



Spatial variation in soil microbial processes as a result of woody encroachment depends on shrub size in tallgrass prairie

R. Kent Connell · Rory C. O'Connor · Jesse B. Nippert · John M. Blair

Received: 13 July 2020 / Accepted: 16 December 2020

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Abstract

Aims As woody plants encroach into grassland ecosystems, we expect altered plant-soil interactions to change the microbial processes that affect soil carbon storage and nutrient cycling. Specifically, this research aimed to address how (1) soil chemistry, (2) microbial nutrient demand, and (3) the rate and source of potential soil C mineralization vary spatially under clonal woody shrubs of varying size within a mesic grassland.

Methods We collected soil samples from the center, the midpoint between the center and edge, the edge, and the shrub-grass ecotone of multiple *Cornus drummondii* shrubs across a shrub-size gradient in infrequently burned tallgrass prairie.

Results Total soil carbon and nitrogen increased with shrub size at every sampling location but the edge. Microbial demand for nitrogen also increased as shrubs increased in size. Across all shrub sizes and sampling locations, potential soil carbon mineralization rates were higher when microbes broke down proportionally more shrub-derived (C₃) organic matter than grass-derived (C₄) organic matter.

Conclusions Our results suggest that the spatio-temporal context of woody encroachment is critical for understanding its impact on belowground microbial processes. In this ecosystem, a longer period of occupancy by woody plants increases potentially mineralizable soil carbon.

Responsible Editor: Yolima Carrillo.

R. K. Connell (✉) · R. C. O'Connor · J. B. Nippert · J. M. Blair
Division of Biology, Kansas State University, Manhattan, KS 66506, USA
e-mail: r.kent.connell@gmail.com

Present Address:
R. K. Connell
School for Environment and Sustainability, University of Michigan, Ann Arbor, MI 48109, USA

Present Address:
R. C. O'Connor
USDA-ARS Eastern Oregon Agricultural Research Center, Burns, OR 97720, USA

Keywords Woody encroachment · Soil microbes · C mineralization · Enzymatic activity · Grasslands · Stable isotopes

Abbreviations

C	carbon
N	nitrogen
P	phosphorus
BG	β-glucosidase
NAG	N-acetyl-glucosaminidase
PHOS	phosphatase
LAP	leucine-aminopeptidase

Introduction

Woody encroachment, the increase in cover and abundance of woody plants in grasslands and savannas, threatens the conservation and maintenance of many grasslands globally (Sala and Maestre 2014; Archer et al. 2017). The change from grass-dominated to woody-dominated communities can significantly impact the cycling of carbon (C) and nutrients in these ecosystems (Knapp et al. 2008; Coetsee et al. 2010; Barger et al. 2011; Pellegrini et al. 2020), although drawing general conclusions across grassland types is challenging. Compared to the grasses they displace, woody plants allocate a greater proportion of biomass aboveground and generally have greater aboveground net primary productivity (ANPP) (Briggs et al. 2005; Barger et al. 2011), whereas grasses usually allocate a large proportion of biomass belowground (Blair et al. 2014). Additionally, woody plants typically invest in larger-diameter roots than grasses and allocate roots deeper in the soil profile than grasses (Jackson et al. 1997; February and Higgins 2010; Kulmatiski et al. 2020). Woody plants can also increase the formation of soil macro-aggregates, which may increase the storage of stable C belowground (Liao et al. 2006).

Whether woody encroachment results in shifts in the cycling of C or nutrients often depends on context such as the interaction between biotic and abiotic factors or the ecosystem properties being measured [e.g., increases in soil organic C and aboveground C but no change in soil respiration (Eldridge et al. 2011)]. Local abiotic factors such as climate (Jackson et al. 2002; Knapp et al. 2008), soil texture (Li et al. 2016), and hydrology (Ludwig et al. 2005) can mediate the impacts of woody plant species on ecosystem function. Biotic factors that also can influence nutrient cycling include association with nitrogen-fixing microbes (Blaser et al. 2014), mycorrhizal interactions (Williams et al. 2013), interactions with saprotrophs (Ochoa-Hueso et al. 2018), and interactions with grazing megafauna (Soliveres and Eldridge 2014; Wigley et al. 2020).

Both biological and physical processes can contribute to an increase in soil moisture and nutrients under and around plants relative to the surrounding area (Garner and Steinberger 1989; Schlesinger et al. 1996; Schlesinger and Pilmanis 1998; Ridolfi et al. 2008). These “islands of fertility” are generally associated with woody plants in dryland ecosystems, but they can also develop in more mesic systems (Rong et al. 2016) and

under other plant growth forms such as bunchgrasses (Derner et al. 1997). Several studies demonstrate that soil properties under islands of fertility exhibit spatial heterogeneity, with higher fertility closer to the center or the trunk of the plant (Bolling and Walker 2002; Eldridge and Wong 2005; Throop and Archer 2008; Rong et al. 2016) or greater fertility under larger shrubs (Ward et al. 2018). However, in the dryland ecosystems where fertile islands are best studied, these patches of fertility develop over many decades (Bolling and Walker 2002) and bare patches of ground between fertile islands are common (Ridolfi et al. 2008). In mesic grasslands like tallgrass prairie, shrub encroachment is a more recent phenomenon (i.e., within the past three decades; Briggs et al. 2005; Ratajczak et al. 2014) and bare patches are rare; spots unoccupied by woody plants are occupied by grasses and forbs. Whether patterns of fertility develop in mesic grasslands that are similar to those in more arid ecosystems and whether these changes in soil properties impact microbial processes such as extracellular enzyme production is unknown.

The consequences of woody plant encroachment and fertile island development are expected to vary with time (Bolling and Walker 2002; Creamer et al. 2011; McCulley and Jackson 2012; Blaser et al. 2014). In the North American tallgrass prairie, for example, woody encroachment can increase ecosystem C stocks, at least in the short term. Encroachment by *Cornus drummondii* C.A. Mey. into tallgrass prairie increased aboveground net primary productivity (ANPP) and decreased soil respiration (Lett et al. 2004). Replacement of tallgrass prairie by another woody species, *Juniperus virginiana* L., increased soil organic C (McKinley and Blair 2008). However, these increases in soil C might be temporary. In another study, McCulley and Jackson (2012) reported that belowground net primary productivity and soil organic C stocks were significantly lower in tallgrass prairie that had been encroached by *Prosopis glandulosa* Torr. and *Gleditsia triacanthos* L. for over sixty years.

