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ORIGINAL ARTICLE

Chronic Nitrogen Additions Decrease Rates of N Recovery and Increase Rates of Soil Inorganic N Availability in A Temperate Grassland

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ABSTRACT

Anthropogenic activities add more reactive nitrogen (N) to the environment than all natural sources combined, and the fate of this N is of environmental concern. If N that is deposited on terrestrial ecosystems through atmospheric deposition is retained in plant tissues or soil organic matter, it could stimulate carbon (C) storage in plant biomass or soils. However, added N also could increase soil inorganic N concentrations and leaching, potentially polluting watersheds, particularly in areas with low-N soils and/or a high propensity for leaching, such as sandy or arid areas. Here, we assessed N allocation and retention across a 13-year experimental N addition gradient in a temperate grassland. We found that N accumulation decreased significantly at mid- to high levels of N addition compared to the Control, such that ecosystem N pools were equivalent across a 10 g m^{-2} $year^{-1}$ range of annual N addition rates (0–10 g N m^{-2} $year^{-1}$), which spans most of the global range of N deposition. Nitrogen addition increased plant tissue percent N, but the total pool of N did not increase because of reduced plant biomass, particularly in roots. Nitrogen addition also increased soil inorganic N concentrations. Our results indicate that N addition is unlikely to increase grassland N pools, particularly in sandy or low-fertility ecosystems with a high potential for leaching because high application rates lead to N saturation, and additional inputs are lost.

Key words: Nitrogen addition; Nitrogen accumulation; Nitrogen deposition; Soil carbon; Soil nitrogen; Nitrogen losses.

HIGHLIGHTS

- We experimentally added multiple rates of N for 13 years to assess N accumulation

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- We found similar rates of N accumulation regardless of N input rate
- Ecosystem N pools may not increase with N supply rate, especially in sandy soils

INTRODUCTION

Eutrophication via nitrogen (N) loading from human activities is putting more reactive N into terrestrial ecosystems than lightning and natural biological N fixation combined (Galloway and others 2008), leaving a fingerprint on the world's natural systems (for example, Stevens and others 2015). Nitrogen is a key element for growth in all organisms, and N addition often, but not always (Fay and others 2015), increases plant growth by reducing N limitation in terrestrial ecosystems (for example, LeBauer and Treseder 2008). Too much N addition, however, can decrease plant diversity and soil microbial activity (Shi and others 2016; Li and others 2017), promote soil acidification (Bobbink and others 2010), and increase N leaching to groundwater and runoff into surface waters (Sparks 2019). Given that N deposition is predicted to increase in many areas of the world, but is decreasing or unchanging in others (Jefferies and Maron 1997; Bebber 2021), understanding the fate of N in ecosystems is critical: If N addition promotes increased plant carbon uptake and growth (for example, growth-dilution, Jarrell and Beverly 1981), it could promote carbon (C) storage, particularly in moist soils (for example, Keller and others 2021). In areas where the potential for plant biomass response to N is constrained by low water availability (for example, Delgado-Baquerizo and others 2013; Wang and others 2014), N addition may lead to increased leaching and potentially groundwater pollution if plant and microbial N uptake are low but soil organic and inorganic N accumulation is high. Furthermore, the rate of N addition can shape C responses, including C uptake and emission, and biomass distribution (for example, Peng and others 2017a; Wilcots and others 2022), and may likely alter N retention as well. Specifically, in grasslands, which make up 30% of the earth's ice-free surface and store between 30 and 90% of C belowground in soil and roots (Scurlock and Hall 1998; Titlyanova and others 1999; Fan and others 2009), understanding the effect of added N on N retention versus N loss and associated effects on ecosystem C pools is important for predicting changes in global C stocks. Furthermore, investigating grassland C and N stocks on marginal grasslands, which are often the areas where grassland restoration takes place (for example, De and others 2020) could also improve estimates of the climate mitigation potential of grasslands.

There are multiple biotic and abiotic pathways through which N might increase or reduce ecosystem N pools in grasslands. Nitrogen addition may lead to N gains in plant pools via biomass gains (with no change in tissue N concentration) (Figure 1, circle 1) or increased tissue N concentration (Figure 1, circle 2), either of which alone or in combination would result in a larger plant N pool. Nitrogen-induced increases in biomass or in leaf tissue N concentration can also affect litter accumulation and decomposition (Figure 1, circle 7); increases in litter mass can reduce light levels and the richness of understory species (Eskelinen and others 2022), especially at relatively high rates of N addition. However, if foliar tissue N increases with increasing N addition (for example, Hao and others 2018; Prager and others 2020; Figure 1, circle 1), litter in high-N areas may become more easily digestible and initially decomposable, leading to increased rates of N mineralization.

Nitrogen addition can shift species composition such that community root:shoot ratios decrease (Figure 1, circles 4 and 10). Optimal allocation theory predicts that, as competition for belowground nutrients is relieved, competition will shift aboveground for light (Newman 1973; Dybzinski and Tilman 2007). Root:shoot ratios can also be sensitive to the experimental rate of N addition (Wilcots and others 2022), with lower root:shoot ratios (that is, lower belowground biomass or higher aboveground biomass) at high rates of N addition. However, if this shift occurs concurrently with a shift toward annual, fast-growing species, belowground plant N inputs would also shift from long-lived, low-N roots (Figure 1, circle 4) toward annual, high-N roots (Figure 1, circle 10; Hendricks and others 1993; Li and others 2015). Thus, shifting community composition and subsequent root turnover may influence soil N pools and fluxes.

Nitrogen retention is also strongly affected by soil processes. Nitrogen addition often slows later stages of microbial litter decomposition (Riggs and others 2015; Riggs and Hobbie 2016; Gill and others 2021, 2022) and decreases soil microbial biomass (Treseder 2008; Verma and Sagar 2020), which could lead to an increased or reduced, respectively, soil N pool (Figure 1, circle 3). Nitrogen addition, however, can increase leaching and/or gaseous N emissions if N supply exceeds plant and microbial demand (Chapin and others 1995; Perakis and Hedin 2002; Lovett and Goodale 2011; Figure 1,

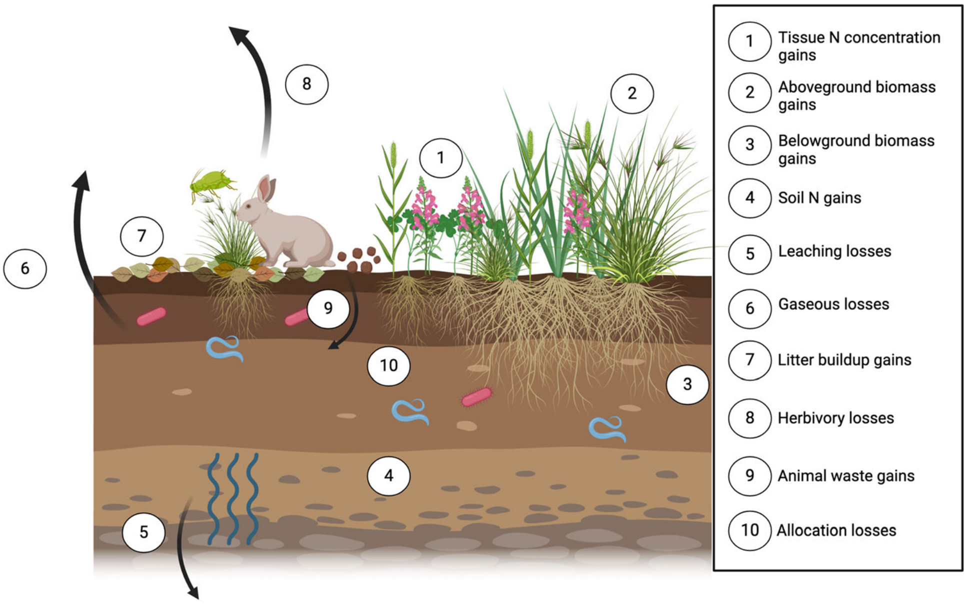

Figure 1. A conceptual figure showing how nitrogen addition may affect total ecosystem N pools through changes in above and belowground biomass, changes in herbivory, or changes in leaching and other pathways of N loss. (1) N-induced increase in tissue N concentration, possibly through changes in species composition; (2) N-induced increase in aboveground biomass; (3) N-induced increase in belowground biomass, via changes in species composition; (4) increases in soil N pool; (5) losses via leaching; (6) losses via denitrification; (7) increase in N via litter buildup; (8) losses via herbivory; (9) N additions via urine and excrement from herbivores; and (10) N-induced decrease in belowground biomass via changes in species composition, or increase in root N

