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Interactions of nitrogen and phosphorus cycling promote P acquisition and explain synergistic plant-growth responses

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Abstract. Plant growth is often co-limited by nitrogen (N) and phosphorus (P). Plants might use one element to acquire another (i.e., trading N for P and P for N), which potentially explains synergistic growth responses to NP addition. We studied a 66-yr-old grassland experiment in South Africa that consists of four levels of N addition with and without P addition. We investigated the response of aboveground net primary production (ANPP) to N and P addition over the last 66 yr. Further, we tested whether phosphatase activity and plant P uptake depend on N availability, and vice versa, whether non-symbiotic N_2 fixation and plant N uptake depend on P availability. We expected that the interaction of both elements promote processes of nutrient acquisition and contribute to synergistic plant growth effects in response to NP addition. We found synergistic N and P co-limitation of ANPP for the period from 1951 to 2017 but the response to N and P addition diminished over time. In 2017, aboveground P stocks, relative rRNA operon abundance of arbuscular mycorrhizal fungi, and soil organic P storage increased with N fertilization rate when N was added with P compared to the treatment in which only N was added. Further, N addition increased phosphatase activity, which indicates that plants used N to acquire P from organic sources. In contrast, aboveground N stocks and non-symbiotic N_2 fixation did not change significantly due to P addition. Taken together, our results indicate that trading N for P likely contributes to synergistic plant-growth response. Plants used added N to mobilize and take up P from organic sources, inducing stronger recycling of P and making the plant community less sensitive to external nutrient inputs. The latter could explain why indications of synergistic co-limitation diminished over time, which is usually overlooked in short-term nutrient addition experiments.

Key words: ecological stoichiometry; N and P co-limitation; N and P trade-offs; non-symbiotic N_2 fixation; phosphatase activity; plant N and P uptake.

INTRODUCTION

In grassland ecosystems, plant communities are predominantly co-limited by N and P and often show a synergistic growth response when N and P are added together (Elser et al. 2007). This was also found by Fay et al. (2015) and Harpole et al. (2016) who showed, based on 40 grassland sites, that plant productivity increased with the number of nutrients added, highlighting the role of multiple resource limitations for plant productivity. Different concepts of N and P co-limitation have been proposed (Harpole et al. 2011, Townsend et al. 2011), but the terminology covering how primary consumers respond to multiple resource addition is often used inconsistently in the literature. In this study, we refer to "co-limitations" when combined N and P addition increase primary productivity to a greater extent than the single addition of N or P (NP > N and NP > P) and we refer to "synergistic effects" and "synergistic co-limitation" when the increase in primary productivity caused by combined N and P addition is larger than the sum of the increases caused by single N and single P addition (NP > N+P; for graphical illustration, see Appendix S1: Fig. S1).

The mechanisms that cause synergistic responses of plant growth to multiple element addition are not well understood (Davidson and Howarth 2007). Potential explanations could be that plants and microbes adapt mechanisms of element uptake or change allocation patterns, in the way that they invest one element they have in excess into the acquisition of a limiting element until their growth is equally limited by both elements (Bloom

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et al. 1985). Recent studies have demonstrated that interactions between N and P may promote plant nutrition (Ohkama-Ohtsu and Wasaki 2010), e.g., through shaping the root architecture via root branching (Chevalier et al. 2003, Desnos 2008), increasing the rates of arbuscular mycorrhiza fungi colonization (Nasto et al. 2014) or upregulating the active transport system for inorganic N and P uptake (Zeng et al. 2012). In addition, plants and microbes might trade one element to acquire another (e.g., trading N for P and P for N). Two important nutrient acquisition processes that may depend on interrelationships of N and P availability are the fixation of atmospheric N_2 and the mineralization of organic P through phosphatase enzymes.

Symbiotic and non-symbiotic N_2 fixation turns the atmospheric N_2 into reactive N, and renders it available for biota (Vitousek et al. 2013). Fixation of N_2 is catalyzed by the nitrogenase enzyme complex (Vitousek et al. 2002, Reed et al. 2011), which requires 16 moles of ATP to reduce one mole of N_2 (Simpson and Burris 1984). Consequently, N_2 fixation goes along with high energetic costs and an increased P demand required to synthesize ATP (Vitousek et al. 2002, Reed et al. 2011). Hence, symbiotic and non-symbiotic N_2 fixation rates have often been shown to increase with higher P availability, whereas N inputs usually decrease N_2 fixation, as it is more economic for plants and microorganisms to utilize reactive N than to fix N_2 (Eisele et al. 1989, Smith 1992, Hartley and Schlesinger 2002). However, for tropical forests it has been found that symbiotic N_2 fixation does not necessarily increase or decrease in response to high P or N availability, respectively, which therefore has been described as a biogeochemical paradox (Hedin et al. 2009). Reasons for this could be that some legumes are not capable of downregulating N_2 fixation (e.g., obligate N_2 fixers) or that symbiotic N_2 fixation is advantageous for P acquisition in P-poor but N-rich ecosystems (Hedin et al. 2009, Nasto et al. 2014).

While N_2 fixation may depend on P availability, the mobilization of organic P depends on available N because exoenzymes that catalyze P mineralization contain N (Spohn 2016). Thus, organisms can use N to synthesize phosphatase enzymes promoting P acquisition. For instance, Nasto et al. (2014) showed for a lowland tropical rainforest that symbiotic N_2 fixers cause higher phosphatase activities than non-fixing plants. Furthermore, several studies reported a higher soil phosphatase activity under elevated N inputs, which might increase the pool of plant-available P through mineralization of organic P (Olander and Vitousek 2000, Allison and Vitousek 2005, Marklein and Houlton 2012, Heuck et al. 2018, Widdig et al. 2019). In contrast, addition of inorganic P to soil decreases phosphatase activity because organisms stop investing into phosphatase production once their P demand is covered by inorganic P (Spiers and McGill 1979, Olander and Vitousek 2000). Taken together, several studies have demonstrated that organisms may trade N for P, and vice versa P for N through adjusting rates of soil N and P acquisition processes. However, whether these interactions of N and P promote aboveground net primary production (ANPP) and thereby cause synergistic growth effects in response to NP addition is less well studied.

The aims of this study were to elucidate the impact of changing soil N and P availabilities on (1) mechanisms of N and P acquisition and (2) their effects on ANPP and plant N and P uptake. For this purpose, we investigated a 66-yr-old nutrient addition experiment in a mesic grassland in South Africa. Previous research has shown that vegetation in this grassland was N and P co-limited with a tendency toward synergistic responses of ANPP to combined NP addition in the period from 1951 to 1980 (Fynn and O'Connor 2005). We hypothesized, first, that ANPP in this grassland shows synergistic N and P co-limitation in the period from 1951 to 2017 (hypothesis 1). Second, we expected that the increase in ANPP upon NP addition is promoted by interactions in N and P acquisition processes. Specifically, we hypothesized that that plants and microorganisms use added N to acquire P from organic P sources in soil by releasing larger amounts of phosphatases or increasing mechanisms of plant P uptake (hypothesis 2). Vice versa, we hypothesized that plants and microbes use added P to acquire increased amounts of N through non-symbiotic N_2 fixation and increased plant N uptake (hypothesis 3).

