



Synergistic effects of four climate change drivers on terrestrial carbon cycling

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Disentangling impacts of multiple global changes on terrestrial carbon cycling is important, both in its own right and because such impacts can dampen or accelerate increases in atmospheric CO₂ concentration. Here we report on an eight-year grassland experiment, TeRaCON, in Minnesota, United States, that factorially manipulated four drivers: temperature, rainfall, CO₂ and nitrogen deposition. Net primary production increased under warming, elevated CO₂ and nitrogen deposition, and decreased under diminished summer rainfall. Treatment combinations that increased net primary production also increased soil CO₂ emissions, but less so, and hence ecosystem carbon storage increased overall. Productivity, soil carbon emissions and plant carbon stock responses to each individual factor were influenced by levels of the other drivers, in both amplifying and dampening ways. Percentage increases in productivity, soil carbon emissions and plant carbon stocks in response to two, three or four global changes experienced jointly were generally much greater than those expected based on the effects of each individual driver alone. Multiple global change drivers had a profound combined influence on observed outcomes that would have been poorly predicted by knowledge of each driver alone. If such interacting impacts of multiple global change drivers on carbon cycling occur widely among ecosystems, accurately projecting biosphere responses to multifactorial global changes will remain a major challenge in the decades ahead.

Ecosystems in the twenty-first century face multiple simultaneous global changes that influence many aspects of their structure and function, including carbon (C) cycling^{1–11}. Moreover, given that changes in C cycling can dampen or accelerate the rate of increase in atmospheric carbon dioxide (CO₂) concentration and of associated climate change, understanding the C cycle responses of terrestrial ecosystems to elevated CO₂ (eCO₂) and climate change is a priority for C cycle science^{1,5}. Understanding the individual and joint impacts of multiple global change drivers from observational studies is difficult, as teasing apart the independent effects of each may not always be possible because of confounding effects among global change factors and interactions with other factors^{2–6}.

Addressing these issues experimentally is also challenging, as ecologically realistic global change experiments with even a single factor are difficult to implement¹², and field experiments with multiple factors are rare. For example, of 1,119 experiments assessed in a recent meta-analysis¹³, only one factorial experiment of more than one year¹¹ included all four of the following manipulations—altered growing season precipitation, eCO₂, increased growing season day-time temperatures by >1 °C and enriched nitrogen (N) supply—and only two others included three of those four manipulations^{9,14} (Methods). Experiments that manipulate two factors (for example, CO₂ crossed with warming, water or N) are more plentiful, but such experiments done in the field for more than two years are rare¹¹.

To advance our understanding of the effects of, and interactions among, multiple global change factors, we conducted an eight-year experiment, TeRaCON (temperature, rainfall, CO₂ and N) in a perennial temperate grassland in Minnesota, United States, with all 16 combinations of two contrasting levels of four global change factors (temperature, rainfall, CO₂ and N)¹⁵, which represents a novel

test of multiple global change interaction theory¹. The experiment used 48 plots, each planted in 1997 with 9 species randomly chosen from a 16 species pool (Methods). From 2012 to 2019, in addition to ambient controls, we employed free-air warming of plants and soils to a 1 m depth^{15,16} by +2.5 °C, removable rain shelters^{15,17} to reduce the total growing season rainfall by ~30%, free-air CO₂ enrichment technology^{17,18} to expose plants to elevated (+180 ppm) levels of CO₂ and ammonium nitrate (NH₄NO₃) fertilization^{17,18} (+4 g N m⁻² yr⁻¹) to simulate the effects of increased atmospheric N deposition. We annually measured above- and below-ground plant biomass and plant C pools and productivity^{17,19–21}. We estimated the mean soil CO₂ flux over each growing season, based on multiple samplings made from early May to late September each year¹⁹. In 2016, we also measured soil C stocks to a 60 cm depth.

Global change context and theory

Hypotheses regarding global change effects on ecosystems can be based on either a resource availability or an environmental optimality framework: changes that increase resources or move conditions closer to their optimum should typically increase productivity, in contrast to those that decrease resources or move away from optimal conditions. As CO₂ and N are key plant resources, moderate increases in atmospheric CO₂ concentrations and N deposition should increase net primary productivity (NPP) in many ecosystems^{1,5,18}. Except in excessively wet ecosystems, increased rainfall should increase productivity, whereas reduced rainfall should decrease it¹. The effects of higher temperatures are more difficult to predict, for two reasons. First, any given plant community might be below its optimum temperature at certain times and be above it at others, so to predict the net effect of changes in mean annual temperatures on the NPP within an environmental optimality

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Table 1 | Changes (%) in the ecosystem C pool and flux components due to the effects of four treatment factors

NPP		Soil CO ₂ flux		Total plant C pool		
Treatment	Observed main effect	Observed main effect	Observed main effect	Observed main effect		
+2.5 °C Temp	+23.3**	+11.2**	+11.2**	+14.2**	+14.2**	
Reduced Rain	-12.8**	-1.2	-1.2	-1.4	-1.4	
+CO ₂	+23.4**	+15.9**	+15.9**	+24.4**	+24.4**	
+N	+16.2*	+3.7	+3.7	+6.2	+6.2	
Joint effects		Joint effects		Joint effects		
Interactions	Expected	Observed	Expected	Observed	Expected	
Temp × Rain	+18.1	+7.7	+1.8	+9.7	+17.0	+13.0
Temp × CO ₂	+66.1	+54.2	+26.2	+28.8	+66.1	+45.3**
Temp × N	+10.0	+40.4**	-3.6	+14.6**	+5.0	+21.6**
Rain × CO ₂	-11.5	+7.1	+0.3	+13.9*	-1.0	+21.2
Rain × N	-20.5	+1.1*	-6.5	+2.3	-12.9	+5.1
CO ₂ × N	+8.3	+40.1**	+11.2	+19.7	-0.8	+30.5**
Temp × Rain × CO ₂	+72.8	+47.3	+8.3	+28.4*	+72.4	+53.1
Temp × Rain × N	-19.0	+22.0	-20.4	+12.9*	+2.8	+24.5
Temp × CO ₂ × N	-20.8	+53.6**	-15.7	+24.8*	-23.5	+33.3**
Rain × CO ₂ × N	-42.6	+21.7	-8.1	+19.1	-37.7	+27.0
Temp × Rain × CO ₂ × N	-75.3	+42.9*	-37.8	+26.0	-44.9	+43.7

