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RESEARCH ARTICLE

Phosphorus mining for ecological restoration on former agricultural land

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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 are 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 species-rich *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 µg P_{Olsen}/g), P-removal rate was high but bioavailable soil-P did not decrease. At lower soil P-concentrations (64 and 36 µg P_{Olsen}/g), 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 (<10 µg P_{Olsen}/g) 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.

Key words: arbuscular mycorrhiza, humic substances, nardus grassland, phosphate-solubilizing bacteria, phytoextraction, plant-growth-promoting rhizobacteria

Implications for Practice

- P-removal will slow down in time. Calculations of the restoration time needed to reach low soil P-concentrations with P-mining need to take that into account.
- The addition of arbuscular mycorrhiza, humic substances, or phosphate-solubilizing bacteria seems not to enhance P-removal.

Introduction

To bring the worldwide loss of species to a halt, habitat restoration is required (Millennium Ecosystem Assessment 2005). However, restoration of species-rich habitat types in industrialized countries is often difficult because of the high soil nutrient content resulting from past fertilization in an agricultural context (Marrs 1993). As phosphorus (P) is one of the least mobile mineral nutrients (Stevenson & Cole 1999), many agricultural soils have large reserves (Sattari et al. 2012). This is problematic for nature restoration because elevated soil P-concentrations have been shown to be detrimental for restoring species-rich grasslands (Gilbert et al. 2009; Ceulemans et al. 2011; Merunkova & Chytry 2012). High biodiversity is related to P-limitation because more endangered species persist in P-limited soils (Wassen et al. 2005) and the total species number seem to be higher in P-limited and P and N co-limited grasslands (Olde Venterink 2011; Ceulemans et al. 2013). Typical postagricultural fields often have bioavailable P-concentrations of more than 80 µg P_{Olsen}/g (Barberis et al. 1996). While the target in this study, species-rich semi-natural *Nardus* grassland mostly has P-concentrations of less than 10 µg P_{Olsen}/g (Gilbert et al. 2009; Raman et al. 2014, in preparation). It remains unclear whether this habitat type might also be established at slightly higher bioavailable P-concentrations (Ceulemans et al. 2014), but severe P-depletion of ex-agricultural fields is essential.

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Several methods are being used in current ecological restoration practice to reduce the P-stocks of former agricultural soils. Topsoil removal is an expensive measure (on average €75,000/ha for restoration projects in Belgium, Anonymous 2012), removing also the seed bank, most of the soil buffer capacity and the soil biota. This might be disadvantageous for successful reestablishment of species-rich grasslands both in the short term and long term (Carbajo et al. 2011; Brinkman et al. 2012). A second technique frequently used to reduce soil fertility is mowing with removal of the cut hay. Grasslands that were intensively fertilized might produce more than 15 ton dry mass of biomass per year (DM/year, Pegtel et al. 1996). After several years of mowing without fertilization, however, biomass production significantly decreases due to limitation of other nutrients than P, namely N (Van Der Woude et al. 1994; Smits et al. 2008) and/or potassium (K) (Oelmann et al. 2009). Then, biomass production generally falls back from 15 to 5 ton DM/year or lower (Berendse et al. 1992; Pegtel et al. 1996; Pavlu et al. 2011). The latter is still too high for creating the right circumstances with enough light for the germination of subordinate, often red-listed target species. Consequently, with lower biomass production, P-removal rates decline from about 45 to 6 kg P/ha (Pegtel et al. 1996) and, therefore, this restoration method might take several decades.

A third method for removing P from the soil is the technique of P-mining, suggested by Marrs (1993) and Crawley et al. (2005). With this adjusted agricultural technique, soils are depleted of P by harvesting crops whereby biomass production, and hence P-removal rate, is optimized by fertilization with nutrients, other than P. This technique is intended to prepare the required abiotic soil conditions for nature restoration, and as such it can be part of a guided transition from agriculture to nature. However, little experimental knowledge is available on how fast P can be removed (MacDonald et al. 2012), if it can be removed at all because modeling analysis suggested limited P-removal might occur over time using this technique (Perring et al. 2009). During the P-mining process, it can be expected that biomass production diminishes as a consequence of depleting the bioavailable soil P-pool. Also, the diffusion of P through the soil is much slower than the rate of uptake into the roots. P becomes depleted in the rhizosphere, limiting plant growth and consequently P-removal (Koopmans et al. 2004).

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 (PSB). Mycorrhiza can provide P to the plant by overcoming the P-depletion zone in the rhizosphere with an extensive hyphal network (Smith & Read 2008). Humic substances are humic and fulvic acids that can mobilize and solubilize P by (1) blocking P-adsorption sites (ligand exchange), (2) oxide dissolution by complexing aluminum (Al) or iron (Fe) held in minerals, (3) mobilization of P held in metal-humic substances, or (4) via alteration of the soil pH (Gyaneshwar et al. 2002; Drouillon & 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 & Bakhashwain 2013). PSB 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 & Fraga 1999; Rosas et al. 2006). 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. 2013).

