



# Differential Resilience of Soil Microbes and Ecosystem Functions Following Cessation of Long-Term Fertilization

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## ABSTRACT

Nitrogen (N) from anthropogenic sources has dramatically increased in terrestrial ecosystems globally. Although belowground microbial processes and events that release N into the atmosphere, such as fire, could support ecosystem resilience to eutrophication, little is known about how these factors might affect N loss following chronic fertilization, thus promoting ecosystem recovery. We studied how N pools, N-cycling potential rates and their affiliated microbial populations, and microbial community composition responded to the factorial effects of cessation of 30 years of chronic N fertilization and annual burning at a tallgrass prairie. Soil N availability in previously fertilized plots recovered by 86% (using never-fertilized control plots as a 100% recovery reference), while plant and microbial biomass did not change. Nitrification potential recovered (80%), and denitrification potential partially recovered (50%), in previously fertilized plots.

There were differential responses among key groups of nitrifiers (archaea vs. bacteria) and denitrifiers (*nosZ* clade I vs. clade II from *Anaeromyxobacter dehalogenans*) to long-term N fertilization and cessation despite the whole microbial community composition not shifting significantly after fertilization ceased. Also, N-cycling potentials were consistently higher in unburned prairie. Together, results suggest that fire is a prominent mechanism for ecosystem N removal in annually burned prairie, while N-cycling microbes will have an important role in the absence of fire; however, the recovery to pre-fertilized condition, with or without fire, will take longer than 1 year. Overall, differential resilience of biotic populations and processes can potentially shape different outcomes of soil N loss and tallgrass prairie ecosystem recovery from long-term N fertilization.

**Key words:** tallgrass prairie; fire; nitrogen fertilization; resilience; microbial diversity; nitrification; denitrification.

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## HIGHLIGHTS

- Available soil N dropped 86% following cessation of a 30-year fertilization treatment

- Potential nitrification, denitrification and immobilization recovered differentially

## INTRODUCTION

Across the globe, nitrogen (N) availability has increased substantially in terrestrial ecosystems that were historically limited in N (Galloway and others 2008; Schlesinger 2009). Elevated atmospheric deposition of N from fossil fuel emissions (Vitousek and others 1997) and increasing rates of N fertilizer application to croplands (Fixen and West 2002) have augmented N availability for plants and soil microbes. This terrestrial “eutrophication” tends to increase aboveground net primary productivity (ANPP; Gough and others 2000; LeBauer and Treseder 2008) but can also lower the richness of plant species (Stevens and others 2004; Clark and Tilman 2008), and cause turnover of plant functional groups (Suding and others 2005), particularly the loss of plants that are more competitive in nutrient-poor soils (Tilman and Wedin 1991). Belowground, the linked composition and function of soil microbial communities is also responsive to N fertilization (Ramirez and others 2012), with a consistent taxonomic response across many grassland soils (Leff and others 2015). Microbial biomass often decreases with N addition in non-agricultural soils (Treseder 2008; Zhang and others 2018), while nitrification and denitrification activities, and the populations that carry out these processes, tend to increase (Lu and others 2011; Ouyang and others 2018). Together, both plant and microbial dynamics underlying ecosystem N-cycling may shift substantially with chronic N addition.

Fire or denitrification could contribute significantly to N removal and ecosystem recovery.

In some terrestrial biomes, like the tallgrass prairie, suppressing natural disturbances such as fire can also increase total soil N by preventing the combustion of organic N from plant litter (Raison and others 1985; Ojima and others 1994). Shifts from high to low fire frequency can concurrently promote net soil N accumulation (Turner and others 1997) and increase N loss over time (Dell and others 2005) from leaching and denitrification, pointing to higher N-cycling rates and changes in the microbiota driving these processes. Although microbes are consistently responsive to N addition in prairie soils with contrasting fire regimes, the magnitude of response is substantially higher in soils with elevated baseline N availability due to fire suppression (Carson and Zeglin 2018). The consequences of interactions between these two com-

mon global change factors, fire suppression and increased N availability, may allow the establishment of woody plant species (Silva and others 2013) and further increase total soil N. Furthermore, although both fire and microbial activity have substantial roles in ecosystem N loss following chronic fertilization, they have rarely been compared as mechanisms for ecosystem recovery from chronic fertilization (Stevens 2016).

Ecosystem resilience to eutrophication can be assessed by manipulating nutrient availability. Ecological states, measured as key population sizes, community metrics, and ecosystem process rates, often respond to altered environmental drivers, including exogenous N addition. The sensitivity of a state to an altered driver reflects the magnitude of responsiveness (Smith and others 2009; Ratajczak and others 2018). The resilience of the state to a driver is assessed by measuring the recovery, defined as the time until, or magnitude of, the sensitive state’s return to a prior or present reference control upon ceasing the driver (Grimm and Wissel 1997; Hodgson and others 2015; Scheffer and others 2015; Angeler and Allen 2016; Ratajczak and others 2018). Thus, a more resilient state recovers more quickly, or rebounds by a greater magnitude in the same period of time. Resistance, the inverse of sensitivity, is the state’s lack of response to a driver (Grimm and Wissel 1997; Hodgson and others 2015; Angeler and Allen 2016). Tracking multiple biotic and abiotic ecological states under current or ceased drivers allows for an understanding of how interactions between drivers, such as fire and fertilization, affect a state’s resilience and for an assessment of potential legacy effects (Gilliam and others 2019). Assessing the sensitivity and recovery of prairie ecosystems with different fire regimes to long-term N fertilization will aid our understanding of the mechanisms underlying ecosystem responses to anthropogenic change, both now and into the future.

Microbial diversity can modulate ecosystem responses to environmental change (Folke and others 1996; Loreau 2001). A recent meta-analysis showed that the compositional or functional properties of soil microbes to abiotic change, while driver- or site-dependent, are often sensitive to both short- and long-term alterations, with a surprisingly low prevalence of recovery (Shade and others 2012). Thus, differential and asynchronous responses of soil microbes to chronic fertilization can contribute to the sensitivity and recovery of ecosystem processes, including N cycling (Bardgett and Caruso 2020). Ammonia-oxidizing archaea

(AOA) and bacteria (AOB), for example, complete the first step in nitrification as a requirement of their chemolithoautotrophic lifestyle, but differences in AOA and AOB sensitivity to added N substrate can moderate rates of nitrate ( $\text{NO}_3^-$ ) production (Verhamme and others 2011; Prosser and Nicol 2012; Carey and others 2016). Because of their differences in growth rate and activity, shifts in AOA and AOB abundances, quantified as the AOA/AOB ratio, can indicate changes to the nitrification potential and  $\text{NO}_3^-$  availability in soil (Prosser 1990; Jung and others 2011; Prosser and Nicol 2012), with AOB abundance more likely to increase after fertilization, and to correlate positively with nitrification potentials, than AOA abundance (Di and others 2010; Carey and others 2016). Denitrifier responses to N fertilization, in contrast, may be weaker and less coherent due to the facultative nature of denitrification, as well as the reliance of some  $\text{N}_2$  producers on other soil microbes for  $\text{N}_2\text{O}$  production (Sanford and others 2012; Hallin and others 2018). Yet, denitrifiers together regulate the loss of soil N as leached  $\text{NO}_3^-$ , inert atmospheric gas, or heat-trapping atmospheric gas. Therefore, in order to understand ecosystem resilience, the sensitivity and potential recovery of soil microbes and their functions need to be addressed, especially following chronic changes to N availability.

Nitrogen loss from a tallgrass prairie ecosystem following long-term fertilization could be underpinned by either fire or microbes. Dominant grasses in tallgrass prairie (for example, *Andropogon gerardii*) can assimilate and retain substantial amounts of N (Tilman and Wedin 1991). Higher N mineralization along with greater plant uptake can increase the aboveground biomass and N content in plant tissue (Tilman and Wedin 1991). Fire, therefore, could ameliorate nutrient eutrophication by combusting plant litter N and preventing its remineralization. However, soil N mineralization in the absence of fire can remain elevated up to 12 years following fertilizer cessation (Clark and others 2009; O'Sullivan and others 2011), during which time subsequent belowground N loss mechanisms depend heavily on soil microbes. Nitrification and denitrification, together with mineralization, would support ecosystem N loss through increased leaching and gaseous N emission. After decades of N addition, a lack of recovery in soil microbial populations and their functions may result in high potential for N loss even in the absence of fertilizer, independent of fire.

We assessed the strength of mechanisms underlying ecosystem resilience to chronic N enrichment

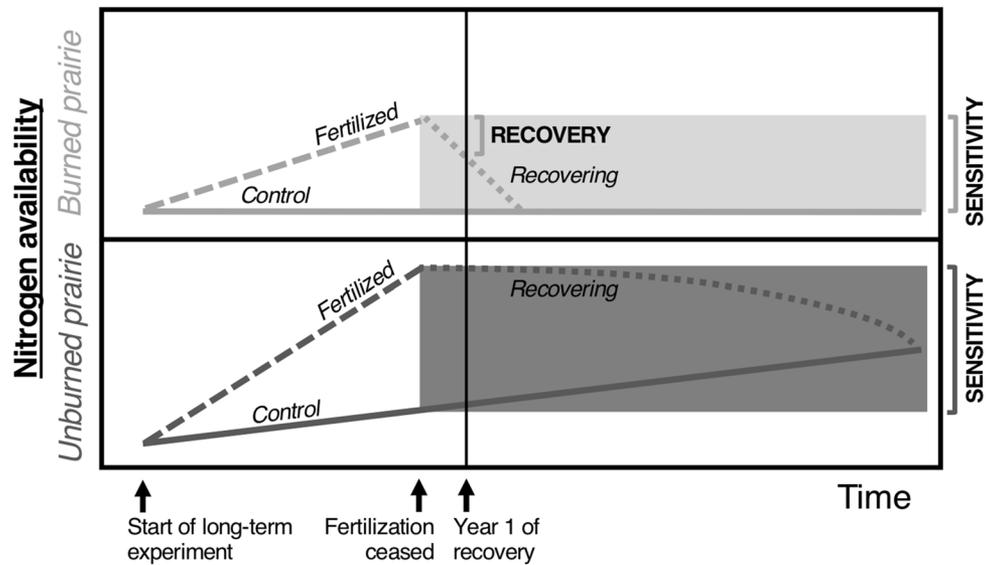
by ceasing long-term N fertilization at a 30-year grassland field experiment with contrasting fire treatments (annual fire versus no fire since 1986). During the first growing season without N application (2017), we measured the sensitivity and recovery of plants and soil microbes in burned and unburned prairie using long-term plant data collected from unfertilized and fertilized soils and by continuing to fertilize subplots of soil to collect microbial data not obtained in previous years. We hypothesized the magnitude of recovery toward an unfertilized state relative to long-term fertilized conditions would depend on how directly each functional group's metabolism and growth relied on the supplemental inorganic N. Thus, we predicted microbial nitrifiers would have the greatest recovery from fertilizer cessation, denitrifiers and plants would recover to some extent, and the total heterotrophic microbial community, reliant primarily on soil organic nitrogen, would recover the least. We also expected greater recovery in the annually burned prairie, due to fire's role in reducing N availability in this ecosystem, and higher reliance of all biota on supplemental N in that more N-limited system (Figure 1).

