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RESEARCH ARTICLE

Realistic rates of nitrogen addition increase carbon flux rates but do not change soil carbon stocks in a temperate grassland

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Abstract

Changes in the biosphere carbon (C) sink are of utmost importance given rising atmospheric CO₂ levels. Concurrent global changes, such as increasing nitrogen (N) deposition, are affecting how much C can be stored in terrestrial ecosystems. Understanding the extent of these impacts will help in predicting the fate of the biosphere C sink. However, most N addition experiments add N in rates that greatly exceed ambient rates of N deposition, making inference from current knowledge difficult. Here, we leveraged data from a 13-year N addition gradient experiment with addition rates spanning realistic rates of N deposition (0, 1, 5, and 10 gNm^{-2} year⁻¹) to assess the rates of N addition at which C uptake and storage were stimulated in a temperate grassland. Very low rates of N addition stimulated gross primary productivity and plant biomass, but also stimulated ecosystem respiration such that there was no net change in C uptake or storage. Furthermore, we found consistent, nonlinear relationships between N addition rate and plant responses such that intermediate rates of N addition induced the greatest ecosystem responses. Soil pH and microbial biomass and respiration all declined with increasing N addition indicating that negative consequences of N addition have direct effects on belowground processes, which could then affect whole ecosystem C uptake and storage. Our work demonstrates that experiments that add large amounts of N may be underestimating the effect of low to intermediate rates of N deposition on grassland C cycling. Furthermore, we show that plant biomass does not reliably indicate rates of C uptake or soil C storage, and that measuring rates of C loss (i.e., ecosystem and soil respiration) in conjunction with rates of C uptake and C pools are crucial for accurately understanding grassland C storage.

KEYWORDS

carbon cycling, grasslands, net ecosystem exchange, nitrogen deposition, soil respiration

1 | INTRODUCTION

Soils store 2.5 and plants store 1.5 times as much C compared to the atmosphere, respectively (Schlesinger & Bernhardt, 2013). Thus,

even small changes to the size of these C sinks, or to rates of photosynthesis or respiration, could have large consequences for atmospheric CO_2 levels. Importantly, other global change drivers, such as nitrogen (N) deposition (Stevens, 2019; Vitousek et al., 1997), may be

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changing rates of ecosystem C uptake and emission and the relative sizes of ecosystem C pools. However, studies that examine changes in C flux rates and C pools across levels of N addition relevant to understanding effects of N deposition are scarce. Given that N deposition will increase in many parts of the world in the coming decades while declining in others (Ackerman et al., 2019), disentangling the effect of N deposition on ecosystem C uptake and respiration will be key to interpreting the effects of continued global change on the terrestrial C sink.

Elevated N deposition is one of the most severe and ubiquitous global change drivers, altering ecosystem function globally (Stevens, 2019; Vitousek et al., 1997). Nitrogen limits plant growth in many ecosystems (Elser et al., 2007; LeBauer & Treseder, 2008), including grasslands (Fay et al., 2015), where ambient N deposition is a more effective predictor of aboveground plant biomass across sites around the world than either climate or soil variables (Stevens et al., 2015). Nitrogen deposition also can acidify soils (Du et al., 2018; Gill et al., 2021; Kimmel et al., 2020; e.g., Roem et al., 2002; Stevens et al., 2010), which can impact soil microbial communities and rates of decomposition (Fierer & Jackson, 2006; Li et al., 2016; Ramirez et al., 2012; Shi et al., 2016; Wang et al., 2018). Therefore, N has the potential to influence grassland C accumulation by concurrently changing plant biomass, detritus, and rates of decomposition. Adding N alone can have positive, negative, and neutral effects on soil C (e.g., Crowther et al., 2019; Keller et al., 2021), even when N increases aboveground plant biomass accumulation (Fay et al., 2015) and reduces the rate of litter and soil organic matter (SOM) decomposition (Gill et al., 2021; e.g., Riggs et al., 2015; Riggs & Hobbie, 2016).