Material and Methods

Study area

The experiment was carried out in a mesic grassland located at the Ukulinga Research Farm close to Pietermaritzburg city, KwaZulu-Natal, South Africa (29°24′ E, 30°25′ S). The vegetation is classified as a southern KwaZulu-Natal moist grassland Mucina and Rutherford 2006), and the dominant plant species are *Themeda triandra* Forssk., *Heteropogon contortus* L., and *Tristachya leucothrix* Trin ex. Nees, which are interspersed with C_3 trees (e.g., *Acacia sieberiana* DC.; Fynn and O'Connor 2005). However, trees were absent in the experimental area (covering about 5,000 m^2). The grassland hosts a relatively high plant diversity with 9 different grass species/m^2 and 14 different forb species/m^2 (Zeglin et al. 2007), and the ANPP averages approximately 310 $g \cdot m^{-2} \cdot yr^{-1}$ considering the period from 1951 to 1980 (Fynn and O'Connor 2005). The mean annual precipitation is 790 mm, and about 80% of the rain falls during the summer months from October to April. Mean monthly minimum and maximum temperatures range between 8.8°C (July) and 26.4°C (February; Fynn and O'Connor 2005). The site is situated on the top of a hilly terrain with an elevation of ~840 m above sea level on a slightly southeast facing slope. According to the world reference base of soils (WRB) developed by the FAO the soils are classified as (plinthic) Acrisols

overlaying shales of the Karoo sedimentary sequence (Fynn and O'Connor 2005, IUSS Working Group WRB 2015). Soils are shallow (0.6–1.0 m) with a silty-clay texture (5% sand, 46% silt, 49% clay), moderately acidic pH values (pH in H_2O 5.5), and high stocks of total organic carbon (TOC; 7.3 kg C/m^2) and total nitrogen (TN; 0.47 kg N/m^2) in the upper 15 cm (Schleuss et al. 2019). The site is mown each year, and the biomass is removed. Even though fire and grazing are frequent and natural components of South African grasslands, both were eliminated since the onset of the nutrient addition experiment.

Experimental design and sampling

The long-term nutrient addition experiment started in 1951, and since then N and P have been added each year. The component of the experiment sampled for this study consists of four N addition levels each with and without P addition resulting in eight treatments, each replicated three times (in total 24 plots). The treatments include a control (N_0P_0, with no addition of any nutrient), a P addition treatment (N_0P_9 = 8.9 g $P \cdot m^{-2} \cdot yr^{-1}$), three N addition treatments without P (N_7P_0 = 7.1 g $N \cdot m^{-2} \cdot yr^{-1}$; $N_{14}P_0$ = 14.1 g $N \cdot m^{-2} \cdot yr^{-1}$; $N_{21}P_0$ = 21.2 g $N \cdot m^{-2} \cdot yr^{-1}$), and three combined N and P treatments with the same nutrient addition rates as stated above (N_7P_9; $N_{14}P_9$; $N_{21}P_9$). Nitrogen was supplied annually as limestone ammonium-nitrate (28% N) and P was added annually as super-phosphate (10.5% P). All experimental plots (9 × 2.7 m) were randomly arranged in a block design with a minimum distance of 1 m between the plots. Aboveground NPP was measured by harvesting a 2.13 m wide strip across the 2.7 m wide plot in winter 2017 (and in the same way for all previous years, too), giving a total area sampled per plot of 5.75 m^2. In February 2017, we took six soil samples per plot from the upper 15 cm based on an equidistant sampling design using a 3.5 cm diameter soil corer. The six soil samples per plot were pooled, resulting in one mixed sample per plot. All soil samples were stored in plastic bags at room temperature and were transferred to the University of Bayreuth within one week after sampling.

Soil, plant, and microbial analyses

Fresh aboveground biomass was dried (60°C) and subsequently weighed. Moist soil samples were sieved (<2 mm) and roots, stolons, and rhizomes were removed. Soil was dried at 50°C. Soil water content and soil water holding capacity (WHC) were determined gravimetrically using field moist samples. For the determination of the maximum WHC, field moist samples were oversaturated, then left for 24 h on a sand bath until maximum WHC was reached, and finally samples were weighed before and after drying at 105°C (Öhlinger 1996). Further, fresh soil of each plot was adjusted to a WHC of 60% and preincubated for 5 d at 15°C before subsequent measurements (i.e., for soil water extracts, phosphatase activities, and non-symbiotic N_2 fixation; see below).

For the determination of total organic C (TOC) and total N (TN) concentrations of soil and aboveground biomass, milled material was measured with an element analyzer (Vario Max Elementar, Hanau, Germany). Total P (TP) concentrations of soil and aboveground biomass were determined using an inductively coupled plasma-optical emission spectroscopy (ICP-OES; Vista-Pro radial, Varian) after a pressure digestion in aqua-regia (HNO_3 + 3 HCl) and concentrated nitric acid (HNO_3), respectively.

For measurements of element concentrations in soil water extracts a dry-mass equivalent of 20 g soil was extracted in 80 mL distilled water and filtered (0.45 μm). The filtrated water extracts were measured for total dissolved N (DN; TOC-TN Analyzer, Jena Analytics, Jena, Germany), dissolved inorganic N (DIN; NH_4^+ measured via flow injection analysis, FIA-Lab, MLE Dresden, Dresden Germany; and NO_3^- measured via ion chromatography, Metrohm 881 Compact IC pro, Herisau, Switzerland), dissolved organic C (DOC; TOC-TN Analyzer, Jena Analytics), and dissolved inorganic P (DIP; UV 1800, Shimadzu, Kyoto, Japan).

Total organic P.—Total organic P (TOP) was determined using a differential method proposed by Saunders and Williams (1955). Milled soil samples were separated in two aliquots, each of 1 g. The first aliquots were directly extracted with 0.5 mol/L H_2SO_4 in a horizontal shaker for 16 h. The other aliquots were ignited at 550°C for 24 h and subsequently extracted in H_2SO_4 in the same way. Inorganic P was measured in the extracts by the molybdenum blue method according to Murphy and Riley (1962) using a spectrophotometer (UV-1800, Shimadzu Corporation). Total organic P was calculated as difference between inorganic P in ignited and non-ignited samples.

Non-symbiotic N_2 fixation.—Atmospheric N_2 fixation by free-living microorganisms was measured based on a stable isotope approach using 99.8 atom% $^{15}N_2$ (Zechmeister-Boltenstern 1996). Soil (4 g dry-mass equivalent) was added to a 12 mL exetainer (Labco, Lampeter, UK). All exetainers were flushed with argon, evacuated, and finally filled with 7.2 mL $^{15}N_2$ and 0.8 mL O_2. For each step, pressure changes were noted to ensure a precise calculation of the artificial atmosphere composition (average composition (v/v): $^{15}N_2$ = 72.5% ± 3.1%; O_2 = 8.2% ± 1.6%; Ar = 19.2% ± 1.5%). Samples were incubated in the dark at 15°C for 72 h and afterward dried at 50°C. Soil samples exposed to $^{15}N_2$ atmosphere and non-exposed controls were milled and analyzed for ^{15}N (Delta plus, Conflo III, Thermo Electron Cooperation, Bremen, Germany). The $\delta^{15}N$ signature was calculated using the isotope ratio of each sample (R_{sample} = $^{15}N/^{14}N$) and calculated as ^{15}N atom%. The $^{15}N_2$ fixation rate (in ng $N \cdot g\ soil^{-1} \cdot h^{-1}$) was estimated using

an isotope mixing model (Zechmeister-Boltenstern 1996):

$$^{15}\mathrm{N}^2\text{fixation rate}\,(\mathrm{ng\,N\,g\,soil^{-1}h^{-1}}) = \mathrm{TN}\,(\mathrm{mg\,N\,g\,soil^{-1}}) \times \frac{(^{15}\mathrm{N}^{\mathrm{labeled}}(\mathrm{atom\%}) - {}^{15}\mathrm{N}^{\mathrm{NA}}(\mathrm{atom\%}))}{100 * t(\mathrm{h})} \times 106$$

where TN is the total soil N (in mg N/g soil), $^{15}N_{labeled}$ (atom%) is the percentage of ^{15}N atoms in the labeled sample, $^{15}N_{NA}$ (atom%) is the percentage of ^{15}N atoms in the control samples, and t is the incubation time (in h).

