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LETTER

Plant diversity shapes microbe-rhizosphere effects on P mobilisation from organic matter in soil

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Abstract

Plant species richness (PSR) increases nutrient uptake which depletes bioavailable nutrient pools in soil. No such relationship between plant uptake and availability in soil was found for phosphorus (P). We explored PSR effects on P mobilisation [phosphatase activity (PA)] in soil. PA increased with PSR. The positive PSR effect was not solely due to an increase in C_{org} concentrations because PSR remained significant if related to PA:C_{org}. An increase in PA per unit C_{org} increases the probability of the temporal and spatial match between substrate, enzyme and microorganism potentially serving as an adaption to competition. Carbon use efficiency of microorganisms (C_{mic}:C_{org}) increased with increasing PSR while enzyme exudation efficiency (PA:C_{mic}) remained constant. These findings suggest the need for efficient C rather than P cycling underlying the relationship between PSR and PA. Our results indicate that the coupling between C and P cycling in soil becomes tighter with increasing PSR.

Keywords

Microorganisms, P mobilisation, phosphatase enzymes, plant diversity, substrate availability, The Jena Experiment.

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INTRODUCTION

Despite the importance of phosphorus (P) as a productivity-limiting element (Vitousek *et al*. 2010), manipulative field experiments on the relationship between plant diversity and nutrient cycling have mainly focused on biomass production and the nitrogen (N) cycle. It has been shown that highly diverse plant communities are more productive than less diverse communities (Naeem *et al*. 1994; Spehn *et al*. 2005; Tilman *et al*. 2014). High diversity of plant communities in terms of plant species and functional group richness promotes niche differentiation and thus, results in a greater exploitation of the total available nutrient pool (Levine & HilleRisLambers 2009). Accordingly, increased aboveground P uptake of highly diverse grassland mixtures was observed (Karanika *et al*. 2007; Oelmann *et al*. 2011). Plants in highly diverse communities may complement each other in mobilising P from soil in space and over time (Lambers *et al*. 2006; Li *et al*. 2014).

P mobilisation mechanisms include phosphatase enzymes that hydrolyse organically bound P (P_{org}) into its inorganic, bioavailable form ($H_2PO_4^-$/HPO_4^{2-}). Because of this biochemical P mineralisation, phosphatases are essential for plant nutrition and for growth of microorganisms (Nannipieri *et al*. 2011). In case of P demand, plant roots and soil microbes increase the exudation of phosphatase enzymes and therefore the transformation of P_{org} into inorganic P (P_i) (Tarafdar & Jungk 1987; Olander & Vitousek 2000). Additionally, arbuscular mycorrhizal fungi (AMF) form symbioses with plant roots to receive plant-assimilated carbon (C) in exchange for mineral nutrients like P and N (Smith *et al*. 2003). The separation of phosphatase exudation by AMF from those of plant roots themselves and of microbes colonising the rhizosphere is challenging. Therefore, direct evidence for phosphatase exudation of AMF is lacking so far. Studies suggest that AMF associated with plant roots exude phosphatase enzymes (Nottingham *et al*. 2013) with a negligible fungal contribution to total soil phosphatase activity ranging between 1 and 10% (Joner & Johansen 2000).

The release of phosphatase enzymes is regulated by the demand for P, which increases with increasing biomass

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productivity of highly diverse plant mixtures (Tilman *et al.* 1996; Oelmann *et al.* 2011). The actual enzyme activity also depends on substrate availability. Enzymes are synthesised from amino acids requiring the simultaneous availability of C and N (Allison & Vitousek 2005). Additionally the availability of C as a substrate is the most limiting factor for growth and activity of the phosphatase-exuding soil microorganisms (Hodge *et al.* 2000). The exudation of phosphatase enzymes to induce P availability is closely associated with the rhizosphere (McGill & Cole 1981; Tarafdar & Jungk 1987; Olander & Vitousek 2000; Lambers *et al.* 2006). An increased belowground biomass in more diverse plant communities, as shown for different biodiversity experiments (Spehn *et al.* 2005; Reich *et al.* 2012; Mueller *et al.* 2013; Ravenek *et al.* 2014) offers a higher root surface area that is in direct contact to the soil.

In addition to plant species richness, nutrient cycling in soil varies with plant functional group identity. For instance, the presence of N_2-fixing legumes increases N availability in soil that can also favour neighbouring non-leguminous species (Temperton *et al.* 2007). Legumes have an increased P demand to synthesise adenosinetriphosphate that is needed for the symbiotic fixation of N_2 (Aerts & Chapin 2000). In a recent review, Li *et al.* (2014) distinguished between P-mobilising and non-mobilising plant species. All six plant species classified as "P mobilising" in their study belong to the functional group of legumes very likely because of the increased P demand associated with N_2 fixation. In mixed plant communities, facilitation between neighbouring P-mobilising and non P-mobilising plant species may explain increased P uptake (Li *et al.* 2014).

Here, we test whether plant diversity (either as plant species or functional group richness) influences phosphatase activities in soil, and how this potential relationship is mediated by soil conditions (substrate availability, plant root and microbial biomass). Secondly, we assess whether plant functional groups (presence or absence of legumes, grasses, small herbs and tall herbs) have an effect on P mobilisation in soil exemplified by phosphatase activities.