arrow 5), particularly in sandy soils (Cameron and others 2013), and can increase nitrate (NO_3^-) leaching via decreases in root biomass (Scherer-Lorenzen and others 2003) or changes in species diversity (Tilman and others 1996, 1997). Enhanced denitrification (Figure 1, arrow 6) may also play a role in N loss from N-enriched ecosystems (Woodmansee 1978; Phoenix and others 2003), albeit a smaller role in ecosystems with well-drained soils (Cameron and others 2013).

Furthermore, N addition may lead to N losses via herbivory and disease (Figure 1, arrows 8 and 9). Large mammals can reduce total plant pools of N, P, and K where water is limited, but can have little impact on aboveground nutrient pools in areas where water is not growth-limiting (Anderson and others 2018). Nitrogen addition can increase both arthropod abundance and size (Lind and others 2017) and can induce increased rates of pathogen and invertebrate damage (Ebeling and others 2021), with herbivory and/or damage increasing as a function of plant biomass (Borer and others 2020). However, both mammalian and insect herbivores can increase ecosystem N inputs via urine and dung, if consumption occurs outside of ecosystem boundaries and N is transferred into the system via animal movement (Frost and Hunter 2007; Cameron and others 2013; Hobbie and Villéger 2015; Le Roux and others 2020), or, conversely, increase ecosystem N losses, through consumption and subsequent movement out of the system. Alternatively, increased herbivory may simply lead to faster rates of N cycling if there is no net animal movement in or out of the ecosystem (for example, Belovsky and Slade 2000; Figure 1, arrow 5).

Crucially, though, these processes and mechanisms may depend on the rate at which N is supplied. If, for example, low rates of N addition do not shift plant community composition toward species with N-rich tissues (for example, Wilcots and others 2021), the plant N pool might grow via increased biomass (Figure 1, circle 2) rather than via more N-rich tissues, with negligible effects on litter N concentration. On the other hand, if the plant N pool grows via increased tissue N (Figure 1, circle 1), litter tissue N would also increase and leaching may become a prevalent loss pathway at higher N supply rates (Chapin and others 1995; Figure 1, arrow 5). Understanding the rate of N addition at which N losses begin to outweigh gains will be crucial for future ecosystem management as N deposition continues to increase in many regions, with potential impacts on biodiversity, carbon storage, and groundwater contamination.

Though the pools and fluxes of N in grasslands have been well-documented (Woodmansee 1978) and the impacts of N on individual pathways of N loss and gain have been well-studied, integrating all loss and gain pathways together can provide a more holistic picture of how N addition may affect the fates of N in grasslands and thus the community and ecosystem consequences of enriched N (for example, Lovett and Goodale 2011). As N deposition is an increasing source of N to ecosystems in many parts of the world, understanding the effect of chronic N addition is crucial for estimating N losses and gains, as well as the critical load of N beyond which ecosystem function is impacted (Jefferies and Maron 1997; Lovett and Goodale 2011). Assessing the impact of N at multiple rates will thus be crucial for predicting how N deposition might impact both N and C stocks. If added N primarily ends up in biomass, it can promote C storage through C:N stoichiometry if plants allocate N to building new structures (for example, Shaver and others 1992), and thus, grassland areas with higher rates of N addition could potentially increase their C stock and help mitigate rising atmospheric CO_2 levels. However, if added N remains as inorganic N in the soil, it could increase leaching, leading to soil acidification and potentially groundwater pollution, or to increased N fluxes to the atmosphere, particularly in mesic environments (for example, Zhang and others 2023).

In light of recent theoretical (Bebber 2021) and empirical (Peng and others 2017a, b; Wilcots and others 2022) work that found nonlinear responses of plant biomass to increasing N addition rates, it is necessary to also assess how N gains and losses change across multiple rates of N supply. Here, we used data from a 13-year N addition experiment to ask how N allocation and retention change across a gradient of N addition. After 13 years of N addition, we quantified N stocks (N pools in plant aboveground biomass, root biomass, plant litter, and soils) and potential N loss pathways (large mammalian and insect herbivory, gaseous N losses, and potential for leaching of soil NO_3^- and NH_4^+) to understand how a gradient of N addition rates similar to N deposition might affect N loading in grasslands. We hypothesized that total N stocks would increase with increasing N addition, and that the trend would be driven both by increased pool size (for example, increasing plant biomass) and increased tissue N concentration. Finally, we hypothesized that N losses (herbivory, gaseous, and leaching) would increase with N addition rate, especially as other limitations, such as water limitation, increase.

MATERIALS AND METHODS

Experimental Design

We collected data from a long-term (13 years) nitrogen and nutrient addition and herbivore exclusion experiment that was established in 2007 in an old field, 57 years after abandonment from agriculture at Cedar Creek Ecosystem Science Reserve (CCESR) in East Bethel, MN, USA (45.4020°N, 93.1994°W, mean annual temperature = 6 °C, mean annual precipitation = 750 mm). CCESR is located on the Anoka Sand Plain, characterized by sandy soils ($> 88\%$ sand) with low nutrient content and negligible carbonates (Grigal and others 1974). For this study, we collected data from two sub-experiments in the same location. The first experiment (E1) is a randomized block design composed of five replicate blocks of four 25-m^2 plots each with treatments as follows: ambient N deposition with 0 (Control), + 1 (N1), + 5 (N5), and + 10 (N10) g N m^{-2} $year^{-1}$ above ambient N deposition. Nitrogen was added to the plots as time-released urea (CH_4N_2O), which has been found to be an effective mimic of N deposition (Jia 2020), in spite of the majority of N deposition being composed of NHx and NOy compounds (Bebber 2021). Fertilizer was added at the end of each growing season. Background N deposition at this site is approximately 0.9 g N m^{-2} $year^{-1}$ (equivalent to 9 kg N ha^{-1} $year^{-1}$; Ackerman and others 2019). The second experiment (E2) is a randomized block design composed of five replicate blocks of four 25-m^2 plots each with treatments as follows: Control, Fertilization (NPK), Herbivore Exclusion (Fence), and Fertilization + Herbivore Exclusion (NPK + Fence). The Control plots from E1 and E2 are the same. The NPK and NPK + Fence treatments received 10 g N, 10 g phosphorus (P; as triple super phosphate), 10 g potassium (K; as potassium sulfate) added per m^2 annually, and a single addition of 100 g m^{-2} mixture of common micronutrients that was added in Year 1 only (see Borer and others 2014). The Fence and NPK + Fence treatments excluded large herbivores (for example, white-tailed deer) using a 2.3 m tall fence with a 1 cm mesh size up to 90 cm and 3 strands of barbless wire above this, equally spaced up to 2 m (see Borer and others 2013). To estimate site-level herbivory while avoiding confounding effects of continued herbivore exclusion (for example, changes in species composition and litter accumulation), we compared data from the fenced and unfenced plots in Year 1 of the experiment.

