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High CO_2 dampens then amplifies N-induced diversity loss over 24 years

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Rising levels of atmospheric carbon dioxide (CO_2) and nitrogen (N) deposition affect plant communities in numerous ways[1–11]. Nitrogen deposition causes local biodiversity loss globally[12–14], but whether, and if so how, rising CO_2 concentrations amplify or dampen those losses remains unclear and is almost entirely unstudied. We addressed this knowledge gap with an open-air experiment in which 108 grassland plots were grown for 24 years under different CO_2 and N regimes. We initially found that adding N reduced plant species richness less at elevated than at ambient CO_2. Over time, however, this interaction reversed, and elevated CO_2 amplified losses in diversity from enriched N, tripling reductions in species richness from N addition over the last eight years of the study. These interactions resulted from temporal changes in the drivers of diversity, especially light availability, that were in turn driven by CO_2 and N inputs and associated changes in plant biomass. This mechanism is likely to be similar in many grasslands, because additions of the plant resources CO_2 and N are likely to increase the abundance of the dominant species. If rising CO_2 generally exacerbates the widespread negative impacts of N deposition on plant diversity, this bodes poorly for the conservation of grassland biodiversity worldwide.

Rising levels of atmospheric CO_2 and N deposition are influencing plant communities now and will continue to do so[1–10]. Elevated levels of N deposition occur in much of the world, and although trends are geographically variable, average global N deposition rates are continuing to rise[11]. This is a major concern for biodiversity because observational and experimental studies indicate that this N pollution decreases the richness of plant communities by as much as 20–30% across herbaceous ecosystems on multiple continents[12–15]. Numerous studies have shown that elevated levels of N availability cause these losses in local species diversity by multiple mechanisms. These mechanisms mostly involve competitive exclusion, but also reduced niche dimensionality and changed ecological stoichiometry[1,5,6,16–20]. Essentially, increased N allows a subset of species to grow faster and reduce the resources available to their neighbours, as well as reducing the number of resources limiting plants, thereby decreasing the number of niches. By contrast, there has been surprisingly little research about how rising CO_2 levels will directly influence species diversity[4,21,22]. The gradual rise in well-mixed atmospheric CO_2 concentrations makes it extremely difficult to evaluate its impact from observations of vegetation change, and there have been very few experimental manipulations of atmospheric CO_2 at decadal timescales to address this question[2,4,21–23].

The effects of elevated CO_2 (eCO_2) on local diversity might mirror or differ from those of N deposition. Just as nutrient enrichment can drive biodiversity loss by increasing biomass production and decreasing light availability[24–26], atmospheric CO_2 may similarly increase biomass and drive biodiversity loss by favouring only the best competitors for light. Alternatively, eCO_2 can alter biogeochemical cycling and strengthen N limitation relative to the carbon supply[27]; if imbalanced stoichiometry results in greater niche dimensionality, this might increase local diversity. Furthermore, atmospheric CO_2 differs from soil resources, such as N, in ways that may influence its effects on diversity. For example, although plants can outcompete one another by depleting soil resources, CO_2 is well mixed in the atmosphere, making it almost impossible for plants to outcompete one another by locally depleting atmospheric CO_2.

We wondered how plant diversity might respond to the combination of eCO_2 and N deposition, which occur together in many regions of the world. Many pairs of global changes are thought to synergistically drive biodiversity loss, such that their combined effects are greater than the sum of their individual effects[8,28]. It remains unclear whether eCO_2 and N enrichment non-additively drive biodiversity loss, because relevant evidence is extremely scarce[2,23]. A 10-year open-air grassland experiment, called Biodiversity, CO_2, and Nitrogen (BioCON), which added N at 4 g m^{-2} yr^{-1}, resulted in an average loss of species richness of 16% at ambient CO_2 but 8% at eCO_2 (ref. 23). The present study extends BioCON by another 14 years, making it the longest study by more than a decade to consider this question.

Here we report the results of 24 years of the BioCON experiment and examine the underlying mechanisms. Surprisingly, the early (the first 12 years) dampening of species loss from N addition by eCO_2 shifted over time to amplify such losses in years 17–24. This reversal was driven by associated changes in light availability. We documented these responses using 108 plots, each 4 m^2, that were planted in 1997 with either 9 (60 plots) or 16 (48 plots) perennial herbaceous species

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Table 1 | Treatment effects on realized species richness over time

	numDF	denDF	*F*-value	*P*-value
Intercept	1	2,476	3,204.23	<0.0001
Planted diversity	1	96	380.85	<0.0001
N	1	96	45.63	<0.0001
CO_2	1	4	0.43	0.5481
Year of study	1	2,476	2,859.21	<0.0001
Planted diversity×N	1	96	1.37	0.2443
Planted diversity×CO_2	1	96	0.23	0.6339
N×CO_2	1	96	0	0.9527
Planted diversity×year	1	2,476	375.23	<0.0001
N×year	1	2,476	0.07	0.7950
CO_2×year	1	2,476	1.44	0.2305
Planted diversity×N×CO_2	1	96	0.71	0.4020
Planted diversity×N×year	1	2,476	35.8	<0.0001
Planted diversity×CO_2×year	1	2,476	2.34	0.1261
N×CO_2×year	1	2,476	33.1	<0.0001
Planted diversity×N×CO_2×year	1	2,476	1.41	0.2357

Mixed model output for the treatment effects of original planted species numbers (9 or 16 species; planted diversity), ambient versus enriched nitrogen, ambient versus elevated CO_2, and time (year) on realized species richness (the number of species observed in the sampled portion of each plot). numDF and denDF indicate numerator and denominator degrees of freedom, respectively.

and treated from 1998 to 2021 with all combinations of ambient CO_2 and eCO_2 (with the addition of 180 μmol mol^{-1} CO_2 delivered using a free-air CO_2-enrichment technique) and ambient N and enriched N (with the addition of N at 4 g m^{-2} yr^{-1})[23,29,30]. Non-target species were removed from plots, so the results reflect changes in the original pool of planted species (Methods). We sampled at neighbourhood scales (0.1–0.5 m^2), that is, scales at which plants in herbaceous communities are likely to interact with their neighbours[31–33]. The focus of our study was the simplification of communities at such scales, rather than the extinctions of rare species in the broader landscape. Some natural and restored grasslands have been losing diversity over time[34–36], although less so in those recovering following disturbance[37,38]. The diversity of our experimentally assembled communities also declined with time, including under ambient conditions. The effects of CO_2 and N treatment were therefore superimposed on a trajectory of declining species richness (Methods). In the following sections, we sequentially do the following: report the impact of treatments on diversity; highlight the treatment-driven changes in resources that caused those changes in diversity; identify the mechanisms by which changes in biomass drove changes in resources and thus diversity; examine the role of individual species in these dynamics; and provide interpretations and implications of these findings.

Treatment effects on realized diversity

The changing effect of added nitrogen (+N) on neighbourhood species richness over time at the contrasting CO_2 levels is illustrated in Table 1, Fig. 1 and Extended Data Fig. 1. Across all years and both CO_2 levels, +N tended to reduce richness (Fig. 1a,b). At ambient CO_2, this reduction was largest initially (around 15%) and diminished thereafter (Fig. 1b). Because richness declined in all treatments, and at both neighbourhood and plot scales (Extended Data Fig. 2), this decrease in the effect of +N at ambient CO_2 does not mean that realized richness increased over time; rather, it declined at a slowing rate in this treatment combination. At eCO_2, the decrease in richness from +N was smaller and shrank faster than at ambient CO_2 for the first eight years of the study, after which it reversed direction (Fig. 1b). Thus, although +N initially had a less negative effect on richness in eCO_2 than in ambient CO_2, over time this flipped (Fig. 1b,c). In the first eight years, +N reduced richness by an average of 13% at ambient CO_2 and by only 5% at eCO_2; that is, eCO_2 eliminated more than half of the loss of richness from +N under ambient CO_2. During the last eight years (years 17–24), +N reduced richness on average by 7% at ambient CO_2 and by 19% at eCO_2, thus eCO_2 nearly tripled the losses from +N during that later period. Statistically supporting these patterns, in a mixed-effects linear model there were significant main effects of N enrichment on species richness, as well as a significant interaction of year × CO_2 × N (Table 1). The plots with 9 and 16 species did not differ significantly in their CO_2 × N interaction over time (Table 1, $P = 0.24$).

A measure of evenness, the Pielou corrected index[39] also showed a significant ($P < 0.0001$) year × CO_2 × N interaction (Extended Data Table 1). In all four combinations of CO_2 and N, evenness (the equity of species' relative abundances in sampled areas) increased for the first seven years and then gradually decreased, especially in the eCO_2 and +N treatment (Fig. 1d,e). +N caused evenness to decline much more markedly over time in eCO_2, such that evenness was around 18% lower in eCO_2 in the last eight years than under ambient CO_2 (Fig. 1e). In essence, eCO_2 initially modestly dampened reductions of evenness from +N, but with time that effect switched to a larger amplification of loss of evenness (Fig. 1e,f).

We can also express the above results in terms of the eCO_2 effect, instead of the +N effect. Doing so reveals that, at ambient N, eCO_2 initially had modest negative effects on richness and evenness that became positive with time, whereas at +N, eCO_2 increased diversity initially but decreased it thereafter (Extended Data Fig. 3a,b). During the last four years (years 21–24), eCO_2 increased richness by 5–10% at ambient N, but decreased richness by 10–20% at +N. Thus, in the long term, differences in N availability changed the direction of the eCO_2 effects on species richness.

