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Woody encroachment increases carbon degradation rates and alters soil microbial community structure in restored and remnant mesic tallgrass prairie

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ABSTRACT

Grasslands store approximately one-third of the global terrestrial carbon stock and microbial communities in grassland soil play an essential role in soil carbon sequestration. Despite being vital carbon reservoirs, grasslands face many challenges to carbon sequestration, perhaps most notably conversion for agricultural use and encroachment by woody plants. We used a temperate grassland system consisting of a reconstructed tallgrass prairie and an adjacent never cultivated remnant prairie both undergoing woody encroachment to understand how management history and woody encroachment influence degradation rates of multiple forms of organic carbon and microbial community structure. We measured rates of hydrolytic carbon-degrading extracellular enzymes and used 16S and ITS amplicon sequence data to categorize bacterial and fungal taxa into microbial groups of potential carbon use efficiency. We found that extracellular enzyme rates were higher in the remnant site and in encroached soils. Additionally, microbial groups with low potential carbon use efficiency—which are expected to contribute to soil C release—were generally more prevalent in the remnant site and in encroached soils. This could suggest that higher rates of organic carbon degradation occur with lower potential microbial carbon use efficiency. Our results contradict the widely held notion that never-cultivated remnant grasslands are reliable carbon sinks and suggest that woody encroachment can promote C release from grassland soils.

1. Introduction

Grasslands cover approximately 40 % of the Earth's land surface and store an estimated third of the terrestrial carbon (C) stock (Bai and Cotrufo, 2022), so even small changes in soil C stocks in grasslands have large implications for greenhouse gases. As in other biomes, soil microbes play an essential role in grassland C cycling. When microbes metabolize C inputs—in the form of leaf and root litter and root exudates—a proportion of C is assimilated into microbial biomass and metabolic products (e.g., enzymes, polysaccharides) while some is mineralized and subsequently respired (Schimel and Schaeffer, 2012). This ratio of C stored in microbial biomass relative to C respired is known as carbon use efficiency (CUE) (Manzoni et al., 2012), and it is an important indicator of soil organic C content (Tao et al., 2023). However, the extracellular enzymes that microbes produce to degrade organic substrates into useable forms are energetically costly, so their production increases microbial respiration (Allison, 2014; Malik et al., 2019; Ramin and Allison, 2019), and reduces growth and assimilation of C into microbial biomass (T.-H. Anderson and Domsch, 1985; Dijkstra et al., 2015). Accumulating evidence suggests that microbial functional groups and taxa vary in CUE and their respective investments into extracellular enzyme production and growth (Anthony et al., 2020; Baldrian, 2009; Midgley and Phillips, 2019; Osburn et al., 2021; Soares and Rousk, 2019; Ullah et al., 2021; Wieder et al., 2015), so environmental factors that shape soil microbial communities may have consequences for soil C stocks.

Although grasslands are important carbon reservoirs, they are some of the world's most heavily managed and altered ecosystems, which brings considerable challenges to carbon sequestration. Conversion to agriculture heavily depletes soil C: across grasslands in the midwestern

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United States for example, approximately 27 % of total C was lost from the whole soil profile (Mann, 1985) and nearly 50 % of total C was lost from surface soil (DeLuca and Zabinski, 2011). Many grasslands are also dependent on fire and/or grazing, and disruptions of historical fire and grazing regimes can have profound effects on grassland ecosystems. Both reduced fire frequencies (Bond et al., 2003; Briggs et al., 2002) and grazing pressure (Auken, 2000; Briggs et al., 2002), combined with rising carbon dioxide concentrations (Bond et al., 2003; Bond and Midgley, 2012), have substantially increased tree and shrub encroachment into the world's grasslands over the past 200 years (Archer et al., 2017).

Meta-analyses have generally found that woody encroachment into grasslands increases soil C (Eldridge et al., 2011; Li et al., 2016), but this may not necessarily indicate C stabilization. Even though an increase in trees and shrubs in grasslands leads to increases in soil organic inputs of structurally complex litter (Filley et al., 2008; Liao et al., 2006a, 2006b), some studies show that woody-derived C accumulates primarily in rapid-cycling and physically unprotected soil fractions (Billings, 2006; Creamer et al., 2011; Liao et al., 2006b). Specifically, highly labile particulate organic matter can increase with woody cover (Mureva and Ward, 2017; Scharenbroch et al., 2010). In contrast, soil organic C derived from non-woody grasslands often accumulates in silt- and clay-sized fractions that are more physically protected and biochemically recalcitrant (Angst et al., 2021; Liao et al., 2006a, 2006b).

The variation in soil organic matter fractions among encroached and un-encroached soils likely interacts with microbial dynamics that underpin C cycling, like CUE. Some studies have documented greater abundances of copiotrophic bacteria—fast-growing taxa that thrive in nutrient-rich conditions and are thought to have low potential CUE—in encroached grasslands compared to un-encroached grasslands (Creamer et al., 2016; Xiang et al., 2018). Additionally, copiotrophic taxa can assimilate more woody-derived C than oligotrophic taxa (slow-growing taxa that thrive in nutrient-poor conditions and are thought to have high potential CUE) (Creamer et al., 2016). Combined with the finding that microbial respiration can be greater in encroached grasslands (Mureva and Ward, 2017), these patterns suggest that woody encroachment may lead to more rapid C cycling. Importantly, most research on woody encroachment is limited to xeric grasslands. Although a recent study of globally distributed grasslands found that microbial CUE generally increased with soil moisture (Risch et al., 2023), it is unclear how woody encroachment may impact microbial C cycling dynamics in mesic grasslands. This is an important knowledge gap to fill because it has been proposed that mesic grasslands have greater C sink potential compared to xeric grasslands (Barger et al., 2011).

In this study, we used paired sites of remnant and previously cultivated, restored mesic grasslands to investigate how woody encroachment and land management influence microbial C cycling dynamics. Although restoration activities often seek to match remnant reference sites, a global meta-analysis found that no ecosystems, including grasslands, fully recovered to reference conditions (Jones et al., 2018). For example, previous work in our field sites documented greater belowground biomass in the remnant site compared to the previously cultivated, restored site, likely because agricultural land use in this area shifted plant growth allocation to aboveground biomass (Jelinski et al., 2011). We measured extracellular enzyme activity to quantify degradation rates of several forms of organic C and characterized soil microbial communities to explore potential CUE of bacterial and fungal communities.