Phosphatase activity

Phosphatase activity was determined according to German et al. (2011). A 1 g portion of moist soil was homogenized in 50 mL of sterile water by shaking for 20 minutes. The soil homogenates were pipetted into microplates and fluorescent substrate solution was added. Samples were preincubated in the dark at 15°C for 30 minutes, and subsequently measured fluorometrically after 0, 30, 60, and 180 minutes using a microplate reader (Infinite 200 PRO, TECAN, Mannedorf, Switzerland). Fluorescence was corrected for quenching of the soil as well as for the fluorescence of substrate and soil (German et al. 2011). Phosphatase activity was calculated from the slope of net fluorescence over incubation time in nmol·g $soil^{-1}·h^{-1}$ according to German et al. (2011).

Predicted relative abundance of AMF.—Abundance of arbuscular mycorrhizal fungi relative to the whole fungal community and predicted abundance of the *nifH* gene, which encodes the nitrogenase iron protein (*nifH*), relative to the prokaryotic gene profile were extracted from an amplicon sequencing-based survey of the same plots (Schleuss et al. 2019). As previously described in more detail (Schleuss et al. 2019), the ITS2 region of fungal rRNA operons and the V4 region of bacterial 16S rRNA genes were sequenced using Illumina amplicon sequencing technology (Illumina, San Diego, CA, USA). Raw reads (accessible as NCBI bioproject PRJNA517390) were trimmed and filtered and sequence variants were determined using the DADA2 workflow (Callahan et al. 2016) before taxonomic annotation against the UNITE v7.2 (Kõljalg et al. 2013) or SILVA v128 (Quast et al. 2012) databases. Functional annotations and predictions were performed using FunGuild and PanFP (Nguyen et al. 2016).

Statistics

Statistical analyses were carried out using SigmaPlot 13 (SYSTAT, San Jose, CA, USA) and R version 3.3 (R Development Core Team 2018). All statistical tests were considered to be significant at $P < 0.05$. Before implementing mixed linear models or ANOVAs, data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene test). If necessary, data were log- or square-root-transformed and retested. We used one-way ANOVA to test effects of single and combined N and P addition on ANPP for each single year and N addition level, separately. To analyze the overall effects of N and P addition on ANPP for the whole period (1951 to 2017) mixed linear models were used. We acknowledged repeated measurements of different years by selecting "year" as random factor. Further, ANPP data were separated into two periods (1, 1951–1979; 2, 1994–2017) to investigate the dynamics of ANPP responses. A Welch test was used to compare both periods to account for differences in sample sizes.

To test for effects on various plant and soil characteristics and to acknowledge the two factorial design (factor 1, N addition and factor 2, P addition) two-way ANOVAs were implemented. Two-way ANOVA was followed by a Tukey post hoc test for multiple comparisons. Two-way ANOVA was used to check for three aspects: (1) if there are significant differences among N levels (N effect), (2) if there are significant differences among P levels (P effect) and, (3) if there are significant differences among N levels within P addition groups (N × P effect).

To identify independent effects of N addition or P addition on phosphatase activity and non-symbiotic N_2 fixation, we applied linear mixed models using the nlme package and the multcomp package for a post hoc test in R. For this purpose, treatments were grouped according to their N addition or P addition levels. Additional variance coming from P addition or N addition was eliminated by selecting "P addition" or "N addition" as random factor, respectively.

Simple regressions (Pearson) were used to identify single relationships between response variables. Further, we implemented multiple regression analyses. According to our hypothesis, we tested how the combinations of N addition, P addition, DN, DIP, phosphatase activity, and non-symbiotic N_2 fixation contributed to explain changes in ANPP for the year 2017. Aboveground NPP was selected as dependent variable in all models. Model fits were evaluated based on the combined contributions of all predictors to explain the variance (R^2) of the total model and the significance level. All data are provided in Metadata S1, Metadata S2, and Data S1, S2).

Results

Aboveground net primary productivity

Aboveground NPP records for the period 1951 to 2017 showed that both single and combined N and P addition increased ANPP and led to synergistic growth responses (Fig. 1a). Aboveground NPP amounted on average to 385 g dry mass·m^{-2}·yr^{-1} in the control and it was increased by 18% in the single P addition treatment. Single N addition increased ANPP by 17%, 27%, and 22% in the low, medium, and high N treatment, respectively, and on average by 22%. The strongest increase in ANPP was observed in response to combined NP

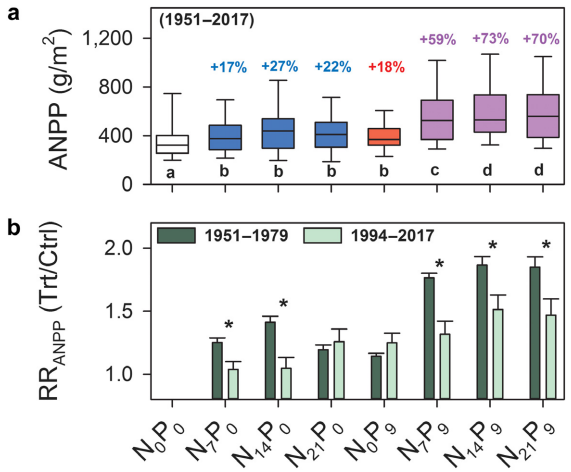

FIG. 1. Effects of single and combined N and P addition on aboveground net primary production (ANPP) between 1951 and 2017. Note that ANPP data are not available for all years (missing years 1980–1993, 1995, 2001, 2010, 2013, and 2016). For panel a, box plots show the median (black line), the 25th and the 75th percentile (box edges) and the 10th and the 90th and percentile (error bars) (n = 48). Different lowercase letters indicate significant differences ($P < 0.05$) between nutrient addition treatments. Repeated measurements of different years were acknowledged by selecting year as random factor in a mixed linear model. For panel b, response ratios (RR; treatment/control) of ANPP (mean and standard error) were calculated for the periods 1951–1979 (n = 29) and 1994–2017 (n = 19), separately. A Welch test was used to compare both periods since groups differed in sample size. Asterisks indicate significance differences between both periods ($P < 0.05$). The x-axis shows the N addition treatments with and without P addition (N addition rates: 0, 7, 14, or 21 g·N·m^{-2}·yr^{-1} and P addition rates: 0 and 9 g·P·m^{-2}·yr^{-2}).

addition, being significantly higher than in all single addition treatments of N or P. In the NP treatments, ANPP increased by 59%, 73%, and 70% due to the low, medium, and high N addition rates, respectively, as compared to the control with a mean of 67% increase (Fig. 1a). The increase in ANPP due to combined N and P addition was significantly higher than the sum of the increases in ANPP caused by single N and single P addition. This was observed for all N addition levels. The difference in the ANPP response between the combined NP treatment and the sum of the increases due to single N and single P addition amounted to 24%, 28%, and 30% in the low, medium, and high N addition level, respectively (Appendix S1: Fig. S2).

