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RESEARCH ARTICLE

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# Neglecting non-vascular plants leads to underestimation of grassland plant diversity loss under experimental nutrient addition

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## Abstract

1. Nutrient availability and grazing are known as main drivers of grassland plant diversity, and increased nutrient availability and long-term cessation of grazing often decrease local-scale plant diversity. Experimental tests of mechanisms determining plant diversity focus mainly on vascular plants (VP), whereas non-vascular plants (NVP, here bryophytes) have been ignored. It is therefore not known how the current models based on VPs predict the rates of total (NVP+VP) losses in plant diversity.
2. Here we used plant community data, including VPs and NVPs, from nine sites in Europe and North America and belonging to the Nutrient Network experiment, to test whether neglecting NVPs leads to biased estimates of plant diversity loss rates. The plant communities were subjected to factorial addition of nitrogen (N), phosphorus (P), potassium with micronutrients ($K_{+\mu}$), as well as a grazing exclusion combined with multi-nutrient fertilization ($NPK_{+\mu}$) treatment.
3. We found that nutrient additions reduced both NVP and VP species richness, but the effects on NVP species richness were on average stronger than on VPs: NVP species richness decreased 67%, while VP species richness decreased 28%, causing their combined richness to decrease 38% in response to multi-nutrient ($NPK_{+\mu}$) fertilization. Thus, VP diversity alone underestimated total plant diversity loss by 10 percentage points.
4. Although NVP and VP species diversities similarly declined in response to N and $NPK_{+\mu}$ fertilizations, the evenness of NVPs increased and that of VPs remained unchanged. NP, $NPK_{+\mu}$ fertilization and $NPK_{+\mu}$ fertilization combined with grazing

exclusion, associated with decreasing light availability at ground level, led to the strongest loss of NVP species or probability of NVP presence. However, grazing did not generally mitigate the fertilization effects.

5. *Synthesis*. In nine grassland sites in Europe and North America, nutrient addition caused a larger relative decline in non-vascular plant (NVP) than vascular plant species richness. Hence, not accounting for NVPs can lead to underestimation of losses in plant diversity in response to continued nutrient pollution of grasslands.

**KEYWORDS**

bryophytes, community diversity, eutrophication, fertilizer, grassland, grazing, light limitation, Nutrient Network (NutNet)

## 1 | INTRODUCTION

Ecological theory predicts that nutrient addition should cause plant diversity loss (Tilman, 1982), and this has been shown experimentally in many studies of plant communities worldwide (Borer, Seabloom, et al., 2014; Harpole et al., 2016; Seabloom, Batzer, et al., 2021). Furthermore, theoretical and empirical work shows that nutrient-induced losses in plant diversity may be mitigated when herbivores increase light at ground level (Borer, Seabloom, et al., 2014; Olff & Ritchie, 1998). Experimental tests of drivers of plant diversity in terrestrial systems typically focus on vascular plants (VP), while non-vascular plants (NVP; here bryophytes), are usually ignored, even though NVPs can commonly represent 20%–40% of total plant diversity in grassland communities (Dengler et al., 2020; Klaus & Müller, 2014; Löbel et al., 2006; Lyons et al., 2022; Tansley & Adamson, 1925). It is therefore not known whether the response of NVP diversity to nutrient addition or grazing is consistent with that of VPs, in which case VP responses could predict NVP responses. However, NVPs could react differently (e.g. decrease to a greater or lesser degree), in which case their inclusion would refine our understanding of plant diversity loss in terrestrial systems.

In grassland ecosystems, NVPs consist mainly of two clades of land plants (Bryophyta (mosses) and Marchantiophyta (liverworts)) that contribute to many ecosystem functions and properties. Bryophytes often make up a large proportion of above-ground biomass (Boch et al., 2018; Hejcman et al., 2010; Wielgolaski, 1972), regulate the microclimate (Jaroszynska et al., 2023), hydrology (Michel et al., 2013), carbon and nutrient cycling (O'Neill, 2000; Turetsky, 2003), nitrogen fixation (Lindo et al., 2013), and affect seed germination of native and exotic vascular plants (Dollery et al., 2022), soil micro-organism diversity (Xiao et al., 2023) and soil multifunctionality (Xiao et al., 2024). Therefore, the presence and diversity of NVPs in terrestrial systems can have far-reaching ecological repercussions even in VP-dominated ecosystems. Important differences between NVPs and VPs, which may affect their ecological role, include that NVPs are an order-of-magnitude shorter than VPs, lack efficient conducting tissues, roots and stomata, and have relatively low photosynthetic rates (Rydin, 2009). Their small stature makes NVPs poor competitors for light, for which reason NVPs likely experience higher species loss rates than VPs, when increased nutrient supply intensifies competition for light (Rydin, 2009).

A handful of empirical studies over the past few decades provide expectations for NVP responses to increased nutrient availability or grazing pressure. Experimental nutrient addition can reduce the diversity of NVPs in various ecosystems (Bergamini & Pauli, 2001; Jägerbrand et al., 2006; Jäppinen & Hotanen, 1990; Jonasson, 1992; Wilson & Tilman, 2002), often due to increased competition for light with VPs (Aude & Ejrnæs, 2005; Bergamini & Pauli, 2001; Boch et al., 2018; Cusell et al., 2014; Jonasson, 1992; van der Wal et al., 2005; Virtanen et al., 2017). Excessive nutrient loads also may have direct negative effects on NVP survival (Armitage et al., 2012; Pearce et al., 2003; Virtanen et al., 2000). While nitrogen (N) is often assumed to be the most influential nutrient affecting NVPs, joint additions of N and phosphorus (P) can result in stronger negative effects on NVPs than additions of single nutrients (Bergamini & Pauli, 2001; Øien et al., 2018). However, nutrient effects are not entirely consistent among studies, with some finding relatively weak effects on NVPs (Stevens et al., 2004, 2006; Sun et al., 2017) or even positive relationships between nutrient levels and NVP abundance or species diversity (Gordon et al., 2001; Ingerpuu et al., 1998; Slavik et al., 2004). Therefore, the responses of NVPs to nutrient addition appear variable, which suggests that the responses are context dependent.

As with nutrient effects, grazing impacts on NVPs have been found to vary from positive (Bernes et al., 2018; Chollet et al., 2013; Ingerpuu & Sarv, 2015; Takala et al., 2012, 2014; Tansley & Adamson, 1925) to neutral (Austrheim et al., 2007) or negative (Boch et al., 2018; Spitale, 2021; Virtanen & Crawley, 2010). Herbivores can enhance plant diversity by promoting local colonization, stimulating germination from the soil diaspore bank, or altering plant competitive interactions (Olff & Ritchie, 1998). Biomass removal by herbivores can alleviate the competitive effects of VPs on NVPs, compensating for the negative effect of fertilization (Aude & Ejrnæs, 2005). On the other hand, intense grazing also may act as a strong disturbance causing loss of NVP species (Downing, 1992). Based on current, scarce and mixed evidence, it remains uncertain how NVP diversity will respond to nutrient addition and changes in grazing pressure separately and in interaction. Importantly, if NVPs respond differently from VPs, this could alter predictions for loss rates of grassland plant diversity.