The values are averaged from 2012 to 2019 and the expected and observed percent changes that resulted from two-, three- and four-way interactions are shown. Pools and fluxes included total NPP (g C m⁻² yr⁻¹; above-ground productivity plus root productivity, 0–20 cm), soil CO₂ flux (μmol CO₂ m⁻² s⁻¹; the mean of growing season soil flux measurements, early May to late September) and plant C pool (g C m⁻²; above + below ground, 0–100 cm). Years are treated as continuous. *0.05 < P ≤ 0.1, **P ≤ 0.05. Calculations for two- and three-way interactions were done across all levels of the other treatments. Expected effects were the additive effects sizes of each treatment when imposed individually. Several of the higher-order joint effects interacted with time (Table 2). Rain, rainfall; Temp, temperature.

framework is context dependent^{4,11,22}. Second, higher temperatures can have multiple indirect effects on resource supply, for example, decreasing soil moisture due to heightened evapotranspiration or increasing soil nutrient availability due to increased soil microbial activity, with opposing effects on productivity^{11,22–24}. The effects of global change on the total ecosystem C pools and fluxes are even more difficult to predict and assess, given that a large fraction of that C is in soil—which has a high spatial variability, slow rate of change and many processes that influence the rate of its decomposition.

Responses to individual global change factors

As this experiment was situated in a temperate ecosystem where growing season length is limited but hot summers can occur, it was unclear whether warming (and associated soil drying) would enhance or reduce NPP. Hypotheses for the main effects of the other drivers were more straightforward; we expected higher levels of CO₂ and N to enhance NPP and reduced rainfall to decrease it. We expected the responses of soil CO₂ flux to directionally reflect the NPP responses¹⁹ because of the importance of root biomass and organic matter inputs to soils in driving root autotrophic and soil heterotrophic respiration, respectively; but we did not necessarily expect NPP and soil CO₂ flux responses to be of the same magnitude. It is possible that the total ecosystem (soil plus plant) C stock responses to treatments might also reflect NPP responses, as inputs drive changes in total biomass and soil C in this ecosystem¹⁹.

We found that all four factors influenced the three C cycling metrics and there were many interactions among the global change drivers (Tables 1 and 2). The main effects of factors on NPP (above-ground productivity plus below-ground productivity to a 20 cm depth) were congruent with the hypotheses and in the same direction as the effects on soil CO₂ flux, as expected. However, warming and eCO₂ had larger effects on NPP and soil CO₂ flux than those of added N, which we did not expect based on past evidence of strong N limitation in these grasslands^{17,18,20}.

An elevated temperature increased NPP by 23.3% on average across other treatments and all years, mean growing season soil CO₂ flux by 11.2% and total (above plus below ground to 1 m depth) plant C pools by 14.2% (Table 1). Elevated CO₂ increased NPP by 23.4%, the mean soil CO₂ flux by 15.9% and the total plant C pools by 24.4% (Table 1). Enriched N increased NPP by 16.2%, whereas reduced rainfall reduced it by 12.8%. These average main effects of each of the four drivers across all eight combinations of the other treatments were generally consistent across the eight years of the experiment (Fig. 1), yet these averages mask large context-dependent variation, as many of these responses were strongly contingent on the levels of the other treatments. We also note that the levels of the four treatments were chosen to test for effects and interactions of potential global changes, rather than simulate specific scenarios of projected changes, so responses should not be taken as a direct prediction of future C cycling.

Responses to multiple global change factors

Interactions among multiple global changes are more difficult to predict^{1,7–11,15,17,18,22–30}. Whether responses to multiple changes are additive, antagonistic (less than additive) or synergistic (greater than additive) depends on (1) whether any given response metric is co-limited by more than one factor (the multiple limitation hypothesis) and/or (2) whether the indirect effects of any one factor on the other resources or environmental conditions influence the response to other global change factors. We hypothesized that greater-than-additive responses to combinations of treatments at their more favourable (for C cycling) levels would occur in TeRaCON; that is, that alleviation of one limitation to NPP would amplify the constraint by another co-limiting factor^{17,18,26,27}; thus, to alleviate multiple limitations simultaneously would result in greater-than-additive responses. Additionally, temperature, rainfall, CO₂ and N deposition can each influence resources, such as soil moisture and soil nutrient supply, and thus indirectly influence the

Table 2 | Mixed effects on annual NPP, soil CO₂ flux (growing season) and plant C pool

Effect	d.f.	NPP		Soil CO ₂ flux		Plant C pool	
		F	P>F	F	P>F	F	P>F
CO ₂	4	32.06	0.0047*	16.90	0.0147*	12.87	0.0230*
yr	319	7.51	0.0065*	7.48	0.0066*	32.67	<.0001*
yr × CO ₂	319	0.15	0.6973	0.06	0.8023	0.79	0.3753
N	28	3.02	0.0930**	0.90	0.3511	0.61	0.4422
yr × N	319	0.68	0.4104	0.13	0.7199	0.08	0.7826
CO ₂ × N	28	5.82	0.0226*	1.53	0.2267	5.19	0.0306*
yr × CO ₂ × N	319	0.59	0.4443	0.11	0.7418	0.23	0.6310
Rain	28	4.36	0.0460*	0.23	0.6361	0.16	0.6963
yr × Rain	319	0.13	0.7140	1.26	0.2620	0.17	0.6817
CO ₂ × Rain	28	2.24	0.1456	3.38	0.0767**	1.35	0.2545
yr × CO ₂ × Rain	319	0.61	0.4371	1.27	0.2599	0.01	0.9373
N × Rain	28	3.39	0.0761**	1.41	0.2445	2.25	0.1452
yr × N × Rain	319	0.73	0.3938	0.25	0.6184	0.20	0.6535
CO ₂ × N × Rain	28	0.30	0.5910	0.20	0.6615	0.03	0.8717
yr × CO ₂ × N × Rain	319	0.12	0.7325	0.33	0.5650	0.03	0.8534
Temp	28	12.84	0.0013*	9.13	0.0053*	5.95	0.0213*
yr × Temp	319	0.26	0.6111	0.31	0.5795	0.05	0.8230
CO ₂ × Temp	28	1.24	0.2745	0.01	0.9328	1.83	0.1874
yr × CO ₂ × Temp	319	1.13	0.2892	2.21	0.1383	5.47	0.0199*
N × Temp	28	4.18	0.0503*	6.96	0.0135*	1.96	0.1730
yr × N × Temp	319	0.51	0.4772	0.20	0.6517	0.12	0.7314
CO ₂ × N × Temp	28	4.39	0.0454*	3.16	0.0864**	5.92	0.0217*
yr × CO ₂ × N × Temp	319	3.68	0.0558**	2.44	0.1196	0.07	0.7969
Rain × Temp	28	0.96	0.3367	1.35	0.2553	0.32	0.5737
yr × Rain × Temp	319	1.26	0.2618	0.52	0.4696	1.72	0.1903
CO ₂ × Rain × Temp	28	2.71	0.1111	0.20	0.6563	1.63	0.2127
yr × CO ₂ × Rain × Temp	319	2.91	0.0889**	2.77	0.0972**	1.79	0.1819
N × Rain × Temp	28	0.00	0.9780	0.03	0.8739	0.39	0.5364
yr × N × Rain × Temp	319	0.21	0.6491	3.01	0.0836**	0.50	0.4787
CO ₂ × N × Rain × Temp	28	3.94	0.0570**	0.03	0.8539	1.08	0.3085
yr × CO ₂ × N × Rain × Temp	319	1.55	0.2140	2.03	0.1553	1.32	0.2513