Predicting grassland C cycle responses to N deposition is difficult because many N addition experiments are not designed to answer questions about the effect of current rates of N deposition. The majority of Earth's surface receives between 0 and 2 gN m^{-2} year⁻¹, though some areas of China and southeastern Asia receive rates of N deposition up to 8.3 gN m⁻² year⁻¹ (Xu et al., 2015). However, a recent metanalysis found that experimental N additions (across all terrestrial ecosystems) add N at an average rate of 12.5 gN m⁻² year⁻¹ (Midolo et al., 2019). If ecosystem productivity increases linearly with increasing N addition, then accurate predictions of the effects of N deposition could be made from existing data. However, studies examining gradients of N addition that included low rates found that N can have saturating (Peng et al., 2017a) accelerating (Bai et al., 2010; Prager et al., 2017), and unimodal (Peng et al., 2017a, 2017b) effects on C fluxes, including productivity. Examining a gradient of N input rates, including realistically low rates, will increase understanding of the likely trajectory of impacts to ecosystem C stocks and fluxes as N input rates increase.

The size of an ecosystem's C stock is ultimately determined by the difference between C uptake via gross primary productivity (GPP) and C loss rates, which are often dominated by autotrophic and heterotrophic respiration (collectively, ecosystem respiration, ER). Nitrogen addition often increases net ecosystem C uptake and storage (Peng et al., 2017b; Prager et al., 2017), which often, but not always (Peng et al., 2017a), correlates with increased aboveground biomass. Soil respiration (from plant roots and soil microbes) can also

change in response to N addition. Nitrogen fertilization generally reduces soil heterotrophic microbial respiration (i.e., SOM decomposition), by decreasing soil microbial biomass (Janssens et al., 2010; Li et al., 2016; Riggs et al., 2015; Wang et al., 2018), possibly because high levels of N addition may cause soil acidification and depletion of base cations (Ning et al., 2021; Roem et al., 2002; Silvertown et al., 2006; Tian et al., 2015). In grasslands, the impact of N may depend on the rate of N addition, as studies with larger N inputs $(>10 \text{ gN m}^{-2} \text{ year}^{-1})$ have found suppression of heterotrophic respiration (Riggs et al., 2015), and those with smaller rates of N addition (<8 gN m⁻² year⁻¹) found stimulation of total soil respiration (Adair et al., 2011; Peng et al., 2017b). However, few studies simultaneously measure both pools (plant biomass, soil C) and fluxes (GPP, ER, net ecosystem exchange [NEE], soil respiration) across a range of N input rates, limiting understanding of N's concurrent impacts on ecosystem C pools and fluxes and their dependence on the rate of N input.

Understanding the relative allocation of biomass (aboveground versus belowground) and the subsequent size of soil and ecosystem C fluxes will be crucial for predicting whether grasslands will gain or lose C under conditions of elevated N supply. Optimal allocation theory dictates plants will allocate resources to acquire the most limiting resource(s) (e.g., Farrior et al., 2013). For example, where soil water is sufficient, elevated N can result in increased aboveground relative to belowground biomass (Cleland et al., 2019; Newman, 1973). This shift to greater relative aboveground investment could increase C stored via products of photosynthesis while concurrently reducing root biomass and other belowground structures such as microbial symbionts, leading to reduced C inputs (Cleland et al., 2019; Keller et al., 2021). However, N addition also can increase C inputs. In this case, if N reduces rates of SOM decomposition (e.g., Riggs et al., 2015; Riggs & Hobbie, 2016), it may leave more C in the soil.

Here, we use an experimental N addition gradient in a low fertility grassland to ask how C pools and fluxes respond across a range of N inputs, focusing on aboveground and belowground plant biomass, soil C, and soil and whole ecosystem C fluxes in a longrunning (>10 years) experimental N gradient. We hypothesized that, consistent with past work and optimal allocation theory (e.g., Farrior et al., 2013), N addition would increase aboveground plant biomass and decrease relative investment in belowground biomass, with this shift in allocation increasing with N input rates (Cleland et al., 2019). We also predicted that soil heterotrophic microbial respiration would be negatively correlated with N input due to reduced pH and microbial biomass (Riggs & Hobbie, 2016), and that the combined effect of reduced belowground plant biomass and soil microbial respiration would reduce total soil respiration, leading to an overall increase in net C uptake (NEE) and higher soil C stocks in our high N treatments.

