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Long-Term Biomass Yield and Species Composition in Native Perennial Bioenergy Cropping Systems

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ABSTRACT

Biomass yield is an important factor when recommending native perennial plants and mixtures for bioenergy production. Our objective was to determine long-term biomass yields in fertilized and unfertilized native plant monocultures and mixtures that show promise for bioenergy across diverse environments in the Upper Midwest. We measured biomass yields, species composition, and diversity annually in monocultures and mixtures ranging from 4 to 24 planted species including grasses, legumes, and other forbs; each managed with and without 67 kg N ha^{-1} fertilizer applied annually at nine locations for 7 yr. Without N fertilization, switchgrass (*Panicum virgatum* L.) monocultures and an eight-species mixture of grasses and legumes produced the most biomass over locations and years (5.1 Mg ha^{-1}). With N fertilizer, switchgrass monocultures and a four-species mixture of grasses produced the highest yields (6.8 and 6.4 Mg ha^{-1}). Over time, biomass yields increased for switchgrass, decreased for Canada wild rye (*Elymus canadensis* L.), and remained stable for the high diversity mixtures. Other mixtures had nonlinear changes in yield, likely related to changes in species composition. Although the relative abundance of individual species changed over time, Shannon diversity was constant except for the four-species legume mixture where it decreased. Contrary to other studies, N fertilization did not decrease species diversity through time. Diversity was positively related to biomass yield following establishment, but the strength of the relationship diminished with stand age. Native plant mixtures managed with and without N fertilizer can yield similar biomass compared with highly productive monocultures in the Upper Midwest.

Production of bioenergy from native perennial biomass crops can reduce fossil fuel use and associated C emissions (McLaughlin et al., 2002; Tilman et al., 2006a; Schmer et al., 2008; Gelfand et al., 2013). Growing native perennial biomass crops in agricultural landscapes results in fewer adverse environmental effects compared to annual row-crops (Asbjornsen et al., 2014) including reductions in runoff and soil erosion (Hernandez-Santana et al., 2013), nutrient loss (Smith et al., 2013; Zhou et al., 2014), and biodiversity loss (Robertson et al., 2011; Werling et al., 2014). Additional environmental benefits accrue when native perennial plants are grown in mixtures (polycultures) compared to monocultures. For instance, polycultures sequestered five times more C in the first meter of the soil profile than native monocultures (Fornara and Tilman, 2008) and had 33% less N leached compared to native monocultures (Dybzinski et al., 2008).

Harvestable biomass from native perennial crops is typically lower than current annual row-crop options available for bioenergy (Jarchow and Liebman, 2012; Wilson et al., 2014). Although the net energy output may be higher in native perennial cropping systems (Tilman et al., 2006a), profitability is strongly influenced by yield and is a major component in native perennial crop adoption (Jensen et al., 2007). Increasing biomass yields of native perennial bioenergy crops could increase producer profits and thereby incentivize commercial production (James et al., 2010). Two primary approaches for increasing biomass yields from native perennial crops include N fertilization (Heaton et al., 2004; Fike et al., 2006; Schmer et al., 2008) and enhancement of plant diversity (Tilman et al., 2006a; Picasso et al., 2011). Nitrogen fertilization has consistently been shown to enhance yields of native perennial plants grown in monoculture and polyculture (Berg, 1995; Lemus et al., 2008; Kering et al., 2012; Jarchow and Liebman, 2013). For instance, relatively modest amounts of N fertilizer (60–80 kg ha^{-1}, nearly half of the average rate applied to corn fields in the United States) increased biomass yields by more than 50% in monocultures and polycultures of native perennial species in Minnesota (Jungers et al., 2015b). Despite short-term yield gains from N fertilizer in polycultures, long-term fertilization can reduce species diversity (Suding et al., 2005) and associated yield increases (Isbell et al., 2013). Therefore, the interactive effects of N fertilizer and species composition on biomass yield need to be analyzed, especially as species composition may change through time with annual N fertilization.

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Abbreviations: CI, confidence interval.

Native polyculture yields can also be enhanced through the process of "overyielding", where mixtures of multiple species produce more biomass than monocultures of the same species (Tilman et al., 2006a; Khalsa et al., 2012). Furthermore, Tilman et al. (2006b) showed that temporal stability of biomass yields was greater in diverse polycultures compared to monocultures, and increased with stand maturity. However, this positive diversity-productivity relationship was not observed in some bioenergy studies (Johnson et al., 2010; Adler et al., 2009, Griffith et al., 2011). The measured effect of plant diversity on biomass yield reported from diversity experiments also varies across environments (Cardinale et al., 2000; Loreau and Hector, 2001), thus there is a need to quantify environmental variation regarding species composition in polyculture bioenergy systems before making regional recommendations.

Spatial and temporal variation influence native species persistence and resulting biomass yield (Isbell et al., 2013), yet most studies comparing monocultures and polycultures of native perennial bioenergy crops were, for any given combination of species, limited to a few environments (Tilman et al., 2006a), short time scales (Jarchow and Liebman, 2012), or were purely observational and lacked replicated experimental treatments (Adler et al., 2009; Johnson et al., 2010). Thus, it is not immediately clear how diverse mixtures that may be planted for bioenergy across many sites might perform across space and time. Our objective was to determine long-term biomass yields of native plant monocultures and polycultures that are candidates for bioenergy production including grasses, forbs, and legumes varying in diversity when grown with and without N fertilization. We also measured changes in species composition of polycultures with time and related those changes to biomass yield. The scope of this study was unique in that species mixture and N fertilizer effects on biomass yields were measured for 7 yr at nine locations in Minnesota and North Dakota. These represent spatial and temporal scales that are likely to be realized for bioenergy production in the Upper Midwest.

MATERIALS AND METHODS

The experiment was conducted at nine locations spanning soil, precipitation, and temperature conditions in Minnesota and North Dakota. (Tables 1 and 2). Sites south of 46° N were seeded in 2006 while those north of 46° N were seeded in 2007. A detailed description of cropping history, site preparation, and establishment year yield and composition is reported by Mangan et al. (2011).

The experiment was a randomized, split-plot design with three replications per location. The main plot treatment was N fertilizer applied at two levels; either 0 or 67 kg N ha^{-1} as ammonium nitrate and broadcast by hand in spring. Although plots were seeded in 2006 and 2007, N application did not begin until 2008. The subplot treatment included 12 mixtures of native plant species that ranged in species and plant functional group number, from monocultures to diverse 24-species mixtures (Table 3). Species selection was not random, but rather based on regional suitability to establish successfully and produce reliable, harvestable biomass. Treatments were

Table 1. Average mean daily temperature (°C) (first number) and mean monthly total precipitation (mm) (second number) from 2007 to 2013.

Location	April	May	June	July	August	September	October
Becker	7- 135	14.1- 219	19.1- 182.2	22.2- 156.1	20.7- 269.7	16.2- 186.3	8.7- 162.3
Crookston	5.3- 31.3	12.5- 58.4	17.9- 51.5	20.7- 47.7	19.4- 47.9	14.8- 45.4	7.2- 75.5
Fargo	6.4- 196.9	14.5- 310.7	20- 407	22.8- 206	20.6- 192	16.2- 255.9	8.7- 236.7
Lamberton	7.2- 114.8	14.8- 216.9	19.8- 270.1	22.6- 99.4	20.9- 198.3	17- 180	10.1- 113.8
Mahnomen	5.3- 96.6	12.6- 179.4	17.5- 291	20.4- 176.3	19.3- 184.4	14.9- 217.8	7.1- 205.9
Red Lake Falls	3.2- 94.4	11.9- 227.3	17.1- 257.6	20.1- 186.7	19- 136.6	13.5- 231.9	7.2- 194.8
Roseau	4.9- 125.4	12- 235.5	17.3- 242.4	20.3- 208.6	19.1- 137.1	14.8- 172	7.3- 165.6
Saint Paul	8.5- 229.7	15.6- 303.6	20.6- 300.1	23.9- 288.6	22.5- 294.3	17.8- 207.8	10.2- 219.9
Waseca	7.3- 66.6	14.2- 71.1	19- 101.1	21.4- 64.2	20.1- 42.3	16.2- 68.2	9.4- 71.9

Table 2. Soil type, description, and characteristics of the top 30 cm at each location before establishment.

