the recovery of the native enzymatically active tetramer (*6*). Alternatively, interaction with the individual chaperones Hsp90, Hsc70, Hsp70, or Cyp-40 does not lead to refolding of the denatured substrate to its native state, but rather leads to an apparent collapse of the denatured β-Gal to a stable proteolysis-resistant nonnative intermediate that is subsequently responsive to the refolding activity of Hsp70 and Hdj-1. We suggest that the interaction between p23 and denatured β-Gal represents a distinct activity that results in the maintenance of the β-Gal in a proteolysis-sensitive, yet soluble, nonnative state that can be converted to the native state upon addition of Hsp70 and Hdj-1. These studies identify new members of the family of proteins that act as molecular chaperones. The involvement of multiple proteins with apparently redundant chaperone activities in heteromeric complexes may provide diversity and specificity in the regulation of the biological activity of associated protein substrates. This may have implications for pathways of hormonal regulation, signal transduction, and immunosuppression (*11*).

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8. The substrate (β-Gal) was denatured at 30°C for 30 min in 6 M guanidinium hydrochloride, diluted 1:125 into refolding buffer [25 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM adenosine triphosphate] to a final concentration of 3.4 nM β-Gal, and supplemented with BSA, Hsp90, Hsp70, p60, Cyp-40, Hdj-1, or p23, or various combinations of chaperones. The refolding reactions were incubated at 37°C for 240 min and aliquots (10 μl) were taken at various time points. The percent enzyme activity was determined after incubation with orthonitrophenyl galactoside and measurement of absorbance at 412 nm. The results are presented as percent enzyme activity relative to the same amount of native β-Gal (3.4 nM) in refolding buffer containing BSA (1.6 μM). The molecular chaperones Hsp90, Hsp70, and Hdj-1 were purified to homogeneity as described (*6*). Cyp-40 [L. J. Kieffer *et al.*, *J. Biol. Chem.* **268**, 12303 (1993)] was purified with a Resource Q column and used as a glutathione-S-transferase (GST) fusion protein. The p60 [D. F. Smith *et al.*, *Mol. Cell. Biol.* **13**, 869 (1993)] and p23 [J. L. Johnson, T. G. Bieto, C. J. Krco, D. O. Toft, *ibid.* **14**, 1956 (1994)] proteins were recombinantly expressed and purified by sequential passages over DEAE, Resource Q, and Superdex-200 columns (Pharmacia Biotech).
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10. The folded state of denatured β-Gal diluted into BSA, Hsc70, p60, Cyp-40, Hdj-1, or p23 was characterized by electrophoresis on native 4% polyacrylamide Na-borate (0.1 M sodium acetate and 0.1 M boric acid) gels at 4°C at 1 mm/min (*6*) or by separation of the soluble and insoluble fractions of β-Gal by centrifugation at 13,000 rpm for 5 min and separation with 10% SDS-PAGE. The β-Gal was detected by protein immunoblot analysis after electroblotting onto nitrocellulose, incubation with an antibody to β-Gal (anti–β-Gal), and detection by ECL (Amersham).
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Influence of Nitrogen Loading and Species Composition on the Carbon Balance of Grasslands

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In a 12-year experimental study of nitrogen (N) deposition on Minnesota grasslands, plots dominated by native warm-season grasses shifted to low-diversity mixtures dominated by cool-season grasses at all but the lowest N addition rates. This shift was associated with decreased biomass carbon (C):N ratios, increased N mineralization, increased soil nitrate, high N losses, and low C storage. In addition, plots originally dominated by nonnative cool-season grasses retained little added N and stored little C, even at low N input rates. Thus, grasslands with high N retention and C storage rates were the most vulnerable to species losses and major shifts in C and N cycling.

Humans have dramatically altered the cycling of nitrogen on Earth, doubling the natural rate of N fixation and causing atmospheric N deposition rates to increase more than tenfold over the last 40 years to current values of 0.5 to 2.5 g N m^{-2} year^{-1} in eastern North America and 0.5 to 6.0 g N m^{-2} year^{-1} in northern Europe (*1*). Because N is the primary nutrient limiting terrestrial plant production, N addition is causing shifts in plant species composition, decreases in species diversity, and changes in food-web structure in terrestrial ecosystems (*2–5*). This N-driven terrestrial eutrophication parallels phosphorus-driven eutrophication in lakes. Increased N deposition may lead to greater C storage in soil organic matter and vegetation, thus providing a sink for CO_2 and potentially explaining the globally "missing C" (*6*). Despite this, almost no experimental data exist on changes in ecosystem C in response to long-term N addition in nonagricultural ecosystems; rather, effects on C stores have been estimated from models, giving divergent predictions (*6*).