Mechanisms driving observed interactions

Several factors influence richness and evenness in grasslands, often through competition for resources such as light, water and nutrients[17,20,26]. These resources may in turn be sensitive to enriched CO_2 and N[17,20,26]. We tested whether changes in these resources influenced richness and how it varies over time by individually adding light availability, soil volumetric water content, soil solution inorganic N concentration or soil pH as a covariate to our full factorial experimental mixed model. Light availability had a significant interactive effect on richness with year × CO_2 × N (year × CO_2 × N × light, $P = 0.007$; Extended Data Table 2), whereas none of the soil metrics did ($P > 0.59$). Moreover, of these variables, only light availability was influenced by CO_2 × N treatment (Fig. 2a–c) in a manner that varied across time (year × CO_2 × N, $P = 0.0027$; Extended Data Table 3). To be clear, light was not the only environmental factor influencing diversity responses to CO_2 × N over the 24 years. In eCO_2, light and soil solution N concentration both influenced how much +N reduced richness, and, in ambient CO_2 treatment, the +N suppression of richness was dependent on light and interactions of light, soil moisture and soil pH (Extended Data Tables 4 and 5). However, here we focus on light for three reasons: light and its interactions explained the most about the richness response to +N (Extended Data Table 4); light was the only driver of richness that itself varied interactively over time at contrasting CO_2 × N (Fig. 2, Extended Data Table 3); and light was the only covariate that had a changing effect on richness over time at contrasting CO_2 × N treatments.

The time period during which +N increased shading the most differed markedly across CO_2 levels. In the first four years, light availability under ambient CO_2 was roughly one-fifth less on average under +N than under ambient N, with little difference thereafter (Fig. 2a). By contrast, light availability under eCO_2 was higher under +N than

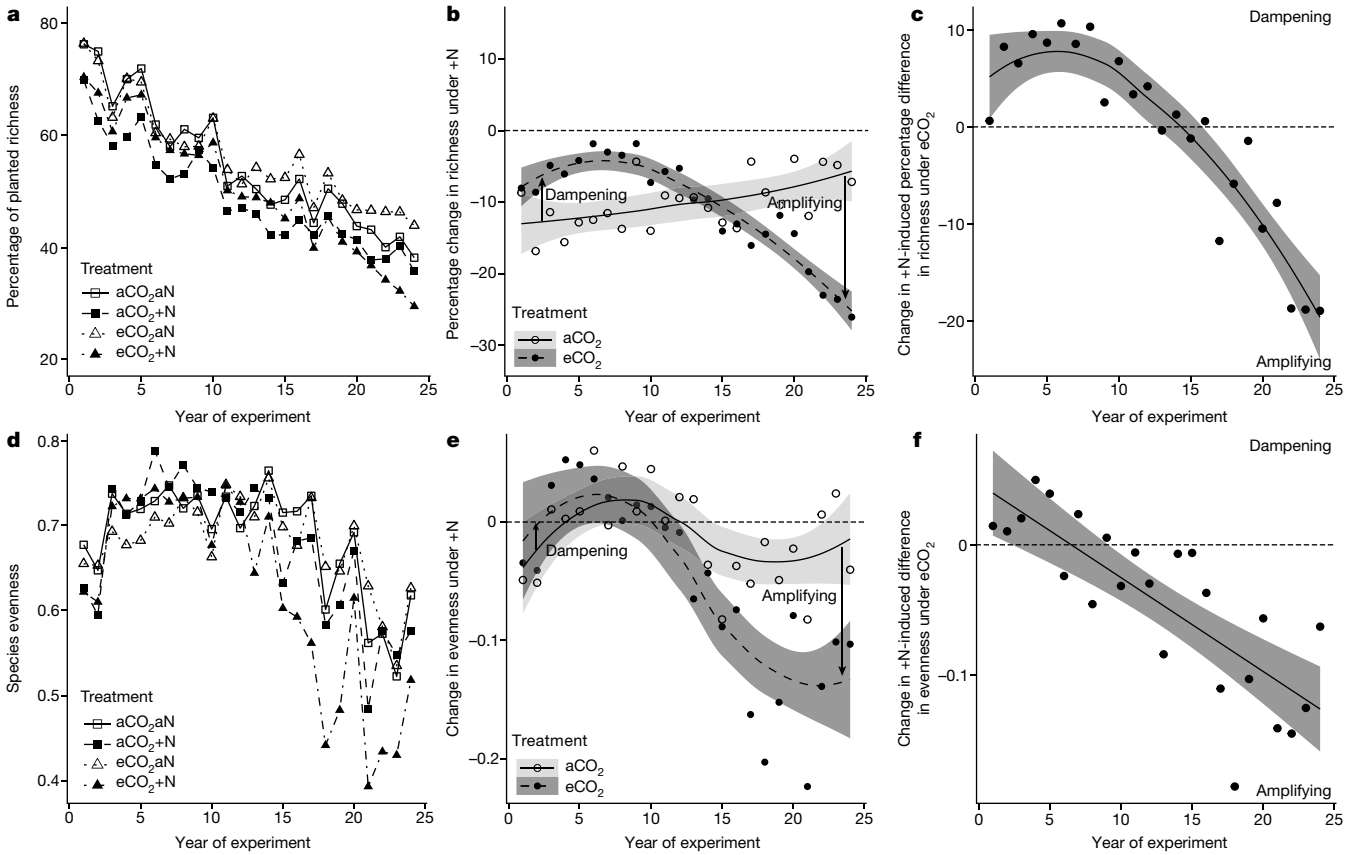

Fig. 1 | Change in species diversity under CO_2 and N. a, Realized species richness (SR) in the sampled area of the plot each year as a percentage of planted richness in the entire plot for four treatment combinations; aCO_2, aN, eCO_2, and +N indicate ambient CO_2, ambient N, elevated CO_2 and enriched N, respectively. **b**, Percentage difference in SR resulting from +N (compared with aN) at two CO_2 levels. The difference under +N was calculated as: SR in +N − SR in aN × 100/SR in aN for each level of CO_2. **c**, eCO_2 mediation of richness loss resulting from +N. This is described by the change in the +N effect on SR under eCO_2 (compared with aCO_2) and calculated as the difference between the +N effect on SR in elevated and ambient CO_2 levels (that is, the difference between the dashed and solid lines in **b**). **d**, Values of the species evenness metric Pielou corrected J for all treatment combinations. **e**, Difference in evenness caused by +N at two CO_2 levels (calculated as the difference between aN and +N for each CO_2 level). **f**, Change in the effect of +N on evenness under eCO_2 (calculated as in **c**). Dampening and amplifying indicate a decrease or increase, respectively, in diversity loss from +N resulting from treatment with eCO_2. The results are supported by linear mixed models (Table 1 and Extended Data Fig. 1), which are based on the full dataset (108 plots over 24 years). For all figures, values are averaged over 9- and 16-species plots ($n = 27$ plots per unique treatment, or 108 plots in total), and for visualization purposes, locally estimated scatterplot smoothing (LOESS) polynomial fits are shown when the patterns are nonlinear. The dashed line at 0 represents no change in response to the treatments, and the shaded region represents the 95% confidence interval.

under ambient N annually from years 3–10 and then lower under +N than under ambient N annually from years 12–24; during the last seven years of the study, light availability under eCO_2 was on average roughly one-tenth less under +N than under ambient N (Fig. 2a). Thus, +N treatment resulted in reduced light availability early in the experiment under ambient CO_2 but not eCO_2, whereas late in the experiment, +N resulted in increased shading in eCO_2 and in decreased shading under ambient CO_2 (Fig. 2b).

Within and across CO_2 treatments, the effects of +N on light were related to its effects on diversity. In both CO_2 treatments considered separately, over the 24 years, the effect of +N on richness was correlated with the effects of +N on light; in other words, when +N reduced light the most, +N reduced richness the most (Extended Data Fig. 4a,e and Extended Data Table 4). Moreover, the period (years 17–24) of amplified shading from +N at eCO_2 (Fig. 2b,c) matched the period of amplified diversity loss by +N at eCO_2 (Fig. 1b,c). In essence, eCO_2 modulation of the +N effect on diversity tracked its modulation of the +N effect on light; regression (Fig. 3) shows that the eCO_2 regulation of the effects of +N on light explained half of the interannual variation in the eCO_2 regulation of the effects of +N on both richness and evenness ($R^2 \geq 0.49$, $P < 0.0001$; Fig. 3). In other words, when eCO_2 amplified shading by added N, eCO_2 also increased the loss of diversity from added N. Furthermore, a model of the 24 years of eCO_2 regulation of the effects of +N on richness was most strongly related to the eCO_2 modulation of the effects of +N on light and soil N, with light being the most important driver (Extended Data Table 5).

The dynamics of the effects of treatments on light were driven by changes in biomass and cascaded to affect species diversity. Over time (Fig. 2d), there were shifts in plant biomass responses under CO_2 and N treatments (year × CO_2 × N, $P = 0.05$); +N consistently increased plant biomass to a greater extent under elevated than ambient CO_2 during the second half of the experiment, but not earlier. As a result, +N reduced light availability more under eCO_2 than under ambient CO_2 in the second half of the experiment (Fig. 2b). Light availability was generally lower in plots with greater above-ground biomass (Fig. 2e), and the effects of the eCO_2 and +N treatments on light were largely manifested by those changes in biomass. This contrasting and temporally varying effect of +N on biomass, and thereby on light availability, at the different CO_2 levels (Fig. 2) echoed the changing effects on richness (Fig. 1). In the first decade of the study, eCO_2 dampened decreases in light transmission in +N plots. This effect subsequently flipped to amplification by eCO_2 of the effects of +N, because +N created shadier conditions under