We hypothesise that species richness and/or functional group richness promote phosphatase release. Secondly, we hypothesise that species richness and/or functional group richness have a twofold positive effect on phosphatase activity consisting of the direct release from the root system and the indirect release from microorganisms because of the increasingly favoured microbial community in the rhizosphere. Thirdly, we hypothesise that legumes have a stronger positive effect on phosphatase activity than other functional groups because of their high P demand.

MATERIAL AND METHODS

Study site

The field site is located near the German city of Jena (50°55′ N, 11°35′ E; 130 m above sea level). Mean annual air temperature is 9.3 °C, and mean annual precipitation amounts to 587 mm (Kluge & Müller-Westermeier 2000). The soil is an Eutric Fluvisol developed from up to 2 m-thick fluvial sediments that are almost free of stones. The systematic variation in soil texture as a consequence of fluvial dynamics is considered in the experimental design by arranging the plots in four blocks at different distance to the river. The study site was converted from grassland to arable land in the early 1960s and used for crops until the establishment of the experiment in 2002. C_{org} concentrations ranged from 13 to 33 g kg^{-1}, C_{org}:N ratios from 8 to 15 and pH (H_2O) from 7.1 to 8.4 at the start of the experiment in 2002.

The experimental design is described by Roscher *et al.* (2004). Briefly, the main experiment comprises 80 plots that were split in different subplots with a core area of ~ 43.5 m^2. Each plot contains a specific combination of plant species (1, 2, 4, 8, 16, 60 species) that belong to different numbers (1, 2, 3, 4) of plant functional groups [grasses, (non-leguminous) small herbs, (non-leguminous) tall herbs, legumes]. The species are chosen from a pool of 60 species typically found in *Molinio-Arrhenatheretea* meadows. Each plant species richness level had 16 replicates except for 14 mixtures with 16 species and 4 replicates of the 60-species mixture. To maintain the sown species richness levels, plots were weeded three times a year by cutting weeds carefully with the roots. This minimises potential bias from weed root contributions. Henceforth, we refer to plant diversity as a surrogate for plant species or functional group richness. The management of all plots was adapted to extensive meadows used for hay production, and all plots were mown twice a year in June and September with the harvest being removed from the plots. Plots were not fertilised during the experimental period.

Soil sampling and analyses

We sampled soil in September 2013 to determine phosphatase activities. Nine soil cores per plot with a diameter of 2 cm were taken at a depth of 0 to 5 cm and combined to a composite sample considered representative for the plot. Moist soil samples were sieved to < 2 mm and stored at 4 °C in a refrigerator until chemical analyses. Roots and other coarse organic material were manually removed from the samples. Because of the alkaline pH of the soil, we measured alkaline phosphomonoesterase (PME) activity according to the modified assay by Eivazi & Tabatabai (1977). For each soil sample one replicate and one blank value were included in the laboratory study. One gram of field moist soil was mixed with toluene, modified universal buffer and p-nitrophenylphosphate (pNP), and incubated at 37 °C for 1 h. Subsequently, we added $CaCl_2$ and NaOH. To blanks pNP was added after incubation. The solution was filtered through P-free filters (MN 619 G ¼, Macherey-Nagel GmbH & Co. KG, Düren, Germany). Phosphodiesterase (PDE) activities were measured after Browman & Tabatabai (1978). One gram of field-fresh soil was mixed with toluene, tris(hydroxymethyl)-aminomethan buffer (THAM) and bis-p-nitrophenylphosphate (bpNP). The following procedure was identical to that in the PME extraction except for $CaCl_2$ + THAM-NaOH addition to all samples and bpNP to the blanks before filtration. Directly after filtration, p-nitrophenol concentrations [μg mL^{-1}] were measured at 400 nm with a spectrophotometer (PU 8675 VIS spectrophotometer, Philips GmbH,

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Hamburg, Germany) for enzyme activities (PME and PDE). For conversion to μg g^{-1} dry matter (DM), the soil moisture was determined gravimetrically, i.e. by weighing before and after drying at 105 °C. For a selected set of samples (n = 11), we additionally measured acid phosphatase activity (acid PME). Acid PME was neither correlated with microbial biomass (r = 0.40; p = 0.22) nor to belowground biomass (r = 0.41; p = 0.21). Furthermore, acid PME activities were much lower than alkaline PME and PDE activities very likely because the pH of our study site soil (7.4–7.9) did not match with the enzyme-specific acid pH optimum (Nannipieri *et al.* 2011). Therefore, we consider the importance of acid PME minor as compared to alkaline PME for our study.

Aboveground biomass was harvested in September 2013 during peak standing biomass by harvesting all plant material 3 – 5 cm above the soil surface in two replicate 0.1 m^2 (0.2 × 0.5 m) subplots. Sown plant species were separated from weeds and dead aboveground biomass and sorted to species. Biomass of each sown species was determined after drying at 70 °C for a minimum of 48 h and data from the two replicate subplots were pooled and extrapolated to 1 m^2.

Aliquots of the same soil samples as for determination of phosphatase activities were used for measurements of soil microbial biomass. The soil samples were first adjusted to a gravimetric soil water content of 25%. Measurements of soil microbial biomass C (μg C g^{-1} DM) were performed using an O_2-microcompensation apparatus *via* substrate induced respiration (SIR) after the addition of D-glucose (Scheu 1992). SIR is a common method for measuring soil microbial biomass, which has successfully been used in the framework of the Jena Experiment (Eisenhauer *et al.* 2010).