2 | MATERIALS AND METHODS

We measured ecosystem C fluxes (GPP, ER, and NEE) and soil respiration across the growing season (early April through mid-November) in 2020 and collected plant aboveground and belowground biomass

and soil cores in July and August 2020 and 2021 along an experimental gradient of long-term N addition plots 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 = 750mm). CCESR is located on the Anoka Sand Plain, characterized by sandy soils (>88% sand) with low nutrient content (Grigal et al., 1974). The experiment was established in 2007 in an old field, 57 years after abandonment from agriculture. Background N deposition is approximately 0.9 gN m⁻² year⁻¹ (equivalent to 9 kgN ha⁻¹ year⁻¹; Ackerman et al., 2019). The experiment is a completely randomized block design composed of five replicate blocks of four 25-m² plots each with treatments as follows: ambient N deposition with 0 (Control), +1 (N1), +5 (N5), and +10 (N10) gN m⁻² year⁻¹ above ambient N deposition. Nitrogen was added to the plots as time-released urea (CH₄N₂O). Time-released urea has been found to be an effective mimic of N deposition (Jia, 2020), in spite of the majority of N deposition being composed of NH, and NO, compounds (Bebber, 2021). Fertilizer was added at the end of each growing season, and herbivores were not excluded from any of the plots. Data are archived with the Environmental Data Initiative (Wilcots, 2022).

2.1 | Biomass measurements

Aboveground biomass was harvested in two 10 cm × 100 cm strips per plot around peak biomass in mid-August 2020. Biomass from the two strips was pooled, dried to a constant mass at 60°C, weighed to the nearest 0.01 g, and scaled to gm^{-2} . The locations of the clipped strips were moved every year to adjacent, but previously unsampled, locations. Belowground biomass (BGB) was harvested in late July 2020 using two 5 cm diameter × 20 cm soil cores per plot. The top 20 cm of soil contains over 80% of root biomass at this site (Knops & Bradley, 2009). To estimate belowground net primary productivity (BNPP), we removed two 5 cm diameter × 20 cm soil cores from each plot 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 2mm 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 BGB 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 0.01g. Root biomass was pooled between cores, and then scaled on an areal basis to gm^{-2} to compare it directly to aboveground biomass. We estimated root mass fraction (RMF) by dividing total root biomass by the sum of aboveground and root biomass.

2.2 | Carbon flux measurements

Soil respiration in field plots was measured biweekly from April through November 2020 (total number of sampling days = 14) using a Licor 6400XT with a soil respiration chamber (Licor). The chamber

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was fitted onto a 10 cm diameter PVC collar installed at a fixed location in each plot. Three measurements of chamber headspace CO₂ concentrations were taken in a fixed location in each plot at each sampling date and then averaged. All aboveground live biomass and litter was cleared from the sampling footprint throughout the season. We measured net ecosystem exchange (NEE) by measuring the change in CO_2 concentration in the headspace of a chamber (a 1 m³ PVC frame covered with 6 mil clear plastic sheeting with 30 cm flaps that lay on ground) placed over a plot, using a Licor 850-3 (Licor) connected to a Lenovo tablet running the "Flux Puppy" software (Carbone et al., 2019). The chamber was large enough to capture a representative mix of species in each plot, including a dominant perennial bunchgrass (Andropogon gerardii) that often exceeds 0.1 m² in areal cover. The chamber was sealed to the ground using two heavy chains placed on the ground flaps, and 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). Chamber CO₂ concentrations were measured for at least 2 min or until they stabilized (up to 5 min). To standardize NEE values across light levels, we used garden shade cloth and tarps to create a gradient of decreasing light environments within the chamber. Two-minute measurements were conducted at 50% ambient light, 25% ambient light, and 0% ambient light (i.e., ecosystem respiration, ER). NEE was then calculated using Equation 1:

$$NEE = (\rho \times V/a) \times dC/dt,$$
(1)

where ρ is the air density (mol air m⁻³) calculated as *P/RT* where *P* is the average atmospheric pressure (Pa), *R* is the ideal gas constant (8.314 m³ Pamol air⁻¹ K⁻¹), T is the average temperature in units of Kelvin (K), and dC/dt is the change in CO₂-C concentration over time (molC mol air⁻¹ s⁻¹). The volume of the cube (V) and ground area (A) sampled were 1 m³ and 1 m², respectively. From the four light measurements, we fit linear and hyperbolic curves to predict NEE at a standard level of photosynthetically active radiation (PAR = 800 µmolm⁻² s⁻¹), NEE₈₀₀, to account for variation in ambient light levels during sampling (Kohli et al., 2020). We used the *R*² values to assess the fit of each curve, and the hyperbolic estimation was always the better fit. Ecosystem respiration (ER) was estimated using the 0% ambient light measurement. Gross primary productivity (GPP) was estimated using Equation 2:

$$GPP = ER - NEE_{800}$$
(2)