Biomass Measurements

Aboveground biomass and litter were harvested in two 10 cm × 100 cm strips per plot around peak biomass in mid-August from 2007 to 2020. Biomass from the two strips was pooled, sorted into species groups, dried to a constant mass at 60 °C, weighed to the nearest 0.01 g, and scaled to g m^{-2}. The locations of the clipped strips were moved every year to adjacent, but previously unsampled, locations. Root biomass was harvested in E1 in late July 2020 only using two 5 cm diameter × 20 cm depth soil cores per plot. The top 20 cm of soil contains over 80% of root biomass at this site (Knops and Bradley 2009). To estimate belowground net primary productivity (BNPP), we removed two 5 cm diameter × 20 cm soil cores from each plot in E1 at the beginning of the growing season, and a 1 cm mesh core was placed in the hole. The soil removed from the plot was immediately sieved at 2 mm to remove roots, and the sieved soil was placed back into the mesh core in the plot. Cores were placed on May 18, 2020, after the soil had completely thawed and removed on September 13, 2020, prior to the first autumn frost. For both root biomass and BNPP, soil was washed off the roots immediately after harvest, and root biomass was dried to a constant mass and weighed to the nearest 0.01 g. Biomass was pooled between cores and then scaled on an aerial basis to g m^{-2} to compare it directly to aboveground biomass. We estimated root turnover by dividing BNPP by total root biomass.

Insect and Disease Damage Measurements

Disease and insect damage severity were assessed as the percent of leaf area visibly damaged by fungus or insects (Mitchell and others 2002; Ebeling and others 2021). In July 2019, we randomly selected ten tillers per species (*Andropogon gerardii* or *Elymus repens*) per plot. Damage data were collected within 2 weeks in a randomized order between plots to minimize rust spread between plots.

Nutrient Content Measurements

In 2020 only, we measured tissue C and N content in E1 in the three main functional groups present at our site: perennial C_4 and C_3 grasses, and non-leguminous forbs (legume forbs were not significantly represented at the site). We pooled dried biomass for each functional group, ground the tissue samples, and analyzed them for C and N using dry combustion on an ESC 4010 Elemental Analyzer (Costech, Valencia, CA, USA). Similarly, litter biomass and root biomass for E1 were ground and analyzed on an Elemental Analyzer for C and N content.

Soil Chemistry, Temperature, Moisture, and Climatic Variables

To assess the effect of N addition on soil chemistry, we collected 20 cm deep soil cores in each plot in E1 in late July 2020. Soil was sieved to 2 mm to remove roots, air dried, ground, and weighed before being analyzed for C and N by dry combustion with an ESC 4010 Elemental Analyzer (Costech, Valencia, CA, USA). Soil bulk density was estimated using previously collected data from the same experiment (that is, Keller and others 2021). To assess soil inorganic N content (ammonium; NH_4^+, and nitrate; NO_3^-) (as an indicator of potential for inorganic N leaching losses), we buried ion resin exchange beads at a depth of 5 cm on June 4, 2019, in E1. Briefly, beads (Dowex Marathon MR-3 H/OH, Sigma-Aldrich) were placed into bags made of fine mesh (pantyhose material) and acid washed with a 10% HCl solution and rinsed several times with deionized water before deployment. Four bags were buried in each plot and were retrieved on October 7, 2019. Bags were immediately frozen until analysis. Beads were removed from the bags, weighed, and air dried for several days. The beads were then extracted with acidified (0.1 M HCl) 2 M NaCl solution; the extractant liquid was filtered through ashless Whatman filter paper, and analyzed colorimetrically for NH_4^+- and NO_3^--N (Hood-Nowotny and others 2010). We measured soil temperature using a Digi-Check soil temperature probe (Cole Palmer, Vernon Hills, IL) and soil moisture using a ProCheck moisture probe (ICT International, New South Wales, Australia) biweekly from April through September 2020 in E1. We averaged temperatures taken during peak season (mid-July through mid-August) for use in the multi-model averaging. We used data from the Cedar Creek Weather Station, collected daily since 1962, to compare climatic conditions during the years of our study to the long-term mean (Liang 2025). The mean growing season minimum and maximum temperature fell within the interquartile range (25th quartile–75th quartile) of mean temperature 1962 (Supplemental Figure 1). Mean growing season precipitation in 2019 fell within the

IQR, but mean growing season precipitation in 2020 fell between the long-term minimum and first quartile, though still well within the long-term 95% confidence interval at our site.

Nitrogen Fluxes

In E1, we measured N_2O and NH_3 fluxes in September 2019 by measuring the change in concentration in the headspace of a closed chamber (a 1 m^3 PVC frame covered with six mil clear plastic sheeting with 30 cm flaps that lay on ground) placed over each plot, using a Gasmet FTIR DX4000 gas analyzer. The chamber was sealed to the ground using two heavy chains placed on the ground flaps, and three small fans were used inside the chamber to ensure air mixing. Light conditions inside the chamber were measured using an Apogee MQ-200X PAR sensor (Apogee Instruments). We measured the flux rate in four different light environments that were manipulated using shade cloth and a black tarp, and each measurement was taken for 2 minutes. Ultimately, there was no difference in flux rate between the light environments, and flux rate for each plot was averaged across the four different measurements. We collected flux data at least 2 days removed from rain events to avoid erroneously high readings (Zhang and others 2023).

Total N Retention

To assess total *Net Ecosystem N Accumulation (Eq. 1)* in E1, we subtracted the total ecosystem N pool (*Ecosystem N pool*$_C$) in the Control treatment from the total ecosystem N pool in the N treatment (*Ecosystem N pool*$_N$) for each block to estimate the net increase in total ecosystem N pools for each experimental treatment, where ecosystem N pools were the sum of N in plant aboveground and belowground biomass, litter, and soil.

$$\text{Net N Accumulation} = \text{Ecosystem N pool}_N - \text{Ecosystem N pool}_{\text{Control}}$$

We then summed cumulative experimental N additions through time for each treatment over the course of the experiment (13 years) to determine *Cumulative N Addition* and divided the net increase in total ecosystem N pool resulting from N treatment by *Cumulative N addition* minus estimated *Cumulative gaseous N losses* to calculate *N Recovery* (Eq. 2).

Neither N_2O nor NH_3, the two measured N gases, differed between treatments or differed from zero (SI Figure 1), so we assumed that gaseous losses were negligible. We were unable to measure N_2, NO, or NO_2 gas flux, but the previous studies have found that N_2O fluxes from grasslands can be 50–100 times larger than NO or NO_2 fluxes (Yamulki and Jarvis 2002), so we assumed negligible N_2, NO, and NO_2 fluxes. Finally, we subtracted *Cumulative N addition* from *Net N accumulation* to calculate the change in total N pools (ΔN, in units of grams of N m^{-2}) to assess how much of the added N was retained in each treatment.

$$\Delta N = \text{Net } N \text{ accumulation} - \text{Cumulative } N \text{ addition}$$

