikkeling Op Landbouwgrond : Herstelmaatregelen. Natuur.focus 13 (1): 31–39

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Book chapter

De Schrijver A, Ampoorter E, van der Burg R, Demey A, **Schelfhout S,** Olsthoorn A, De Smedt P, Vangansbeke P, Van Nevel L, Hommel P, De Frenne P, Mertens J, Verheyen K. 2018. Groeiplaatsverbetering. In: Jansen P, Boosten M, Cornelis J, Thomassen E, Winnock M. Praktijkboek Bosbeheer. Stichting Probos, Wageningen

Scientific activities

Participation in symposia with oral presentation

Schelfhout S. 2018. Kleine landschapselementen kiezen: de bijenweide upgraden in agrarisch gebied. In Koplopers bijeenkomst: De meerwaarde van Bestuivers & agrobiodiversiteit. Ghent, Belgium

Schelfhout S, Raman M, De Schrijver A. 2016. Restoration of Species-rich *Nardus* Grasslands on Former Fertilized Land by Reinitiating Mowing and Grazing Is Inadequate. In SER conference Best Practice in Ecological Restoration: 10th European Conference on Ecological Restoration. Freising, Germany

Schelfhout S. 2015. Restoration of Species-rich Grasslands on Former Agricultural Land via P--mining? In Interlife. Natuurpunt. Kasterlee, Belgium

Schelfhout S, De Schrijver A, Verheyen K, Haesaert G, Mertens J. 2013. Restoration of Species-rich *Nardus* Grasslands: Exploring the Technique of Phosphorus Mining. In Open Landscapes : Ecology, Management and Nature Conservation. Hildesheim, Germany

Mertens J, **Schelfhout S**. 2014. Natuurrestoratie Op Voormalige Landbouwgronden via Uitmijnen Van Fosfor: Eerste Resultaten in Natuurgebieden 'Landschap De Liereman' (Oud-Turnhout) En Het 'Vrieselhof' (Oelegem). In "(A)biotisch" En Toch Actief!: Ontmoetingsdag Ankona. Antwerpen, Belgium

Schelfhout S. 2013. Afgraven, maaien en uitmijnen. In Studiedag Fosfaat En Graslandbiodiversiteit. Brussels, Belgium

Schelfhout S. 2011. De voorbereidende fase: fosfor uitmijnen of niet? In Ecologische bosuitbreiding op sterk bemeste gronden: van wetenschap naar praktijk. Gontrode, Belgium

Schelfhout S, De Schrijver A, Mertens J, Vesterdal L, Verheyen K. 2011. Het boomsoorteffect op regenwormpopulaties in Deense en Vlaamse bossen. In Startersdag in het Bos- en Natuuronderzoek. Brussels, Belgium

Participation in symposia with poster presentation

Demey A, **Schelfhout S**, De Schrijver A, Mertens J, de la Pena E, Viaene N, De Sutter N, Verheyen K. 2015. Impact of Bioavailable Phosphorus on Plant and Soil Microbial Communities in Grassland Under Restoration Management. In Ecology of Soil Microorganisms (ESM). Prague, Czech Republic

Heyman T, **Schelfhout S**. 2016. Fosforuitmijning: Gewasselectie ter verschraling van voormalig landbouwgebied voor natuurontwikkeling. In Startersdag in het bos- en natuuronderzoek. Brussels, Belgium

Bracke J, **Schelfhout S**, Debersaques F, Baert G, Mertens J. 2015. Verkennende studie van de regenworm als bodemingenieur in private moestuinen. In Startersdag in het bos- en natuuronderzoek. Brussels, Belgium

De Beelde R, **Schelfhout S**, De Schrijver A, Verheyen K, Haesaert G, Mertens J. 2013. Uitmijnen van fosfor in functie van natuurontwikkeling op voormalige landbouwgronden: Potexperiment met twaalf gewassen op een bodem-P-chronosequentie. In Startersdag in het bos- en natuuronderzoek. Brussels, Belgium

Schelfhout S, Du Pré T, De Schrijver A, Verheyen K, Haesaert G, De Bolle S, Mertens J. 2012. Phytoextraction of Phosphorus for Ecological Restoration: Application of Soil Additives. In 4th International EcoSummit. Ohio, USA