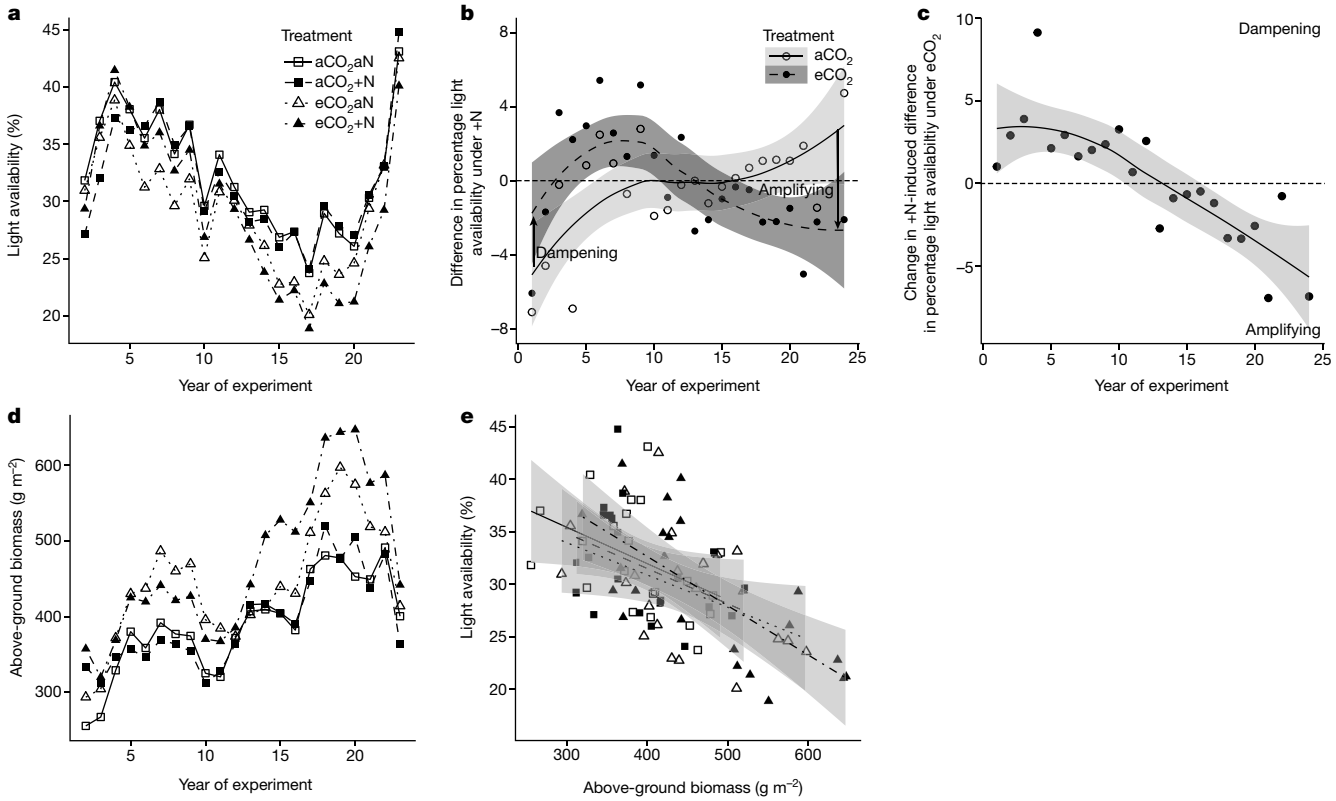

Fig. 2 | Change in light availability under CO_2 and N. a, The three-year moving average of average May–July percentage light availability beneath the plant canopy for four treatment combinations. **b**, Annual difference in percentage light availability for +N relative to aN at two CO_2 levels. **c**, Annual change in +N percentage light availability under eCO_2 relative to aCO_2. Data for **b** and **c** were calculated in the same way as Fig. 1d,e, respectively. **d**, The three-year moving average of the total above-ground biomass for the four treatment combinations, centred around the middle year. **e**, Relationship between the three-year moving average percentage light availability and above-ground biomass. In **b** and **c**, annual values are shown, not three-year moving averages. The dashed lines at 0 represent no difference in light availability in response to the treatments, and the shaded region represents the 95% confidence intervals. Three-year moving averages are shown for **a**, **d** and **e** to increase data visibility, but all mixed models used annual data. These results are supported by a linear mixed model reported in Extended Data Table 2 that is based on the full dataset (108 plots and 23 years). Data on light availability are not available for 2020.

eCO_2 than under ambient CO_2 in the last decade of the study. Thus, the changing response of diversity to CO_2 × N over time was driven, at least in part, by the shifting interactive effect of CO_2 × N on biomass (Fig. 2) and thus on light (Fig. 2 and Extended Data Table 3), a resource that had a strong influence on richness and evenness (Fig. 3 and Extended Data Table 2).

The responses of individual species can help to explain these results. Some of those that cast more shade (such as *Amorpha*, *Andropogon*, *Lespedeza* and *Lupinus* species) in monoculture during some or most of the time periods (Methods) tended to be more dominant in mixtures, whether averaged across the entire experiment, examined early ($R^2 = 0.26$, $P < 0.0001$; Fig. 4) versus late in the experiment ($R^2 = 0.42$, $P < 0.0001$; Fig. 4), or in every year of the study. Thus, increased shading leading to reduced richness in certain treatment combinations was also associated with the increasing dominance of species that probably reduced light availability to other species in mixtures. In particular, *Andropogon gerardii* became the dominant species in mixtures over time, and as its relative abundance increased, both shading and loss of richness increased too ($P < 0.0001$).

Species that disappeared from specific combinations of CO_2 × N treatments (Extended Data Fig. 5) show the complexity of changing diversity in these plots. Species differed in whether, how and when they were lost, potentially as a result of varying environmental filters imposed by the different global change treatments. For example, *Amorpha* tended to disappear under +N at both CO_2 levels, whereas at +N, *Solidago* was lost frequently under eCO_2 but much less so under ambient CO_2. Furthermore, the sensitivities of species and functional groups to global change treatments also varied with time, and this may indirectly affect temporal patterns in diversity.

Interpretation and take-home messages

In summary, losses of diversity from adding N became larger under eCO_2 than under ambient CO_2 in the second half of the 24-year study, because this treatment combination led to greater biomass and thus to greater competition for light. Because of this heightened light competition, +N drove more species to local extinction under elevated than ambient CO_2 in the later stages of the experiment. The combination of eCO_2 and +N treatment resulted in 22% lower species richness in the last two years of the study than in the plots under completely ambient conditions.

Whether the changing interaction of CO_2 and N on species diversity we observed is representative of what occurs elsewhere is uncertain, for several reasons. First, there has been an almost complete lack of previous experimental or observational data relevant to this question, so we have almost no evidence to compare our data with. Second, our experiment chronicled the consequences of interactions among planted communities of a pool of 16 native and naturalized grass and forb species. Non-target species were removed from the plots, and this could bias our assessment of the relative effects of CO_2 and N by preventing colonization from species that could otherwise have been present in control or treatment plots. However, these diverse communities were relatively resistant to colonization each year (new colonizer plant biomass averaged about 1% of resident biomass), and neither +N nor the CO_2 × N interaction ($P > 0.05$) influenced colonizer

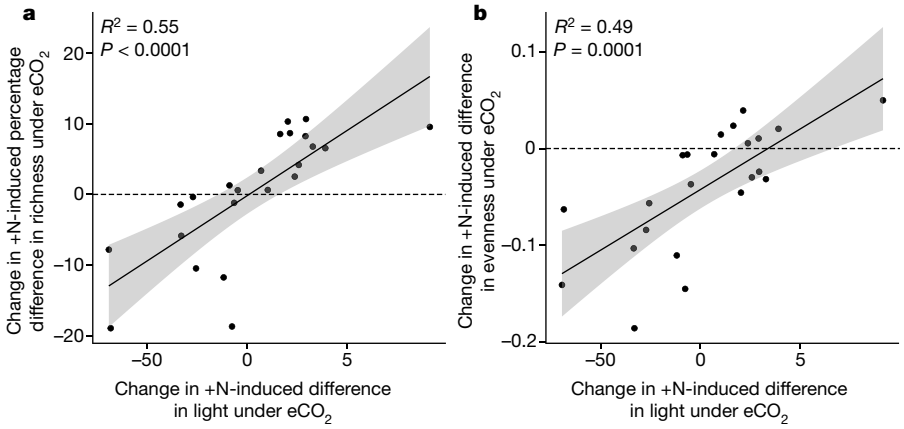

Fig. 3 | Shifts in N-induced impacts on species richness and evenness under contrasting CO_2 are due to similar shifts in light availability. a,**b**, Relationship between the effects of the shift in +N on species richness (**a**) and species evenness (**b**) resulting from eCO_2 versus the shift in +N effect on light resulting from eCO_2. Each data point represents a year. When +N resulted in shadier conditions under eCO_2 than under aCO_2 it also resulted in greater losses in species richness and evenness under eCO_2 than under aCO_2. The dashed line represents the no-effect line and the shaded regions represent 95% confidence intervals. Simple linear models were used to statistically test the relationships in this figure (n = 23 and the test of significance is based on the F-statistic).

productivity for the 11-year period (years 1–11) when data were available. Moreover, after year 11, the combination of +N and eCO_2, which had lower richness and evenness than all other treatments, was the most light-limited (Fig. 2), and thus would have been the least likely to be successfully colonized by new species, given that the large majority of grassland species in this site are light-demanding and that their richness declines with shade[24,32,40,41]. There is therefore no evidence that removing non-target species had an impact on the observed shift over time towards reduced diversity from added N under eCO_2, and, based on existing evidence, allowing non-target species to persist would have been more likely to amplify rather than dampen this trend. Third, because N deposition blankets landscapes in a rather inescapable manner, and eCO_2 does so even more, the effects of treatments may have been underestimated in our study if diversity in resource-enriched plots was subsidized by source populations in nearby control plots[42] that in a non-experimental context would probably be further away. Fourth, climate change associated with rising CO_2 could alter the way CO_2 and N deposition interact to affect diversity, but whether and how it will is currently unknown. Despite these limitations, this experiment offers a unique opportunity to assess the long-term effects of N deposition and eCO_2, and provides a baseline for expectations in natural systems. It should also motivate the development of theory on the interactive effects of global changes.

In our study, the shifting dynamics we observed may have partly depended on the stage of ecosystem development, with the CO_2 amelioration of N-induced species loss during the first decade reflecting the response of a community in a transitional successional phase. By contrast, if the greater-than-additive loss of richness through enhanced resource competition that occurred under combined CO_2 and N enrichment during the second half of the study represents the response of well-established grassland communities, this is of general concern for intact communities. However, our results may be more likely to occur in ecosystems in which N deposition and eCO_2 exacerbate light limitation than in systems primarily limited by other factors.