The effects of woody encroachment over time may be inferred by investigating the heterogeneity of soil processes along two gradients: (1) a horizontal spatial gradient under individual clonal shrubs that expand radially from the center (Throop and Archer 2008), or (2) a shrub size gradient when age correlates with size. Clonality is characteristic of several species contributing to woody encroachment of grasslands around the world (Ratajczak et al. 2011; Case et al. 2020). In the absence

of disturbance or management, a single clonal shrub can grow to occupy a large area of the landscape. One example is *Cornus drummondii* C.A. Mey. (hereafter, “dogwood”), a woody shrub currently increasing in abundance and cover in many areas of North American tallgrass prairie (Briggs et al. 2002). After dogwood establishes, it spreads radially through vegetative reproduction to create discrete shrub islands that have distinctive aboveground properties (e.g. ANPP and leaf-area index) compared to nearby open grassland (Ratajczak et al. 2011). The clonal nature and radial growth pattern of dogwood makes it ideal for exploring the effects of encroachment over time, since island size is related to the age of the dogwood clone (Ratajczak et al. 2011), and soils under the center of the island are assumed to have been impacted by woody plants the longest while soils near the edges of the island are more recently impacted (sensu Throop and Archer 2008). Soil is a complex medium in which heterogeneity exists at both coarse and fine spatial scales (Jackson and Caldwell 1993; Lehmann et al. 2008). Woody encroachment via clonal shrubs has the potential to alter fine-scale heterogeneity in soil properties and microbial processes as shrubs increase in size, though this has not been well studied, especially in mesic grasslands such as tallgrass prairie.

An additional advantage to studying encroachment by dogwood, a C_3 plant, into areas previously dominated by C_4 grasses is that we can use natural abundance of stable C isotopes to determine the source of C respired by soil microbes during organic matter decomposition at various locations within the clonal shrub islands. Previous analysis of heavily encroached tallgrass prairie has found that the soil under woody plants has a C isotopic signature that reflects a large background of soil organic matter derived from C_4 grasses with varying amounts of more recent C_3 -derived C (Connell et al. 2020a). According to the preferential substrate utilization hypothesis, microbes preferentially break down organic matter from newer sources before decomposing older, more stabilized organic matter (Blagodatskaya et al. 2011). Based on this hypothesis, the isotopic C signature of respired soil CO_2 from under encroaching shrub islands should indicate proportionally more microbial decomposition of recently derived C_3 shrub-derived organic matter relative to older and potentially more stabilized C_4 grass-derived organic matter. To our knowledge, this is the first study to investigate microbial preference of shrub- versus grass-

derived organic inputs within heavily encroached tallgrass prairie.

By examining the properties of soils collected from multiple spatial locations within dogwood islands of varying size in a heavily encroached area of tallgrass prairie, we aimed to answer the following three questions: (1) Does soil chemistry vary predictably across sampling location within shrub islands and across a range of shrub sizes in woody encroached areas? (2) How does microbial biomass and nutrient demand (as indicated by enzymatic activity) vary spatially as shrubs increase in size within woody encroached areas? and (3) How do patterns of potential C mineralization and sources of respired soil C change across sampling location within a dogwood island? Specifically, we hypothesized that total soil C and nitrogen (N) content would increase with shrub size as a result of higher ANPP and lower soil respiration (Lett et al. 2004), but since range-expanding plants tend to increase nutrient availability as a strategy for occupying new territory (Zhou and Staver 2019), soil C:N would be lower in locations further from shrub center due to higher quality inputs from newer roots. This would result in lower microbial demand for N and increased demand for C and phosphorus (P) [i.e., lower N-acquiring extracellular enzymatic activity and higher C and P-acquiring extracellular enzymatic activity (Sinsabaugh et al. 2002, 2009; Sinsabaugh and Follstad Shah 2012)] in locations furthest from the center. Second, at the scale of individual shrubs, we hypothesized that lower C:N ratios and greater N availability would increase potentially mineralizable C in locations furthest from the center of the island. Because we expected soils under the center of dogwood to be influenced by woody plant encroachment the longest (sensu Throop and Archer 2008), we predicted that the stable isotopic C signature of respired CO_2 would be relatively lower at the center, reflecting greater reliance on C_3 -derived organic matter, and would become relatively higher in locations further from the center.

Methods

Study site

To assess spatial patterns of soil properties under shrub islands encroaching into tallgrass prairie, soils were sampled in the lowland topographic region of an infrequently burned treatment area at the Konza Prairie

Biological Station (KPBS) near Manhattan, Kansas, USA. KPBS experiences a temperate mid-continental climate. Over the past 37 years, the mean annual temperature has been 12.8 °C, and the mean annual precipitation has been 811 mm. The mean temperature in the middle of the growing season (July) has been 26.1 °C, and the mean growing season (May – September) precipitation has been 510 mm (Nippert 2020). KPBS is a preserve of unplowed tallgrass prairie primarily dominated by *C₄* grasses. The site is divided into large landscape units that vary in fire frequency. Areas that are infrequently burned (fire return interval ≥ 4 years) are undergoing woody encroachment into what were *C₄* grass dominated prairies (Ratajczak et al. 2014). The site used in our study has a prescribed fire interval of every 20 years. Since 1978, it has burned only twice, once in 1991 and once in 2012. Grazing megafauna have been excluded from this site since 1977. The dominant woody species in this area are *Cornus drummondii*, *Juniperus virginiana*, *Gleditsia triacanthos*, and *Prunus americana*.