Location	Soil type	Soil description	pH	Organic matter	NO_3	S	P	K
				g kg^{-1}	— kg ha^{-1} —		— mg kg^{-1} —	
Becker	Hubbard	sandy, mixed, frigid Entic Haploboroll	6.6	14	8	11	30	108
Crookston	Wheatville	coarse-silty over clayey, mixed over smectitic, superactive, frigid Aeric Calciaquoll	8.3	27	40	na†	14	145
Fargo	Fargo	fine-silty, mixed, superactive, frigid Typic Calciaquoll	8.0	3.4	18	23	9	457
Lamberton	Normania	fine-loamy, mixed superactive mesic Aauic Hapludoll	5.7	36	14	18	71	601
Mahnomen	Barnes	fine-loamy, mixed, superactive, frigid Calcic Hapludoll	7.7	33	8	19	14	162
Red Lake Falls	Wheatville	coarse-silty over clayey, mixed over smectitic, superactive, frigid Aeric Calciaquoll	7.2	21	4	16	20	115
Roseau	Bearden	fine-silty, mixed, superactive, frigid Aeric Calciaquoll	7.2	16	7	24	18	121
Saint Paul	Waukegan	fine-silty over sandy, mixed, superactive, mesic Typic Hapludoll	6.9	44	24	34	127	373
Waseca	Webster	fine-loamy, mixed, superactive, mesic Typic Endoaquoll	6.5	43	30	31	18	189

† na, not applicable.

the same across all sites except for some polyculture mixtures, where species substitutions were made for sites north of 46° N to accommodate geographical range boundaries. Seeding rates for each species within each treatment are reported in Mangan et al. (2011). A total of 648 subplots were maintained, each 9 m^2 in size.

A preventative application of a pre-emergence herbicide, acetochlor [2-Chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide], was applied at all locations in late April in 2008 and 2009. Afterward, weeds never represented a significant portion of the biomass. Boundaries around each experiment were mowed to prevent encroachment of weeds. At some sites, local outbreaks of perennial weeds developed and were controlled by herbicide and/or removing individuals by hand. For example, sporadic invasions of Canada thistle [*Cirsium arvense* (L.) Scop.] at Lamberton required manual removal and spot-spraying of glyphosate [N-(phosphomonomethyl) glycine].

Plant species composition was determined by visually assessing the relative abundance of each species within a subplot as percent aerial coverage in late August or early September. A careful survey of the presence of all plant species within the subplot boundary was first conducted. Then, each observed species was assigned a percentage of the total area within the plot for which its biomass covered. Weed and native volunteer species were not identified to species, but rather categorized together along with bare ground and included in the percent cover survey. The same observer measured relative abundance in all plots during all years of this study.

Biomass yield was measured annually following a killing frost (−2°C) from a 0.75 m^2 sample quadrat within each subplot. Biomass was cut by hand to a stubble height of approximately 5 cm. Harvested biomass was collected, dried at 60°C until constant weight, and then weighed to determine dry matter (DM) yield.

Statistical Analysis

We hypothesized that biomass yield would vary with planted species mixture, fertilizer treatment, and stand age (i.e., years since establishment). To test for differences in total biomass

Table 3. List of species mixture treatments, number of species planted in the mixture (species richness), and the species planted in each mixture treatment.

Treatment	Species richness	Species sown	ID	Common name	Latin name	Functional group
Switchgrass	1	A	A	Switchgrass	*Panicum virgatum* L.	Grass
Big Bluestem	1	B	B	Big bluestem	*Andropogon gerardii* Vitman	Grass
Indiangrass	1	C	C	Indiangrass	*Sorghastrum nutans* L.	Grass
Canada wild rye	1	D	D	Canada wild rye or Virginia wild rye	*Elymus canadensis* L. or *E. virginicus* L.	Grass
Grass mix	4	A, B, C, D	E	Little bluestem	*Schizachyrium scoparium* Michx.	Grass
Legume mix	4	I, J, K, L	F	Slender wheatgrass	*Elymus trachycaulus* Link	Grass
Forb mix	4	Q, R, S, T	G	Sideoats grama	*Bouteloua curtipendula* Michx.	Grass
Grass/Legume	8	A, B, C, D, I, J, K, L	H	Virginia wild rye or Canada wild rye	*Elymus virginicus* L. or *E. canadensis* L.	Grass
Grass/Forb	8	A, B, C, D, Q, R, S, T	I	Canada milkvetch	*Astragalus canadensis* L.	Legume
Legume/Forb	8	I, J, K, L, Q, R, S, T	J	Wild blue indigo or showy tick trefoil	*Baptisia australis* L. or *Desmodium canadense* L.	Legume
12-species mix	12	A, B, C, D, I, J, K, L, Q, R, S, T	K	Purple prairie clover	*Dalea purpurea* Vent.	Legume
24-species mix	24	A through X	L	Lead plant	*Amorpha canescens* Pursh	Legume
			M	Perennial lupine or American licorice	*Lupinus perennis* L. or *Glycyrrhiza lepidota* Pursh	Legume
			N	Partridge pea or pale pea	*Chamaecrista fasciculate* Michx. or *Lathyrus ochroleucus* Hook.	Legume
			O	Showy tick trefoil or white prairie clover	*Desmodium canadense* L. or *Dalea candida* Michx.	Legume
			P	Roundheaded bushclover or American vetch	*Lespedeza capitata* Michx. Or *Vicia Americana* Muhl.	Legume
			Q	Butterfly milkweed or purple coneflower	*Asclepias tuberosa* L. or *Echinacea purpurea* L.	Forb
			R	Maximillian sunflower	*Helianthus maximiliani* Schrad.	Forb
			S	Stiff goldenrod	*Solidago rigida* L.	Forb
			T	Yellow coneflower	*Ratibida pinnata* (Vent.) Barnhart	Forb
			U	Rough blazing star or northern bedstraw	*Liatris aspera* Michx. or *Galium boreale* L.	Forb
			V	Wild bergamot	*Monarda fistulosa* L.	Forb
			W	Cup plant or black eyed Susan	*Silphium perfoliatum* L. or *Rudbeckia hirta* L.	Forb
			X	Golden Alexander	*Zizia aurea* L.	Forb

yield associated with individual treatments, we used mixed-effects ANOVA with species mixture, N fertilizer, and stand age as main effects. Additionally, we tested for two-way and three-way interactions among the three main treatments. In all analyses, we controlled for pseudo-replication, within-plot, and within-year autocorrelation by allowing the intercept to vary randomly as a function of year, nested within plot, nested within site. We conducted our tests in the R statistical programming environment (R Development Core Team, 2014) using the nlme package (Pinheiro and Bates, 2013).