We found that synergistic effects due to combined N and P addition decreased over time, as indicated by negative linear relationships of the difference in effect size (NP − N + P) over time for the low and high N addition rate (Appendix S1: Fig. S3). Further, we found that during the first 30 yr of the experiment (1951–1979), years in which a significant N and P co-limitation occurred more frequently than in the second period of the experiment (1994–2017; Appendix S1: Fig. S4, Appendix S2: Table S1). Accordingly, mean ANPP response ratios to nutrient addition were significantly lower in the second period of the experiment in most nutrient addition treatments (except for N_0P_9 and $N_{21}P_0$, Fig. 1b). The decrease in the ANPP response over time was especially visible in all NP treatments; the ANPP response decreased on average by 45%, 35%, and 38%, in the low, medium, and high N addition rate, respectively, in the period 1951–1979 compared to the period 1994–2017 (Fig. 1b).

In the year 2017, ANPP ranged between 267 to 442 g DM/m^2 across all treatments, with lowest ANPP found in the control (Table 1, Appendix S1: Fig. S5). Two-way ANOVA indicated a positive effect of P addition, but subsequent post-hoc tests (Tukey) revealed no significant difference between treatments. Further, N addition alone had no effect on ANPP ($P > 0.05$) in the year 2017 (Table 1). Multiple regression analyses indicated that P addition and phosphatase activity were the best predictors of ANPP in 2017 (Appendix S2: Table S2). After eliminating insignificant variables from the model, P addition explained 25% and phosphatase activity explained 13% of the variance in ANPP, respectively. In contrast, N addition, DN concentrations, and non-symbiotic N_2 fixation had no effect on ANPP in each of three models (Appendix S2: Table S2).

Aboveground N and P stocks and concentrations in 2017

Aboveground N stocks did not differ significantly between element addition treatments, but we observed a positive correlation between aboveground N stocks and N addition in treatments without P addition ($R^2 = 0.37$, $P < 0.05$, Appendix S1: Fig. S6a). Aboveground N concentrations in the control amounted to 11.5 mg N g/dry mass and were significantly higher in all single N addition treatments as compared to the control (Table 1). In the N treatments without P addition, N concentrations in aboveground biomass were strongly correlated with the N addition rate ($R^2 = 0.89$, $P < 0.001$, Appendix S1: Fig. S6b). In contrast, in the NP treatments, we found no significant difference in aboveground N concentrations between N levels (Table 1) and no correlation with N addition rate (Appendix S1: Fig. S6a, b). Phosphorus addition significantly reduced aboveground N concentrations in all NP treatments as compared to respective N treatments without P addition (Appendix S2: Table S1).

Aboveground P stocks were significantly higher in all treatments receiving P compared to treatments without P addition (Table 1), and the aboveground P stocks were higher the more N was added in combination with P ($R^2 = 0.32$, $P < 0.05$, Appendix S1: Fig. S6c). Single P addition significantly elevated P concentrations in the aboveground biomass by a factor of 2.6 as compared to the control, while single N addition did not change P concentrations in aboveground biomass independently of how much N was supplied (Table 1). However, when P was added together with N, aboveground P concentrations were consistently higher than in all corresponding single N treatments. Most important, the aboveground P

TABLE 1. Effects of N and P addition on aboveground net primary production, aboveground N and P concentrations, and stocks and soil biochemical properties.

	Two-way ANOVA			Effect of N addition (among groups)				Without P			
Variable	N	P	N x P	N_0	N_7	N_{14}	N_{21}	N_0P_0	N_7P_0	$N_{14}P_0$	$N_{21}P_0$
ANPP† (2017)	n.s.	**	n.s.	A	A	A	A	$267^a \pm 79$	$292^a \pm 109$	$371^a \pm 47$	$326^a \pm 135$
Abg. N_{stock}	n.s.	n.s.	n.s.	A	A	A	A	$3.1^a \pm 0.8$	$4.3^a \pm 1.7$	$6.6^a \pm 1.1$	$5.9^a \pm 2.5$
Abg. N_{conc}†	***	**	**	A	B	B	B	$11.5^a \pm 0.7$	$14.7^b \pm 0.7$	$17.6^c \pm 0.7$	$18.1^c \pm 1.4$
Abg. P_{stock}	n.s.	***	n.s	A	A	A	A	$0.31^a \pm 0.03$	$0.25^a \pm 0.10$	$0.35^a \pm 0.05$	$0.37^a \pm 0.11$
Abg. P_{conc}	**	***	**	A	AB	AB	A	$1.21^a \pm 0.31$	$0.84^a \pm 0.09$	$0.94^a \pm 0.16$	$1.18^a \pm 0.12$
TOP‡	**	***	***	A	B	AB	AB	$0.25^a \pm 0.02$	$0.22^a \pm 0.01$	$0.25^a \pm 0.03$	$0.23^a \pm 0.01$
PASE	***	**	**	A	A	A	B	$114^a \pm 30$	$249^a \pm 181$	$417^a \pm 183$	$917^b \pm 211$
Nfix†	**	n.s.	n.s.	A	B	B	AB	$12.4^a \pm 2.5$	$5.2^a \pm 5.0$	$4.6^a \pm 3.2$	$6.7^a \pm 3.4$
AMF	n.s.	***	n.s.	A	A	A	A	$16.2^a \pm 7.4$	$6.5^a \pm 4.6$	$6.7^a \pm 2.6$	$5.6^a \pm 4.4$
nifH	***	n.s.	**	A	A	B	C	$122^a \pm 6$	$114^a \pm 5$	$85^b \pm 10$	$49^c \pm 8$

Notes: Values are means ± SD. Separate and combined effects of N and P addition were tested by two-way ANOVA followed by a Tukey post hoc test. Some variables were transformed to maintain requirements on normal distribution and variance homogeneity. Different uppercase letters show significant differences among N levels ($P < 0.05$), and asterisks indicate significant differences among P levels ($P < 0.05$). Different lowercase letters after means indicate significant difference between N addition rates within P groups (with and without P addition). Abbreviations are N addition rate (N_{add}; g $N \cdot m^{-2} \cdot yr^{-1}$), P addition rate ($P_{add}$; g $P \cdot m^{-2} \cdot yr^{-1}$), aboveground net primary production (ANPP; g/m^2), aboveground N stock (abg. N_{stock}; g N/m), aboveground N concentration (abg. N_{conc}; mg/g), aboveground P stock (agb. P_{stock}; g P/m^2), aboveground P concentration (abg. P_{conc}; mg P/g), total organic P concentrations (TOP; g kg/soil), phosphatase activity (PASE; nmol·g $soil^{-1} \cdot h^{-1}$), predicted relative gene abundance of arbuscular mycorrhiza fungi (AMF; ppm), non-symbiotic N_2 fixation (Nfix; ng·g $soil^{-1} \cdot d^{-1}$), predicted relative *nifH* abundance (*nifH*; ppm). All variables are graphically illustrated with bar plots in Appendix S1: Fig. S5.
*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.
†log-transformed.
‡Square-root-transformed.

concentrations increased with N addition rate when N was added in combination with P ($R^2 = 0.56$, $P = 0.003$, Appendix S1: Fig. S6d). Accordingly, in the NP treatments, aboveground P concentrations were significantly higher by a factor of 1.30, 1.28, and 1.40 in the low, medium, and high N addition level, respectively, as compared to the single P addition level.