Belowground biomass was sampled down to 5 cm depth in June 2011. Data on deeper depths is available as well (Ravenek *et al.* 2014), but because of the match with all other variables, we constrained the data set to 0–5 cm. Nevertheless, including deeper soil layers did not change the overall results (data not shown). Three soil cores of 3.5 cm diameter were taken per plot and combined to one composite sample that was stored at 4 °C. The bulk samples were soaked in water and then rinsed with tap water over a 0.5 mm sieve. Remaining soil particles were removed by hand. Roots were dried at 60–70 °C and weighed subsequently (Ravenek *et al.* 2014).

C_{org} concentrations in soil were determined as described by Steinbeiss *et al.* (2008). In short, three soil samples were taken randomly located per plot in 2011 (4.8 cm in diameter, 5 cm deep) using a split tube sampler. All three cores were combined to a single homogenously mixed sample. In this way, we minimised the potential bias caused by spatial heterogeneity. Soil was dried, sieved (2 mm mesh) and ball-milled (4 min, frequency 30 s^{-1}). Total C concentrations were determined on ground samples by an elemental analyser (Elementaranalysator vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany). C_{org} concentrations were calculated by subtracting inorganic C concentrations (determined after removal of C_{org} at 450 °C in a muffle furnace) from total C concentrations.

To determine P partitioning in soil solid phase which usually does not change markedly in time periods of several years, soil samples collected in September 2007 were used (Oelmann *et al.* 2011). Five replicates per plot with a diameter of 1 cm were bulked to one composite sample. A stepwise sequential P fractionation after Hedley *et al.* (1982) was used to determine the amount of labile ($NaHCO_3$ extractable) and moderately labile (NaOH extractable) P_{org} and P_i. First, 20 mL 0.5M $NaHCO_3$ (adjusted to pH 8.5) was added to 0.5 g soil, shaken for 20 min, centrifuged at 2500 rpm (ROTANTA 460 rs, Hettich Lab Technologies, Tuttlingen, Germany), and filtered through P-free filters (MN 619 G ¼, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The same was done afterwards with 30 mL 0.1 M NaOH addition to the same soil.

Calculations and statistical analyses

We calculated the enzyme activity of PME and PDE and the specific activities per mass C_{org} (PME:C_{org}, PDE:C_{org}). The enzyme exudation efficiency was calculated as activity per mass microbial biomass (PME:C_{mic}, PDE:C_{mic}) and represents the proportional allocation of microbial biomass into enzyme synthesis (Stone *et al.* 2014). Furthermore, we approximated the carbon use efficiency of soil microorganisms by using the ratio between C_{mic} and C_{org} (Kuzyakov & Blagodatskaya 2015).

All data sets were tested for outliers and extreme values with exploratory data analyses and boxplots before statistical analyses. One plot had to be excluded as outlier because of an extremely high biomass (> 2 times the mean + SD). Eight plots from different blocks and plant diversity levels contained missing values in the soil microbial biomass data set (measurements failed). Because this is approximately 10 % of all plots, we used the maximum likelihood criterion to complete the data set on the basis of the existing values using Amos 22.0.0 (IBM Development Corporation, Meadville, USA) (Hox 1999).

All statistical analyses were realised with R studio (RStudio, Version 0.98.501, RStudio Inc., Boston, Massachusetts, USA) with the free statistics software R (R version 3.0.2 (2013–09–25), The R Foundation for Statistical Computing, Vienna, Austria) including the packages ggplot2 (version 0.9.3.1, 2013-03-02, Wickham (2009)), lavaan (version 0.5-16) and outliers (version 0.14), Rosseel (2012).

To test for effects of plant diversity on phosphatase activities, specific activities and carbon use efficiency we used a sequential ANOVA (type I sum of squares). PME, PDE and all ratios except for the PDE:C_{mic} ratio showed variance homogeneity (Levene Test; $p > 0.05$) and were normally distributed (Shapiro-Wilk normality Test, $p > 0.05$; visually through histograms and qq-plots). PDE:C_{mic} ratios had to be square-root transformed to meet the criterion of normal distribution and variance homogeneity. The ANOVA was performed with plant species richness and functional group richness as explanatory variables, and additionally the block effect was included as random factor first to take the block wise arrangement parallel to the river into account. For each of the plant functional groups we ran one separate model including the presence/absence of a specific plant functional group as between-subject factor. Additionally, we assessed the interaction effects of plant functional group identity with plant species and plant functional group richness. We calculated five separate models (one model without plant functional group identity effects and

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four models each with one plant functional group). Linear regressions with PME and PDE activity as dependent variables and labile P (organic and inorganic), moderately labile P (organic and inorganic), sum of labile and moderately labile P, C_{org}, microbial, plant and belowground biomass as independent variables were conducted.

We constructed structural equation models (SEM) to test indirect or direct relationships between plant diversity, potential explanatory variables and phosphatase activities in a multivariate approach. In an SEM, hypotheses about causal connections in systems can be evaluated (Grace *et al*. 2012). We used it to analyse the relationships of several variables with PME or PDE comprising the above- and belowground compartment. Compared to other statistical analyses e.g., multiple regressions, SEM are advantageous because directions can be assigned to several relationships yielding multiple explanatory as well as multiple response variables in one model (Grace *et al*. 2006). Furthermore, the structure of such models can reveal whether a significant bivariate relationship derives from a significant relationship of these two variables with a third variable. In this context, it is worth noting that significant bivariate relationships might no longer stay significant in an SEM. To evaluate the fit of the models we used the root mean squared error of approximation (RMSEA) after Steiger (1990), Chi-square (χ^2) standardised root mean residuals (SRMR) and the comparative fit index (CFI). The RMSEA estimates the amount of approximation error per degree of freedom in the model and is sensitive against different numbers of parameters in a model. SRMR is similar to the RMSE but estimates the standardised difference between an observed correlation and the predicted correlation. Both are "badness of fit" values and thus values close to zero indicate a good fit. Chi-square "assesses the magnitude of discrepancy between the sample and fitted covariance matrices" (Hu & Bentler 1999) and tests whether there are differences between the observed and expected covariance matrix of the model. Also in this case, values close to zero indicate less differences and thus a good fit. The CFI is a "goodness-of-fit index" and therefore with values close to one the model fits the data well (Hu & Bentler 1999). See Appendix S5 for all indices of our conducted SEMs.