## MATERIALS AND METHODS

### Site Description and Experimental Design

This work was performed at Konza Prairie Biological Station (KPBS), a 3,487-hectare tallgrass prairie reserve in the Flint Hills region of Northeastern Kansas near Manhattan, KS, USA (39° 05' N, 96° 35' W). The Belowground Plots (BGP) experiment, located at KPBS, was established in 1986 by the Konza Prairie Long-Term Ecological Research (LTER) program to evaluate linked plant–soil responses to nutrient amendments and fire. This experiment is situated on Irwin series (fine, mixed, mesic, pachic Arguistolls) silty clay loam soil, has a mean annual precipitation of 835 mm and mean annual temperature of 26.6°C, and is dominated by perennial  $\text{C}_4$  grasses (*A. gerardii*, *Panicum virgatum*) in burned treatments and encroaching woody plants (*Juniperus virginiana*, *Cornus drummondii*) in unburned treatments (Carson and others 2019).

The BGP experiment includes eight 50 m × 25 m field blocks with split-strip plots. Blocks have either burned annually each spring (March–April), or remain unburned since the experiment's inception. A mowing treatment to mimic grazing originally split all the blocks, and N and phosphorus fertilizers were applied at the



**Figure 1.** Our conceptual model of tallgrass prairie ecosystem sensitivity and resilience to long-term nitrogen (N) fertilization under contrasting fire treatments. Ecological components may change differentially with increasing N availability, thus regulating the sensitivity and recovery of ecosystem states. Annual fertilization (height of the shaded box) can increase independently in unburned prairies because of the higher baseline level of ecosystem N. Following the cessation of fertilization, the magnitude of recovery (difference between fertilized and recovering prairies) is hypothesized to depend on the prairie's fire treatment. We predict that ecological states of burned prairies will recover quicker than unburned prairies.

beginning of each growing season (June) as strips. For this project, we focused on responses in the unmowed, annually N fertilized ( $10 \text{ g N m}^{-2} \text{ y}^{-1}$  as  $\text{NH}_4\text{NO}_3$ ) versus unfertilized control plots ( $12.5 \text{ m} \times 12.5 \text{ m}$ ). In 2017, fertilizer application at the BGP ceased, while long-term plant biomass and community data collections continued, allowing for a comparison of plant recovery. To enable recovery assessment of the soil microbial parameters not monitored prior to 2017, we continued to fertilize four  $1 \text{ m} \times 1 \text{ m}$  subplots randomly within each historically fertilized plot. Thus, with each field treatment having four separate replicate plots ( $n = 4$ ), we compared three fertilization treatments (control, recovering, and fertilized), crossed with burned and unburned treatments, for soil chemistry and microbial responses.

### Soil Collection

Soils were collected every five weeks between April and September 2017. In June, samples were collected one week after fertilizer application on the subplots. Four 2 cm diameter, 20 cm depth mineral soil cores were randomly sampled from each plot and combined for one composite sample per plot and treatment. Fertilized treatment soils were collected similarly by extracting one core from each fertilized subplot and combining them into one

composite plot-scale sample. Soils were aseptically sieved to remove particles larger than 4 mm and as many roots as possible, then a  $\sim 15 \text{ g}$  subsample of each composite sample was stored at  $-80^\circ\text{C}$  for molecular work, a  $\sim 50 \text{ g}$  subsample was stored at  $-20^\circ\text{C}$  for soil chemistry and microbial biomass, and remaining soil was kept at  $5^\circ\text{C}$  before N-cycling activity potential assays. All response variable measurements are described below, with more methodological detail available in the Supplemental Text (Appendix 1).

### Soil Chemistry and Microbial N-Cycling

Soil inorganic N available for plant and microbial uptake was estimated using resin bags buried from June to September (Baer and Blair 2008). The integrated amount of resin-sorbed ammonium ( $\text{NH}_4^+-\text{N}$ ) and  $\text{NO}_3^--\text{N}$  was quantified using a modified indophenol method and  $\text{VCl}_3$ /Griess reagent method (Hood-Nowotny and others 2010) and measured spectrophotometrically with a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Soil %C and %N were measured by the Kansas State University Soil Testing Laboratory in Manhattan, KS, USA, using a LECO TruSpec CN Combustion Analyzer (LECO Corporation, St. Joseph, MI, USA), and soil pH was

measured in a 1:1 slurry of field-moist soil and DI H<sub>2</sub>O, on soils collected in September 2017.

Gravimetric water content (GWC) of fresh soil was estimated by drying about 5 g of soil at 105°C overnight. Microbial biomass C (MBC) and N (MBN) were estimated using the chloroform fumigation-extraction method (Brookes and others 1985; Vance and others 1987; Zeglin and Myrold 2013). For fumigated and unfumigated samples, dissolved organic C (DOC) was quantified via combustion using a Shimadzu TOC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), and organic N was converted to NO<sub>3</sub><sup>-</sup>-N using a persulfate digestion and quantified as noted above. Difference in DOC and extractable total inorganic N between fumigated and unfumigated samples was used to estimate microbial biomass C and N.

Nitrogen cycling activity potential assays were run on fresh soil samples within 48 h of collection. Nitrification potential (NP) was estimated as the rate at which a saturating concentration of NH<sub>4</sub><sup>+</sup> was oxidized to NO<sub>3</sub><sup>-</sup> in 24 h in an aerobic soil slurry (Taylor and others 2010). Denitrification potential (DNP) and denitrification enzyme activity (DEA) were measured together. DEA assays were supplemented with glucose and KNO<sub>3</sub> (Groffman and others 1999) to estimate the maximum enzymatic potential for NO<sub>3</sub><sup>-</sup> reduction in each soil sample, while DNP assays were not amended with nutrients, to estimate the NO<sub>3</sub><sup>-</sup> reduction potential attainable under substrate-limited field conditions. Activities were estimated as the rate at which NO<sub>3</sub><sup>-</sup> was reduced to nitrous oxide (N<sub>2</sub>O) over 1 (DEA) or 4 (DNP) hours in an anaerobic soil slurry, in the presence of acetylene to prevent N<sub>2</sub>O reduction to N<sub>2</sub>. N<sub>2</sub>O-N was measured using a Shimadzu 2014 GC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

### Molecular Microbial Analysis

Genomic DNA (gDNA) was extracted from 0.5 g soil using a modified CTAB approach (DeAngelis and others 2010), and yields were estimated using Quant-iT PicoGreen assay kits (Life Technologies, Grand Island, NY, USA). The abundance of archaeal and bacterial *amoA* genes (Rotthauwe and others 1997; Mosier and Francis 2008), the bacterial *nosZ* clade I (Henry and others 2006) and clade II genes (clade II from *Anaeromyxobacter dehalogenans* only; Sanford and others 2012), and bacterial 16S rRNA genes (Fierer and others 2005) were estimated using quantitative polymerase chain reactions (qPCR), with non-template controls,

melting curves and standard curve efficiencies to assure target specificity (detail in Appendix 1). Gene abundance was calculated from standard curves and normalized against the gDNA yield kg<sup>-1</sup> dry soil. The bacterial and archaeal V4 region of the 16S rRNA gene was PCR amplified using an established one-step protocol (Caporaso and others 2012), with the exception that only 25 cycles were used instead of 35 (Carson and Zeglin 2018). Sequencing was performed with Illumina MiSeq Technology (Illumina, San Diego, CA, USA) using 2 × 150 paired end cycles at the Kansas State University Integrated Genomics Facility. QIIME1 bioinformatics software was used to process the raw data collected from the Illumina sequencing run (Caporaso and others 2010; detail in Appendix 1). All raw sequence data are deposited with links in the NCBI BioProject database under accession PRJNA577961 (<https://www.ncbi.nlm.nih.gov/bioproject/>).

### Aboveground Plant Responses

Aboveground biomass of grasses, forbs, and woody plants was collected annually from 1986 to 2017 at the end of the growing season (September–October) for all plots. Plant mass was clipped in the field at two 0.1 m<sup>2</sup> quadrats per plot, dead litter removed, live tissue sorted into the functional groups, oven-dried, and weighed. Aboveground net primary production (ANPP) was estimated for each functional group by converting the dry weight accumulated in 1 year to g m<sup>-2</sup>. To evaluate whether plant biomass changed following fertilizer cessation, the 2013–2017 data (4 years before and 1 year after fertilizer cessation) were compared. All data are available at the Konza LTER Data Portal in dataset PBB01 (Blair and Zeglin 2020).