Element concentrations in soil and soil water extracts

While soil TOC and TN concentrations were independent of N and P addition, soil TP concentrations strongly increased in treatments with P addition (Appendix S2: Table S3, Appendix S1: Fig. S7). DN concentrations increased with N addition rate but the increase was less strong when N was added in combination with P. The DOC concentrations were less responsive to N and P addition, and DOC was only significantly increased compared to the control in the high N addition level without P addition (Appendix S2: Table S3, Appendix S1: Fig. S7). The DIP concentration was significantly higher in all treatments with P addition than in the treatments without P addition, and it increased with N addition rate when N and P were added in combination (Appendix S2: Table S3, Appendix S1: Fig. S7). Effects of single and combined N and P addition on C, N and P concentrations in soil and soil water extracts are described in more detail in Appendix S3.

Soil TOP concentrations

In the control, the soil TOP concentration amounted to 0.25 g P kg/soil. Single N addition did not change TOP concentrations independently of application rate. In the NP treatments, TOP concentrations significantly increased by a factor of 2.2, 1.7, and 2.0 in the low, medium, and high NP addition level, respectively, compared to the single P addition treatment without N addition (Fig. 2a). Phosphorus addition significantly increased the TOP concentration in all N addition treatments (Fig. 2b). Further, we observed that TOP concentrations significantly increased with N addition rate in the treatments, in which N was added in combination with P as compared to the P treatment without N addition (Fig. 2a). The TOP concentrations were positively correlated with aboveground P concentrations ($R^2 = 0.83$, $P < 0.001$, Fig. 2c) and aboveground P stocks ($R^2 = 0.68$, $P < 0.001$, Fig. 2d).

Phosphatase activity

Phosphatase activity amounted to 114 nmol·g $soil^{-1} \cdot h^{-1}$ in the control and was significantly higher in the high N level without P addition by a factor of 8.1 (Table 1). The low and medium N level without P addition did not significantly differ from the control. Further, no significant differences were found for

Effect of N and P addition (within groups)			
With P			
N_0P_9	N_7P_9	$N_{14}P_9$	$N_{21}P_9$
$378^{a} \pm 104$	$408^{a} \pm 110$	$430^{a} \pm 91$	$442^{a} \pm 77$
$4.6^{a} \pm 1.2$	$5.9^{a} \pm 1.2$	$6.3^{a} \pm 2.0$	$6.0^{a} \pm 1.3$
$12.2^{a} \pm 0.3$	$14.7^{a} \pm 1.5$	$14.5^{a}* \pm 1.9$	$13.5^{a}* \pm 1.2$
$1.20^{a}* \pm 0.32$	$1.69^{a}* \pm 0.45$	$1.75^{a}* \pm 0.39$	$1.99^{a}* \pm 0.44$
$3.19^{a}* \pm 0.24$	$4.14^{b}* \pm 0.47$	$4.07^{b}* \pm 0.33$	$4.48^{b}* \pm 0.23$
$0.36^{a}* \pm 0.01$	$0.78^{b}* \pm 0.14$	$0.62^{b}* \pm 0.08$	$0.73^{b}* \pm 0.14$
$178^{a} \pm 45$	$186^{a} \pm 99$	$193^{a} \pm 108$	$369^{a}* \pm 132$
$12.7^{a} \pm 4.4$	$7.0^{ab} \pm 5.5$	$2.1^{b} \pm 1.2$	$4.9^{ab} \pm 2.2$
$38.0^{a}* \pm 7.8$	$57.3^{a}* \pm 19.5$	$55.9^{a}* \pm 41.2$	$80.6^{a}* \pm 16.2$
$99^{a} \pm 6$	$106^{a} \pm 9$	$98^{a} \pm 6$	$67^{b} \pm 15$

phosphatase activity between N addition levels in all NP treatments (Table 1). Phosphatase activity in the treatments without N addition amounted to 146 nmol·g soil^{-1}·h^{-1} (Fig. 3a). Phosphatase activity increased with N addition rate and was significantly elevated by a factor of 4.4 in the high N level as compared to the treatments without N addition (Fig. 3a). Phosphatase activity decreased with P addition and was significantly lower in treatments with than without P addition (Fig. 3b).

The positive effect of N addition on phosphatase activity was also indicated by a strong positive correlation with the DN concentration ($R^2 = 0.56$, $P < 0.001$, Fig. 3c), and the partly negative effect of P addition was supported by a negative correlation between phosphatase activity and log-transformed DIP concentrations ($R^2 = 0.13$, $P < 0.05$, Fig. 3d). Further, we observed a strong relationship between phosphatase activity and DOC : DN ratio. Phosphatase activity decreased exponentially as the DOC : DN ratio increased ($R^2 = 0.59$, $P < 0.001$, Fig. 3e), and it increased exponentially as the log-transformed DIN : DIP ratio increased ($R^2 = 0.38$, $P < 0.001$, Fig. 3f).

Non-symbiotic N_2 fixation

The non-symbiotic N_2 fixation rate was 12.5 ng N·g soil^{-1}·d^{-1} in treatments without N addition and was decreased by a factor of 0.49, 0.25 and 0.46 in the low, medium, and high N addition level, respectively. However, N_2 fixation rates showed large variations within the same N addition level; only the medium N level significantly differed from the reference N level without N addition (Fig. 4a). We observed no significant differences in N_2 fixation between the treatments with and without P addition (Fig. 4b). The non-symbiotic N_2 fixation rate in the control plots was on average 12.4 ng N·g soil^{-1}·d^{-1} (Table 1), which corresponds to 6.6 kg N·ha^{-1}·yr^{-1} in the top 15 cm of the soil. No significant difference was found among N treatments without P addition, while for the NP treatments, only the medium N level was significantly lower, by a factor of 0.17 compared to the P treatment without N addition (Table 1). Single correlation analysis revealed that the non-symbiotic N_2 fixation rate was negatively related to the DN concentration ($R^2 = 0.35$, $P < 0.01$, Fig. 4c), while it was independent of DIP concentration (Fig. 4d). Non-symbiotic N_2 fixation was positively correlated with the DOC : DN ratio ($R^2 = 0.32$, $P < 0.01$, Fig. 4e) and negatively with the log-transformed DIP : DIN ratio ($R^2 = 0.20$, $P < 0.05$, Fig. 4e).

AMF and nifH *gene abundance*

The predicted relative abundance of rRNA operons of the arbuscular mycorrhizal fungi (AMF) families *Glomeraceae*, *Archaeosporales*, *Paraglomeraceae*, *Glomeromycetes*, and *Claroideoglomeraceae* was significantly higher in the treatments with P than without P addition in all N levels (Table 1), and increased with N addition rate in the NP treatments ($R^2 = 0.32$, $P < 0.01$, Appendix S1: Fig. S8). Yet, AMF gene abundance was