Linear repeated measures model probabilities (P>F). All the models used log₁₀ values for the response variables. The ring was nested within CO₂, and the plot was nested within CO₂, N, Rain and Temp as random effects. *P≤0.05, **P≤0.1. yr, year; d.f., degrees of freedom; F, F ratio; Rain, rainfall; Temp, temperature.

C cycle responses to other treatments. As eCO₂ routinely increases leaf-level water-use efficiency¹², it often has water-savings effects at the ecosystem scale²⁸, leading to the CO₂-induced water-savings hypothesis, that reduced rainfall has fewer negative effects when CO₂ is elevated. In some short-term studies, this water-savings effect of eCO₂ alleviated the negative effects of rising temperatures or reduced rainfall on soil moisture^{7,11,14,24}. Higher temperatures can also influence responses to the other global change factors by reducing soil moisture as a result of heightened evapotranspiration²². Thus, reduced rainfall could have more negative effects on C cycling processes when temperature is elevated, but fewer negative effects at eCO₂.

The NPP and soil CO₂ flux responses found in this study supported the hypothesis that this ecosystem is limited by multiple factors. When the four treatments were grouped into their levels assumed to be more limiting (ambient T, ambient CO₂, ambient N and reduced rainfall) versus less limiting to productivity (+2.5°C, eCO₂, enriched N and ambient rainfall), an increase in the

number of factors that were at less-limiting levels increased NPP (by 70% across years) and soil CO₂ flux (by 20%) (Fig. 2).

Available soil C data from 2016 (after five years of treatments) enabled us to examine whether the effects of global change factors on productivity might translate into effects on total ecosystem C storage in this perennial grassland system. Among the 16 factorial combinations of the 4 treatments, those with a higher NPP tended to have greater plant, soil and total (plant+soil) ecosystem C pools (Fig. 3a–c). Similarly, plant, soil and total ecosystem C pools also increased with the number of treatments at their elevated level (Fig. 3d–f), which also corresponded with a higher NPP (Fig. 2). Pairwise treatment combinations that involved eCO₂, elevated temperature and enriched N tended to have larger positive effects on both NPP and C stores, whereas pairwise combinations that involved reduced rainfall tended to dampen the effects on productivity and C storage (Fig. 3g–i).

For the three measures of C cycling and stocks measured from 2012 to 2019 (NPP, soil CO₂ flux and total plant C pools),

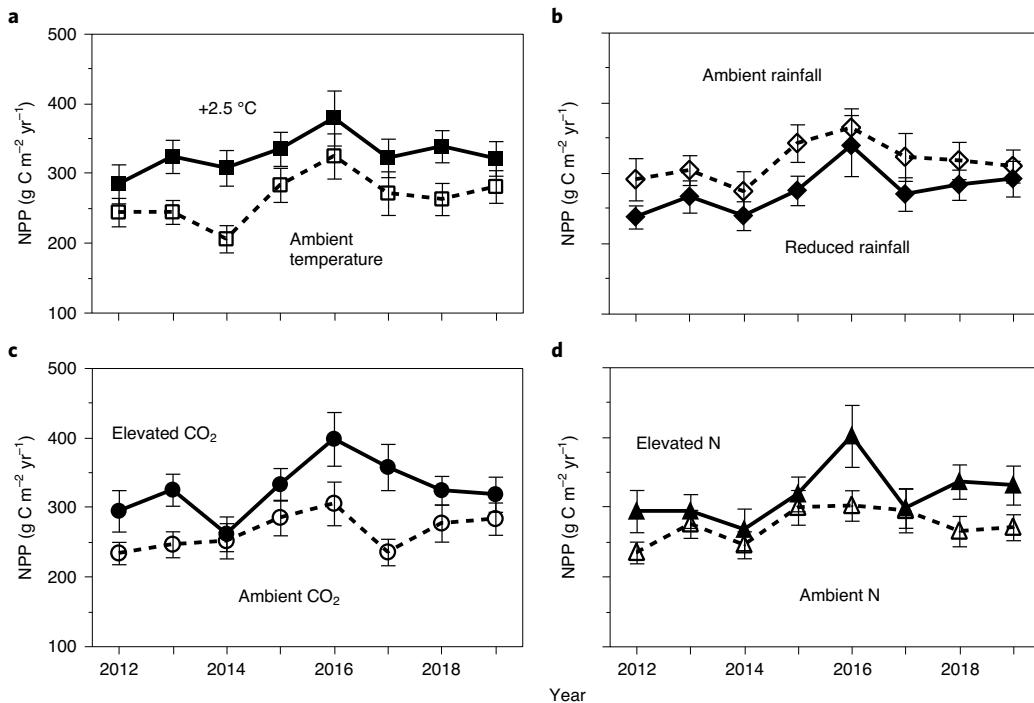


Fig. 1 | NPP over time in relation to each of the four treatments. **a–d**, Shown are the main effects of each treatment averaged across all levels of the other three treatments for the eight years of the experiment: temperature (+2.5 °C) (**a**), reduced rainfall (**b**), elevated CO₂ (**c**) and enriched N (**d**). Open symbols indicate the ambient conditions, filled symbols indicate the treatment modification. NPP was estimated as the sum of annual above-ground and below-ground productivity from 0 to 20 cm depth. Error bars represent ± 1 standard error of the mean.