We developed three hypotheses to assess both the individual and interactive effects of woody encroachment and land management. We hypothesized that remnant soils would have faster organic C degradation rates compared to previously cultivated soils undergoing restoration (hereafter referred to as "restoration") (Hyp. 1). We expected this because of the possibly greater amount of root exudation and C inputs from the higher root biomass in the remnant site (Blagodatskaya and Kuzyakov, 2008; Zhou et al., 2021). Given that woody-derived C tends to accumulate in rapidly cycling soil fractions (Billings, 2006; Creamer et al., 2011; Liao et al., 2006b), we also hypothesized that soils with woody encroachment would have faster C degradation rates compared to soils without encroachment (Hyp. 2). Lastly, integrating Hyp. 1 and 2 we hypothesized that remnant soils with woody encroachment would have the highest rates of C degradation, and un-encroached, restoration soils would have the lowest C degradation rates (Hyp. 3). Further, we categorized bacterial and fungal taxa into relevant CUE functional groups to explore whether patterns in microbial CUE community composition accompanied microbial extracellular enzyme activity. We predicted groups with generally low potential CUE (fungi relative to bacteria, saprotrophs relative to other fungi, and oligotrophic relative to copiotrophic bacteria) would be prevalent in remnant and encroached soils.

2. Methods

2.1. Study system & site description

The tallgrass prairie of North America is a prime example of a grassland ecosystem that has been heavily impacted by land use conversion. Once encompassing 68 million hectares of the eastern portion of the Great Plains (Samson and Knopf, 1994), the tallgrass prairie was supported by Indigenous stewardship and the natural disturbances of frequent fire and grazing (R. C. Anderson, 2006; Kimmerer and Lake, 2001; McClain et al., 2021). Euro-American colonization of the region that began in the early 19th century then set in motion a period of intensive and widespread ecosystem alteration. Ultimately, more than 90 % of the tallgrass prairie was converted to other uses, especially row crop agriculture (Samson and Knopf, 1994). As a result, the tallgrass prairie ecosystem lost 40–60 % of its soil C (Kucharik et al., 2001; Parton et al., 2005). Today, the landscape is still dominated by agriculture, but intermixed with scattered fragments of remnant prairies and restorations. There is growing interest to understand the C sequestration potential of tallgrass prairie restorations, and particularly how it compares to C storage in remnant tallgrass prairie (Jelinski and Kucharik, 2009; Kucharik and Brye, 2013; Lal et al., 2007; Stocker et al., n.d.; von Haden and Dornbush, 2017). Tallgrass prairies in the Upper Midwest are also ideal for studying woody encroachment because they occur within a transition zone between North America's more arid grasslands and more mesic forests (Axelrod, 1985) where woody encroachment has been occurring for hundreds of years.

The Faville Prairie State Natural Area and the Faville Grove Sanctuary in southern Wisconsin contains over 300 ha of wet and wet-mesic tallgrass prairie remnants and restorations, prairie-forest, and wetlands and it is owned and managed by the University of Wisconsin-Madison Arboretum and the Southern Wisconsin Bird Alliance, respectively. This includes the 35-ha remnant Faville Prairie and the 18-ha previously cultivated, restored Tillotson Prairie in which our study took place. This region of North America was first inhabited by the Peoria, Meskwaki, Myaamia, Očhéthi Šakówiŋ, Hoocąk, and Kiikaapoi American Indian Nations (Native Land Digital; native-land.ca). Euro-American colonizers began arriving and working the land around 1840 and within 100 years, most of the area's prairies had been converted for row crop agriculture and grazing (Hawkins, 1940). However, the land that would come to be known as Faville Prairie "escaped both the plow and cow" and was used solely for mowing hay and occasional prescribed burning to remove dead grass (Rooney and Leach, 2010). Many native tallgrass prairie plants persisted as the mowing and burning somewhat imitated the prairie's historical disturbances of fire and grazing (Rooney and Leach, 2010). The conservationist Aldo Leopold helped to acquire the 60-ha in 1941 to save the best remaining prairie remnants from cultivation. Several years later the land was deeded to the University of Wisconsin-Madison, designated as a state natural area in 1952 (McCabe, 1978), and is thought to be the world's first publicly preserved prairie.

The previously cultivated Tillotson Prairie, directly south adjacent to the remnant Faville Prairie underwent a corn-soybean rotation for over 50 years, after which it was seeded with native prairie plants in 1999 (Jelinski et al., 2011). Seven years after restoration began at the Tillotson Prairie, total nitrogen by area was indistinguishable between the remnant Faville Prairie and the Tillotson Prairie (Jelinski et al., 2011). By our 2022 soil sampling, the Tillotson Prairie had been undergoing restoration for approximately 23 years. *Populus tremuloides* (quaking aspen), *Salix* spp. (willow), and *Cornus* spp. (dogwood) were recorded as encroaching into the Sanctuary's prairies in the mid-1970s (McCabe, 1978). Over the past several decades land managers have periodically employed prescribed burning, herbicide treatments and manual removal to limit woody encroachment in both the remnant Faville Prairie and the previously cultivated Tillotson Prairie (Rooney and Leach, 2010) (B. Herrick, University of Wisconsin-Madison Arboretum; D. Harry, Southern Wisconsin Bird Alliance; personal communication).

Bounded on the east by the Crawfish River, both sites overlie glacial lake sediment, and the soil texture is Wacousta silty-clay loam (fine-silty, mixed, superactive, Endoaquolls, wet Mollisol) (Jelinski et al., 2011). Soil pH is relatively neutral (~ 7), and the area's soils contain approximately 10 % organic matter, according to chemical and physical analysis of soils from a prairie restoration bordering the south boundary of Tillotson Prairie (D. Harry, Southern Wisconsin Bird Alliance; personal communication). This area has a temperate climate with a mean annual temperature of 8.2 °C and mean annual precipitation of 874 mm (mostly in September–November and March–May) (Jelinski et al., 2011). Both sites were inundated in the early summer of 2008 by a historically unprecedented extreme flood (Zedler and Herrick, 2023).