To assess the impact of N addition on heterotrophic microbial respiration, we conducted a 143-day laboratory incubation of soils from each plot, sampled in July 2021. Briefly, 50g of fresh 2-mm-sieved soil was sampled from the top 10 cm of the mineral soil and adjusted to 70% field capacity. Microbial respiration rate (mgC gsoil⁻¹ day⁻¹) was measured with a Licor 7000 by sampling the headspace at the beginning and end of a 24-h period of a sealed 1 L jar containing a soil sample. Between measurements jars with samples were stored in the dark at 20°C, which is the mean temperature during the growing season at our site (e.g.,

Kothari et al., 2018), and covered with low-density polyethylene film, allowing CO_2 and O_2 to diffuse freely while retaining water (see Riggs et al., 2015). Respiration was measured for each sample 14 times over the 143-day period. At the beginning of that period, respiration was measured daily to capture the quickly declining decay rate as labile substrates were metabolized; as decay rate slowed as labile substrates were consumed, respiration was measured fortnightly (e.g., Riggs & Hobbie, 2016). Cumulative microbial respiration was calculated by averaging microbial respiration rates from consecutive measurements, multiplying by the number of days elapsed between measurements, and adding the previous cumulative measurement.

2.3 | Soil chemistry and C stocks

To assess the effect of N addition on soil chemistry, we collected 20 cm deep soil cores in each plot in late July 2020. Soil was sieved to 2 mm to remove roots, dried, ground, and weighed before being analyzed for cation exchange capacity (CEC), pH (1:1 v/w in water), and common exchangeable micronutrients using the Mehlich-3 extraction method (Mehlich, 1984) (Waypoint Analytical). Soil C and N content were measured on air-dried soils by dry combustion with an ESC 4010 Elemental Analyzer (Costech). Soil C stocks were calculated using bulk density measurements taken from microbial respiration soil cores.

2.4 | Statistical analyses

All statistical analyses were performed in R (v.4.1.1) and RStudio. We used either nonlinear or linear regression models to assess the shape of the response curve across the N gradient. We fit a linear and a nonlinear regression to each response variable and used loglikelihood ratio tests and Akaike's information criterion (AIC) values to determine which model best fit the data. Because of our small sample size, we report AICc values, which are better suited for smaller samples (Hurvich & Tsai, 1989). With four N addition levels, we were able to test for nonlinearities; a design with more N addition levels would be better suited to test for more complex nonlinearities or a more exact location of maxima and minima. We modeled nonlinear regressions similarly to Peng et al. (2017a) using the nls() function in R (R Core Package, 2017) (Equation 3):

$$y = a + b \times \frac{x^c}{e^{d \times x}},\tag{3}$$

where *y* equals the response variable (e.g., GPP, aboveground biomass, pH), *x* equals the quantity of N added, *a* (intercept) equals the mean value from the unmanipulated Control treatment, and *b*, *c*, and *d* were parameters fit to the model using convergence. Depending on the values of the fitted parameters, this function can converge to a linear function (e.g., when c = 1 and d = 0), exponential growth (c > 1 and d = 0), and exponential decay (c = 0 and d < 1) while also allowing for

unimodal responses. A full table of model comparisons is found in the Supporting Information (Tables S1-S3).

We analyzed C pools and fluxes using linear mixed-effect models (R package "nlme"; Pinheiro et al., 2018) with N added as a fixed effect and block and plot as random effects to account for the repeated sampling over time in the same plot (i.e., our seasonal C flux data). To assess treatment differences at each sampling date (n = 9for ecosystem fluxes, n = 14 for soil respiration), we used linear mixed-effects models and ANOVA with N added as a factor and fixed effect, and block and plot as random effects, which account for the inherent correlation among samples taken in the same plot.

3 | RESULTS

3.1 | Effects of N addition on plant biomass

Nitrogen addition of up to $1.69 \text{ g Nm}^{-2} \text{ year}^{-1}$ increased aboveground plant biomass, with higher rates resulting in no change from control levels (Figure 1a). Aboveground biomass was best described by a non-linear model (Tables S1–S3) that peaked at $1.69 \text{ g Nm}^{-2} \text{ year}^{-1}$, close to the N1 treatment rate. Nitrogen addition caused a linear decrease in belowground biomass (Figure 1b; $R^2 = 0.18$, p = 0.034), with the lowest amounts of BGB in the highest N addition treatment. As with AGB, annual belowground production (BNPP) was best described by a nonlinear model (Tables S1–S3), with highest rates of BNPP production around $2.63 \text{ g Nm}^{-2} \text{ year}^{-1}$ (Figure 1d). Root mass fraction (RMF) was best described by a nonlinear model (Tables S1–S3), with the lowest RMF value at around $2.96 \text{ g Nm}^{-2} \text{ year}^{-1}$ (Figure 1c).