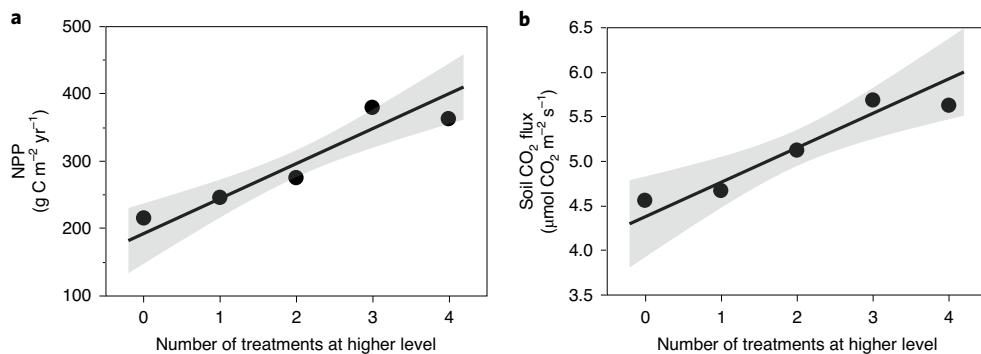


Fig. 2 | NPP and soil CO₂ flux. **a,b**, Mean NPP (**a**) and soil CO₂ flux (**b**) in relation to the number of factors at their less-limiting levels. The filled circles represent averages across all eight years of the study. For relationships with NPP and soil CO₂ flux, $R^2 = 0.88$ and 0.91, respectively ($P = 0.0187$ and 0.0122, respectively). The shaded area shows a 95% confidence interval for the fit.

there was evidence for 15 of the 18 possible pairwise interactions ($0.0001 < P < 0.10$), some of which varied among years or with other treatments (Table 1). Moreover, all four global change factors were involved in these interactions.

Many of these interactions were consistent with existing theory and our hypotheses. Pairwise interactions that involved temperature, CO₂ and N tended to be greater than additive, in support of the multiple limitation hypothesis (for example, Fig. 4a,b,d,e). For example, across all the years, warming increased NPP by 38% at enriched N versus 8% at ambient N (Fig. 4a), and eCO₂ increased NPP by 39% at enriched N versus 7% at ambient N (Fig. 4b). Interactions that involved rainfall \times CO₂ were less than additive, in that reduced rainfall had fewer negative effects when CO₂ was elevated, consistent with the CO₂-induced water-savings

hypothesis (Fig. 4c,f) and in contrast to the multiple limitation hypothesis. For example, lower rainfall reduced NPP by 23% at ambient CO₂, but by only 4% at eCO₂.

The observed effects of multiple drivers in combination were often markedly greater than the expected effects of multiple global change drivers based on their impacts in isolation (Table 1). This was true for both synergistic (greater-than-additive) and antagonistic (less-than-additive) interactions, because simultaneous manipulation of other global change treatments tended to amplify the positive effects (such as from eCO₂) and dampen the negative effects (such as from reduced rainfall). For two- and three-way interactions, the effect sizes were calculated across all levels of the other treatments not part of that particular interaction. For 27 of the 33 cases that involved NPP, total plant C and soil CO₂ flux, which