Statistical Analyses

All statistical analyses were conducted using R (v.4.1.1). We used mixed-effects models with rate of N addition as a continuous, fixed effect and block as a random effect (R package 'nlme,' with function lme(), Pinheiro and others 2018) to assess the effect of N addition on total ecosystem N, N concentration, N recovery, and ΔN. We used the lsmeans package to calculate marginal ($R_m{}^2$) and conditional ($R_c{}^2$) R-squared values, which represent the percentage of error explained by the fixed or fixed and random variables, respectively (Lenth 2016). We used Shapiro–Wilk test to test assumptions of normality, and total ecosystem N data were natural log-transformed to meet assumptions of normality. We assessed the influence of covariates on total N using multi-model averaging with the dredge() and get.models() functions in the MuMIn package in R (Bartón 2020). The initial model included litter mass, soil moisture, rate of annual N addition, total soil %C, soil pH, C_3 and C_4 grass biomass, aboveground biomass, root biomass, BNPP, root turnover, and block as predictor variables. The final averaged model ($R^2 = 0.97$, $p < 0.0001$) included total soil %C, litter mass, and soil pH. We used predicted R^2 to assess overparameterization; the predicted R^2 value was 0.94, indicating our approach is robust to overparameterization. We used an *F*-test to assess differences in variance between N addition treatments (Snedecor and Cochran 1989). Statistical significance was assessed at $p < 0.05$. In Figures 2, 3, and 5, model fits are displayed as solid lines with a 95% confidence internal.

$$N\text{ Recovery} = \frac{(\text{NetNAccumulation})}{(\text{Cumulative } N \text{ addition} - \text{Cumulative gaseous } N \text{ losses})}$$

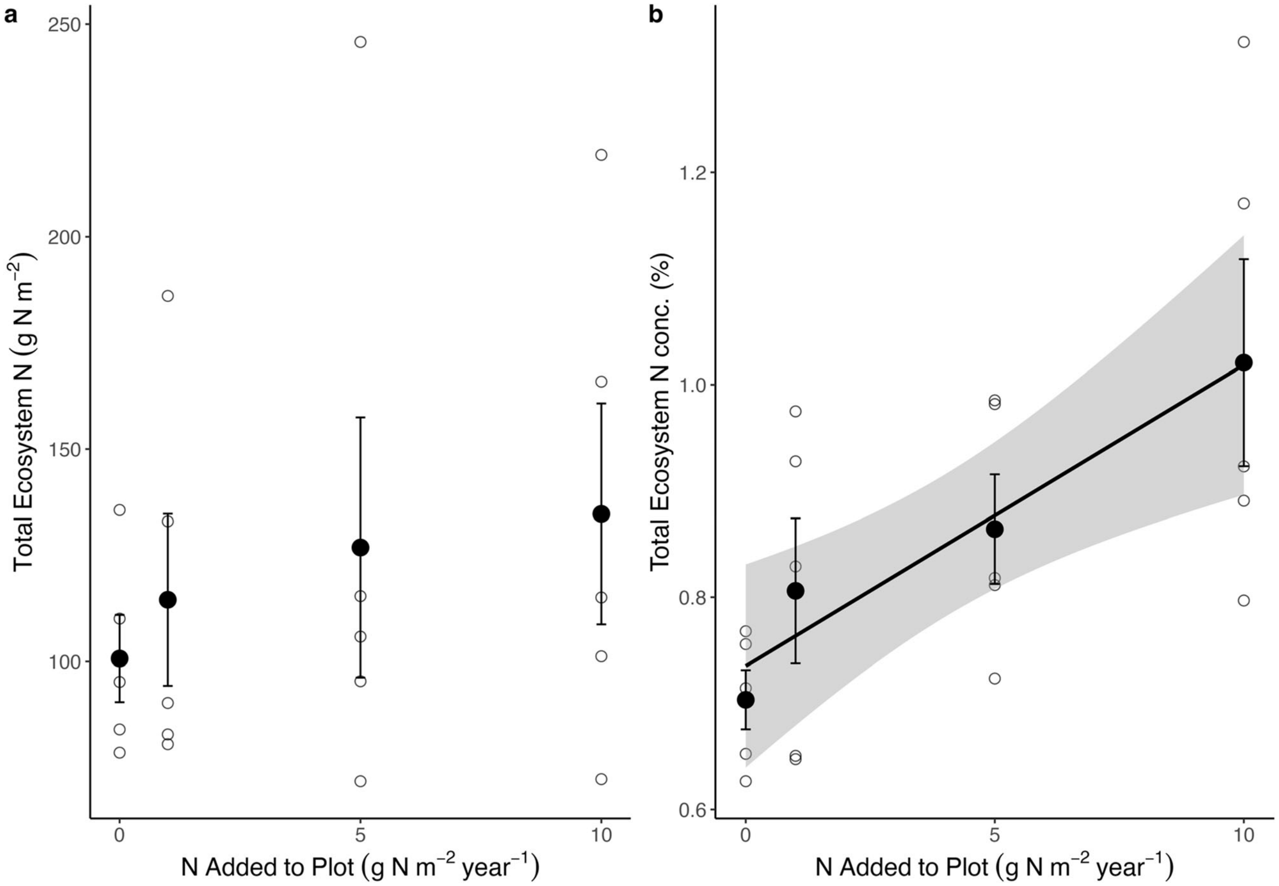

Figure 2. Trends in ecosystem N with increasing levels of N addition. Total ecosystem N was variable across the N gradient (left) and total ecosystem N concentration (%; right) increased with increasing N addition. The black line is a line of best fit, and the gray shaded region represents the 95% confidence interval. Solid black points indicate treatment-level means, black error bars denote ± 1 SE, and the open points are individual data points for each plot

RESULTS

Total N Retention

Total ecosystem N pools were highly variable across the experiment, ranging from 71.8 g N m^{-2} to 245.8 g N m^{-2} (Figure 2), with an average of 119.27 g N m^{-2} across all treatments. However, we found no relationship between annual N addition rate and total N pools ($R_m^2 = 0.06$, $R_c^2 = 0.46$, $p = 0.18$; Supplemental Table 2). Plot-scale plant biomass %N increased with increasing N addition, with the highest %N values (averaged across aboveground and belowground plant biomass, litter, and soil) in the N10 treatment (Figure 2; Supplemental Table 2). Nitrogen addition, on average, increased net N accumulation by 24.7 g N m^{-2} compared to Control, but there was no difference in net N accumulation among the N addition treatments ($R_m^2 = 0.02$, $R_c^2 = 0.83$, $p = 0.29$) (Figure 3a). When regressed against cumulative N addition over the entire experimental duration, the change in N pools (ΔN) declined with increasing cumulative N added (Figure 3b; $R_m^2 = 0.26$, $R_c^2 = 0.88$, $p = 0.0004$); on average, the N10 treatment only retained 34 g N m^{-2} of the 130 g of cumulative N added, and the N5 treatment only 26.9 g N m^{-2} of the 65 g of cumulative N added over the 13-year experiment (Supplemental Table 3). However, ΔN in the N1 treatment was 0.8 g N m^{-2} more than cumulative inputs, or approximately 106% of cumulative N inputs, indicating that there may have been small N gains beyond the experimentally added N. Conversely, the N5 treatment retained an average of 40% of inputs, and the N10 treatment retained an average of 26% of inputs (Figure 4), most of which was in the soil pool. However, variances within the replicate plots for each of the three N addition treatments differed; N1 had more variability in N recovery than both N5 ($F_{4,4} = 13.94$, $p = 0.03$) and N10 ($F_{4,4} = 83.97$, $p = 0.0008$); N5 and N10 had similar variances ($F_{4,4} = 6.023$, $p = 0.11$) (Supplemental Table 4).