Here we present 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 toward 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.

Methods

A pot experiment was conducted to test the effect of (1) the soil P-concentration and (2) additions of mycorrhiza, humic substances and P-solubilizing bacteria on biomass production, P-concentrations, P-removal, and final plant available P-concentrations.

Soil Collection and Experiment Initiation

The soil originated from three sites located within a 500-m 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 & Janák 2008). Non-degraded *Nardus* grasslands are closed dry or mesophile perennial grasslands on oligotrophic soils that have a bioavailable P-concentration of less than 10 µg P_{Olsen}/g (Raman et al. 2014, in preparation); this will be considered as the target in this paper. The three sandy soils were selected based on their bioavailable P-concentrations: 111 µg P_{Olsen}/g further referred to as "High-P," 64 µg P_{Olsen}/g further referred to as "Mid-P" and 36 µg P_{Olsen}/g further referred to as "Low-P" (Table 1), and represented three phases in the development toward P-poor soil conditions (10 µg P_{Olsen}/g), that is a soil P-chronosequence. The three sites are currently managed as hay meadows and annually cropped as they were taken out of agriculture. In the low-P site, fertilization stopped about 20 years ago. The mid-P and high-P sites were taken out of agriculture 5 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 to 15 cm soil layer to avoid the plant roots from the top 0 to 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

Table 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′.2″E	51°19′58.4″N 5°0′57.4″E	51°20′0.1″N 5°1.7″E
P_{CaCl2} (µg/g)	7.00	0.93	0.23
P_{Olsen} (µg/g)	111	64	36
P_{Ox} (µg/g)	414	196	59.4
Al_{Ox} (µg/g)	619	543	380
Fe_{Ox} (µg/g)	349	246	175
PSD (%)	46	26	11
P_{Total} (µg/g)	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} (µg/g)	16.2	10.2	14.7
Ca_{BaCl2} (µg/g)	741	355	128
Mg_{BaCl2} (µg/g)	62.4	30.2	9.85
Na_{BaCl2} (µg/g)	2.49	2.49	4.00
Al_{BaCl2} (µg/g)	30.0	52.0	136
CEC (meq/kg)	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).

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.) was sown on 18 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.

Fertilization

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 Table S1 in Appendix S1, Supporting Information). In total, 351 mg N and 468 mg K were added per pot, corresponding with 198 kg N/ha and 319 kg K_2O/ha 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, mid-P 434 mg/pot, and low-P 602 mg/pot).

Biostimulant Additions

Next to the control that only received N- and K-fertilization, four treatment levels of biostimulants were applied on day 1 of the experiment: (1) the application of arbuscular mycorrhiza, (2 and 3) addition of humic substances at two levels (HS1 and HS2), and (4) application of PSB. For the mycorrhiza treatment, the commercially available product *INOQ Agri* was used (from INOQ GmbH, Schnega, Germany). This contained three species of the family 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 L/ha, the recommended dose by the producer) and HS2 with 7 mL Humifirst per pot (7,000 L/ha, 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 PSB was done as described by De Bolle et al. (2013) and consisted of a mixture of *Bacillus brevis* (ATCC 8246), *Pseudomonas 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) per gram of dry soil, based on the population size of PSB in soils as found by Hu et al. (2009).

Germination

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

Root-Colonization by Arbuscular Mycorrhiza

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

Survival of PSB in Soil

To determine the prevalence of the PSB 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 *Pseudomonas 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 S2). Overall, the amount of CFUs per gram of soil decreased from circa 3×10^7 to 2×10^5 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 & Yu et al. 2011).

Sampling and Chemical Analysis

To get an insight in how the three soils differ in chemical composition, initial soil characteristics were assessed on subsamples 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, United Kingdom, 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) 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). Furthermore, we quantified the original soil P-concentrations by analyzing bulk samples on:

(1) Soluble and readily soluble P extracted in $CaCl_2$ (P_{CaCl_2}; Simonis & Setatou 1996) and measured by ICP (iCAP 6000 series, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA);
(2) Bioavailable P that 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);
(3) Active 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_{Ox}, Al_{Ox} and Fe_{Ox}; 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
(4) 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 (2). Grass was cut 2 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 also obtained 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 1,150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Hanau, 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 S3).