We present results of 12 years of experimental N addition to 162 grassland plots in three N-limited Minnesota grasslands that varied in successional age, total soil C, and plant species composition (*7, 8*). The youngest field (Field A) was dominated by vegetation with the C_3 photosynthetic pathway, primarily nonnative "cool-season" grasses and forbs, whereas the two older fields (Fields B and C) were dominated by native C_4 "warm-season" prairie grasses. Because other potentially limiting nutrients were supplied and soil pH was controlled, our study addresses the eutrophication effects of N loading while controlling for acidification and related biogeochemical effects that might also affect natural ecosystems (*9, 10*).

Nitrogen loading dramatically changed plant species composition, decreased species diversity, and increased aboveground productivity in these plots (*2, 7, 11*). After 12

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years of N addition, species richness declined by more than 50% across the N addition gradient (Fig. 1A), with the greatest losses at 1 to 5 g N m^{-2} $year^{-1}$—levels spanning current atmospheric deposition rates in eastern North America and northern Europe (*1*). This loss of diversity was accompanied by major shifts in composition, with C_4 grasses (predominantly the native bunchgrass *Schizachyrium scoparium*) declining and the weedy Eurasian C_3 grass *Agropyron repens* becoming dominant at high N addition rates (Fig. 1B) (*2, 7, 11*).

As the vegetation shifted with increasing N inputs from C_4 species to C_3 species, the C:N ratios of aboveground and belowground plant tissues decreased (Fig. 1, C and D) (*12*). Two analyses indicate that interspecific differences in tissue chemistry together with the observed species shifts can account for most of this shift in biomass C:N ratios across the experimental N gradient. First, nitrogen-use efficiency (NUE), the ratio of plant production to N use [estimated following (*13*)], averaged 203 across the N addition gradient for *S. scoparium* (*14*). The high NUE of *S. scoparium* and other perennial C_4 grasses is well documented (*15, 16*). In contrast, *Poa pratensis* and *A. repens*, the dominant C_3 grasses, had mean NUE values of 107 and 78, respectively. Intraspecific plasticity for NUE—the shift in tissue chemistry within species across the N addition gradient—was small relative to the large interspecific differences among the three species (*14*). In addition, multiple regression showed that the best correlate, after the rate of N fertilization, for the C:N ratio of dead biomass in a plot was the *S. scoparium* abundance in the plot (*17*).

At N addition rates of <5 g N m^{-2} $year^{-1}$, soil NO_3^- concentrations were significantly lower in the older fields dominated by *S. scoparium* (Fields B and C) than in the C_3-dominated youngest field (Field A) (Fig. 2A) (*18, 19*). This parallels results from experimental monocultures of these prairie and old-field grasses (*20*). Soil NO_3^- did not respond significantly to N addition at rates <5 g N m^{-2} $year^{-1}$ (*19*), but NO_3^- concentrations increased by a factor of ten at higher N addition rates (Fig. 2A). With the exception of two treatments in Field B, annual net N mineralization rates also showed relatively little change at low N addition rates, but increased linearly with increased N addition at rates >5 g N m^{-2} $year^{-1}$ (Fig. 2B).

At low N addition rates (1 to 2 g N m^{-2} $year^{-1}$), the two C_4-dominated fields retained approximately all of the N inputs after 12 years (Fig. 2C) (*21*). Nitrogen retention in these fields dropped as N addition increased, converging on an N retention of 35% of N inputs at the two highest N addition rates. Similar results are reported for N-loading studies in European forests, where, on average, 43% of N inputs were retained at N inputs ranging from 2.5 to 7.5 g N m^{-2} $year^{-1}$ (*22*). However, N retention varied greatly from site to site in those studies, supporting the conclusion of Aber *et al.* (*23*) that "N retention will vary non-linearly depending on the internal state of the system." In contrast to the two older fields, the C_3-dominated Field A retained essentially none of the added N at low input rates (Fig. 2C) (*24*). Although the mechanisms of N loss in Field A are unresolved, our grassland result contrasts with that of forest research, where early successional stands are hypothesized to have higher nutrient retention (*25, 26*).