Interactions and insignificant variables were removed based on model comparisons using Akaike's information criteria (AIC) and ANOVA of nested comparisons (Zuur et al., 2009). The best-fit model was used to estimate coefficients from 9999 bootstrapped data sets. The distribution of the resulting coefficients was used to determine the mean effect size (hereafter model-based estimates) and 95% confidence intervals (CIs) for all treatment levels. Significant differences among species mixtures within N fertilization treatments was concluded by comparing CIs. Species mixtures with overlapping CIs were considered similar in terms of their affect on biomass yield.

Table 4. Fixed-effects results from analysis of variance of log transformed biomass yield.

Source of variation	df†	F statistic	P value
Species treatment (S)	11, 3302	78.3	<0.001
N fertilization (N)	1, 3302	31.7	<0.001
Year since establishment (Y)	1, 38	1.7	0.2
S × N	11, 3302	4.7	<0.001
S × Y	11, 3302	13	<0.001
N × Y	1, 3302	3.8	0.051
S × N × Y	11, 3302	1.2	0.302

† Numerator and denominator.

For each species mixture, we explored changes in biomass yield through time. We fit a global mixed-effects model that included N fertilizer treatment and stand age as a cubic predictor. The global model was reduced using the "stepAIC" function in the MASS package (Venables and Ripley, 2002). This function reduced the global model one predictor at a time until a reduced model was less supported than the previous. The best-fit model was used to predict biomass yield during each year for fertilized and unfertilized plots of each species mixture treatment. Each best-fit model was fit to 9999 bootstrapped data sets to determine mean effect size and 95% CIs.

We tested for a relationship between plant diversity and biomass yield using linear mixed effects models. We used two metrics of plant diversity, planted species richness and the Shannon diversity index $H' = -\Sigma p_i \log(p_i)$ of each polyculture species mixture. Shannon diversity (H') accounts for both the number of species within a community and how even the community is in terms of relative abundance based on percent cover of each species (p_i). Our measure of Shannon diversity was based on the proportion of each species within the group of species planted in the mixture treatments. Weed and volunteer species were not separated and therefore omitted from this analysis.

Planted species richness was the number of plant species sown into each treatment at the start of the experiment. Planted species richness did not change from year to year, while the Shannon diversity index did to account for changes in the relative abundance, and thus evenness, among plant species in each plot. Therefore, planted species richness and Shannon diversity were tested with independent models. Similar to the regression models used for determining effects of species treatment, we fit models that predicted changes in biomass yield based on planted species richness, N fertilization, and stand age with all possible interactions. We estimated 95% CIs based on the bootstrap method described above, and used these

Table 5. Average biomass yield (and SE) of N fertilized and unfertilized switchgrass, grass mix, grass/legume, 12-, and 24-species mixture treatments at all study locations.

Location	N rate	Switchgrass	Grass mix	Grass/legume	12-species mix	24-species mix
	kg N ha^{-1}	Mg ha^{-1}				
Becker	0	3.4 (0.2)	2.8 (0.2)	2.9 (0.2)	2 (0.2)	2.1 (0.2)
	60	4.7 (0.5)	4.7 (0.4)	4.8 (0.5)	2.6 (0.4)	3.2 (0.4)
Crookston	0	4.4 (0.5)	5.5 (0.3)	5.5 (0.5)	4.1 (0.4)	4.8 (0.5)
	60	6.9 (0.5)	8.4 (0.7)	5.5 (0.6)	4.9 (0.5)	5.2 (0.4)
Fargo	0	3.7 (0.4)	2.8 (0.3)	4.2 (0.5)	3.7 (0.3)	4 (0.4)
	60	6.1 (0.8)	4.4 (0.6)	4.6 (0.4)	4.9 (0.5)	4.3 (0.5)
Lamberton	0	8.7 (0.4)	9 (0.4)	6.4 (0.5)	5.7 (0.6)	5.4 (0.3)
	60	9.9 (0.4)	10.3 (0.5)	5.2 (0.3)	5.9 (0.5)	4.9 (0.3)
Mahnoman	0	3.1 (0.3)	2.7 (0.3)	4.5 (0.3)	4.4 (0.4)	3.8 (0.2)
	60	4.6 (0.3)	5.1 (0.3)	4.3 (0.3)	5 (0.3)	5 (0.3)
Roseau	0	4 (0.4)	3.8 (0.3)	5.9 (0.4)	4.5 (0.3)	4.8 (0.5)
	60	7 (0.8)	6.2 (0.5)	6.4 (0.7)	6 (0.6)	5.7 (0.4)
Red Lake Falls	0	5.8 (0.4)	4.6 (0.4)	5.9 (0.7)	4.8 (0.4)	5.5 (0.5)
	60	7.7 (0.7)	6.6 (0.5)	6.6 (0.7)	5.4 (0.6)	5.7 (0.5)
Saint Paul	0	5.5 (0.7)	6 (0.6)	5.1 (0.4)	4.7 (0.5)	5.1 (0.4)
	60	4.6 (0.6)	4.3 (0.5)	4.8 (0.5)	6.5 (0.6)	5.7 (0.5)
Waseca	0	6.5 (0.4)	3.7 (0.2)	5.5 (0.4)	5 (0.5)	4.4 (0.4)
	60	9 (0.5)	6.5 (0.6)	7.1 (0.8)	6.6 (0.8)	5 (0.5)
Mean	0	5.1 (0.2)	3.5 (0.2)	4.6 (0.2)	5.1 (0.2)	4.4 (0.2)
	60	6.8 (0.2)	4.8 (0.2)	6.4 (0.2)	5.5 (0.2)	5 (0.2)

estimates to determine significance. The same procedure was conducted with Shannon diversity as a predictor variable in place of planted species richness.

Finally, we measured changes in species composition of each species mixture that included more than one species. We modeled the change in relative abundance of each species within all polyculture species mixtures using the same global model and model selection process for yield dynamics described above.

RESULTS AND DISCUSSION

The effect of species mixture and N fertilization on biomass yield varied substantially across environments and through time (Tables 4 and 5). We account for this variation by modeling environment and year as random effects (Hector et al., 2011; Hautier et al., 2014) to produce model-based estimates of "typical" treatment effects across locations and years (hereafter "model-based" estimates). Model-based estimates serve as regional estimates independent of stand age to compare biomass yields across species mixtures and N fertilizer treatments.

In both N fertilized and unfertilized plots, model-based yield estimates of switchgrass monocultures were either the highest, or among the highest from all species mixture treatments (Fig. 1). This was not surprising given that switchgrass has been selected as a model bioenergy crop because of its high yield potential across a range of temperature and moisture conditions (Jefferson and McCaughey, 2012). Nevertheless, there was substantial between-site variation, and switchgrass yields within locations ranged from 3.1 Mg ha^{-1} yr^{-1} at Mahnoman to 8.7 Mg ha^{-1} yr^{-1} at Lamberton. The relatively higher yield of switchgrass compared to other treatments was also not consistent over locations. For example, at Roseau, switchgrass yields were similar to 24-species polycultures and less than the grass/legume mixture when unfertilized (Table 5).

Biomass Yields of Grass Monocultures

Switchgrass was the highest yielding unfertilized grass monoculture followed by big bluestem (*Andropogon gerardii* Vitman), indiangrass (*Sorghastrum nutans* L.), and Canada wild rye. Switchgrass, big bluestem, and indiangrass are warm-season grasses, and each has been evaluated as a potential bioenergy feedstock grown in monoculture (Kaiser et al., 2011). Similar to our observations, Hong et al. (2013) measured higher switchgrass yields compared to big bluestem and indiangrass monocultures at two of three locations in South Dakota and Minnesota.