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 $PSD = [P_{ox}/0.5 \times (Al_{ox} + Fe_{ox})] \times 100(\%)$ with P_{ox}, Al_{ox}, and Fe_{ox} representing the numbers of moles of P, Al, and Fe per kg of soil extracted with ammoniumoxalate-oxalic acid (see section 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/L 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/L 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) at day 0 and day 123 were calculated by multiplying the bioavailable P-concentration (in µg P_{Olsen}/g) at that time with the amount of soil per pot (1.4 kg).

Table 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. 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.

	Soil P-Concentration		*Biostimulant Addition*		*Soil P-Concentration**		
	f	p	f	p	f	p	*Error*
Total biomass production	144.8	***	21.7	***	11.2	***	
Mean P-concentration	450.8	***	18.7	***	1.3	ns	
Total P-removal	1,675.5	***	5.0	**	21.9	***	
Final bioavailable P-concentration	4,284.9	***	5.4	***	1.5	ns	
Initial–final bioavailable P-stock	65.7	***	5.9	***	1.9	ns	
Degrees of freedom	2		4		8		88

Table 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. All pots received N- and K-fertilization, more additions were as follows: arbuscular mycorrhiza; humic substances at 50 L/ha; humic substances at 7,000 L/ha; 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 less than 10 µg P_{Olsen}/g (Raman et al. 2014, in preparation).

	Soil P-Concentration		*P-Mining*	*P-Mining + Arbuscular Mycorrhiza*	*P-Mining + Humic Substances (50 L/ha)*	*P-Mining + Humic Substances (7,000 L/ha)*	*P-Mining + Phosphate-Solubilizing Bacteria*
			B	*B*	*AB*	*A*	*C*
Total biomass production (g DM/pot)	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 ± 0.66a	3.89 ± 0.53a	3.86 ± 0.53a	4.16 ± 0.59a	1.70 ± 0.43b
	Low-P	C	3.09 ± 0.48b	2.64 ± 0.44bc	2.76 ± 0.30bc	4.11 ± 0.37a	1.88 ± 0.27c
			BC	BC	C	B	A
Mean biomass P-concentration (mg P/g DM)	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
			B	C	C	C	A
Final bioavailable P-concentration (µg P_{Olsen}/g dry soil) at 123 days	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

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 analysis of variance (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.

Results

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 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 2 & 3). The further decrease in P-removal from mid-P to low-P was not significant (Fig. 1).

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. 1). In

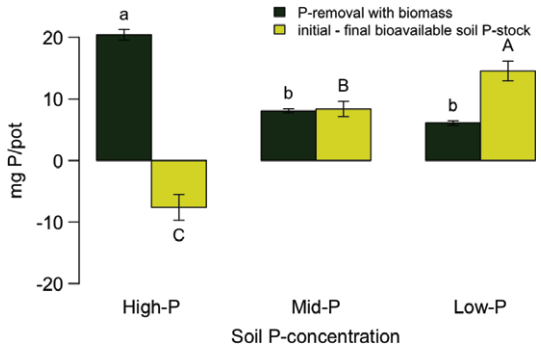

Figure 1. In high-P, P-removal did not result in a decline of bioavailable soil-P, whereas in mid-P, P-removal was equal to the decrease in bioavailable soil-P. In low-P, bioavailable soil P-stocks decreased more than the amount of P that was removed with biomass. Mean ± SE ($n = 7$) of change in bioavailable soil P-stocks (initial−final bioavailable soil P-stock) and total P-removal with *Lolium perenne* over 123 days (mg P/pot). Upper- and lowercase letters, respectively, indicate significant differences between mean changes in bioavailable soil-P stocks and mean P-removal with biomass (Table 2).

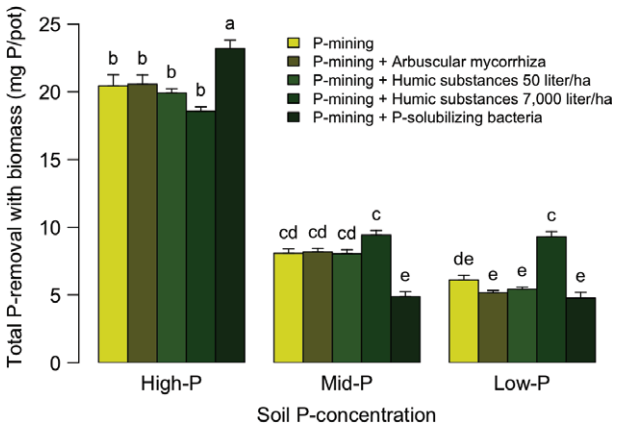

Figure 2. Mean + SE ($n = 7$) of the total P-removal with *Lolium perenne* over 123 days (mg P/pot). High-P contains 111 µg P_{Olsen}/g, mid-P 64 µg P_{Olsen}/g and low-P 36 µg P_{Olsen}/g. Letters indicate significant differences between all means ($p < 0.05$) based on a Tukey HSD post hoc test following a two-way ANOVA (Table 2).