Based on the ANOVA results, we chose plant species richness to be the only exogenous variable. As endogenous variables, we included microbial and belowground biomass, illustrating the two possible origins of phosphatase enzymes, C_{org}, as sources of hydrolyzable substrate, and aboveground biomass, explaining a higher nutrient demand because of higher productivity. Plant species richness was suggested to influence all other variables and phosphatase activities were thought to be affected by all included variables. Belowground biomass was linked with C_{org} because of providing organic material after root turnover and also with microbial biomass because the rhizosphere serves as habitat for soil microorganisms. Furthermore, C_{org} supplies substrate for microorganisms so that C_{org} and soil microbial biomass were also linked. We related soil microbial biomass to aboveground biomass because it has been shown that the microbial community is more active under plant communities with high productivity. We did not simplify our default model by excluding non-significant paths because the changes were negligible according to the Akaike Information Criterion (AIC), which is a modification of the likelihood criterion including a correction index to take into account that with increasing number of parameters in the model, the model fit generally increases (Grace *et al*. 2006).

RESULTS

Plant diversity effects on phosphatase activities: bivariate considerations

Plant species richness had significantly positive effects on PME and PDE activities (Fig. 1a and b). 35 and 37% of the variance of PME and PDE activities could be explained, respectively (Table 1).

Plant functional group richness did not significantly affect phosphatase activities when fitted after plant species richness ($p > 0.05$). Fitting functional group richness before plant species richness in the ANOVA resulted in significant effects of both functional group and plant species richness on phosphatase activities (Appendix S2). This indicates that plant species richness can explain an additional proportion in the variance of phosphatase activities. Functional group richness cannot explain an additional proportion of the variance in phosphatase activities.

The enzyme exudation efficiency of microorganisms (PA: C_{mic}) was not related to plant species richness (Appendix S3, Appendix S7 a and b). Specific enzyme activities and the proxy for carbon use efficiency increased with increasing plant species richness (Appendix S3 and Appendix S7 c, d for PME/PDE:C_{org}; Appendix S4 and Appendix S8 C_{mic}:C_{org},).

Variables potentially mediating plant diversity effects on phosphatase activities

PME and PDE activities correlated positively with belowground and microbial biomass (both $p < 0.001$; Fig. 2a–d). Microbial biomass explained 34 % of the variation of PME and 33 % of the variation of PDE activity (Fig. 2c and d). Belowground biomass explained a significant but smaller proportion of the variation in PME and PDE activities (PME: 14%, $p < 0.001$; PDE: 16%, $p < 0.001$) (Fig. 2a and b).

C_{org} concentrations in soil ranged between 16.2 and 31.7 g kg^{-1} with a mean of 24.2 $\pm$ 0.4 g kg^{-1}. They correlated with PME and PDE activities, explaining 55% and 38% of their variations, respectively (both $p < 0.001$; Fig. 3a and b).

We did not detect significant relationships between PME/PDE activities and labile P_i ($NaHCO_3$-P_i), moderately labile P_i (NaOH-P_i), or P_{org} fractions in soil ($NaHCO_3$-P_{org}, NaOH-P_{org}, $NaHCO_3$-P_{org} + NaOH-P_{org}). For minimum, maximum, mean, standard deviation and standard error of all variables see appendix in supporting information (Appendix S1). For linear regressions see Table 2.

Plant diversity effects on phosphatase activities: complex interactions with controlling variables

Using different model fit tests we found that our SEMs for PME and PDE activity can be accepted as a potential

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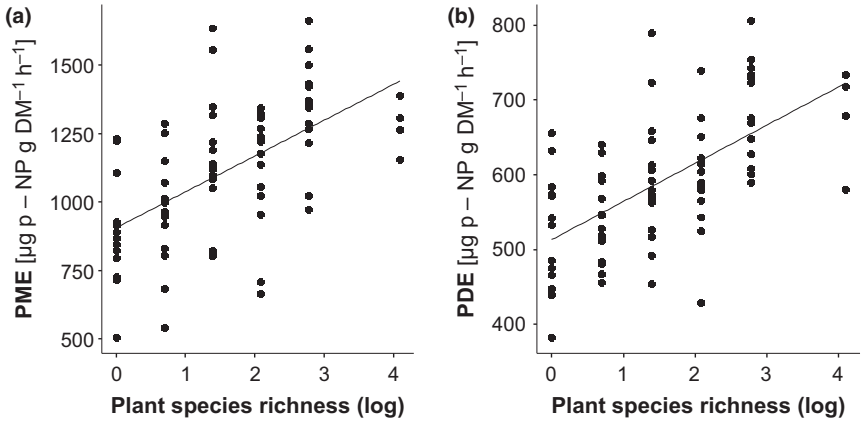

Figure 1 Relationship between PME and PDE activity and plant species richness (log transformed) (PME: a, PDE: b). Significance of effects are given in Table 1. Note different y axes scales for PME and PDE.

