Increasing plant species richness by seeding has marginal effects on ecosystem functioning in agricultural grasslands

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Abstract

1. Experimental evidence shows that grassland plant diversity enhances ecosystem functioning. Yet, the transfer of results from controlled biodiversity experiments to naturally assembled ‘real world’ ecosystems remains challenging due to environmental variation among sites, confounding biodiversity ecosystem functioning relations in observational studies. To bridge the gap between classical biodiversity-ecosystem functioning experiments and observational studies of naturally assembled and managed ecosystems, we created regionally replicated, within-site gradients of species richness by seeding across agricultural grasslands differing in land-use intensity (LUI) and abiotic site conditions.

2. Within each of 73 grassland sites, we established a full-factorial experiment with high-diversity seeding and topsoil disturbance and measured 12 ecosystem functions related to productivity, and carbon and nutrient cycling after 4 years. We then analysed the effects of plant diversity (seeded richness as well as realized richness), functional community composition, land use and abiotic conditions on the ecosystem functions within (local scale) as well as among grassland sites (landscape scale).
3. Despite the successful creation of a within-site gradient in plant diversity (average increase in species richness in seeding treatments by 10%–35%), we found that only one to two of the 12 ecosystem functions responded to realized species richness, resulting in more closed nitrogen cycles in more diverse plant communities. Similar results were found when analysing the effect of the seeding treatment instead of realized species richness. Among sites, ecosystem functioning was mostly driven by environmental conditions and LUI. Also here, the only functions related to plant species richness were those associated with a more closed nitrogen cycle under increased diversity.

4. The minor effects of species enrichment we found suggest that the functionally-relevant niche space is largely saturated in naturally assembled grasslands, and that competitive, high-functioning species are already present.

5. Synthesis: While nature conservation and cultural ecosystem services can certainly benefit from plant species enrichment, our study indicates that restoration of plant diversity in naturally assembled communities may deliver only relatively weak increases in ecosystem functioning, such as a more closed nitrogen cycle, within the extensively to moderate intensively managed agricultural grasslands of our study.

**KEYWORDS**

biodiversity-ecosystem functioning experiments, complementarity, ecological restoration, land-use intensity gradient, plant functional traits, seed addition, species pool effect

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1 | INTRODUCTION

Concerns about ongoing biodiversity loss have led to many studies investigating relationships between biodiversity and the functioning of ecosystems (Cardinale et al., 2006; van der Plas, 2019). Currently, most knowledge on the relationships between plant biodiversity and ecosystem functioning (BEF) comes from controlled experiments (Cardinale et al., 2012; Hooper et al., 2012). These usually assess the functioning of randomly assembled communities by manipulating initial or “sown” species richness (e.g. Roscher et al., 2004; Tilman et al., 1996). Such diversity manipulations have generally shown that initially diverse assemblages promote ecosystem functioning, with positive but saturating BEF relationships being consistently observed (Cardinale et al., 2012; Hooper et al., 2012; Isbell et al., 2015). At the same time, BEF experiments have often been criticized for their artificial nature and lack of realism (e.g. Srivastava & Vellend, 2005), leading to questions regarding their relevance to ecosystem management and conservation (Klaus et al., 2020; Manning et al., 2019). This critique has been further substantialized by observational BEF studies that showed contrasting results between experimental and non-experimental systems (reviewed in van der Plas, 2019). Thus, the transferability of findings from controlled experiments to managed real-world grassland ecosystems, remains challenging. We detail three reasons for this below:

First, BEF experiments are criticized for representing species loss as a random process (Lepš, 2004; Wardle, 2016), while in real-world-communities species loss or gain is non-random and species with certain functional traits are more likely to go extinct or increase in abundance (Lisner et al., 2023; Pywell et al., 2003; Saar et al., 2012). Both simulations and experiments underpin this criticism, showing that productivity is largely unaffected by species loss if the least competitive and least productive species are most extinction prone (Gross & Cardinale, 2005; Smith & Knapp, 2003). Thus, not only biodiversity per se (i.e. how many species or functional types occur within a community) but also functional community composition (i.e. which species are present and/or dominant within a community) need to be considered in the analysis of BEF relations.

A second factor complicating the comparison of BEF relationships found in biodiversity experiments and observational studies with self-assembled communities is the distinction between initial and realized biodiversity (Hagan et al., 2021). Complementarity and selection effects are considered the main mechanisms underlying the positive relationship between initially sown species-richness and ecosystem functioning of classical BEF experiments, (Loreau & Hector, 2001). Importantly, both theory and experimental evidence show that these complementarity and selection effects (and hence overall effects of biodiversity on ecosystem functioning) are more strongly related to initially sown biodiversity, than to realized biodiversity. The local species pool as an analogue to the initially sown biodiversity in experiments is often unknown in observational studies (Hagan et al., 2021). Therefore, in these studies BEF relations are typically based on ‘realized’ biodiversity while the opposite is
true for experiments, where the species pool, corresponding with the initially sown diversity levels, is easy to determine. Third, divergent findings between experimental and observational BEF studies can be attributed to specificities of these two contexts. Experiments usually directly manipulate local species diversity at one site while controlling for environmental conditions. Conversely, observational studies typically correlate local species diversity across multiple sites with ecosystem functions. As sites differ in environmental conditions, species diversity and/or ecosystem functions may be confounded with gradients of land-use intensity (LUI) and abiotic site conditions in observational studies and thus overshadow their BEF relationship (De Laender et al., 2016; Srivastava & Vellend, 2005; see overview in Table 1). To resolve this problem, observational studies often use covariates to control for differences in environmental conditions (e.g. nutrient availability, soil moisture). However, it is often unclear which covariates are required to isolate effects of species diversity on ecosystem functioning, which can result in an incomplete set of covariates and hence incorrect conclusions.