Soil collection, preparation, and handling

Ten distinct dogwood islands were randomly selected within the previously described treatment area. Two 5 cm diameter \times 15 cm deep soil cores were taken at four locations within each dogwood island along a linear transect: the center, the midpoint, the edge, and the ecotone with grassland. We only sampled to 15 cm depth since the objective of this study was to assess the response of soil microbial processes to dogwood occupation, and most soil microbial activity occurs in the topsoil (Taylor et al. 2002). The center was defined as the intersection of perpendicular transects running the length and width of the dogwood island. The midpoint was defined as the halfway point between the center and the furthest edge. The edge was defined as the outer perimeter of the dogwood island canopy, and the ecotone was defined as the halfway point between the edge of the dogwood island of interest and the nearest neighboring dogwood island. The median distance between the edge and the ecotone was 0.875 m. While above-ground biomass in the ecotone was dominated by grasses and forbs, dogwood roots can extend several meters beyond the canopy edge (pers. obsv. R.C. O'Connor), so even soils in the ecotone could be influenced by the dogwood clone. All soil cores were kept on ice until they could be placed in short-term storage at

4 °C. Shrub island size was calculated by ellipse area equation using the lengths of the two perpendicular transects. The ten islands varied in size from 85 m² to 830 m².

On the day after sampling, soils were passed through a 4 mm sieve. A small subsample of soil from each location was dried at 60 °C for 48 h to determine gravimetric water content. One core from each location was used for nutrient, microbial, and enzymatic analyses. Soil from the other core was used to assess potential C mineralization rates and isotopic signatures. Soil used for nutrient analyses was stored at 4 °C. Soil used for extracellular enzymatic activity analyses was stored at –20 °C.

Soil C, N, P, and organic matter

Total C and N content of each soil sample was determined via dry combustion using a LECO TruSpec CN combustion analyzer. Total organic matter was determined by a slightly modified loss on ignition protocol outlined in Combs and Nathan (1998). Briefly, 1 g of soil was dried at 150 °C for two hours and then combusted at 400 °C for three hours. Available P was determined by the Mehlich-3 procedure (Mehlich 1984). The above analyses were performed at the Soil Testing Lab at Kansas State University (Manhattan, KS, USA). Fifty ml of 2 N KCl was added to 12 g of field moist soil and placed on an orbital shaker table at 200 rpm for 60 min to extract soil NH₄⁺ and NO₃[–] (Bremner and Keeney 1966). Extracts were passed through a 0.45 μ m polycarbonate filter and stored at –20 °C until they were analyzed colorimetrically for NO₃[–] and NH₄⁺ in a flow analyzer at the Kansas State University Soil Testing Lab (Manhattan, KS, USA).

Microbial biomass carbon and nitrogen

Microbial biomass C was determined using the chloroform fumigation-extraction method (Jenkinson and Powlson 1976). A subsample of each soil sample was placed into a chamber, fumigated by boiling chloroform under a vacuum, and kept in the fumigation chamber under a vacuum for 48 h. A vacuum pump was used to remove all chloroform from the chamber after fumigation was completed. Fumigated and unfumigated samples were extracted by combining 15 g of field moist soil and 75 ml 0.5 M K₂SO₄

and placing on an orbital shaker table at 200 rpm for 60 min, then passing through a 0.45 μm polycarbonate filter. Extracts were stored at $-20\text{ }^{\circ}\text{C}$ until they were analyzed for total organic C with a Shimadzu TOC-L. Microbial biomass C was defined as the difference in dissolved organic C between fumigated and unfumigated subsamples.

Total nitrogen content in microbial biomass was determined by taking a subsample of the K_2SO_4 extracts and subjecting them to a persulfate digest (D'Elia et al. 1977; Cabrera and Beare 1993). This oxidizes all forms of nitrogen to NO_3^- . After being reduced to NO_2^- by a cadmium coil, N concentrations in the extracts were determined colorimetrically using an Alpkem OI Analytical Flow Solution IV. Microbial biomass N was defined as the difference in N between fumigated and unfumigated subsamples.

Potential extracellular enzymatic activity assays

We tested the potential activity of four extracellular enzymes (Sinsabaugh et al. 1999; German et al. 2011): β -glucosidase (BG; a C-acquiring enzyme), N-acetylglucosaminidase (NAG; a N-acquiring enzyme), phosphatase (PHOS; a P-acquiring enzyme), and leucine-aminopeptidase (LAP; a N-acquiring enzyme). We used 200 μM solutions of 4-methylumbelliferone-b-D-glucoside, 4-methylumbelliferone-N-acetyl-b-glucosaminide, 4-methylumbelliferone-phosphate, and L-leucine 7-amido-4-methylcoumarin as substrates, respectively. For each soil sample in each assay, we created a slurry of 1 g of soil in 100 ml of 50 mM acetate buffer (pH 5). In 96-well plates, we pipetted 200 μl of the soil slurry with 50 μl of the substrate solution. There were six analytical replicates for each sample in each assay as well as a blank, a negative control, a reference standard, three quench standards, and six soil blanks. For BG, NAG, and PHOS assays, we incubated the plates in the dark at room temperature for 2 h. Assays for LAP activity were incubated for 16 h. Once the incubations were complete, we added 10 μl of 0.5 N NaOH solution to raise the pH and stop the assays. Finally, we used a FilterMax F5 microplate reader (Molecular Devices, San Jose, CA) to collect fluorescence data. Since extracellular enzymatic activity increases with substrate availability (Sinsabaugh et al. 2008), all potential extracellular enzymatic activity data were standardized by weight (g) of organic matter content.