Switchgrass was also the highest yielding grass species under fertilized conditions (Fig. 1). Big bluestem produced more biomass than indiangrass and Canada wild rye, which had similar biomass yields. We observed a 40, 38, 26, and 30% increase in fertilized switchgrass, big bluestem, indiangrass, and Canada wild rye yields compared to their unfertilized counterparts, respectively. These biomass yield increases were similar to those estimated for switchgrass and prairie polyculture mixtures where biomass yields plateaued in response to range of N fertilizer rates (Jungers et al., 2015b), thus suggesting that our results describe a "best-case" scenario for fertilization benefits. However, Lee et al. (2009) found that higher N fertilizer rates were needed to obtain similar yield increases of 30% in switchgrass and big bluestem (150 kg N ha^{-1} compared to our 67 kg N ha^{-1}).

Biomass Yields of Unfertilized Mixture Treatments

In unfertilized plots, the eight-species grass/legume mixture produced similar yields as switchgrass (Fig. 1). Without N fertilizer, N fixation by legumes reduces N limitation for co-occurring grass species, which allows the mixture to overyield compared to other monocultures and mixtures without legumes (Fornara and Tilman, 2008). The grass/legume mixture had higher yields than the four-species grass and four-species legume mixtures, indicating that facilitation likely occurred.

The unfertilized four-species grass mixture, 12-, and 24-species mixture treatments produced similar model-based yields, but yields were lower than those for switchgrass and the grass/legume mixture (Fig. 1). Other unfertilized mixtures produced less biomass than the grass/legume mixture because they lacked both components grown together (e.g., forb and legume only mixtures), or

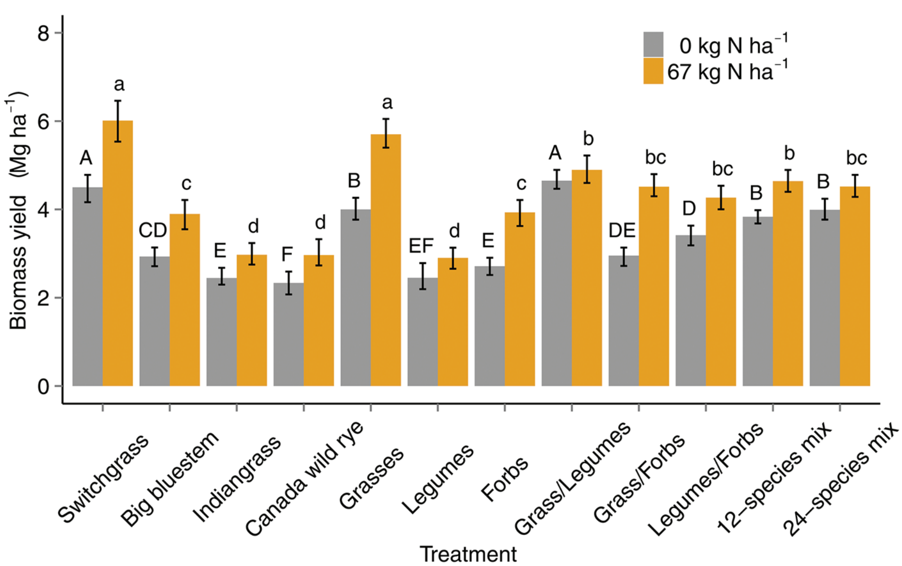

Fig. 1. Model-estimated biomass yield (± 95% confidence intervals, CI) from 12 species treatments grown with and without N fertilizer averaged across time and environments. Error bars indicate 95% CIs and are used to determine significant differences between treatments. Bars sharing the same upper- and lower-case letters indicate similar average yields in unfertilized and fertilized plots, respectively.

because other forb species limited the abundance of the grass and legumes (e.g., 12- and 24-species mixtures). However, this does not explain why switchgrass monocultures were so productive.

Biomass Yields of Nitrogen Fertilized Mixture Treatments

In fertilized plots, model-based biomass yield estimates of the four-species grass mixture and switchgrass were similar (Fig. 1). These treatments produced more biomass than the eight-species grass/legume, grass/forb, 12-species, and 24-species mixture treatments, which had similar yields. The ranking of fertilized species mixture treatments by model-based yield was not consistent in all locations (Table 5). For example, fertilized four-species grass mixtures produced less mean biomass at St. Paul than the 12- and 24-species mixtures.

All mixture treatments except the four-species legume mixture and the grass/legume mixture produced more annual biomass when fertilized compared to their unfertilized counterparts, but the relative response varied among species treatments (Tables 4 and 5). Nitrogen fertilization of the four-species legume mixture and the grass/legume mixture treatments had no effect on biomass yield, likely because legume N-fixation increased N availability sufficiently to remove N limitation. However, the forb/legume, 12-, and 24-species mixture treatments also contained legumes yet still produced more biomass when fertilized with N compared to those without N fertilizer. Lower legume abundance in these mixtures, or differential N uptake ability by non-legume forbs, may have widened the yield gap between fertilized and unfertilized mixtures that included non-legume forbs.

Effects of Treatments through Time

The relationship between biomass yield and stand age varied by species mixture and fertility (Fig. 2). When controlling for environmental variation as a random effect, biomass yield of the unfertilized switchgrass, big bluestem, and indiangrass monocultures increased nonlinearly during 7 yr of production following establishment. With fertilization, switchgrass and big bluestem yields increased until the sixth production year after which yield plateaued. Indiangrass yield increased from Year 6 to 7. From the third to sixth year, yields of all grass monocultures and the grass mixtures were greater in fertilized than unfertilized treatments; but by the seventh year, biomass yields in fertilized and unfertilized switchgrass, big bluestem, and indiangrass monocultures were similar. This resulted both from (i) greater biomass increases through time in unfertilized compared with fertilized plots, and (ii) decreases in biomass yield in later years for fertilized plots. A potential reason for the decrease in biomass yield in fertilized plots with stand age could be related to a shift in nutrient limitation. Macro- and micronutrients are removed from the cropping system during biomass harvest at rates relative to yield (Jungers et al., 2015b). Therefore, the higher biomass yields in fertilized treatments compared to unfertilized treatments in earlier years of this study could have resulted in nutrient deficiency in the fertilized plots. This is consistent with previous research on fertilized (Isbell et al., 2013) and unfertilized (Reich et al., 2012) mixtures, and suggests that N fertilization alone may not be a viable strategy for increasing yields for timespans longer than those that we considered here. Continued research is needed to understand long-term yield limitations related to soil nutrients in grassland bioenergy cropping systems.

Fertilized and unfertilized Canada wild rye yields decreased nonlinearly during the study and were lower than all warm-season grass monocultures by the last year. This was expected, as Canada wild rye is an early successional cool-season grass with low persistence. Fertilized four-species grass mixture yields increased until Year 4, and then remained constant. In unfertilized four-species grass mixtures, yields declined slightly after establishment until Year 4, and then increased to Year 7. Yields of the four-species legume and grass/legume mixtures