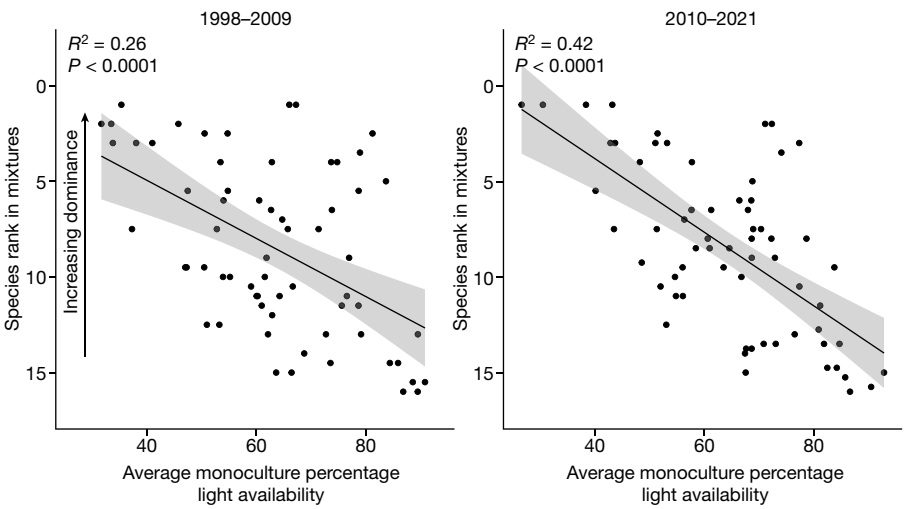

Fig. 4 | Light competition ability and species dominance in mixtures. Association between average light levels in monocultures and mean species abundance rank based on cover in mixtures for all species in all four treatment combinations. The percentage light availability in monocultures was calculated for all four CO_2 × N combinations as May–July averages over each period of the experiment of light levels near the ground. Species ranks were calculated on mean cover for each CO_2 × N × plant richness combination. Each symbol is a species in one of the four CO_2 × N combinations averaged over the years shown and averaged across 9- and 16-species plantings, over 12 years for **a** and over 11 years for **b** because light data were not available for 2020. The shaded regions around the trend lines represent 95% confidence intervals, and simple linear models provided the R^2 and P-values using F-statistics based on sample sizes of n = 64 (each species in all CO_2 × N combinations).

That CO_2 concentrations will remain elevated over historical levels, and will continue to increase for several decades at least, if not for the entire century, is not in dispute[9], and these elevated concentrations will affect vegetation everywhere on Earth. Levels of atmospheric N deposition remain elevated over much of the globe, although the trends and impacts of this are decreasing in some regions and increasing in others[11,27]. Our broad concerns about biodiversity changes, including those resulting from habitat loss, change in fire regimes, extirpation of historic large grazers, and climate change[34–36,43], need to be viewed in the context of rising CO_2 and varying N deposition, which probably also have considerable effects in many ecosystems. If rising CO_2 generally exacerbates the already substantial negative effects of N deposition on established community-scale species richness[12–14] over relevant ecological time scales, this bodes poorly for biodiversity conservation, especially given the myriad other threats to biodiversity. Calls for biodiversity preservation and restoration are already at fever pitch, and results such as those shown here only add to the chorus.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-024-08066-9.

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Methods

Experimental design

The complete BioCON experiment includes 371 plots, each measuring 2 m × 2 m, in six circular areas 20 m in diameter called rings, and is located at the Cedar Creek Ecosystem Science Reserve in Minnesota, USA. The plots were established on secondary successional grassland on a sandy outwash soil after removing the existing vegetation. The BioCON project includes several overlapping and nested experiments. The main biodiversity × CO_2 × N experiment (n = 296 plots) consisted of a split-plot arrangement of treatments in a completely randomized design. CO_2 treatment (ambient CO_2 or +180 μmol mol^{-1} CO_2) is the whole-plot factor (ring scale) and is replicated three times in the six rings. Each ring contains about 60 plots, and on the exterior boundary along its circumference are some perforated vertical poles that discharge either ambient air or CO_2-enriched air during the day. The N treatment (ambient or enriched with 4 g m^{-2} yr^{-1} N) was a subplot factor (plot scale) assigned randomly and replicated in half of the individual plots in the six rings[29,30,44]. Planted richness (1, 4, 9 or 16 species) was a subplot factor (plot scale) assigned randomly among plots in the six rings[29,30,44]. All 16 species were planted individually in 8 monoculture plots (2 per unique CO_2 and N treatment) and all together in 12 plots per unique CO_2 and N treatment. There were 15 plots per unique CO_2 and N treatment planted with either 4 or 9 species, with the individual species assignment in each plot drawn at random from the full pool of 16 species.

The present study focuses on 108 plots drawn from the main biodiversity × CO_2 × N experiment, all of which were originally planted with 9 or 16 species and experimentally treated with the complete factorial combination of CO_2 and N levels. Thus, each ring, contained 5 and 4 plots planted with 9 and 16 species, respectively, with ambient N treatment and another 5 and 4 plots planted with 9 and 16 species, respectively, with enriched N treatment. In total, therefore, there were 15 and 12 plots planted with 9 or 16 species, respectively, for each unique combination of CO_2 and N treatment. Beginning in 2007, 2 of the 5 plots with 9 species at each N treatment level in each ring began to be treated annually with a rainfall reduction, and in 2012, 2 of the 5 plots with 9 species at each N treatment level in each ring (one of which had rainfall reduction) began to be treated annually with warming treatments[30]. As we describe later in the Analyses section, we conducted statistical analyses both removing plots that eventually had altered rainfall and/or temperature treatments from the entire analyses and also retaining all plots in the analyses. The results were similar for analyses done in these two ways. We also tested whether rainfall and/or temperature treatments influenced the CO_2 × N × year interaction (that is, testing for those four- or five-way interactions) using data from 2007–21 onwards for rainfall treatments and 2012–21 onwards for temperature. None of those four- or five-way interactions had significant results (P > 0.05). Hence, responses of species richness to CO_2 × N and how that changed over time were not influenced by treatment-induced variation in rainfall or temperature (that was in any case balanced across CO_2 × N treatments). Given the similar results for including or removing plots treated with rainfall and temperature, we used all the plots in the analyses presented here.

The 16 species used in this study were all native or naturalized to the Cedar Creek Ecosystem Science Reserve. They include four C4 grasses (*Andropogon gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium* and *Sorghastrum nutans*), four C3 grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata* and *Poa pratensis*), four N-fixing legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis* and *Petalostemum villosum*) and four non-N-fixing herbaceous species (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa* and *Solidago rigida*). Since the experiment began, *A. repens* has been renamed *Elymus repens* and *K. cristata* has been renamed *Koeleria macrantha*. For consistency with previous publications from this experiment, we continue to use the previous name here. Each 16-species plot was planted in 1997 with 12 g m^{-2} of seed partitioned equally among the 16 species. For each 9-species plot, the plants were drawn at random from all 16 species, with 12 g m^{-2} of seed partitioned equally among the 9 species. All the BioCON plots were weeded annually to remove species that were not in the initial planting, but the 9- and 16-species plots resisted invasion and so needed little weeding. Enriched N treatments on unweeded grassland plots elsewhere at Cedar Creek exhibited similar effects on species richness as those found in this study, indicating that the overall patterns observed here are likely to be representative of the unmanipulated, as well as the manipulated, assemblages. Figure 4 also includes data from the 128 monoculture plots, which were compared (by averages from species, CO_2 and N combinations) with species performance in the 108 mixed-species plots that we focused on.

Beginning in 1998, the equivalent of 4 g m^{-2} yr^{-1} N in the form of NH_4NO_3 was added to all the plots assigned to the enriched-N treatment, in three doses during the growing season (in May, June and July). This N addition is similar to, or slightly larger than, the average annual net N mineralization rate in similar secondary grasslands on these soils. Beginning in 1998, a free-air CO_2-enrichment system was used during each growing season to maintain the CO_2 concentration at an average of +180 μmol mol^{-1} in elevated treatments (three rings) during all daylight hours from spring (early April) to autumn (late October to mid-November) each year. The three ambient-CO_2 rings were treated identically but without the additional CO_2.

Species composition, richness, biomass and biogeochemistry

In each year (unless otherwise noted), plant species composition and richness, above- and below-ground biomass, percentage soil moisture, percentage light transmission, plant C and N, and soil solution N concentration were assessed in every plot[23,29,30]. Soil solution N concentrations (total) were measured in each plot every year with four cores 2.5 cm in diameter taken from a depth of 0–20 cm during early to mid-summer (typically late June). The cores were composited, sieved (2 mm) and extracted with 1 M KCl. Extracts were analysed for NO_3^- and NH_4^+ on an Alpkem auto-analyser (OI Analytical). Percentage soil moisture and light transmission were measured repeatedly throughout each growing season in every plot. Light transmission was measured using an 80-sensor linear array, an AccuPAR LP-80 (Decagon Devices). Each sensor measured photosynthetic photon flux density in the 400–700 nm range. For each measurement, the sensors were arrayed above (one measurement) and below (average of three measurements) the live vegetation in each plot, with the latter divided by the former ×100 to obtain the percentage light transmission, a proxy for light availability. Soil moisture was measured using time-domain reflectometry at a depth of 0–20 cm. Average light transmission and percentage soil moisture data measured between 1 May and 31 July each year were used to assess the effects of treatments on these environmental variables, as well as their relations with species richness. Presence and estimates of percentage cover were made visually in July for each of the 16 species in a permanent zone of 0.50 m^2 (50 cm × 100 cm) for each plot that throughout the experiment was neither sampled for biomass nor had soil cores removed. Above-ground biomass was collected elsewhere for every plot in early August by clipping a strip 10 cm × 100 cm just above the soil surface; these locations rotated year by year among ten such locations in each plot. All biomass was collected, sorted to live material and senesced litter, dried and weighed. Live material was considered to be current plant biomass, sorted to species and used to assess species richness and the relative abundance of each species (defined as a fraction of the total above-ground biomass). The two independent estimates of species richness for each plot (from sorting of clipped biomass and from visible estimates of presence and percentage cover, done in different areas of the plot) were averaged for each plot and year. The average was used for three reasons: there was no a priori reason to consider one measure to be more reliable than the other (they are

well correlated anyway); because they were done in different parts of the plot, this doubled the sampling intensity; and because each was done by different researchers within and among years, the use of both measurements together helped to smooth out any observer bias. In cases for which clipped and sorted biomass were missing (in 2005 and 2006 for all nine-species plots, and in 2020 for low-rainfall nine-species plots), we used only the percentage-cover data.