Potential carbon mineralization

Approximately 300 g of soil from each sample was placed into an 8 cm wide \times 15 cm deep mason jar (Day 0). Total CO_2 respired and the $\delta^{13}\text{C}\text{-CO}_2$ value was measured at 1, 3, 5, 7, 10, 34, and 77 days after the start of the incubations. Starting at Day 0, soils were wetted to 60% water-filled pore space. Throughout the duration of the incubation, each jar was regularly weighed and rewetted to maintain constant soil moisture. Before each measurement, the time at which the lids were sealed was recorded. Total CO_2 concentration and the $\delta^{13}\text{C}\text{-CO}_2$ value was determined by taking a gas sample of the headspace of each jar through a rubber septum (Zeglin and Myrold 2013). Two blanks were also measured on each day to account for background CO_2 of the room. The CO_2 concentration and stable isotopic ratio of the gas sample was analyzed with a Picarro Cavity Ringdown Spectrometer (model G2101-i, Picarro Inc., Santa Clara, CA). The isotopic ratio of samples was calculated using delta notation as:

$$\delta = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) * 1000 \right]$$

where R is the ratio of the heavy to light isotope for the sample and standard, respectively. Delta values were calculated relative to the international standard for carbon, VPDB. For measurements of CO_2 concentration, the spectrometer had a precision of ± 200 ppb for $^{12}\text{C}\text{-CO}_2$ and ± 10 ppb $^{13}\text{C}\text{-CO}_2$. The precision for $\delta^{13}\text{C}$ values was $\pm 0.3\%$. Total mineralizable C pools were estimated by integrating the total C respired during the 77-day incubation.

Statistical analyses

All response variables fit either normal or lognormal distributions. Generalized linear mixed models were used to analyze the effect of categorical sampling location (i.e., center, midpoint, edge, ecotone), shrub size, and their interaction. In each model, location and shrub size were treated as fixed effects and the identity of the dogwood island was treated as a random effect. Potential extracellular enzymatic activity was analyzed as ratios in order to determine how relative microbial demand for C, N, and P changes along location within shrub islands and shrub island size (i.e., microbial C/N demand = $\ln(\text{BG activity}) / [\ln(\text{NAG}) + \ln(\text{LAP})$

activity)], microbial N/P demand = $[\ln(\text{NAG}) + \ln(\text{LAP activity})] / \ln(\text{PHOS activity})$, and microbial C/P demand = $\ln(\text{BG activity}) / \ln(\text{PHOS activity})$. The model was simplified for potential C mineralization rates: $\text{Rate} \sim \delta^{13}\text{C-CO}_2 + (1|\text{Island})$. This model was selected from all possible models using shrub size, location, day of incubation, and $\delta^{13}\text{C-CO}_2$ as fixed effects using AIC (Supplementary Appendix 1). We checked that the residuals were normally distributed and that there was no heteroscedasticity for all models. For our analysis of potentially mineralizable C, we removed one outlier data point to meet model assumptions (ecotone, $892 \mu\text{g C g soil}^{-1}$). All means comparisons were conducted using Tukey HSD comparisons of least square means. In the case of a significant interaction between sampling location and shrub size, slope comparisons were conducted using the *lstrends* function of the *lsmeans* package. All statistics were performed within the R statistical computing software (R Core Team 2019) using the packages *car* [version 3.0–3; (Fox and Weisberg 2019)], *lsmeans* [version 2.30.0 (Lenth 2016)], *multcomp* [version 1.4.11 (Hothorn et al. 2008)], and *lme4* [version 1.1–21; (Bates et al. 2015)]. Figures and tables were generated with the packages *tidyverse* [version 1.2.1; (Wickham 2017)], *ggeffects* [version 0.14.1 (Lüdtke 2018)], and *cowplot* [version 1.0.0 (Wilke 2019)].

Results

Changes in total soil C and N with shrub size varied with spatial location

There was a significant interaction between sampling location and shrub size on total soil C and N (Table 1), such that the direction of change in soil C and N at a specific sampling location varied as a function of shrub island size. In general, both total C and total N concentrations tended to increase with shrub island size across all sampling locations except the edge, where total C and N tended to decrease as shrub islands increased in size (Fig. 1). There was no significant effect of sampling location or shrub size on any other soil nutrient or microbial biomass property measured (inorganic N, extractable P, C:N, organic matter content, microbial biomass C, or microbial biomass N; Table 1).

An interaction between shrub size and spatial location influenced microbial demand for C, N, and P

Sampling location, shrub size, and their interaction differentially affected microbial demand for C, N, and P as indicated by the potential activity of the four enzymes we assayed (Table 1). Microbial demand for C relative to microbial demand for N (microbial C/N demand) was affected by a significant interaction between sampling location and shrub size. As shrubs increased in size, microbial C/N demand decreased at the edge (Fig. 2a; Table 1). Microbial demand for N relative to P (microbial N/P demand) increased as shrub size increased independent of sampling location (Fig. 2b; Table 1). There was a weak effect of the interaction between sampling location and shrub size on microbial demand for C relative to P (microbial C/P demand; Table 1). As shrub size increased, microbial C/P demand increased in the center (Fig. 2c).

More CO₂ was respired when microbes broke down proportionally more shrub-derived organic matter

According to the top model based on AIC, there was a negative logarithmic relationship between $\delta^{13}\text{C-CO}_2$ values and potential C mineralization rate across all sampling locations and shrub island sizes ($t = -4.796$, $r^2_{\text{marginal}} = 0.654$, $p < 0.001$; Fig. 3). Potential C mineralization rates decreased by 9% for every 1‰ increase in the $\delta^{13}\text{C}$ value of the respired CO₂ indicating that potential C mineralization rates were lower when $\delta^{13}\text{C-CO}_2$ was closer to the $\delta^{13}\text{C}$ value of C₄ grasses. The effect of day of incubation was not included in our top model and had no statistically significant effect in any of the models in which it was included.