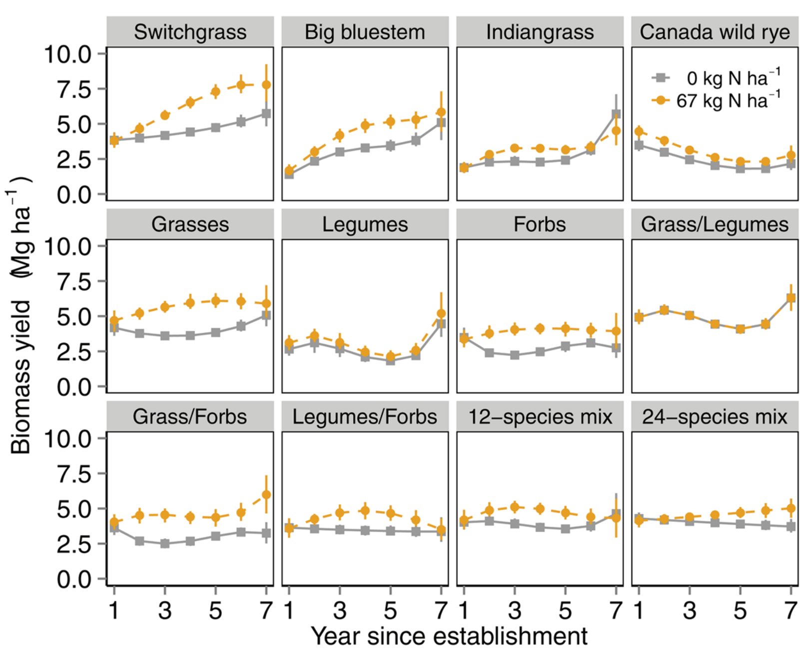

Fig. 2. Change in model-estimated biomass yield (± 95% confidence intervals, CI) of 12 species mixture treatments grown with and without N fertilizer from the first year following establishment to the end of the study.

exhibited a similar, cubic response with stand age. The grass/forb, legume/forb, and 12-species mixture treatments showed nonlinear trends in biomass yield through time with less fluctuation and net change compared to the grass monocultures, legume, and grass/legume treatments (Fig. 2).

The 24-species mixture was the only treatment with a linear biomass yield response through time, but the slope was small for both fertilized (slope = 0.03) and unfertilized (slope = −0.01) treatments, indicating little variation in biomass yield through time. The 24-species mixture contained early successional species like Canada wild rye (Table 3), which contributes biomass early during stand establishment, as well as late successional species like big bluestem, which replace biomass lost by species that decrease in abundance with stand maturity. This "portfolio effect" is one explanation for why biomass yields are more stable in mixtures with more species (Lehman and Tilman, 2000).

Across years, N fertilizer effects on yield were positive for most treatments except the legume, grass/legume, and 24-species mixtures. The N effect was strongest between Years 3 and 6, and by the seventh year biomass yields were similar between N fertilized and unfertilized plots for all treatments. Isbell et al. (2013) showed that N fertilization increased yields of grass monocultures and native polycultures, but the net positive effect of N on yields declined with time especially under high N fertilizer rates. It is not clear whether fertilized grasslands maintain high yields in years following fertilizer cessation, or if species loss due to fertilization will lead to chronic declines in biomass yield. Both scenarios have been observed in the Netherlands under varying soil conditions (Olff and Bakker, 1991).

Model-based estimates of biomass yield by year are useful for comparing yield trends across treatments grown at a "typical" site in the study region. However, it is important to note that biomass yield dynamics varied greatly among location (Fig. 3). The response functions that best explained changes in biomass yield through time were different among treatments and locations. The source of this environmental variation could be related to a combination of factors including soil type, soil fertility, temperature, precipitation, and identity of the species within mixtures.

Changes in Shannon Diversity and Plant Species Composition

The relative abundance of species and overall Shannon diversity of polycultures changed (Fig. 4). Changes in Shannon diversity and species composition can be related to patterns in biomass yield dynamics. Below, we first compare patterns in changes in diversity and plant species composition of mixture treatments through time, and then associate species composition and diversity to changes in biomass yield.

The grass, grass/legume, and grass/forb mixtures increased in Shannon diversity throughout the experiment in both fertilized and unfertilized plots (Fig. 4). The unfertilized 24-species mixture also increased in diversity, but the fertilized 24-species plots did not. Diversity did not change in fertilized or unfertilized plots of forb, legume/forb, and 12-species mixture treatments. The only treatment that experienced a significant decrease in diversity was the legume mixture, and this decrease occurred in both fertilized and unfertilized treatments.

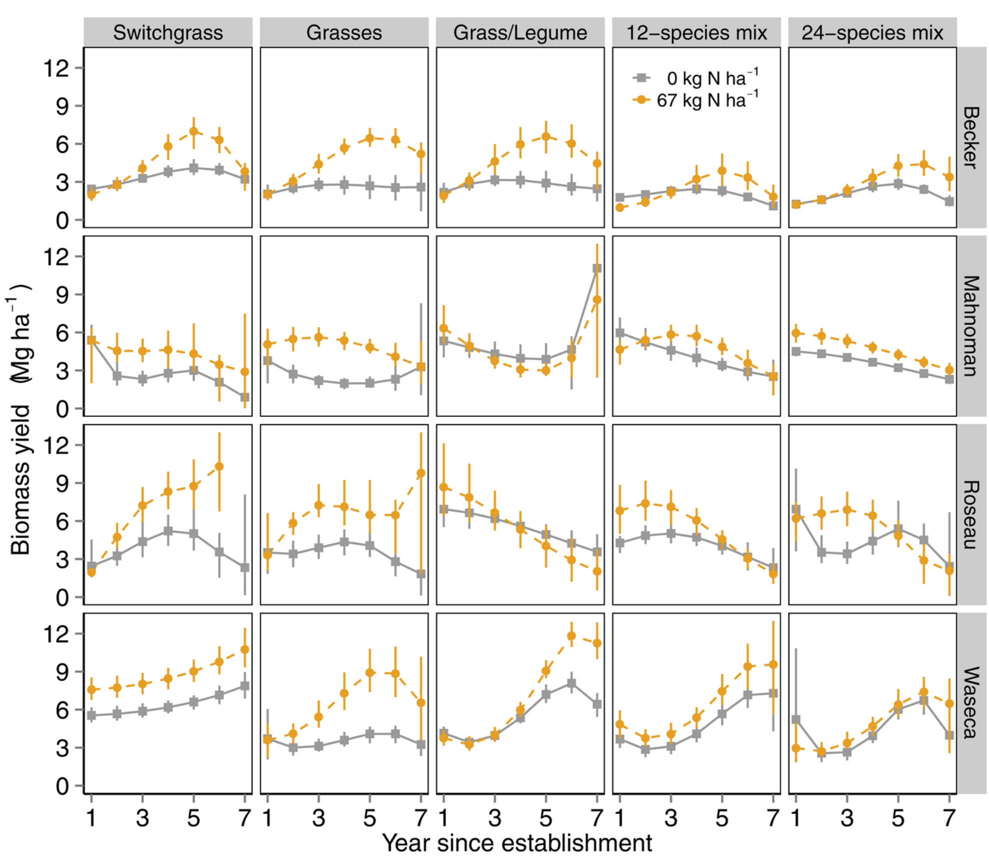

Fig. 3. Change in model-estimated biomass yield (± 95% confidence intervals, CI) in 5 of 12 species treatments at 4 of 9 study locations from the first year following establishment to the end of the study.

Both N fertilization and harvest regime have been shown to affect species diversity in native polyculture grasslands. In unharvested grasslands, species diversity decreases with N fertilization in the short- and long-term because certain species disproportionally benefit from nutrient enrichment and therefore outcompete other species (Tilman, 1987; Suding et al., 2005; Clark and Tilman, 2008; Yang et al., 2012). However, annual mowing of fertilized grasslands can prevent species loss by increasing light availability (Yang et al., 2012; Collins et al., 1998). We harvested annually in autumn, which may explain why we did not observe consistent decreases in Shannon diversity in fertilized treatments compared to unfertilized treatments. Other studies have reported maintained diversity in mowed grasslands for bioenergy (Jungers et al., 2015a), while some have reported *increases* in diversity under mowed and fertilized conditions (Jarchow and Liebman, 2012).