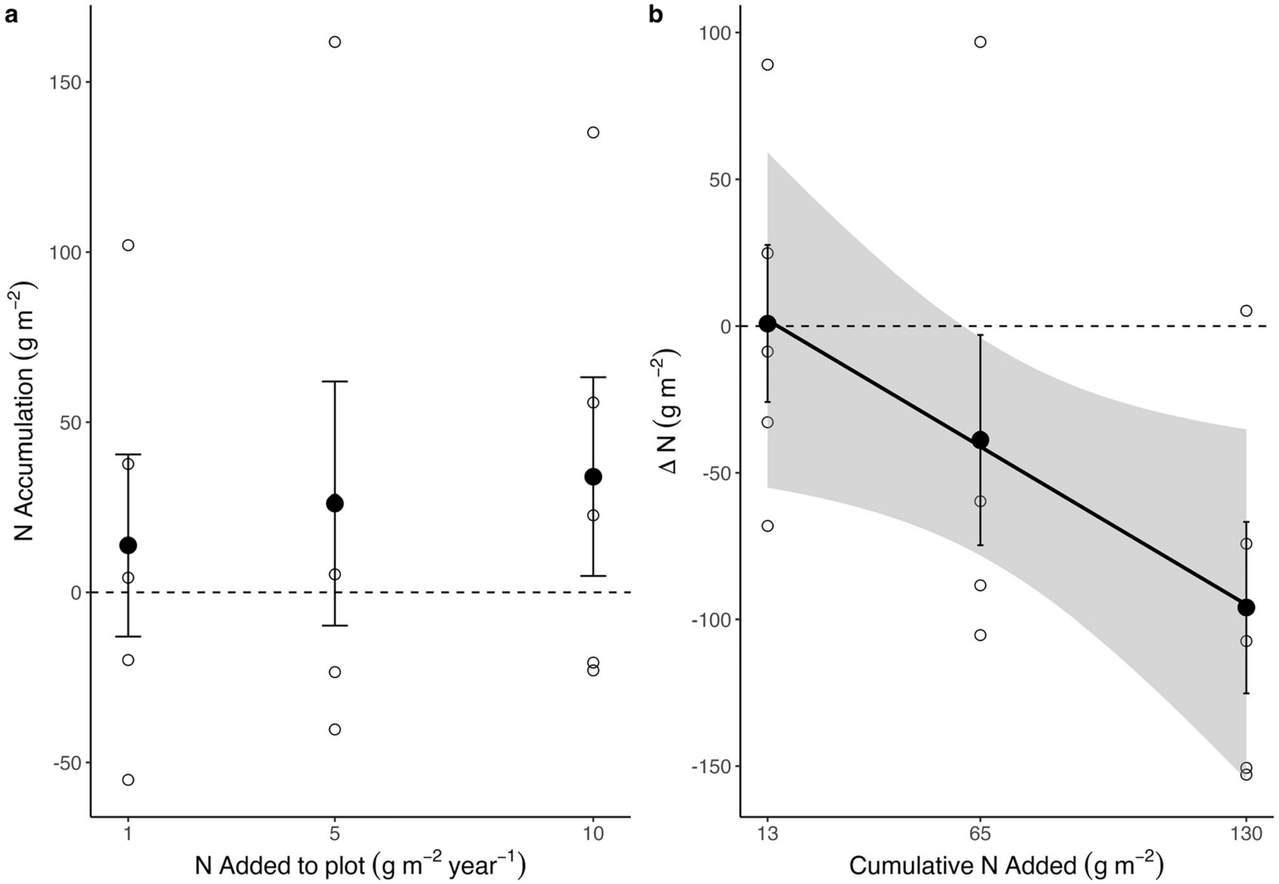

Figure 3. Nitrogen accumulation (**a**; N $\text{pools}_{\text{treatment}}$ − N $\text{pools}_{\text{Control}}$) varied within each treatment, but there was no change in N accumulation across the N addition gradient. Change in N (ΔN; the difference in N accumulation minus cumulative N added) decreased with increasing cumulative N addition (**b**). The *x*-axis in panel **b** represents the cumulative amount of N addition (annual rate of N addition multiplied by the length of the experiment). Solid black points are treatment-level means, the open points are individual data points for each plot, error bars are ± 1 SE, the regression line in (**b**) is a line of best fit, and the shaded region represents the 95% confidence interval

Similarly, we found a large range in net N accumulation among treatments (Table 1), from 152.9 g m^{-2} of N lost to 96.8 g m^{-2} of N gained, which suggested unmanipulated variables were driving N accumulation, in part. To better understand how N was being lost from the system, we first assessed which other factors drove N accumulation or loss. We then assessed pathways for N gain (increase in pool N concentration, increase in total pool mass, and increase in total N pools) and N loss (herbivory, leaching, and gaseous losses) across the N gradient.

Effect of Covariates on Total Ecosystem N

Total soil %C was the strongest (positive) determinant of total ecosystem N (Supplemental Table 5). Litter mass had a small, but significant, negative effect on total N, and soil pH had a small negative effect on total N (SI Table 4). In the model, total C had the largest standardized effect size (1.03), and litter mass and soil pH had similarly sized standardized effect sizes (-0.15 and -0.10, respectively) (SI Figure 2).

N Gains

Nitrogen addition did not increase total N in aboveground biomass ($F_{1,18}$ = 1.1, p = 0.31), litter ($F_{1,18}$ = 0.45, p = 0.51), belowground biomass ($F_{1,18}$ = 0.01, p = 0.92), or soil ($F_{1,18}$ = 1.15, p = 0.30) pools, despite 13 years of fertilization (Supplemental Tables 6–8). Aboveground biomass N concentration increased with N addition ($F_{1,18}$ = 13.82 p = 0.0016), as did root percent N ($F_{1,18}$ = 25.36, p < 0.0001; Figure 5a, b). Neither

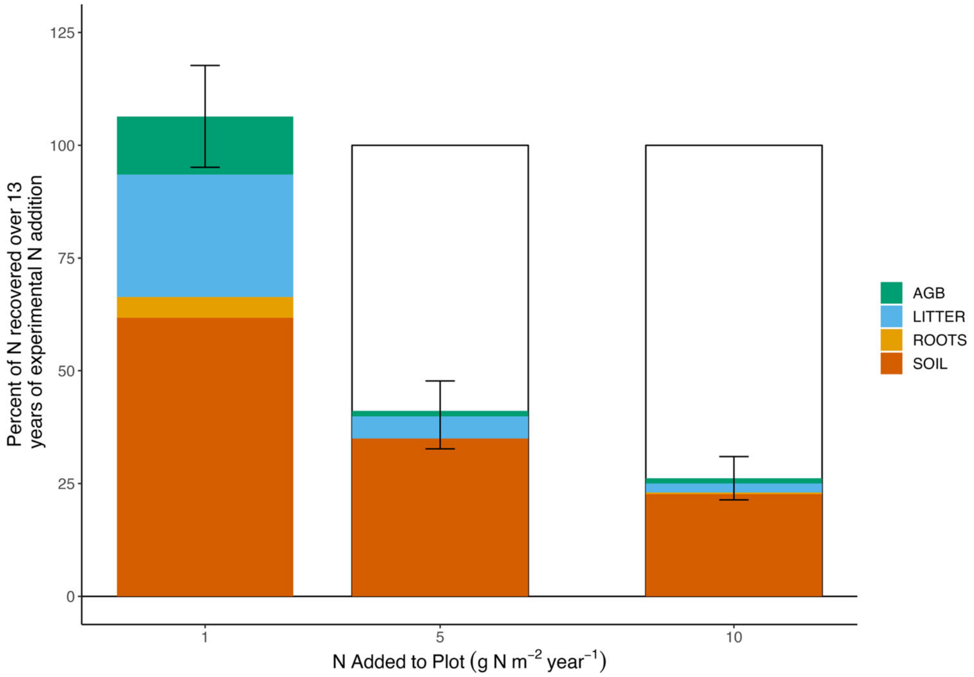

Figure 4. Percent of N recovery, defined as net N accumulation ($\mathrm{Npools_{treatment}} - \mathrm{Npools_{Control}}$) divided by cumulative N inputs (annual N addition rate times length of the experiment) in each N addition treatment. The colored bars represent the amount that each category (aboveground biomass, AGB, litter, roots, or soil) contributed to the overall treatment-level proportion of N recovered. The white bars with black outline represent the percentage of N lost. The error bars show the mean ± one SE of the total percentage of N retained for each N addition treatment

