Soil Biology and Biochemistry 189 (2024) 109247

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Product-inhibition feedbacks, not microbial population level tradeoffs or soil pH, regulate decomposition potential under nutrient eutrophication

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ARTICLE INFO

Keywords:
Nitrogen
Phosphorus
Extracellular enzyme activity
16S rRNA gene
Soil organic matter
Copiotrophic hypothesis

ABSTRACT

Global eutrophication of growth-limiting nutrients for plants and microbes, including nitrogen (N) and phosphorus (P), can cause enzymatic feedbacks at different levels of biological organization that in turn drive soil organic matter (SOM) decomposition. Because land management practices, such as fire in mesic grasslands, also impact nutrient availability, it is important to understand how soil feedbacks may manifest under multifactorial change. Here, using a thirty-year field experiment that crosses N- and P-fertilization (alone and in combination) with contrasting fire treatments (annual burning and fire suppression) in a tallgrass prairie ecosystem, we evaluated evidence for three possible feedbacks (product-inhibition, enrichment of fast-growing copiotrophic taxa, and soil pH shift) by measuring soil extracellular enzyme activities (EEAs) and microbial community composition (16S rRNA genes) monthly for a year. Consistent with product-inhibition feedback predictions, both N-fertilization and fire suppression (which increases soil N availability) decreased N-acquiring relative to C-acquiring EEAs, suggesting reduced N limitation. P-fertilization did not lower acid phosphatase activity, thus the product-inhibition feedback hypothesis was not supported under P eutrophication conditions. Although N- and P-fertilization, alone and in combination, did decrease putatively oligotrophic populations in favor of putatively copiotrophic populations, the hypothesized tradeoff for faster growth, a concomitant decrease in all soil EEAs, was only observed in unburned soils fertilized with N. Finally, observed changes in EEAs were not driven by acidification, in contrast to other fertilization experiments. Overall, results suggest that higher N availability affects enzymatic feedbacks primarily through product-inhibition, but a threshold-like growth tradeoff (copiotrophic) response is possible under the combination of fire suppression and N-fertilization. Inherent soil properties may modulate site-specific responses to fertilization, as evidenced by weak pH and EEA responses to N- and P-fertilization, respectively. Finally, soil microbial community change following chronic fertilization did not reliably predict the “copiotrophic” feedback of reduced SOM decomposition potential.

1. Introduction

Soil decomposer communities redistribute energy and nutrients from the aboveground ‘green’ world to form the base level of belowground ‘brown’ foodwebs (Hairston et al., 1960; Allison, 2006). However, modern global industrialization risks disrupting microbial feedbacks that shape carbon (C) and nutrient cycling though soil organic matter (SOM) decomposition. Specifically, inputs of bioavailable nitrogen (N) and phosphorus (P) to ecosystems have soared (Cordell et al., 2009; Kanakidou et al., 2016), due largely to the rise in atmospheric deposition from fossil fuel combustion and fertilizer application (Vitousek et al., 1997; Fixen and West, 2002), and, as a result, elevated soil N and P availability has increased net primary production as plants fix more C (Elser et al., 2007; Harpole et al., 2011). Prior to contemporary eutrophication, plants relied on soil microbes to release nutrients from SOM, and over decades to centuries, positive feedbacks between litter input and decomposition would enhance soil fertility, leading to higher net primary production (Hobbie, 2015). Following anthropogenic augmentation of nutrient availability, microbial feedbacks that maintain soil fertility may be altered.

Soil microorganisms (including bacteria, archaea, and fungi) produce an arsenal of extracellular enzymes that attack unprotected SOM to release labile C, N, and P compounds needed by microbes and plants (Burns, 1982; Sinsabaugh, 1994). The synthesis and activity of

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https://doi.org/10.1016/j.soilbio.2023.109247
Received 10 March 2023; Received in revised form 9 September 2023; Accepted 10 November 2023

extracellular enzymes reflects microbial demand for particular nutrients, in that soil microbes reallocate intracellular resources to increase the production and extracellular release of enzymes to target a limiting nutrient (Chróst, 1991; Sinsabaugh et al., 2008). In turn, as extracellular nutrient concentrations increase, the synthesis and activity of enzymes linked to the release of those nutrients may decrease (Olander and Vitousek, 2000; Schimel and Weintraub, 2003; Sinsabaugh and Follstad Shah, 2012). Thus, this cellular level product-inhibition feedback of extracellular enzymes responsible for SOM decomposition explains observations of lower soil N- or P-acquiring enzymatic activity following the addition of N or P, respectively (Olander and Vitousek, 2000; Schimel and Weintraub, 2003; Allison and Vitousek, 2005). In addition, the copiotrophic hypothesis states that, under conditions of higher nutrient availability, microbial communities become dominated by populations with faster growth rates, but lower synthesis of extracellular enzymes (i.e., copiotrophs), that outcompete populations with slow growth rates and higher energy investment in enzyme synthesis to acquire limiting nutrients from the SOM (i.e., oligotrophs). This population-level tradeoff between growth and resource acquisition predicts that fertilization will shift the relative abundance of functionally important taxa within soil microbial communities, with consequences in terms of lower SOM decomposition (Fierer et al., 2007; Ramirez et al., 2012). However, while these mechanisms have been well supported in lab experiments, results from field fertilization experiments show weaker support for both cellular- and population-level feedbacks (Riggs and Hobbie, 2016; Keller et al., 2022). Also, other studies have found mixed responses of soil extracellular enzyme activities to exogenous nutrient additions, and while the copiotrophic hypothesis is based on studies of N-fertilization, it is not clear whether P-fertilization invokes similar mechanisms and feedbacks (Allison and Vitousek, 2005; Hernández and Hobbie, 2010; Jian et al., 2016; Ochoa-Hueso et al., 2020).

Lower SOM decomposition potential following fertilization can be mediated by other ecosystem-scale feedbacks as well, such as inhibition of microbial activity due to decreased soil pH (Treseder, 2008; Riggs and Hobbie, 2016). Fertilization often lowers soil pH, which can decrease soil enzyme activity by altering enzyme structure or affecting the adsorption of extracellular enzymes on mineral surfaces (Leprince and Quiquampoix, 1996). Microbial biomass also decreases at low soil pH levels that can result from chronic fertilization (Treseder, 2008), so that enzyme activity may be impaired if functionally important microbial taxa cannot survive under acidic soil conditions. Furthermore, the magnitude of enzymatic responses to fertilization can be constrained by inherent soil properties (Zeglin et al., 2007), such that even if microbial enzymatic feedbacks are universal, the impact of exogenous nutrient input on enzyme activity and decomposition may vary among ecosystems with different biotic and abiotic baseline conditions.