Ignoring NVPs may lead to no effect, over- or underestimation of species loss rates under nutrient addition and/or changes in grazing in grasslands. More specifically, these outcomes can arise under the following conditions: (i) No effect on species loss rate estimates. This is the case if NVP and VP respond similarly to nutrient addition and grazing exclusion, or if NVPs are too scarce, and diversity is too low to have any effect. (ii) Overestimation of species loss rates. This could occur if the loss of VPs is compensated for by increased NVP diversity due to direct NVP diversity benefits of nutrient addition or altered level of grazing. (iii) Underestimation of species loss rates. This could occur if NVP diversity declines more than VP diversity due to nutrients directly suppressing NVPs or due to increased competition for light with VPs.

Here, we test the effects of nutrient addition and grazing exclusion on NVP and VP diversities at nine sites that are part of the globally distributed Nutrient Network experiment (Borer, Harpole, et al., 2014). The sites differ in their species composition and environmental context, which enables the quest to find generalities in the responses and mechanisms driving plant diversity. Specifically, we compare the responses of NVP and VP species richness, diversity and evenness to factorial addition of different nutrients (N, P, potassium (K)) and to NPK$_{+\mu}$ and grazing exclusion treatments to uncover whether accounting for NVP responses alters the overall estimates of changes in grassland plant diversity currently based on VPs only.

## 2 | MATERIALS AND METHODS

### 2.1 | The NutNet experiment and plant sampling

The Nutrient Network (NutNet) is a distributed experiment designed to address consumer and nutrient controls of grassland productivity and diversity. The network coordinates collection of consistent plant community data from experiments being conducted identically across a broad range of sites spanning continents (http://www.nutnet.umn.edu/; Borer, Harpole, et al., 2014). The network's standard field protocol includes sampling of vascular plant community data at species level from experimental plots every year, but the protocol excludes sampling of NVPs at species level. Therefore, we conducted an additional sampling on NVPs in the same way as for VPs. For this study, we selected nine sites that were aimed to make a representative, yet logistically feasible set of grasslands in different climatic biomes, including arctic-alpine, mediterranean and temperate regions of northern hemisphere (Table 1). These nine sites are the only NutNet sites from which NVPs have been sampled at species level. The sites fulfil the definition of grasslands 'as herbaceous dominated ecosystems' used by the network, and some main characteristics of the sites are shown in Figure S1. The mean

TABLE 1 NutNet sites sampled with the duration of experimental treatment years at the time of sampling in the parentheses.

| | | | | | | | Above-ground biomass (DW g m$^{-2}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| Site_code (years) | Habitat | Grazers excluded | Elevation, m a.s.l. | Latitude (°N), longitude (°E) | MAT | MAP | VP | NVP | Total |
| kilp.fi (3) | Tundra grassland | Reindeer, mountain hare, small mammals | 730 | 69.06, 20.87 | −4.1 | 523 | 96.0 | 126.6 | 250.5 |
| saana.fi (2) | Montane grassland | Reindeer, mountain hare, small mammals | 605 | 69.04, 20.84 | −2.6 | 490 | 199.0 | 20.4 | 386.7 |
| valm.ch (7) | Alpine grassland | Chamois, ibex | 2320 | 46.63, 10.37 | 0.3 | 1098 | 322.0 | 7.4 | 337.9 |
| hero.uk (8) | Mesic grassland | Deer, rabbit | 60 | 51.41, −0.64 | 9.9 | 692 | 316.9 | 13.8 | 407.1 |
| jena.de (2) | Mesic grassland | NA | 320 | 50.93, 11.53 | 8 | 610 | 692.7 | 3.4 | 814.8 |
| rook.uk (8) | Mesic grassland | Deer, rabbit | 60 | 51.41, −0.64 | 9.8 | 706 | 39.0 | 54.6 | 101.4 |
| hopl.us (8) | Annual grassland | Elk, deer, rabbits, feral pigs | 598 | 39.01, −123.06 | 12.3 | 1127 | 154.4 | 0.5 | 331.2 |
| mcla.us (8) | Annual grassland | DEER, rabbits, feral pigs | 641 | 38.86, −122.41 | 13.5 | 867 | 318.4 | 0.0 | 800.8 |
| sier.us (8) | Annual grassland | Elk, rabbits, deer, feral pigs | 197 | 39.24, −121.28 | 15.6 | 935 | 180.8 | 0.1 | 278.6 |

*Note*: Site jena.de has only nutrient treatments and no grazer exclosures. Biomass for vascular plants (VP) and non-vascular plants (NVP; bryophytes) before treatments. Total biomass includes litter.

annual temperature (MAT) of the sites ranges from −4.1°C to 15.6°C, mean annual precipitation (MAP) from 490 to 1127 mm and above-ground plant biomass from 101 to 818 $\mathrm{g\,m^{-2}}$ (Table 1; climate data from Hijmans et al., 2005).

Two experiments were established at each site: (1) multiple nutrient addition (9 sites) and (2) herbivore exclusion by nutrient addition (8 sites). In each case, the experimental units were 5 × 5 m plots, and treatments were assigned as a randomized block design (3–5 blocks per site). The multiple nutrient experiment was composed of controls and factorial combinations of three treatments: nitrogen (N; 10 $\mathrm{g\,N\,m^{-2}\,year^{-1}}$), phosphorus (P; 10 $\mathrm{g\,P\,m^{-2}\,year^{-1}}$) and potassium with micronutrients ($K_{+\mu}$; 10 $\mathrm{g\,K\,m^{-2}\,year^{-1}}$) combined with micronutrients (Borer, Harpole, et al., 2014). The N addition rate (10 $\mathrm{g\,m^{-2}}$) was chosen to overcome N limitation (Seabloom, Batzer, et al., 2021). The N fertilizer was applied as granular time-released urea, P fertilizer as triple super phosphate and K fertilizer as potassium sulphate. The micronutrient treatment (+μ; 100 $\mathrm{g\,m^{-2}}$) was applied only in the first year of treatments and was composed of boron (B; 0.1 $\mathrm{g\,m^{-2}}$), calcium (Ca; 6 $\mathrm{g\,m^{-2}}$), copper (Cu; 1 $\mathrm{g\,m^{-2}}$), iron (Fe; 17 $\mathrm{g\,m^{-2}}$), magnesium (Mg; 3 $\mathrm{g\,m^{-2}}$), manganese (Mn; 2.5 $\mathrm{g\,m^{-2}}$), molybdenum (Mo; 0.05 $\mathrm{g\,m^{-2}}$), sulphur (S; 12 $\mathrm{g\,m^{-2}}$) and zinc (Zn; 1 $\mathrm{g\,m^{-2}}$). The herbivore exclusion experiment was composed of controls and factorial combinations of two treatments: nutrient addition (fertilized) and herbivore exclusion (fenced). The fertilization treatment was the same as the all-nutrients-added treatment ($NPK_{+\mu}$) in the multiple nutrient experiment. The fences were designed to prevent entry by nonclimbing, medium to large herbivores. They were 110–230 cm tall, with the lower 80–90 cm surrounded by 1 cm mesh (except for site valm.ch where fences had 5 cm mesh size). The fence mesh was bent outward at the base in a 30 cm flange and stapled to the ground to prevent animals from digging under the fence (Borer, Harpole, et al., 2014). The permit to conduct the experiment at the Finnish sites (kilp.fi and saana.fi) was granted by Metsähallitus (MH 6083/2013), and permissions were not needed for the other sites.