Table 1 Sequential ANOVA results of effects of plant species and functional group richness and the presence or absence of functional groups on PME and PDE activities

	PME				PDE			
Factor	d.f.	SS [%]	*F*	Sig.	d.f.	SS [%]	*F*	Sig.
Block	**3**	**19.65**	**10.97**	**< 0.001**	**3**	**10.54**	**5.13**	**0.003**
Plant species richness	**1**	**34.55**	**57.86**	**< 0.001**	**1**	**37.07**	**54.09**	**< 0.001**
Functional group richness	1	1.62	2.71	0.104	1	1.68	2.46	0.121
Legumes	1	0.01	0.02	0.881	1	0.19	0.29	0.592
Grasses	1	0.26	0.44	0.511	1	0.06	0.08	0.780
Tall herbs	1	0.02	0.03	0.875	1	0.59	0.86	0.358
Small herbs	1	0.56	0.97	0.329	1	0.32	0.46	0.501
Species richness × legumes	**1**	**3.23**	**5.84**	**0.018**	**1**	**2.70**	**4.17**	**0.045**
Species richness × grasses	1	1.53	2.57	0.114	1	0.25	0.35	0.559
Species richness × tall herbs	1	0.28	0.45	0.506	1	0.91	1.31	0.256
Species richness × small herbs	**1**	**2.65**	**4.60**	**0.035**	1	0.00	0.001	0.501
Functional group richness × legumes	1	1.63	2.95	0.090	1	1.87	2.90	0.093
Functional group richness × grasses	1	0.01	0.02	0.891	1	0.01	0.02	0.889
Functional group richness × tall herbs	1	0.01	0.01	0.911	1	0.02	0.03	0.870
Functional group richness × small herbs	1	0.05	0.09	0.767	1	0.18	0.25	0.619

Plant species richness was log transformed. SS [%] refers to the explained proportion of Sum of Squares, d.f. to degrees of freedom, and *F* represents the *F* value of the corresponding factor. Significant effects (Sig.) on a level of 0.05 are given in bold.

explanation of the observed covariance matrix (for all model fit indices see Appendix S5). SEMs explained 65 and 57% of the variance in PME and PDE activities, respectively (Fig. 4 a and b). Plant species richness showed significantly positive relationships with all included variables. Phosphatase activities were correlated with all variables but only plant species richness, C_{org}, and microbial biomass showed significantly positive relationships. The effects of plant root and aboveground biomass were not significant. Aboveground biomass was positively affected by plant species richness, but showed no significant effects on other included variables. Furthermore, the link between belowground biomass and C_{org} was positive but not significant, but belowground biomass showed a significantly positive effect on microbial biomass. Soil microbial biomass was also positively influenced by C_{org} concentrations.

Plant functional group effects on phosphatase activities

The presence of individual plant functional groups did not significantly affect phosphatase activities ($p > 0.05$; Table 1), but there were significant interactions between plant species richness and the presence of legumes for both PME and PDE activity ($p < 0.05$; Table 1). Furthermore, there was a significant interaction between plant species richness and the presence of small herbs for PME activity ($p < 0.05$; Table 1). However, the interaction effects showed no clear pattern i.e., pronounced functional group effects were either absent for all plant species richness levels or were not associated with particularly low or high plant species richness (Appendix S6). For example, in monocultures the presence of legumes significantly increased PDE activity (with legumes 601.20 ± 27.17, without legumes 482.60 ± 19.75; $t = 3.312$, $p < 0.05$) which was not

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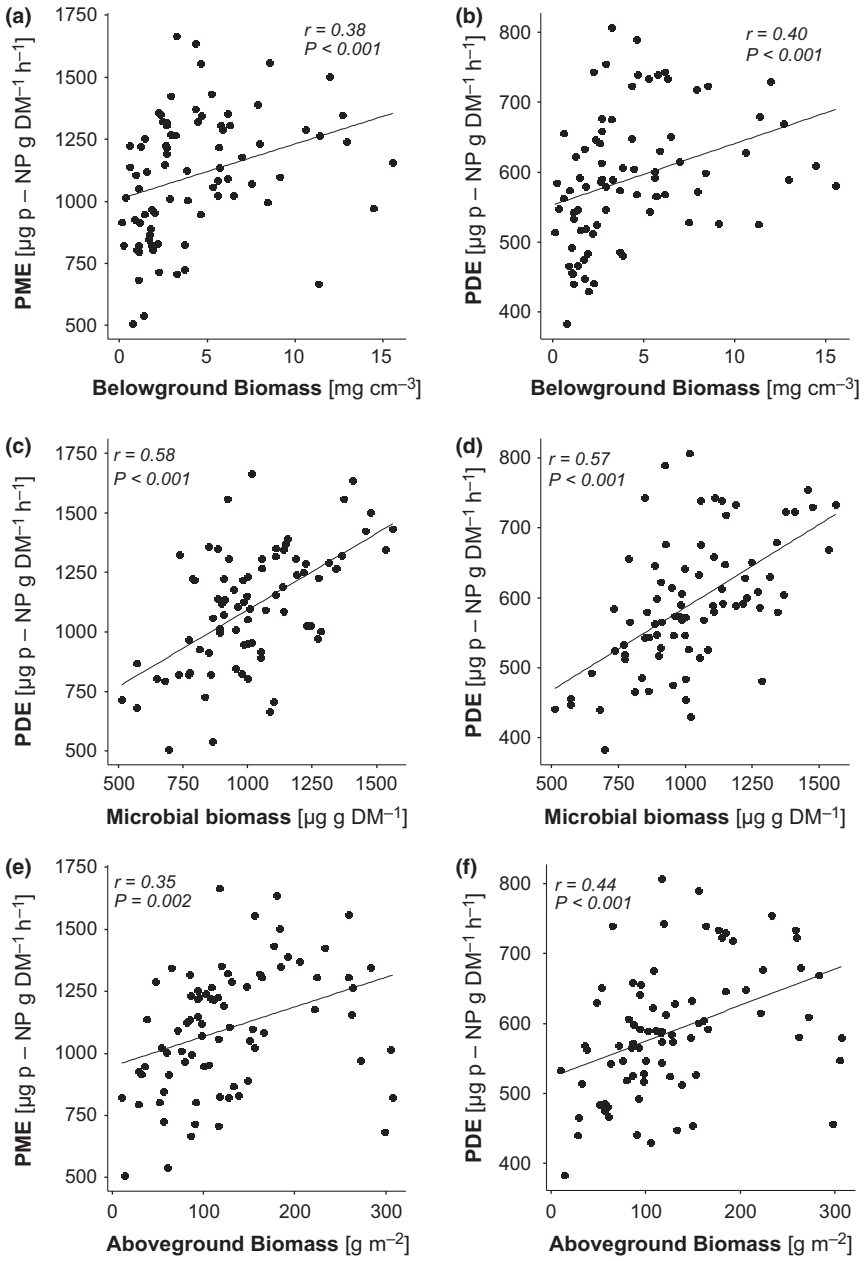