In grasslands, which cover over a third of the planet's land surface (White et al., 2000; Bai and Cotrufo, 2022), local land management practices such as fire suppression, or changes to grazing regime, have altered grassland nutrient dynamics concurrently with the global enrichment of bioavailable N and P. Historically, the frequent occurrence of fire in mesic grasslands combusted N stored in senesced plant litter and limited its return belowground, supporting an N-limited ecosystem (Ojima et al., 1994; Blair, 1997). In contrast, fire suppression allows litter-N accumulation and decomposition, and positive feedbacks develop, as lower litter C:N promotes faster litter breakdown and higher plant N availability (Parton et al., 2007; Hobbie, 2015). In addition, fire suppression limits P availability as P is immobilized in biomass and detritus and not subsequently released through pyromineralization (Seastedt, 1988; Butler et al., 2018). Also, long-term fire suppression shifts plant community composition to reduced dominance of C_4 grasses and can increase the cover and abundance of woody plants (i.e., woody encroachment; Briggs et al., 2002b, 2002a; Ratajczak et al., 2011). Woody encroachment is a widespread and growing concern in the tallgrass prairie ecosystem of North America (Beilmann and Brenner, 1951; Bragg and Hulbert, 1976; Ratajczak et al., 2014), and impacts local C and nutrient cycles through changes to litter quality and soil microclimate (Acharya et al., 2017; Connell et al., 2020). Fire suppression also shifts soil bacterial, archaeal, and fungal community composition (Carson and Zeglin, 2018; Carson et al., 2019), but it is not known if these community shifts result in changes in soil enzymatic activity. Thus, due to multiple feedbacks operating at different scales, SOM decomposition responses to concurrent anthropogenic eutrophication and land management change are difficult to predict. To begin disentangling these mechanisms, we tested hypotheses related to soil enzymatic feedbacks using a long-term field experiment that crosses fertilization treatments with contrasting fire management practices in a tallgrass prairie ecosystem.

Specifically, we evaluated the product-inhibition, copiotrophic, and soil pH shift hypotheses, collecting evidence to discern which of the soil enzymatic feedbacks that affect SOM decomposition are most prevalent in soils that have been fertilized with N and P (alone and in combination) under contrasting fire management treatments (annual burning and fire suppression) for thirty years. Recent work at this field experiment supported the copiotrophic hypothesis, in that soils chronically fertilized with N had a higher relative abundance of microorganisms putatively classified as copiotrophs, with greater shifts in the unburned prairie soils possibly because the baseline level of N availability is 3–5 times higher that of burned prairie soils (Carson and Zeglin, 2018; Nieland et al., 2021). However, we do not know whether soil enzymatic activity has been simultaneously affected, or whether chronic P-fertilization in this P-limited system (Wilson et al., 2009; Johnson et al., 2015) has similar effects. We predicted that the product-inhibition feedback would be supported if just N- and P-acquiring enzyme activities decrease following N- and P-fertilization, respectively; and that the copiotrophic feedback would be supported if all soil enzyme activities decrease concomitant with microbial community turnover towards higher copiotroph abundance under eutrophic conditions (Ramirez et al., 2012). Further, we predicted that the soil pH feedback would be supported if decreases in soil enzyme activity strongly correlate with decreases in soil pH. Using this framework to understand how cellular, population, and ecosystem level feedbacks to SOM decomposition may respond to terrestrial eutrophication, we tested all three non-mutually exclusive hypotheses by collecting soils from the field experiment monthly for a year and measuring soil extracellular enzyme activities (EEAs), bacterial and archaeal community composition, and soil chemistry.

2. Methods

Field experiment description and sample collection – The Belowground Plot (BGP) experiment was established in 1986 in an ungrazed area of the Konza Prairie Biological Station (KPBS) near Manhattan, KS, USA (39° 05′ N, 96° 35’ W) (Seastedt et al., 1991). Mean annual precipitation at KPBS is 835 mm, but total precipitation in the study year (2015) was considerably wetter at 1002.5 mm (Carson and Zeglin, 2018). As a strip-plot design, the BGP experiment crosses fertilization across four blocks that are burned annually in early spring (burned prairie) and another four that are not burned (unburned prairie). Strips within blocks are either not fertilized (control) or fertilized with N (10 g N m^{-2} yr^{-1} as NH_4NO_3), P (1 g P m^{-2} yr^{-1} as $Ca(H_2PO_4)_2$), or N and P, totaling eight fire × fertilization field treatments, each with four 12.5 m × 12.5 m replicate plots ($n = 4$). At the BGP experiment, soils are deep and silty clay loams belonging to the Irwin series (fine, mixed, mesic, pachic Arguistolls) with no organic horizon.

Plot-scale samples were aseptically collected as the composite of four 2 cm diameter, 15 cm deep mineral soil cores within each plot in control and N-only treatments in January 2015 and in all fertilization treatments from March to December 2015. Samples were kept on ice until stored at −20 °C in the laboratory for further processing. Soils were collected one week after the prescribed fire in April 2015 and one week after fertilizer application in June 2015.

Soil chemistry – Soil total C and N were quantified using a FlashEA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Soil C, N, and pH (pH in 1:1 ratio of soil and deionized water) were measured only in soils collected in July because we did not expect these parameters to change monthly. Soil C and N were converted to molar amounts to calculate soil C:N ratios. Gravimetric water content (GWC) was measured in all samples by drying soils overnight at 105 °C.