Shrub encroachment increased potentially mineralizable C

There was a significant effect of sampling location on the potentially mineralizable C pool and mean respired $\delta^{13}\text{C-CO}_2$ (Fig. 4, Table 1) indicating that, on average, the microbial community was not breaking down the same proportions of woody- and grass-derived organic matter across the spatial gradient within shrub islands. Potentially mineralizable C was greatest in soil under the center of the dogwood island and decreased with distance from center (Fig. 4a). On average, the respired $\delta^{13}\text{C-CO}_2$ was significantly lower in soil collected from

Table 1 Effect of sampling location (i.e., center, midpoint, edge, ecotone), shrub size, and their interaction on soil chemical properties, microbial biomass properties, microbial elemental demand, potentially mineralizable C, and the $\delta^{13}\text{C}$ -CO₂ respired during thepotential C mineralization assays. All *p* values < 0.1 are bolded. (*L* Location, *S* Size, *MBC* Microbial biomass C, *MBN* Microbial biomass N)

Response	X ²	<i>p</i>	Response	X ²	<i>p</i>
Total C			MBC		
Location	2.22	0.527	Location	1.55	0.672
Size	1.16	0.283	Size	1.55	0.214
L * S	9.60	0.022	L * S	2.34	0.505
Total N			MBN		
Location	2.94	0.400	Location	2.06	0.560
Size	1.38	0.241	Size	0.078	0.781
L * S	7.94	0.047	L * S	1.37	0.714
C/N			C/N demand		
Location	2.26	0.521	Location	1.48	0.686
Size	0.115	0.735	Size	2.25	0.134
L * S	3.82	0.282	L * S	9.78	0.021
Inorganic N			N/P demand		
Location	4.78	0.189	Location	1.11	0.775
Size	1.63	0.202	Size	6.13	0.013
L * S	3.35	0.341	L * S	4.50	0.212
Extractable P			C/P demand		
Location	3.89	0.274	Location	2.22	0.529
Size	0.030	0.863	Size	1.38	0.240
L * S	5.87	0.118	L * S	7.26	0.064
Organic matter			$\delta^{13}\text{C}$-CO₂		
Location	1.69	0.640	Location		
Size	0.563	0.453	Size	0.126	0.723
L * S	4.73	0.193	L * S	6.17	0.104
Potentially mineralizable C					
Location	11.0	0.011			
Size	0.725	0.394			
L * S	6.03	0.110			

the center and midpoint than soil collected from the edge or ecotone of shrub islands (Fig. 4b).

Discussion

By measuring soil characteristics and microbial processes at multiple locations under individual dogwood clones of various sizes, we found that woody encroachment predictably altered variation in nutrient cycling at finer scales than are often measured in studies of woody plant encroachment. Our results demonstrate that heterogeneity develops at the scale of individual clonal

dogwood shrub islands and that within-shrub-island heterogeneity is moderated by shrub size. Throughout this discussion we use our results to infer the impacts of dogwood encroachment over time since shrub island size increases with age (Ratajczak et al. 2011) and soils under the center of a shrub have been occupied longer than soils under the island edge.

Soil C and N varies predictably with shrub size and within-shrub location

In contrast to our initial hypothesis that roots further from the center may contribute higher quality inputs

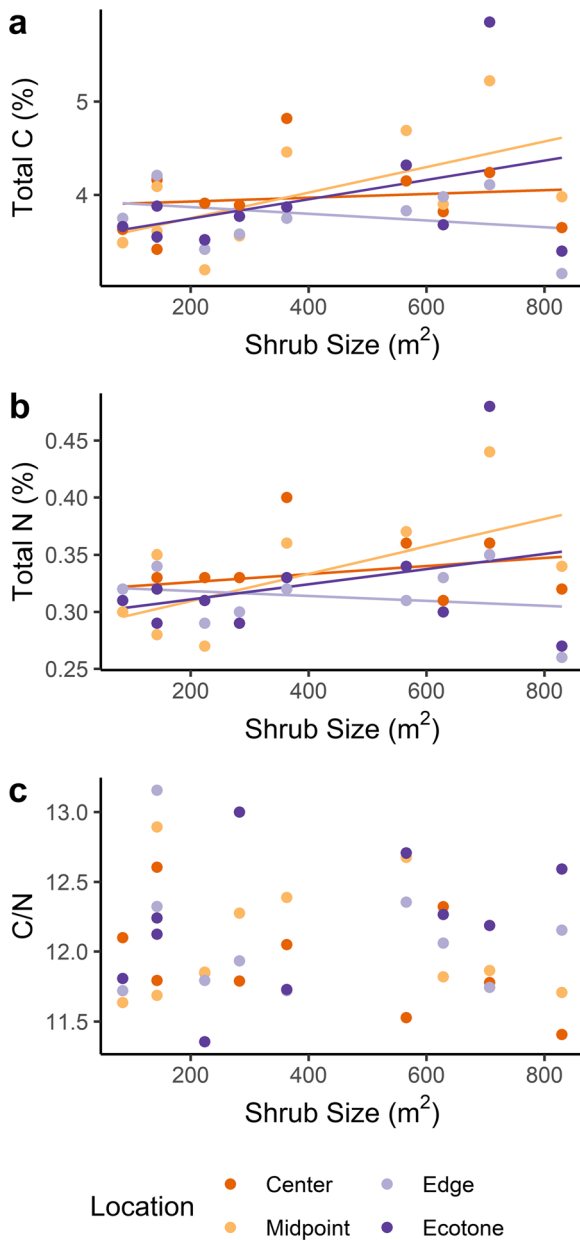


Fig. 1 Interaction plots displaying the marginal effect of the interaction between sampling location (i.e., center, midpoint, edge, ecotone) and shrub size as lines on a) Total C, b) Total N, and c) C/N ratio. Marginal effects are calculated from mixed effect models using the formula: $\text{Response} \sim \text{Location} * \text{Size} + (1 | \text{Island})$. The raw data are also displayed as points. The color of the lines and points indicate sampling location. We do not show lines on panel c because no significant effects were detected in our model

such that soil C:N ratios would decrease with distance from the center of dogwood islands, both soil C and soil N were lower at the edge of the canopy under larger dogwood islands (Fig. 1a and b), and there was no