We sampled above-ground plant biomass at peak biomass of vascular plants (in May–August, depending on local site level characteristics) by clipping at ground level and removing all above-ground vegetation (live and dead) from two 0.1 × 1 m strips, sorting the current year's VP and NVP biomass from the previous year's biomass (dead litter), drying the biomass to a constant mass at 60°C and weighing it to the nearest 0.01 g. NVP biomass was not sampled from valm.ch, but was estimated from NVP cover using regression (unpublished analyses). Except for two sites (heron.uk and rook.uk), we also measured photosynthetically active radiation (PAR) at the ground surface and above the grassland canopy at the time of peak biomass and calculated the proportion of transmitted light to ground level.

The percent cover of each VP and NVP species was estimated visually within a 1 × 1 m core sampling plot. The NVP and VP cover sampling was conducted in March–August 2016, except for heron.uk and rook.uk, which had been sampled for VPs in 2013. For the smallest NVPs, shoots were collected for microscopic keying in the laboratory. NVPs were mostly identified to species, but in absence of necessary diagnostic characters (capsules, other reproductive organs, distinctive gametophytic features), some specimens were identified at morphospecies group, subgenus or genus level. In the following text, we refer to the unique taxa as 'species.'

We calculated three plant diversity indices for NVPs, VPs and total (NVPs and VPs combined) in each plot. First, *species richness* ($S$) is the number of species per 1 $\mathrm{m^2}$ for NVPs and VPs. For plots having no NVPs, NVP richness is zero. Second, for plots having at least one NVP, we calculated Inverse Simpson's index of diversity (referred to as *species diversity*), which is equivalent to the Probability of Interspecific Encounter or Effective Number of Species ($ENS_{PIE}$) (Chase & Knight, 2013; Oksanen, 2022; Roswell et al., 2021). Third, we calculated Simpson's evenness ($E = ENS_{PIE}/S$; referred to as *evenness*), which was expected to reflect changes in species' dominance. Species richness was positively correlated with species diversity for NVPs, VPs and total ($r = 0.71$ for NVPs, $r = 0.69$ for VPs, and $r = 0.67$ for total, $p < 0.001$, df = 222 for all tests), whereas species richness was either negatively correlated ($r = -0.54$ for NVPs, $p < 0.001$, df = 222) or uncorrelated with evenness ($r = 0.05$ for VPs, $p = 0.43$, and $r = -0.03$ for total, $p = 0.6$, df = 222 for both tests). Species diversity was either uncorrelated ($r = -0.02$ for NVPs, $p = 0.8$, df = 222) or positively correlated with evenness ($r = 0.72$ for VPs and $r = 0.68$ for total, $p < 0.001$, df = 222 for both tests).

## 2.2 | Data treatment and statistical analyses

We computed the compositional turnover of NVPs, VPs and total plant communities based on dissimilarity of community compositions between control plots and $NPK_{+\mu}$-fertilized plots. We aggregated community data by sites and by control vs. $NPK_{+\mu}$-fertilized plots and calculated Jaccard dissimilarities between controls and $NPK_{+\mu}$-fertilized plots based on occurrence data (using TBI function of R package adespatial; Dray et al., 2022). The TBI function partitions turnover in three components (losses, gains and their sum). The indices were computed for data from eight sites, because mcla.us lacked NVPs in $NPK_{+\mu}$-fertilized plots.

The next statistical analyses were conducted in two steps. We first tested whether NVPs and VPs differ in their responses of species richness, species diversity or evenness to nutrient treatments and grazing. After this, we tested whether or how much the observed significant differences mattered for estimating the responses of Total species richness, species diversity or evenness (NVP and VP combined). In conducting these tests, we used mixed effects models followed by contrast analyses. In the mixed effects models, we used appropriate error structures depending on the response variable. The response variables included (1) species richness which was considered best fitted using a negative binomial error structure suitable for data with over-dispersed Poisson distributions; (2) species diversity was best fitted using log-transformation with Gaussian errors, and the same error structure was found suitable for (3) evenness and change in species richness. The change of species richness was obtained by subtracting the site mean of species richness within control plots from observed species richness in treatment plots.

In the Multiple Nutrient experiment models, fixed factors included N, P and $K_{+\mu}$ nutrient treatments, plant group [NVP, VP] and their interactions. In the Herbivore Exclusion by Nutrient Addition experiment models, $NPK_{+\mu}$ fertilization and grazing exclusion treatments, plant group [NVP, VP] and their interactions were treated as fixed variables. In all mixed effects models, the inclusion of random factors was specified to match the spatial hierarchy of the experimental design (i.e. block nested within site). The full models included all variables and their interactions as predictors. We simplified these models using a stepwise-backward selection procedure (omitting terms with $p > 0.05$; Crawley, 2005). For the models, we used R statistical software (packages brms version 2.19.0; Bürkner, 2017; nlme version 3.1-162; Pinheiro et al., 2022; and lme4 version 1.1-29; Bates et al., 2015). The brms (function brm) models performed better than lme4 (function glmer) or nlme (function lme) models, and for consistency, the final models used for predictions were ran using brms, and these brms models were used also in emmeans analyses (see below). The final brms model fits were examined using posterior predictive checking, which compares observed data to data simulated from the posterior distribution (pp_check function; Bürkner, 2017). We also used leave-one-out cross-validation to test for the presence of influential data points (loo function in R package loo; Vehtari et al., 2024), which showed no indications of any highly influential data points. Simplified models with interactions were subjected to estimation of marginal means and contrast analyses to summarize the responses of NVP, VP and total diversity to treatments (functions emmeans and contrast of R package emmeans; Lenth, 2022). Here we used both interaction and simple pairwise contrasts (Dean et al., 2017; Lenth, 2022).

We also quantified the changes of NVP and VP and total species richness, species diversity and evenness resulting from nutrient addition and grazing exclusion using log response ratio (LRR) LRR = log(value in treatment plot/site-wise mean value in controls). LRRs could not be calculated for plots with zero NVPs or for sites where controls had zero NVPs. We then asked if the magnitude of change estimated using LRR was predicted by the change in light availability at ground level in response to the treatments. Due to strong non-linearities, these models were fitted using generalized additive models (GAM, R package mgcv; Wood, 2017). In these analyses, we excluded sites with no light measurements (heron.uk, rook.uk) and omitted plots in which NVPs were not present (some plots from mcla.us and sier.us). In the case of evenness, plots with only single species also were omitted because evenness for monocultures is undefined. Thus, for all response variables, models examining the impact of light included at least 171 plots (out of 304; 56%). Finally, because the probability of the presence or absence of NVPs could depend on the nutrient addition treatments and consequent changes in the amount of light transmitted to ground level, we tested this using two additional mixed effects logistic regression analyses using R packages brms (Bürkner, 2017) and marginaleffects (Arel-Bundock, 2023).

## 3 | RESULTS

In 304 plots included in the data set, we recorded a total of 464 plant species of which 140 species were NVPs (30% of total) and 324 VPs (70%, Tables S1 and S2). Across all sites, the number of NVP species in control plots ($n = 31$) was 89 and that of VPs was 204, for a total of 293 species under ambient conditions. Across all sites, the $NPK_{+\mu}$ plots ($n = 31$) contained 203 species, which was 31% lower than under ambient conditions. However, losses were distributed unequally among species groups: while $NPK_{+\mu}$-fertilized plots contained 159 VP species (22% lower than in control plots), there were only 44 NVP species across all $NPK_{+\mu}$-fertilized plots (51% lower than in control plots), indicating greater species loss for NVP species than for VPs with elevated nutrients.