Extracellular enzyme activities – Soil extracellular enzyme activity potentials were measured using short-term, room temperature assays with fluorometric organic substrates (methylumbelliferone (MUB) or methylcoumarin (MC)) for hydrolytic enzymes and a colorimetric substrate (L-3,4,-dihydroxyphenylalanine (L-DOPA)) for the oxidative enzyme (Saiya-Cork et al., 2002; Zeglin et al., 2013). Activities of β-glucosidase (BG; EC 3.2.1.21), cellobiohydrolase (CBH; EC 3.2.1.91), *N*-acetylglucosaminidase (NAG; EC 3.2.1.14), leucyl aminopeptidase (LAP; EC 3.4.11.1), acid phosphatase (PHOS; EC 3.1.3.1), and phenol oxidase (POX; EC 1.10.3.2) were measured in 96-well plates with 6–8 technical replicates per sample and enzyme combination. All assays were run in 50 mM sodium acetate buffer (pH 5) with a final substrate concentration at either 40 μM (hydrolytic enzymes) or 5 mM (POX) for 2 (BG and PHOS), 4 (NAG and CBH), or 18 h (LAP and POX) before raising the pH > 8 with 0.5 M NaOH to measure MUB and MC fluorescence at 360/450 nm (excitation/emission) and absorbance of L-DOPA at 450 nm using a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Blanks and quench controls were included in each assay plate. Hydrolytic EEAs below detection were reported as 0 nmol g^{-1} dry soil h^{-1}, and POX activities were reported as 60 nmol g^{-1} dry soil h^{-1}, if below detection. To assess relative microbial investment towards acquiring certain nutrients, we calculated ratios between ln (BG) and the sum of N-acquiring enzyme activities ln (NAG + LAP) for C relative to N, ln (BG) and ln (PHOS) activities for C relative to P, and ln (NAG + LAP) and ln (PHOS) activities for N relative to P. While soil enzyme ratios can be effectively used as indices of relative microbial nutrient limitation (Sinsabaugh and Follstad Shah, 2012), as we did in this study, they should not be used to identify the one growth-limiting nutrient in any system, and they are also best interpreted conservatively and with consideration of local biological and edaphic conditions (Zeglin et al., 2007; Fierer et al., 2021; Mori et al., 2023).

Microbial community composition – Soil genomic DNA was extracted using a modified CTAB method and allowed to precipitate overnight in PEG 6000, followed by an ethanol wash the next day (DeAngelis et al., 2010). Polymerase chain reaction (PCR) was performed to target the 16S rRNA gene V4 region using 515F/806 R primers with existing protocols, except 25 cycles were run instead of 35 to minimize PCR bias (Carson and Zeglin, 2018). Libraries were gel-purified, and three separate libraries were sequenced with 2 × 150 paired-end Illumina MiSeq runs (Illumina, San Diego, CA, USA).

Sequence data were processed using QIIME2 (Bolyen et al., 2019). Sequence reads from demultiplexed data were trimmed to 150 bp based on the lowest library quality, denoised using DADA2 (Callahan et al., 2016), and then classified taxonomically as amplicon sequence variants (ASVs) using the Ribosomal Database Project classifier V2.13 (Wang et al., 2007; Callahan et al., 2017). Afterwards, we merged the libraries. ASVs were removed if they had less than three reads (Bokulich et al., 2013) and if they were classified as mitochondria or chloroplasts. This quality control ended in a dataset with 19,251,474 sequence reads and 21,816 ASVs. Because of the uneven distribution of reads among samples, we first removed samples that had less than 15,000 reads, losing 30 of the 340 samples, and then normalized the data by proportionally transforming each ASV read within a sample using *phyloseq* (McMurdie and Holmes, 2013). Raw sequence data used in this study can be found in the NCBI BioProject database under the accession PRJNA398249 and PRJNA577961 (https://www.ncbi.nlm.gov/bioproject/).

Statistical analyses – We assessed whether fire management and fertilization affected soil chemistry and soil EEAs by using a linear modeling approach in R V4.2.1 (R Core Team 2022) in the RStudio environment V2022.07.0 (RStudio Team 2022). Fire, N-fertilization, and P-fertilization treatments were set as interacting fixed effects in all models. For GWC, soil EEAs (natural log-transformed +1), and EEA ratios, we used linear mixed effect models that included month as an interacting fixed effect and block as a random intercept to account for repeated sampling with the *lmerTest* package (Kuznetsova et al., 2017). For soil C, N, molar C:N ratio, pH, we used linear models. Also, correlations between soil chemistry and EEAs were tested using linear models. We checked for homoscedasticity and residual normality for every model; in cases where model assumptions were not satisfied, outlier data were removed if they fell outside the 1.5 × interquartile range from the 25 and 75% quartiles and new models were applied, though inference was similar between models that included outliers and those without them. All models except for soil chemistry and soil EEA correlations were assessed using analysis of variance (ANOVA) in the *stats* package (R Core Team, 2020), and pairwise comparisons between months or for significant interactions were further evaluated using Tukey's post-hoc analyses with the *emmeans* package (Lenth, 2016). Significance was set at $\alpha \leq 0.05$.

We assessed whether fertilization differentially affected soil EEAs and putative copiotroph and oligotroph relative abundances between fire management treatments by testing their log response ratios (LRRs) against zero using LMMs that included blocks as random intercepts. LRRs were calculated at each month using the equation

$$\mathrm{LRR} = \frac{\ln\left(R_F\right)}{\ln(R_C)}$$

where R_F is the soil EEA or normalized abundance of the microbial group in the N- and P-fertilized (alone and in combination) plot, and R_C is the soil EEA or normalized abundance of the microbial group in the control plot within the same block. Putative copiotrophic or oligotrophic phyla and classes were defined based on nutrient responses identified in previous studies (Ramirez et al., 2012; Leff et al., 2015). We assessed whether microbial community composition shifted in response to fire management, N-fertilization, or P-fertilization treatments, and within the year, by using a permutational analysis of variance (PERMANOVA) that included field treatments and month as interacting fixed effects based on the Bray-Curtis dissimilarity in the normalized dataset. Based on the PERMANOVA, we compared microbial community composition between combined field treatments using the *pairwiseAdonis* package (Martinez Arbizu, 2020) and visualized microbial community composition using non-metric multidimensional scaling (NMDS) ordination with the lowest stress value from twenty iterations.

3. Results

Soil chemistry – Chronic fertilization had minimal effects on most soil chemistry parameters (Table 1, Table S1). Soil pH was not different among fertilization or fire management treatments, and neither soil total C nor N increased following chronic fertilization or fire suppression. Yet, N-fertilization in unburned prairie soils lowered the mean soil C:N ratio from 13.6 to 12.7 ($P = 0.029$), and burned prairie soils fertilized with P had a higher mean soil C:N ratio than the N- or N- and P-fertilized burned prairie soils (post-hoc: all $P < 0.05$). GWC differed monthly (Fig. S1), ranging from 0.192 g water g^{-1} dry soil in October to 0.322 g water g^{-1} dry soil in December, with a time by fire interaction ($F = 6.50$, $P < 0.001$) reflecting the lower water content in unburned prairie soils relative to burned prairie soils in August, September, and November (post-hoc: all $P \leq 0.01$).