To help bridge the gap between observational and experimental BEF studies we combined both approaches in a study design that manipulated within-site diversity by seeding species-rich mixtures, along larger scale gradients of LUI and abiotic site conditions (Figure 1a—seeding treatment; Figure 1b—integration of LUI and abiotic site conditions). This approach avoids some major drawbacks of classical BEF experiments such as the rather artificial maintenance of unnaturally low diversities and allow the creation of diversity gradients under more realistic environmental settings. The studied agricultural grasslands comprise a variety of land-use intensities typical of Central Europe ranging from unfertilized sheep pastures to highly fertilized silage meadows and cover broad range of environmental conditions (Blüthgen et al., 2012). Seed addition experiments are ideal for studying BEF relationships while acknowledging the effect of other covariates on both biodiversity and ecosystem functioning, and allow distinction between the effects of realized and initially sown biodiversity in realistic settings as well as considering non-random biodiversity loss (Bannar-Martin et al., 2018; Manning et al., 2019). Furthermore, seed addition experiments are of high applied value, as alongside high soil nutrient levels impoverished local species pools are often a key factor in limiting successful grassland biodiversity restoration (Myers & Harms, 2009). From a functional perspective, such impoverishment may prevent high-functioning species from colonizing a site (Hagan et al., 2021; Leibold et al., 2017). Thus, by performing a regionally distributed seed addition experiment, we could jointly study the effects of non-random biodiversity loss and realized biodiversity at the between sites level as well as the within-site effects of randomly added sown biodiversity, and compare their effects. Such a design allows us to identify positive within-site BEF relationships that may be obscured by larger environmental and management drivers of ecosystem functioning at the among-site scale (Figure 1).

Across 73 sites, we manipulated vascular plant diversity in a fully factorial seed addition experiment in which we crossed high-diversity seeding of native species and topsoil disturbance treatments (to facilitate colonization). At the same subplots, we measured 12 ecosystem functions related to above-ground primary productivity, carbon (C)- and nutrient cycling after 4 years that have shown to influence BEF relationships in previous studies (see Table 1 for the list of ecosystem functions) and investigated their response to seeding treatments. The seeding and disturbance treatments successfully established a species richness gradient within grassland sites (Freitag et al., 2021). After 4 years runtime of the experiment, species richness increased on average by 2.5 species per m^2 (10%) on the seeding-only subplots and by 9.0 species per m^2 (35%) on the seeding-and-disturbance subplots compared to the control. Moreover, there was an overall 10% increase in the effective number of species (S_{pie}, Jost, 2006) in the seeding-and-disturbance subplots, indicating that some newly sown species became major constituents of their communities. We therefore expected complementarity effects of the moderately increased species richness to prevail, with—among all functions included—particularly positive effects on functions related to C and nutrient cycling that have shown high responsiveness in previous studies (Allan et al., 2015; Kleinebecker et al., 2014; Oelmann et al., 2021). Besides species-richness we also tested for the effects of alternative parameters that may covary with species richness and have direct or indirect effects on ecosystem functioning, such as functional composition, LUI and abiotic conditions.

We tested the following hypotheses: (i) positive relations between realized plant species richness and ecosystem functioning exist within sites (Hypothesis I, blue line in Figure 1); (ii) however, within sites, the seeding treatment shows a stronger effect on ecosystem functioning than the actual change in realized richness (Hypothesis II) and (iii) positive BEF relations of realized among sites are obscured by variation in LUI and abiotic site conditions, the dominant controls of functioning at these scales (Hypothesis III, dark grey line in Figure 1).

2 | MATERIALS AND METHODS

2.1 | Study design

Our plant-diversity-restoration experiment was performed in 73 grasslands as part of the large-scale and long-term Biodiversity Exploratories project (Fischer et al., 2010). They are distributed across three German regions (Biosphere Reserve Schwäbische Alb, 48.4°N, 9.4°E; National Park Hainich-Dün and surroundings, 51.1°N, 10.4°E; Biosphere Reserve Schorfheide-Chorin, 53.0°N, 14.0°E). These regions span gradients in soil characteristics, elevation and climate that are representative of large parts of Central Europe. The 23–25 agricultural grassland sites per region were selected to represent gradients of LUI. They range from low input systems such species-rich calcareous grasslands used as unfertilized sheep pasture to moderately high-input systems such as fertilized silage meadows with up to four cuts per year. To measure LUI farmers were interviewed annually between 2014 and 2018 to establish the amount of fertilizer used, mowing frequency and...
The 12 ecosystem function indicators (henceforth ecosystem functions) measured 4 years after the set-up of the experiment. Most ecosystem functions were measured in all 73 grasslands (N = 292 subplots), but sample sizes vary due to constraints in fieldwork logistics and lost samples. See section 2. Ecosystem function measurements in Supporting Information for more details on ecosystem-function-indicator measurements. BEF evidence was compiled from grassland studies and related experiments in similar, sown communities.