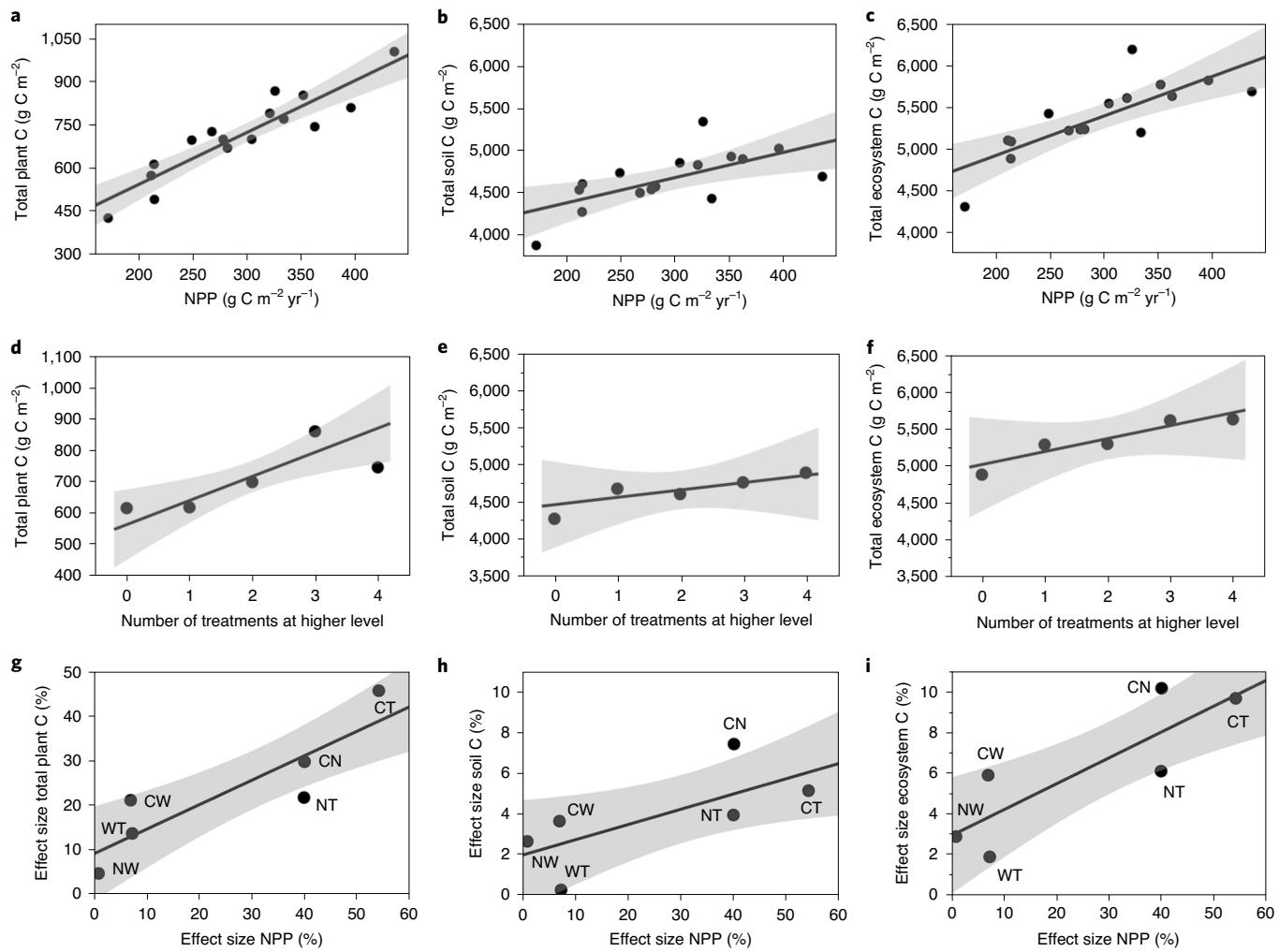


Fig. 3 | Relationships of C pools, NPP and treatments. **a–f**, Shown are the mean (across plots) total plant (**a,d**), soil (**b,e**), and ecosystem (**c,f**) C pools in relation to the mean NPP (**a–c**) of all 16 combinations of the 4 treatments and to the number of factors at their less limiting level (**d–f**). **g–i**, Effect size of pairwise treatment combinations on total plant (**g**), soil (**h**) and ecosystem (**i**) C pools shown in relation to their effect sizes on NPP. As soil C pool data were only available for 2016, estimates are averaged across all eight years for the other variables. $R^2 > 0.42$, $P < 0.05$ (**a–c,e–g,i**), $R^2 = 0.61$, $P = 0.12$ (**d**) and $R^2 = 0.49$, $P = 0.12$ (**h**). Shaded areas show 95% confidence intervals for the fits.

included 13 of the 14 significant ($0.0001 \leq P \leq 0.1$) cases (Table 1), the observed effects of joint global change treatments were more positive than the additive expectation. In seven of eight pairwise interactions ($P < 0.1$), the percentage increases in observed NPP, total plant C and soil CO_2 flux rates were at least four times greater than expected (and with two changed directions) based on the additive effects of each driver alone (Table 1).

In essence, for temperature, CO_2 and N, the ability of plants to produce more biomass at higher levels of each variable was generally enhanced by higher levels of the others; whereas for $\text{CO}_2 \times$ water, the water savings of eCO_2 dampened the negative impacts of low rainfall, which also leads to more positive net joint effects than expected from their impacts in isolation. This tendency for joint treatments to have greater effects than expected from each factor alone was true for the three- and four-way interactions. For the five significant ($0.0001 \leq P \leq 0.1$) three-way interactions, increases due to joint global changes were large (average of +31%), and always much greater than the expected effect from the addition of the individual effects, which averaged a 14% reduction (Table 1). The four manipulations together increased NPP by 43% and soil CO_2 flux by 26%, whereas the expected effects based on responses to each

driver alone were strongly negative (Table 1). Clearly, for this system the responses to multiple global change drivers would not be well predicted from even the best knowledge of the impacts of each driver considered alone.

The role of species composition

Responses to the four global change drivers were largely independent of species composition or its changes over time or among treatments. The dominant species, *Andropogon gerardii*, made up about two-thirds of the above-ground biomass on average across years and all treatments in the 27 (of 48) plots in which it was planted. However, its relative abundance (percentage of total above-ground biomass) varied modestly with treatments, which suggests that C cycling responses to treatments were driven in minimal-to-modest ways by shifts towards a greater abundance of this more productive species. For example, *Andropogon* made up 66 versus 70% of above-ground biomass for ambient versus warmed plots, 71 versus 66% for ambient versus low rainfall, 65 versus 71% for ambient versus eCO_2 and 62 versus 72% for ambient versus +N. Moreover, including the presence or absence of *Andropogon* in our model did not influence the response to any of the treatments; there was no