2.2. Experimental design and soil collection

We sampled soil from plots along two transects in both sites, using vegetation sampling plot grids initially established by Partch (1949) in the remnant Faville Prairie and extended by the Zedler lab group at the University of Wisconsin-Madison to the previously cultivated, restored Tillotson Prairie. In both sites, we selected two transects spaced 92 m apart. Sampling plots are 2 × 2 m, separated by 30.5 m, and the 15 plots in each row occur along a soil moisture gradient, with moisture increasing eastward toward the river (Partch, 1949). On 6–7 June 2022, we used scoopulas to collect soil from the top 15 cm from five haphazardly-selected points in each plot. The five samples were pooled by plot and mixed in zip-top plastic bags. Scoopulas were cleaned with ethanol wipes after sampling each plot to minimize microbial cross-over between plots. At each plot, we measured soil moisture to a depth of 20 cm with an Extech MO750 soil moisture meter. Soil samples were kept on ice for two days and then stored at −80 °C. We used on-the-ground vegetation surveys and images from Google Earth to assign plots as encroached ($n = 21$) or un-encroached ($n = 36$) by woody shrubs, saplings, or trees. One plot in Faville Prairie and two plots in Tillotson Prairie were inaccessible due to standing water, yielding 57 total soil samples (29 plots in Faville Prairie, 28 in Tillotson Prairie).

2.3. Soil extracellular enzyme assays

To assess soil microbial activities, we measured the rates of five hydrolytic C-degrading enzymes: α-glucosidase (AG; degrades starch), β-glucosidase (BG; degrades glucose), β-xylosidase (XYL; degrades hemicellulose), cellobiohydrolase (CBH; degrades cellulose), and *N*-acetyl-β-D-glucosaminidase (NAG; degrades chitin). We followed the protocol described in Bell et al. (2013). For each sample, 2.75 g of thawed, field-wet soil was blended in a 50 mM sodium acetate buffer (pH ~ 6) for 1 min. While soil slurry was stirring, 800 μl was transferred to two 96-well plates: one empty and one filled with 200 μl of 4-methylumbelliferone (MUB), with concentration varying by plate row (0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM). Then 200 μl of each of the five substrates was added by row to the soil slurry only plate. Solution in each well was pipetted up and down several times to mix and ensure soil microbes interacted with MUB and the substrates.

Plates were then sealed with ThinSeal™ film and incubated in the dark at 25 °C for 3 h. After incubation, 250 μl of solution from each well was transferred to black 96-well plates, and 10 μl of NaOH was added to each well to stop the reaction. Fluorescence was measured on a SpectaMax iD5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, U.S.) using an excitation wavelength of 365 nm and emission wavelength of 450 nm. Prior to calculating activity rates, soils were dried at 50 °C for 48 h to quantify soil dry weight. Extracellular enzyme activity rates are expressed as nmol of activity per gram of dry soil per hour (nmol g^{-1} h^{-1}).

2.4. DNA extraction, amplification, and amplicon sequencing

We extracted DNA from 0.25 g of thawed field-wet soil per sample by first lysing microbial cells and extracting the supernatant using the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Venlo, Netherlands). All remaining steps of the DNA extraction protocol were performed using the Qiagen QIAcube machine. We amplified the bacterial V4 region of the 16S rRNA gene and the fungal ITS2 gene region. Initial PCR products were sent to the Genomics and Microbiome Core Facility at Rush University Medical Center (Chicago, IL, U.S.) for purification, second PCR, and sequencing using the Illumina MiSeq V3 platform to produce 2 × 300 bp paired-end amplicons. Detailed methods on PCR and library prep are described in the Supplementary Materials.

2.5. Bioinformatics and microbial community characterization

All processing of 16S and ITS2 sequences, including primer removal, was performed with the software package DADA2 (Callahan et al., 2016). We clustered sequences into amplicon sequence variants (ASVs, 100 % similarity) and assigned taxonomy using the RDP (Wang et al., 2007) and UNITE (Abarenkov et al., 2010) databases for bacteria and fungi, respectively. This resulted in a total of 36,174 bacterial ASVs and 7664 fungal ASVs. After removing singletons to account for spurious presence, 75 % (27,263) of bacterial ASVs and >99 % (7657) of fungal ASVs were retained. We assessed sequence read depth among samples, and there were no significant differences in the number of bacterial ASVs detected among remnant prairie samples and previously cultivated prairie samples ($p = 0.9$). There were slightly more fungal ASVs detected in remnant prairie samples compared to previously cultivated prairie samples ($p = 0.02$), but this was less than a 1.1 magnitude of difference. For analysis, raw counts were converted to proportion of total ASVs and total sequence abundance as described below.

We categorized a portion of the bacterial ASVs into oligotrophic or copiotrophic life strategies. Oligotrophic bacteria generally specialize in the degradation of recalcitrant forms of C (Fierer et al., 2007; Trivedi et al., 2018), and as such should be expected to invest more into extracellular enzyme production and have lower CUE relative to copiotrophic bacteria, which specialize in degrading labile forms of C. We assigned oligotrophic and copiotrophic strategies using recent findings from Stone et al. (2023) which used stable isotope probing to measure growth responses of individual bacterial taxa to isotopically-labeled C sources. This is a more accurate method of identifying oligotrophic and copiotrophic strategies compared to approaches that base classifications at the phylum level, because it is well understood that taxa within phyla vary in metabolic strategies (Ho et al., 2017; Martiny et al., 2015; Morrissey et al., 2016; Philippot et al., 2010). From the ASVs in the Stone et al. (2023) dataset, we selected ASVs for which there was a minimum of six replicates (twelve replicates possible) and that were classified as either oligotrophic or copiotrophic for >50 % of the replicates. This resulted in 382 "taxon IDs" within the Stone dataset. Because species level was rarely identified in this dataset, we used genus as the finest level of taxonomic resolution and removed duplicate genera. This resulted in a total of 112 bacterial genera and 69 families identified as oligotrophic or copiotrophic (Supplementary Materials; Table S1). In our dataset, taxa that were assigned to one of these functional groups accounted for approximately 9 % and 19 % of the total bacterial richness and abundance, respectively.