Our result showing that fertilization did not decrease diversity in mixed species treatments suggests that polyculture bioenergy crop production can complement other environmental goals related to native grassland expansion. Accounting for C emissions related to N fertilizer production and application, Gelfand et al. (2013) showed that fertilizing polyculture grasslands for bioenergy increased the greenhouse gas emissions offsets compared to unfertilized grasslands. If diversity can be sustained through annual harvest of fertilized polycultures, further greenhouse gas offsets can accrue in root biomass (Tilman et al., 2006a). Our results support previous findings that polycultures can be managed with N fertilizer for bioenergy without detrimental effects to species diversity (Jarchow and Liebman, 2013).

Changes in the relative abundance of species within mixture treatments can explain changes in Shannon diversity. In the grass mixture treatment where Shannon diversity increased with and without N fertilizer, switchgrass dominated in terms of relative abundance soon after establishment, but declined throughout the study. In both fertilizer treatments, big bluestem abundance increased through time as switchgrass decreased (Fig. 5). Others have shown that a single grass species can become dominant within a four-species grass mixture, and the species that dominates is also dependent on the environment (Griffith et al., 2011). However, in our study the four species grass mix became more uniform as species abundances in that treatment became more equal.

Despite a similar change in Shannon diversity through time, the relative abundance of species differed for the fertilized and unfertilized grass mixture (Fig. 5). In unfertilized plots, indiangrass increased in abundance until Year 5, while the fertilized plots experienced a similar increase in Canada wild rye. The difference in species' responses through time across fertilizer treatments in the grass mixture could be related to plant phenology and the timing of fertilization. Cool-season species such as Canada wild rye have the advantage of accessing spring-applied N fertilizer before warm-season grasses because they begin annual growth earlier in the year. Without the annual spring N source, Canada wild rye was not able to increase in abundance therefore allowing other species like indiangrass to increase.

Model-based estimates of species' abundances within the grass/legume mixture show that Canada milkvetch (*Astragalus canadensis* L.) and showy tick trefoil (*Desmodium canadense* L.) were the most abundant legumes in the mix, with switchgrass being the most abundant grass during most years (Fig. 5). Canada milkvetch and showy tick trefoil were similarly abundant during the first 4 yr following establishment, but showy tick trefoil emerged as the more dominant legume in both fertilized and unfertilized plots. Canada wild rye increased in abundance enough to exceed switchgrass cover during Year 5 in the fertilized grass/legume mixtures, but Canada wild rye abundance decreased to less than switchgrass by Year 7. Canada wild rye abundance remained less than switchgrass during all years of the study in unfertilized grass/legume mixtures.

Comparing species' abundances within the grass/legume mixture across locations shows that final species composition in this treatment was not similar to the model-estimated outcome at all locations. For instance, legumes dominated the

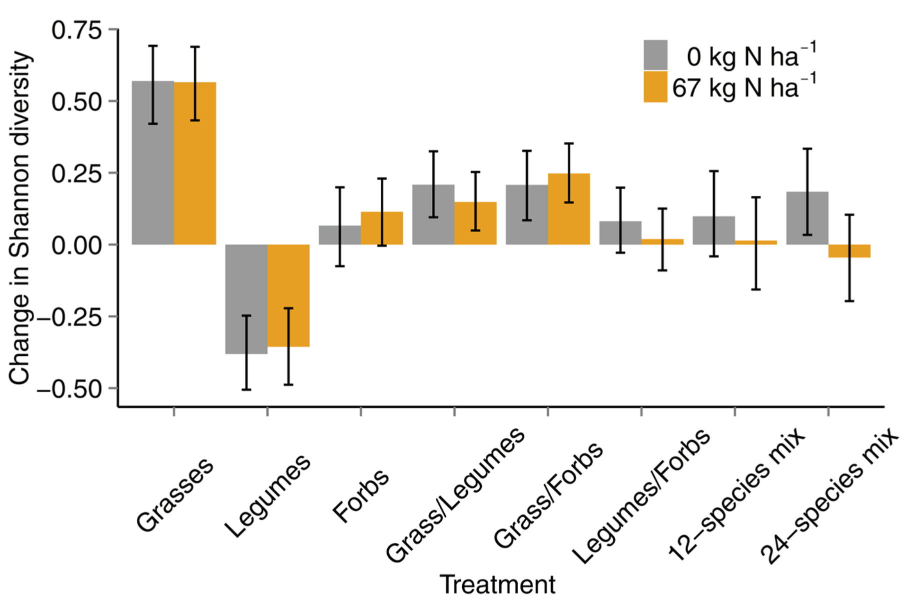

Fig. 4. Change in model-estimated Shannon diversity (± 95% confidence intervals, CI) from the first year following establishment compared to the final study year in fertilized and unfertilized polyculture treatment mixtures.

mixture at Mahnoman, Crookston, and Fargo (Table 6; only Mahnoman data shown). For locations north of 46° N, big bluestem was more abundant than switchgrass in unfertilized plots. In fertilized grass/legume mixtures, switchgrass was the most dominant warm season grass at all locations except for Fargo, which corroborates the model-based estimates.

In the legume mixtures, a rather dramatic turnover in species abundances occurred in both fertilized and unfertilized plots (Fig. 5). During the first year following establishment, Canada milkvetch was the most abundant legume in the legume mixture treatment, but decreased in abundance during the first few years of the experiment. During the decline in Canada milkvetch abundance, showy tick trefoil increased in abundance and eventually dominated both fertilized and unfertilized legume mixtures (Fig. 5). This dominance explains the decrease in Shannon diversity within the legume treatment (Fig. 4).

In the 12-species mixture, forbs and legumes were always more abundant than grasses (Fig. 6b). Switchgrass and Canada wild rye were the only warm- and cool-season grasses to cover more than 3% in fertilized or unfertilized 12-species mixture treatments in any given year. Yellow coneflower [*Ratibida pinnata* (Vent.) Barnhart] and Maximilian sunflower (*Helianthus maximilianii* Shrad.) were the most abundant forbs. These forbs were among the top three most abundant species in fertilized and unfertilized 12-species mixtures at all locations during the final year, except Waseca and unfertilized plots at Red