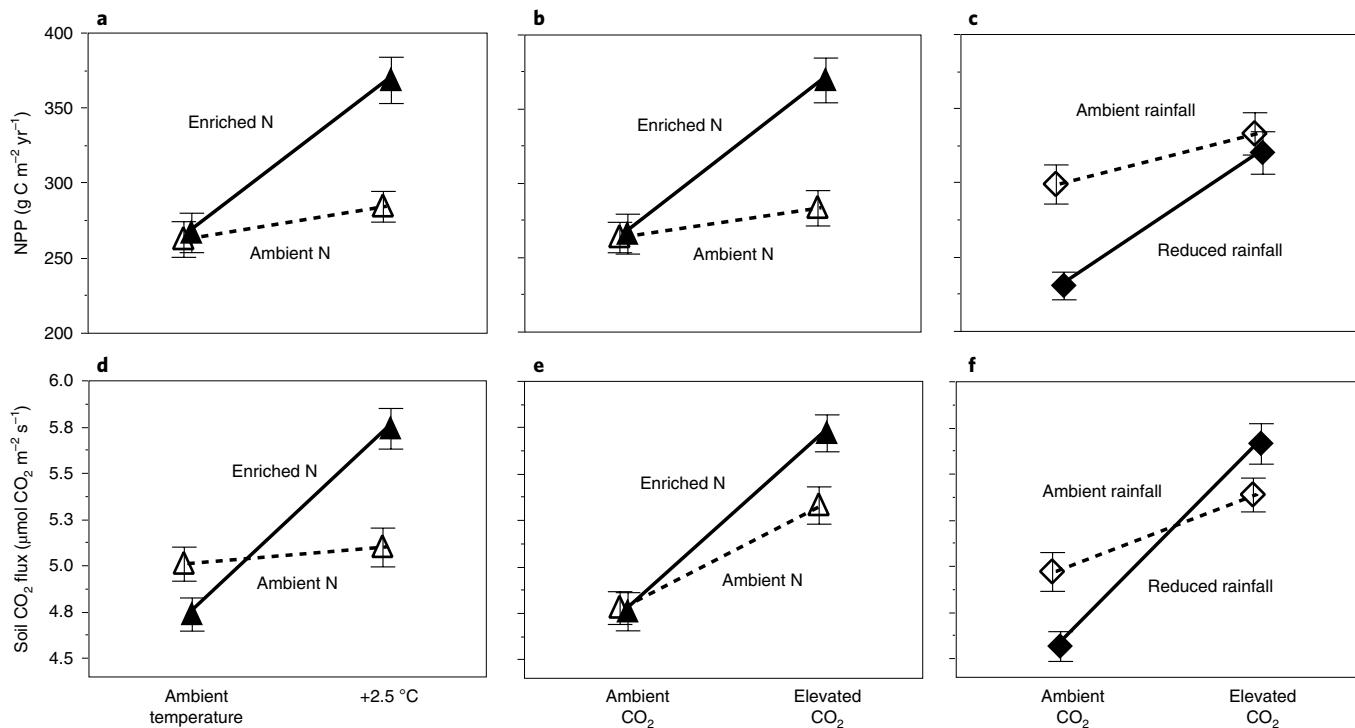


Fig. 4 | Mean NPP and soil CO₂ flux, averaged across all eight years, in relation to pairs of treatment factors. **a–f**, Synergistic interactions were consistent with the multiple limitation hypothesis (**a, b, d, e**) and antagonistic interactions were consistent with the CO₂-induced water-savings hypothesis (**c, f**). Error bars represent ± 1 standard error of the mean. See Tables 1 and 2 for statistical results.

significant ($P \leq 0.1$) interaction of *Andropogon* presence or absence with any combinations of the four treatments that were testable. Thus, plots with *Andropogon* did not respond differently to treatment than those without it. Similar results were found for the other ten most-abundant species, which had average relative abundances of between 9 and 19% in plots in which each was planted (as each species was on average in 27 of 48 plots, the average relative abundance across species adds up to more than 100%). Finally, the composition of all the plots changed appreciably over time from 2012 to 2019 (Extended Data Fig. 1). The temporal changes in composition that occurred were not mirrored by any similar shifts over time in the treatment main effects on C cycling. Thus, it appears that relatively stable effects on C cycling occurred over the eight years of the treatments (for example, Fig. 1) and their interactions, despite the communities becoming continuously more C₄ grass and legume dominated and less C₃ dominated. Hence, the global change effects on the composition played a minor (if any) role in the responses we noted.

Implications for C cycling and efforts to model it

Models that simulate future ecosystem C cycling responses must make implicit or explicit assumptions about the relative impacts of and interactions among different global change drivers, despite a lack of general understanding. Even with repeated calls for better empirical tests of such interactions^{1,13,18,24} they remain rare. Shorter-term and less-complex experiments often report a lack of evidence for interactions¹³, which, if a general phenomenon, would greatly simplify C cycling models. However, reports of interactions often emerge from long-term, more-complex and ecologically realistic studies^{7,11,18,23}. The results presented here also show many interactions that involve treatment pairs, and three-way and four-way treatment combinations.

Several of the observed interactions have important implications for terrestrial C cycling. Smaller responses to either eCO₂ or warmer

temperatures when N was at ambient compared with an enriched N supply suggest that regions with considerable N deposition from pollution might see a greater, more positive response to rising CO₂ or higher temperatures; whereas the near-ubiquitous nutrient limitation in natural soil might constrain the capacity of plants, across much of the land surfaces on Earth, to acquire and use C in a warmer, higher CO₂ world^{18,30}. This natural nutrient limitation would dampen the ability of terrestrial ecosystems to slow climate change by accelerating C uptake and storage in a warmer, higher CO₂ world. In contrast, the dampening of the negative impacts of low rainfall by eCO₂ suggests that higher CO₂ could enhance the resistance of biomass production to drought.

Our results strongly suggest that the responses of different ecosystem types to multiple global change drivers will probably not be a simple additive sum of their effects in isolation. In fact, in our study ecosystem, the magnitude of impacts of global changes when they occurred jointly were profoundly different from predictions based on their effects in isolation. Whether this would be a general trend in other ecosystems is almost entirely unknown, given the extreme rarity of well-replicated long-term experiments that include multiple global change factors and a lack of observational approaches that can cleanly assess such interactions. Thus, there is still a great need for a better understanding not only of how and when interactions among global change drivers might occur, but also of the conditions under which they occur and of their net impact when combined¹³. Accelerating such a knowledge advancement is essential for developing meaningful projections of future C cycling feedbacks of multiple global changes.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of

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Methods

This work was conducted at the TeRaCON experiment within the Cedar Creek Ecosystem Science Reserve in East Bethel, Minnesota^{17,18,20}. The region has a continental climate with warm summers (long-term July average temperature of 22 °C) and cold winters (January average temperature of -11 °C), and a long-term mean annual precipitation of 660 mm (refs^{17,18}). TeRaCON was established as a subexperiment within the BioCON (biodiversity, CO₂ and nitrogen) experiment²⁰, which was set up on a sandy N-poor outwash soil (94.4% sand and 2.5% clay) in a secondary successional grassland²⁰. Within six FACE (free-air CO₂ enrichment) rings (three ambient and three elevated), a total of 371 plots (2 × 2 m) were established in 1997 with a distance of 20 cm between each other and separated by metal barriers below ground (30 cm deep)^{15,20}. TeRaCON is composed of 48 of these 371 plots chosen with a stratified random approach (to balance the treatments and locations among experimental blocks) from 64 plots within the greater BioCON experiment that were initially planted with nine perennial grassland species^{15,20}. The TeRaCON experiment is a complete factorial design with two temperature (ambient and about +2.5 °C soil and surface warming), two growing-season rainfall (ambient and about -30% April through September), two atmospheric CO₂ (ambient and +180 ppm) and two soil N (ambient and +4 g N m⁻² yr⁻¹) treatments. In 1997, plots were planted with 9 species randomly selected from a pool of 16 possible species that belonged to 4 herbaceous plant functional groups: C₃ grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata* and *Poa pratensis*), C₄ grasses (*A. gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium* and *Sorghastrum nutans*), non-leguminous forbs (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa* and *Solidago rigida*) and N-fixing legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis* and *Petalostemum villosum*).