3.2 | Effects of N addition on soil chemistry

Bulk soil C (Figure 2b) did not change in response to N addition. However, N addition significantly reduced soil pH (Figure 2a; $R^2 = 0.44$, p = 0.0009). Despite reduced soil pH, Mehlich-3extracable base cation concentration (K⁺, Ca²⁺, Mg²⁺) did not differ across the N gradient, either summed together (Figure 2c, $R^2 = 0.015$, p = 0.27) or considered separately (Figure S1). Nitrogen addition caused linear decreases in soil microbial biomass C (MBC) (Figure 2d, $R^2 = 0.24$, p = 0.02). Soil pH was correlated with BGB (r = 0.49, p = 0.027), MBC (r = 0.42, p = 0.079), and base cations (r = 0.43, p = 0.056) (Figure S2).

3.3 | Effects of N addition on carbon fluxes

Nitrogen addition also caused linear decreases in soil heterotrophic respiration measured in laboratory incubations ($R^2 = 0.38$, p = 0.0023; Figure 3a), which also was correlated with soil pH (r = 0.51, p = 0.027; Figure S2). Consistent with this decline, total soil respiration from field plots was highest at low N addition levels (Figure 3b). Annual plant growth (AGB and BNPP, summed) explained around



FIGURE 1 Aboveground (a) and belowground (b) plot biomass, and root mass fraction (c) and belowground net primary productivity (d) at peak biomass. AGB, RMF, and BNPP were best described by nonlinear functions, which peaked at 1.69, 2.96, and 2.63 gN m^{-2} year⁻¹, respectively. BGB was best described with a linear function ($R^2 = 0.14$, p = 0.034)

17% of the variance in soil respiration, and plots with more annual biomass had higher rates of soil respiration (Figure S3). Soil respiration was best described by a nonlinear function that peaked around 1.25 g N m⁻² year⁻¹. Similarly, ER (plant + soil respiration) was best described by a nonlinear model with a peak around $1.5 \text{ gNm}^{-2} \text{ year}^{-1}$ added (Figure 4a), and gross primary productivity (GPP) was best described by a nonlinear model with a peak at 1.4 gNm^{-2} year⁻¹ added (Figure 4b). Over the course of the growing season, rates of GPP were significantly different among treatments in mid-August only ($F_{3,12} = 3.73$, p = 0.04). ER was not significantly different among treatments in mid-August ($F_{3,12} = 2.98$, p = 0.07), though rates of ER were high in the N1 treatment. While ER and GPP both showed unimodal responses with increasing N addition rate, particularly at peak season, these fluxes were counterbalancing, resulting in no change in NEE across the experimental N gradient (Figure 4c; $R^2 = 0.047$, p = 0.18) at any point in the growing season (Figure 5a-c). In contrast, rates of soil respiration were different among N addition treatments beginning in early May ($F_{3,12} = 11.97$, p = 0.0006) and continuing through September ($F_{3,12} = 3.54$, p = 0.048; Figure 5d).

4 | DISCUSSION

In this study, when N was added at rates spanning the range of global atmospheric N deposition rates, it increased the rate of

ecosystem-level C fluxes at low rates of N addition, and at the peak of the growing season (mid-August). However, soil respiration was consistently higher in the lowest (1 g N m⁻² year⁻¹) treatment compared to ambient conditions from mid-May through October. Both GPP and ER peaked at low N supply, around 1.5 g N m⁻² year⁻¹, but contrary to our hypotheses, these fluxes offset one another, such that there were no observed effects of N on NEE. Despite significant negative impacts of N addition on belowground plant biomass, microbial biomass C, and soil microbial respiration, we found no effect of N addition on soil C after 13 years of N fertilization. Our findings highlight the importance of concurrently measuring both pools and fluxes of C, as we found strong effects of low rates of N addition on plant C uptake and ecosystem C loss, but not on net ecosystem C uptake or storage.