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Grazing intensity per grassland site (Vogt et al., 2019). We averaged fertilization (kgNha⁻¹year⁻¹), mowing (no. of cuts year⁻¹) and grazing (livestock units × grazing days ha⁻¹) intensities across years and standardized to their global means before calculating a LUI index according to Blüthgen et al. (2012) using the LUI tool (Ostrowski et al., 2020) in BExIS (http://doi.org/10.17616/R32P9Q). Low LUI corresponds to unfertilized sheep pastures or annually cut meadows, while high LUI indicates highly fertilized and frequently disturbed meadows and pastures.
To create an experimental plant species diversity gradient within each of the 73 grasslands (Figure 1), we established a 2×2 factorial experiment in autumn 2014 in which high-diversity seeding and topsoil disturbance were the treatments. This resulted in four 7×7 m subplots (control, seeding-only, seeding-and-disturbance, disturbance-only) per site (see Figure S1 for the experimental design). We applied the disturbance by tilling the topsoil down to 10 cm with a rotary harrow or rotary cultivator in October 2014. Fragments of the former sward were left on the disturbance subplots so that plants could potentially regrow. For the seeding treatment, we compiled seed mixtures of commercially available regional seeds from common and less-common native species of the species pools of each region (see Table S1), including grasses (12–15 spp., depending on region), legumes (0–7 spp.) and forbs (35–44 spp.) and used the same regional seed mixture for all sites within a region. The mixtures were applied to the seeding subplots twice, in October 2014 and March 2015. Seeding density per species depended inversely on seed mass. In total, we sowed 5.37 g m⁻² and 66 species in Schwäbische Alb, 4.11 g m⁻², 52 species in Hainich-Dün and 3.47 g m⁻² and 47 species in Schorfheide (see Klaus et al., 2017 for further details).

We used soil pH and a topographic wetness index (TWI) to characterize the abiotic site properties of the grasslands, as these have been shown to influence local plant diversity (Le Provost et al., 2021) and ecosystem functioning (Allan et al., 2015; Le Provost et al., 2022). For pH, in May 2017, we took a composite sample of 14 soil cores of 10-cm depth in the direct vicinity of the experiment, dried the soil and measured pH in a 0.01 M CaCl₂ solution (1:2.5 soil:solution ratio). The TWI was calculated as log(a/tanθ) where α is the specific catchment area (cumulative upslope area which drains through a Digital Elevation Model (DEM, http://www.bkg.bund.de) cell, divided by per unit contour length) and tanθ is the slope gradient in radians calculated over a local region surrounding the cell of interest (Gessler et al., 1995). TWI therefore combines both the upslope contributing area (determining the amount of water received from upslope areas) and slope (determining the loss of water to downslope areas). We calculated TWI from raster DEM data with a cell size of 25 m using ArcGIS tools (flow direction and accumulation tools of the hydrology toolset and raster calculator) and averaged the TWI values of a 4×4 m window centred on the grassland site, that is 16 DEM cells (100×100 m in total).

### 2.2 Plant diversity and functional composition

We surveyed plant diversity and functional composition in May 2018 and 2019, ~4-years after the set-up of the experiment. We recorded plant diversity developed after seeding as realized species richness, i.e. the number of all vascular plant species on one 2×2 m quadrat per treatment subplot. To characterize the functional plant community composition along resource-acquisition (specific leaf area [SLA]; Reich, 2014) and mycorrhizal collaboration gradients (root diameter; Bergmann et al., 2020), we: (i) quantified taxonomic community composition by visually estimating the percentage cover of each species in each quadrat, and (ii) and extracted functional species trait information using species-specific means of SLA from TRY (Kattge et al., 2020) and mean log-transformed measurements of fine root diameter from Lachaise et al. (2021). For both traits t, we calculated community-weighted means (CWMs) for each community c with S species as $CWM_{ct} = \sum_{i=1}^{S} p_{ci} \times t_{i}$, where $p_{ci}$ is the relative...
abundance of species $i$ in community $c$ and $t_i$ is the mean trait value of species $i$, using the R package FD (Laliberté & Legendre, 2010) in R v4.0.5 (R Core Team, 2021).

2.3 | Ecosystem functions

Between 2018 and 2019, we measured 12 above- and below-ground ecosystem function indicators (hereafter ‘ecosystem functions’) related to productivity and nutrient cycling on all four subplots of each site (Table 1). See section 2. Ecosystem function measurements in Supporting Information for more details on measurements. We selected variables that we hypothesised would respond to diversification within the timeframe of the experiment, based on previous BEF studies (Allan et al., 2015; Kleinebecker et al., 2014; Oelmann, Buchmann, et al., 2011; Oelmann, Richter, et al., 2011; Oelmann et al., 2021) and our knowledge of the study system. In Table 1, we compiled existing information to show which biodiversity effects can be expected for the measured functions considering both experimental as well as observational studies. These previous results clearly show that similar responses may be found in experimental as well as in observational approaches, but in general evidence is weaker and much more limited in observational studies.

2.4 | Analyses

We used two complementary diversity indices, namely species richness and effective number of species $S_{\text{PIE}}$, which accounts for community evenness by weighing abundant species more than rare species. We calculated evenness as the probability of interspecific encounter $\text{PIE} = \frac{\sum_{i=1}^{S} p_i^2}{i}$, where $S$ is species richness and $p_i$ is the relative cover of species $i$, and transformed $\text{PIE}$ into an effective number of species $S_{\text{PIE}} = 1/(1-\text{PIE})$ (Chase et al., 2018; Jost, 2006). $S_{\text{PIE}}$ equals species richness when all species have equal abundance, but $S_{\text{PIE}}$ decreases when cover is more unevenly distributed across species.

To analyse within-site effects of realized richness (Hypothesis I) and seeding (Hypothesis II), and among-site (Hypothesis III) effects of realized species richness on ecosystem functioning we performed three separate analyses.