### Statistical Analysis

Mixed-effect models were used to test the null hypotheses that soil chemistry, microbial, and plant variables had no responses to N fertilization or contrasting fire treatments, or to the cessation of chronic fertilizer application. The mixed-effect models, with a plot within block random effect, were performed for variables with repeated sampling to test the fire, fertilization, and date-of-sampling (that is, Month or Year) fixed effects by using the lme4 package in RStudio version 1.2.1335 (Bates and others 2015; RStudio Team 2018). Shapiro–Wilk tests and QQ-plots were used to assess the distribution of the response variables, and data were log<sub>10</sub> transformed (except for plant biomass which was square root transformed) to

meet normality assumptions when needed. The assumption of homoskedasticity for the mixed-effect models was tested by plotting the residual versus fitted values prior to analysis of variance (ANOVA). Tukey's honest significant difference (HSD) post hoc tests were used to distinguish differences between the levels in the fixed effects or their interactions using the *lsmeans* package (Lenth 2016). The contribution of the contrasting treatments to variation in soil bacterial and archaeal 16S rRNA gene community composition was assessed using indirect ordination and permutational analysis of variance (PERMANOVA). Ten iterations of multivariate non-metric multidimensional scaling (NMDS) visualized the Bray–Curtis dissimilarity of microbial communities using the *vegan* package (Oksanen and others 2019); the model of best fit (stress = 0.1409) was accepted. PERMANOVA was performed with the Bray–Curtis dissimilarity matrix and comparing the sampled communities to 999 null communities drafted from the composition data. Null hypotheses were rejected when  $P \leq 0.05$ . More detail for the statistical analysis is found in the Supplemental Text (Appendix 1).

We considered *sensitivity* to chronic fertilization as the change of measured variables in the long-term fertilized treatment relative to the unfertilized control. To quantify the relative level of *recovery* from chronic fertilization, we calculated the percent recovery of the variable of interest using this equation

$$\left[ 1 - \left( \frac{\frac{\bar{X}_R}{\bar{X}_C} - 1}{\frac{\bar{X}_F}{\bar{X}_C} - 1} \right) \right] \times 100$$

where  $\bar{X}$  is the mean value of the variable in control (C), recovering (R), or fertilized (F) soils.

## RESULTS

### Soil Available Nitrogen

Total available soil inorganic N was always higher in unburned than annually burned treatments and dropped substantially after fertilization ceased in both fire treatments (Table 1, Figure 2). Average  $\text{NH}_4^+\text{-N}$  fell from 4.6 and 20.2 mg  $\text{NH}_4^+\text{-N}$  per kg resin in the chronically fertilized burned and unburned soils, respectively, to 1.4 and 0.6 mg  $\text{NH}_4^+\text{-N}$  per kg resin in the recovering soils, which was comparable to control soils ( $P < 0.001$ ; Figure 2A). Between the fertilized and recovering soils, average  $\text{NO}_3^-\text{-N}$  dropped from 19.8 to 3.5 mg  $\text{NO}_3^-\text{-N}$  per kg resin in annually burned prairie and from 59.4 to 10.2 mg  $\text{NO}_3^-\text{-N}$  per kg resin in the unburned

prairie, but these levels were still higher than control soils (0.7 and 2.0 mg  $\text{NO}_3^-\text{-N}$  per kg resin in annually burned and unburned treatments, respectively;  $P < 0.001$ ). The control, annually burned treatment soils had the lowest available  $\text{NO}_3^-\text{-N}$  and total inorganic nitrogen (Figure 2B). The unburned treatment soils had higher  $\text{NO}_3^-\text{-N}$  availability than the annually burned treatment soils ( $P = 0.001$ ), and the ratio of  $\text{NO}_3^-\text{-N}/\text{NH}_4^+\text{-N}$  was highest in the recovering soils of the unburned treatment ( $P = 0.044$ ; Table 1, Figure 2B). Total inorganic N availability showed no interaction between fire and fertilization treatment effects, recovered significantly by 86.0% across all soils, yet remained 5 times higher in the recovering versus control treatment soils ( $P < 0.001$ ; Table 1).

### Soil Nitrification Potential Rates and Functional Composition

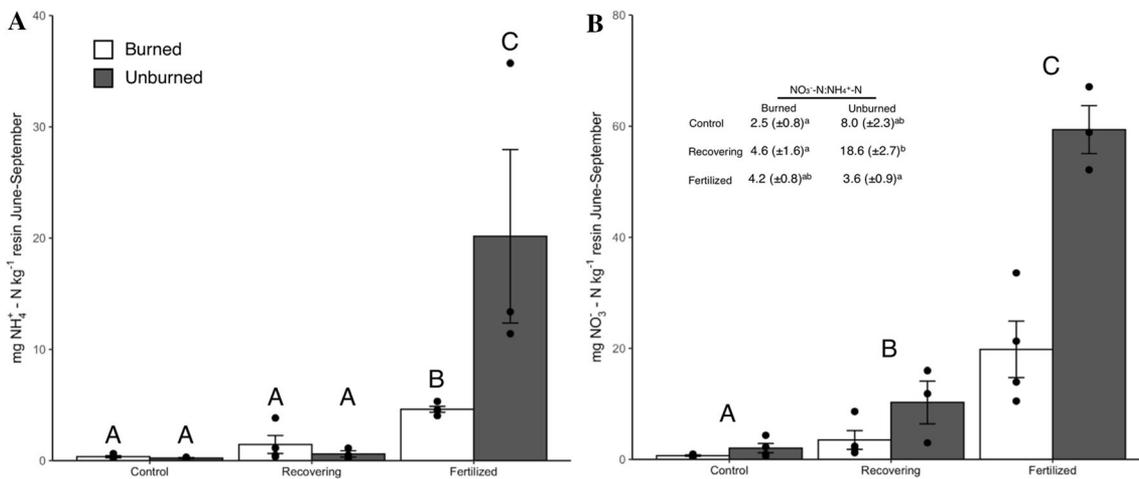
Nitrification potential (NP) rates were higher in both unburned and fertilized soils, and rates significantly declined in the first year of ceasing chronic fertilization to a level comparable to the control soils (Table 2, Figure 3). Comparing the fertilized and recovering soils, seasonal average NP rates dropped from 1.30 to 0.59 mg N  $\text{kg}^{-1}$  dry soil  $\text{h}^{-1}$  and from 3.07 to 1.25 mg N  $\text{kg}^{-1}$  dry soil  $\text{h}^{-1}$  in burned and unburned prairie soils, respectively ( $P < 0.001$ ; Figure 3A), corresponding to a 79.9% recovery in both fire treatments. A significant three-way interaction between fire, fertilization, and month reflects temporal variation in NP ( $P = 0.038$ ): In fertilized, unburned treatment soils, rates were significantly higher than all other treatments in June after fertilizer was added, and remained high; in contrast, NP was higher in September than June in all other treatments (Table 2, Figure S1a,b).

Soil archaeal *amoA* gene abundance (AOA) and bacterial *amoA* gene abundance (AOB) differentially responded to fire regime and the cessation of long-term N fertilization (Table 2, Figure 4A, B). AOB was consistently higher in unburned than annually burned treatment soils ( $P = 0.042$ ), and their abundance in recovering soils was significantly similar to control soils, but lower than chronically fertilized soils, for a 68.3% recovery magnitude ( $P < 0.001$ ; Figure 4B). In contrast, AOA did not change with fire regime, was higher in fertilized soils, and showed no significant recovery from chronic fertilization ( $P < 0.001$ ; Figure 4A). The AOA/AOB ratio did not differ between fertilization treatments. AOA was higher than AOB by about an order of magnitude, and the AOA/AOB

**Table 1.** Statistical Results for Two-way ANOVA on the Direct and Interactive Effects of Fertilization ("Fertilizer") and Burning ("Burn") Treatments on Soil Chemistry Response Variables

Soil chemistry variables	$F_{(\text{numDF}, \text{denDF})}$		
	Fertilizer	Burn	Fertilizer $\times$ Burn
$\text{NH}_4^+-\text{N}^{\dagger}$	<b>74.49</b> <sub>(2, 13.2)</sub>	3.27 <sub>(1, 13.8)</sub>	<b>10.00</b> <sub>(2, 13.2)</sub>
$\text{NO}_3^--\text{N}^{\dagger}$	<b>60.23</b> <sub>(2, 13.2)</sub>	<b>15.87</b> <sub>(1, 13.8)</sub>	0.81 <sub>(2, 13.2)</sub>
$\text{NH}_4^+-\text{N} + \text{NO}_3^--\text{N}^{\dagger}$	<b>72.25</b> <sub>(2, 13.6)</sub>	<b>13.29</b> <sub>(1, 14.2)</sub>	1.50 <sub>(2, 13.6)</sub>
$\text{NO}_3^--\text{N}:\text{NH}_4^+-\text{N}^{\dagger}$	3.56 <sup>+</sup> <sub>(2, 12.8)</sub>	<b>13.46</b> <sub>(1, 13.5)</sub>	<b>4.04</b> <sub>(2, 12.8)</sub>
%C	<b>4.25</b> <sub>(2, 14.1)</sub>	<b>21.30</b> <sub>(1, 14.1)</sub>	<b>5.14</b> <sub>(2, 14.1)</sub>
%N	<b>11.96</b> <sub>(2, 14.0)</sub>	<b>35.45</b> <sub>(1, 14.0)</sub>	3.18 <sup>+</sup> <sub>(2, 14.0)</sub>
C:N	<b>5.41</b> <sub>(2, 17.0)</sub>	0.55 <sub>(1, 17.0)</sub>	<b>4.41</b> <sub>(2, 17.0)</sub>
pH	<b>14.68</b> <sub>(2, 15.0)</sub>	1.12 <sub>(1, 15.0)</sub>	0.31 <sub>(1, 15.0)</sub>

Superscript "<sup>†</sup>" denotes variables that were log-transformed to meet assumptions of normality. Values bolded signify treatments in which  $P \leq 0.05$ , and superscript "+<sup>+</sup>" indicates where  $P = 0.10-0.051$ .