We found consistent nonlinear relationships between N addition and plant-driven processes; AGB, BNPP, GPP, ER, and soil respiration all peaked between 1 and 3 gNm⁻² year⁻¹, and root mass fraction had a valley in the same range. N addition up to about 3 gNm⁻² year⁻¹ generally increased plant C uptake and emission processes, but these effects declined back to Control levels with further increases in N addition rate. Similar hump-shaped responses in plant biomass have been found across other N gradient experiments, notably on the Tibetan plateau (Peng et al., 2017a, 2017b), which could indicate broad applicability of our results across global grasslands. The shapes of our results are remarkably similar to the Peng



FIGURE 2 Soil pH (a; 1:1 w/v in water), soil C (b), base cation concentration (c), and microbial biomass carbon (d: MBC) in the soil at peak biomass. Both pH and MBC decreased linearly with N addition, but neither soil C nor base cation concentrations varied with N addition

and colleagues' studies, despite very different soil textures (>88% sand vs. 61% sand), soil pH values (5.5 vs. 9.5), and background and historical N deposition rates (~1 vs. 4g Nm⁻² year⁻¹), respectively. Thus, the unimodal response shapes (e.g., Peng et al., 2017b) may be common across global grasslands where N is often a limiting resource. Our study site is characterized by poorly buffered, N-poor, acidic, and sandy soils (Grigal et al., 1974); thus, negative effects of high rates of N addition may accumulate more quickly than in wellbuffered or more alkaline or neutral soils such as those found on the Tibetan plateau. Although the shape of the response curve to increasing N addition may be broadly similar across grasslands, the degree to which high rates of N addition reduce plant C uptake and emission processes (i.e., the slope or curvature of the response) may depend in part on a site's soil characteristics. The consistent unimodal responses across many of our response variables suggest that negative consequences of N addition eventually counteract the direct effects of N on plant growth and C fixation; we propose several biotic and abiotic mechanisms that may underlie this trend.

Unimodal effects on plant biomass may arise from increased consumer offtake with increasing N supply rates. Biomass loss via herbivory or pathogen infection also may have led to the decreases in aboveground biomass at high compared to low levels of N addition. Across grassland soils, N addition can alter tissue stoichiometry, resulting in more nutrient-rich tissues that are more attractive to herbivores

(Anderson et al., 2018; Firn et al., 2019; Hillebrand et al., 2009; Mattson, 1980). Specifically, insect, pathogen, and fungal damage on leaves can increase at high levels of N addition in grasslands (Ebeling et al., 2021; Lind et al., 2017; Throop & Lerdau, 2004); high rates of pathogen damage with N addition have previously been found in the highest N addition plots of this experiment (Ebeling et al., 2021). Additionally, Seabloom et al. (2017) found that, across a spectrum of plant diversity, pathogens and fungi removed a constant proportion of plant biomass. In the context of our results, this could mean that biomass reduction from herbivory or infection negates biomass gain from high rates of N fertilization. Mammalian herbivores also can remove significant amounts of biomass; across global grasslands, large vertebrate herbivory increases in fertilized grasslands, although offtake is proportionally less compared to non-fertilized conditions (Borer et al., 2020). Pocket gophers (Geomys bursarius), which are abundant at our site, can have large effects on soil nutrient cycling and plant productivity (Eviner & Chapin, 2005; Reichman & Seabloom, 2002), and have been recorded to increase the rate at which they build mounds and disturb the soil as N input increases (Huntly & Inouye, 1988). Thus, we hypothesize that increased vertebrate herbivore, insect, pathogen, and fungal damage could be contributing to the reduction of AGB at higher N input rates compared to low N input rates.

Unimodal effects of increasing N on soil respiration may be due to a tight linkage between this belowground response and live



FIGURE 3 Soil microbial respiration (a) and total soil respiration (b) at peak season. Soil microbial respiration (after 143 days of incubation) decreased linearly with N addition ($R^2 = 0.38$, p = 0.002), and soil respiration was best described by a nonlinear function with a peak around $1.25 \text{ gNm}^{-2} \text{ year}^{-1}$

biomass. Soil respiration, the only belowground response with a unimodal shape, is often strongly controlled by rates of photosynthesis and fresh aboveground C inputs from that support root respiration, priming, and belowground fungal symbionts (Janssens et al., 2010). Belowground net primary productivity followed a similar pattern to soil respiration, which could indicate that the observed trends in soil respiration may be associated with root respiration. Taken together with the response of AGB, around 17% of the variance in soil respiration was explained by annual plant production. Given that rates of soil respiration decreased steeply at rates of N addition higher than about 1.5 gNm⁻² year⁻¹, and that heterotrophic respiration decreased across all N levels, it could be that rates of plant production at higher N levels were negatively affected by factors that directly affect heterotrophic microbial respiration, such as soil chemistry, non-N nutrient availability, or by microbial pathogen infection (Kohli et al., 2020).