Across the study sites, Jaccard dissimilarity-based turnover (gains + losses) was greater for NVPs than VPs, primarily because losses of NVPs tended to be greater than VPs (Figure S2). Although the gains of NVP species exceeded losses at a single site (jena.de, a mesic grassland in Germany), $NPK_{+\mu}$ fertilization caused more losses than gains at 7 out of 8 sites, with losses contributing an average of 86% to the total dissimilarity, while gains contributed only 14% (TBI: paired permutation $t = -3.46$, $p = 0.02$ for the difference between losses and gains). $NPK_{+\mu}$ fertilization caused more VP losses than gains at all sites and, on average, the contribution of species losses to the total dissimilarity was 72% and that of gains 28% (TBI: paired permutation $t = -4.80$, $p = 0.006$). Total community composition more strongly reflected VP losses and gains, with a 77% contribution of losses to the total dissimilarity but only 23% gains (TBI: permutation $t = -5.28$, $p = 0.009$).

Both NVP and VP species richness decreased in response to N, P and $K_{+\mu}$ additions, but NVP species richness decreased more strongly than VP species richness under N addition (Figure 1a; plant group × N interaction in a mixed effects model; Table 2). For NVP species richness, the contrast control–N was 0.42 (95% highest posterior density interval (HPD) [0.27–0.55]; medians on the natural log scale) and that of VP species richness was 0.12 (95% HPD [0.03–0.22]; natural log scale). NVP species richness also responded more strongly than VP species richness to $NPK_{+\mu}$ addition (Figure 1b; plant group × $NPK_{+\mu}$ interaction in a mixed effects model; Table 3). For NVP species richness, the contrast control–$NPK_{+\mu}$ was 1.11 (95% HPD [0.84–1.34]; natural log scale) and that for VPs was 0.32 (95% HPD [0.15–0.48]; natural log scale). The responses of both NVP and VP species diversity to N and $NPK_{+\mu}$ addition were consistently negative (mixed effects model results in Tables 2 and 3). The responses of evenness to additions of N, P and $NPK_{+\mu}$ differed between NVPs and VPs (mixed effects model results in Tables 2 and 3; Figure 1c–e). NVP evenness increased with single and multiple nutrient additions as shown by the following contrast comparisons: control–N contrast −0.09, 95% HPD interval [−0.14 to −0.04], control–P contrast −0.09, 95% HPD interval [−0.14 to −0.04] and control–$NPK_{+\mu}$ contrast −0.24, 95% HPD interval [−0.34 to −0.15]. Differing from NVPs, VP evenness did not respond to the nutrient treatments (Figure 1c–e; HPDs of contrasts overlapped zero). Grazing exclusion (Fence)

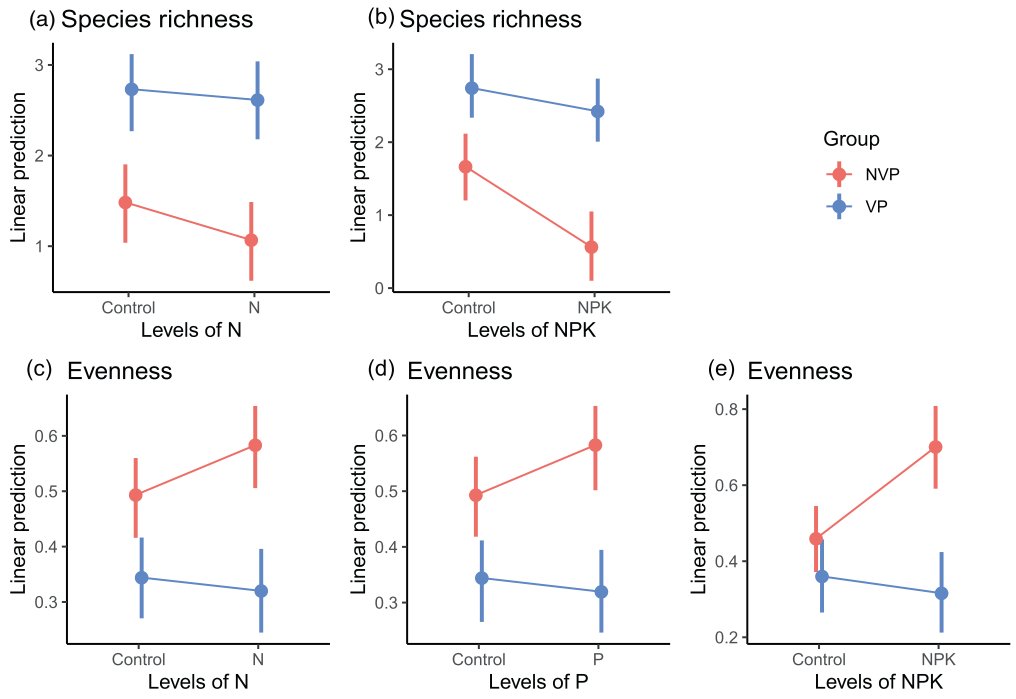

**FIGURE 1** Interaction plots of estimated marginal means (medians on the natural log scale (species richness) or on the linear scale (evenness) with 95% highest posterior density (HPD) intervals) showing differential responses of non-vascular plants (NVPs) and vascular plants (VPs) to nutrient addition treatments. Effects of N addition on species richness (a), effects of $NPK_{+\mu}$ addition on species richness (b), effects of N, P or $NPK_{+\mu}$ addition on evenness (c–e). Note that only treatments that showed different effects on NPVs and VPs (i.e. plant group × treatment interactions) are shown.

**TABLE 2** Summary output of the mixed effects models testing for differential effects of nutrient additions on species richness (spp $m^{-2}$), diversity and evenness depending on plant group.

| | **Species richness** | | **Species diversity (log-transformed)** | | **Evenness** | |
|---|---|---|---|---|---|---|
| **Predictors** | **Log-mean** | **CI (95%)** | **Estimates** | **CI (95%)** | **Estimates** | **CI (95%)** |
| Intercept | 1.60 | 1.15–2.02 | 0.78 | 0.43–1.12 | 0.45 | 0.37–0.52 |
| Plant group | 1.25 | 1.14–1.36 | 0.82 | 0.73–0.92 | −0.09 | −0.15 to −0.04 |
| N | −0.42 | −0.56 to −0.28 | −0.14 | −0.24 to −0.04 | 0.09 | 0.04–0.14 |
| P | −0.15 | −0.23 to −0.07 | | | 0.09 | 0.04–0.14 |
| $K_{+\mu}$ | −0.08 | −0.16 to −0.00 | | | | |
| Plant group × N | 0.30 | 0.13–0.47 | | | −0.11 | −0.19 to −0.04 |
| Plant group × P | | | | | −0.11 | −0.19 to −0.04 |
| Random effects | | | | | | |
| $\sigma^2$ | 53.51 | | 0.22 | | 0.03 | |
| $\tau_{00}$ | 48.26 | | $0.23_{\text{site}}$ | | $0.01_{\text{site}}$ | |
| | | | $0.00_{\text{site:block}}$ | | $0.00_{\text{site:block}}$ | |
| ICC | 0.53 | | 0.52 | | 0.21 | |
| *N* | $5_{\text{block}}$ | | $4_{\text{block}}$ | | $4_{\text{block}}$ | |
| | $9_{\text{site}}$ | | $8_{\text{site}}$ | | $8_{\text{site}}$ | |
| Observations | 496 | | 362 | | 316 | |
| Marginal $R^2$/conditional $R^2$ | 0.50/0.78 | | 0.32/0.60 | | 0.27/0.36 | |

*Note*: NVPs were used as reference group. Also shown are the random effect variance, $\sigma^2_i$, represents the mean random effect variance of the model, as well as random intercept variance describing variance attributable to site and blocks within sites ($\tau_{00}$). Intra-class correlation (ICC) can be interpreted as the proportion of the variance explained by the grouping structure 'blocks within sites'. *N* is the number of blocks and sites. Marginal $R^2$ is concerned with variance explained by fixed factors, and conditional $R^2$ is concerned with variance explained by both fixed and random factors. CIs are Highest Posterior Density (HPD) intervals. The species diversity and evenness models exclude the site mcla.us.