We assigned fungal ASVs to one of ten functional guilds using the FUNGuild database of fungal taxa with known or suspected ecological functions (Nguyen et al., 2016): saprotrophs, arbuscular mycorrhizae, ectomycorrhizae, orchid mycorrhizae, root endophytes, foliar endophytes, lichenized fungi, plant pathogens, mycoparasites, and “other” (including animal pathogens and animal endosymbionts). Fifty-eight percent of fungal ASVs (4444 ASVs) were assigned to a fungal guild. For statistical analysis, we only included ASVs that were assigned to a fungal guild and that had a FUNGuild assignment with a confidence score of “probable” or “highly probable,” as recommended by Nguyen et al. (2016). Of those assigned to a guild, 80 % of ASVs had a confidence score of “probable” or “highly probable.” Ultimately, 46 % of all fungal ASVs met the selection criteria (assigned to a guild and had strong confidence score).

For the purposes of this study, we focused on saprotrophs, arbuscular mycorrhizae and ectomycorrhizae. As the principal decomposers in soil microbial communities, saprotrophs have high investment in extracellular enzyme production (Baldrian, 2009; Midgley and Phillips, 2019), which may suggest low CUE. Arbuscular mycorrhizae, on the other hand, mostly degrade structurally simple plant litter (Lin et al., 2017; Midgley et al., 2015; Phillips et al., 2013) compared to ectomycorrhizae which generally target structurally complex litter (Craig et al., 2018; Lin et al., 2017; Midgley et al., 2015; Phillips et al., 2013). As such, a greater of proportion of arbuscular mycorrhizae (relative to ectomycorrhizae) may suggest high CUE. Of the ASVs confidently assigned to a guild, saprotrophs accounted for 61 % of the ASVs, ectomycorrhizae accounted for 15 %, and arbuscular mycorrhizae accounted for 13 %. In terms of abundance, saprotrophs accounted for 67 % of the sequences, ectomycorrhizae accounted for 20 %, and arbuscular mycorrhizae accounted for 2 %. We also used the ASV sequence data to compare amounts of fungi relative to bacteria because fungi:bacteria ratios are negatively correlated with CUE (Soares and Rousk, 2019; Ullah et al., 2021).

2.6. Statistical analyses

All analyses were conducted in R (v 4.3). Prior to running the enzyme activity rate models, we assessed normality. We log transformed the activity rates for BG and NAG to meet assumptions of residual normality and homoscedasticity. We built generalized linear models with the glm function in the *MASS* package for the AG and BX activity rates because normality assumptions were not met with log transformation. We built a linear model using the lm function in the built-in *stats* package for CBH because the activity rates were normally distributed. For each enzyme model, we specified the activity rate as the dependent variable and encroachment status, management treatment, and their interaction as independent variables.

Prior to analysis, we generated the following metrics to assess microbial potential CUE: 1) ratios of the richness and abundance of arbuscular mycorrhizal fungi to ectomycorrhizal fungi (AMF:ECM), 2) proportion of saprotrophic fungi richness and abundance relative to whole fungal community, 3) ratios of the richness and abundance of oligotrophic to copiotrophic bacteria (O:C). For the saprotroph metrics, and O:C ratios, a higher value is interpreted as lower potential CUE. For the AMF:ECM ratios, a lower value is interpreted as lower potential CUE. For each richness ratio metric, we summed all ASVs that had a non-zero sequence count and then divided the respective ASV sums. For each abundance ratio metric, we summed all sequence counts for all ASVs and then divided the respective sequence sums. For example, O:C richness and abundance ratios were calculated as the following:

$$\text{Oligotroph} : \text{Copiotroph (richness)} = \frac{\text{number of unique oligotrophic bacterial ASVs with sequence count} \geq 1}{\text{number of unique copiotrophic bacterial ASVs with sequence count} \geq 1}$$

$$\text{Oligotroph} : \text{Copiotroph (abundance)} = \frac{\text{total number of oligotrophic bacterial sequences}}{\text{total number of copiotrophic bacterial sequences}}$$

To explore microbial community composition (i.e., potential CUE metrics), we built negative binomial generalized linear models using the glm.nb function in the *MASS* package (Venables and Ripley, 2002). For each potential CUE metric model, we specified the numerator of the ratio metric as the dependent variable. Independent variables were encroachment status (encroached or un-encroached), management treatment (remnant or restored), and their interaction. We included log of the denominator of the ratio metric as an offset. We visually assessed residual normality and homogeneity of variance to validate models.

For all enzyme activity and microbial composition models we evaluated independent variables using the Anova function in the *car* package (Fox and Weisberg, 2019), specifying type III tests. We conducted Tukey-adjusted pairwise comparisons at the 95 % confidence level using the emmeans package (Lenth, 2024). Finally, we tested for correlations between the potential CUE microbial metrics and microbial enzyme activity rates using the rcorr function in the *Hmisc* package (Harrell Jr, 2024).

3. Results

3.1. Soil extracellular enzyme activity

Activity rates of all but one of the enzymes were significantly higher in encroached plots compared to un-encroached plots, with *N*-acetyl-β-D-glucosaminidase activity rates slightly higher in encroached plots (Table 1, Fig. 1). Three of the five enzymes responded to land management (Table 1): activity rates for β-glucosidase (Table 1, Fig. 2A) and β-xylosidase (Table 1, Fig. 2B) were significantly higher in remnant plots compared to restoration plots. Activity rates for *N*-acetyl-β-D-glucosaminidase were slightly higher in remnant plots compared to restoration plots (Table 1, Fig. 2C). There was no interactive effect of woody encroachment and management type on microbial extracellular enzyme activity (Table 1).