Added N had different impacts on most plant versus soil microbial responses, and negative impacts of N addition on soil may have contributed to the impacts on plant responses at high rates of N addition. Reductions in soil pH or microbial biomass C with high levels of N addition may have impacted plant nutrient acquisition such that plant growth was reduced compared to low N conditions, for example. A past study in this same experiment found no difference in MBC effects of N addition after 9 years (Widdig et al., 2020), but we found a strong negative effect of N on MBC after 13 years of N addition. Furthermore, soil pH was similar across all treatments after 10 years of N addition (Wilcots et al., 2021), but here we found significant declines in soil pH after 13 years of N addition, similar to the MBC response. Thus, we have evidence that soil acidification caused soil MBC to decline over time with added N, as has been found in many other grassland N addition studies (e.g., Liu et al., 2014; Liu & Zhang, 2019; Zong et al., 2017). Decreasing soil MBC also can be associated with changes in bacterial and fungal community composition, which can affect soil nutrient cycling and ecosystem functioning (Liu & Zhang, 2019; Zeng et al., 2016). Soil acidification has further been shown to greatly reduce rhizosphere respiration and plant C inputs to the soil (Janssens et al., 2010), and recent work indicates that soil microbes are often C-limited under elevated N conditions (Ning et al., 2021). We also found significant decreases in heterotrophic microbial respiration with N addition, which was highly correlated with soil pH, similar to previously observed results (Ning et al., 2021; e.g., Riggs & Hobbie, 2016). Thus, lower microbial respiration at higher rates of N addition may be due to cumulative negative effects of acidification on belowground communities. Studies all over the world have similarly found decreases in soil pH as a result of N addition (Bowman et al., 2008; Liu & Zhang, 2019;



FIGURE 4 GPP (a), ER (b), and NEE (c) at peak biomass. GPP and ER were both best described by nonlinear functions that peaked at 1.4 and 1.5 gNm⁻² year⁻¹, respectively. NEE, however, was not affected by N addition

e.g., Roem et al., 2002), so understanding how soil acidification impacts C uptake and emission will be important to predicting future grassland C stocks globally.

The negative effect of N supply on pH could help to explain the decrease in AGB at higher N addition levels. Many studies have shown that decreasing soil pH reduces soil fungi: bacteria ratios (Ning et al., 2021; Widdig et al., 2020), including by reducing arbuscular mycorrhizal fungi (AMF) biomass (Ning et al., 2021; Pan et al., 2020). Given that AMF increase plant phosphorus (P) uptake, we hypothesize that decreasing soil pH may be indirectly leading to P-limitation of plant function in this experiment. This may explain the discrepancies in response between this study and similar studies from an adjacent field (Clark & Tilman, 2008; Seabloom et al., 2021; e.g., Wedin & Tilman, 1996) that added N in conjunction with P, potassium (K), and many commonly limiting micronutrients. In these other studies, the addition of micronutrients and liming also prevented base cation depletion, and even alkalized the soil at high input rates (Seabloom et al., 2021). It is possible that higher rates of plant biomass accumulation in these studies were due to a relaxation of P, K, or micronutrient co-limitation, rather than an effect of N addition alone (Seabloom et al., 2021). Furthermore, a recent study from the first 10 years of same experiment described here found increases in AGB at all N input rates, and no changes in soil pH (Wilcots et al., 2021). A recent metanalysis of many global change-focused experiments found that

many experiments only begin to show strong treatment effects after 10 years of manipulation (Komatsu et al., 2019). In the context of this past work, we hypothesize, based on our results here, that the negative consequences of acidification at higher N addition levels added over more than a decade may override possible increases in productivity from fertilization (Kimmel et al., 2020; Silvertown et al., 2006).