In 2019, in a separate study, species richness was assessed in 70 of the 9- and 16-species plots using the identical percentage-cover method in 324 grid cells of 10 cm × 10 cm in the central zone of 1.8 m × 1.8 m for each 2 m × 2 m plot. The total aggregate number of observed species among all of the 324 sampled cells in each plot was significantly related for the 70 plots to the number of species originally planted (9 or 16) and the observed neighbourhood richness ($P < 0.001$, $R^2 = 0.73$). We applied the coefficients of this model to the neighbourhood richness data in ambient plots for all years to obtain an estimate of total richness at almost whole-plot scale. We used this to compare changes over time in richness at plot scale with other published studies of changing grassland richness over time, which were usually at a similar scale. This is relevant because, as observed in some grasslands[34–36], but not those recovering from disturbance[37,38], the diversity of our experimentally assembled communities declined with time, including under ambient conditions at both neighbourhood and plot scales (Extended Data Fig. 2). Richness measured at the neighbourhood scale was one-quarter to one-half less than the estimated available species pool at almost whole-plot scale (3.2 m^2), showing that neighbourhoods did not contain the full available species pool. Moreover, the fraction of that pool observed at the neighbourhood scale declined over the course of the experiment, indicating increased control of realized neighbourhood richness by species interactions over time (Extended Data Fig. 2).

Gains in all treatments in neighbourhood species richness from new species recruits would dampen the degree of reduction in species richness over the 24 years, and if different combinations of CO_2 and N availability led to different magnitudes of gains in species richness, this could alter the contrasts in their interactive effects. Data on new colonizers were acquired in 10 of the first 11 years of the study in each plot by removing, drying and weighing all individuals of all species not originally planted there. Because we seeded at a relatively high density and had successfully established most species, the 9- and 16-species mixtures were fully stocked and dense, and proved difficult to colonize from the beginning of the experiment right through to the end. For example, in monocultures of all 16 species, new recruit biomass (of any of the other 15 species or of species not included in the experiment) averaged 14.2% (median) of total plot biomass. By contrast, these values were much smaller in plots planted with 9 (1.2% median) and 16 (0.15%, median) species, respectively. Thus, 9- and 16-species plantings were on average much harder to invade than plots planted with the same species in monocultures and were generally resistant to invasion. More germane to this issue, in plots planted with 9 and 16 species, neither main effects of CO_2 or N nor their interaction had significant effects on the biomass of non-target species. There was a CO_2 × year interaction ($P < 0.001$); plots under eCO_2 had decreasing proportions of non-focal biomass over time, at both N treatment levels. If the numbers of new species gains were associated with the magnitude of new-recruit biomass, the lack of CO_2 × N interactions indicates that even modest gains in species richness from recruitment that would have occurred without species removals were unlikely to influence the observed effects and interactions of CO_2 and N. It is possible that the above-ground and below-ground biomass in these diverse plots was sufficiently dense, regardless of CO_2 or N treatment, to prevent these treatments from having a major impact on colonization. Furthermore, across the 24 years of the study, total biomass in 9- and 16-species plots grew larger with time, indicating that resistance to colonization was unlikely to have weakened (and may even have strengthened) in the second half of the study during which no data on removed recruitment are available. Moreover, the eCO_2 and +N treatment, which through light pre-emption reduced species richness the most in the second half of the experiment compared with all other treatment combinations, also tended to reduce light the most. Thus, if new recruits had been allowed, this treatment would have been the least likely to be successfully colonized, given that the vast majority of local grassland species require a lot of light. Overall, these data indicate that allowing new recruitment would probably not have confounded our interpretation of diversity changing on the basis of CO_2 and N over time in this system.

Analyses

Statistical analyses were done using R v.4.2.2 (ref. 45) and JMP Pro v.16.2. All the statistics shown in this paper were derived from R analyses. To test the effect on species richness of adding N and CO_2, we ran a linear mixed effects model in the nlme package[46] with species richness as the dependent variable, N treatment (ambient and enriched), CO_2 treatment (ambient and elevated), planted diversity (9 and 16), and experimental year (24 years, 1998–2021 as a continuous linear variable) as the fixed effects, and included plot nested within ring as random effects to account for the split-plot design of the experiment. We also included an AR1 correlation structure to account for repeated sampling from the plots over the 24-year period. Furthermore, to better understand any environmental variables that may be influencing species richness, we ran a second set of linear mixed models that included covariates (light, soil solution N, moisture and pH), each individually, and their full interactions with year, CO_2, N and plant species richness. In these models, only light had significant interactions with CO_2 × N × year, so only this output is shown in Extended Data Table 2. The model including light was more likely than the model without it, on the basis of Akaike information criteria (AIC) values. We also ran models that included the covariates together to evaluate the effects they had on species-richness responses to +N at the two CO_2 levels independently. Soil solution N, light availability, soil water and soil pH were extremely weakly related to one another, so collinearity of these variables was not an issue. We transformed the dependent variable where it was useful to make distributions closer to normal and to reduce issues with heteroscedastic residual patterns. We also tested whether responses of richness to treatments were influenced by interannual variation in temperature or rainfall, or the gradual and steady decline in richness among all plots, and found that none were significant.

To better visualize the effects of the +N and eCO_2 treatments, Figs. 1 and 2 show temporal changes in three parameters (richness, evenness and light) for each of the treatments, change in those parameters resulting from +N treatments at the two CO_2 levels, and CO_2 modulation of the +N effect as the difference in +N effect at the two CO_2 levels. Because the effects of treatments were sometimes nonlinear, we show the polynomial fit in the figures as appropriate, generated using the LOESS method with span=1 for a smooth fit in the ggplot2 package[47] (LOESS is a non-parametric fitting method that fits multiple local regressions to provide a smooth curve[48]). Decisions about whether to show a nonlinear fit were based on AIC, patterns of residuals, and overall fit. Finally, to better visualize temporal trends in light and above-ground biomass, which show a lot of interannual variation, we present three-year moving average values for temporal trends in Fig. 2a,d,e. All other temporal trends are presented as yearly values.

To test whether responses to CO_2 × N in the 9-species plots were influenced by extra rainfall or temperature treatments that began in mid-experiment, we ran several tests. First, for the 14 years of rainfall-removal treatments, a model of only 9-species plots including rainfall treatments found a year × CO_2 × N interaction for realized species richness (year × CO_2 × N, $P < 0.0001$) and no evidence that the interaction was modified by rainfall treatment (year × CO_2 × N × rainfall, not significant), indicating that the CO_2 × N interaction, and how it changed over time, was not altered by rainfall treatment. Similarly, for the nine years of rainfall removal and temperature treatments, a model of just

9-species plots including rainfall and/or temperature found a year × CO_2 × N interaction for realized species richness (year × CO_2 × N, $P < 0.0001$) and no evidence that the interaction was modified by temperature or rainfall treatment or their interaction (year × CO_2 × N × rainfall, not significant). We also ran the full mixed model of the experimental treatments and their impacts on species richness, using both a full dataset retaining all plots and a dataset removing all plots that had altered rainfall and/or temperature treatments during all 24 years. The main effects and interactions were similar in both analyses. Hence, we used the complete data set because it provided more power for examining and detecting the effects and interactions of CO_2 and N.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the data and the reproducible research document are publicly available at https://doi.org/10.6073/pasta/bc1e3e405c7d49e3880ca626e54425fa.

Code availability

All the code used in this paper is available at https://www.google.com/url?q=https://doi.org/10.6073/pasta/bc1e3e405c7d49e3880ca626e54425fa&source=gmail-imap&ust=1728237675000000&usg=AOvVaw29979cAyd_X4xoXFN019cw.

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Author contributions P.B.R. led the design and implementation of the experiment, conceived the questions addressed, led the data collection and analysis, wrote the first draft of the paper and led the subsequent editing, writing and revisions. N.M. contributed to the analysis and visualization. S.E.H. assisted in experiment implementation. P.B.R, N.M., S.E.H., F.I. and E.E.B. contributed to data interpretation, editing, review and writing.

Competing interests The authors declare no competing interests.