**Figure 2.** Estimated concentrations of **A**  $\text{NH}_4^+-\text{N}$  and **B**  $\text{NO}_3^--\text{N}$  sorbed to resin bags buried in soils under contrasting fertilizer treatments and fire regimes. The height of the bars (mean  $\pm$  standard error [SE]) corresponds to the plot and subplot means for each treatment combination and each point representing the individual plot and subplot concentration means during the course of the growing season. Tukey's honest significant difference (HSD) post hoc analysis results are shown with different letters indicating field treatment groups that differed at the  $P < 0.05$  level.

ratio was higher in the annually burned than unburned prairie soils ( $17.4 \pm 14.0 > 12.4 \pm 9.3$ ;  $P = 0.015$ ; Table 2). Like NP in control and recovering soils, both AOA and AOB abundance increased as the growing season progressed ( $P < 0.001$ ; Table 2, Figure S2a-d).

### Soil Denitrification Potential Rates and Functional Composition

Maximum denitrification enzyme activity potential (DEA) rates were low in April and peaked in July and August, but were not affected by fire or chronic fertilization (Table 2, Figure S1e, f). However, with only field-available levels of soluble C and  $\text{NO}_3^-$ , substrate-limited denitrification potential (DNP)

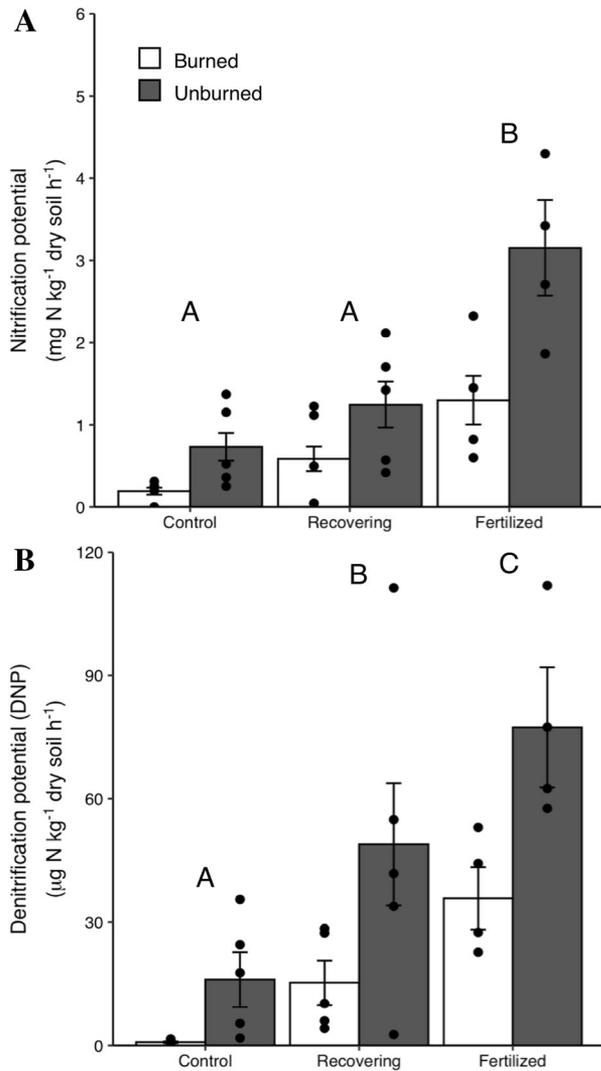
rates were higher in all unburned treatment soils ( $P = 0.007$ ), and rates were similar in the recovering and fertilized soils ( $P < 0.001$ ), with no fire by fertilizer interaction (Table 2, Figure 3B). Average DNP rates for both burned and unburned fire treatments, respectively, were  $35.8$  and  $77.4 \mu\text{g N}_2\text{O}-\text{N kg}^{-1}$  dry soil  $\text{h}^{-1}$  in fertilized soils,  $15.2$  and  $48.9 \mu\text{g N}_2\text{O}-\text{N kg}^{-1}$  dry soil  $\text{h}^{-1}$  in recovering soils, and  $0.8$  and  $16.0 \mu\text{g N}_2\text{O}-\text{N kg}^{-1}$  dry soil  $\text{h}^{-1}$  in control soils.

Bacterial *nosZ* gene abundances were consistently higher in annually burned than unburned treatment soils (clade I:  $P = 0.036$ ; clade II:  $P = 0.016$ ), but clade I and clade II responses differed in fertilizer response and temporal change

**Table 2.** Statistical Results for Three-way ANOVA on the Direct and Interactive Effects of Fertilization (“Fertilizer”), Burning (“Burn”), and Time (“Month” or “Year”) on Soil Microbial and Plant Response Variables