On a plot-by-plot basis, net N losses (as g N m^{-2}) (*21*) were highly correlated with the average growing-season concentration of soil NO_3^- (Fig. 3A). Soil NO_3^- is highly mobile, and high soil NO_3^- concentrations frequently lead to large leaching losses of N, as presumably happened in this study (*10, 27*). We cannot partition N losses, however, because N leaching, ammonia volatilization, dissolved organic N losses, and denitrification were not measured (*28*).

Soil NO_3^- concentrations were highly correlated with biomass C:N ratios (Fig. 3B) (*29*). A comparable relationship existed between soil NO_3^- and the C:N ratio of either belowground biomass or aboveground litter. At biomass C:N ratios greater than 30, soil NO_3^- concentrations were low (<1 mg/kg). As C:N ratios dropped below 30, the immobilization sink for mineral N provided by dead organic matter disappeared, rates of net N mineralization increased, soil NO_3^- increased sharply, and overall N retention rates decreased (Fig. 2). Thus, our results

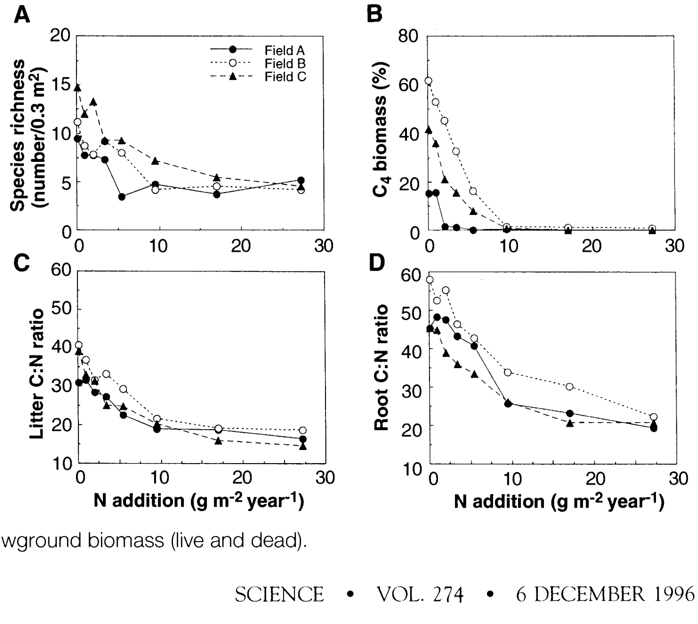

Fig. 1. Vegetation responses to 12 years of N addition. Points represent treatment means (6 replicates per N addition level, 12 for controls) for each of three fields. (**A**) Number of vascular plant species found in 0.3-m^2 vegetation samples. (**B**) Biomass of grasses with the C_4 photosynthetic pathway as a proportion of aboveground live biomass at mid–growing season. One species, *S. scoparium*, contributed >95% of the C_4 biomass in the plots. Biomass C:N ratios for (**C**) aboveground dead biomass (both recent and old) and (**D**) belowground biomass (live and dead).