Table 1. Net *N* Accumulation (Ecosystem $\mathrm{N_{treatment}}$–Ecosystem $\mathrm{N_{control}}$) in Each *N* Addition Treatment

Treatment	N accumulation range	N accumulation (mean ± 1 SE)
N1	− 68.1–89.0 g N m^{-2}	0.83 ± 26.7 g N m^{-2}
N5	− 105.2–96.8 g N m^{-2}	− 38.9 ± 35.8 g N m^{-2}
N10	− 152.9–5.2 g N m^{-2}	− 96.0 ± 29.2 g N m^{-2}

The second column is the range between the lowest and highest amount of N accumulation among replicates within a treatment, with negative values indicating that the Control plot in a block had more total N than the N addition treatment plot; the third column lists the average N pool size and one standard error

litter percent N ($F_{1,18} = 0.49$, $p = 0.49$) nor soil percent N ($F_{1,18} = 1.15$, $p = 0.30$) was affected by N addition (Supplemental Tables 7 and 8). Within all plant functional groups, aboveground biomass percent N increased with N addition (Figure 5a), and forbs had higher biomass percent N (1.36%) compared to C_3 (1.14%) and C_4 (0.995%) grasses (Supplemental Table 6). Similarly, N addition increased biomass percent N for both shallow (0–10) and deeper (10–20 cm) roots (Figure 5b). As previously reported (Wilcots and others 2022), aboveground biomass peaked at low rates of N addition, and belowground biomass decreased with N addition (Supplemental Table 6). Litter mass was not affected by N addition ($F_{1,18} = 0.95$, $p = 0.34$) (SI Table 6).

Potential for N Losses

The total inorganic N accumulated on soil ion-exchange resins (IER) over 125 days increased with N addition (NH_4^+: $F_{1,18} = 7.20$, $p = 0.0006$; NO_3^-: $F_{1,18} = 12.66$, $p = 0.0022$; Figure 6). IER-NH_4^+ concentration was twenty times higher in the N10 treatment compared to the Control, and IER-NO_3^- concentration was over fifty times higher in N10 compared to Control, indicating higher potential for nitrate leaching with N addition. There were negligible N_2O losses via denitrification, and neither rates of N_2O nor NH_3 emissions differed based on N fertilization rate (SI Figure S1). Leaf damage by insects increased slightly with N addition for C_3 grasses, but the effect of N was not significant

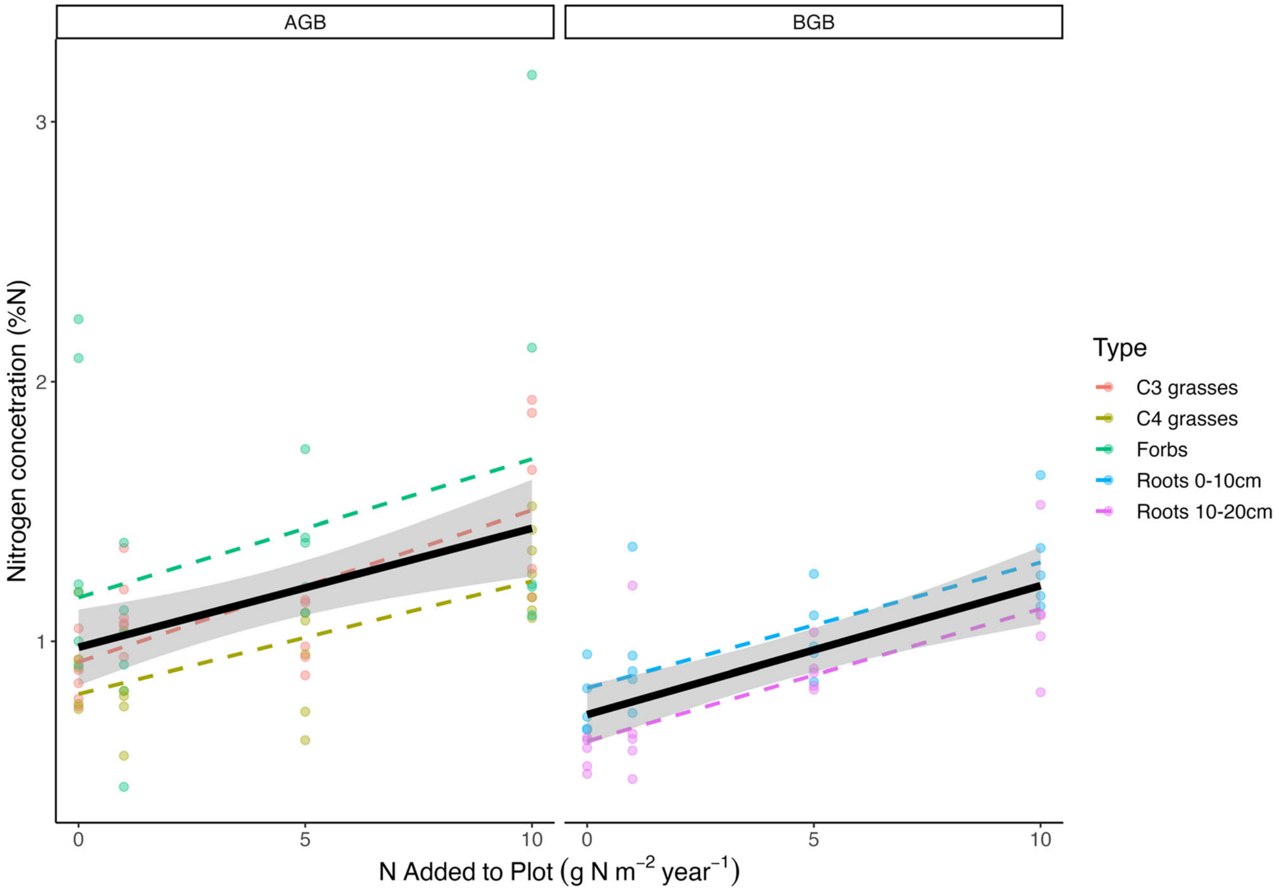

Figure 5. Biomass, N concentrations, and total N pools in aboveground plant, root, and litter biomass, and N concentration and total N pool in soil (to 20 cm). Litter biomass did not change across the N gradient (**a**); root biomass decreased and AGB increased at low rates of N only (a; as previously reported in Wilcots and others 2022). N concentration increased in roots and AGB (**b**), but not in litter (**b**) or soil (**c**). Total N pools did not increase across the N gradient in any category (**d** and **e**). For all panels, filled points represent plot-level measures of biomass, %N, and N pool sizes. Solid lines represent lines of best fit for significant regressions between N added to plot and biomass, %N, or total N, respectively. Dashed lines represent lines of best fit for regressions with $p > 0.05$. Shaded areas represent 95% confidence intervals

($R_m^2 = 0.05$, $R_c^2 = 0.35$, $p = 0.28$; Supplemental Fig. 3).