TABLE 3 Summary output of the mixed effects model testing for differential effects of grazing exclusion (Fence) and NPK$_{+\mu}$ additions on species richness (spp/m$^2$), species diversity (log-transformed inverse Simpson's index) and evenness depending on plant group.

| | Species richness | | Species diversity (log-transformed) | | Evenness | |
|---|---|---|---|---|---|---|
| **Predictors** | **Log-mean** | **CI (95%)** | **Estimates** | **CI (95%)** | **Estimates** | **CI (95%)** |
| Intercept | 1.70 | 1.25–2.17 | 0.95 | 0.65–1.22 | 0.46 | 0.37–0.55 |
| Plant group | 1.08 | 0.90–1.27 | 0.86 | 0.61–0.93 | −0.10 | −0.17 to −0.02 |
| Fence | −0.08 | −0.22–0.06 | −0.08 | −0.24–0.09 | | |
| NPK$_{+\mu}$ | −1.10 | −1.35 to −0.85 | −0.37 | −0.54 to −0.20 | 0.24 | 0.15–0.34 |
| Plant group × NPK$_{+\mu}$ | 0.78 | 0.49–1.09 | | | −0.29 | −0.42 to −0.16 |
| Random effects | | | | | | |
| $\sigma^2$ | 45.79 | | 0.26 | | 0.03 | |
| $\tau_{00}$ | 49.45 | | 0.09$_{\text{site}}$ | | 0.01$_{\text{site}}$ | |
| | | | 0.00$_{\text{site:block}}$ | | 0.00$_{\text{site:block}}$ | |
| ICC | 0.49 | | 0.27 | | 0.25 | |
| *N* | 5$_{\text{block}}$ | | 4$_{\text{block}}$ | | 4$_{\text{block}}$ | |
| | 8$_{\text{site}}$ | | 7$_{\text{site}}$ | | 7$_{\text{site}}$ | |
| Observations | 224 | | 150 | | 128 | |
| Marginal $R^2$/conditional $R^2$ | 0.49/0.74 | | 0.37/0.50 | | 0.33/0.43 | |

*Note*: For details, see Table 2. The species richness models exclude the site jena.de that had not grazer exclosure treatment, and species diversity and evenness models exclude also the site mcla.us.

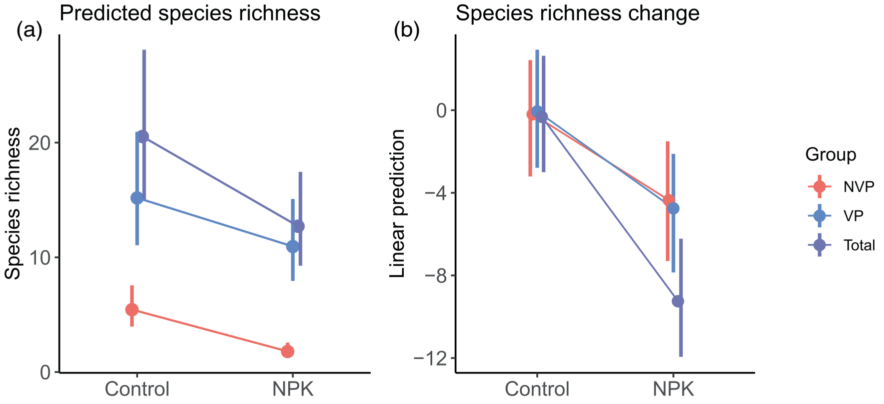

FIGURE 2 The predicted responses of non-vascular plant (NVP), vascular plant (VP) and total species richness (medians with 95% highest posterior density interval (HPD) intervals) to NPK$_{+\mu}$ fertilization (a). Interaction plot of species richness changes as a response to NPK$_{+\mu}$ fertilization (b). In (b), the points are estimated marginal medians of the difference in species richness in NPK$_{+\mu}$-fertilized plots as compared to controls (bars show 95% HPD intervals). The predictions are shown only for species richness or its change that showed the strongest responses to the fertilization treatment.

had only a weak effect on NVP and VP species richness (mixed effects model result; Table 3), and the groups did not differ in their response to the joint treatment of nutrients and fencing (the plant group × NPK$_{+\mu}$ × Fence interaction term was not retained in the final mixed effects models). The mixed effects model regression coefficients of other plant group × nutrient addition and grazing exclusion treatment interactions overlapped with zero (Tables 2 and 3) and were not considered further in contrast analyses.

Changes in total species richness depended on differential responses of NVPs and VPs. On average, NVPs decreased from six species in controls to two species in NPK$_{+\mu}$-fertilized plots (Figure 2a), corresponding to a 67% decline in NVP species richness. VP species richness decreased on average from 17 to 12 (Figure 2a), corresponding to a 28% decrease in VP species richness. Taken together, total species richness decreased, on average, from 23 to 14 (Figure 2a), corresponding to a 38% decrease in total species richness. NPK$_{+\mu}$ fertilization reduced species richness of NVPs, VPs and total community richness at different rates (interaction contrast was −0.15, 95% HPD [−0.31, 0.02], mixed effects model results presented in Table S3). Changes in total species richness (the difference between control plots and NPK$_{+\mu}$-fertilized plots) in response to NPK$_{+\mu}$ fertilization were more negative, on average, than that of VPs (interaction contrast −4.17, 95% HPD [−6.57, −1.91]; Figure 2b, mixed effects model results presented in Table S3).

Analyses of individual sites revealed considerable variation in the responses of NVP, VP and total species richness to treatments. Under ambient conditions (1 m$^2$ in control plots), mean species richness of NVPs across all sites was 6 (ranging from zero to 20 among sites), and that of VPs was 17 (ranging from 4 to 34), and that of total was 23 (ranging from 4 to 45) (Figures S3–S8). Under ambient conditions, NVP and VP species richness were positively correlated (0.60; Figure S9), whereas NVP and VP species diversity and evenness showed only moderate positive correlations (0.37 and 0.36, respectively; Figure S9). The responses of NVPs to nutrient addition or grazing exclusion treatments also varied among sites (Figures S3 and S6). For instance, in an annual-dominated grassland in California United States, mcla.us, NVPs were scarce and were only encountered in grazing exclosures (both NPK$_{+\mu}$-fertilized and unfertilized), P and NK$_{+\mu}$ fertilized plots (Figures S3 and S6). Single or multi-nutrient additions affected NVP species richness at five of nine sites, with NPK$_{+\mu}$ additions generally reducing NVP species richness (Figure S6). In a mesic, perennial grassland in the United Kingdom (rook.uk), the tendency of increased NVP species richness in the NPK$_{+\mu}$+Fence treatment resulted from ant colonies inside fences that built up elevated hummocky microsites favouring NVPs (Figure S1).