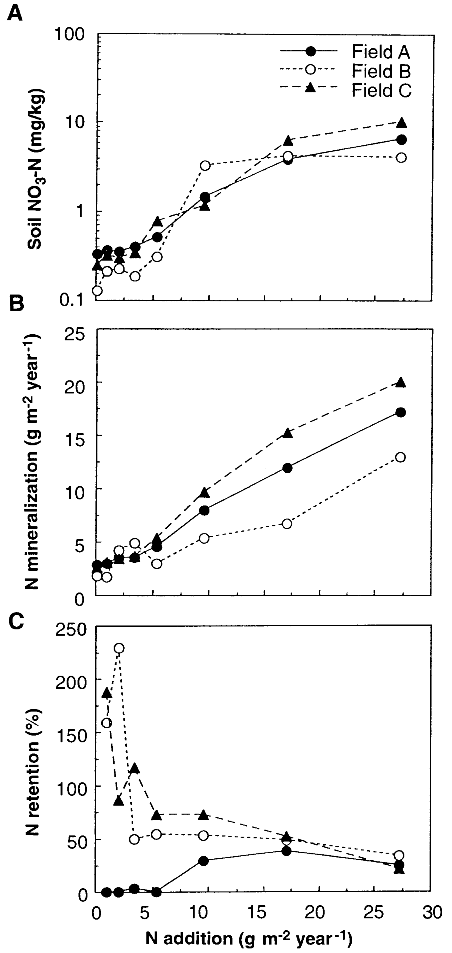

Fig. 2. Nitrogen dynamics after 12 years of N addition. (**A**) Seasonal average extractable soil NO_3^- concentrations, (**B**) annual in situ net N mineralization, and (**C**) net N retention after 12 years estimated as the change in total system N (relative to controls) divided by the sum of experimental N additions.

support the conclusion that microbial immobilization of mineral N is a major factor regulating N retention (*10, 23, 25, 30*).

Our analyses indicate another potentially important factor regulating soil NO_3^- pools in these grasslands. Plant species diversity remained a significant negative correlate of soil NO_3^- in a multiple regression model that accounted for the effects of litter C:N ratio and N addition rate (*29, 31*). This suggests that complementary spatial and temporal patterns of nutrient uptake associated with high plant-species diversity or functional group diversity also play a significant role in ecosystem N retention (*32*).

We conclude that the shift from N immobilization to mineralization, a threshold determined by microbial resource requirements and the C:N ratio of an ecosystem's detrital biomass, creates an inherent nonlinearity in the response of these grasslands to chronic N loading. In our study, species shifts in the vegetation at low levels of N loading appear to be driving such a nonlinear response of the N cycle (*4, 15*). In addition to shifts in species composition, the loss of diversity, per se, during eutrophication may contribute to decreased N retention in grassland ecosystems subjected to atmospheric N deposition (*31*).

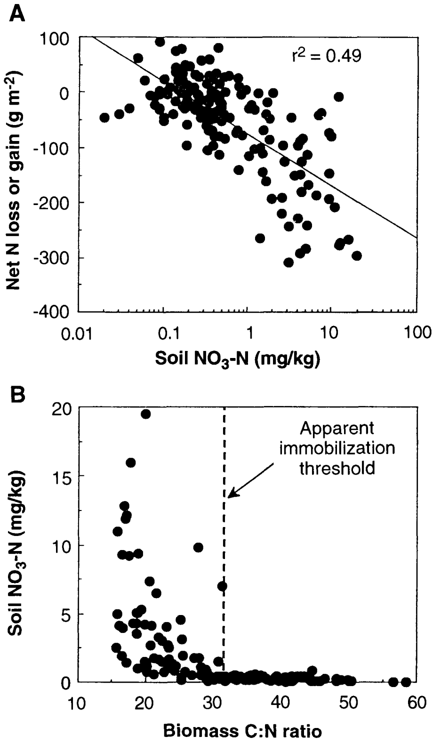

Fig. 3. (**A**) The relationship between net N losses or gains (the change in total system N minus the sum of experimental N additions) and seasonal average soil NO_3^- concentrations in 162 experimental plots. The equation for the fitted curve (note log scale) is $N_{lost} = (-75.56) - [94.89 \times \log(NO_3)]$. (**B**) The relationship between soil NO_3^- and the C:N ratio of plant biomass (aboveground dead biomass plus belowground biomass). Vertical line represents a biomass C:N ratio of 32.

Two patterns emerged for the net change in total ecosystem C stores after 12 years (*12, 21*). First, although total C stores differed significantly among the three fields across the experimental N gradient, differences were greater at the low end of the gradient (*33*). At the high end of the N addition gradient, all fields were converging on total C stores of roughly 4000 to 5000 g C m^{-2}. Second, total C stores increased significantly at low N addition rates in the C_4-dominated fields (Fields B and C) but not in the C_3-dominated field. Averaging across the three lowest N addition levels (1, 2, and 3.4 g N m^{-2} $year^{-1}$), total ecosystem C increased 21% (545 g C m^{-2}) in Field B, which had lower soil C initially, 10% (445 g C m^{-2}) in Field C, and only 1% (27 g C m^{-2}) in Field A. In contrast, theoretical estimates of C storage for humid temperate grasslands in response to climate change, direct CO_2 enrichment, or both range from 3% to −3% (*34*). Carbon storage resulting from anthropogenic N inputs, although highly dependent on grassland type, may be markedly greater than C storage in response to other components of global change.