Microbial variables	$F_{(numDF, denDF)}$			Fertilizer × Burn × Month			Fertilizer × Burn × Month			Fertilizer × Burn × Month		
	Fertilizer	Burn	Fertilizer × Burn	Fertilizer × Burn	Month	Fertilizer × Burn × Month	Fertilizer × Burn	Month	Fertilizer × Burn × Month	Fertilizer × Burn	Month	Fertilizer × Burn × Month
NP <sup>1</sup>	<b>17.34</b> <sub>(2, 15.2)</sub>	<b>20.34</b> <sub>(1, 14.9)</sub>	0.62 <sub>(2, 15.2)</sub>	0.62 <sub>(2, 15.2)</sub>	<b>10.27</b> <sub>(4, 64.7)</sub>	<b>3.10</b> <sub>(7, 64.8)</sub>	0.69 <sub>(4, 64.7)</sub>	0.69 <sub>(4, 64.7)</sub>	<b>2.28</b> <sub>(7, 64.8)</sub>	0.69 <sub>(4, 64.7)</sub>	0.69 <sub>(4, 64.7)</sub>	<b>2.28</b> <sub>(7, 64.8)</sub>
DNP (Subs.-lim.) <sup>1</sup>	<b>13.87</b> <sub>(2, 15.4)</sub>	<b>9.90</b> <sub>(1, 15.1)</sub>	0.43 <sub>(2, 15.4)</sub>	0.43 <sub>(2, 15.4)</sub>	<b>4.21</b> <sub>(4, 64.6)</sub>	1.43 <sub>(7, 64.6)</sub>	2.03 <sub>(4, 64.6)</sub>	2.03 <sub>(4, 64.6)</sub>	0.42 <sub>(7, 64.6)</sub>	2.03 <sub>(4, 64.6)</sub>	2.03 <sub>(4, 64.6)</sub>	0.42 <sub>(7, 64.6)</sub>
DEA (+ CN) <sup>1</sup>	0.07 <sub>(2, 15.4)</sub>	0.10 <sub>(1, 15.1)</sub>	1.09 <sub>(2, 15.4)</sub>	1.09 <sub>(2, 15.4)</sub>	<b>4.18</b> <sub>(4, 61.4)</sub>	1.26 <sub>(7, 61.4)</sub>	1.86 <sub>(4, 61.4)</sub>	1.86 <sub>(4, 61.4)</sub>	1.36 <sub>(7, 61.4)</sub>	1.86 <sub>(4, 61.4)</sub>	1.86 <sub>(4, 61.4)</sub>	1.36 <sub>(7, 61.4)</sub>
Archaeal <i>amoA</i> <sup>1</sup>	<b>15.72</b> <sub>(2, 15.3)</sub>	0.26 <sub>(1, 15.1)</sub>	1.34 <sub>(2, 15.3)</sub>	1.34 <sub>(2, 15.3)</sub>	<b>12.05</b> <sub>(4, 55.6)</sub>	1.55 <sub>(7, 55.5)</sub>	0.67 <sub>(4, 55.6)</sub>	0.67 <sub>(4, 55.6)</sub>	1.38 <sub>(7, 55.5)</sub>	0.67 <sub>(4, 55.6)</sub>	0.67 <sub>(4, 55.6)</sub>	1.38 <sub>(7, 55.5)</sub>
Bacterial <i>amoA</i> <sup>1</sup>	<b>11.54</b> <sub>(2, 15.7)</sub>	<b>4.90</b> <sub>(1, 15.5)</sub>	0.70 <sub>(2, 15.7)</sub>	0.70 <sub>(2, 15.7)</sub>	<b>33.22</b> <sub>(4, 63.7)</sub>	0.83 <sub>(7, 63.6)</sub>	0.39 <sub>(4, 63.7)</sub>	0.39 <sub>(4, 63.7)</sub>	1.13 <sub>(7, 63.6)</sub>	0.39 <sub>(4, 63.7)</sub>	0.39 <sub>(4, 63.7)</sub>	1.13 <sub>(7, 63.6)</sub>
AOA <sup>1</sup> :AOB <sup>1</sup>	2.85 <sup>+</sup> <sub>(2, 15.7)</sub>	<b>8.89</b> <sub>(1, 15.1)</sub>	2.34 <sub>(2, 15.7)</sub>	2.34 <sub>(2, 15.7)</sub>	<b>15.76</b> <sub>(4, 57.8)</sub>	1.40 <sub>(7, 57.4)</sub>	0.66 <sub>(4, 57.8)</sub>	0.66 <sub>(4, 57.8)</sub>	1.24 <sub>(7, 57.4)</sub>	0.66 <sub>(4, 57.8)</sub>	0.66 <sub>(4, 57.8)</sub>	1.24 <sub>(7, 57.4)</sub>
<i>nosZ</i> clade I <sup>1</sup>	3.34 <sup>+</sup> <sub>(2, 15.2)</sub>	<b>5.31</b> <sub>(1, 15.1)</sub>	1.15 <sub>(2, 15.2)</sub>	1.15 <sub>(2, 15.2)</sub>	<b>104.48</b> <sub>(4, 63.1)</sub>	1.40 <sub>(7, 63.2)</sub>	0.14 <sub>(4, 63.2)</sub>	0.14 <sub>(4, 63.2)</sub>	1.10 <sub>(7, 63.2)</sub>	0.14 <sub>(4, 63.2)</sub>	0.14 <sub>(4, 63.2)</sub>	1.10 <sub>(7, 63.2)</sub>
<i>nosZ</i> clade II ( <i>A.deh</i> ) <sup>1</sup>	<b>14.71</b> <sub>(2, 15.8)</sub>	<b>7.27</b> <sub>(1, 15.6)</sub>	3.00 <sup>+</sup> <sub>(2, 15.8)</sub>	3.00 <sup>+</sup> <sub>(2, 15.8)</sub>	<b>181.90</b> <sub>(4, 63.7)</sub>	<b>3.20</b> <sub>(7, 63.7)</sub>	0.80 <sub>(4, 63.7)</sub>	0.80 <sub>(4, 63.7)</sub>	1.70 <sub>(7, 63.7)</sub>	0.80 <sub>(4, 63.7)</sub>	0.80 <sub>(4, 63.7)</sub>	1.70 <sub>(7, 63.7)</sub>
Clade I <sup>1</sup> /clade II <sup>1</sup>	1.67 <sub>(2, 15.1)</sub>	1.61 <sub>(1, 15.0)</sub>	0.21 <sub>(2, 15.1)</sub>	0.21 <sub>(2, 15.1)</sub>	<b>370.42</b> <sub>(4, 63.1)</sub>	<b>3.87</b> <sub>(7, 63.1)</sub>	1.21 <sub>(4, 63.1)</sub>	1.21 <sub>(4, 63.1)</sub>	0.48 <sub>(7, 63.1)</sub>	1.21 <sub>(4, 63.1)</sub>	1.21 <sub>(4, 63.1)</sub>	0.48 <sub>(7, 63.1)</sub>
Bacterial 16S rRNA	<b>4.20</b> <sub>(2, 15)</sub>	0.051 <sub>(1, 15.0)</sub>	0.85 <sub>(2, 15.0)</sub>	0.85 <sub>(2, 15.0)</sub>	<b>20.83</b> <sub>(4, 1 595 320)</sub>	0.25 <sub>(7, 1 400 142)</sub>	0.53 <sub>(4, 1 771 442)</sub>	0.53 <sub>(4, 1 771 442)</sub>	0.36 <sub>(7, 1 735 891)</sub>	0.53 <sub>(4, 1 771 442)</sub>	0.53 <sub>(4, 1 771 442)</sub>	0.36 <sub>(7, 1 735 891)</sub>
MBC <sup>1</sup>	<b>4.15</b> <sub>(2, 15.7)</sub>	0.12 <sub>(1, 15.7)</sub>	2.74 <sub>(2, 15.7)</sub>	2.74 <sub>(2, 15.7)</sub>	<b>37.26</b> <sub>(3, 51.8)</sub>	1.88 <sub>(6, 51.7)</sub>	1.00 <sub>(3, 51.8)</sub>	1.00 <sub>(3, 51.8)</sub>	0.72 <sub>(6, 51.7)</sub>	1.00 <sub>(3, 51.8)</sub>	1.00 <sub>(3, 51.8)</sub>	0.72 <sub>(6, 51.7)</sub>
MBN <sup>1</sup>	<b>5.88</b> <sub>(2, 16.9)</sub>	3.12 <sub>(1, 18.0)</sub>	0.88 <sub>(2, 17.2)</sub>	0.88 <sub>(2, 17.2)</sub>	<b>19.40</b> <sub>(3, 47.9)</sub>	2.26 <sub>(6, 47.2)</sub>	1.99 <sub>(3, 46.8)</sub>	1.99 <sub>(3, 46.8)</sub>	0.61 <sub>(5, 46.6)</sub>	1.99 <sub>(3, 46.8)</sub>	1.99 <sub>(3, 46.8)</sub>	0.61 <sub>(5, 46.6)</sub>
MBC/MBN <sup>1</sup>	0.85 <sub>(2, 17.8)</sub>	1.00 <sub>(1, 18.2)</sub>	0.10 <sub>(2, 17.9)</sub>	0.10 <sub>(2, 17.9)</sub>	<b>9.27</b> <sub>(3, 46.2)</sub>	<b>2.94</b> <sub>(6, 45.5)</sub>	0.79 <sub>(3, 45.3)</sub>	0.79 <sub>(3, 45.3)</sub>	1.16 <sub>(5, 45.0)</sub>	0.79 <sub>(3, 45.3)</sub>	0.79 <sub>(3, 45.3)</sub>	1.16 <sub>(5, 45.0)</sub>
<b>Plant variables</b>	<b>Fertilizer</b>	<b>Burn</b>	<b>Fertilizer × Burn</b>	<b>Fertilizer × Burn</b>	<b>Year</b>	<b>Fertilizer × Year</b>	<b>Burn × Year</b>	<b>Fertilizer × Year</b>	<b>Burn × Year</b>	<b>Fertilizer × Burn × Year</b>	<b>Fertilizer × Burn × Year</b>	<b>Fertilizer × Burn × Year</b>
Grass ANPP <sup>s</sup>	<b>8.81</b> <sub>(1, 9.0)</sub>	<b>81.63</b> <sub>(1, 9.0)</sub>	<b>7.94</b> <sub>(1, 9.0)</sub>	<b>7.94</b> <sub>(1, 9.0)</sub>	<b>5.05</b> <sub>(4, 48.0)</sub>	1.37 <sub>(4, 48.0)</sub>	1.46 <sub>(4, 48.0)</sub>	1.37 <sub>(4, 48.0)</sub>	1.46 <sub>(4, 48.0)</sub>	0.78 <sub>(4, 48.0)</sub>	0.78 <sub>(4, 48.0)</sub>	0.78 <sub>(4, 48.0)</sub>
Forb ANPP <sup>s</sup>	0.13 <sub>(1, 9.0)</sub>	0.0021 <sub>(1, 9.0)</sub>	<b>11.01</b> <sub>(1, 9.0)</sub>	<b>11.01</b> <sub>(1, 9.0)</sub>	1.66 <sub>(4, 48.0)</sub>	0.95 <sub>(4, 48.0)</sub>	1.48 <sub>(4, 48.0)</sub>	0.95 <sub>(4, 48.0)</sub>	1.48 <sub>(4, 48.0)</sub>	0.77 <sub>(4, 48.0)</sub>	0.77 <sub>(4, 48.0)</sub>	0.77 <sub>(4, 48.0)</sub>
Woody plant ANPP <sup>s</sup>	0.12 <sub>(1, 9.0)</sub>	<b>10.44</b> <sub>(1, 9.0)</sub>	0.12 <sub>(1, 9.0)</sub>	0.12 <sub>(1, 9.0)</sub>	<b>3.79</b> <sub>(4, 48.0)</sub>	1.27 <sub>(4, 48.0)</sub>	<b>3.79</b> <sub>(4, 48.0)</sub>	1.27 <sub>(4, 48.0)</sub>	<b>3.79</b> <sub>(4, 48.0)</sub>	1.27 <sub>(4, 48.0)</sub>	1.27 <sub>(4, 48.0)</sub>	1.27 <sub>(4, 48.0)</sub>

Superscript <sup>1</sup> denotes variables that were log-transformed and <sup>s</sup> that were square root-transformed to meet assumptions of normality. Values bolded signify comparisons with  $P \leq 0.05$ , and superscript <sup>+</sup> indicates where  $P = 0.10-0.051$ .



**Figure 3.** Measured **A** nitrification potential (NP) and **B** denitrification potential (DNP) rates of the soils from contrasting fertilizer and fire treatments. Bar heights (mean  $\pm$  SE) are the means of the NP and DNP rates from each plot and subplot at each month with the points showing the monthly average of each (sub)plot. Tukey's HSD post hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the  $P < 0.05$  level. Both NP and DNP rates were affected by the burning treatment, but no interaction between fire and fertilization.

(Table 2, Figure 4C, D, Figure S2). After post hoc comparison, *nosZ* clade I gene abundance did not shift in response to long-term fertilization or its cessation, but did increase between April and September like NP, AOA, and AOB. In contrast, after long-term addition of N ceased, the abundance of *nosZ* clade II in *A. dehalogenans* in recovering soils dropped below that in control soils ( $P < 0.001$ ). *NosZ* clade II abundance was an order

of magnitude greater than clade I and showed a distinct peak in July in all treatments; thus, the clade I/clade II ratio was most strongly influenced by sampling month ( $P < 0.001$ ; Table 2, Figure S2e–h).