In E2 (see *Experimental Design* in Materials and Methods), we compared aboveground biomass offtake by large mammalian herbivores (Fenced–Unfenced plots) between the unfertilized and fertilized (with N, P, K, and μ) treatments. Averaged across time, fencing without fertilization caused a greater difference in biomass (Fenced–Unfenced) compared to the effect of fencing in fertilized plots (t = 2.78, $p = 0.006$; Supplemental Figure 4). Herbivore effects in unfertilized conditions (Fenced–Unfenced) reduced biomass by 78 g more than under fertilized conditions.

Climatic conditions.

DISCUSSION

After 13 years of chronic N addition, ecosystem N pools did not increase, regardless of N addition rate. Rather, all treatments maintained similarly sized N pools, and thus, plots subjected to the higher rates of N addition became saturated and were not able to retain all the experimentally added inputs. These findings indicate that for ecosystems with similar characteristics to those at our site (for example, well-drained, low-fertility soils), higher rates of N deposition may lead to increased N losses. Within each ecosystem pool (aboveground plant biomass, plant litter, belowground plant biomass, and soil), total N was similar across treatments, despite increased percent N in plant aboveground biomass

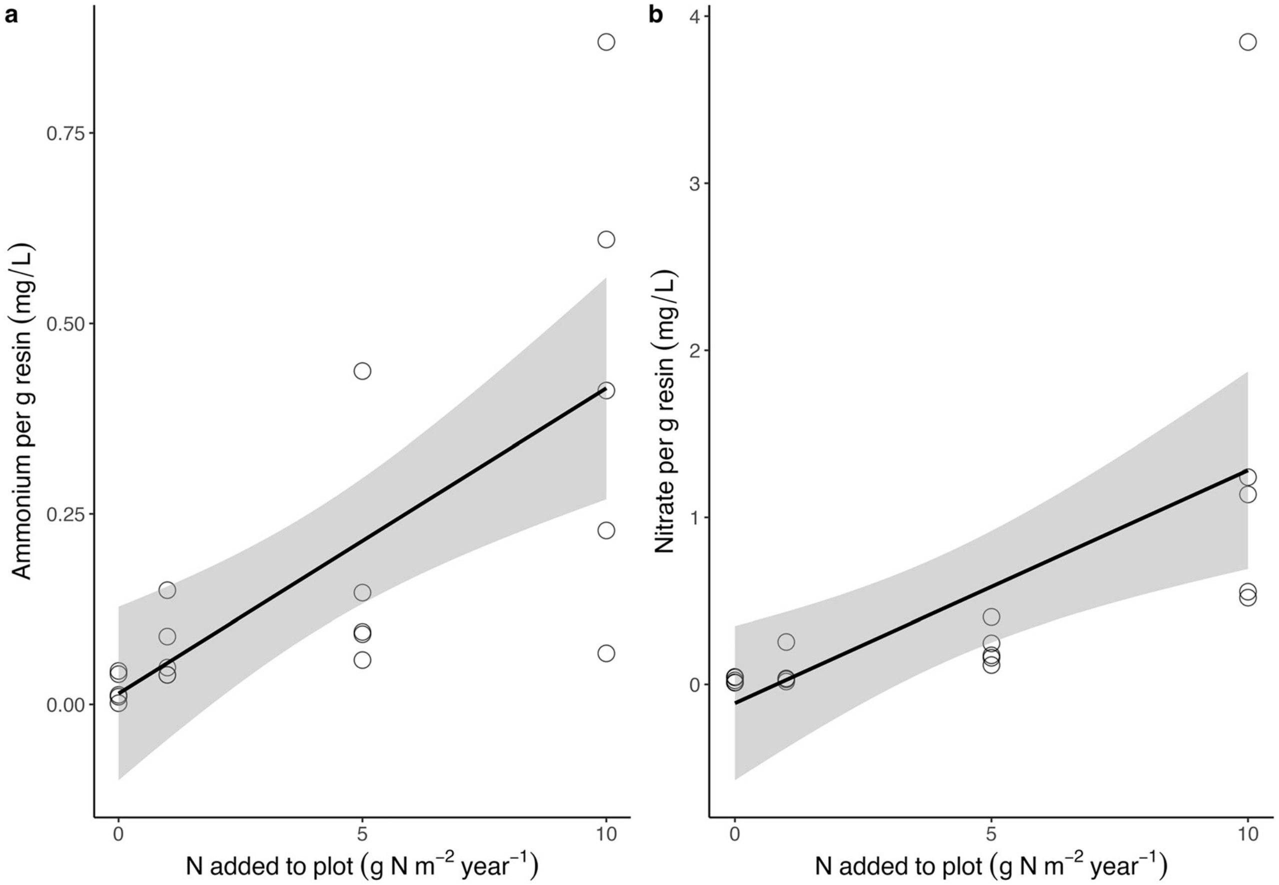

Figure 6. Ammonium **a** and nitrate **b** concentrations accumulated on ion-exchange resins over 126 days increased with increasing N addition rates. Ammonium and nitrate concentrations on resins were negligible in the Control treatments, and increased more than 20-fold (ammonium) and 50-fold (nitrate) between the Control and N10 treatments. Open points are replicate-level concentrations of ammonium and nitrate, black lines are lines of best fit from a linear model, and shaded regions are ± 1 SE

and roots at elevated N supply. Our results suggest greater potential for leaching at high rates of N addition; both soil IER-NH_4^+ and IER-NO_3^- levels increased with N addition rates. Nitrate is particularly susceptible to leaching in soils that have high cation exchange capacity (but low anion adsorption capacity; see Grigal and others 1974).

In this study, multiple lines of evidence suggest that increasing N addition may not lead to more ecosystem N storage, and that increasing N addition increases N losses. First, ΔN, net N accumulation minus cumulative N addition, decreased significantly with N addition, with cumulative N additions being nearly 100 g m^{-2} higher than the total ecosystem N pool in the N10 treatment. Nitrogen addition rate was not included in the multi-model average predicting total ecosystem N. Total C was the strongest determinant of total ecosystem N; the majority of ecosystem N was contained in the soil pool in all treatments. As laid out conceptually by Lovett and Goodale (2011), SOM, and soil C content generally, seem to provide a key mechanism for N retention in this ecosystem, potentially via stoichiometric relationships or N-induced shifts in species composition. Past work has indicated that N addition on its own may not change soil C stocks (Keller and others 2021; Wilcots and others 2022), and thus, if N retention depends on soil C, an increased soil N sink in grasslands may not be possible without concurrent changes in soil C stocks. The soil C stock could also depend on non-N nutrients, such as K, Mg, Ca, and other micronutrients that control C accumulation in soils (Crowther and others 2019; Seabloom and others 2021).

Soil C accumulation in response to N addition also can be tied strongly to mean annual precipitation and soil texture, with soils with higher silt or clay content accumulating more C (Dungait and others 2012; for example, Cai and others 2016; Egan and others 2018), which, in turn, could increase N accumulation. In this study, we only measured roots and soil C to a depth of 20 cm, but increased N storage at greater depths than this is unlikely, given that past work from nearby fields (Knops and Bradley 2009) found the majority of the soil C and N pool was contained in the top 20 cm of the soil; however, this distribution of soil C and N can be related to shallow-rooted, non-native plant dominance and a loss of legumes after old field abandonment (Yang and Knops 2023). Thus, decreased N recovery with increased N inputs could be expected if total N (numerator) is limited in size but N addition (denominator) increases. We found that four of the 20 experimental plots (two N1 and one each N5 and N10) had a ΔN value higher than 0, indicating a net gain of N to the plot, and per gram of N added, N1 had the highest proportion of N recovery (1.06), though this was not significantly different than 1. This indicates that N inputs into the system could be possible, due to increased waste inputs from herbivores from biomass consumed outside the plot, or that the plants were able to access soil N from depths deeper than 20 cm and redistribute that N into the top layers of soil or into plant or microbial tissues. Increased rates of biological N fixation (BNF) are unlikely given the rarity of legumes at this site (see Materials and Methods); fertilization can also decrease rates of BNF (Keuter and others 2014).