Finally, we determined the net long-term change in total ecosystem C per unit of added N over our 12-year study. In regression analysis, there was significantly lower C storage (g C/g N) at N addition rates <5 g N m^{-2} $year^{-1}$ for Field A than for Fields B and C, as well as a significant effect of N addition rate and a significant field-by-N-addition interaction (Fig. 4) (*35*). Without field as a categorical variable, plot C_4 biomass was the best single predictor of C storage (*35*).

At the lowest N addition rates (1 and 2 g N m^{-2} $year^{-1}$), the C storage rate averaged 24.3 g C/g N ($n = 24$, SE = 7.6) in Fields B and C (Fig. 4). Although we know of no comparable values from other long-term experiments, our value of 24.3 g C/g N is low compared to most model estimates of net C storage in response to atmospheric N deposition, which range from 17 to >100 g C/g N (*6*). This difference probably relates to ecosystem type. In our two C_4-dominated grasslands, 63% of the long-term C storage was in soils, which had a C:N ratio of roughly 11. Globally, woody vegetation with a higher C:N ratio becomes a more significant C sink.

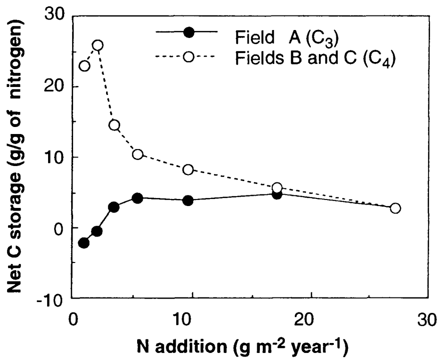

Fig. 4. Net C storage per unit experimentally added N after 12 years. Because C storage rates (g C/g N) did not differ significantly between Fields B and C (*34*), overall treatment means for the two C_4-dominated fields are presented.

Estimates of C storage in response to N loading are the product of two terms: net C storage per unit N retained and the N retention rate. In simulations with the CENTURY model of long-term C budgets for *S. scoparium* monocultures in our soils and climate, we found a long-term C storage rate of 22 g C/g N input from atmospheric deposition (*36*). Thus, our empirical and modeling estimates of C storage (g C/g N) were very similar for low N addition plots in Fields B and C, where N retention rates were ~100%.

In contrast, the model (*36*) did a relatively poor job of predicting C storage rates for Fields B and C at medium to high N inputs and Field A across the N gradient. CENTURY simulations predicted a long-term C storage rate of 10 g C/g N for *A. repens* monocultures, the dominant C_3 grass in Field A and in high-N plots. However, no net C storage was observed for Field A at low N inputs, and at the high end of the gradient, net long-term C storage across all fields converged on roughly 4 g C/g N (Fig. 4). These results underscore the need for a clearer understanding of why N retention rates differ among ecosystems if ecologists are to make reasonable estimates, whether on local or global scales, of C sequestration in response to N loading.

The grassland types best able to retain added N and sequester C were also the types most vulnerable to N eutrophication through losses of diversity, changes in plant species composition, and the resultant changes in C and N cycling. Thus, N-caused shifts in species composition limit the ability of temperate grasslands to serve as significant long-term C stores. In our fields dominated by C_4 prairie grasses, shifts in species composition at relatively low N addition rates led to decreased biomass C:N ratios and decreased N immobilization potential, and, consequently, increased soil NO_3^- concentrations, high N loss rates, and low C sequestration rates (g C/g N). The nonlinear or threshold-dependent response that we observed in response to chronic N loading appears to have two causes: species shifts in response to N eutrophication and an N mineralization or immobilization threshold for the decomposition of litter and soil organic matter. Our results show that N loading is a major threat to grassland ecosystems, causing loss of diversity, increased abundance of nonnative species, and the disruption of ecosystem functioning, and that these responses are tightly linked.