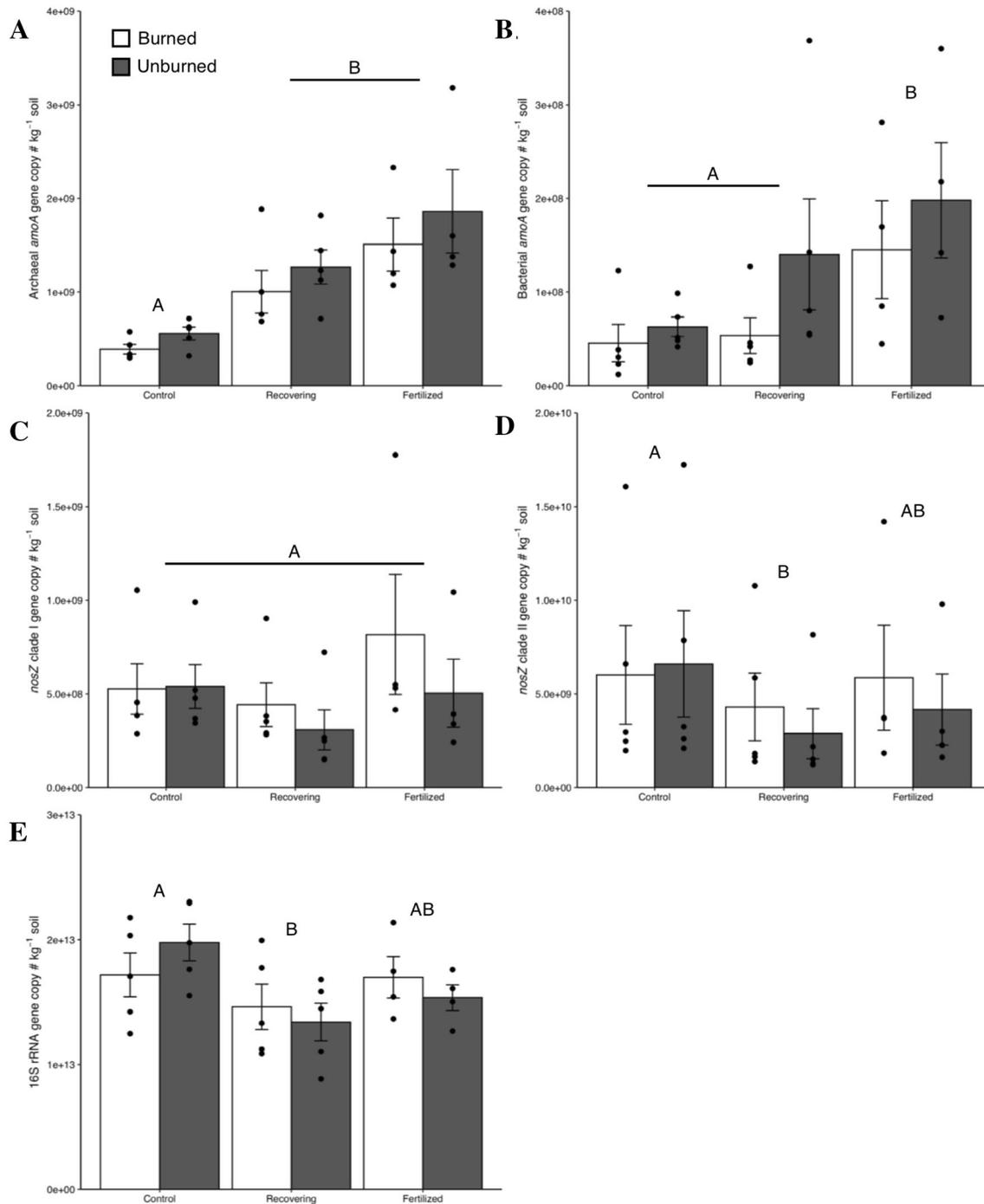
### Soil Microbial Biomass and 16S rRNA Gene Abundance and Composition

Microbial biomass C (MBC) and N (MBN) were lower in fertilized and recovering soils relative to control soils ( $P = 0.02$ ; Table 2, Figure S3a–d). Both MBC and MBN peaked in August for all fire and fertilization treatments ( $P < 0.001$ ). The MBC/MBN ratio was variable and high, but it matches other MBC/MBN ratios reported from Konza Prairie (Baer and Blair 2008; Zeglin and others 2013). Also, the MBC and MBN extraction efficiencies were likely different and could explain the variability (Vance and others 1987). Bacterial 16S rRNA gene abundance was also highest in the control soils and lowest in recovering soils, like the *nosZ* clade II gene ( $P = 0.035$ ; Table 2, Figure 4E). Soil 16S rRNA abundance was not affected by fire regime and increased through the growing season in all field treatments (Table 2, Figure S2i, j).

Soil bacterial and archaeal 16S rRNA gene composition responded to chronic fertilization, and more strongly so in the unburned treatment (Table 3, Figure S4), but the soil microbial community composition did not recover following fertilizer cessation. PERMANOVA indicated that more (11.5%) of the variation in community composition was attributed to the fertilizer treatments alone than the fire treatments (5.9%), their interaction (3.4%), or sampling month (6.8%; Table 3). The relative abundance of many common ( $\geq 0.10\%$  relative abundance) phyla or classes changed in response to long-term fertilization, fire treatment, or their interaction; however, no group's relative abundance shifted toward recovery in the first year following cessation of chronic fertilization (Table S1).

### Plant Aboveground Biomass

Aboveground net primary productivity (ANPP) changed substantially in response to long-term fire and fertilization treatments, but did not respond to the cessation of fertilization in 2017 (Table 2, Figure 5). Grass ANPP in annually burned prairie increased by an average of 156% in response to chronic fertilization ( $P = 0.020$ ), and while post hoc comparisons showed no significance, forb ANPP tended to be highest in the unburned fertil-



**Figure 4.** Gene abundance of **A** archaeal and **B** bacterial *amoA*, **C** clade I and **D** clade II (*A. dehalogenans*) of *nosZ*, and **E** bacterial 16S rRNA in soils with contrasting treatments of burning and fertilization. The bar height (mean ± SE) represents the mean gene abundance at each plot and subplot for each given treatment combination during the span of the growing season. Points are the monthly average for each (sub)plot. Tukey's HSD post hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the  $P < 0.05$  level.

ized prairie, but neither changed in the year fertilization was ceased (Figure 5A, B). Annual fire suppressed woody plant growth ( $P = 0.010$ ), but

neither fertilization nor its cessation affected woody plant ANPP (Figure 5C).

**Table 3.** PERMANOVA Results on the Direct and Interactive Effects of Fertilization (“Fertilizer”), Burning (“Burn”), and Sampling Month (“Month”) for the 16S rRNA Gene Composition of Microbial Communities Based on the Bray–Curtis Dissimilarity

Factor	DF	$R^2$	Pseudo- $F$	$P$ value
Burn	1	<b>0.059</b>	<b>7.15</b>	<b>0.001</b>
Fertilizer	2	<b>0.115</b>	<b>7.01</b>	<b>0.001</b>
Month	4	<b>0.068</b>	<b>2.08</b>	<b>0.001</b>
Burn × Fertilizer	2	<b>0.034</b>	<b>2.08</b>	<b>0.001</b>
Burn × Month	4	0.022	0.67	0.973
Fertilizer × Month	7	0.044	0.76	0.862
Burn × Fertilizer × Month	7	0.037	0.64	1.000
Residuals	76	0.622	–	–

Observed composition of communities were compared by permutating 999 null communities, resulting in a pseudo- $F$  score and variation explained ( $R^2$ ) by treatments and their interactions. Bolded rows signify the comparisons in which  $P \leq 0.05$ .

## Soil Chemistry

Soil %C, %N, and pH responded to chronic fertilization, and soil %C and %N also responded to the fire treatments, but no recovery in soil pH, %C, or %N was detected in the first year of fertilizer cessation (Table 1, Figure S5a, b, d). After 30 years of fertilization, soil pH decreased from an average of 6.9 in control soils to 6.3 in recovering and fertilized soils ( $P < 0.001$ ; Table 1, Figure S5d). Soil %C was highest in unburned and fertilized soils ( $P = 0.21$ ), and soil %N increased in response to both long-term fertilization ( $P < 0.001$ ) and fire suppression ( $P < 0.001$ ; Table 1). C/N was variable, but tended to be highest in the annually burned and unfertilized prairie soils. Variation among field replicates also reflects sub-plot scale heterogeneity that is typical of this and other grassland soils.

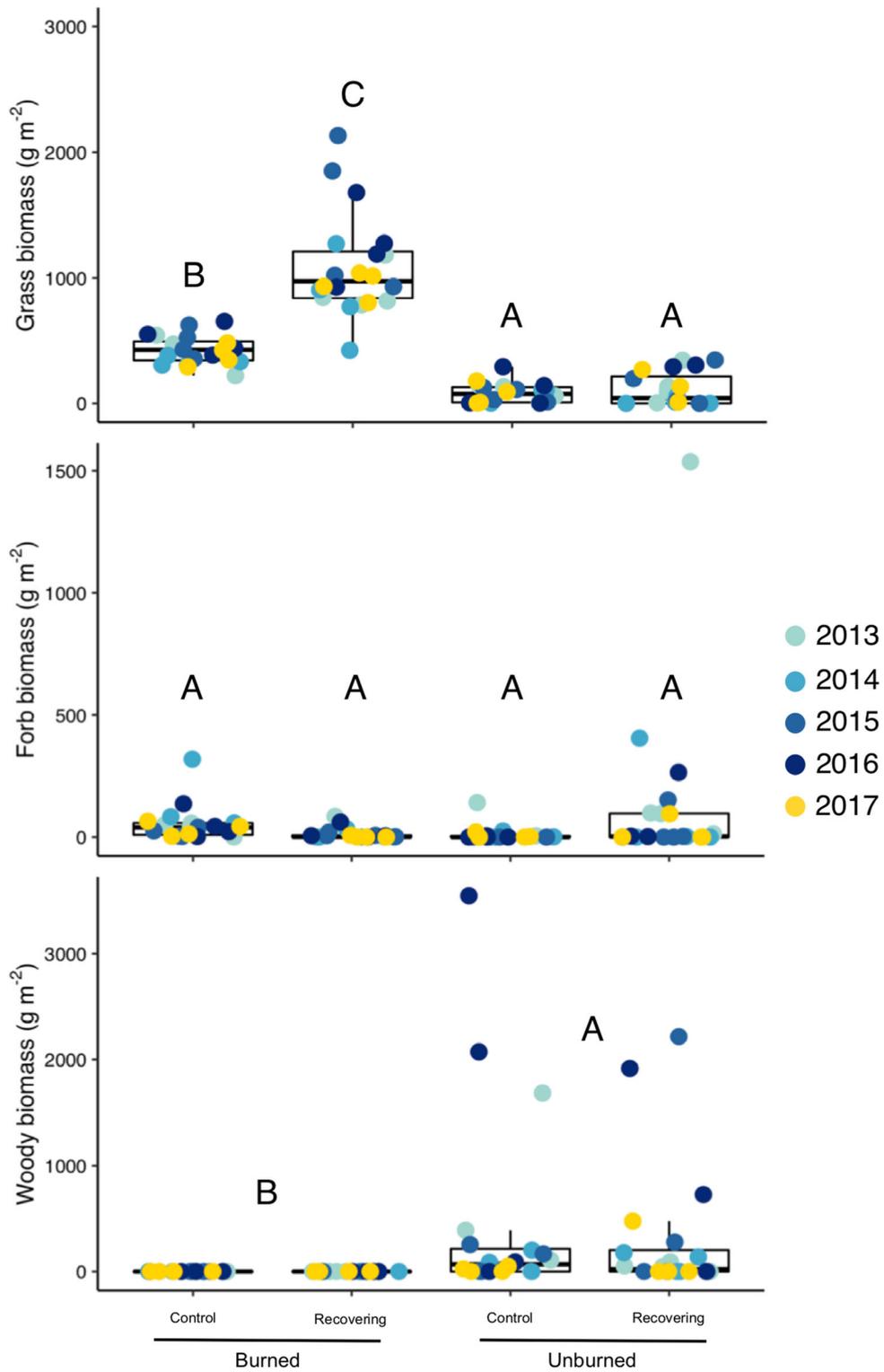
## DISCUSSION

We predicted that ecosystem N-cycling processes, and the organisms supporting these functions, would show levels of recovery from chronic (30 years) fertilization that reflect how directly that process or population was supported by supplemental inorganic N. Further, we expected that recovery would be greater in the annually burned, more N-depleted, tallgrass prairie relative to the unburned prairie treatment. Nitrogen availability for both plants and microbes was indeed independently higher in both unburned (3 times greater) and fertilized (30 times greater) treatments than the burned and control treatments, respectively, and most response variables were also sensitive to long-term fertilization. (That is, differences between control and fertilized treatments were sta-