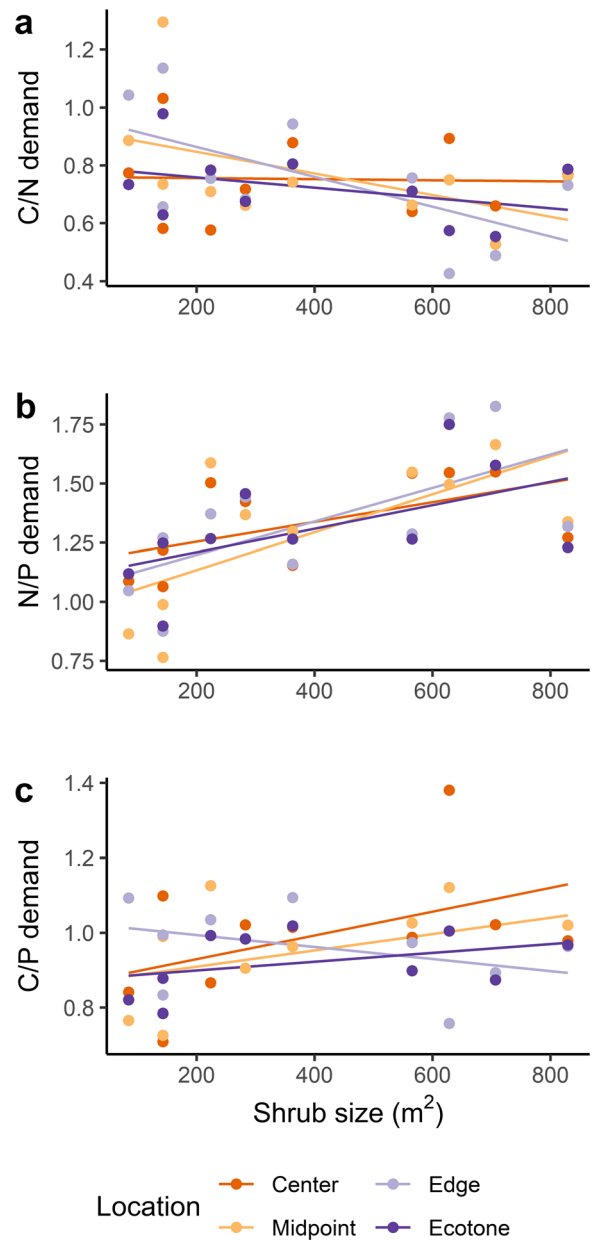


Fig. 2 Interaction plots displaying the marginal effect of the interaction between sampling location (i.e., center, midpoint, edge, ecotone) and shrub size as lines on a) microbial C/N demand, b) microbial N/P demand and c) microbial C/P demand. Marginal effects are calculated from mixed effect models using the formula: $\text{Response} \sim \text{Location} * \text{Size} + (1 | \text{Island})$. The raw data are also displayed as points. The color of the lines and points indicate sampling location

significant change in soil C:N ratios with sampling location or shrub island size (Fig. 1c). Previous studies of spatial gradients resulting from woody encroachment by other species in more arid systems have also

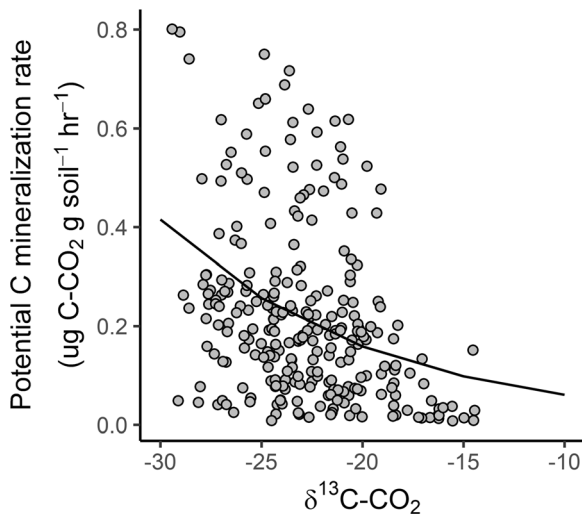


Fig. 3 The potential C mineralization rate was negatively correlated with the $\delta^{13}\text{C}$ signature of respired CO_2 across all shrub islands and locations. The line represents the calculated marginal effect of $\delta^{13}\text{C}\text{-CO}_2$ on potential C mineralization using a mixed effects model. The formula for the mixed effects model was: $\ln(\text{C mineralization rate}) \sim \delta^{13}\text{C}\text{-CO}_2 + (1|\text{Island})$. The raw data are also displayed as points

observed that soil C and N decreases with distance from the center of a shrub (Throop and Archer 2008; Li et al. 2008) and increases with shrub size (Wheeler et al. 2007; Ward et al. 2018). Using shrub size as a proxy for age, the root system in the central portion of a dogwood island has had more time to contribute greater amounts of C and N to the soil pool. Additionally, aboveground litter inputs would increase as the shrub grows larger since size is positively related to shrub age in dogwood. On the other hand, soil at the edge of the canopy would have received fewer aboveground and belowground inputs over a shorter period of time. Interestingly, as shrub size got larger, total soil C and N also increased in the ecotone (Fig. 1), potentially indicating persistence of organic inputs from grasses to soil organic matter as well as greater inputs from shrub roots during exploration for unoccupied areas to produce new ramets. Enhanced resource availability (e.g., greater total soil C and N in the ecotone) is often a result of the activity of range-expanding and invasive plants and is implicated as a mechanism for their success (Zhou and Staver 2019). Finally, larger shrub islands in dry and patchy landscapes are able to accumulate a larger amount of resources through physical processes such as aeolian deposition (Whitford et al. 1997) or runoff interception (Ludwig et al. 2005). However, the tallgrass prairie site used in our study is a more mesic system that