At the lowest light availability at the ground level, NVP, VP and total species richness declined most strongly in response to the treatments (Figure 3a). The effect of nutrient additions on NVP species diversity loss did not depend on light availability, whereas the effect of nutrient additions on VP species diversity and total species diversity increased with declining light availability (Figure 3b). In contrast, the evenness of VP and the total community in response to nutrient addition did not depend on light availability, while the evenness of NVPs in response to nutrient addition increased with decreasing light availability at ground level (Figure 3c). With increasing above-ground VP biomass, less light was transmitted to the ground (Figure S10), and the NPK$_{+\mu}$+Fence treatment most strongly suppressed light transmission (Figure S11). Although the percent transmitted light declined most strongly with increasing vascular plant biomass (Figure S12), species richness, diversity and evenness of NVP, VP and the total community weakly tracked the above-ground vascular plant biomass gradient (Figure S13).

The mixed effects logistic regression analysis showed that the presence of NVPs in plots became less likely in NP, NPK$_{+\mu}$ and NPK$_{+\mu}$+Fence plots (odds ratios 0.04–0.07; Figure 4; Table S4), whereas in other treatments the presence of NVPs remained approximately as likely as in controls (Figure 4). Using inverse logit conversions (using R function plogis) for the intercept and coefficients, we found that the probability of NVP presence, on average, decreased with the NPK$_{+\mu}$+Fence (30%), NP (36%) and NPK$_{+\mu}$ (44%) treatments, while the other treatments had negligible effects on the probability of NVP presence (on average, range from −8% (N) to +1.3% (Fence)). The probability of NVP presence was related to light transmitted to ground level (Figure 5; Table S5). Based on inverse logit conversions for the intercept and coefficients, we found that the probability of NVP presence decreases on average by 1.4% by every one percentage point decrease in the proportion of light transmitted to ground level. The strongest effect of the change in light transmitted to ground on the probability of NVP presence occurred at around 20% of transmitted light (Figure S14).

## 4 | DISCUSSION

Our results show that the estimates of grassland plant diversity loss rate resulting from nutrient addition underestimate total plant diversity loss rates when they are based only on declines of vascular plants (VPs). Our experimental results demonstrate that non-vascular

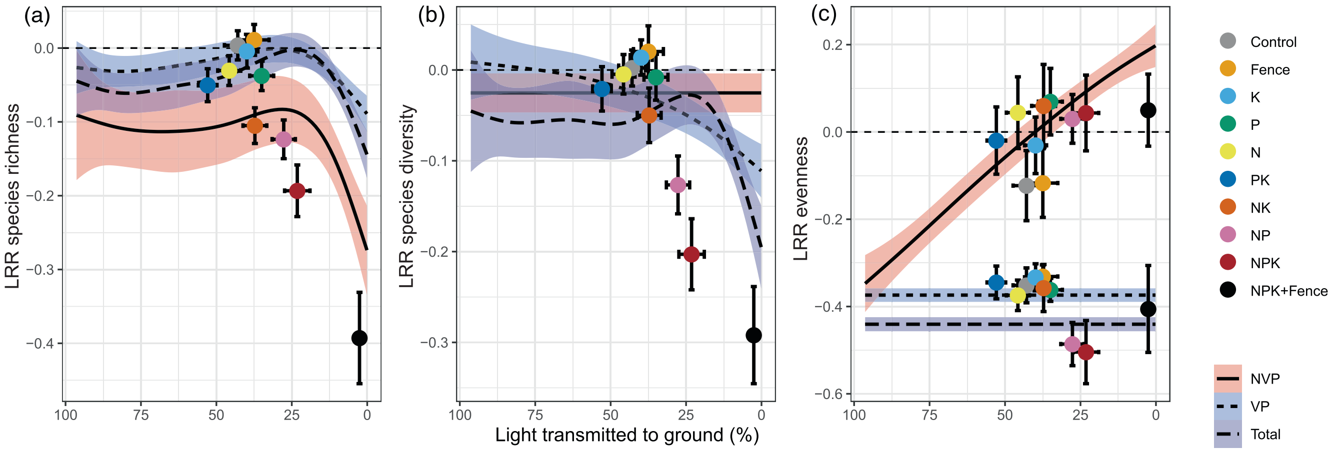

FIGURE 3 Species richness (a), species diversity (b) and evenness (c), all based on log response ratios (LRR), in relation to light transmitted to ground level. The means of light transmitted to ground (%) and LRRs (pooled by group) in different treatments are shown (error crossbars). The *x*-axis describes the increase of light limitation (based on % of ambient photosynthetically active radiation (PAR) transmitted to ground level). A negative LRR indicates that a treatment reduces the value of the response variable. The smooth fits are based on cubic spline smoothing with 75% confidence bands. For evenness (c), the mean LRRs in different treatments are shown separately for non-vascular plants (NVPs) and vascular plants (VPs). For clarity, the plot-level data points are not shown.

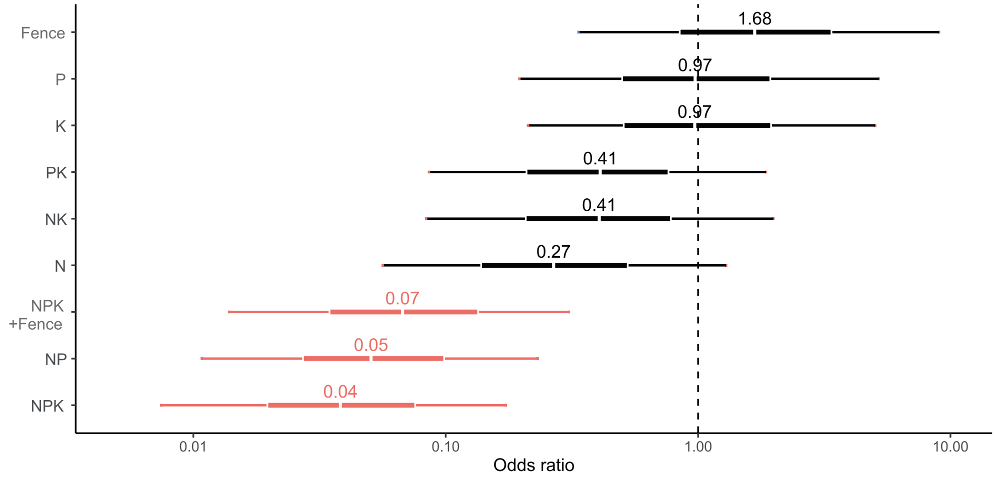

FIGURE 4 Effect of treatments on the presence and absence of non-vascular plants (NVPs) in different treatments based on logistic regression. Odds ratios >1 indicate an increase in the likelihood of NVP presence (relative to that in controls which is indicated by the vertical dash line at odds ratios = 1); odd ratios <1 indicate a reduction in the likelihood of NVP presence. Values show point estimates (median) and 50% and 89% highest posterior density interval credible intervals. Negative effects are shown in red, weak effects in black.