Schelfhout S, Du Pré T, De Schrijver A, Verheyen K, De Bolle S, Mertens J. 2012. Uitmijnen van fosfor: een haalbare maatregel voor natuurontwikkeling op voormalige landbouwgronden? Optimalisatie met chemische en biologische bodemaddities. In Startersdag in het Bos- en Natuuronderzoek. Brussels, Belgium

Participation in symposia or specialist trainings without presentation

20/08-24/08/2018	Best Practice in management and restoration of European dry grasslands. SER Europe Summer School on Ecological Restoration 2018. Hungary. By Melinda Halassy, Katalin Török (SER, MTA Centre for Ecological Research)
15/05/2018; 21, 28-29/06/2018	GrasGoed – graslandbeheer. Interreg project GrasGoed, Natuurlijk groen als grondstof. Zwarte beek, Koersel, Belgium and Blues in the Marshes, Cromvoirt, The Netherlands. By Willy Verbeke (Inverde)
13/02/2018	Het nieuwe natuurbeheerplan. By Liselot Ledene (Inverde)
26-27/08/2016	The role of soil seed banks in restoration. SER Europe student course. Freising, Germany. By Peter Török (University of Debrecen), Harald Albrecht (TUM Freising)
20/04/2016	An introduction to dplyr for R users. Flames workshop. Ghent, Belgium.

16/09/2015	Heideontwikkeling op voormalige landbouwgrond. Nationaal Park Dwingelderveld, Dwingeloo, Nederland.
20/11/2014	Netherlands Annual Ecology Meeting (NERN). Wageningen, the Netherlands.
07/11/2014	Demonstratie: gras maaien voor vergisting? Deinze, Belgium. By Inverde.
05-06/06/2014	Graslanden in de Franse Maasvallei. Stenay, France. By Willy Verbeke (Inverde)
25/02-1/03/2013	Workshop on taxonomy and systematic of arbuscular mycorrhizal fungi. Ghent, Belgium. By Chris Walker

Participation to other trainings

2nd semester 2018	Advanced Academic English: Writing skills – (Bioscience) Engineering. Ghent, Belgium. By Tom De Moor
2nd semester 2018	Grow Your Future Career. Ghent, Belgium. By Robin Lefebvre
26/03/2014	Doeltreffend presenteren. Leuven, Belgium. By Jean-Luc Doumont
04/02/2015	Basisassistententraining. Ghent, Belgium.
12/02/2015	Practicumtraining. Ghent Belgium.
14, 28/11/2014	Schrijven voor niet-vakgenoten en pers. Ghent, Belgium. By Ann De Ron
11-15/02/2013	What you need to know to be effective in writing and publishing your work. Louvain-La-Neuve, Belgium. Michael Hochberg (Monpellier University)

Other activities and relevant memberships

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Consulting experience

Provoost S, Vangansbeke P, Raman M, D'Hulster F, **Schelfhout S**, Verheyen K, De Schrijver A. 2018. Referentieonderzoek nutriënten voor bepalen van de kwaliteit van Europees beschermde duinhabitats. Hoe problematisch zijn fosfaten aan de kust? Opdrachtgever: ANB

Mertens J, **Schelfhout S**, De Schrijver A. Maart 2018. Advisering over natuurherstel in het militair domein Tielenkamp. Opdrachtgever: ANB

Schelfhout S, Vangansbeke P, De Schrijver A, Mertens J. 2017. Bondige rapportage: Herstel van heischrale graslanden en droge heide via uitmijnen in Maasmechelen. Opdrachtgever: ABO group

Schelfhout S, Vangansbeke P, De Schrijver A, Mertens J. 2017. Advisering over Het Herstel Van Soortenrijke Graslanden via Maaien En Uitmijnen in De Oude Kale Vallei. Opdrachtgever: Natuurpunt Lovendegem

Schelfhout S, Vangansbeke P, De Schrijver A, Mertens J. 2017. Advisering over Het Herstel Van Soortenrijke Graslanden via Maaien En Uitmijnen in De Lange Velden En Drongen. Opdrachtgever: Stad Gent

Vangansbeke P, De Schrijver A, **Schelfhout S**, Verheyen K. 2017. Onderzoek Naar Methodes Voor Abiotisch Herstel Van Soortenrijke Graslanden in Het LIFE-project Pays Mosan. Opdrachtgever: Life Pays Mosan