Extracellular enzyme activities – Annual burning decreased soil C-acquiring BG and CBH activities, but not POX activity, with no interactive effects (Table S1; Figure S2). Supporting the product-inhibition hypothesis, chronic N-fertilization consistently reduced N-acquiring NAG and LAP activities by 11 and 14%, respectively (NAG: $F = 7.84$, $P = 0.012$; LAP: $F = 19.9$, $P < 0.001$); but, there was no analogous PHOS

Table 1
Soil chemistry in soils with different fire and fertilization treatments.

Fire	Fertilization	GWC	Soil %C	Soil %N	Molar soil C:N	Soil pH
Burned	Control	0.259 (±0.057)^{a}	2.45 (±0.05)$^{\circ}$	0.21 (±0.00)$^{\circ}$	13.6 (±0.3)$^{\circ a}$	5.50 (±0.23)
	+N	0.258 (±0.060)^{a}	2.44 (±0.45)	0.22 (±0.04)	13.1 (±0.4)^{b}	5.05 (±0.12)
	+P	0.272 (±0.080)^{a}	2.81 (±0.29)$^{\circ}$	0.23 (±0.02)$^{\circ}$	14.1 (±0.4)$^{\circ a}$	5.34 (±0.06)$^{\circ}$
	+N + P	0.269 (±0.061)^{a}	2.52 (±0.16)$^{\circ}$	0.22 (±0.02)$^{\circ}$	13.4 (±0.3)$^{\circ b}$	5.24 (±0.25)
Unburned	Control	0.237 (±0.066)^{b}	2.66 (±0.33)	0.23 (±0.03)	13.5 (±0.5)^{a}	5.23 (±0.19)$^{\circ}$
	+N	0.224 (±0.042)^{b}	2.93 (±0.77)	0.27 (±0.07)	12.7 (±0.2)^{b}	5.36 (±0.67)
	+P	0.233 (±0.056)^{b}	2.34 (±0.24)	0.20 (±0.03)	13.5 (±0.3)^{a}	5.40 (±0.12)
	+N + P	0.236 (±0.065)^{b}	3.00 (±0.44)	0.27 (±0.04)	13.2 (±0.1)^{b}	5.40 (±0.66)

Values are given as means (± standard deviation). GWC is gravimetric water content in g water g^{-1} dry soil.
+N, nitrogen-alone; +P, phosphorus-alone; +N + P, nitrogen- and phosphorus-fertilized.
$^{\circ}$denotes only three plot samples instead of four.
Letters within the same column indicate differences between fire treatments (GWC) and N-fertilization (molar soil C:N) with different letters indicating significant differences based on Tukey post-hoc tests.

decrease under P-fertilized conditions, and no N- by P-fertilization interaction. Contrary to predictions supporting the copiotrophic hypothesis, neither N- nor P-fertilization lowered all soil EEAs (Fig. 1, Figure S2). Instead, fertilization effects were consistently stronger in unburned prairie soils, in that all EEAs except for POX tended to decrease (i.e., all LRR means were negative) under nutrient fertilization, and the only LRRs that differed from zero were in unburned prairie soils (Fig. 1, Table S2). Interestingly, P-fertilization effects on soil EEAs always emerged with a temporal interaction (Table S1; Figure S2) that was not consistent with any of our predictions. Post-hoc tests showed that P-fertilization increased soil CBH activities in July and December, and decreased them in November; and decreased soil NAG activities in August (Figure S2).

Microbial enzymatic investment for C relative to N, an index of microbial N limitation, was lower (i.e., more N-limited) in burned prairie soils, and higher (i.e., less N-limited) under N-fertilization with no interactive effects (Fig. 2, Table S1), both consistent with the product-inhibition hypothesis. N-fertilization also decreased microbial enzymatic investment for C relative to P and N relative to P, both indices of microbial P limitation; and P-limitation relative to N was higher in the burned prairie (Fig. 2, Table S1). However, these P-limitation indices did not respond to P-fertilization, inconsistent with the product-inhibition hypothesis. Instead, P-fertilization had a significant interaction with time; post-hoc tests showed that, in soils not fertilized with P during June and July, microbial enzymatic investment for C relative to N was higher, and enzymatic investment for N relative to P was lower, but relative enzymatic investment for nutrients did not change in the P-fertilized soils (Figure S3).

Plot-scale soil pH and EEAs of soils sampled in July were not correlated (Figure S4), though P-acquiring PHOS activities tended to be higher at lower soil pH ($R^2 = 0.13$, $P = 0.05$). Soil BG activities and soil total N were weakly positively correlated ($R^2 = 0.18$, $P = 0.03$), but no other enzyme activities were correlated to soil C or N. Across the time series, BG, NAG, and PHOS activities were weakly positively correlated to GWC (Figure S4), reflecting a trend of higher values in August and September with subsequent decreases in October, particularly in burned prairie soils (Table S3; Figure S1, S2).

Microbial community composition – Data on the relative abundance of

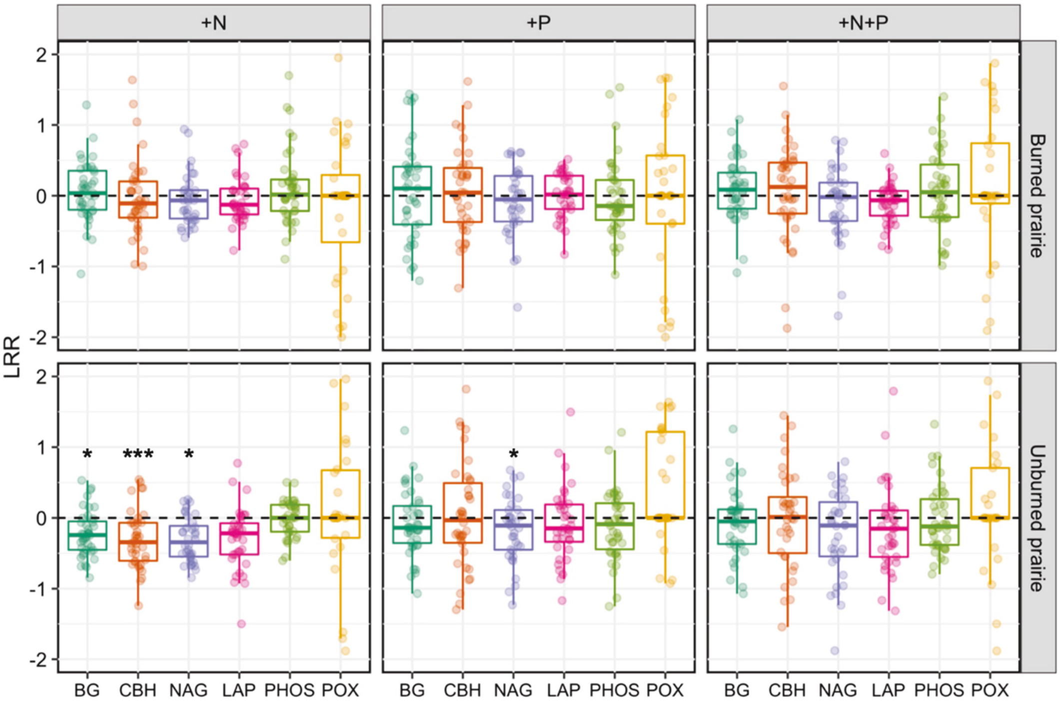

Fig. 1. Log response ratios (LRRs) of extracellular enzyme activities from fertilization separated by fertilization (+N, N-alone; +P, P-alone; +N + P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at the $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ thresholds.