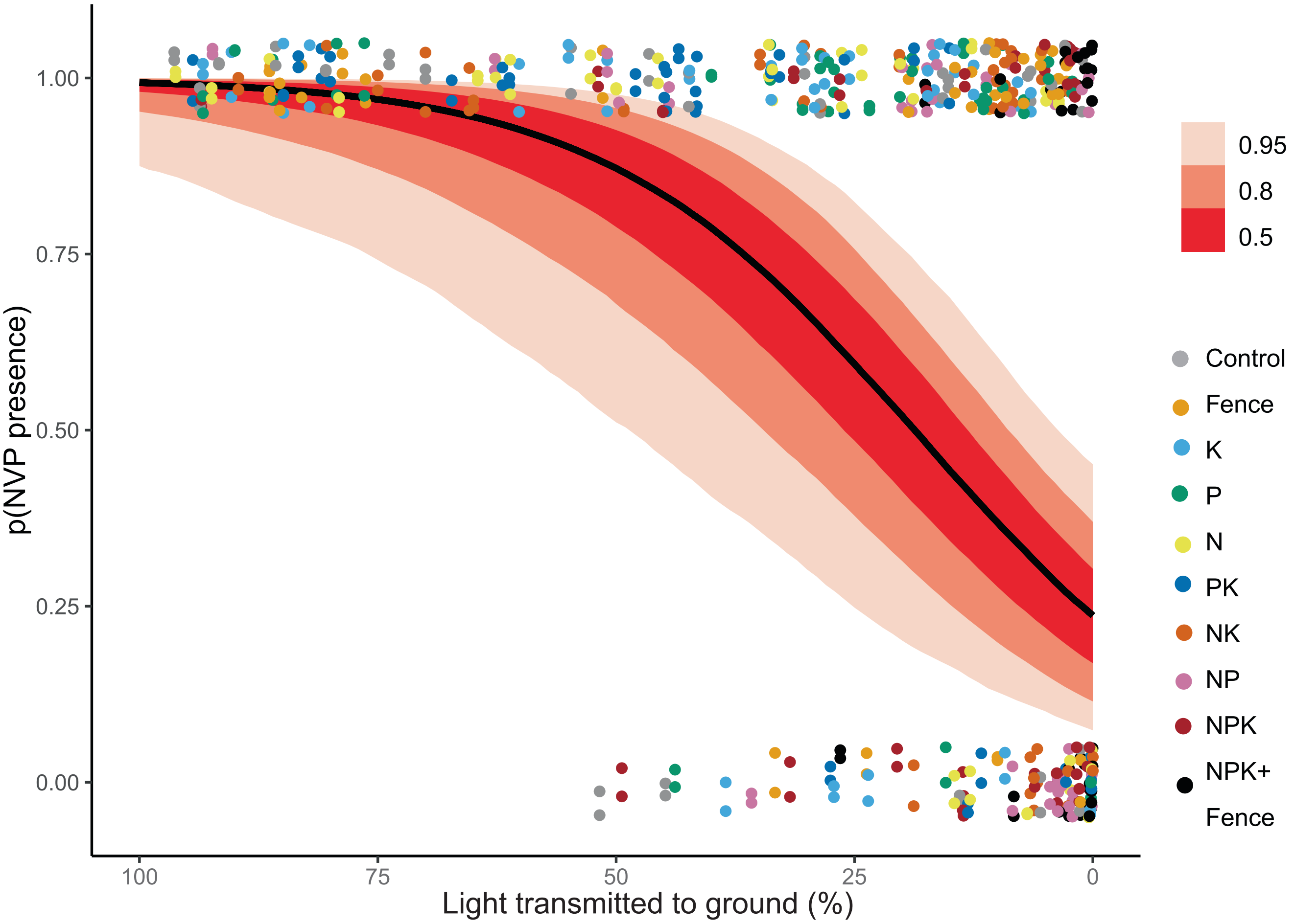

FIGURE 5 Logistic regression of non-vascular plant (NVP) presence in relation to light transmitted to ground level (% of ambient). Presences (1) and absences (0) in different treatments are shown as points, which are jittered vertically. Bands show 50%, 80% and 95% probability intervals drawn from the logistic regression model posterior distribution.

plants (NVPs), although representing a smaller proportion of total plant diversity, are lost at higher rates in response to multi-nutrient additions. Our results further indicate that reduced light availability due to nutrient-induced increase of VP biomass is a strong driver of NVP species loss. Our experimental data from nine grassland sites demonstrate the importance of NVPs amplifying plant diversity loss under nutrient enrichment and highlight the need to include NVPs to generate more accurate estimates of plant species loss rates under nutrient enrichment.

We found that both NVPs and VPs lost on average 4–5 species per 1 $m^2$ under multi-nutrient $NPK_{+\mu}$ addition (Figure 2b) which, because of the overall lower species richness of NVPs, shows that NVP species disappear at a higher relative rate (on average 67% decrease in species richness) than VPs (on average 28% decrease in species richness). As the decrease of total species richness was 38%, considering only VPs underestimates the total species loss by 10 percentage points (on average). Counter to the first two hypothetical scenarios presented in the introduction, nutrient addition does not induce the same relative effect on NVP and VP species richness, NVPs do not compensate for VP losses, and NVPs are not too scarce to impact total plant diversity. Instead, our results support the third scenario that considering VPs alone underestimates total plant species loss in response to multi-nutrient enrichment, due to the relatively great loss of NVP species compared to VPs. The contribution of NVPs to the total species losses is surprising given that NVPs comprised on average 21% (range 0%–46%) of total plant diversity in the grasslands studied by us. The finding suggests that total grassland plant diversity might be more threatened by nutrient deposition than has been thought on the basis of previous studies including only VPs. For example, Borer, Seabloom, et al. (2014) reported the loss of two VPs and Seabloom, Adler, et al. (2021) the loss of 2–3 VP species, on average, under multi-nutrient $NPK_{+\mu}$ enrichment, while our current analyses show losses of approximately 3–4 NVP and VP species, on average. Although species loss rates can also reflect the duration of experimental fertilization treatments (Harpole et al., 2016; Leverkus & Crawley, 2020; Seabloom, Adler, et al., 2021) or the difference in the numbers of sites included (Borer, Seabloom, et al., 2014 had 40 sites vs. 9 sites in this study), our results strongly suggest that VP loss rates underestimate total plant loss rates, and the inclusion of NVPs is needed to generate more accurate estimates of plant loss rates under nutrient enrichment.

The decrease of both NVP and VP species richness in plots receiving NP or $NPK_{+\mu}$ multi-nutrient additions was associated with a reduction of light transmitted to ground level due to increased live and dead VP biomass (Figure 3a). The strongest NVP and VP species losses took place at the lowest levels of light availability (0%–10% PAR transmitted to ground level at time of VP peak biomass) in plots with multiple nutrient addition. The decrease of NVP species richness was consistent with the decreased probability of NVP presence at the lowest levels of light transmission. These findings agree with earlier studies emphasizing the role of competition for light as a mechanism leading to decreased plant richness for VPs (Borer, Seabloom, et al., 2014; Eskelinen et al., 2022; Harpole et al., 2017; Hautier et al., 2009). Since reductions in light transmission were associated with increased VP biomass under increased resource availability, it is likely that increasing VP biomass suppressed light available for NVPs, a mechanism discussed in other studies (Aude & Ejrnæs, 2005; Cornelissen et al., 2001; Cusell et al., 2014; van der Wal et al., 2005; Virtanen et al., 2017). Further, according to contrast comparisons of the treatment effects, we found that NVP diversity decreased less under N addition than under NP or $NPK_{+\mu}$ additions, suggesting that especially multi-nutrient additions likely intensify VP competition for light (Tilman, 1982), which leads to a strong decline or even complete disappearance of the short-statured NVPs (see also Øien et al., 2018; Wilson & Tilman, 2002).