Second, concentrations of inorganic soil N (NH_4^+ and NO_3^-) accumulated on ion-exchange resins increased significantly with N addition. Concentrations of both NH_4^+ and NO_3^- in the Control plots were essentially zero, indicating that combined, microbial N immobilization and plant N uptake match N mineralization in this ecosystem, and thus, leaching is likely not a significant N loss pathway under ambient conditions. Importantly, the small inorganic N pools in the Control plots indicate that the current rate of N deposition at our site (0.9 g N m^{-2} $year^{-1}$) likely has not significantly impacted aqueous N losses, at least during the growing season. This N deposition rate is approximately the same as our lowest experimental addition rate (1 g N m^{-2} $year^{-1}$), and thus, our findings suggest that this ecosystem could absorb a doubling of N deposition rates without increases in N losses. Given that N deposition rates are decreasing in the Upper Midwestern USA region (for example, the US states of Minnesota, Wisconsin, Michigan, as well as parts of Canada) (Benish and others 2022), and are flat or declining at our site specifically (NADP 2022), N deposition may not increase future leaching rates in this area. However, leaching of dissolved organic N (DON), which we did not measure in this study and which may be less influenced by plant and microbial demand (Perakis and Hedin 2002), may comprise a large component of N leaching (for example, Phoenix and others 2003), though past work near our study site indicates that dissolved inorganic N losses are larger than DON by an order of magnitude (Dijkstra and others 2006, 2007). We measured IER-soil NO_3^- concentration at a depth of 5 cm during the summer months, often the time of year with the lowest leaching losses (Cameron and others 2013). Inorganic N pools might have responded more to N treatment than measured here, if non-growing season months were considered, which would imply greater N-induced leaching of N at those times.

Plant tissue percent N increased both aboveground and belowground, but that did not translate to increases in total N pools because increases in tissue %N were offset by decreases in biomass. This suggests that N accumulation in biomass in this ecosystem happens via a stoichiometric sink (Lovett and Goodale 2011), wherein N accumulates without concurrent increases in C, which may result from low stoichiometric plasticity in certain species, or limitations in growth due to light or water (Lovett and Goodale 2011); mean growing season precipitation was typical during the years of our study (Supplemental Figure 1), but soil moisture retention is low at our site given the sandy soil texture (Grigal and others 1974). Within each plant functional group (C_3 and C_4 grasses and non-leguminous forbs), N supply increased tissue N concentration, and tissue %N also increased at both depths (0–10 cm and 10–20 cm) of belowground biomass. However, increases in N concentrations did not lead to increases in the total plant N pool due to decreases in biomass, perhaps because of increased herbivory, particularly belowground. This may indicate that shifts in species composition could lead to changes in N pools in the future as N addition shifts communities toward domination by more palatable species with N-rich leaves (Chapin and others 1987; Tilman 1987; Wedin and Tilman 1996) (but see Schmitz 1994). However, fast-cycling herbaceous plant pools are not necessarily long-term sinks for N (for example, Clark and others 2009), and an increase in N-rich aboveground tissues may lead to more N-rich litter (for example, Parton and others 2007). More N-rich litter releases N more rapidly (for example, Clark and others 2009) in early stages of decomposition (Parton and others 2007; Gill and others 2022), and could increase rates of N cycling without necessarily changing the size of the N pool and may lead to N loss.

Relative to the amount of cumulative N added, the change in N pools (ΔN) decreased across the experimental gradient, which could be due to a combination of leaching (as discussed above) and herbivory at higher rates of N addition. We found slight increases in rates of aboveground insect herbivory in C_3 grasses only, a trend similar to past studies that found N effects on insect herbivory or density (Lind and others 2017; Ebeling and others 2021). However, we found no evidence that multiple nutrient enrichment increased large mammalian herbivory at our site, which, given the importance of nutrient co-limitation at our site (Wilcots and others 2025), would indicated that N addition alone would likely have a similar or smaller effect on N retention. Though we could not measure the rate of N input by herbivores (Cameron and others 2013), any increase in inputs of herbivore-derived N would further exacerbate the extent to which N recovery decreased with N addition. Furthermore, recent work from a nearby field showed that effects of large mammalian herbivores, arthropods, and fungal pathogens together on plant aboveground biomass were three times higher when multiple nutrients were added (Zaret and others 2023). Therefore, the decrease in N recovery observed at higher N addition levels could be due to increased consumption. When considering the change in total N (ΔN) across all treatments, only the N1 treatment retained more N than the total cumulative N added across the 13 years of the experiment: after 13 g of total N inputs, the N1 treatment had, on average 13.8 g of N more than the Control treatment, a 0.8 g N gain, whereas the N5 treatment only had 26.1 g N more than Control despite 65 g of cumulative N inputs (a 34.9 g N m^{-2} loss), and the N10 treatment had 34 g N more than Control despite 130 g of cumulative added N, a 96 g N m^{-2} loss. Thus, the two highest N addition treatments lost N when compared to what was added, likely a direct result of increased herbivory, leaching, or both. Though we did not measure nitric oxide (NO) flux rate, research from a neighboring field has shown NO fluxes 1–2 orders of magnitude lower than the N_2O fluxes we measured, which were negligible (Clark 2007). Areas with less sandy soils with lower rates of leaching, or areas with lower herbivory pressure, may, therefore, see less of a discrepancy in ΔN if conditions are more favorable for N retention.

After 13 years of N addition, we found no difference in total ecosystem N pools across a gradient of N addition in a temperate grassland. We found evidence of decreasing proportional N retention and increased potential for N loss via leaching as N addition increased, and increases in tissue %N were offset by losses in total mass, particularly belowground. This implies a mechanism through which net N retention decreases with increasing N addition, either biotically (through changes in mass driven by altered species composition, herbivory, or root:shoot ratios; see Cleland and others 2019; Borer and others 2020; Wilcots and others 2021, 2022) or through changes in stoichiometry due to water or other resource limitation (for example, Lovett and Goodale 2011). Here, we find clear linear increases in N losses and decreases in ΔN in response to N addition, and despite increases in plant tissue %N, no response in the overall plant or soil N pool. Ultimately, these findings suggest that N-induced tissue N increases may not increase the total ecosystem N pool if there are concurrent decreases in biomass. These results may differ in areas with less sandy, leaching-prone soils, or wetter areas where herbivores remove a smaller percentage of biomass compared to Control (Borer and others 2020). The context of our work is particularly relevant to grassland restoration efforts, because most restoration efforts are aimed at marginal cropland (De and others 2020) that may not accumulate C at rates necessary for climate change mitigation efforts (for example, Knops and Tilman 2000). In these key areas for restoration, increasing N deposition might exacerbate environmental stresses via leaching or groundwater contamination, especially in areas with sandy soils. Taken together, we find evidence that small rates of N addition, similar to rates of N deposition across much of the globe (Bebber 2021), may neither increase N pools nor increase N losses. However, higher rates of N addition, similar to rate of N deposition in the most polluted areas of the world or to agricultural fertilizer runoff, may lead to increased ecosystem N losses through leaching.

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DATA AVAILABILITY

Data used in this experiment are available on the Environmental Data Initiative: https://doi.org/10.6073/pasta/7c815175fc378270b6848351e43275b9.

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