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8. Methods are described in detail (*7*). The experimental fields are at the Cedar Creek Natural History Area in east-central Minnesota (mean annual temperature = 5.5°C, mean annual precipitation = 726 mm). In 1993, field A had been abandoned from agriculture for 25 years, Field B for 36 years, and Field C for 59 years. Each field contained 54 plots of 4 m by 4 m. All plots were randomly assigned to one of nine treatments: no nutrient addition, addition of macro- and micronutrients other than N, or macro- and micronutrients plus one of seven N addition treatments ranging from 1 to 27 g N m^{-2} year^{-1} applied as NH_4NO_3 fertilizer in mid-May and mid-June each year. Crushed limestone was added to the plots as necessary to maintain constant pH. Because neither aboveground biomass nor species composition differed significantly between the two non-N treatments, we considered them as a single control treatment here.
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12. Methods used to measure total C and N in each plot: for soils, three cores (2 cm diameter) were divided into 0- to 15-cm, 15- to 30-cm, and 30- to 50-cm sections; composited; and sieved (1 mm); for belowground biomass, three cores (4.7 cm diameter) were divided into 0- to 15-cm and 15- to 30-cm sections, composited, handwashed over a 1-mm screen, and dried at 40°C; for aboveground biomass, a 0.3-m^2 area was clipped at the soil surface, sorted into litter and live biomass, the live biomass sorted by species, and dried (*7*). Belowground biomass contained both live and dead fractions. Portions of each sample (composites for rare grasses and forbs in each plot) were ground prior to tissue analysis. All soil and plant samples ($n = 1610$) were analyzed for total C and N with a Carlo-Erba NA1500. Samples were collected between 1 July and 15 August 1993.
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14. We estimated NUE (g biomass/g N) as the inverse of the N concentration of newly senesced aboveground tissue (*13*) for three dominant grasses (sampled in October 1994). In a general linear modeling (GLM) model predicting NUE, the species effect ($F = 137.81$, $P < 0.0001$) and the species-by-N-addition rate interaction ($F = 6.07$, $P = 0.0038$) were significant, whereas the N addition rate effect ($F = 0.438$, $P = 0.511$) was not significant ($r^2 = 0.95$, $n = 72$). The significant interaction was caused by *A. repens*, for which NUE decreased significantly at high N addition rates, whereas NUE for *S. scoparium* and *P. pratensis* did not.
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17. In a multiple regression model predicting the C:N ratio of dead biomass (midseason aboveground dead biomass, both recent and old, plus belowground biomass), the effect of N addition rate ($F = 167.46$, $P < 0.0001$), the proportional abundance of the C_4 grass *S. scoparium* (based on midseason live aboveground biomass, $F = 62.6$, $P < 0.0001$), and total soil N (g N m^{-2}, $F = 18.32$, $P < 0.0001$) were all significant ($r^2 = 0.791$, $n = 162$). All *F* values are partial *F*s, that is, corrected for the correlated effects of other terms in the model.
18. Monthly net N mineralization was measured with in situ incubations (polyvinyl chloride pipes 2.5 cm in diameter and 15 cm deep) from April to October 1993. Pre- and postincubation soil samples were extracted with 1 M KCl and analyzed for NH_4^+-N and NO_3^--N colorimetrically with an Alpkem autoanalyzer (*15*). Soil NO_3^- concentrations presented are means for each plot of preincubation concentrations from May to August ($n = 4$). To estimate total annual N supply (the sum of N mineralization, N fertilizer inputs, and atmospheric N deposition), we assumed annual atmospheric N deposition equaled 0.6 g N m^{-2} year^{-1}, the mean wet and dry deposition for 1985–94 measured at our site by the Minnesota Pollution Control Agency.
19. In a two-way analysis of variance testing the effects of field, the four low N-addition treatments (0, 1, 2, and 3.4 g N m^{-2} year^{-1}), and the field-by-N-addition interaction on the seasonal average soil NO_3^- concentration (ln transformed), the field effect was significant ($F = 11.93$, $P < 0.0001$), whereas the N addition ($F = 2.65$, $P = 0.051$) and field-by-N-addition interaction ($F = 0.723$, $P = 0.632$) were not. In a comparable analysis with the three medium N-addition treatments (3.4, 5.4, and 9.5 g N m^{-2} year^{-1}), the field effect was not significant ($F = 0.68$, $P = 0.51$), whereas the N addition ($F = 32.40$, $P < 0.0001$) and field-by-N-addition interaction ($F = 2.69$, $P = 0.043$) were.