We did not find consistent evidence for herbivory mitigating negative effects of nutrient addition on NVP or VP diversity. This result may reflect inconsistent effects of herbivory on light levels across sites (Figure S11; Borer, Seabloom, et al., 2014). The mitigation of plant diversity loss under nutrient enrichment by herbivory may be restricted to conditions where the offtake rate of biomass by herbivores is relatively high, increasing light at the ground level, while at the same time grazers do not cause excessive disturbance, which could impose a negative impact on NVPs. In this experiment, grazers only partly counterbalance VP biomass accumulation under fertilization (Borer et al., 2020), and partial thinning of VP cover may not generally benefit NVPs. Even if there were no strong effects of grazer exclusion on NVP species richness across sites or in individual sites, their effects may still become important in longer time scales and/or in sites with higher grazing intensities. These effects could arise from physical effects of mammalian grazers on microsites (runways, mounds of bare ground, droppings) suitable for NVPs (Takala et al., 2014). These processes could prevent the development of species-poor grass sward with litter layers smothering NVPs. Our results are concordant with other studies showing that grazing may not generally conserve NVPs under nutrient enrichment (Boch et al., 2018; Molina et al., 2021; Virtanen et al., 2024). However, such mitigation may be possible under lower rates of nutrient enrichment (Bergamini & Pauli, 2001; Takala et al., 2014), or in conditions with relatively intense grazing pressure by multiple herbivores that create open gaps in vegetation and promote the colonization of locally rare NVP species (Virtanen et al., 2022).

The differences in the responses of species richness, diversity and evenness to the nutrient addition treatments help us to interpret the processes related to plant diversity loss under nutrient enrichment: NVP and VP species diversity consistently declined under N and $NPK_{+\mu}$ addition, suggesting that relatively abundant NVP and VP species respond similarly to fertilization. An increase in NVP evenness, while species richness decreased, implies that rare species were lost, which may be the most sensitive to changes in nutrient levels (Fernández-Martínez et al., 2020). Along with the disappearance of rare species under N, P and $NPK_{+\mu}$ fertilization, only a few tolerant NVP taxa persisted, which is reflected in increased evenness. These results align well with earlier findings on the vulnerability of rare species to nutrient deposition (Haworth et al., 2007). This finding is also compatible with much higher NVP species losses than gains under NPK$_{+\mu}$ fertilization, suggesting that the majority of NVPs are relatively intolerant of increased rates of nutrient supply (During & Willems, 1986; Roth et al., 2013), and relatively few nutrient or shade tolerant NVPs may co-exist with vascular plants in grasslands (Roth et al., 2013; Stevens et al., 2004; Watts et al., 2019).

Although the study of the role of NVPs in ecosystem functions has increased (Cornelissen et al., 2007; Cornwell et al., 2008; Porada et al., 2023), the consequences of NVP losses on ecosystem function remain unclear (Klaus & Müller, 2014; but see Chen et al., 2022). NVPs are a heterogeneous group, and their contributions to ecosystem functioning may be particularly important in cool–humid ecosystems where they are common (Lett et al., 2021). Based on the current evidence, it is plausible that along with diverse VP assemblages, the presence of diverse NVP assemblages, or even the presence of any NVPs, could be used as an indicator of natural oligotrophic grassland ecosystems, and the absence or low diversity of NVPs could point to elevated nutrient deposition. In this respect, our experimental findings are consistent with observational studies showing that nutrient availability reduces NVP diversity (Boch et al., 2018; Roth et al., 2013; but see Stevens et al., 2006). Further, we did not consider another group of non-vascular photoautotrophs, lichens, that are remarkably diverse in some low productivity grasslands (Dengler et al., 2020) and might be even more responsive to nutrient availability. Their inclusion could further improve estimates of diversity loss rates.

In sum, our experiment distributed at nine grassland sites in Europe and the United States improves estimates of grassland plant species loss rates under nutrient enrichment and advances mechanistic understanding of drivers of plant diversity change. Based on systematically collected data from grasslands spanning a wide range of biotic and abiotic conditions, we show that increased nutrient supply rate leads to a relatively large loss of NVP diversity that is linked to increased VP biomass and reduction of light availability near ground level. Importantly, as NVP loss rates are higher than VP loss rates, our results highlight that VP-based analyses of diversity loss are consistently underestimating plant species loss rates in altered environmental conditions. Preserving high grassland diversity, rich in NVPs and VPs, requires reducing ongoing nutrient enrichment.

## AUTHOR CONTRIBUTIONS

Risto Virtanen, Stan Harpole and Eric Seabloom designed the study. All authors have established experimental sites and conducted sampling of vascular plant and other co-variate data. Risto Virtanen conducted bryophyte sampling, identification, analysed data and wrote the first draft of the manuscript. All authors have commented and edited the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data are available through the data repository Environmental Data Initiative: https://doi.org/10.6073/pasta/4df0b585d58fc6778b7ff00833e9546d (Virtanen et al., 2025).

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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:** Photos of the sites.

**Figure S2:** Species turnover of community compositions of non-vascular plants (NVPs), vascular plants (VPs) and total (NVP+VP) resulting from NPK$_{+\mu}$-fertilization treatment.

**Figure S3:** NVP species richness in relation to N, P and K$_{+\mu}$ additions.

**Figure S4:** VP species richness in relation to N, P and K$_{+\mu}$ additions.

**Figure S5:** Total species richness in relation to N, P and K$_{+\mu}$ additions.

**Figure S6:** NVP species richness in relation to grazing exclusion (Fence) and NPK$_{+\mu}$ addition.

**Figure S7:** VP species richness in relation to grazing exclusion (Fence) and NPK$_{+\mu}$ addition.

**Figure S8:** Total species richness in relation to grazing exclusion (Fence) and NPK$_{+\mu}$ addition.

**Figure S9:** Correlations between NVP and VP species richness, diversity and evenness in control plots across all nine study sites.

**Figure S10:** The percentage of ambient light transmitted to ground level and above-ground vascular plant biomass.

**Figure S11:** The percentage of ambient light transmitted to the ground level as responses to treatments.

**Figure S12:** Above-ground biomass in multi-nutrient addition and grazing exclusion treatments.

**Figure S13:** Log response ratios of NVP, VP and total species richness, species diversity and evenness to above-ground vascular plant biomass.

**Figure S14:** Slopes of the effects of light transmitted to ground on the probability of NVP presence in experimental plots.

**Table S1:** Species numbers of NVPs, VPs and total in all plots across all sites, per site and in different treatments.

**Table S2:** Bryophyte taxa observed in experimental sites.

**Table S3:** Summaries of the mixed model outputs of the responses of NVP, VP and total species richness and change in species richness as response to NPK$_{+\mu}$ fertilization.

**Table S4:** Summary of the mixed logistic regression analysis of NVP presence in plots with different treatments.

**Table S5:** Summary of the mixed logistic regression analysis of NVP presence in relation to light transmitted to ground level (% of ambient).

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