tistically significant.) During the first growing season in 30 years with no supplemental N addition, N availability in recovering soils recovered by 86%, still 5 times greater than that of the controls, nitrification activity potential recovered by 80%, to levels statistically indistinguishable from that of controls, and while denitrification activity potential decreased 51%, it did not show statistically significant recovery. N-cycling microbial functional populations also showed differential recovery, in that nitrifying bacterial populations recovered by 68%, but nitrifying archaeal populations did not, and both nitrous oxide reducing populations and total bacterial populations were insensitive to the chronic fertilization. Soil microbial biomass, which decreased in response to long-term fertilization, did not recover, and neither did the 16S rRNA gene community composition. Finally, grass biomass was also sensitive to chronic fertilization, but did not recover in the first year of its cessation. The unburned prairie treatment had elevated soil available N, NP, and DNP relative to the burned treatment, but a comparatively weak plant biomass response to chronic fertilization. This suggests that microbially mediated N loss could constrain ecosystem recovery in the absence of fire, while plant litter combustion may remain a primary driver for recovery after chronic fertilization in annually burned prairie.

## Soil Available N Sensitivity and Recovery

The whole growing season’s accumulation of resin-sorbed N dropped significantly in the first year of ceasing N fertilizer application, such that N availability in the recovering soils, relative to the control and fertilized soils, recovered more than 80% in



◀**Figure 5.** Yearly variation of aboveground net primary productivity (ANPP), estimated from end-of-growing season biomass, between 2013 and 2017 of **A** grasses, **B** forbs, and **C** woody plants. Data are shown as boxplots with the central line representing the median, and the upper and lower quartiles being the lines above and below the median, respectively. The lines extending from the quartiles end at the upper and lower fences of the data distribution. Yearly plot means are shown as points; each color represents a different year. Tukey's HSD post hoc analysis results are shown with dissimilar letters indicating Fertilizer  $\times$  Burn treatment groups for grasses and forbs, and burning treatment groups for woody plants, that differed at the  $P < 0.05$  level.

both the annually burned and unburned treatments (Table 1, Figure 2). This suggests that after 30 years of chronic fertilization, the large majority of available N for plant and microbial uptake, nitrification, and denitrification was derived directly from the annual fertilizer application. In addition, available  $\text{NO}_3^-$ -N remained five times higher in the recovering versus control soils, reflecting elevated N mineralization and nitrification associated with the legacy of fertilization. Nutrient addition experiments at a Minnesota grassland showed evidence for elevated net N mineralization rates up to 12 years after ceasing long-term N application (Clark and others 2009). Similarly, net N mineralization in a Netherlands grassland took 20–45 years to decline following fertilizer cessation (Olff and others 1994). Although we did not directly measure N mineralization rates, a legacy of higher net N mineralization might be supported by the larger total soil N pool in chronically fertilized soils. Notably, the resin-sorbed  $\text{NO}_3^-$ -N/ $\text{NH}_4^+$ -N ratio was highest in the unburned recovering treatment, despite substantially recovered nitrification potential rates in the first year of ceasing N fertilization. This is related to the larger excess of  $\text{NH}_4^+$ -N in the unburned, chronically fertilized soils, and points to possible saturation of plant uptake or denitrification activity in the unburned treatment, as we will address later in the discussion. With all of this in mind, plant and microbe available N in recovering tallgrass prairie soils could remain elevated for an extended period and subsequently feed ecosystem N loss by promoting  $\text{NO}_3^-$  leaching, supporting gaseous emission from denitrification, and supplying N to plant biomass that is combusted eventually by fire.

## Sensitivity and Recovery of Soil Nitrification Potential Rates and Ammonia Oxidizers

Nitrification potential (NP) rates also significantly declined in the first year of ceasing chronic fertilization, recovering approximately 80%, and reaching a statistically similar level to the control soils (Table 2, Figure 3A). At other N cessation experiments, nitrification activity uniformly decreased as  $\text{NH}_4^+$  availability declined over time (Olff and others 1994; Stienstra and others 1994), and the clear drop of NP in our experiment is also likely explained by the reduced input of soil  $\text{NH}_4^+$ . Interestingly, the significant three-way interaction highlights the increase in NP rates later in the growing season in all but one combinatory fertilization and fire treatment (Table 2, Figure S1a, b). Plant uptake of N early in the growing season, when shoot and root production is highest in annually in grasses (Knapp 1984, 1985) and new biomass production in woody plants also peaks, can limit available  $\text{NH}_4^+$  for nitrifiers and prevent nitrification (Schimel and Bennett 2004). This pattern, indicating plant success over soil nitrifiers in the competition for limiting levels of ammonium, was apparent in all treatments except the unburned, chronically fertilized treatment, suggesting that N may no longer be limiting plant uptake under those conditions. However, because our data do not directly target the interaction, it is not clear whether plant competition for N is related to the recovery of NP rates following cessation of long-term fertilization.

Ammonia-oxidizing archaea (AOA) and bacteria (AOB) differentially recovered in the first year of N fertilization cessation. Although both groups were sensitive to long-term N fertilization, AOA showed only a 38% recovery, in contrast with the significant 68% recovery of the AOB (Table 2, Figure 4A, B), under both burned and unburned conditions. Although AOA and AOB are both considered poor competitors against plants and soil microbial heterotrophs for  $\text{NH}_4^+$  (Verhagen and others 1995; Kaye and Hart 1997; Schimel and Bennett 2004), previous work suggests AOA and AOB differ in response to supplemental N. Increased  $\text{NH}_4^+$  availability from N addition can support greater AOB abundance than AOA (Di and others 2010; Wertz and others 2012). Other studies detect an increase in AOA abundance when organic N, but not inorganic N, is added (Levičnik-Höfferle and others 2012; Ouyang and others 2018). In this study, AOB could have been more directly reliant on fertilizer for substrate, thus recovered to a

greater extent when long-term fertilization ceased, or the lack of AOA recovery could be linked to the incomplete recovery of  $\text{NO}_3^-$  availability or the relatively small but significant changes in pH in the historically fertilized soils (Table 1, Figure S5d). Although some AOA isolates have higher affinity for  $\text{NH}_4^+$  than AOB and are thus better competitors at lower N availability (Martens-Habbena and others 2009), this cannot be extrapolated to all AOA taxa; similarly, we cannot assume that all AOA prefer low soil pH, though some do (Prosser and Nicol 2012). Finally, though we did not measure the contribution of AOA versus AOB to NP rates, group-specific contributions to nitrification activity are possible (Taylor and others 2010). Thus, if AOA abundance remains elevated after fertilizer cessation, the complete recovery of nitrification could be prolonged until AOA abundance declines to levels similar to control soils.

### Partial Recovery of Denitrification Potential and Resistance of Denitrifiers

Denitrification potential (DNP) rate, representing activity under ambient (possibly limiting) levels of N and C, was higher in both the recovering and fertilized soils, and was therefore sensitive but less resilient than nitrification to long-term N fertilization (Table 2, Figure 3B). Substrate-saturated denitrification enzyme activity (DEA) did not change following, and therefore was resistant to, long-term N fertilization. Together, this suggests that soil  $\text{NO}_3^-$  or DOC availability, not enzyme abundance, controls denitrification rates in these soils (Firestone and others 1980; Wallenstein and others 2006). A lower  $\text{NO}_3^-$  supply due to recovery of NP could have reduced DNP; however, ample amounts of  $\text{NO}_3^-$  must have been present to maintain the still-elevated DNP rates in recovering soils. In fact, NP rates remained substantially higher than DEA and DNP rate through the study season, suggesting that DNP was close to its maximum and that N loss through nitrate leaching continued, despite cessation of fertilization. This phenomenon was more likely in the unburned treatment, where the resin-sorbed  $\text{NO}_3^-$ -N/ $\text{NH}_4^+$ -N was highest. The resistance of DEA to chronic fertilization means ecosystem N loss through denitrification might be limited by enzyme abundance when N availability remains elevated.