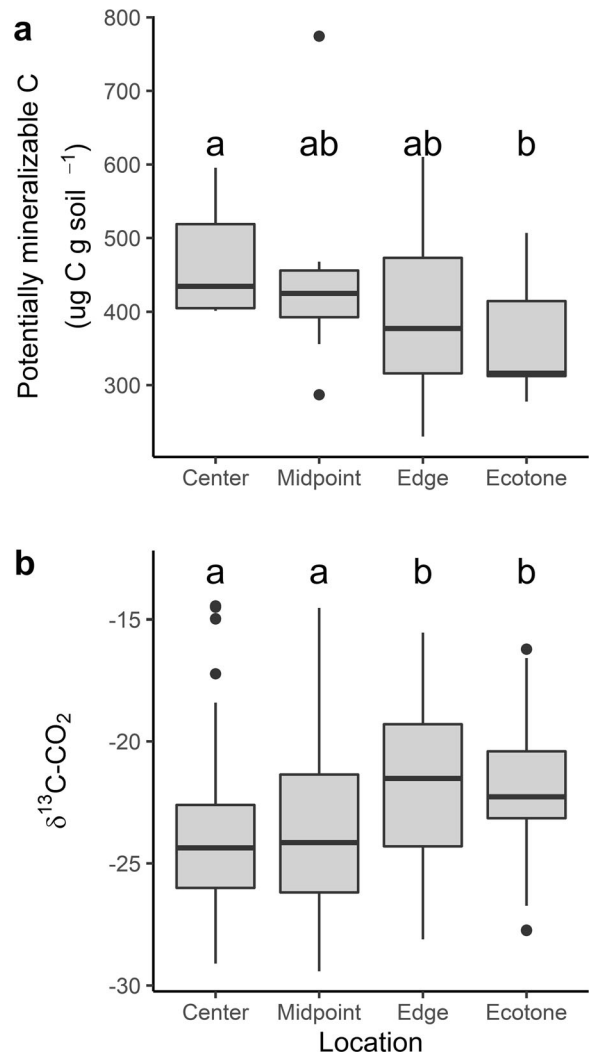


Fig. 4 Boxplots of a) potentially mineralizable C and b) mean $\delta^{13}\text{C}$ of respired CO_2 from each sampling location (center, midpoint, edge, and ecotone) from the potential C mineralization incubations. The dark line represents the median and the box represents the first and third quartiles. Whiskers extend to the maximum and minimum of the data, excepting outliers (represented by points). Letters represent significant differences in Tukey's comparisons of least square means. The potentially mineralizable C pool was smaller and the average $\delta^{13}\text{C}$ signature of respired CO_2 was higher in soils collected further away from the center of the dogwood shrub

has low rates of aeolian deposition and has experienced relatively recent woody encroachment (~30 years). While it is possible that physical resource accumulation played a role in determining the heterogeneity of soil properties in our study, we suggest that biological mechanisms played a larger role.

Shrubs impact microbial elemental demand across space and time

Our results did not support the hypothesis that microbial N demand would vary with spatial location within shrub islands, though it did vary with shrub island size. Microbial demand for N relative to P increased as shrubs increased in size, suggesting that microbes ultimately became more limited by N with clonal vegetative expansion (Fig. 2b). Differences in organic matter quality between grass and shrub inputs or increased soil aggregation due to shrub growth could reduce the amount of microbially-available N under larger shrubs despite the increase in total soil N pools. Additionally, deep-rooted shrubs can translocate P from deeper in the soil profile to the surface (Zhou et al. 2018), so it follows that microbial N/P demand will be higher in locations that have been occupied by shrubs for the longest amount of time.

Also, we found that microbial demand for C (relative to N) at the shrub island edge decreased as shrubs got larger (Fig. 2a). In contrast to the product inhibition pathway well established to regulate N- and P-acquiring enzyme production, BG activity can be stimulated by substrate (cellulose) addition (Allison and Vitousek 2005), and herbaceous litter (i.e., litter originating from a non-woody source) may contain more available cellulose than the more chemically complex woody plant litter (McKee et al. 2016). Further, the biomass of herbaceous litter decreases from the edge of a dogwood island to the center (Ratajczak et al. 2011). Therefore, the center of large dogwood islands could be more C-limited, despite having higher total soil C concentrations, because of fewer new herbaceous inputs originating from above- and belowground. Understanding the patterns of microbial extracellular activity is important because the degree to which microbial communities are limited by C or N is a major driver behind priming effects of soil organic matter decomposition (Wang et al. 2015).

Microbial preference for shrub-derived organic matter impacts C fluxes

Potential soil C mineralization rates did not increase with distance from the center of shrub, but the source of the C being mineralized was an important factor affecting potential C mineralization rates. Across all shrub island sizes and spatial locations, potential C mineralization rates were higher if the isotopic

composition of CO₂ indicated a higher proportion of C₃-derived organic matter (i.e., dogwood-derived organic matter; Fig. 3) used by soil microbes. Since plant communities in this study were growing in soils previously dominated by C₄ grasses (Connell et al. 2020a), we could use an isotopic mixing model to estimate the relative contribution of grass- or shrub-derived organic matter decomposition to soil CO₂ flux (Kuzyakov 2006). Therefore, we conclude that the soil microbes underneath dogwood islands preferentially use newer C₃ shrub-derived organic matter as a carbon source rather than older C₄ grass-derived organic matter. Microbial preference between grass and shrub-derived organic matter may be system specific as C-sources for respiration were primarily grass-derived in an African savanna (February et al. 2020).

Potential C mineralization rates were higher when microbes decomposed proportionally more woody-derived organic matter for several potential reasons. First, according to the preferential substrate use hypothesis, microbes break down newer organic inputs before shifting to older soil organic matter (Blagodatskaya et al. 2011). This has been observed in Australian savannas in which the most recent inputs were preferentially decomposed regardless of source (Wynn et al. 2020). Secondly, newer carbon inputs from dogwood might initially be going to physically unprotected fractions of the soil which would increase microbial access to woody-derived organic matter (Creamer et al. 2011). Thirdly, C₃-derived organic matter might be intrinsically more decomposable than C₄-derived organic matter. However, this contrasts with previous research in a mixed C₃/C₄ system showing that C₄-derived organic matter decomposes more quickly (Wynn and Bird 2007). Finally, shrubs may select for unique microbial communities that have greater levels of activity (Ochoa-Hueso et al. 2018).

Our results suggest that dogwood shrubs increase the potentially mineralizable C pool and that this pool increases with time since establishment (i.e., more potentially mineralizable C in the central locations of the shrub compared to the edge; Fig. 4a). Accordingly, the stable isotopic values of respired CO₂ in the central locations of the shrub were lower than values on the shrub edge (Fig. 4b), indicating that microbes may become more reliant on shrub-derived organic matter for carbon sources as woody encroachment progresses. A similar spatial pattern in soil δ¹³C has been observed in a Texas shrubland (Bai et al. 2012) indicating that spatial heterogeneity in the stable isotopic composition

of respired soil CO₂ may be observed in other systems besides the tallgrass prairie.