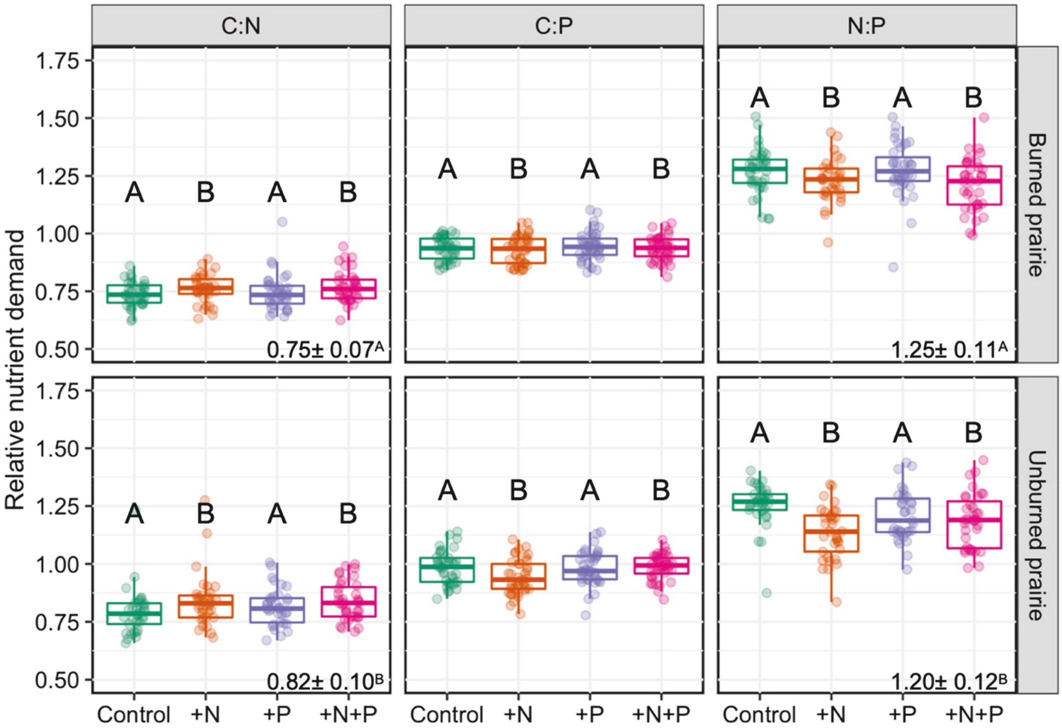

Fig. 2. Relative soil microbial demand for C:N, C:P, and N:P during the study time course summarized by boxplots. Each point is the ratio between C-, N-, and P-acquiring enzymes for each plot corresponding to the fertilization treatment (+N, N-alone; +P, P-alone; +N + P, N- and P-fertilized), represented by color. Capital letters within boxplots or near mean values for fire treatments indicate group differences at $P \leq 0.05$.

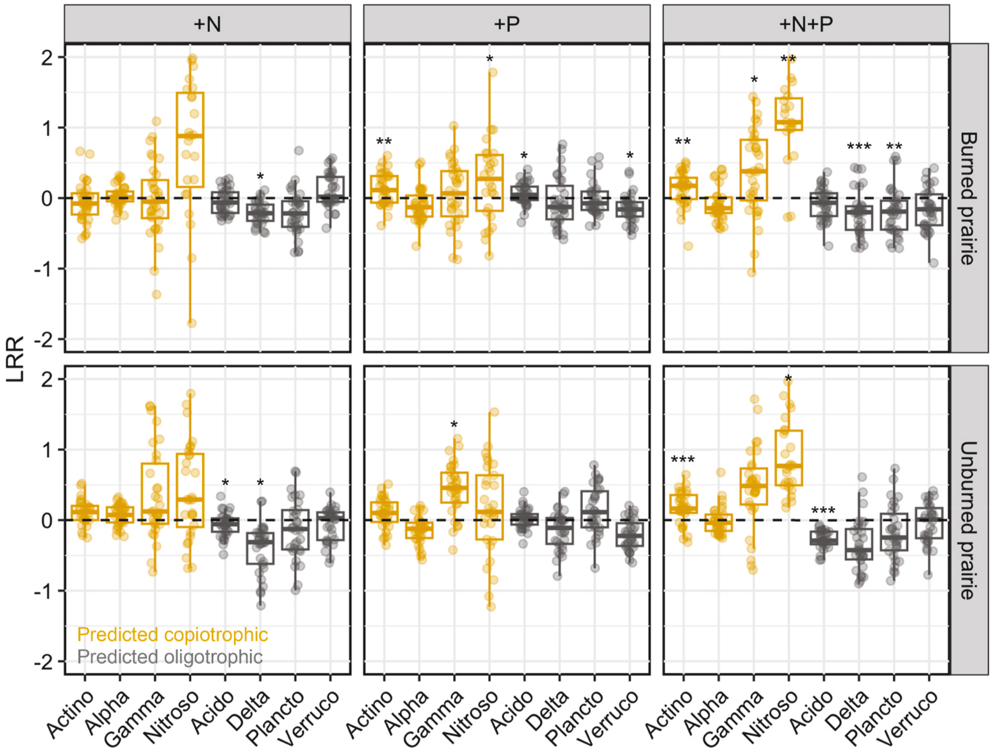

Fig. 3. Log response ratios (LRRs) of microbial phyla and classes predicted to be copiotrophs or oligotrophs, separated by fertilization (+N, N-alone; +P, P-alone; +N + P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ thresholds. Labels on x-axis are shortened for clarity [Actino, Actinomycetota (formerly Actinobacteria); Alpha, Alphaproteobacteria; Gamma, Gammproteobacteria; Nitroso, Nitrososphaera (formerly Thaumarchaeota); Acido, Acidobacteriota (formerly Acidobacteria); Delta, Deltaproteobacteria; Planco, Planctomycetota (formerly Planctomycetes); Verruco, Verrucomicrobiota (formerly Verrucomicrobia)].