Treatments were implemented for different time periods; we restricted this analysis to the eight years (2012–2019) in which all four treatments were imposed. CO₂-enrichment and N-fertilization treatments began in 1998. Half of the rings received ambient air and half received air enriched in CO₂ during daylight hours throughout the growing season^{17,18,20}. Within each ring, half of the plots received an N-fertilization treatment, applied as slow-release ammonium nitrate in three equal fractions over the growing season. This quantity roughly doubled the natural N availability in the system^{17,18,20}. The rainfall reduction treatment began in 2007 and was administered to half of the 48 plots that compose the TeRaCON experiment. Portable 2 × 2 m rainout shelters were used to intercept select rain events^{15,17}; intercepted rain was moved away from the plots via gutters. Rainout shelters were manually installed only just before and during rain events to minimize their influence on the plots. The goal of the rainfall treatment was to intercept select rain events during the middle period of the growing season to achieve a growing season rainfall reduction of ~30%. Across the years, the realized rainfall removals averaged 44% (a reduction of 16 cm) over the 1 May to 10 August period, which corresponds to a 28% reduction of rainfall over the 1 April to 30 September growing season. The warming treatment began in 2012 and was also administered to half of the 48 plots that compose the TeRaCON experiment. Heating was applied prior to, during and after the growing season (~7 months per year), but not in winter. For both above- and below-ground warming, the power to each plot was controlled with an integrated microprocessor-based feedback control to provide a fixed temperature difference both above and below ground¹⁶. Above ground, four 1,000 W heaters (240 V, 245 mm × 60 mm; FTE-1000, Mor Electric Heating Assoc., Inc.) warmed each plot. Below ground, soil warming to a 1 m depth was accomplished through a hybridization of buried heating cable systems and deep soil-heating techniques^{15,16}. This technique used 85 cm vertical warming 'pins' made from resistance cable and inserted at 3 per m² to achieve a temperature increase of the entire soil profile with minimal site disturbance and low energy requirements. Soil warming was controlled via in-ground thermocouples at a 10 cm depth positioned immediately adjacent to the warming pins. The realized mean increases in temperature imposed by the warming treatment compared with the ambient temperature for May through September were 2.33 ± 0.06 °C above ground and 2.61 ± 0.03 °C below ground to 1 m for 2012–2018. Preliminary data for 2019 show similar warming treatments. The average of the above-ground and below-ground warming was +2.47 ± 0.06 °C above ambient, near the +2.5 °C target, although above ground and below-ground means were slightly further from the target.

The above-ground biomass in each plot was sampled annually in early August, with a 10 × 100 cm clipping^{17,18,20}. Peak above-ground biomass in perennial grasslands is considered a reasonable proxy for annual above-ground net primary production. We also estimated the annual root production at a 0–20 cm depth using in-growth root cores²¹. We combined the above-ground biomass and the in-growth root biomass as our estimate of net primary production for every plot in every year. We also estimated the total plant biomass pools by combining the above-ground data described above with measurements of the below-ground standing biomass (independent of the in-growth root-core measurements), derived from three 5 cm diameter (0–100 cm deep) cores per plot. We also measured the %C of the roots and above-ground biomass in every plot in every year from 2012 to 2018 (ECS 4010 CHNSO Analyzer, Costech Analytical Technologies Inc or NA 1500 CNS Analyzer, Carlo-Erba Instruments) and used these data to express biomass and productivity from 2012 to 2019 on a C rather than total biomass basis (total plant C pool and NPP, respectively). In 2016, we sampled soils in the

0–10, 10–20, 20–40 and 40–60 cm horizons in each plot. We used measurements of soil bulk density and %C to estimate the total soil C pool from 0 to 60 cm depth. We combined the 2016 soil C pool data with the mean 2012–2019 total plant C pool data to estimate the total ecosystem C pools. In every plot, we measured the soil CO₂ flux approximately 12–15 times per year between early May and late September using a LI-COR 6400 gas exchange system with a LI-COR 6400-09 soil respiration chamber (LI-COR)^{19,21}. To best match the time period during which all the treatments were imposed, here we used soil CO₂ flux data from 1 May to 31 July; moreover, we averaged these data to derive a single mean soil CO₂ flux value for each plot each year.

The total plant C pools, NPP and soil CO₂ flux data were log₁₀-transformed prior to statistical analysis to normalize the distribution and improve the residual patterns. Effect sizes (as percentage change) are shown based on the absolute (untransformed) values (effect sizes are very similar if transformed values are used and back transformed prior to making such calculations). In the analyses of the treatment effects and interactions on NPP, plant C pools and soil CO₂ flux, we used a repeated measures mixed model (with both fixed and random effects) that deploys a maximum likelihood approach (JMP Pro 14). Year was a continuous variable, whereas treatments were nominal variables; ring and plot were included as random effects. For soil and total ecosystem C pools, we used a plot average (across the experiment), so similar models but without year.

Our statistical approach generally mirrored that of other prior publications from this experiment^{17,18,20}. We did, however, directly test two distinct aspects of temporal variation; whether the effect of treatments changed directionally over time, and whether responses in any given year differed from those in any and every other year, irrespective of their temporal relationship. We ran the models both ways—tests of main effects and their interactions were almost identical, and interactions with time also differed very modestly. Given that Akaike information criterion and Bayesian information criterion values were considerably lower (and thus preferred) for the model using year as the continuous variable, and because we were more interested in directional changes over time than in pure year-to-year variation, we used year as continuous in all of the analyses shown in this article. The model used to generate the results in Table 2 was a standard least squares personality with REML (maximum likelihood method) and unbounded variance components in JMP Pro 14.2.0.