As the DEA data suggest, bacterial populations with the *nosZ* gene were largely resistant to 30 years of fertilization (Figure 4C, D). Under the rationale that these organisms are predominantly facultative, not obligate, denitrifiers, this fits our

predictions; yet, the resistance to long-term fertilization was a bit of a surprise given the significant shift in the total bacterial community. Further, patterns of sensitivity to N fertilization and intra-seasonal dynamics were very different between taxa with the *nosZ* clade I and clade II genes (Table 2, Figure S2e–h). While the *nosZ* clade I abundance did not respond significantly to long-term N addition, but peaked in September concurrent with NP, the abundance of *nosZ* clade II from *A. dehalogenans* tended to be lower in fertilized and recovering soils and peaked in July. Considering that at least 50% of taxa with the *nosZ* clade II gene lack the genes for the full denitrification pathway (Sanford and others 2012; Hallin and others 2018), and because this variant of *nosZ* appears dominant in the study soils (mean ratio clade II/clade I = 14.5), we might predict a lower  $\text{N}_2\text{O}$  sink and higher losses of N as a potent greenhouse gas in soils recovering from chronic fertilization (Jones and others 2014), especially late in the growing season. However, although *nosZ* gene abundance often responds positively to N fertilizer addition, it is not a reliable predictor of denitrification rates (Rocca and others 2015; Ouyang and others 2018), so we can only speculate on  $\text{N}_2\text{O}$  sink or efflux potentials. Measurement of in situ  $\text{N}_2\text{O}$  emission may be a priority for a follow-up study.

### Sensitivity and Lack of Recovery in Total Microbial Biomass and Community Composition

Total microbial biomass carbon (MBC) and nitrogen (MBN) varied more by month than between the fertilization treatments, but tended to be lower in the recovering and fertilized soils than the control soils, which agrees with observations from others (Treseder 2008; Zhang and others 2018). The peak in MBC and MBN in August is likely related to microbes growing more on labile C root exudates and experiencing less competition with plants for inorganic N as the growing season progresses, as also observed by others (Figure S3a–d; Garcia and Rice 1994; Ajwa and others 1999; Kuzakov and Xu 2013). While bacterial abundance at this site is higher through the plant growing season (Figure S2i–j; Carson and Zeglin 2018), this peak was asynchronous with the MBC peak, and the tendency toward lower bacterial 16S rRNA gene abundance in the recovering and fertilized soils was weak (Table 2, Figure 4E), in contrast with the MBC pattern. Gene abundance cannot be equated directly to cell abundance due to variation in 16S rRNA gene copies per genome of different taxa, and

cell abundance does not equate to biomass due to the orders of magnitude of range in cell size among diverse soil microorganisms. Total soil microbial biomass may be dominated by larger but less abundant fungi, not smaller but more numerous bacteria, as also suggested by the high MBC/MBN ratios for all field treatments (Figure S3e, f; Rice and others 1998; Strickland and Rousk 2010). There was no recovery in microbial biomass or 16S rRNA gene abundance from fertilizer cessation; instead, the total 16S rRNA gene abundance declined slightly further in the recovering soils. This could be related to a mild shift in the microbial community composition, since 16S rRNA gene numbers in genomes of different taxa, or even the same bacterial population, can vary (Klappenbach and others 2000; Větrovský and Baldrian 2013). In the context of ecosystem resilience from chronic fertilization, a legacy of lower microbial biomass in chronically fertilized soils indicates that the potential for microbial N immobilization remains lower after long-term N fertilization ceases, making plant assimilation and coupled nitrification–denitrification more important fates of N as whole-ecosystem recovery progresses.

The soil bacterial and archaeal 16S rRNA gene community composition was also sensitive to N fertilization, but did not change significantly in the first year of cessation (Table 3, Figure S4). The compositional shift, including directionality of change in certain high-level taxonomic groups (Table S1), is consistent with many other inorganic fertilization studies (Leff and others 2015; Carson and Zeglin 2018), and thus seems to support the “copiotrophic hypothesis.” This hypothesis predicts that under high-N conditions, fast-growing microbial taxa dominate in relative abundance over slower-growing taxa that are more competitive in nutrient-poor conditions (Ramirez and others 2012). If this is true, the lack of recovery is interesting, given that N availability dropped so dramatically with cessation of fertilizer addition. A legacy effect from long-term N fertilization could result from the increase in soil total N pools (Table 1, Figure S5) and associated higher N mineralization, or slower growth of putative oligotrophic populations could create a lag in turnover toward their recovered dominance. Alternatively, the pH drop, while less extreme than measured in other experiments, might also constrain microbial recovery (Liu and others 2020). Finally, the potential for relic DNA to obscure temporal change is a concern; however, relic DNA proportion was very low in samples collected from this study site, in association with the relatively high base satura-

tion in local soils (Carini and others 2017). While the mechanism is not immediately clear, the legacy of microbial community shift for at least 1 year after ceasing N application suggests that there is no longer a direct link between supplemental inorganic N and many soil microbial heterotrophs after 30 years of fertilization (Shade and others 2012; Bardgett and Caruso 2020).

## Plant Aboveground Sensitivity and Lack of Recovery

Aboveground net primary productivity (ANPP) increased with fertilization by more than double in the annually burned treatment, but did not decline in the first year after 30 years of fertilization was stopped (Table 2, Figure 5). Nitrogen limits the growth of perennial grasses in the tallgrass prairie (Seastedt and others 1991; Blair 1997), and to help overcome this limitation, many grasses retranslocate assimilated N belowground during the annual plant senescence period (Ojima and others 1994; Dell and others 2005). Thus, perennial grasses may represent an N reservoir that slows ecosystem recovery from chronic fertilization, despite the recovery of soil  $\text{NH}_4^+$ -N availability. Although grass and forb biomass responded to long-term fertilization, woody biomass did not; the increase in woody biomass in unburned prairie reflects the well-documented plant community shift toward woody dominance that occurs in prairies following fire suppression (Briggs and others 2005; Bond 2008). While N remains elevated in plant biomass, fire will be a significant factor for ecosystem N removal in the annually burned prairie. If grass tissue in fertilized treatments was 0.4–0.7% N (Norris and others 2001; Reed and others 2005; Roley and others 2018), and all the N in aboveground biomass was combusted, 2.1–3.7 g N m<sup>-2</sup> y<sup>-1</sup> in excess of the unfertilized treatment would be lost to fire. This loss will depend on N concentration in plant litter, and will be less than the senesced tissue amount, since ~10% of litter N can be incorporated into the soil organic matter in one winter before the typical springtime prescribed fire application (Cotrufo and others 2015). Still, this substantial N efflux reduces total ecosystem N, and likely underpins differences in total N accumulation due to chronic fertilization (Figure S5), and recovery of the N cycle following its cessation, between burned and unburned prairie.

## Fire History Effects on Microbial N Fertilization Sensitivity

Many N-cycling rate potentials and microbial populations were higher in unburned treatment soils, independent of fertilization (Table 2). While we were somewhat surprised to see no interactions in N-cycling attributes, given the significant interaction detected in the plant and microbial community change (Tables 2, 3; Carson and Zeglin 2018; Carson and others 2019), this shifted baseline is important. The unburned unfertilized treatment had more than double the available N, almost 4 times the NP and 20 times greater DNP than the burned unfertilized treatment (Figures 2, 3). It is also important to note that in addition to increasing N availability, fire suppression contributes to soil C retention through litter accumulation (Figure S5), especially in woody-encroached areas (McKinley and Blair 2008), which could additively support higher denitrification (Firestone and others 1980; McCulley and others 2004; Wallenstein and others 2006). If the DNP rates in unburned recovering soils, integrated across the whole sampling season (late April–late September), were attained in the field,  $\sim 22.7 \text{ g N m}^{-2} \text{ y}^{-1}$  could be lost through microbes to the atmosphere. This estimate is an order of magnitude greater than the estimated N loss to fire in annually burned prairie.

## CONCLUSION

In summary, our observations suggest that competition between plants and microbes for available N, with or without fire in the ecosystem, will constrain the lag time and primary mechanisms of N loss via fire or microbial activity, and thus the resilience of this tallgrass prairie ecosystem to chronic fertilization. Many of the soil, plant, and microbial responses to fertilizer cessation fit our predictions and agree with our hypothesis and conceptual model (Figure 1), particularly in that the mediating effect of fire was associated with greater recovery in the first year after ceasing fertilization. Yet, both plant and soil N pools will remain important reservoirs of N as the recovery process proceeds, such that they could represent an “N bank” somewhat analogous to that now hypothesized to underlie lags in N-cycling processes in successional forest ecosystems (Lovett and others 2018). As a final projection, soil total N would have increased by 0.14% if the 30 years of added N ( $300 \text{ g N m}^{-2}$ ) was retained in the top 20 cm of soil, with an estimated bulk density of  $1.097 \text{ g cm}^{-3}$  of dry soil (Carson 2013). We mea-

sured a mean difference of 0.03 and 0.06% in soil total N between the historically fertilized and never-fertilized soils in the burned and unburned treatments, respectively, so up to 21% (with fire) and 44% (without fire) of the total added N has been retained in the soil organic matter. Soil microbes will partially support ecosystem N release in the burned prairie, but will exclusively drive N loss in the unburned prairie. Our rough, yet reasonable, estimates suggest that it could take 17–30 years (with fire alone) or  $\sim 6$  years (with denitrification in the absence of fire) for the extraneous N in this ecosystem to be depleted. However, the long-term rate of ecosystem recovery may not fit our predictions. If the soil microbial biomass or plant tissues return to a more retentive state (Dell and others 2005), if nitrification/denitrification potentials continue to drop, or if soil organic N is not biologically available, recovery will take longer or may never be attained (Gilliam and others 2019). In conclusion, we show that chronic N addition results in different potential legacy mechanisms for soil N loss and ecosystem recovery because of the differential resilience of biotic populations and processes shaped by contrasting fire management regimes. Furthermore, suppressing fire can cause some similar belowground effects as chronic N fertilization in burned prairies, so it is essential to consider the relative importance of, and interaction between, the two factors when assessing future global change scenarios. Further, because of the shifting dynamics during the rapid period of change post-fertilizer cessation, more study is needed to ascertain the fate of N in this manipulated prairie ecosystem.

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## DATA AVAILABILITY

Data are available at <http://lter.konza.ksu.edu/> and <https://www.ncbi.nlm.nih.gov/>.

## Declaration

**Conflict of interest** The authors declare that they have no conflict of interest.

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