We used multilevel multivariate regression, which allowed us to account for the experimental design (‘multilevel’ part) and model all responses simultaneously (‘multivariate’ part). Multivariate models allowed us to estimate residual correlations between responses, because responses measured in the same site might not be independent. To be able to estimate residual correlations, we chose a StudentT error distribution for all responses, which is robust in modelling outliers. During model fitting, we also simultaneously predicted missing values of ecosystem functions via posterior prediction because we otherwise had to remove observations with missing data to estimate residual correlations (Bürkner, 2017; see Table 1 for sample sizes).

Explanatory power of predictors was assessed using a Bayesian version of $R^2$ values (Gelman et al., 2019). We used marginal and conditional $R^2$ values to discriminate the variation explained by the fixed effects alone (marginal $R^2$) and in concert with both fixed and varying (i.e. random) effects (conditional $R^2$). Values of conditional $R^2$ can thus be expected to be much higher than values of marginal $R^2$ because they also consider the among-site variation (e.g. varying intercepts for site and varying disturbance estimate), which comes with much larger variation in e.g. ecosystem functioning.

2.4.1 | Species richness and functional composition effects within grassland sites (Hypothesis I)

First, we examined how realized species richness and associated functional composition is linked to ecosystem functions within sites (Hypothesis 1, Figure 1 blue lines). We centred the log-transformed realised species richness and CWMs of SLA and root diameter to the grassland mean (i.e. $x_{ij} = x_{ik} - \bar{x}_g$ for subplot $j$ in grassland $k$; see Figure S2). The site-mean-centred changes are independent of the global means as well as other site-level variables and allow to estimate the within-site effects on ecosystem functions independent from among-site effects (Enders & Tofghi, 2007). We then standardized all response and predictor variables to zero mean and unit standard deviation (SD) to obtain comparable effect sizes (i.e. $x = (x - \bar{x}) / SD_x$).

We modelled all 12 ecosystem functions with site-mean-centred species richness (log-transformed), SLA and root diameter as predictors, included varying intercepts for grassland sites and added a dummy-coded disturbance covariate (allowed to vary between grasslands) to account for the disturbance treatment. We log-transformed above ground plant biomass, root biomass, soil microbial C concentration, N and P uptake and soil N leaching risk to meet distributional assumptions. Gains in species richness by combined seeding and topsoil disturbance tended to be higher in already species-rich grasslands (Figure S3a), but within-site changes (i.e. site-mean-centred) of log-transformed species richness were independent of control treatment species richness (Figure S3b). The establishment of new species caused a small shift towards average CWMs of SLA and root diameter (Figure S3c,d), but within-site changes of CWM traits were independent of within-site gradients of species richness (Figure S3e,f).

Because we expected diversity effects on ecosystem functioning to be strongest at low (pre-treatment) species richness (Cardinale et al., 2012), we included an interaction term between site-mean-centred richness and control-treatment richness (the same control richness for all subplots within a site). We compared models with and without the interaction based on the expected log pointwise predictive density (elpd, derived from leave-one-out cross-validation; Vehtari et al., 2017) to judge whether the interaction improved our predictions of ecosystem function.

We also checked for an interaction of within-site species richness with LUJ. However, LUJ did not improve model predictions for
2.4.2 | Increased species pool effects of seeding on ecosystem functions

Second, to assess the effect of the increased species pool by seeding (as opposed to realized species richness) on ecosystem functioning within sites (Hypothesis II), we performed a similar modelling approach as under Hypothesis I, but replacing realized species richness by a dummy-coded seeding covariate as predictor. The single other modification was that we added the interaction of disturbance with seeding to the set of predictor variables in the new models because seeding success (and thus richness effects) are expected to differ depending on the level of soil disturbance (Freitag et al., 2021; Myers & Harms, 2009). This interaction was only needed in the new models as the seeding treatment was the same in disturbed and undisturbed plots, while realized richness in the previous models already was the result of the interactive effects of seeding and disturbance treatments. Thus, in the models with realized richness the disturbance effects are sufficiently accounted for as an additive predictor, without interaction-term.

2.4.3 | Species richness, land use and environmental effects on ecosystem functions among sites (Hypothesis III)

Third, we used a path analysis to test how realized species richness, functional composition and environmental drivers relate to differences in ecosystem functioning among sites (see grey line in Figure 1b, and the path diagram in Figure 3). While land use and environmental drivers were measured on site-level, species richness and functional composition were measured on the four subplots within sites. To also obtain among-site effects of species richness and functional composition that are estimated independently from within-site gradients, we replaced the measurements of variable x on plot j within site k with their respective site means: \( x_{jk} = \bar{x}_k \) (Enders & Tofghi, 2007). We further centred all response and predictor variables to zero global mean and unit SD to obtain comparable effect sizes (i.e. \( x = (x - \bar{x}) / SD_x \)).

We modelled all 12 ecosystem functions as a function of land-use intensity, TWI and soil pH and site-means of realized species richness and CWMs of SLA and root diameter. The path analysis allowed us to account for mediating (indirect) effects of site-level environmental drivers via the plant community; for this we also modelled the effects of land use, TWI and soil pH on realized species richness and CWMs of SLA and root diameter in the same multivariate model (see path diagram in Figure 3). We included a variable intercept for grassland site and added a dummy-coded disturbance covariate (allowed to vary among sites) for all responses to account for the disturbance treatment and log-transformed above-ground plant biomass, root biomass, soil microbial C concentration and soil N leaching risk to meet distributional assumptions (see Figures S4 and S7 for predictor variable distributions). Because we included all possible links between responses and predictors as well as residual correlations between ecosystem functions in our among-site path analysis, this multivariate model represents a Bayesian implementation of a saturated path analysis. Conceptually, it would also have been possible to estimate these among-site relationships together with the within-site relationships (Hypothesis I) for realized richness in one model, yet, computational constraints forced us to estimate the relationships in two separate models.