Figure 2 Relationship between PME and PDE activity and the amount of belowground biomass (PME: a, PDE: b), microbial biomass (PME: c, PDE: d) and aboveground biomass (PME: e, PDE: f). Note different y axes scales for PME and PDE. For belowground biomass the untransformed data are shown to ease interpretation. r and p refer to square root transformed data.

the case for PME activity (with legumes 939.80 ± 95.96, without legumes 863.93 ± 64.60; $t = 0.637$, $p > 0.05$).

DISCUSSION

Here, we assessed species richness, functional group richness and functional composition effects on phosphatase activities as P mobilisation mechanisms. We found that phosphatase activities increased with increasing plant species richness, which could be explained by C_{org} concentrations (indicative of substrate availability) and microbial biomass. Therefore, plant diversity effects on P mobilisation in soil were driven by interactions between microorganisms in soil, C_{org} as a substrate for microorganisms and roots as a habitat for microorganisms. Our results further suggest that the coupling between C and P cycling in soil becomes tighter with increasing plant diversity.

Plant diversity effects on phosphatase activities

Plant species richness significantly increased both PME and PDE activity (Table 1, Fig. 1). Our SEM revealed that these effects at least in part were mediated by other controlling factors. One third of the variability in phosphatase activity could

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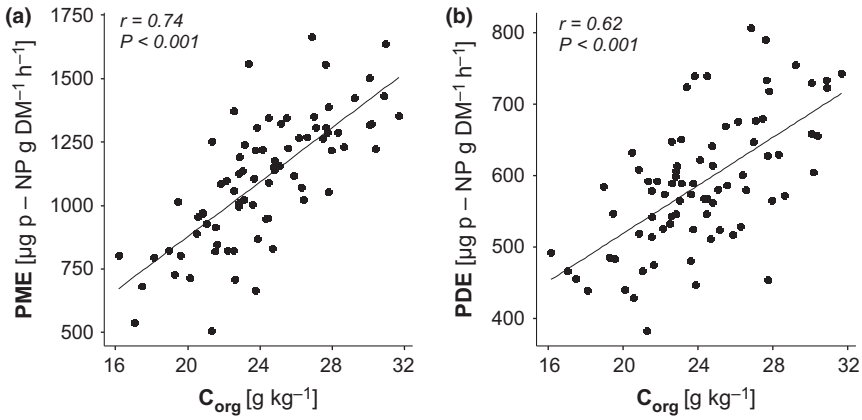

Figure 3 Relationship between PME (a) and PDE activity (b) and C_{org} concentrations in soil. Note different y axes scales for PME and PDE.

Table 2 Linear regressions with PME and PDE as dependent variables and microbial, aboveground and belowground biomass, C_{org}, $NaHCO_3$-P_i, NaOH-P_i, $NaHCO_3$-P_{org}, NaOH-P_{org}, $NaHCO_3$-P_{org} + NaOH-P_{org} as independent variables

	PME					PDE				
Factor	rse	d.f.	*r*	*F*	Sig.	rse	d.f.	*r*	*F*	Sig.
Microbial biomass	206.2	78	0.58	39.31	**< 0.001**	77.03	78	0.57	38.32	**< 0.001**
Aboveground biomass	237.1	77	0.35	10.570	**0.002**	85.03	77	0.44	15.24	**< 0.001**
Belowground biomass	234.1	78	0.38	13.000	**0.001**	86.37	78	0.40	14.51	**< 0.001**
C_{org}	170.8	78	0.74	93.03	**< 0.001**	73.76	78	0.62	48.85	**< 0.001**
$NaHCO_3$-P_i	247.7	78	0.2	3.262	0.075	92.99	78	0.15	1.818	0.181
NaOH-P_i	248.5	78	0.19	2.752	**0.101**	93.60	78	0.10	0.783	0.379
$NaHCO_3$-P_{org}	245.7	78	0.24	4.621	0.035*	92.07	78	0.21	3.426	0.068
NaOH-P_{org}	252.8	78	0.03	0.055	0.816	93.24	78	0.13	1.389	0.242
$NaHCO_3$-P_{org} + NaOH-P_{org}	252.1	78	0.08	0.490	0.486	94.03	78	0.03	0.061	0.805