3.2. Potential CUE microbial metrics

Two of the six potential CUE microbial metrics responded to woody encroachment. AMF:ECM richness and abundance were significantly lower in encroached plots compared to un-encroached plots (richness: Table 2, Fig. S1A; abundance: Table 2, Fig. S1B). Four of the six potential CUE microbial metrics responded to land management. Remnant plots had higher saprotroph richness (Table 2, Fig. 3A), saprotroph abundance (Table 2, Fig. 3B), and O:C abundance (Table 2, Fig. 3E) compared to restoration plots. Remnant plots had lower AMF:ECM richness (Table 2, Fig. 3C) and AMF:ECM abundance (Table 2, Fig. 3D) compared

Table 1

The effect of woody encroachment status (encroached vs. un-encroached), management type (remnant vs. restored), and their interaction on microbial extracellular enzyme activity. Two-way ANOVA (type III) results are represented with either likelihood ratio Chi-square values or F-values and their accompanying *p*-values. Statistically significant results are indicated in bold and marginally significant results are indicated in italics. AG = α-glucosidase (degrades starch); BG = β-glucosidase (degrades glucose); XYL = β-xylosidase (degrades hemicellulose); CBH = cellobiohydrolase (degrades cellulose); NAG = *N*-acetyl-β-D-glucosaminidase (degrades chitin).

		AG (starch)		BG (glucose)		XYL (hemicellulose)		CBH (cellulose)		NAG (chitin)	
Effect	df	LR χ^2	p	F	p	LR χ^2	p	F	p	F	p
Encroachment status	1	4.7	0.030	6.5	0.014	4.9	0.026	7.3	0.009	3.8	0.056
Management type	1	0.8	0.383	6.2	0.016	5.5	0.019	1.9	0.175	3.8	0.056
Encroachment x Management	1	0.7	0.417	1.0	0.331	0.3	0.584	0.1	0.745	0.5	0.491

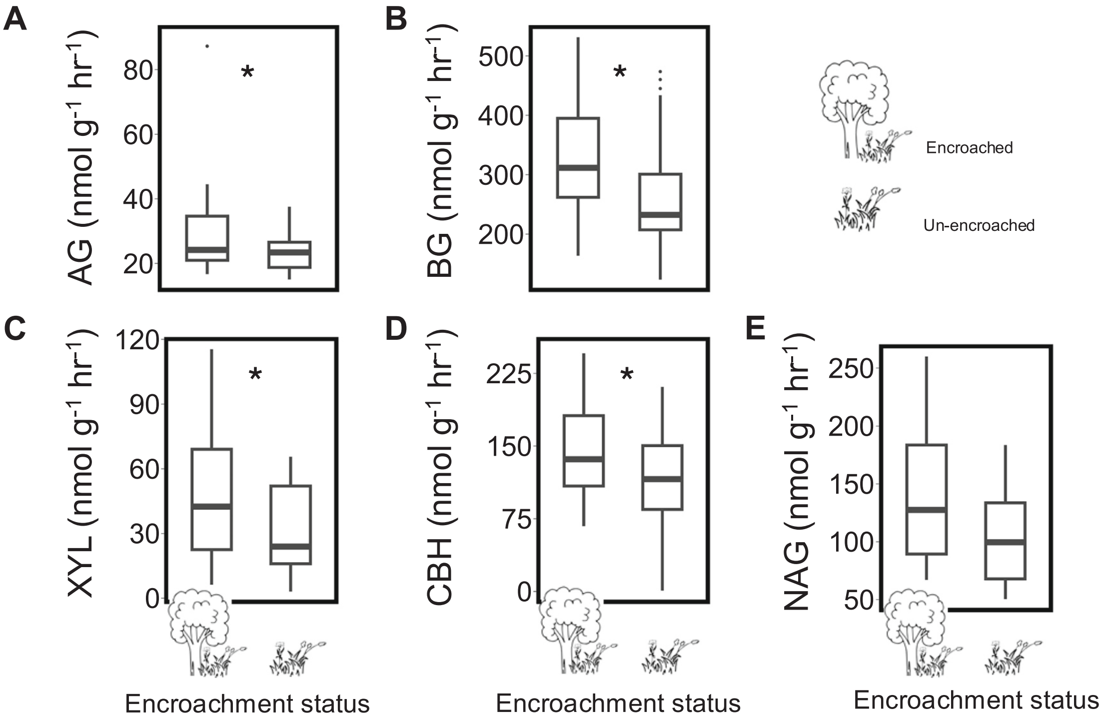

Fig. 1. Difference in microbial extracellular enzyme activity rates among encroached and un-encroached plots. Asterisks denotes statistically significant difference ($p \leq 0.05$). A) α-glucosidase (starch degradation), B) β-glucosidase (glucose degradation), C) β-xylosidase (hemicellulose degradation), D) cellobiohydrolase (cellulose degradation), E) *N*-acetyl-β-D-glucosaminidase (chitin degradation).

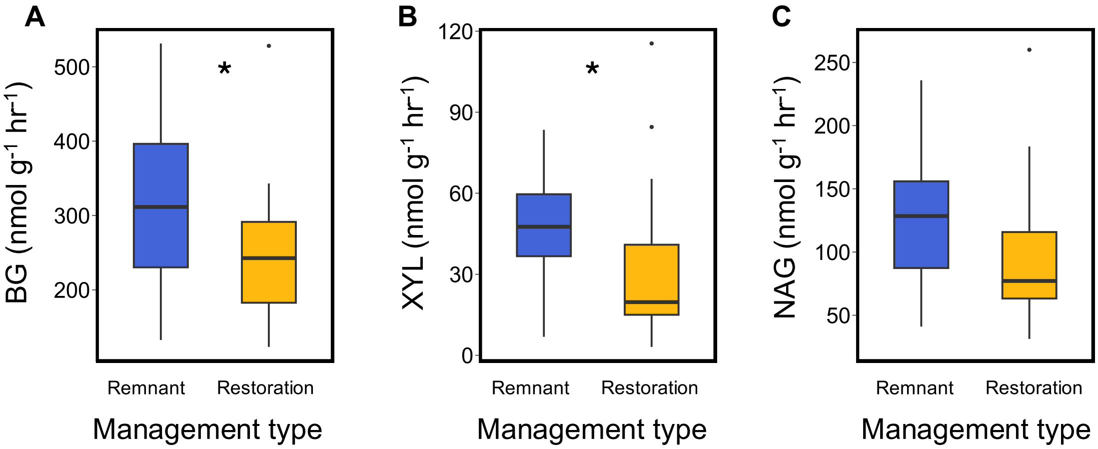

Fig. 2. Difference in microbial extracellular enzyme activity rates among remnant and restored plots. Asterisks denotes statistically significant difference ($p \leq 0.05$). Remnant plots are shown in blue and restored plots are shown in yellow. A) β-glucosidase (glucose degradation), B) β-xylosidase (hemicellulose degradation), C) *N*-acetyl-β-D-glucosaminidase (chitin degradation).