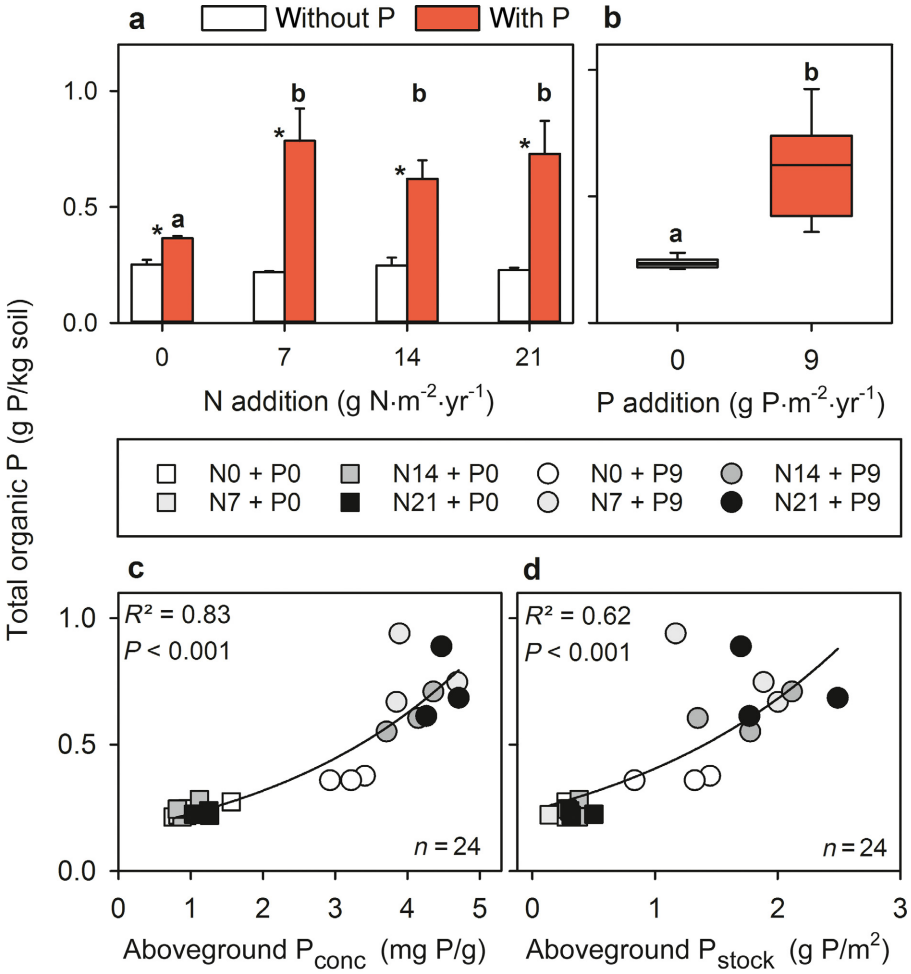

FIG. 2. Total soil organic P (TOP) concentration as a function of (a) N addition, (b) P addition, (c) aboveground P concentration, and (d) aboveground P stock. For panel a, bars show means and SD (n = 3). From a two-way ANOVA, different lowercase letters indicate significant differences ($P < 0.05$) between N addition levels in the NP treatments. Asterisks indicate significant differences between treatments with and without P addition separately for each N addition level ($P < 0.05$). For panel b, box plots show the median (black line), the 25th and the 75th percentile (box edges), and the 10th and the 90th and percentile (error bars) (n = 12). Mixed linear models with N addition as random factor were applied to identify significant differences between P addition levels. For panels c-d, single regression analysis (Pearson) was implemented (n = 24).

independent of N addition in the N addition treatments without P ($P > 0.05$, Appendix S1: Fig. S8).

Predicted relative gene abundance of the nitrogenase iron protein (*nifH*), which is a marker for the N_2 fixing bacteria community, asymptotically decreased with N addition rate ($R^2 = 0.95$, $P < 0.001$, Appendix S1: Fig. S8), but the response was less strong when N and P were added together ($R^2 = 0.77$, $P < 0.001$, Appendix S1: Fig. S8). In the N treatments without P addition, predicted relative *nifH* gene abundance was significantly lower in the medium and high N addition level as compared to the control (Table 1). In the N treatments with P addition only the high N level significantly differed from the single P addition treatment without N addition. Overall, two-way ANOVA revealed no significant changes in the predicted relative abundance of the *nifH* gene upon P addition in all respective N levels (Table 1).

Discussion

Synergistic N and P co-limitation

Combined addition of N and P led to larger increases in ANPP than the single addition of either N or P in the period from 1951 to 2017, which indicates a NP co-limitation. The increase in ANPP due to combined NP was even higher than the sum of the increases observed due to separate N and separate P addition. Thus, the results demonstrate a synergistic effect, confirming our first

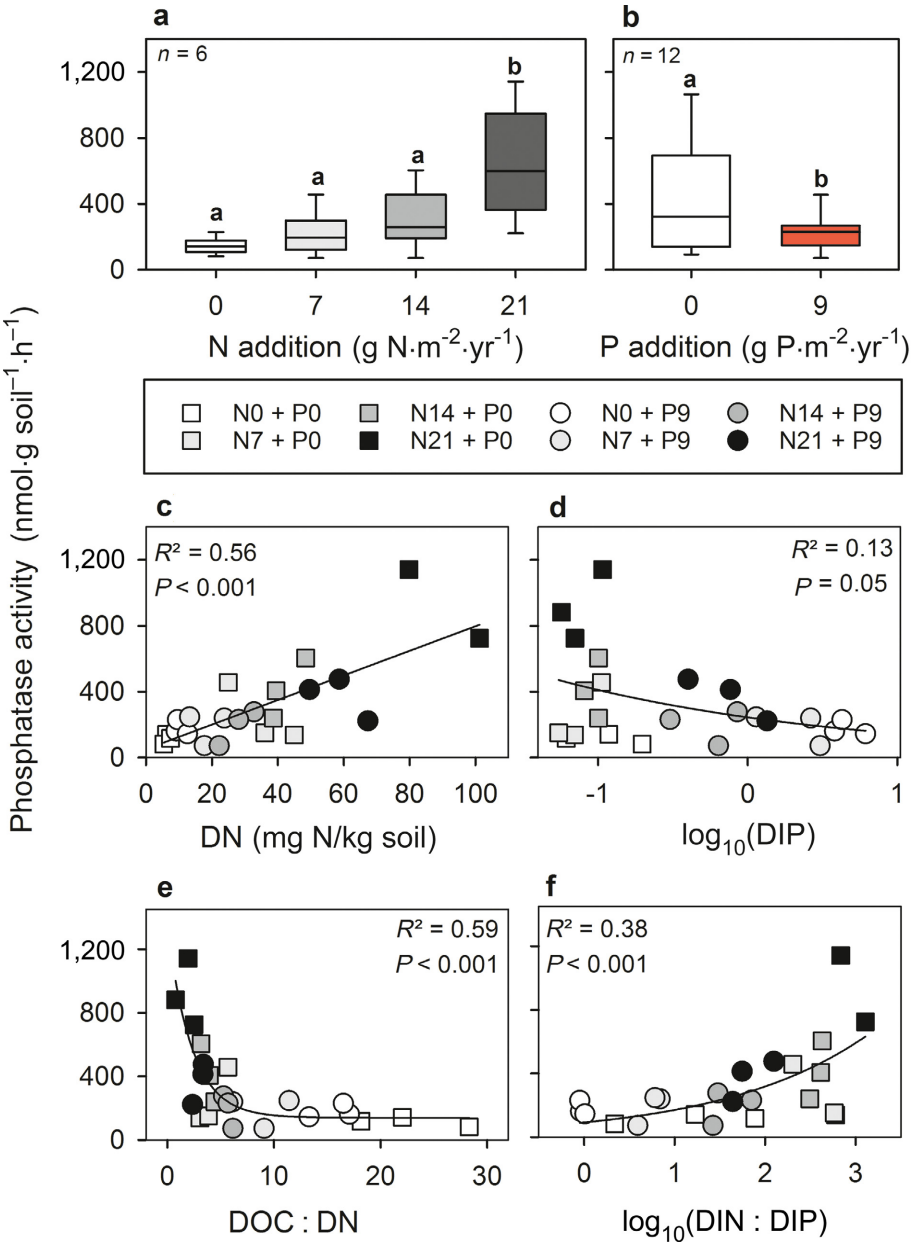

FIG. 3. Phosphatase activity as a function of (a) N addition, (b) P addition, (c) dissolved total nitrogen concentration (DN), (d) $\log_{10}$-transformed dissolved inorganic phosphorus concentration (DIP), (e) DOC:DN ratio, and (f) $\log_{10}$-transformed DIP:DIN ratio. For panels a and b, box plots show the median (black line), the 25th and the 75th percentile (box edges), and the 10th and the 90th percentile (error bars). Mixed linear models were applied to identify significant differences between N addition levels and P addition levels, separately. For this purpose, the variance derived from P addition or vice versa the variance from N addition was eliminated by selecting P addition or N addition, respectively as random factor. Panels c–f show single regression analyses (Pearson).

hypothesis. Our finding is largely consistent with meta-analyses reporting increased plant growth in response to single and especially to combined N and P addition (Elser et al. 2007, Harpole et al. 2011). However, as pointed out by Davidson and Howarth (2007), the data analyzed in Elser et al. (2007) were mainly derived from short-term experiments, while we demonstrate synergistic N and P co-limitation of ANPP in a grassland experiment based on data that cover a period of 66 yr.