Future directions

In light of our results, we suggest that future research should investigate the degree to which dogwood root systems exhibit ‘heterorhizy’ [i.e., differences in root function within individual plants due to ontogenetic differences of individual roots within a whole root system (Hishi 2007)], and how that may correlate with distance from the center of dogwood shrubs. Clonal shrubs commonly have both a shallow lateral root system that is more extensive than many of their herbaceous neighbors (Casper and Jackson 1997) as well as deep taproots (Schenk and Jackson 2002). The roots in the oldest, more developed central parts of a dogwood shrub island grow deeper in the soil profile than grasses (Canadell et al. 1996), facilitating access to deeper water sources (Nippert and Knapp 2007; Nippert et al. 2013; Logan and Brunsell 2015), which alleviates competition between dogwood and the surrounding herbaceous vegetation (Ratajczak et al. 2011; Muench et al. 2016) and allows for the roots further from the center to explore new territory in which to produce ramets (Ratajczak et al. 2011). In other systems, researchers have found that the older roots of woody plants respire less (Bouma

et al. 2001), have lower N concentrations (Hishi and Takeda 2005), and have slower fine root turnover (Hishi and Takeda 2005). We suggest investigating if older dogwood roots associated with deep soil water uptake and distribution function differently from newer roots associated with expansion. Additionally, future studies would benefit from more refined data on clonal shrub demography, how belowground biomass of woody plants varies spatially, how live roots impact in situ CO₂ efflux, and how woody plants affect soil microbial processes deeper in the soil profile in order to more accurately predict ecosystem responses to woody encroachment. Finally, since woody encroachment impacts small mammal abundance and communities (Matlack et al. 2008) and small mammal activity can influence microbial enzyme activity (Moorhead et al. 2017), we suggest investigating how altered plant-animal interactions due to shrub encroachment may impact soil biogeochemistry.

Conclusion

Using shrub size as a proxy for shrub age, we found that shrubs increase the amount of soil C and N in the central, and presumably older, parts of the island but not at the edge (Fig. 5). The heterogeneity in shrub

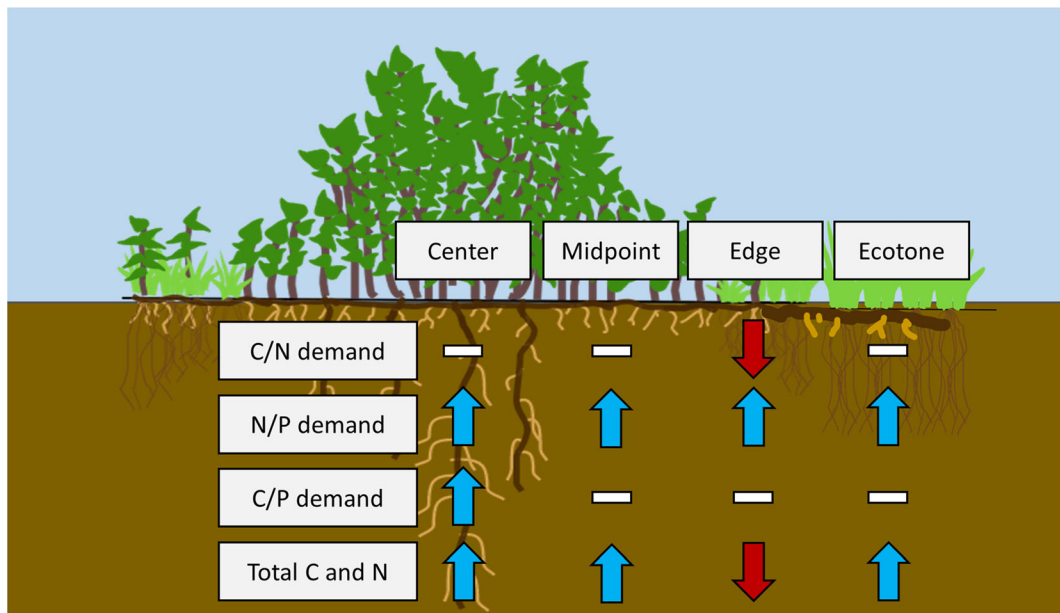


Fig. 5 The response of soil properties and microbial demand for C, N, and P as dogwood shrub islands grow larger. An upward arrow indicates that the property increases as dogwood shrubs

grow larger while a downward arrow indicates the opposite. A horizontal line indicates no change

inputs results in spatial heterogeneity in microbial extracellular enzymatic activity that is dependent on shrub size. As dogwood shrubs continue to expand, microbial demand for N increases across all locations while demand for C increases in the center of the dogwood island but decreases at the edge (Fig. 5). Thus, soil properties and processes vary in space under clonal woody shrubs in the tallgrass prairie and change as islands grow larger, indicating that the biogeochemical impact of woody encroachment does not remain constant over time or space and occurs over finer scales than are usually measured. Finally, we conclude that dogwood occupancy in tallgrass prairie soil increases the amount of potentially mineralizable C and that this newer C is respired at a higher rate than older C₄-grass derived organic matter.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11104-020-04813-9>.

Acknowledgments We thank Dr. Lydia Zeglin for her assistance with the methodology, collection, and interpretation of the potential extracellular enzymatic activity assays and Dr. Anthony Joern for providing funds for reagents. Dr. Lydia Zeglin and Emily Wedel provided feedback which greatly increased the quality of this manuscript. We also thank Aditi Arun and Paige Humphrey for their assistance in the field and lab and Emily Wedel for her artistic contributions to Fig. 5. This research was supported by the NSF Long-Term Ecological Research Program at Konza Prairie Biological Station. JBN was supported by DOE award # DE-SC0019037. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship under Grant No. GGV005607-3145-50010. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. This is publication #21-141-J from the Kansas Agricultural Experiment Station. All data used in this study can be found on the Konza Prairie LTER website (Connell et al. 2020b).

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