Demey A, De Schrijver A, **Schelfhout S**, Verheyen K. 2014. NIP Fondatie-Heernisse: Expertenadvies Vegetatieontwikkeling. Opdrachtgever: ANB

De Schrijver A, **Schelfhout**, Verheyen K. 2013. Onderzoek Naar Mogelijkheden Voor Creatie Van Open Water in De Depressie Van De Moervaart (zone Wulfsdonk) in Relatie Tot Fosfor. Opdrachtgever: ANB

De Schrijver A, **Schelfhout S**, Verheyen K. 2013. Bodemonderzoek naar de potenties voor herstel en ontwikkeling van soortenrijk grasland Bos van Aa. Opdrachtgever: Waterwegen & Zeekanaal

De Schrijver A, **Schelfhout S**, Verheyen K. 2013. Onderzoek Naar De Potenties Voor Herstel En Ontwikkeling Van Glanshavergrasland Met Grote Pimpernel in De Gebieden Pikhaken–Hollaken En Dorent. Opdrachtgever: ANB

De Schrijver A, **Schelfhout S**, Verheyen K. 2012. Onderzoek Naar Mogelijkheden Voor Natuurontwikkeling in De Depressie Van De Moervaart in Relatie Tot Fosfor. Opdrachtgever: ANB

Teaching experience

Excursions and practica as Teaching Assistant

Master	In Master of Science in de Biowetenschappen: land- en tuinbouwkunde optie landschaps- en groenbeheer
2012-Present	Natuurontwikkeling by Jan Mertens **Groenbeheer** by Jan Mertens **Bosbeheer** by Jan Mertens **Bodemecologie** by Jan Mertens
2012, 2014, 2015	Organisation of international student excursions
Bachelor	In Bachelor of Science in de biowetenschappen (2nd year)
2012-Present	**Ecologie** by Jan Mertens

Tutor of master dissertations

2018	Lars Allein: Natuurhersteltechnieken op voormalige landbouwgrond
2016	Frederik Van Daele: Below-ground effects on plant traits, competition and community composition in the restoration of *Nardetea* grasslands (guidance practical work)
2016	Thierry Heyman: Optimale gewaskeuze en –rotatie voor het uitmijnen van fosfor
2015	Jolien Bracke: Verkennende studie van de regenworm als bodemingenieur in private moestuinen
2014	Kjell Bastiaenssens: Optimalisatie van een mesocosm naar effecten van fosfor en bodemleven op plantengemeenschappen
2014	Pieter Joos: Ontwikkeling van blauwgrasland: het effect van stikstofbemesting op de fosforafvoer
2013	Christophe Cousaert: Verhoogde vatbaarheid van Pleiocheita setosa bij lupines tijdens het uitmijnen?
2013	Robbe De Beelde: Uitmijnen van fosfor in functie van natuurontwikkeling op voormalige landbouwgronden: gewasselectie
2013	Tom Du Pré: Natuurherstel via fosfor-uitmijnen: effect van bodemaddities over een bodem-fosfor-chronosequentie

Tutor of bachelor dissertations

2017	Sander Bombeke, Sofie Dejaegher. Herintroductie van een dotterbloemgrasland in het buitenlabo Overmeersen
2017	Tomas Boone, Emma Duthoo, Berdien Van De Velde. Nutriëntenstromen in (moes)tuinen
2015	Bert Thienpondt, Corneel Martens, Joachim Van De Steen: *Op naar een groene stad met leefstraten: potproef*
2014	Nina Sarens, Anthony Denayer and Wietse Bas: Kieming en overleving van flora uit heischrale graslanden
2014	Eline Glorieux, Emmy Decadt and Herlinde Vanheule: Uitmijnen van fosfor uit de bodem: effect van 12 gewassen op de drogestofproductie en fosforafvoer van Engels raaigras
2014	Evelien Stautemans. De overleving van fosfaatsolubiliserende bacteriën in bodems met verschillend fosfaatgehalte
2012	Christophe Cousaert, Robbe De Beelde, Korneel Neysens. Uitmijnen van fosfor in functie van natuurontwikkeling op voormalige landbouwgronden: effect van AMF en PSB's

Miscellaneous

Teaching course for beekeepers: *De Bijenweide* (translated: *Flora for bees*). In Deinze (2015), Gent (2014, 2015, 2016, 2017 and 2018), Laarne (2018).

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