We fitted the multilevel multivariate models in a Bayesian framework using the Stan probabilistic language (Stan Development Team, 2020) accessed via the package brms v2.15.0 (Bürkner, 2017) in R v4.0.5 (R Core Team, 2021) and used the package LOO for leave-one-out cross-validation (Vehtari et al., 2020). For all models, we specified weakly regularizing normal priors (\( \mu = 0, \sigma = 2 \) for fixed parameters; \( \mu = 0, \sigma = 0.5 \) for intercepts; Gelman et al., 2017). We fixed the Student-T freedom parameter to \( v = 4 \), because the sample size (\( N = 292 \)) is too low to reliably estimate \( v \). We ran four parallel chains and 4000 iterations (2000 discarded as burn-in) and ensured convergence with R-hat values ≤ 1.01 for all parameters. Model fit was assessed with posterior-predictive checks for all responses using the package bayesplot v1.7.2 (Gabry et al., 2019; Figures S5 and S8). Explanatory power of models and fixed effects was estimated using the Bayesian version of R² (marginal and conditional R²) as implemented via the package brms v2.15.0 (Bürkner, 2017) in R v4.0.5 (R Core Team, 2021).

3 | RESULTS

3.1 | Within-site effects of realized species richness and functional composition on ecosystem functions (Hypothesis I)

The effects of experimentally created within-site variation in species richness (realized richness) and functional traits on 12 ecosystem functions (Figure 2) were mostly insignificant or weak. The two exceptions were above-ground plant \( ^{15}N \), which decreased with site-mean-centred species richness (\( \beta = -0.04, 95\% \text{ CrI: } -0.07 \) to \(-0.01, \text{ Table S2} \)) and above-ground plant biomass (log-transformed), which marginally increased with higher species richness (\( \beta = 0.02, 95\% \text{ CrI: } -0.02 \) to \(0.06\)). This implies an average increase in above ground biomass of only 2.7%, given an average 34% increase in species richness in the combined seeding and disturbance treatment. The inclusion of an interaction with control-treatment species richness or land-use intensity did not improve model predictions for any ecosystem function (loo-derived expected log pointwise predictive density \( \text{elpd} \) of interaction model lower or within standard errors of \( \text{elpd} \) difference). This indicates that, in contrast to our assumptions, plant species richness effects, or the lack of them, were largely independent of pre-treatment species richness or land-use intensity.
For all combinations of traits and functions studied, we only found three significant trait-function relationships. The first of these was that SLA increased above ground biomass C:N ratio ($\beta = 0.04$, 95% CrI: 0.00 to 0.08). The second and third was that high fine root diameter was associated with slower decomposition of low-quality litter ($\beta = -0.11$, 95% CrI: -0.19 to -0.02) and an increase in the soil microbial C:N ratio ($\beta = 0.04$, 95% CrI: 0.00–0.08). Topsoil disturbance had a lasting effect on several ecosystem functions; it strongly reduced root biomass ($\beta = -0.43$, 95% CrI: -0.65 to -0.21), and increased above-ground plant $\delta^{13}$C ($\beta = 0.10$, 95% CrI: 0.02–0.19) and $\delta^{15}$N ($\beta = 0.16$, 95% CrI: 0.09–0.23), which indicates increased drought stress and nitrogen losses at disturbed sites.

3.2 | Species-pool effects of seeding treatments on ecosystem functions (Hypothesis II)

Within sites, the 12 ecosystem functions were hardly affected by the seeding treatment (Table S3). Only above-ground plant N uptake showed a positive response to the seeding × disturbance interaction.
(β=0.25, 95% CrI: 0.01–0.47, Table S3) and a marginally significant negative response (β=−0.13, 95% CrI: −0.32 to −0.05, Table S3) to disturbance alone. This indicates enhanced N-uptake in disturbed and sown subplots but increased N-losses in disturbed subplots without seeding. Similarly, but less clear, above-ground plant δ\(^{15}\)N weakly (and non-significantly) decreased in the seeding×disturbance treatment (β=−0.09, 95% CrI: −0.2 to 0.02, Table S3) pointing towards a more closed N-cycle in disturbed and sown subplots. Overall, the effects of seeding treatments, which created an enlarged species pool, on ecosystem functions were similarly weak as those of realized richness, but also indicated enhanced nitrogen uptake and a more closed N-cycle in disturbed sites and sown sites.

### 3.3 Among-site effects of species richness on ecosystem functioning (Hypothesis III)

The path analysis revealed strong effects of land-use intensity and TWI on plant diversity and functional composition (Figure 3). Land-use intensity was associated with decreasing species richness (Figure 3a, β=−0.37, 95% CrI: −0.56 to −0.18, Table S4) and increasing SLA (β=0.30, 95% CrI: 0.07–0.52). Regarding changes in functions, species richness was negatively related to above-ground plant δ\(^{15}\)N, soil microbial C and soil N concentration (Figure 3e,g,k), indicating a more closed N-cycle. Meanwhile, land-use intensity and TWI had positive effects on above-ground plant biomass and above-ground plant δ\(^{15}\)N, further accompanied by positive indirect effects via SLA (Figure 3b,e). In line with our findings for within-site effects, also among sites we found a positive relation between plant species richness and functions indicating a more closed N-cycle (Figure 3e,k). Our results support hypothesis III, clearly showing that environmental drivers and land use, not plant species richness, are the main drivers of ecosystem functioning among sites.