The mechanisms that cause synergistic responses of ANPP are poorly understood. Here, we show that processes of organic P storage, P recycling, and plant P uptake increased with N addition in the combined NP

treatments (see discussion on organic P formation, recycling, and uptake, below). We argue that these processes have contributed to synergistic growth responses, as hypothesized before in conceptual studies (Bloom et al. 1985, Davidson and Howarth 2007, Craine and Jackson 2010).

Addition of N and P control organic P formation and recycling

Soil TOP concentrations increased due to P addition, especially when P was added in combination with N. The most plausible explanation for this is that the higher

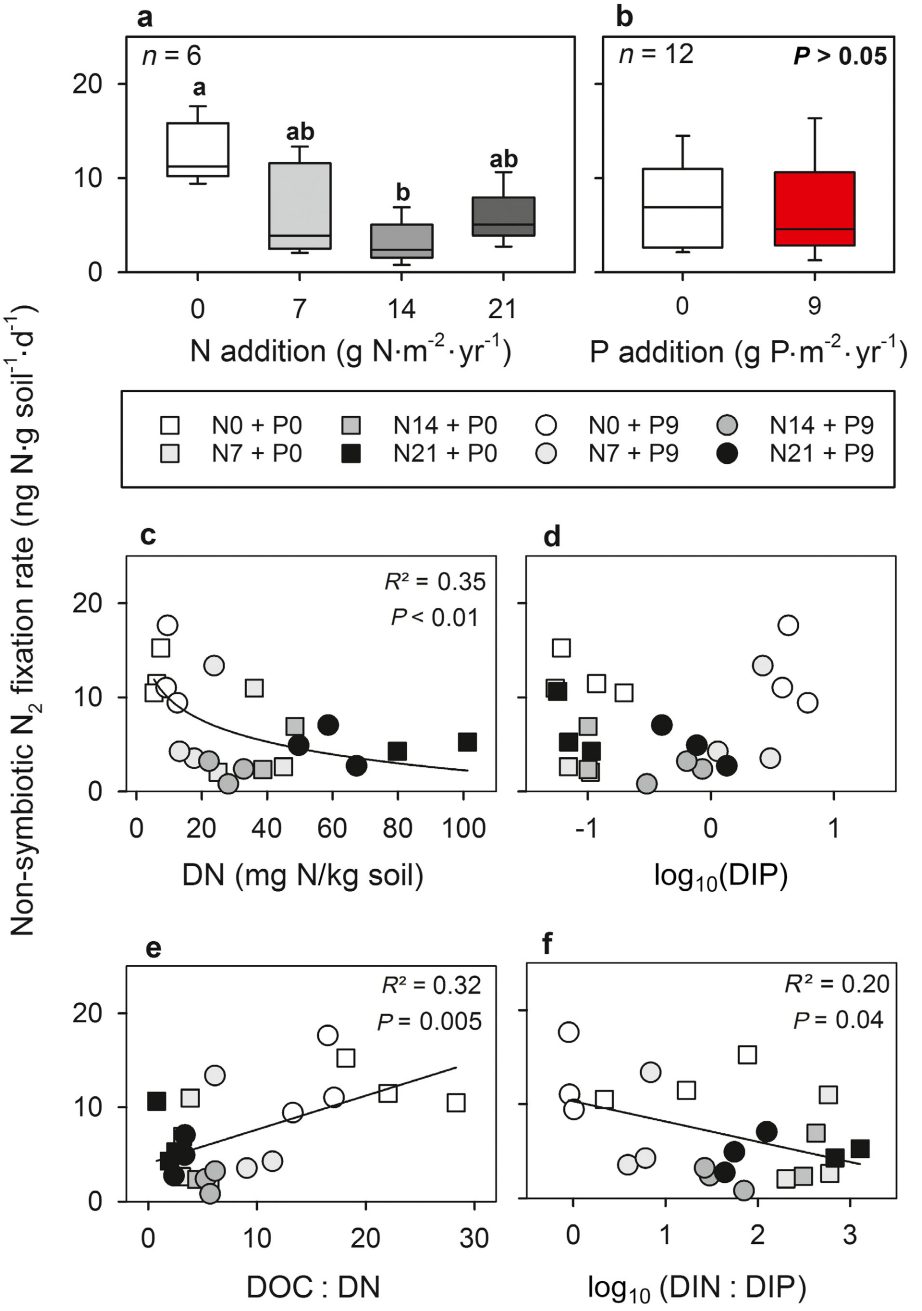

FIG. 4. Non-symbiotic N_2 fixation rate as a function of (a) N addition, (b) P addition, (c) dissolved total nitrogen concentration (DN), (d) $\log_{10}$-tranformed dissolved inorganic phosphorus concentration (DIP), (e) the DOC : DN ratio, and (f) the $\log_{10}$-transformed DIP:DIN ratio. For panels a and b, box plots show the median (black line), the 25th and the 75th percentile (box edges), and the 10th and the 90th percentile (error bars). Mixed linear models were applied to identify significant differences between N addition levels and P addition levels, separately. For this purpose, the variance derived from P addition or, vice versa, the variance from N addition was eliminated by selecting P addition or N addition, respectively, as random factor. Panels e and f show single regression analyses (Pearson).

plant productivity and the higher aboveground P concentrations in the NP treatments have increased soil TOP stocks over the 66 yr of the experiment. In agreement with this, we found a strong correlation between soil TOP concentrations and aboveground P stocks. Similarly, it has been observed in an Inner Mongolian grassland-steppe that combined N and P addition increased TOP concentrations by 8% compared to the control (Tian et al. 2016). Although organic P is not directly plant available, it is highly relevant for plant nutrition because most of the soil organic P can be rendered available through phosphatases that catalyze the hydrolysis of organic P (Tiessen et al. 1994, Helfenstein et al. 2018). Evidence for this comes from a study by Richter et al. (2006) who demonstrated a strong depletion of soil organic P 28 yr after secondary forest establishment, and suggested that most of the organic P had been taken up by trees. Accordingly, Margalef et al. (2017) concluded that the soil organic P reservoir in combination with phosphatase activity often provides a better indicator of P availability than direct measurements of available P forms.