Additional information

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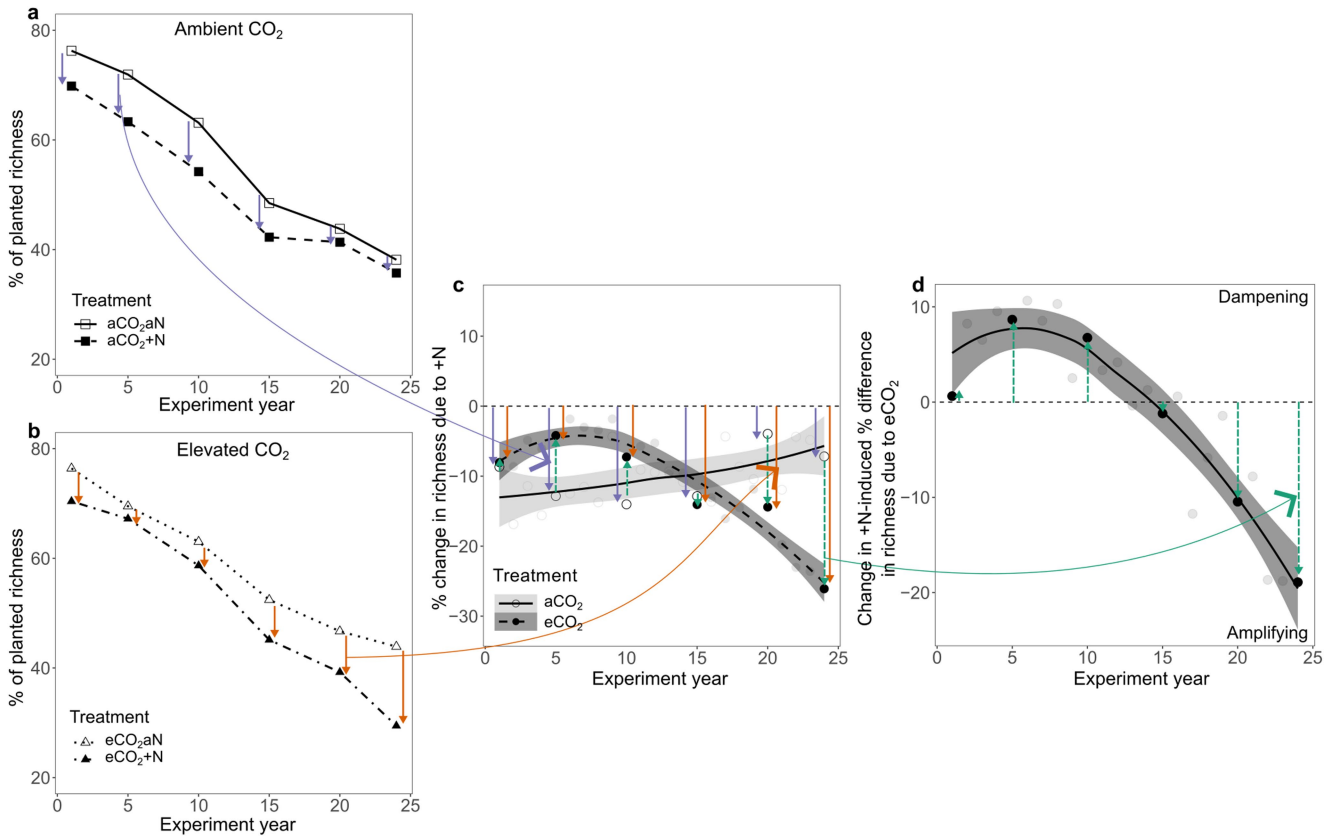
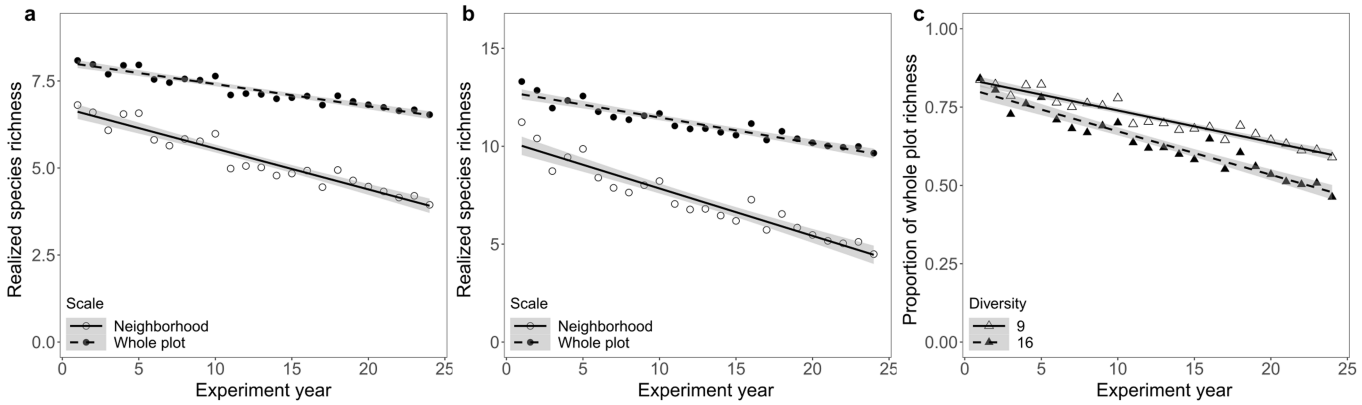
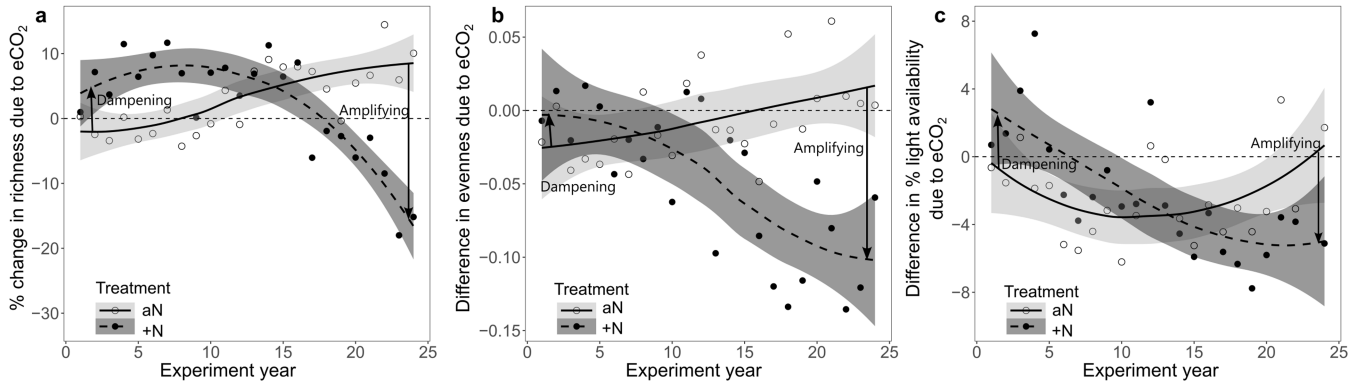


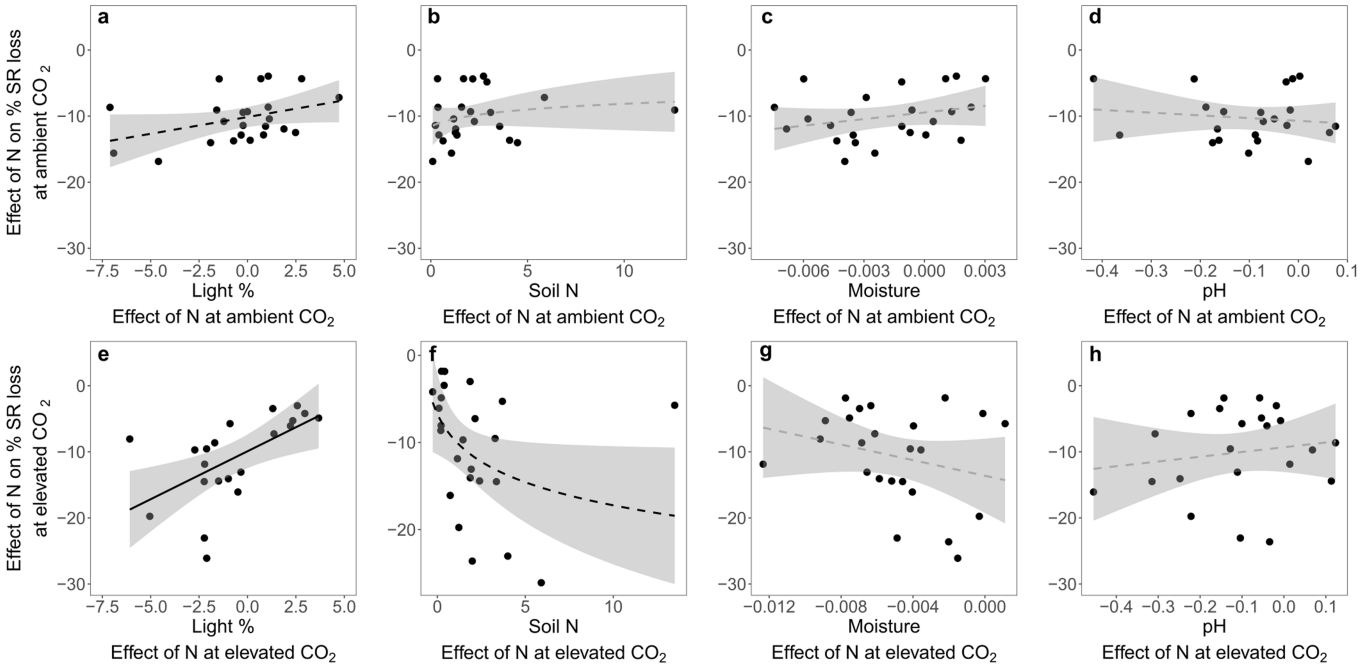
Extended Data Fig. 1 | Simplified illustration of change in species richness under CO_2 and N to aid in interpretation of Fig. 1. a,**b**, Realized species richness (SR) in the sampled area of the plot as % of planted richness in the entire plot at (**a**) ambient CO_2 and (**b**) elevated CO_2. **c**, % difference in SR due to +N (compared to ambient N [aN]) at two CO_2 levels. Percent difference in SR under +N in **c** was calculated as [SR in +N- SR in aN]*100/SR in aN] for each level of CO_2. **d**, eCO_2 mediation of richness loss due to +N; described by the change in +N effect on SR under eCO_2 (compared to ambient CO_2[aCO_2]) and calculated as the difference between +N effect on SR in elevated and ambient CO_2 levels (i.e, difference between dashed and solid lines in Fig. 1b). The straight purple and orange arrows indicate change due to N addition under ambient and elevated CO_2 conditions, respectively, and straight green arrows indicate impact of CO_2 on SR loss due to +N. The long-curved lines illustrate where a specific contrast in values in one type of comparison is located in another type of comparison. All values in Figs. 1–4 averaged over 9- and 16-species plots. For visualization purposes here and in other figures, loess polynomial fits are shown when the patterns are non-linear[43]. The dashed line at 0 represents no change in response to the treatments and the shaded region represents 95% confidence interval.