bacterial and archaeal soil populations provided moderate support for the copiotrophic hypothesis. Of the 48 total comparisons of putative copiotroph and oligotroph relative abundance, 16 groups showed significant responses, 15 of which changed as expected due to chronic fertilization (i.e., increase in copiotrophs, decrease in oligotrophs); the exception was Acidobacteriota, a putative oligotrophic group which increased in burned P-fertilized prairie soils (Fig. 3, Table S2). Relative abundance within all putative copiotrophic groups across the N-, P-, and dual- (N and P) fertilized treatments increased by an average 29% ($t = 4.49$, $P = 0.003$), opposed to relative abundance within putative oligotrophic groups which declined by 11% ($t = -5.01$, $P = 0.002$). Interestingly, P-fertilization had a greater impact in burned prairies, where relative abundances of four groups changed significantly in burned P-alone prairie soils in contrast to one in unburned P-alone prairie soils, and five groups in burned dual-fertilized prairie soils compared to three in unburned dual nutrient prairie soils. Also, dual-fertilization resulted in higher-magnitude LRRs and eight significantly responding groups, in contrast to the three or five significantly responding groups in the N- or P-alone treatments, respectively (Fig. 3, Table S2).

Soil bacterial and archaeal community composition also changed in response to chronic N- and P-fertilization. Independently, N- and P-fertilization explained 2.8 and 3.5% of the variation in the PERMANOVA, respectively, while their interactive effect was weaker (0.7% of variation; Table 2). Fire management treatment also explained 2.3% of the variation, and interactions among the three field treatments together explained 4.3%. Pairwise PERMANOVAs showed that, except for control soils between fire management treatments, community composition was different among all combined field treatments (Table S4), and the NMDS supported these findings in community structure change (Figure S5). Also, a relatively large amount of variation in community composition was attributed to monthly variability (4.5%), and this further depended on whether or not prairies were fertilized with P (3.5% of variation; Table 2). From January through May, microbial community composition in soils not chronically fertilized with P was distinct, then converged towards higher similarity with P-fertilized soils as the growing season progressed, and remained similar through fall and winter (Figure S6).

4. Discussion

By measuring soil chemistry, extracellular enzyme activities, and microbial community composition monthly for one year at a thirty year long field experiment that crossed N- and P-fertilization with different fire management treatments in a tallgrass prairie ecosystem, we found strongest support for the product-inhibition hypothesis as a result of higher N conditions in response to N-addition or fire suppressison, conditional support for the copiotrophic hypothesis to N-fertilization, weaker support for both hypotheses to P-fertilization, and weakest support for the soil pH shift hypothesis. The cellular level product-inhibition feedback, in which higher bioavailability of a nutrient lowers the synthesis of enzymes that catalytically release the nutrient from SOM, was broadly supported under higher N conditions from N-fertilization or fire suppression, but not under P-fertilized conditions. The population level copiotrophic feedback, in which fertilization increases relative abundance of microbial populations that tradeoff growth over extracellular enzyme synthesis, had moderate support in population relative abundance responses under both N- and P-fertilization, but evidence for resource acquisition tradeoffs was only seen in the enzyme activities measured under N-fertilized conditions in unburned prairie soils. Soil pH response to fertilization was weak, and there were no strong correlations between enzyme activity and soil pH to implicate in mediating the biological responses. Consequently, based on this study and previous research that is discussed below, we suggest that SOM-decomposing enzyme responses to eutrophication are more consistently regulated by the nutrient and energy demand of microbial cells under local environmental conditions, not by a universal tradeoff in resource acquisition for faster growth of copiotrophic populations, and that soil pH mediated feedbacks are more likely to occur in different soil types than the one found at this site.

Table 2
Permutational analysis of variance (PERMANOVA) results for soil microbial communities under different fixed effects.

Fixed effects	Degrees of freedom	Pseudo-*F*	R^2	*P*-value
Fire (F)	1	7.93	0.023	0.001
Nitrogen (N)	1	9.67	0.028	0.001
Phosphorus (P)	1	12.45	0.036	0.001
Month (M)	10	1.56	0.045	0.001
F × N	1	4.57	0.013	0.001
F × P	1	4.39	0.013	0.001
N × P	1	2.31	0.007	0.008
F × M	10	0.96	0.028	0.617
N × M	10	0.98	0.028	0.562
P × M	9	1.35	0.035	0.005
F × N × P	1	4.07	0.012	0.001
F × N × M	10	0.82	0.024	0.979
F × P × M	9	0.85	0.022	0.944
N × P × M	9	0.88	0.022	0.907
F × N × P × M	9	0.65	0.017	1.000
Residuals	227		0.649	

Strongest support for the product-inhibition hypothesis, only under higher N conditions – The cellular level product-inhibition feedback mechanism was supported under higher N conditions, where N-acquiring enzyme activities, but not other functional enzyme groups, declined as bioavailable N increased due to N-fertilization or fire suppression. A recent meta-analysis of experiments in a variety of biome types also supports the generality of this phenomenon by finding that N-fertilization rates at or above 10 g N m^{-2} yr^{-1} often result in lower soil NAG activities, and that soil BG and PHOS activities tend to increase at the same N-fertilization rates (Jia et al., 2020), suggesting soil microbes become less N-limited, relative to other elements, with N enrichment. This mechanism has also been broadly supported across varying soil types, and a variety of aquatic and terrestrial habitats (Zeglin et al., 2007; Sinsabaugh et al., 2009). Importantly, our study shows that this product-inhibition response to N is not exclusive to the context of mineral N-fertilization, since microbial investment to acquire N was also lower (i.e., C relative to N increased) in unburned prairie soils. In tallgrass prairie, fire suppression is known to increase bioavailable N (Blair, 1997; Connell et al., 2020), including at this experiment where soil N availability is 3–5 times higher under fire suppression (Nieland et al., 2021); thus, product-inhibition feedback on N-acquiring soil microbial enzyme activities was apparent regardless of the driver of increased soil N availability.