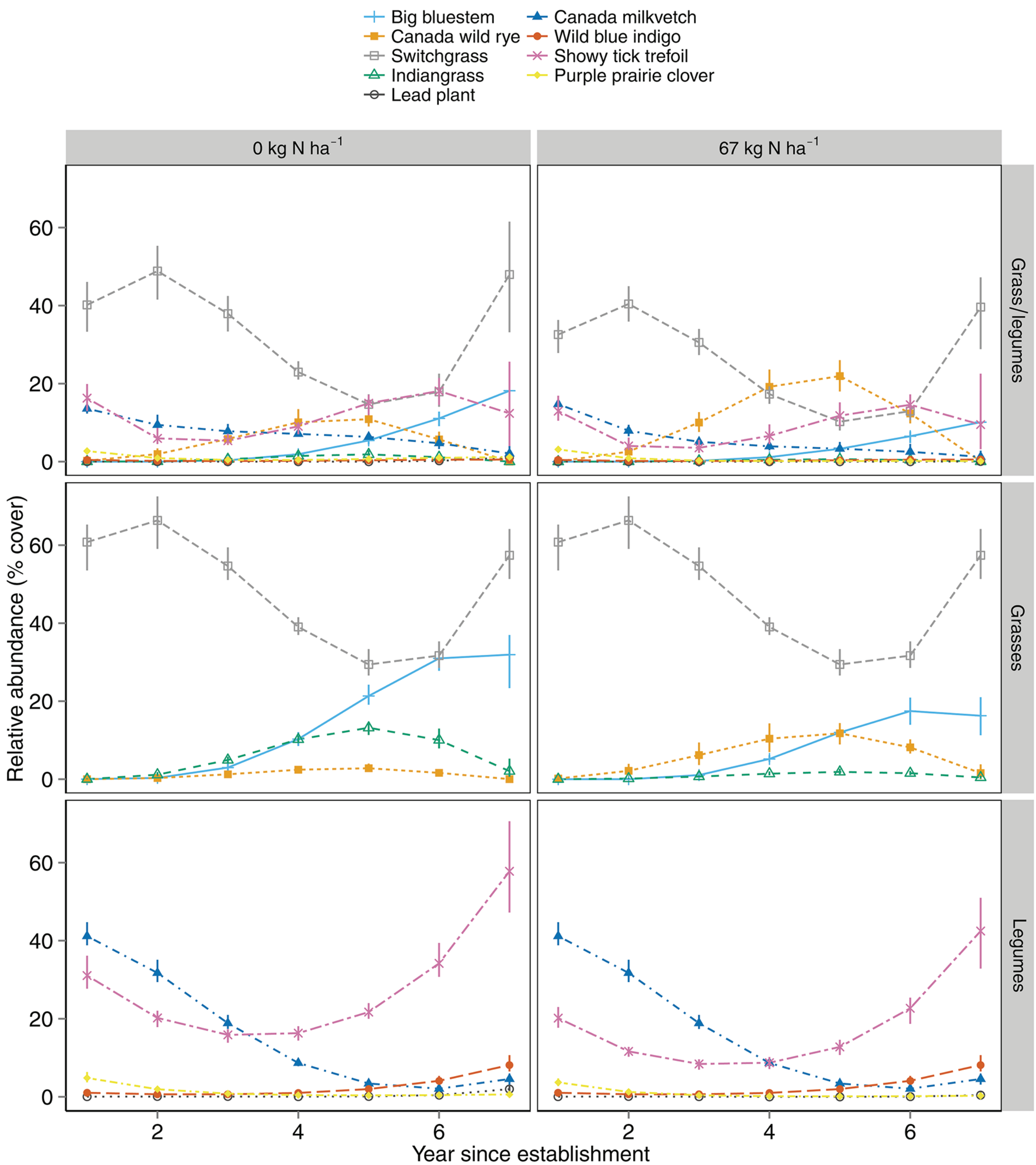

Fig. 5. Change in the model-estimated relative abundance (± 95% confidence intervals, CI) of species planted in fertilized and unfertilized grass, legume, and grass/legume mixtures from the first year following establishment to the end of the study.

Table 6. Top three most abundant species and associated mean percent cover in fertilized and unfertilized grass, grass/legume, 12-, and 24-species mixture treatments during the final year of surveys (2013) at four of nine locations.

Location	Grass mixture				Grass/legume				12-species mixture				24-species mixture			
	0 kg N ha^{-1}		67 kg N ha^{-1}		0 kg N ha^{-1}		67 kg N ha^{-1}		0 kg N ha^{-1}		67 kg N ha^{-1}		0 kg N ha^{-1}		67 kg N ha^{-1}	
Becker	Big bluestem	35	Switchgrass	78	Switchgrass	35	Switchgrass	63	Yellow coneflower	33	Yellow coneflower	33	Little bluestem	42	Switchgrass	45
	Switchgrass	33	Big bluestem	13	Big bluestem	35	Big bluestem	25	Big bluestem	17	Switchgrass	27	Yellow coneflower	15	Yellow coneflower	22
	Indiangrass	18	Indiangrass	3	Canada milkvetch	17	Indiangrass	4	Canada milkvetch	12	Big bluestem	18	Showy tick trefoil	12	Little bluestem	6
Mahnoman	Big bluestem	70	Big bluestem	78	Showy tick trefoil	47	Showy tick trefoil	47	Yellow coneflower	37	Yellow coneflower	49	Yellow coneflower	28	Yellow coneflower	42
	Switchgrass	12	Switchgrass	13	Big bluestem	32	Switchgrass	25	Showy tick trefoil	21	Showy tick trefoil	20	Showy tick trefoil	21	Wild bergamot	13
	na†	0	na	0	Switchgrass	18	Big bluestem	18	Big bluestem	15	Big bluestem	9	Wild bergamot	12	Showy tick trefoil	11
Roseau	Big bluestem	68	Switchgrass	57	Big bluestem	28	Switchgrass	27	Showy tick trefoil	30	Maximillian sunflower	23	American licorice	42	Maximillian sunflower	50
	Switchgrass	17	Big bluestem	38	Canada milkvetch	27	Big bluestem	17	Maximillian sunflower	15	Canada wild rye	18	Golden Alexander	12	Golden Alexander	15
	Canada wild rye	7	Canada wild rye	3	Switchgrass	12	Canada milkvetch	10	Purple prairie clover	15	Showy tick trefoil	12	Maximillian sunflower	10	Canada milkvetch	9
Waseca	Big bluestem	73	Switchgrass	62	Big bluestem	37	Switchgrass	52	Wild blue indigo	40	Switchgrass	25	Golden Alexander	38	Golden Alexander	53
	Switchgrass	12	Big bluestem	35	Switchgrass	37	Big bluestem	37	Stiff goldenrod	20	Big bluestem	20	Switchgrass	8	Stiff goldenrod	7
	Indiangrass	8	Indiangrass	2	Wild blue indigo	18	Wild blue indigo	9	Big bluestem	12	Maximillian sunflower	7	Stiff goldenrod	8	Cup plant	7

† na, not applicable.

Table 7. Estimated coefficients (beta) and associated standard errors (SE) of variables affecting biomass yield including planted species richness and Shannon diversity.

Fixed effects	Beta	SE	*t* value	*P* value
Species richness model				
Intercept	2.399	0.060	39.76	<0.001
N fertilization (N)	0.115	0.034	3.37	<0.001
Planted species richness (S)	0.014	0.002	7.24	<0.001
Year since establishment (Y)	0.019	0.009	2.08	0.043
N×S	–0.006	0.003	–2.06	0.04
N×Y	0.003	0.007	0.39	0.698
S×Y	–0.002	<0.001	–3.97	0.001
N×S×Y	0.001	0.001	1.09	0.277
Shannon diversity model				
Intercept	2.421	0.060	40.09	<0.001
N fertilization (N)	0.095	0.035	2.75	0.006
Shannon diversity (H)	0.121	0.021	5.80	<0.001
Year since establishment (Y)	0.015	0.009	1.64	0.109
N×H	–0.022	0.030	–0.73	0.463
N×Y	0.007	0.007	1.02	0.307
H×Y	–0.015	0.005	–3.14	0.002
N×H×Y	0	0.007	0.04	0.972