### 4 DISCUSSION

To overcome the limitations of both observational and strongly controlled BEF studies, we created within-site plant species richness gradients via seeding of multiple sites. This enabled us to separate the effects of among-site (covarying with environmental drivers) and within-site effects of species richness on ecosystem functioning (Enders & Tofghi, 2007). This demonstrated only weak evidence for changes in ecosystem functioning with increased realized plant species richness (Hypothesis I) and seeding (Hypothesis II) within these agricultural grasslands (within-site effects). In contrast, among sites species richness was clearly associated with three out of 12 ecosystem-functions along confounding gradients of abiotic environmental conditions and land-use intensity (Hypothesis III).

Besides marginal changes in above-ground biomass, the only function affected by within-site increases in (realized) species richness was above-ground plant δ\(^{15}\)N. Above-ground plant δ\(^{15}\)N became more negative with higher species richness, indicating reduced N losses in species-rich communities. This finding is consistent with results of BEF experiments (Gubsch et al., 2011) and has been previously reported in an observational study for a large set of grasslands including those studied here (Apostolakis et al., 2022; Kleinebecker et al., 2014). Increased resource partitioning in diverse communities may have led to a more complete use of available N resources, which minimizes the risk of N losses due to denitrification or leaching, processes that cause residual enrichment in δ\(^{15}\)N (Kahmen et al., 2006).

Variation in the functional community composition within sites was weakly related to ecosystem functioning. Contrary to species richness, increases in SLA were positively related to above-ground plant δ\(^{15}\)N. This suggests a more open N-cycle in ‘fast’ resource-acquisitive communities characterized by high SLA, possibly because fast resource uptake goes along with lower leaf and root lifespan and faster N mineralization (Reich, 2014), which increases N turnover and the risk of N losses. This association of fast biogeochemical cycling with ‘fast’ plant communities is consistent with that seen for plant traits, soil C and soil microbial properties in English agricultural grasslands (de Vries et al., 2012; Manning et al., 2015), and in other studies of trait-function relationships in these German grasslands (Allan et al., 2015; Boedinghaus et al., 2019).

Above-ground plant δ\(^{15}\)N also tended to show a weak decrease as response to the seeding treatment, when seeding was combined with topsoil disturbance, which is consistent with increased above-ground biomass N content in the same treatment and a more efficient N cycle likely due to better resource partitioning as observed for realized species richness (Gubsch et al., 2011; Kahmen et al., 2006). Beyond these effects, we could not find further evidence for more efficient N use in other N-related functions such as the soil N leaching risk or microbial C:N. Against our expectation (Hypothesis II), using the species pool approach through focussing on seeding in the analysis of BEF relationships did not reveal further BEF relations and those found were not stronger than for realized richness.

When focussing on variation in diversity and ecosystem functions among sites, we again found a negative correlation between species richness and above-ground plant δ\(^{15}\)N, but also negative associations between species richness and soil microbial C and the soil N leaching risk. The former result indicates that this relationship operates both among and within sites. Still, we are cautious to interpret among-site effects of species richness as causal relationships. The negative effect of species richness on N leaching risk, for example, may also reflect that species richness is a better indicator of local soil conditions and long-term nutrient inputs on site (resulting in competitive exclusion; Harpole et al., 2016) than our measurements of recent land-use intensity (Kleinebecker et al., 2018).

In sum, our results suggest that plant species enrichment of on average nine species per 4 m\(^2\) via the combined treatment has only minor effects on ecosystem functioning in our traditionally to moderately intensive managed agricultural grasslands, with minor differences between effects of seeding and realized richness. There are three possible and interlinked explanations for our findings:

First, BEF relationships quickly approach saturation (Cardinale et al., 2012), often strongly levelling off at around 10 species per
community (Jochum et al., 2020; Tilman et al., 1996). In even the least diverse grasslands of our study, we recorded 10 species per 4 m² on the control treatments. While this represents diversity levels where saturation starts to occur, BEF relationships should remain observable. Yet, we found no evidence that ecosystem functioning in these species-poor communities was clearly affected by enhanced species richness. Although species gains perhaps led to a more complete exploitation of mineral N, this indication of enhanced resource partitioning appeared not to translate into changes in other ecosystem functions, except for a minimal increase in above-ground plant biomass or a higher above-ground biomass N content in the seeding-and-disturbance treatment. Thus, it seems that strong biodiversity effects, based on considerable increases in complementarity via resource partitioning, might be confined to even lower diversity levels. The functional benefits of grassland species enrichment may be greatest in very species poor grassland systems, such as those sown for silage cropping (Finn et al., 2013; Nyfeler et al., 2009). Alternatively, complementarity effects might take a long time to manifest, and there is evidence from BEF experiments that biodiversity–ecosystem functioning relationships become stronger with experimental duration, and associated community maturation (Guerrero-Ramírez et al., 2017). Thus, long-term monitoring grassland experiments like the one of this study should be encouraged to enable investigating such temporal dynamics.

Second, species most relevant to ecosystem functioning might have been present already. In a previous analysis of establishment success in the same experiment (Freitag et al., 2021) we found small-statured species with low SLA had higher average establishment success than high-SLA species, and low-SLA species preferably established in low-productivity grasslands. This may explain why increases in richness did not substantially raise productivity, as high productivity, ‘fast’ functioning species (i.e. species with high SLA) from the local species pool were already present. According to the mass ratio hypothesis especially dominant species are of key importance for ecosystem functioning, while the directed loss of subordinate species was shown to not matter for grassland productivity (Lisner et al., 2023). Stabilizing effects of newly established low-SLA species on ecosystem functioning, however, may become visible in the long term through the buffering of extreme events such as exceptional droughts (Craven et al., 2018; Tilman & Downing, 1994).