Table 2
The effect of woody encroachment status (encroached vs. un-encroached), management type (remnant vs. restored), and their interaction on potential CUE microbial metrics. Two-way ANOVA (type III) results are represented with likelihood ratio Chi-square values and their accompanying p-values. Statistically significant results are indicated in bold and marginally significant results are indicated in italics. Sapro (rich) = the number of unique saprotroph ASVs relative to the number of unique fungal ASVs; Sapro (abund) = the total number of saprotroph sequences relative to the total number of fungal sequences; AMF:ECM (rich) = the number of unique arbuscular mycorrhizal ASVs relative to the number of unique ectomycorrhizal ASVs; AMF:ECM (abund) = the total number of arbuscular mycorrhizal sequences relative to the total number of ectomycorrhizal sequences; O:C (rich) = the number of unique oligotrophic bacterial ASVs relative to the number of unique copiotrophic bacterial ASVs; O:C (abund) = the total number of oligotrophic bacterial sequences relative to the total number of copiotrophic bacterial sequences.

		Sapro (rich)		Sapro (abund)		AMF:ECM (rich)		AMF:ECM (abund)		O:C (rich)		O:C (abund)	
Effect	df	LR χ^2	p	LR χ^2	p	LR χ^2	p	LR χ^2	p	LR χ^2	p	LR χ^2	p
Encroachment status	1	0.0	0.978	0.3	0.598	15.7	<0.0001	7.5	0.006	0.0	0.868	3.4	0.067
Management type	1	25.1	<0.0001	8.0	0.005	6.7	0.010	11.3	0.001	0.0	0.901	3.7	0.055
Encroachment x Management	1	1.0	0.331	1.1	0.294	0.4	0.525	4.6	0.031	2.5	0.113	2.5	0.116

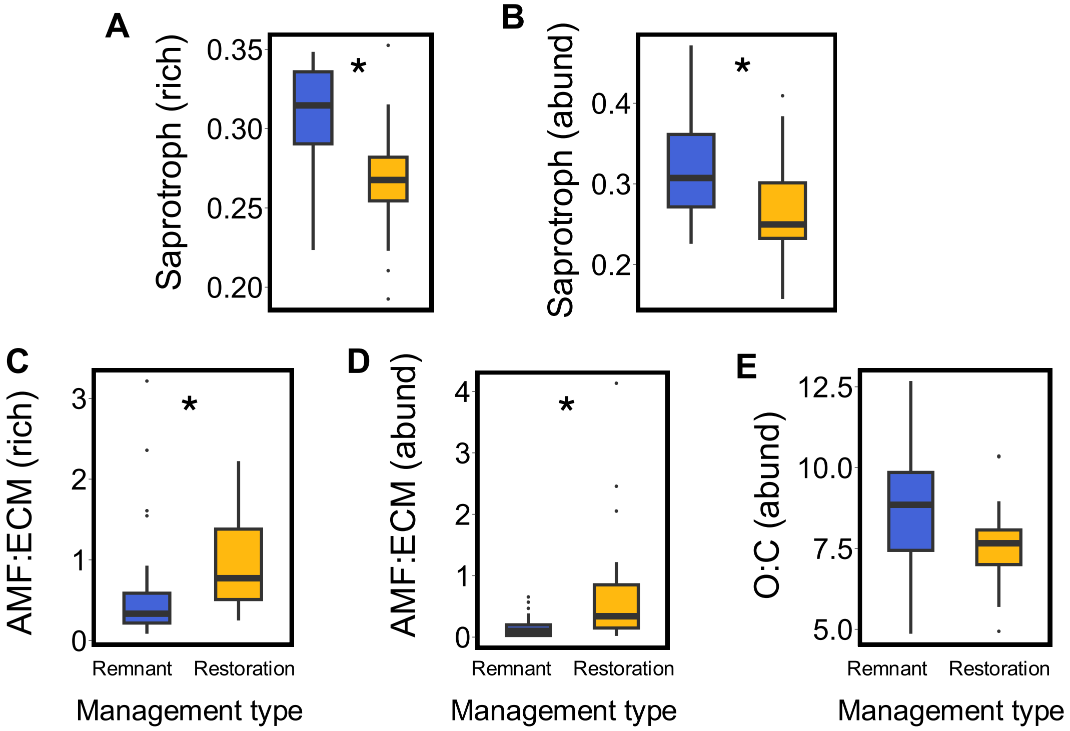

Fig. 3. Difference in potential CUE microbial metrics among remnant and restored plots. Asterisks denotes statistically significant difference ($p \leq 0.05$). Remnant plots are shown in blue and restored plots are shown in yellow. A) relative proportion of saprotroph richness, B), relative proportion of saprotroph abundance, C) arbuscular mycorrhizae:ectomycorrhizae richness ratio, D) arbuscular mycorrhizae:ectomycorrhizae abundance ratio, E) oligtrophic bacteria:copiotrophic bacteria richness ratio.

to restoration plots. There was an interactive effect of woody encroachment and management type for AMF:ECM abundance (Table 2). In restoration plots, encroachment reduced AMF:ECM abundance (Fig. S2).

3.3. Relationship between enzyme activity and potential CUE microbial metrics

Four of the five enzymes were significantly correlated with at least two potential CUE microbial metrics. Beta-glucosidase activity was positively correlated with saprotroph richness (Figs. 4; S3A) and negatively correlated with AMF:ECM richness and abundance (Figs. 4; S3B–C). Richness and abundance ratios of AMF:ECM were also negatively correlated with beta-xylosidase activity (Figs. 4; S3D–E), cellobiohydrolase activity (Figs. 4; S4C), and *N*-acetyl-β-D-glucosaminidase activity (Figs. 4; S4A–B). Although we did not detect relationships for all the CUE microbial metrics, this consistent pattern suggests that higher rates of glucose, hemicellulose, cellulose, and chitin degradation are associated with low potential CUE.