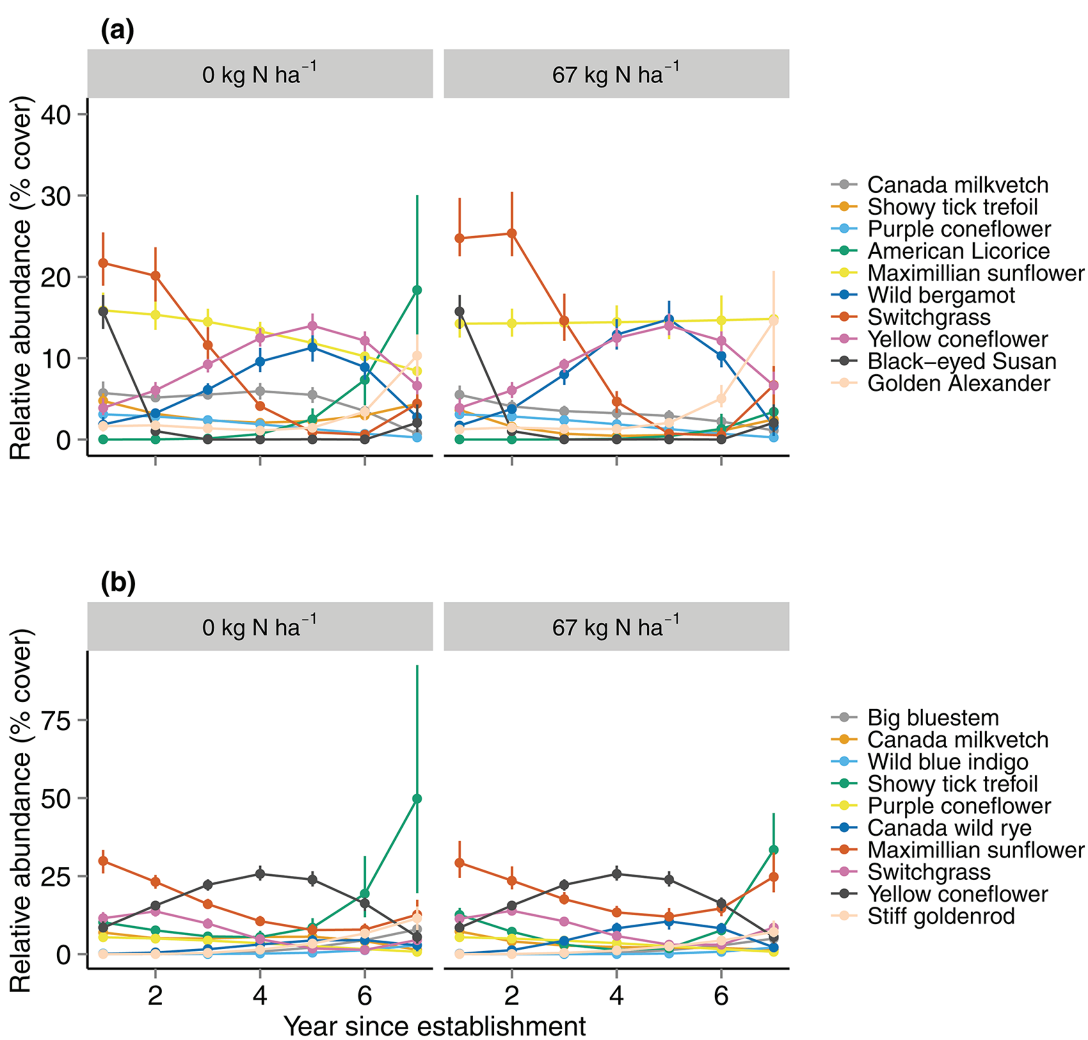

Fig. 6. Change in the model-estimated relative abundance (± 95% confidence intervals, CI) of species planted in fertilized and unfertilized (a) 24-species and (b) 12-species mixture treatments from the first year following establishment to the end of the study. Shown are species that occupied more than 3% cover in at least one fertilizer/year combination. Less abundant species were omitted from this figure for clarity.

Lake Falls (Table 6). Showy tick trefoil was the most abundant legume in the mix, especially by the last year of the study.

In the 24-species mixture, switchgrass was the most abundant species in both fertilized and unfertilized plots during the first 2 yr following establishment, but declined with stand age (Fig. 6a). Wild bergamot (*Monarda fistulosa* L.), yellow coneflower, and Maximilian sunflower were abundant during Year 4 and later. In the unfertilized 24-species mixture, the legume American licorice (*Glycyrrhiza lepidota* Pursh) was the most abundant species based on the model-estimated abundance by the final year. However, this species was only planted at locations north of 46° N, and was especially dominant at the Roseau location (Table 6). No single species was especially dominant in the fertilized 24-species mixture by the end of the experiment.

Associations between Biomass Yield and Plant Diversity

We used planted species richness and the Shannon diversity index as metrics of plant diversity to test for relationships between diversity and biomass yield. The species selected for each mixture treatment were not chosen at random, but instead assembled for high, consistent bioenergy potential. Since this was not a diversity experiment where all species were grown in monoculture and mixtures at various diversity levels, we cannot separate the causal effects of diversity and species composition on biomass yield. Our results should be interpreted to show that switchgrass monocultures differed from most of the polycultures we tested in terms of yield, but this does not necessarily mean that the entire effect can be attributed to diversity.

Planted species richness was positively correlated with biomass yield in unfertilized species treatments but not correlated when fertilized (Table 7). Thus, while diversity may have contributed to increased productivity in unfertilized plots, it did not in fertilized plots. The positive relationship in unfertilized plots diminished through time. Lower yields in the grass monocultures compared to the polycultures in the early years of the experiment led to the positive species richness-productivity relationship, but as grass monoculture yields caught up and later exceeded those of polycultures, the relationship weakened. In other words, increasing monoculture yields through time dampened the positive relationship between species richness and biomass yield. The species richness effect was weaker in fertilized treatments because lower diversity species treatments including the monocultures yielded more when fertilized than their unfertilized counterparts compared to mixed-species treatments.

Patterns in Shannon diversity and biomass yield were similar to those observed for species richness and yield (Table 7). We tested for a relationship between Shannon diversity and biomass yield to account for changes in the relative abundance of species within treatment mixtures. For instance, if the four species grass mixture was dominated by switchgrass, that treatment would still be considered more diverse than the switchgrass monoculture based on species richness because it was planted with more species. Shannon diversity, on the other hand, measures both the number and the relative abundance of species, and therefore polyculture mixture treatments that are dominated by a few species are considered less diverse than mixture treatments with species abundances more evenly distributed. Because we calculate Shannon diversity separately for each year, it also accounts for any changes in species composition through time. Similar to the species richness model, we observed a positive relationship between Shannon diversity and yield, which dampened through time. This suggests that those treatments that experienced an increase in Shannon diversity through time (Fig. 4) did not experience yield increases at the same rate. Unlike the species richness model, there was no interaction between Shannon diversity and N fertilizer treatment. This suggests that N fertilizer did not alter the relationship between Shannon diversity and biomass yield.

CONCLUSIONS

We measured biomass yield from 12 different native grassland mixtures grown for bioenergy ranging from monocultures to high-diversity polycultures, with and without N fertilizer, for 7 yr at nine locations. Switchgrass fertilized with N had higher biomass yields than big bluestem, indiangrass, and Canada wild rye monocultures. Some polycultures mixtures produced similar biomass yields as switchgrass. When fertilized, a four-species grass mixture produced as much biomass as switchgrass, and without N fertilizer, an eight-species grass/legume mixture produced as much biomass as switchgrass. Biomass yields of the 12- and 24-species mixtures were similar and less than the fertilized grass mixtures and unfertilized grass/legume mixtures. There was considerable variation among treatments in biomass yield through time. Biomass yields increased for switchgrass, decreased for Canada wild rye, and remained stable for the 12- and 24-species mixtures. Other treatments had nonlinear changes in yield through time, likely related to changes in species composition. Species diversity of mixtures was positively related to biomass yield during early years of the experiment, but the relationship diminished with stand age. Nitrogen fertilizer did not decrease species diversity of polyculture mixtures. Based on large environmental variation we observed, regional recommendations of perennial bioenergy crop mixtures should not be based on results from single locations. Data from replicated experiments comparing perennial bioenergy crop yields across multiple environments and years should be combined with other metrics of environmental sustainability to inform regional bioenergy and land-use decisions.

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