In contrast, chronic P-fertilization did not decrease relative PHOS activities. This was somewhat surprising since soils at this site have low levels of bioavailable P relative to N (Johnson et al., 2003), and P-fertilization at other sites, including grasslands, can decrease microbial enzymatic investment for P (Allison and Vitousek, 2005; Riggs and Hobbie, 2016). However, there are possible soil chemical and biological reasons for this result. First, the P-fertilization rate at this experiment is an order of magnitude lower than that of N, and soil P availability is low in these soils due in part to low inorganic P solubility and rapid sorption to calcium or iron minerals (Ippolito et al., 2010; Pierzynski, 2016); thus, it is possible that chronic P-fertilization did not relieve limitation of microbial P requirements. Second, increased P availability has minimal effects on aboveground net primary productivity at this tallgrass prairie site (Avolio et al., 2014) since the dominant grasses here rely heavily on arbuscular mycorrhizal (AM) fungi to uptake P from the soil solution, such that AM colonization may be more important for P-acquisition than phosphatases for plant growth (Wilson and Hartnett, 1997; Johnson et al., 2015). Reduction of root-AM relationships also decreases soil aggregation and SOM accumulation (Wilson et al., 2009). Further, PHOS responses to P-fertilization may not have been observed because EEAs were measured in bulk soil rather that the rhizosphere, where PHOS activities are higher (Chen et al., 2002) and more sensitive to plant-soil feedbacks (Ochoa-Hueso et al., 2017). In sum, soil microbial responses to P-fertilization in this experiment may be restricted by soil mineralogy, and feedbacks on SOM dynamics are more likely related to loss of plant-fungal symbioses following P-fertilization than to enzyme product inhibition in bulk soils.

Weaker, conditional support for the copiotrophic hypothesis – The copiotrophic response in community composition was supported most strongly under the combined N- and P-fertilization treatment, which produced more significant and stronger changes in the relative abundance of putative copiotrophic and oligotrophic groups than did N- or P-fertilization alone. Similar soil microbial groups across different ecosystems have positively responded to N-fertilization (Ramirez et al., 2012; Leff et al., 2015), so greater N availability appears to support the growth of certain taxa with a competitive advantage under non-limiting growth conditions (Fierer et al., 2007). But, in contrast to this hypothesis, there was no evidence for a tradeoff between microbial population growth and resource acquisition, since soil EEAs did not decrease in the combined N- and P-fertilization treatment. Only under fire suppression combined with chronic N fertilization did all cellulolytic (BG and CBH) and NAG activities decrease significantly, despite weaker shifts in copiotroph and oligotroph relative abundance. Thus, in contrast to results of laboratory experiments in which N-addition decreased soil EEAs concurrent with shifts in microbial community composition (Ramirez et al., 2012), we observed “copiotrophic” turnover in bacterial and archaeal community composition independent of soil enzymatic feedbacks, and vice versa. Furthermore, across the field experiment, soil N availability was highest in the fire-suppressed, N-fertilized treatment (approximately 40 times higher than in the burned, unfertilized treatment) (Nieland et al., 2021), suggesting that a threshold of high N load must be crossed before a decrease in overall enzyme production occurs. These observations may help explain why chronic N-fertilization does not change soil total C concentrations across many grasslands (Keller et al., 2022) despite some consistency in microbial taxonomic responses among the same distributed experiments (Leff et al., 2015).

Notably, for a soil microbial response mechanistically based on faster growth to manifest, soil available C, N, and P must all meet the demand for biomass synthesis. In this study ecosystem, plant primary production is co-limited by N and P. The root-AMF symbiosis allows dominant grasses to meet increased P demand following N addition; to support this interaction, this also means that new C is fixed by grasses into the soil under chronic-N addition (Wilson et al., 2009). Under dual N- and P-fertilization, then, soil microorganisms will also experience a relief from C-limitation, promoting the strongest “copiotrophic” response in terms of taxonomic turnover towards populations with the highest possible growth rates. In contrast, N-fertilization alone also increased bulk soil microbial P-limitation relative to both C and N (Fig. 2), which would in turn constrain growth rates and related taxonomic turnover. In a related study, microbial biomass C was higher in burned, unfertilized soils than unburned or N-fertilized soils, but we did not measure biomass in P-fertilized soils (Nieland et al., 2021); higher microbial biomass may not be correlated with growth rates. The complex and critical interactions that exist between plants and soil microorganisms *in situ* also make it challenging to predict ecosystem responses based on mechanisms observed in laboratory experiments.

Weakest support for the soil pH shift hypothesis – Soil enzyme activity was not correlated with soil pH, suggesting that the small increase in soil acidity from chronic N-fertilization is not a primary driver of SOM decomposition feedbacks at this experiment. Recent meta-analyses compile evidence that long-term N-fertilization often lowers soil pH (Ouyang et al., 2018; Jia et al., 2020), which can in turn decrease the activity of soil enzymes by moving away from their pH optima for maximum catalytic activity or by decreasing soil microbial biomass and its activity (Treseder, 2008; Turner, 2010; Sinsabaugh and Follstad Shah, 2012). For example, chronic N-fertilization in hardwood and pine forests lowered soil pH by 0.4 units and led to a decrease in soil microbial biomass and soil microbial respiration (Bowden et al., 2019). However, this study site might be different because of soil characteristics. Limestone parent material and base cation levels near saturation in these soils (Ransom et al., 1998) help to buffer soil pH for longer periods of time. Therefore, soil pH change has been minimal at this experiment relative to other locations, and thus becomes a weaker factor in regulating ecosystem functional shifts measured after 30 years of fertilization.

Temporal dynamics of soil microbial structure and function – There was no consistent monthly shift in soil microbial community structure or extracellular enzyme activity over the sampling time course, despite the strong seasonality of temperature and plant growth in this temperate ecosystem (Knapp et al., 1998). This observation contrasts with Mediterranean or snowpack-dominated ecosystems, where strong links between soil microbial taxonomic turnover and C and N cycling can occur (Schmidt et al., 2007; Cruz-Martínez et al., 2009). During the same time course of sampling at this experiment, we observed a large increase in bacterial gene copy number during June, July, and August, across all control and N-fertilized field treatments (Carson and Zeglin, 2018), which we attributed to higher labile C availability from root exudation during the plant growing season. The lack of seasonality in C-cycling enzyme activities suggests that root exudate C may be rapidly assimilated and sequestered into enzyme-protected pools following bacterial death (Bai and Cotrufo, 2022), or that a large proportion of active soil enzymes are stabilized and not rapidly responsive to changes in substrate availability (Burns, 1982). In support of the latter possibility, there was no transient decrease in N-acquiring enzyme activity following the experimental fertilizer application in June, despite a pulse in nitrification potential at that time (Nieland et al., 2021), and there was a consistent increase in enzyme activity across all field treatments through the sampling time course, perhaps in response to increased root tissue input through what was a relatively wet year (Carson and Zeglin, 2018). Interestingly, however, both soil microbial communities and enzyme activities varied through time in a manner dependent on whether or not the soils were fertilized with P. While most interactions between P-fertilization and time on soil enzyme activities were idiosyncratic, soil microbial communities not fertilized with P clearly shifted across the year, converging after the fertilizer application in June (Fig. S6). This pattern was a surprise, and it deserves future exploration and interpretation: There are likely to be factors beyond fertilization and fire management playing out at different time scales that affect soil microbial community composition and soil enzymatic feedbacks, not necessarily in a synchronous manner.