4. Discussion

We measured the activity of hydrolytic extracellular enzymes and examined soil microbial communities in remnant and restored tallgrass prairies to understand how management history and woody encroachment influence C cycling dynamics in a mesic tallgrass prairie ecosystem. We found that extracellular enzyme rates were higher in the remnant site and in encroached soils. We also found distinct differences in microbial composition related to both encroachment and land management. Microbial groups with low potential CUE were generally more prevalent in the remnant site and in encroached soils. This included saprotrophic and ectomycorrhizal fungi and oligotrophic bacteria. Additionally, multiple extracellular enzyme activity rates were correlated with potential CUE metrics, suggesting that higher rates of C degradation may occur with lower potential microbial CUE. Below we discuss in detail these patterns and their potential implications for C sequestration in remnant and restored grasslands.

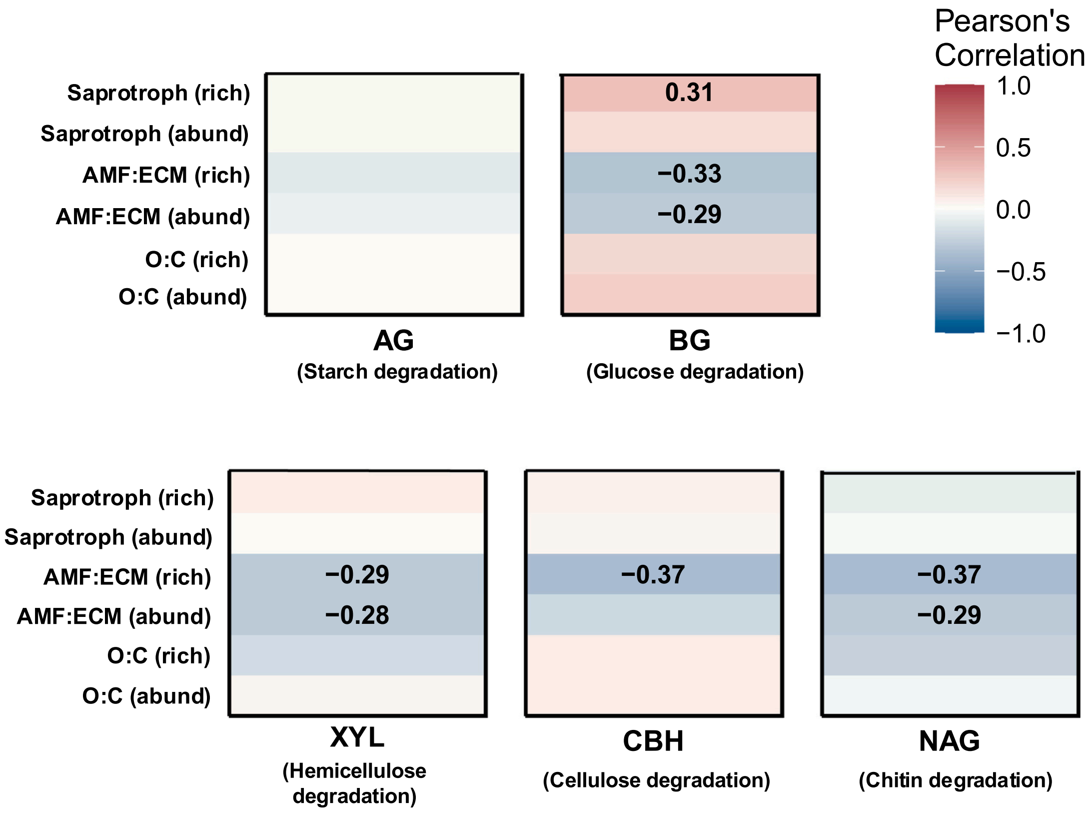

Fig. 4. Pearson's correlation coefficients between potential CUE microbial metrics and microbial extracellular enzyme activity rates. Only statistically significant correlation values are displayed ($p < 0.05$). AG = α-glucosidase (starch degradation); BG = β-glucosidase (glucose degradation); XYL = β-xylosidase (hemicellulose degradation); CBH = cellobiohydrolase (cellulose degradation); NAG = *N*-acetyl-β-D-glucosaminidase (chitin degradation).

4.1. *Encroached soils have faster carbon degradation rates and may contain microbial groups with low potential CUE compared to un-encroached soils*

In support of our hypothesis, encroached soils consistently had accelerated rates of C degradation, with activity of all five of the extracellular enzymes higher than in un-encroached soils. Only the mycorrhizal metrics responded to encroachment. In both sites, encroached soils had lower richness and abundance of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi compared to un-encroached soils. Encroachment only reduced abundance of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi in the restored site.

These findings may be a result of the difference in plant litter quality among encroached and un-encroached soils. High quality herbaceous litter is structurally simple, has a relatively low C:N ratio, and can be efficiently incorporated into microbial biomass and products (Castellano et al., 2015; Cotrufo et al., 2013). In contrast, low quality woody litter is structurally complex and has a relatively high C:N ratio (Castellano et al., 2015; Cotrufo et al., 2013). The degradation of woody litter requires microbes—namely those with low potential CUE—to depolymerize the structurally complex polymers so that they can be converted into simpler forms and incorporated into microbial biomass (Cotrufo et al., 2013). A similar trend was found in a recent study (Sun et al., 2022), in which microbial CUE decreased and extracellular enzyme activity increased with the re-vegetation of abandoned croplands by woody shrubs and trees.

4.2. *Prairie remnants have faster carbon degradation rates and may contain microbial groups with low potential CUE compared to prairie restorations*

As we hypothesized, the remnant site generally had faster organic C degradation rates. Glucose, hemicellulose, and chitin were higher in the remnant site compared to the restoration site. Furthermore, regardless of encroachment, the remnant site had higher richness and abundance of saprotrophic fungi, lower richness and abundance of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi, and slightly higher abundance of oligotrophic bacteria relative to copiotrophic bacteria compared to the restored site. The remnant site had lower abundance of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi compared to the restored site, but only in un-encroached soils.