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.

Effects of Biostimulants on P-Removal

Although biostimulants had little overall effects on P-removal in biomass (Fig. 2), there were some interesting effects of biostimulants at specific soil P-concentrations. In High-P, the addition of PSB resulted in the highest P-removal compared with 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 PSB.

Effects of Biostimulants on Bioavailable P in Soil

Final bioavailable P-concentrations on day 123 were significantly affected by the addition of biostimulants (Tables 2 & 3). Over all soil P-levels, addition with PSB resulted in higher bioavailable P-concentrations than the control. With the other biostimulants, final bioavailable P-concentrations were lower.

Discussion

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 (Table S2 in Appendix S3). 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 *Lolium 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.

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 immobilization 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 (remineralization, release of microbial-P after cell death) and an important sink (immobilization, 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.

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 PSB. The biostimulants significantly increased P-removal in only two cases: PSB 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 PSB 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 & Graham 2002) and that inoculation is not always successful. Therefore, the experiment was repeated using a different application method for mycorrhiza and PSB. 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 S4, Table S4). In contrast to our results, a sterile low-P soil (37 µg P_{Olsen}/g) inoculated with *Glomus intraradices* and sown with *Lolium 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, whereas 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 *Glomus mosseae* in a soil very poor in P (4.5 µg P_{Olsen}/g) 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 & 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 L/ha, much higher than the standard dose (HS1: 50 L/ha), and therefore, very expensive. This low dose neither improves 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 and Kaplan (2003) with strawberries, but in contrast with field experiments where positive effects of low HS dosage on biomass production and P-uptake were observed (Sharif 2002; Verlinden et al. 2009, 2010; Daur & 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 PSB in our experiment. Recently, a pot experiment gave insight in the positive effects of adding *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. 2013). However, we should be cautious with concluding that PSB addition can have beneficial effects on crop growth, as 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 to 23.2 g P/pot) by the addition of PSB. 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 PSB addition. But these organisms may be a useful tool at soils with high P-concentrations. However, the addition of PSB to fields may still be unrealistic as 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.

P-Mining as an Ecological Restoration Technique

Whether P-mining is the best technique to restore species-rich habitat types on former agricultural land depends on two factors: (1) the initial directly (P_{Olsen}) and indirectly (P_{Ox}) bioavailable soil P-concentrations and (2) the targeted P-concentration. Uncertainty exists around the target P-concentration of species-rich habitat types because there is some evidence that high biodiversity can also be obtained at slightly higher P-concentrations (Ceulemans et al. 2014). Neverteless, in the context of restoration on ex-agricultural fields, severe

P-depletion is essential because due to fertilization history soil P-concentrations are enriched significantly. Assuming that the original P-poor conditions for *Nardus* grassland (<10 µg P_{Olsen}/g, Raman et al. 2014, in preparation) should be reached during restoration, e.g. on a high-P soil, 1,500 kg P/ha should be removed from the 0–30 cm soil layer (see also Appendix S5). When mowing without any fertilization is used as a nutrient-depleting technique, the restoration time will be 150 years (1,500 kg P/ha to remove, annual P-removal of 10 kg/ha; Oelmann et al. 2009). In contrast, with P-mining, that is mowing plus N- and K-fertilization to retain high biomass production, the theoretical restoration time will be only 33 years, assuming P-removal would not slow down over time (1,500 kg P/ha to remove, annual P-removal of 45 kg/ha; Gallet et al. 2003). However, from our results, it is clear that the actual P-removal time with P-mining 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. Calculations of the time needed to reach low soil P-concentrations with P-mining need to take this into account. We do not know how long exactly how long it would take to restore low P-concentrations with P-mining; to estimate 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.

Aside from that, next to time needed for the restoration of P-poor soil conditions, also time for the restoration of the specific belowground (Kardol et al. 2009; Maltz & Treseder 2015, in press) and aboveground biota is necessary. Therefore, we suggest that in later, less-efficient, stages of P-mining, it would be better to change the management to mowing with removal of biomass and without any further fertilization. A change to less productive species better adapted to P-poor soil conditions is essential and can happen naturally through succession or through seeding or hay transfer from donor fields (Kiehl et al. 2010).

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Supporting Information

The following information may be found in the online version of this article:

Appendix S1. Nitrogen and potassium fertilization.
Appendix S2. Additional pot experiment for testing the survival of added phosphate-solubilizing bacteria (PSB) in the three soil P-concentrations.
Appendix S3. Nutrient limitations in the main pot experiment.
Appendix S4. Additional pot experiment designed for inoculation testing.
Appendix S5. Estimation of time needed to restore the P-poor conditions on a P-rich ex-agricultural field.

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