In addition to elevated N availability and lower N-acquiring enzyme activities, chronic fire suppression also caused higher cellulolytic enzyme activities, likely driven by the multiannual accumulation of aboveground litter that is not consumed by fire (Ajwa et al., 1999; Pellegrini et al., 2020). However, POX activity did not respond to fire suppression, despite this increase in litter input and a concurrent shift toward greater woody plant cover, which adds more litter with higher lignin content (Norris et al., 2001; Carson et al., 2019). One reason for this may be related to the limited change in the soil fungal community following fire suppression. Fungal communities in unburned prairie soils are not dominated by the lignin-decomposing Basidiomycetes typical of forest soils, despite concurrent woody encroachment (Morrison et al., 2016; Carson et al., 2019). This stability in the fungal community has implications for decomposition responses to N-fertilization in grasslands pressed by global change. In forests, higher N availability slows decomposition because of the decline in basidiomycete relative abundance and soil oxidative enzyme activity (Fog, 1988; Frey et al., 2004; Edwards et al., 2011), while in other grassland soils, N-fertilization tends to either increase or not change oxidative enzyme activity (Keeler et al., 2009; Riggs and Hobbie, 2016). In our study, N-fertilization also did not affect POX activity even under fire suppression and woody encroachment, showing that soil microbial communities, even with significant changes to their structure, can maintain “grassland-like” functioning in prairies that have transitioned from a grassland state to a woodland state (Ratajczak et al., 2014).

Summary and conclusions – The product-inhibition feedback (cellular level), copiotrophic feedback (population level), and soil pH shift feedback (ecosystem level) mechanisms we evaluated are likely active over different time scales in response to eutrophication, particularly with N, so there is a possibility that soil microbial community function and composition responses emerged at different points in time during the three decades of treatment. On the cellular level, increased N availability from fertilizer application in soils with no fertilization history may quickly repress N-acquiring enzyme synthesis, but soil microbial response to one pulse of N is likely short-lived as immobilization and plant uptake also quickly reduces N availability in the soil solution (Dell and Rice, 2005). Any changes to microbial community composition from early fertilization events may have also been ephemeral, as there was no strong shift in response to a one-time fertilization event of N in a switchgrass field with no recent fertilization history (Chen et al., 2019). But, as soil N pools begin to increase due to accumulation of higher quality plant litter following multiple growing seasons of N-fertilization, or from total accumulated aboveground litter resulting from fire suppression (Johnson and Matchett, 2001; Connell et al., 2020), the alleviation of microbial N-limitation could transition from being transient to persistent, which might explain the lack of fertilizer event responses to soil enzyme activities in this experiment after thirty years of fertilization. Moreover, elevated N availability from internal mineralization rather than supplemental addition could also support the growth of copiotrophs over time (Hart et al., 1994; Blaško et al., 2013), and their associated smaller biomass pool could explain the small decrease in soil microbial biomass in N-fertilized and unburned prairie soils at this experiment and others (Nieland et al., 2021). Although soil pH is not a leading mechanism for altered SOM decomposition potential at our experiment, this mechanism can shape soil microbial responses at other sites on a faster trajectory; for example, soil pH declined within a decade of annual N-fertilization at a different tallgrass prairie with sandy soil that resulted in significant shifts in the soil bacterial and archaeal community composition (Widdig et al., 2020). Given the potential for strong changes to abiotic (i.e., SOM pools and pH) and biotic (i.e., microbial populations) soil properties from fertilization, soil microbial responses may reverse in a similar manner if chronic fertilization is ceased (e.g., Clark et al. 2009; Bowman et al. 2018; Nieland et al. 2021), but with significant, and potentially asynchronous, time lags for recovery of both soil microbial community function and composition (Gilliam et al., 2019). In addition, fire suppression may continue to constrain these microbial responses and recovery.

Our repeated measurements of the soil microbial community composition and enzyme activities found cellular level product-inhibition feedbacks tied to microbial nutrient demand can vary between nutrients, with their sensitivities to fertilization depending on baseline nutrient levels. Specifically, N-fertilization reduced microbial investment to acquire N because it alleviated N-limitation and the need to produce N-acquiring enzymes, but this phenomenon did not occur under P-fertilization. Additionally, there was limited evidence for a tradeoff between microbial population growth and nutrient acquisition; therefore, mild shifts to the soil microbial community may not result in drastic changes to enzymatic feedbacks tied to SOM decomposition. Instead, a threshold-response dynamic may be more widespread. Soil properties at this site prevented soil pH to decrease, and thus had minimal effect on soil enzyme activities. In conclusion, changes to nutrient availability induced by global change drivers and land management practices at the local level will affect the N and P cycles in different ways since microbial nutrient demand is constrained by local environmental conditions (Burns et al., 2013), but soil microbial community change may not always portend a slower breakdown of organic matter supplied by the aboveground world.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data can be found through the Konza Prairie LTER repository or NCBI.

Acknowledgments

We thank personnel at Konza Prairie Biological Station and the Konza Prairie LTER program for maintaining the Belowground Plot experiment; Kiona Freeman, Becky Malanchuck, and Sean Morris for helping collect samples and laboratory assistance; Dr. John Blair for use of laboratory space and equipment; Dr. Alina Akhunova and team at the Kansas State Integrated Genomic Facility for sequencing services; and Dr. Caitlin Broderick and Dr. Emily Wedel for statistical advice. We acknowledge the named and unnamed peoples, including most recently the Kaá^{n}ze (Kaw) People, who were stewards of the lands on which this research was conducted prior to European settlement; we pledge to respect and honor the past and current legacy, cultural history, and knowledge of the Kaw Nation. This work was supported by the National Science Foundation [DEB-1440484] and Kansas State University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109247.

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