We found that phosphatase activity increased with N inputs, confirming our second hypothesis. Our results indicate that plants and microbes use additional N to produce and release phosphatases that render P available from soil organic P pools. Thus, it seems likely that in the combined NP treatments, P is more intensively re-cycled from organic pools than in the treatments that receive only P. The elevated phosphatase activity and the increased soil TOP concentrations in the NP treatments have likely contributed to plant P uptake, because both enhance the probability that a phosphatase enzyme meets an organic P compound and catalyzes its hydrolysis. Thus, the enhanced hydrolysis of organic P could be an important mechanism explaining synergistic growth responses of ANPP. Consistently, multiple regression analysis indicated that 38% of the variance of ANPP variance was explained by the combination of phosphatase activity (explaining 13%) and P addition (explaining 25%) in the year 2017. Most likely, added N allowed plants and microbes to synthesize N-costly phosphatases (Olander and Vitousek 2000, Marklein and Houlton 2012, Margalef et al. 2017, Widdig et al. 2019), which then rendered OP available for plant uptake (Treseder and Vitousek 2001). Further, we found that P addition decreased phosphatase activity. The most plausible reason for this observation is that plants and microorganisms downregulate the production of phosphatases once their P demands are covered as reported in previous studies (Spiers and McGill 1979, Olander and Vitousek 2000, Marklein and Houlton 2012, Heuck et al. 2018).

Interactions of N and P on plant P uptake

While aboveground P stocks were positively correlated with the N addition rate, soil DIP concentrations decreased with N addition rate in all NP treatments. This indicates that N addition facilitated plant P uptake (as long as DIP was sufficiently available) and thereby led to depletion in soil DIP, which is consistent with our second hypothesis. Our findings are in line with Long et al. (2016) who demonstrated that P concentrations of nine different plant species in an Inner Mongolian grassland were significantly higher in response to combined NP addition than in response to the separate addition of either N or P. Similarly, Deng et al. (2017) showed that N addition increased plant P uptake in aboveground biomass across a broad range of terrestrial ecosystems. Several mechanisms may explain why plant P uptake benefits from higher N availability. First, plants might use N for upregulating P uptake transport systems (Zeng et al. 2012). Since DIP concentrations are much higher in plant cells than in soil, plants use proton-ATPase transporters for active phosphate transport across the plasma membrane (Smith and Jackson 1987, Zeng et al. 2012). Addition of N can enhance the pumping activity of the anion/H^+ co-transport because NH_4^+ requires the release of H^+ (Ullrich-Eberius et al. 1984, Jing et al. 2010). Second, elevated N and P inputs might have changed root traits in a way that promotes plant nutrient uptake. For example, nitrate and phosphate both serve as a signal molecule that initiates root branching, which causes an intensification of small scale soil exploration (Desnos 2008). Other studies showed that root biomass can increase under N and P addition; e.g. a meta-analysis on changes in root production due to N and P addition revealed that single N addition, single P addition and combined NP addition increased fine root production by 11%, 31% and 53%, respectively, relative to the control in tropical grasslands, respectively (Yuan and Chen 2012). Third, N and P addition can either negatively or positively affect the abundance and colonization of AMF (Treseder and Allen 2002, Treseder 2004, Camenzind et al. 2016). In our study, we observed that relative gene abundance of AMF increased with N addition rate, when N was applied in combination with P, indicating that AMF might have contributed to nutrient uptake upon combined NP addition. Increasing AMF abundances with NP addition were similarly observed in the South Ecuadorian Andes (Camenzind et al. 2016) as well as in alpine grasslands on the Tibetan Plateau (Xiang et al. 2016).

Controls on non-symbiotic N_2 fixation and plant N uptake

Contrary to our third hypothesis, P addition did not increase non-symbiotic N_2 fixation rates and plant N uptake. In addition, other processes involved in N acquisition such as leucine-aminopeptidase activity and net N mineralization rates were also unaffected by P addition (Schleuss et al. 2019). While previous studies observed that P addition elevated non-symbiotic N_2 fixation as it provides P for ATP production (Simpson and Burris 1984, Eisele et al. 1989, Reed et al. 2010), our study

indicates that P does not limit N_2 fixation in this grassland. The reason for this might be that elements other than P (e.g., molybdenum) limit non-symbiotic N_2 fixation, as shown previously for different ecosystems (Wurzburger et al. 2012). However, we found that N addition reduced non-symbiotic N_2 fixation, which might have different reasons. First, non-symbiotic N_2 fixers might reduce nitrogenase activity when supplied with N, since it is less energy consuming for them to take up reactive N than to fix N_2 (Smith 1992). Consistently, we observed that increased DN concentrations were associated with lower N_2 fixation rates. Second, N_2 fixation is a highly energy-requiring process and consequently non-symbiotic N_2 fixing communities rely on available C sources to meet their energy demands. Long-term N addition reduced C availability with respect to N as shown by the low DOC: DN ratios in the N addition treatments (see Schleuss et al. 2019), and thus might have induced energy limitations for N_2 fixation. We found that non-symbiotic N_2 fixation negatively corresponded with decreasing DOC: DN ratios. Our explanations agree with previous studies showing that low C:N ratios in growth media and direct inorganic N applications strongly decreased nitrogenase activity in lab experiments (Drozd et al. 1972, Bühler et al. 1987). Furthermore, elevated N inputs could have constrained the abundance and diversity of the N_2 fixing bacterial community (Fani et al. 2000, Reed et al. 2011) as revealed by decreased *nifH* gene abundance. The relatively high N availability compared to other subtropical and tropical grasslands might also be the reason why the non-symbiotic N_2 fixation rate (6.6 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$ in the control) was in the lower range of the rates reported for savanna grasslands (3.0–30 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$; Reed et al. 2011), and was about two times lower than rates reported for tropical grasslands (11 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$; Cleveland et al. 1999).

Similar to non-symbiotic N_2 fixation rates, plant N uptake (measured as aboveground N concentrations and stocks) was independent of P addition. This is in agreement with results of Craine et al. (2008) who found that single P addition did not changed aboveground N concentrations in five different South African grasslands.

Dynamics of N and P co-limitation

We observed a decreasing response of ANPP to NP addition over time. One explanation for this could be that NP addition lead to the formation of a relatively large organic P stock in the soils and intensified processes of P recycling, which turned organic P plant available. Further, long-term N and P additions have caused strong changes in the plant community composition and a 30% decrease in plant species richness in the experiment studied here (Fynn and O'Connor 2005). Similarly, Isbell et al. (2013) found that the magnitude of ANPP responses to elevated N inputs decreased over time in a North American grassland due to strong changes in plant community composition and loss of dominant species. The plant communities that developed over time due to long-term nutrient inputs might be better adapted to the nutrient-rich conditions, but do not produce more biomass than the previous community when provided with increased amounts of N and P. Thus, our results indicate that plant-growth responses due to increased nutrient availability might fade out over time due to changes in plant community composition.

In conclusion, we show that ANPP in this mesic grassland has been synergistically co-limited by N and P during the last 66 yr, and we found several indications that interactions between N and P promoted processes of plant P acquisition and uptake. These interactions likely have a positive effect on ANPP, explaining synergistic growth responses of ANPP to combined NP addition. The combined addition of N and P enhanced the accumulation of organic P in soil and its re-mobilization through phosphatases. Further, combined NP addition increased relative AMF gene abundance and elevated plant P uptake compared to treatments with single P addition. In contrast, the studied N cycling processes were mainly independent of NP interactions, and neither aboveground biomass N stocks nor non-symbiotic N_2 fixation increased in response to elevated P availability.

Our results show that interactions between N and P promote nutrient recycling, which might explain synergistic plant-growth responses under combined NP addition. The latter has often been observed in grasslands exposed to short-term NP additions. However, long-term responses (>50 yr) are hardly investigated and our data indicate that synergistic growth responses to combined NP addition become less strong over time, likely due to changes in plant community composition. Thus, our study gives important insights into long-term co-limitation dynamics that are often neglected.

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