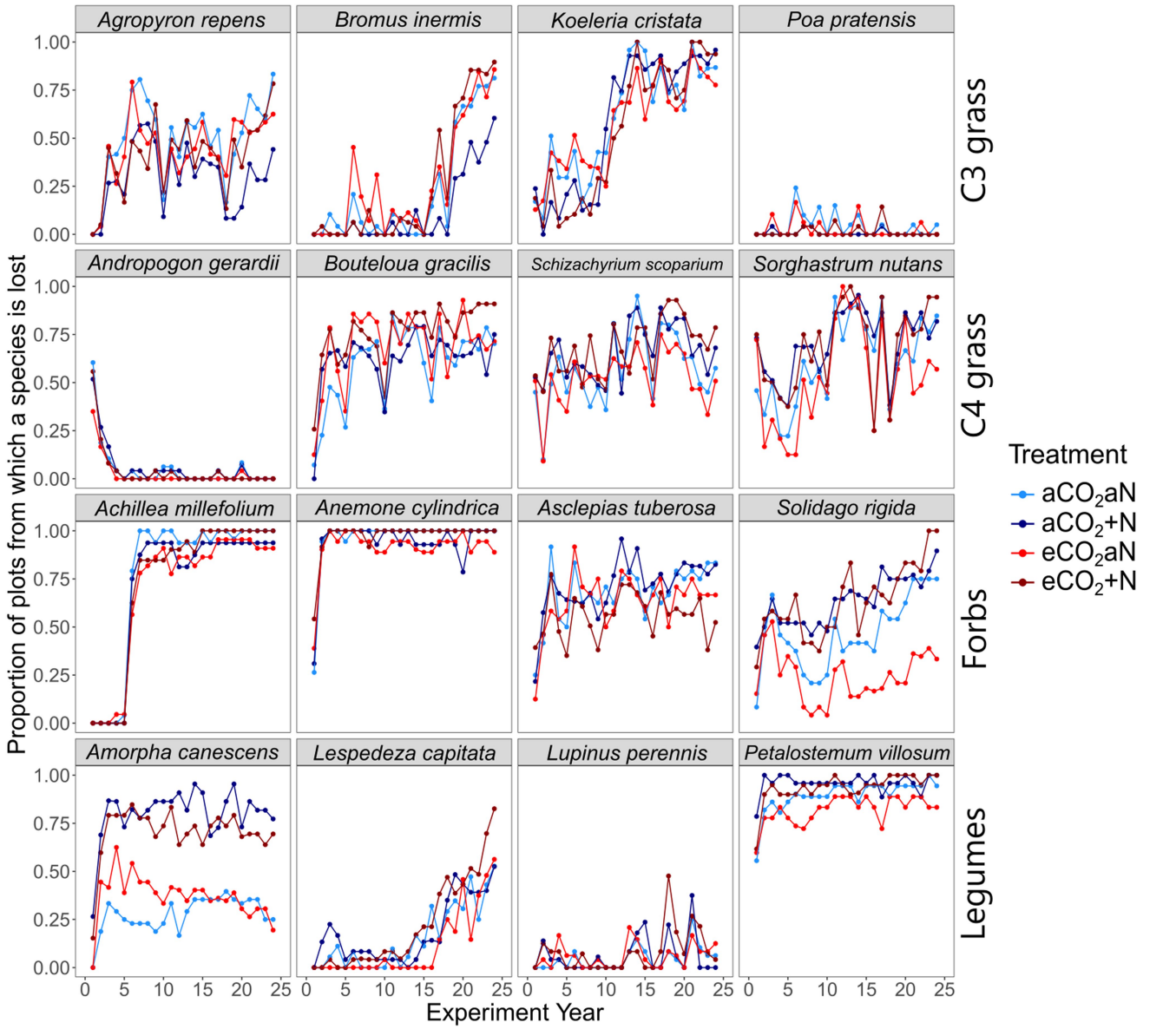
Extended Data Fig. 2 | Realized species richness measured at neighborhood scale (0.1–0.5 m²) and estimated at near-whole plot scale (3.24 m²) for ambient treatments. a, For plots originally planted with 9 species. **b**, For plots originally planted with 16 species. **c**, Neighbourhood richness as a proportion of whole plot richness. The shaded regions around the trend lines represent the 95% confidence intervals.



Extended Data Fig. 3 | Change in species diversity and light availability expressed as a function of CO_2 treatment. Same data as in Fig. 1 but examined from the perspective of CO_2 effects. **a**, % difference in species richness due to eCO_2 (compared to ambient CO_2) at two N levels. Percent difference in SR under eCO_2 in A was calculated as [SR in eCO_2- SR in aCO_2]*100/SR in aCO_2] for each level of N and averaged over 9- and 16-species plots. **b**, Effect of CO_2 on species evenness, calculated as its difference between eCO_2 and ambient CO_2 at each N level. **c**, Effect of CO_2 on percent light availability, calculated as its difference between eCO_2 and ambient CO_2 at each N level. The dashed line at 0 represents no change in response to the treatments and the shaded region represents 95% confidence interval. The results are supported by linear mixed models presented in Table 1, Extended Data Figs. 1–3.



Extended Data Fig. 4 | Effect of N on percent species richness (richness) loss at contrasting CO_2 levels along different environmental gradients. **a**–**h**, Associations between effect of added N on species richness percent loss at ambient or elevated CO_2 (top and bottom row, respectively) and effect of added N on: **a**,**e**, light; **b**,**f**, log Soil N (mid-summer soil solution N concentration); **c**,**g**, moisture (soil volumetric water content averaged across the growing season); and **d**,**h**, soil pH. The effect of N for the environmental variables were calculated as a difference between control and treatment at each CO_2 level whereas effect of N on SR was calculated as a proportional difference as in Fig. 1. Solid black lines represent significant ($p < 0.05$) associations in a multiple regression model, dashed black lines represent marginally significant associations ($0.05 < p < 0.1$) and dashed grey lines represent insignificant associations ($p > 0.1$). The shaded regions around the trend lines represent the 95% confidence intervals. The results are supported by linear models in Extended Data Table 4 based on F statistic.



Extended Data Fig. 5 | Species-specific losses over time due to global change treatments. Temporal trends in species lost in sampled neighborhoods under different global change manipulations. The proportion lost was calculated as the number of plots in which a planted species had 0 cover divided by the total number of plots the species was planted. For 9 species plots, not all species were planted in every plot. The treatments are aCO_2aN: ambient CO_2 and ambient N; aCO_2 + N: ambient CO_2 and enriched N; eCO_2aN: elevated CO_2 and ambient N; and eCO_2 + N: elevated CO_2 and enriched N. Because we sample 12.5% of the plot each year, species missing in any sampled plot in any one year might have been present elsewhere in the plot and able to recolonize the sampled cover area in a subsequent year. Species can also recolonize a plot from seed from nearby plots. Hence, proportion of plots from which a species was lost in not necessarily unidirectionally increasing. Each species by treatment combination has n = 24 data points.

Extended Data Table 1 | Treatment effects on species evenness over time

	numDF	denDF	F-value	p-value
Intercept	1	2468	13967.97	<.0001
Planted diversity	1	96	0.95	0.3311
N	1	96	11.85	0.0009
CO2	1	4	6.09	0.0691
Year	1	2468	142.36	<.0001
Planted diversity x N	1	96	2.28	0.1341
Planted diversity x CO2	1	96	0.09	0.7613
N x CO2	1	96	4.69	0.0329
Planted diversity x Year	1	2468	5.17	0.0230
N x Year	1	2468	21.05	<.0001
CO2 x Year	1	2468	1.85	0.1743
Planted diversity x N x CO2	1	96	1.84	0.1786
Planted diversity x N x Year	1	2468	2.93	0.0872
Planted diversity x CO2 x Year	1	2468	0.00	0.9675
N x CO2 x Year	1	2468	11.24	0.0008
Planted diversity x N x CO2 x Year	1	2468	6.61	0.0102

Linear mixed model output for the treatment effects of original planted species numbers (9 or 16 species; "Planted Diversity"), ambient versus enriched nitrogen (N), ambient versus elevated CO_2 treatment, and time (Year) on species evenness, Pielou's corrected J.

Extended Data Table 2 | Effect of light and its interactions on species richness

	numDF	denDF	F-value	p-value
Intercept	1	2352	3070.70	<.0001
Planted Diversity	1	96	391.51	<.0001
N	1	96	44.24	<.0001
CO2	1	4	0.52	0.5121
Year	1	2352	2861.95	<.0001
Light	1	2352	0.59	0.4411
Planted Diversity x N	1	96	1.78	0.1849
Planted Diversity x CO2	1	96	0.12	0.7264
N x CO2	1	96	0.09	0.7648
Planted Diversity x Year	1	2352	378.79	<.0001
N x Year	1	2352	0.21	0.6489
CO2 x Year	1	2352	0.51	0.4762
Planted Diversity x Light	1	2352	17.00	<.0001
N x Light	1	2352	0.66	0.4181
CO2 x Light	1	2352	0.09	0.7583
Year x Light	1	2352	0.91	0.3405
Planted Diversity x N x CO2	1	96	0.40	0.5280
Planted Diversity x N x Year	1	2352	33.27	<.0001
Planted Diversity x CO2 x Year	1	2352	1.93	0.1649
N x CO2 x Year	1	2352	29.34	<.0001
Planted Diversity x N x Light	1	2352	1.68	0.1952
Planted Diversity x CO2 x Light	1	2352	0.05	0.8284
N x CO2 x Light	1	2352	0.74	0.3895
Planted Diversity x Year x Light	1	2352	0.20	0.6539
N x Year x Light	1	2352	0.59	0.4440
CO2 x Year x Light	1	2352	0.03	0.8602
Planted Diversity x N x CO2 x Year	1	2352	0.19	0.6667
Planted Diversity x N x CO2 x Light	1	2352	0.06	0.8027
Planted Diversity x N x Year x Light	1	2352	2.40	0.1213
Planted Diversity x CO2 x Year x Light	1	2352	0.39	0.5311
N x CO2 x Year x Light	1	2352	13.78	0.0002
Planted Diversity x N x CO2 x Year x Light	1	2352	1.90	0.1681

Linear mixed model analyses testing the association of light and its interactions with the treatments- planted diversity (9 and 16 species), N (ambient and enriched), and CO_2 (ambient versus elevated) with species richness. Some values for light availability were missing for the year 2020 and those values were excluded prior to the analysis.