Faster C degradation in the remnant site may be due to variation in the form of C inputs and variation in microbial communities among the remnant and restored sites. Root exudates are a labile form of C input into the soil (Panchal et al., 2022), and in some scenarios rhizodeposition can induce a positive priming effect such that the decomposition of native soil organic matter is stimulated by the input of fresh organic C (Shahzad et al., 2015; Zhou et al., 2021). This positive priming can occur when slow-growing microbial taxa (e.g., K-strategists with low potential CUE) use fresh organic matter as an energy source to produce enzymes that in turn degrade recalcitrant organic matter (Blagodatskaya and Kuzyakov, 2008; Chen et al., 2014; Fontaine et al., 2003; Zhou et al., 2021). Although fast-growing taxa (e.g., r-strategists with high potential CUE) may also be targeting this fresh organic matter, they may be outcompeted by K-strategists (Chen et al., 2014; Fontaine et al., 2003; Pascault et al., 2013). This phenomenon could be occurring in our tallgrass prairie ecosystem considering that the remnant site was more heavily dominated by microbial groups predicted to have low CUE (i.e., lower amounts of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi, greater amounts of oligotrophic bacteria relative to copiotrophic bacteria, and higher proportion of saprotrophs).

Additionally, the quantity and frequency of rhizodeposition can increase with root biomass (Baptist et al., 2015; Preece and Peñuelas, 2016). Previous research found that this remnant site had greater root biomass compared to surrounding areas that had undergone cultivation, such as our restored site (Jelinski et al., 2011). As such, it is possible that the remnant soils receive greater C inputs in the form of diverse root exudates (McLaughlin et al., 2023), and that positive priming is driving faster C degradation in the remnant site.

There were a few cases in which remnant and restored soil microbial communities responded differently to woody encroachment, as mentioned above. Contrary to our prediction, woody encroachment did not further accelerate C degradation rates in the remnant site. Similarly, encroachment did not increase the amount of low potential CUE microbial groups in the remnant site. Rather, encroachment reduced the abundance of arbuscular mycorrhizal fungi relative to ectomycorrhizal fungi in the restored site. Nonetheless these interactive effects of encroachment and management on mycorrhizal fungi did not correspond to differences in extracellular enzyme activities, which were consistently and independently driven by both variables.

4.3. Considerations and implications

Throughout this study, we have assumed that the differences in remnant and restored soils are primarily due to plant inputs. But microbial communities and extracellular enzyme activities are also affected by soil abiotic properties (Sinsabaugh et al., 2008). There are, however, surprisingly few differences in soil properties between these two adjacent sites. Despite their diverging management history, they have similar soil pH, soil texture and taxonomy, and N levels (Kucharik and Brye, 2013). Additionally, soil moisture was similar among the sites and did not vary with encroachment. As we hypothesized above, root density could be an important factor driving microbial differences among the sites, and it may simply take a very long time to develop the high root density that characterizes ancient grasslands, which underscores their conservation value.

We did not directly measure soil organic C stocks or soil respiration, so we cannot assume that degradation rates equate to C release (Margenot and Wade, 2023). In some scenarios, accelerated degradation can indicate that plant C inputs are being efficiently incorporated into microbial biomass and subsequently contributing to stabilized soil organic C formation (e.g., mineral associated organic matter) (Sokol et al., 2019a; Sokol et al., 2019b; Villarino et al., 2021). However, this is less likely the case here since 1) there was a high prevalence of low potential CUE microbial groups in treatments that had accelerated C degradation (i.e., remnant site and encroached soils) and 2) C degradation rates were positively associated with these low potential CUE microbial groups.

The combination of accelerated C degradation and greater prevalence of microbial groups with low potential CUE in encroached soils and in the remnant site begs two central questions: does woody encroachment into grasslands promote C loss and are remnant grasslands reliable C sinks? Other research in tallgrass prairie experiencing woody encroachment found that CO_2 respiration was higher when microbes broke down proportionally more woody-derived compared to herbaceous-derived organic matter (Connell et al., 2021). Taken together with our findings of accelerated C degradation in encroached soils, it is possible that woody encroachment can promote C release from grassland soils. This underscores the importance of management strategies to reduce woody cover in grasslands where past human activities have allowed or promoted encroachment, especially in sites where C sequestration is a management objective. It is important to note however, that woody plants themselves are temporary C reservoirs. Whether woody encroachment indeed promotes C release from grasslands in large part depends on the relative amounts of C stored in woody biomass relative to the amount of C released from grassland soils over the lifetime of the woody biomass.

We found that the remnant site had faster C degradation rates and greater prevalence of low potential CUE microbial groups compared to the restored site. If the accelerated degradation rates are indicative of C release, this finding would contradict the notion that never-cultivated remnant grasslands are C sinks. However, this does not necessarily mean that the restored site has greater C sequestration potential. Despite the significant labor and resources that go into reconstructing prairies (Rowe, 2010; Smith et al., 2010), their soil organic C stocks can fall short of reference remnants. For instance, an assessment of nineteen tallgrass prairie reconstructions ranging in age from 1 to 21 years after restoration establishment found that even after 20 years, the reconstructed prairies contained 36 % less soil organic C compared to a nearby remnant prairie (Bugeja and Castellano, 2018). Similarly, another study found that nearly 30-year-old restored prairies contained 66 % less soil organic C relative to nearby remnant sites (von Haden and Dornbush, 2017). Importantly, one of these studies also found that while fast-cycling coarse particulate organic matter increased with time since prairie restoration, slow-cycling microaggregate-stabilized soil organic matter did not (Bugeja and Castellano, 2018), which suggests that whatever soil C that is rebuilt through restoration may be impermanent. A full construction of the C budget, including the relative sizes of the slow- and fast-cycling organic matter fractions in these soils and across a woody encroachment gradient would contribute to verifying our results here and provide land managers and restoration practitioners with a more detailed assessment of the C sequestration potential for grasslands.

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CRediT authorship contribution statement

Kendall K. Beals: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cooper Rosin:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Bradley M. Herrick:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Paul H. Zedler:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Isaac Bailey-Marren:** Writing – review & editing, Methodology, Investigation, Data curation. **Zheng Yao:** Writing – review & editing, Methodology, Investigation, Data curation. **Nicholas A. Barber:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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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

Data will be made available on request.

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