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21. The net change in C and N pools after 12 years of N addition was calculated as the difference between total C or N stores (g m^{-2}) of each plot and the mean for the 12 control (no N addition) plots in that field. Although insufficient data existed to calculate initial C and N stores in each plot, analyses of total C and N in archived soils (the largest C and N pool) found no significant pretreatment difference between controls and N addition treatments. Nitrogen retention (as a percent) is the ratio of net change in total system N and the sum of experimental N additions over 12 years. Net N lost or gained (as g N m^{-2}) is the former term minus the latter. Because control and treatment plots both received atmospheric N inputs, these were not included in estimates of net change. At the lowest N addition rates, where changes in total C and N average <3% of total C and N pools, considerable variance exists in our estimates of net change.
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24. In GLM analyses predicting percent N retention (Fig. 2C) (*21*), the effect of N addition rate was not significant (ln transformed, $F = 0.671$, $P = 0.414$), whereas effects of field (categorical variable, $F = 18.97$, $P < 0.0001$) and the field-by-N-addition interaction ($F = 10.322$, $P < 0.0001$) were significant ($r^2 = 0.261$, $n = 126$). In a stepwise multiple regression model predicting N retention without field as a categorical effect, N addition rate was not significant ($F = 0.585$, $P = 0.446$), whereas the effects of C_4 aboveground biomass ($F = 20.85$, $P < 0.0001$), biomass C:N ratio (litter and roots, $F = 11.25$, $P = 0.001$), soil C:N ratio ($F = 8.93$, $P = 0.003$), soil NO_3^- (ln transformed, $F = 6.24$, $P = 0.014$), and the C_4-biomass-by-N-addition interaction ($F = 6.42$, $P = 0.013$) were significant ($n = 126$, $r^2 = 0.300$). All *F* values are partial *F*'s.
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28. In contrast to forests, where nitrification (the proportion of net N mineralization ending up as NO_3^-) often increases with increased N loading (*10, 23*), nitrification exceeded 90% in all treatments here. Because of the high nitrification rates, sandy texture, and consistently aerobic status of our soils, our assumption that N losses are dominated by NO_3^- leaching is reasonable.
29. In a multiple regression model predicting the seasonal average soil NO_3^- concentration (ln transformed), biomass C:N ratio (aboveground litter and roots; slope $= -0.0648$, partial $F = 59.7$, $P < 0.0001$), N supply rate (N addition plus net N mineralization; slope $= 0.0367$, partial $F = 33.78$, $P < 0.0001$), and plant species diversity (Shannon-Wiener index calculated from aboveground proportional biomass, slope $= -0.1701$, partial $F = 14.25$, $P = 0.0002$) were significant ($n = 162$, $r^2 = 0.768$). Root biomass, live biomass, root:shoot ratio, and soil C:N ratio were not significant correlates of soil NO_3^-.
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33. Total ecosystem stores (g C m^{-2}) in control plots differed significantly among fields ($F = 53.18$, $P < 0.0001$; means: Field A = 3639, Field B = 2537, Field C = 4619). Differences among fields were significant but smaller for the highest N addition treatment ($F = 6.59$, $P = 0.0088$; means: Field A = 4509, Field B = 3897, Field C = 5094).
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35. In GLM analyses predicting C storage per unit N input (Fig. 4), the effects of field (categorical variable, $F = 7.04$, $P = 0.0013$), N addition rate (ln transformed, $F = 6.99$, $P = 0.009$), and the field-by-N-addition interaction ($F = 3.48$, $P = 0.034$) were significant ($r^2 = 0.16$, $n = 126$). In a GLM model predicting C storage per unit N without the field effect, the effects of C_4 aboveground biomass ($F = 18.15$, $P < 0.0001$), soil C:N ratio ($F = 12.39$, $P = 0.0006$), and root biomass (ln transformed, $F = 11.13$, $P = 0.0011$) were significant ($r^2 = 0.332$, $n = 126$). All *F* values are partial *F*s.
36. CENTURY is a grassland simulation model of productivity and soil organic matter dynamics that has been used extensively and is described in [W. J. Parton, D. S. Schimel, C. V. Cole, D. S. Ojima, *Soil Sci. Soc. Am. J.* **51**, 1173 (1987); see also (*34*)]. The long-term monoculture simulations for *S. scoparium* and *A. repens* used species-level data on productivity, allocation, and litter quality from (*15, 20*).
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