Despite increases at low N levels in growth (AGB, BNPP, and GPP), increases in respiration (ER, soil respiration) offset plant C uptake such that there were no changes in net ecosystem C uptake rates or in soil C stocks. The peak in both GPP and ER between 1 and 2 g N m⁻² year⁻¹ added indicates that the stimulation of both C uptake and emissions in this ecosystem occur at around the same N input rate, and are likely mechanistically linked, as discussed previously. This further demonstrates the importance of measuring both C inputs and outputs when estimating C uptake or storage under N addition; measures such as AGB or ANPP that have been previously used to infer how ecosystem C stocks might change with added N are not sufficient (e.g., Wedin & Tilman, 1996). Furthermore, our results indicate that current rates of N deposition are not likely to provoke significant changes in the size of the grassland C sink. Recent evidence from this and other North American grassland sites also indicate that 10+ years of high rates of N addition do not trigger changes in soil C pools (Keller et al., 2021). Our results clarify the role N may play in soil C storage in this ecosystem: previous studies near the site of this study have added N in



FIGURE 5 GPP (a), ER (b), NEE (c), and soil respiration (SR) (d) across the growing season. Throughout much of the growing season, total ecosystem fluxes (GPP, ER, and NEE) were not different between treatments; however, in mid-August GPP was significantly different between treatments (p = 0.04). On the other hand, soil respiration was significantly different between treatments beginning in late May (DOY = 150) until mid-September (DOY = 258). Solid points are the mean flux rate from sampling date for each treatment, and open points are data from each plot on each sampling date. All fluxes are in units of μ mol CO₂ m⁻² s⁻¹

combination with other nutrients and have observed increases in soil C storage (Fornara & Tilman, 2012; Seabloom et al., 2021; e.g., Wedin & Tilman, 1996). These findings likely reflect synergistic effects of N added with other nutrients that cause greater increases in biomass than N alone, as well as potential effects of cations on soil C stabilization. In contrast to these previous findings, our results indicate that N alone does not increase soil C storage in this ecosystem. Broadly, this could indicate that plant growth stimulated by N deposition may not ultimately increase soil C, limiting the ability of grasslands to counteract increases in atmospheric CO_2 .

Our work highlights the need for more studies across gradients of realistic input levels of N (Bebber, 2021). The observed response to N of AGB, BNPP, and ecosystem and soil C fluxes were all nonlinear, indicating that predicting ecosystem responses at lower N addition rates is not always possible from high N addition studies. Importantly, our results demonstrate that most N addition studies may underestimate N effects, especially in areas with poorly buffered or low N soils such as our site. In the context of a recent metaanalysis showing that the average N addition rate in experiments was $12.5 \text{ gNm}^{-2} \text{ year}^{-1}$ (Midolo et al., 2019), we should exercise caution in interpreting potential effects of N deposition from these studies given that global N deposition rates rarely exceed 2 g N m⁻² year⁻¹. In a similar vein, recent work from N gradient experiments that include very low rates of N input in Inner Mongolia (Bai et al., 2010; Pan et al., 2020; Song et al., 2011) and Tibet (Peng et al., 2017a, 2017b) also found nonlinear effects of N addition on biomass, ecosystem C fluxes, and plant and microbial community composition. Taken in this context, the current work indicates that nonlinear ecosystem responses to N enrichment are common, and that work focused on high rates of N addition is likely underestimating the impact of N supply to C flux and storage in grassland ecosystems.

Grasslands cover 40% of Earth's ice-free surface and store approximately one-third of all terrestrial C (Scurlock & Hall, 1998); thus, a better understanding of controls on grassland C uptake versus C emission is critical for understanding the role of grasslands in feedbacks to atmospheric CO_2 concentrations. Here, we found that increases in plant biomass and gross primary productivity at low rates of N addition were counteracted by increases in respiration, resulting in no net change in C uptake, and that high rates of N addition can reduce plant growth and C uptake via soil acidification. By measuring both the C fluxes and pools across an N gradient that encompasses the global range of N deposition rates, we can more accurately predict

how ecosystems may be responding to N deposition (Bebber, 2021). Our work indicates that rates of N deposition between 1 and $3 \text{ gNm}^{-2} \text{ year}^{-1}$ may increase plant biomass and ecosystem C fluxes in grasslands, but in the absence of other environmental changes, these increases will ultimately not change the size of the grassland C sink.

AUTHORS' CONTRIBUTIONS

ETB and EWS designed the experiment, all authors contributed to collecting data, MEW, KMS, SJK, and LCD performed the preliminary analyses, MEW wrote the manuscript, and all authors contributed edits to the manuscript.

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DATA AVAILABILITY STATEMENT

Data are archived through the Environmental Data Initiative. Data can be accessed at: 10.6073/pasta/85348706ff48588b2b3262111c 22c63e, 10.6073/pasta/e731badf4f4453ccc3c408bf2893d72c, and 10.6073/pasta/ff00b3d3fe927098df8d0d03403be293

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