Third, among grassland sites, the strong influence of environmental drivers has obviously masked and overruled diversity effects. Here, land-use intensity and TWI strongly affected both species richness and plant community traits. Environmental drivers also strongly influenced ecosystem functions, for example, above-ground plant production, above-ground plant C:N ratio (biomass quality), and N uptake. Observational studies comparing BEF relationships among grassland sites therefore typically find weak diversity effects, often because diversity and functional composition are the result of land use and abiotic conditions (van der Plas, 2019).

In summary, our results showed only very limited increases in ecosystem functioning as a response to seeding-induced gains in species richness. This begs the question as to under which circumstances restoring plant diversity can be more functionally beneficial. Enhancing ecosystem functioning by restoration of plant diversity might be confined to previously degraded or extremely species-poor systems on ex-arable land, where enhanced plant species richness by seeding indeed has led to higher productivity (Bullock et al., 2007), higher biomass quality (Savage et al., 2021), accelerated litter decomposition (Zirbel et al., 2019), and reduced soil inorganic N losses (Klopf et al., 2017; Mueller et al., 2013). A recent synthesis further supports this and contradicts generally positive effects of seed addition on above-ground biomass production (Ladouceur et al., 2020). The results of this meta-analysis indicate a context-dependency of BEF relationships, especially regarding the degree of ecosystem degradation before seeding (e.g. permanent grasslands vs. ex-arable fields). Many grassland BEF experiments were created on ex-arable soils, which are, unlike permanent grasslands soils, degraded in view of low soil Corg content and reduced soil fauna (Lange et al., 2015). Increased root and microbial biomass inputs as well as an improved soil structure via plant-soil feedbacks have been identified as main mechanisms that may strongly contribute to complementarity effects observed in experiments (Barry et al., 2019; Eisenhauer et al., 2017; Klopf et al., 2017). Yet, in contrast to BEF experiments on ex-arable land, the soils of the permanent agricultural grasslands in this study are certainly less degraded due to a long history of grassland use and hence do not benefit as much from increased plant species richness in terms of soil organic C accumulation and other soil properties (Lange et al., 2015). This indicates that some BEF relationships may be of greater applicability to degraded grasslands with a strongly depleted species pool, where the addition of high-performing species could strengthen complementarity and improve soil quality. In grasslands with less degraded soils and species communities, expectations of considerable increases in ecosystem functioning after plant diversity restoration might not be met, even if the species pool of a grassland was significantly enriched, as in this study.

Yet, other functions or cultural and regulating ecosystem services that more directly depend on plant diversity, such as pollination, and aesthetic appeal, are more likely to improve with plant diversity restoration (Allan et al., 2015; Le Provost et al., 2022). From our among-site analysis we further conclude that the control of both plant species richness and community assembly by environmental drivers and especially land use can override BEF relationships and limit the transferability of experimental BEF results to the maintenance and restoration of real-world grassland ecosystems.

More such experiments that manipulate biodiversity under realistic settings of community assembly and ecosystem management are needed if we are to gain robust insights in the transferability of BEF relationships from classical experiments to ‘real-world’ ecosystems (e.g. Lisner et al., 2023). This will help us identify the situations in which the protection and restoration of biodiversity has its greatest benefits and will identify the circumstances in which functional or more traditional arguments for conservation and restoration are best applied.
AUTHOR CONTRIBUTIONS

Norbert Hözel, Ute Hamer, Till Kleinebecker, Valentin H. Klaus and Daniel Prati designed the experiment and Valentin H. Klaus, Martin Freitag, Norbert Hözel, Lena Neuenkamp, Fons van der Plas and Peter Manning conceived ideas for this study. Anna Abrahão, Ralph Bolliger, Martin Freitag, Norbert Hözel, Ute Hamer, Mark van Kleunen, Ellen Kandeler, Till Kleinebecker, Klaus-Holger Knorr, Valentin H. Klaus, Peter Manning, Sven Marhan, Daniel Prati and Gaëtane Le Provost contributed data. Martin Freitag analysed the data and prepared a first draft of the manuscript with substantial input from Norbert Hözel and Valentin H. Klaus. All authors contributed to revising the manuscript.

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REFERENCES


Peer REVIEW

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DATA AVAILABILITY STATEMENT

All data and code used for this study are archived within the BEXIS database of the Biodiversity Exploratories project: https://doi.org/10.25829/bexis.31496-8 (Freitag et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Design of the seeding and disturbance experiment on the 73 grassland sites under study. The four treatments (control, seeding, seeding and disturbance, disturbance) were arranged in a rectangle in most cases. Within each treatment, we surveyed vegetation on 2 x 2 m in 2018 and 2019. The symbols depict the locations where we sampled biomass and soil or placed resin bags and tea bags to measure ecosystem functions.

Figure S2. Conceptual figure showing the separation of among- and within-site effects of species richness on ecosystem functions. In (a), all (hypothetical) datapoints are shown, with four treatments (i.e. plots with the same symbol) at each site, highlighting the overall gradient in species richness (grey line) and the individual within-site gradients in richness (blue lines) as induced by the treatments. In (b), plots have been aggregated to site-mean centred variables. Here, we expect neutral or even negative correlations between plant species richness and ecosystem functioning, as these respond, in contrasting ways, to grassland management intensity. In (c) the within-site richness levels have been centred around the site mean to be independent of the among-site gradients. In that case, we expect positive relationships between within-site richness and ecosystem functioning, due to processes such as resource partitioning that can promote species complementarity.