*This regression is significant only if values below the detection limit (see Appendix S1) are set to zero.
Root biomass was square root transformed. rse refers to residual standard error, d.f. to degrees of freedom, *r* to the regression coefficient and *F* represents the *F* value of the corresponding variable. Significant regressions at $p < 0.05$ are displayed in bold.

not be explained by variables introduced in the SEM as illustrated by the direct path between plant species richness and phosphatase activities being retained in the final SEM. Therefore, other variables (e.g., trace element concentrations) not captured in our study might further contribute to explaining plant species richness effects on phosphatase activities. Nevertheless, among variables included in the SEM, substrate availability (C_{org}) was the primary mediating factor of plant species richness effects on phosphatase activities (Fig. 4). It is well-known that phosphatase activities are closely related to the presence of hydrolyzable substrate (Speir & Ross 1978; Santruckova *et al.* 2004). Even if P or other macronutrients limit organism growth, C (and N) must be supplied as a major component of amino acids for enzyme synthesis (Allison & Vitousek 2005). Gradients in C_{org} concentrations or stocks were previously observed in grassland plant diversity experiments where greater plant species richness resulted in a higher accumulation of soil organic matter (SOM) (Steinbeiss *et al.* 2008; Fornara & Tilman 2009). In general, more diverse grassland communities have a higher demand for P and simultaneously offer a greater amount of hydrolyzable substrate resulting in a higher enzyme exudation and activity. With increasing plant species richness the hydrolysis of SOM seems to gain importance for meeting the P demand of microorganisms and plants. This suggests that particularly in diverse plant mixtures, plants and microbes rely on SOM rather than P_i sources (sorbed and mineral-bond P). Whereas C and N cycling are driven by biological processes, both biological and physicochemical processes contribute to P cycling. Therefore, an increase in SOM content will shift the contributions towards biological processes (Achat *et al.* 2013) ultimately resulting in a closer link between C and P cycling. Plant species richness effects even remained significant if enzyme activities were normalised to C_{org} concentrations i.e., expressed as specific enzyme activities (Appendix S3, Appendix S7 c and d). Therefore, positive plant species richness effects on PME and PDE activity not only derive from an increase in hydrolyzable substrate.

More enzyme activity per unit substrate (C_{org}) increases the probability of the temporal and spatial match between substrate, enzyme, and microorganism in soil potentially serving as an adaption to competition e.g., between microorganisms and plants. In accordance, substrate availability (C_{org}) also influenced microbial biomass in soil (Appendix S4, S8) because of the heterotrophic C metabolism. With increasing plant species richness the ratio C_{mic}:C_{org} i.e., carbon use

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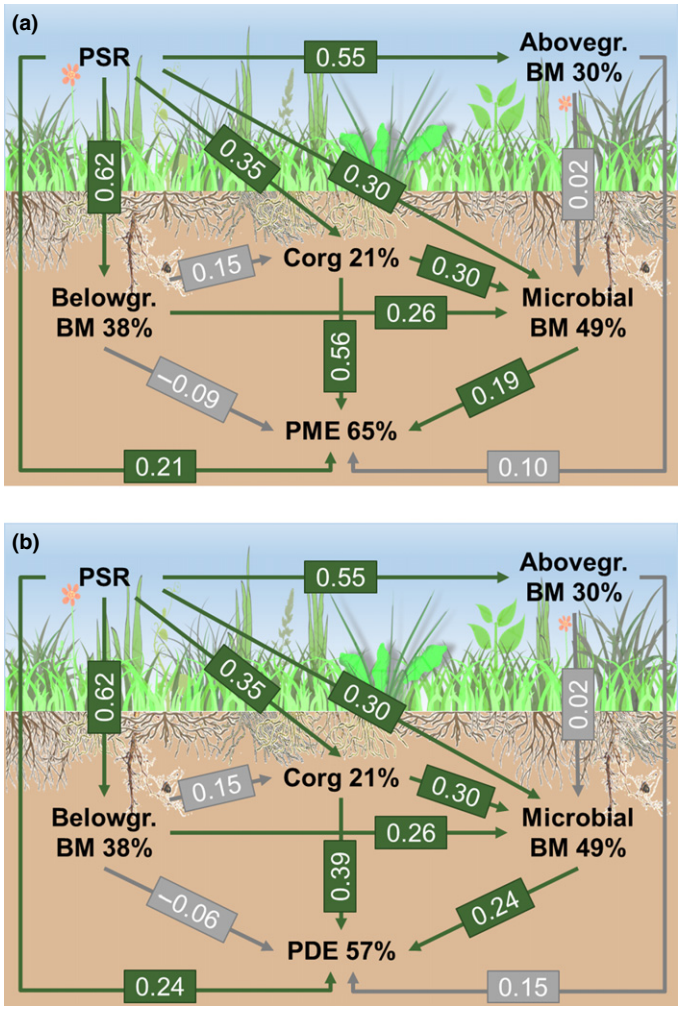