Finally, we placed our study within the experimental and methods context. Of the 1,119 experiments assessed in a recent meta-analysis¹³, only three factorial experiments of more than one year included either three or four of the following manipulations: altered growing season precipitation, eCO₂, increased growing season daytime temperatures by >1 °C and enriched N supply. The most comparable experiment to TeRaCON is the long-running Jasper Ridge annual grassland study in California with a similar four-factor design¹. The two other studies that met the above multifactor criteria were open-top chamber studies in grasslands. One, by Song et al.¹⁴ manipulated four factors for six years, which included night-time-only passive warming of 0.7 °C; as there was no daytime warming, theirs was a more useful test of the three-way interactions among the other three factors. The other study⁹ examined CO₂ × warming × precipitation for four years. Several other three-factor experiments ran for more than a year, but cannot be used to test growing-season global change interactions, as they either did not use a factorial design³¹, were only warmed at night (and at <1 °C even then)³² or warmed minimally (<1 °C), or were manipulated winter rather than growing season precipitation³³. Experiments that manipulated two factors (for example, CO₂ × warming, CO₂ × water, CO₂ × warming and CO₂ × N) are more common, but those done in open air for more than two years are quite rare (for example, PHACE, CO₂ × above ground warming, seven years⁷ and TasFACE, CO₂ × above-ground warming, ten years³⁴). Thus, our eight-year multifactorial grassland experiment represents a novel test of multiple global change interaction theory¹.

Data availability

All the data used in this article are available through the Environmental Data Initiative data repository at <https://go.nature.com/3843kVz> and <https://go.nature.com/38f8s9c>.

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Author contributions

P.B.R. designed the study with assistance from S.E.H., T.D.L. and R.R., and supervised the overall experiment and measurements. R.R. designed the warming facility. R.R. and K.W. conducted the warming manipulations. K.W. supervised the plant and ecosystem

measurements and the rainfall treatments. M.A.P. conducted the soil C analyses. P.B.R. analysed the data, with assistance from the other authors. P.B.R. wrote the first draft; all the authors jointly revised the manuscript.

Competing interests

The authors declare no competing interests.

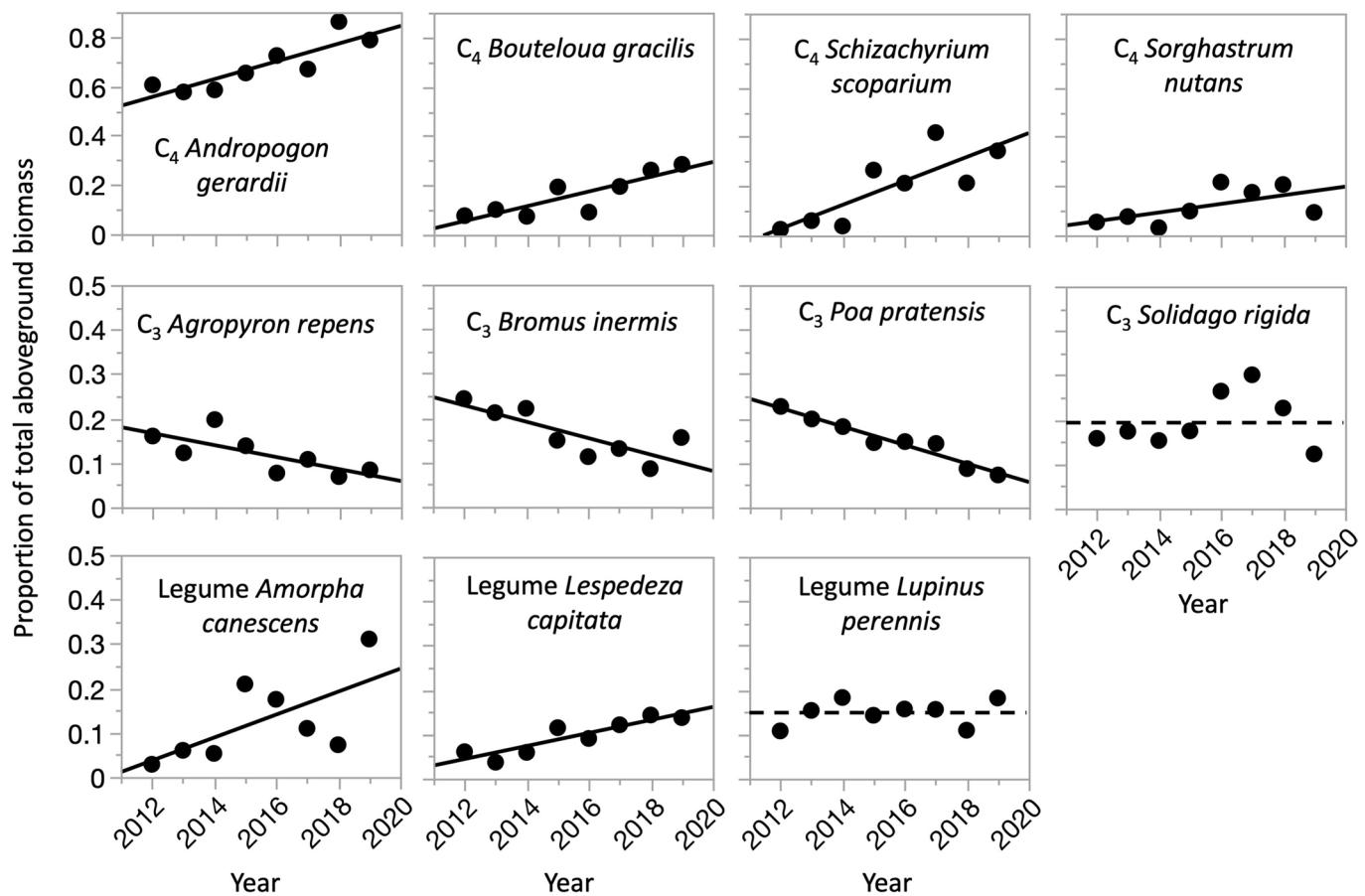
Additional information

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Extended Data Fig. 1 | Proportion of total aboveground biomass across all treatments for every year for each of the most dominant 11 species.

Species were planted on average in 9/16th of all plots. Solid lines show linear change over time for 9 species ($0.11 < P < 0.0001$; $0.38 < R^2 < 0.94$); dashed lines show mean values for two species with no evidence of consistent temporal change ($0.5 < P$; $R^2 < 0.07$). Note that the proportion axes differ for the C₄ grasses versus all of the other species.