Extended Data Table 3 | Effect of global change drivers on environmental covariates

a) Light	numDF	denDF	F-value	p-value
Intercept	1	2141	2251.7112	<.0001
Planted Diversity	1	96	26.0169	<.0001
N	1	96	0.0699	0.7921
CO2	1	4	4.3017	0.1067
Year	1	2141	217.1149	<.0001
Planted Diversity x N	1	96	5.1170	0.0259
Planted Diversity x CO2	1	96	0.4722	0.4936
N x CO2	1	96	0.0921	0.7621
Planted Diversity x Year	1	2141	0.5875	0.4435
N x Year	1	2141	0.9060	0.3413
CO2 x Year	1	2141	6.4532	0.0111
Planted Diversity x N x CO2	1	96	0.2646	0.6082
Planted Diversity x N x Year	1	2141	0.8670	0.3519
Planted Diversity x CO2 x Year	1	2141	0.3503	0.5540
N x CO2 x Year	1	2141	9.0360	0.0027
Planted Diversity x N x CO2 x Year	1	2141	4.1371	0.0421

b) Soil Solution N	numDF	denDF	F-value	p-value
Intercept	1	2141	10102.8140	<.0001
Planted Diversity	1	96	1.3640	0.2457
N	1	96	234.6900	<.0001
CO2	1	4	5.0150	0.0887
Year	1	2141	299.9300	<.0001
Planted Diversity x N	1	96	4.8360	0.0303
Planted Diversity x CO2	1	96	0.5260	0.4701
N x CO2	1	96	3.0580	0.0835
Planted Diversity x Year	1	2141	0.7630	0.3824
N x Year	1	2141	20.0030	<.0001
CO2 x Year	1	2141	3.1790	0.0747
Planted Diversity x N x CO2	1	96	0.1270	0.7227
Planted Diversity x N x Year	1	2141	0.6750	0.4115
Planted Diversity x CO2 x Year	1	2141	0.0950	0.7582
N x CO2 x Year	1	2141	1.4960	0.2215
Planted Diversity x N x CO2 x Year	1	2141	0.0030	0.9544

c) Moisture	numDF	denDF	F-value	p-value
Intercept	1	2141	1200.2128	<.0001
Planted Diversity	1	96	0.8114	0.3700
N	1	96	3.9727	0.0491
CO2	1	4	0.2446	0.6469
Year	1	2141	6.7115	0.0096
Planted Diversity x N	1	96	0.8668	0.3542
Planted Diversity x CO2	1	96	1.8453	0.1775
N x CO2	1	96	1.0778	0.3018
Planted Diversity x Year	1	2141	4.7009	0.0303
N x Year	1	2141	0.0127	0.9102
CO2 x Year	1	2141	0.8348	0.3610
Planted Diversity x N x CO2	1	96	0.6755	0.4132
Planted Diversity x N x Year	1	2141	0.2939	0.5878
Planted Diversity x CO2 x Year	1	2141	0.0269	0.8698
N x CO2 x Year	1	2141	0.0003	0.9872
Planted Diversity x N x CO2 x Year	1	2141	0.1863	0.6661

d) pH	numDF	denDF	F-value	p-value
Intercept	1	2141	13367.1460	<.0001
Planted Diversity	1	96	8.9090	0.0036
N	1	96	12.5160	0.0006
CO2	1	4	0.2890	0.6195
Year	1	2141	129.0470	<.0001
Planted Diversity x N	1	96	1.3510	0.2480
Planted Diversity x CO2	1	96	0.1420	0.7076
N x CO2	1	96	0.0120	0.9129
Planted Diversity x Year	1	2141	3.7070	0.0543
N x Year	1	2141	4.4720	0.0346
CO2 x Year	1	2141	0.0830	0.7737
Planted Diversity x N x CO2	1	96	0.0500	0.8241
Planted Diversity x N x Year	1	2141	0.0240	0.8759
Planted Diversity x CO2 x Year	1	2141	0.0010	0.9698
N x CO2 x Year	1	2141	0.2520	0.6155
Planted Diversity x N x CO2 x Year	1	2141	2.1040	0.1471

Results from linear mixed models testing the effect of CO_2 and N on different environmental covariates: **a**, light; **b**, soil solution N; **c**, moisture; and **d**, pH.

Extended Data Table 4 | Effect of N and environmental covariates on species richness at ambient and elevated CO_2

a) Ambient CO2	Df	Sum Sq	Mean Sq	F-value	p-value
Soil N	1	10.08	10.08	0.8208	0.3999
Moisture	1	29.08	29.08	2.3682	0.1748
Light	1	50.82	50.82	4.1378	0.0882
pH	1	8.87	8.87	0.7221	0.4281
Soil N x Moisture	1	9.96	9.96	0.8114	0.4024
Soil N x Light	1	1.41	1.41	0.1150	0.7460
Soil N x pH	1	1.58	1.58	0.1286	0.7322
Moisture x Light	1	33.92	33.92	2.7623	0.1476
Moisture x pH	1	2.99	2.99	0.2432	0.6394
Light x pH	1	1.29	1.29	0.1048	0.7571
Soil N x Moisture x Light	1	8.66	8.66	0.7050	0.4333
Soil N x Moisture x pH	1	0.69	0.69	0.0560	0.8208
Soil N x Light x pH	1	48.62	48.62	3.9592	0.0938
Moisture x Light x pH	1	0.97	0.97	0.0789	0.7882
Residuals	6	73.69	12.28		

b) Elevated CO2	Df	Sum Sq	Mean Sq	F-value	p-value
Soil N	1	77.06	77.06	4.2763	0.0841
Moisture	1	1.16	1.16	0.0644	0.8081
Light	1	383.16	383.16	21.2630	0.0036
pH	1	42.46	42.46	2.3562	0.1757
Soil N x Moisture	1	24.31	24.31	1.3491	0.2895
Soil N x Light	1	21.85	21.85	1.2125	0.3130
Soil N x pH	1	1.95	1.95	0.1080	0.7536
Moisture x Light	1	6.02	6.02	0.3340	0.5843
Moisture x pH	1	0.69	0.69	0.0383	0.8513
Light x pH	1	9.36	9.36	0.5193	0.4982
Soil N x Moisture x Light	1	33.46	33.46	1.8569	0.2219
Soil N x Moisture x pH	1	0.25	0.25	0.0141	0.9094
Soil N x Light x pH	1	5.31	5.31	0.2949	0.6066
Moisture x Light x pH	1	0.00	0.00	0.0002	0.9892
Residuals	6	108.12	18.02		

Linear model results for variation in effect of N on percent species richness loss as influenced by N effect on environmental covariates: soil N (log-transformed), moisture, light, pH and their interactions under: **a**, ambient CO_2 and **b**, elevated CO_2 conditions.

Extended Data Table 5 | Influence of environmental covariates in CO_2 modulation of N effect on species richness

	Df	Sum Sq	Mean Sq	F-value	p-value
ΔSoil N	1	235.34	235.34	6.5994	0.0501
ΔMoisture	1	2.17	2.17	0.0608	0.8151
ΔLight	1	463.23	463.23	12.9899	0.0155
ΔpH	1	69.99	69.99	1.9628	0.2201
ΔSoil N x ΔMoisture	1	74.17	74.17	2.0800	0.2088
ΔSoil N x ΔLight	1	101.18	101.18	2.8372	0.1529
ΔSoil N x ΔpH	1	20.49	20.49	0.5746	0.4826
ΔMoisture x ΔLight	1	2.36	2.36	0.0661	0.8074
ΔMoisture x ΔpH	1	0.01	0.01	0.0003	0.9864
ΔLight x ΔpH	1	51.00	51.00	1.4301	0.2854
ΔSoil N x ΔMoisture x ΔLight	1	89.76	89.76	2.5170	0.1735
ΔSoil N x ΔMoisture x ΔpH	1	3.87	3.87	0.1086	0.7551
ΔSoil N x ΔLight x ΔpH	1	4.59	4.59	0.1286	0.7345
ΔMoisture x ΔLight x ΔpH	1	2.40	2.40	0.0674	0.8055
ΔSoil N x ΔMoisture x ΔLight x ΔpH	1	20.15	20.15	0.5650	0.4861
Residuals	5	178.30	35.66		

Linear model results showing associations between eCO_2 modulation of +N effect (denoted as Δ) of percent species richness loss and Δ soil N, Δlight, Δmoisture, ΔpH and their interactions.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used for data collection
Data analysis	All data analysis was done in R 4.2.2 and JMP Pro v16.2. The R code for all the statistical models and plotting figures is available along with the data on https://portal.edirepository.org/nis/mapbrowse?scope=knb-lter-cdr&identifier=732&revision=1

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Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	NA
Reporting on race, ethnicity, or other socially relevant groupings	NA
Population characteristics	NA
Recruitment	NA
Ethics oversight	NA

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	The study reports results from a 24 year grassland experiment that manipulates species richness, N and CO2 factorially in a split-plot design
Research sample	Species-specific plant biomass data from clippings and cover data from visual observation were collected for most years of the experiment. There are a total of 16 species in the experiment and all 16 were present in the 16 species plots and a subset of species were present in the 9 species plot (randomized subset sown in 1997).
Sampling strategy	All plots were intended to sampled every year
Data collection	Data on species-specific plant biomass and cover have been collected annually from all 108 plots from 1998-2021 (except for 9 species plots, species specific biomass data for 2005 and 2006, and a subset of plots in 2020, were missing due to resource limitations and/or COVID restrictions).
Timing and spatial scale	The experiment spans 24 years at one site, Cedar Creek Ecosystem Science Reserve, Minnesota, USA
Data exclusions	The experiment includes monocultures and 4 species plots as well as the 9 and 16 species plots used herein, but given our focus on global change impacts on richness and evenness it seemed appropriate to use only plots originally seeded with 9 and 16 species.
Reproducibility	The experiment per se is not reproduced.
Randomization	The initial planting of species in 9 species plots were randomized.
Blinding	*Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.*

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	The experiment is situated in a North American prairie ecosystem (i.e., a temperate grassland)) with a mean annual temperature of 7C and mean annual rainfall of 800mm. The study is located on primarily sandy soils which are relatively poor in nitrogen.
Location	Cedar Creek Ecosystem Science Reserve, Minnesota, USA

Access & import/export	NA
Disturbance	The site has been burned half of the years between 2000 to 2012 and every fall since 2013 as a part of grassland management strategy.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☐	☒ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Dual use research of concern

Policy information about dual use research of concern

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Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

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Does the work involve any of these experiments of concern:

No	Yes
☒	☐ Demonstrate how to render a vaccine ineffective
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☒	☐ Enhance the virulence of a pathogen or render a nonpathogen virulent
☒	☐ Increase transmissibility of a pathogen
☒	☐ Alter the host range of a pathogen
☒	☐ Enable evasion of diagnostic/detection modalities
☒	☐ Enable the weaponization of a biological agent or toxin
☒	☐ Any other potentially harmful combination of experiments and agents

Plants

Seed stocks	Planted in 1997 from seeds collected in Minnesota by commercial native plant vendors
Novel plant genotypes	NA
Authentication	NA