Figure 4 Structural equation model (SEM) of influences of plant species richness (PSR) on PME (a) and PDE activity (b) mediated by aboveground biomass (abovegr. BM), belowground biomass (belowgr. BM), C_{org} concentrations in the soil, and soil microbial biomass (Microbial BM). Numbers following the included variables show the explained percent of its variance by its predictors. Numbers on arrows are standardised path coefficients. Green arrows indicate significant effects. Both models fits the data well; model fits are given in appendix S5.

efficiency, increased illustrating increased microbial biomass produced per unit substrate (Appendix S4). Either complementary effects among different microorganism groups or an increased contribution of microorganisms particularly efficient in using carbon sources might serve as an explanation. In addition to the substrate-driven effect, microbial biomass itself also mediated the significantly positive plant species richness effects on phosphatase activities (Fig. 4). In this case, the normalisation of enzyme activities to microbial biomass yielded comparable exudation efficiencies among plant species richness levels (Appendix S3, Appendix S7 a and b). Therefore, microorganisms invest the same amount of resources into enzyme synthesis irrespective of plant species richness. Similar exudation efficiencies of microorganism indicate a proportional increase of microbial biomass and enzyme activities with increasing plant species richness. In combination, these findings suggest the need for efficient C rather than P cycling underlying the positive relationship between plant species richness and PME and PDE activity. The increased enzyme exudation and P release might thus be seen as a byproduct of the decomposition forming part of the microbial heterotrophic C metabolism (Spohn & Kuzyakov 2013; Stone *et al.* 2014). This again highlights the strong link between the C and P cycle gaining importance with increasing plant species richness.

Contrary to our expectation that increased belowground biomass (Ravenek *et al.* 2014) will contribute to increased enzyme exudation in mixtures of high plant species richness, we found no direct path between belowground biomass and phosphatase activities. Because of this missing link and the positive correlation between soil microbial biomass and phosphatase activities, we infer that microorganisms dominate the exudation of enzymes to mobilise P in the studied grassland plant communities.

Although our results show no direct effect of belowground biomass on phosphatase activities, the SEM highlights that plant roots play an important role in plant P uptake. Increased plant diversity is likely to be associated with an increased quantity and more variable quality of root exudates (Griffiths *et al.* 1999; Wardle *et al.* 2004). This builds the basis for microbial colonisation of the rhizosphere, which is more pronounced in plant mixtures of high plant species richness (Eisenhauer *et al.* 2010). Root exudates stimulate the microbial activity (Lange *et al.* 2015) and thus the exudation of phosphatase enzymes (Prober *et al.* 2015). Thus, a plant species richness-dependent increase of belowground biomass alone would not result in increased phosphatase activities. Instead, the linkage between roots and microbes in the rhizosphere ultimately control phosphatase activities.

Plant functional group effects on phosphatase activities

Our results show no effects of plant functional group identity on phosphatase activities (Table 1) not even if leguminous and non-leguminous monocultures were contrasted – with the exception of PDE activity. On the one hand, this is surprising as our legume species pool was greater ($n = 10$) than the six legume species classified as "P mobilising" by Li *et al.* (2014). On the other hand, the species pool of the Jena Experiment comprises legume species typical for meadows with comparatively low productivity fluctuating from year to year whereas five of six legume species mentioned by Li *et al.* (2014) are used as grain legumes in tropical and subtropical agriculture optimised for constantly high yield – and accordingly high P demand. Therefore, our results indicate that the effect of P-mobilising but low-yielding legume species under temperate climate are not as pronounced as in sub-tropical agricultural systems. Some authors argued that facilitative interactions between P mobilising and non P-mobilising plant species might be responsible for increased P uptake of diverse plant communities (Lambers *et al.* 2006; Li *et al.* 2014). In an earlier study, we indeed found an increased P uptake of diverse plant communities (Oelmann *et al.* 2011), but the results of the current study illustrate that this cannot be explained by a

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"P mobilising trait" associated with a certain plant functional group such as legumes in the Jena Experiment. In conjunction with the importance of microbes for the positive plant diversity effect on enzyme exudation mentioned above, not only plant roots but also the microbial colonisation of the rhizosphere form part of the species/functional group-specific ability to mobilise P.

CONCLUSION

We could show that plant species richness increased phosphatase activities in soil. Plant species richness effects on phosphatase activities were mediated by soil microorganisms which themselves depended on the rhizosphere as habitat and on C for their metabolism. Positive plant species richness effects on specific phosphatase activity (normalised to C_{org}) indicate the need of increased exudation per unit substrate very likely due to the associated increased probability of a temporal and spatial match among substrate, enzyme, and microorganism in soil. This pattern might be attributable to increased competition e.g., between plants and microorganisms. Carbon use efficiency of microorganisms (C_{mic}:C_{org}) increased with increasing plant species richness while resources allocated to enzyme synthesis by microorganisms (enzyme exudation efficiency = phosphatase activity:C_{mic}) remained constant across plant species richness levels. These findings suggest the need for efficient C rather than P cycling underlying the positive relationship between plant species richness and phosphatase activity. Furthermore, absence of plant functional group richness and identity effects on phosphatase activities suggest that the microbial community colonising the rhizosphere should be considered as a trait of P-mobilising plant species.

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AUTHORS CONTRIBUTIONS

YO and WW developed the idea for the study; NH, YO and WW designed the study; AE, AG, GG, OGM, HdK, ML, LM, NE, JR, SS, AW and CW provided data and associated methods; NH wrote the first draft of the manuscript and all authors contributed substantially to revisions.

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