Table S1. List of plant species sown in the 73 grassland sites in three regions. Seed mixtures differed by region depending on regional species pools (Klaus et al., 2017).

Figure S3. Overview of variation in species richness and functional composition four years after the set-up of the experiment (N = 292). (a) Gains in species richness by combined seeding and topsoil disturbance tended to be higher in already species-rich grassland sites (control plots not shown; Freitag et al., 2021), but within-site gradients (i.e. site-mean-centred) of log-transformed species richness was independent of control treatment species richness. (c, d) the establishment of new species caused a small shift towards average community-weighted means (CWMs) of specific leaf area (SLA) and root diameter, but within-site gradients of CWM traits were independent of within-site gradients of species richness.

Figure S4. Assessment of regional variation in within-site effects of realised plant richness on ecosystem functions (H1). Distribution (diagonal), pairwise scatterplots (lower panels) and correlations of predictor variables among regions (upper panels) as used in the analysis of the effects of site-mean-centred plant species richness and community weighted means of specific leaf area and root diameter on ecosystem functions.

Table S2. Summary of model estimates of within-site effects of realised plant richness on ecosystem functions (H1). Effects of site-mean-centred plant species richness and community weighted means of specific leaf area and root diameter on 12 ecosystem functions (95% credible intervals). For comparison, we also show results for the effective number of species $S_{PIE}$ (inverse Simpson index, Chase et al., 2018; Jost, 2006) instead of species richness. We modelled ecosystem functions as StudentT-distributed and estimated residual correlations between functions (see Figure S7). Random intercepts for grassland sites and a fixed effect for disturbance was included (allowed to vary among sites) to account for the experimental design. Ecosystem functions as well as predictors were scaled to unit standard deviation and some functions were log-transformed to meet distributional assumptions. Credible intervals which do not overlap zero are shown in bold (discrepancies with displayed 2.5% and 97.5% quantiles due to rounding errors). We calculated conditional $R^2$ ($\text{cR}^2$) taking all fixed and varying effects into account and marginal $R^2$ ($\text{mR}^2$) with fixed effects only.

Figure S5. Diagnostic plots for models testing within-site effects of realised plant richness on ecosystem functions (H1). Kernel density estimates of observations ($Y$) and 15 draws from the posterior predictive distribution ($Y_{\text{rep}}$) for the scaled ecosystem functions with site-mean-centred species richness and functional traits as predictors (and other predictors, see Methods).

Figure S6. Assessment of multicollinearity for models testing within-site effects of realised plant richness on ecosystem functions (H1). Residual correlations of ecosystem functions after accounting for site-mean-centred plant species richness and functional traits. See Table S2 for the model parameters.

Table S3. Summary of model estimates of within-site effects of
initially sown plant richness on ecosystem functions (H2). Effects of within-site seeding and disturbance treatments and their interaction on ecosystem functions (95% credible intervals). We modelled ecosystem functions as StudentT-distributed, included random intercepts for grassland sites and estimated residual correlations between functions. Ecosystem functions were scaled to unit standard deviation and some functions were log-transformed to meet distributional assumptions (see Methods). Credible intervals which do not overlap zero are shown in bold (discrepancies with displayed 2.5% and 97.5% quantiles due to rounding errors). We calculated conditional $R^2$ ($cR^2$) taking all fixed and varying effects into account and marginal $R^2$ ($mR^2$) with fixed effects only.

**Figure S7.** Assessment of regional variation in among-site effects of realised plant richness on ecosystem functions (H3). Distribution (diagonal), pairwise scatterplots (lower panels) and correlations of predictor variables among regions (upper panels) as used in the analysis of the effects of site means of plant species richness and community weighted means of specific leaf area and root diameter, as well as land-use intensity, soil pH and topographic wetness (TWI) on ecosystem functions.

**Table S4.** Summary of model estimates of among-site effects of realised plant richness on ecosystem functions (H3). Effects of site means of plant species richness and community weighted means of specific leaf area and root diameter, as well as land-use intensity, soil pH and topographic wetness (TWI) on 12 ecosystem functions (95% credible intervals). For comparison, we also show results for the effective number of species $S_{PIE}$ (inverse Simpson index, Chase et al., 2018; Jost, 2006) instead of species richness. We modelled ecosystem functions as StudentT-distributed and estimated residual correlations between functions (see Figure S9). Random intercepts for grassland sites and a fixed effect for disturbance was included (allowed to vary among sites) to account for the experimental design. Ecosystem functions as well as predictors were scaled to unit standard deviation and some functions were log-transformed to meet distributional assumptions. Credible intervals which do not overlap zero are shown in bold (discrepancies with displayed 2.5% and 97.5% quantiles due to rounding errors). We calculated conditional $R^2$ ($cR^2$) taking all fixed and varying effects into account and marginal $R^2$ ($mR^2$) with fixed effects only.

**Figure S8.** Diagnostic plots of models testing among-site effects of initially sown plant richness on ecosystem functions (H3). Kernel density estimates of observations ($y$) and 15 draws from the posterior predictive distribution ($\gamma_{rep}$) for the among-sites scaled ecosystem functions with site-means of species richness and functional traits as predictors (and other predictors, see Methods).

**Figure S9.** Check of multicollinearity for models testing among-site effects of realised plant richness on ecosystem functions (H3). Residual correlations of site-scaled ecosystem functions after accounting for site-means of plant species richness and functional traits. See Table S4 for the model parameters.