Bioenerg. Res. (2015) 8:1636–1646
DOI 10.1007/s12155-015-9613-2

Environment Has Little Effect on Biomass Biochemical Composition of *Miscanthus*×*giganteus* Across Soil Types, Nitrogen Fertilization, and Times of Harvest

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Published online: 21 April 2015

Abstract Efficient conversion of lignocellulosic feedstocks to ethanol will benefit from a consistent composition of supplied biomass. While composition or quality for a given feedstock is known to vary, the influence of environment, rather than genotype, has rarely been separated for mature field-grown material. Replicated trials of a single sterile hybrid clone of *Miscanthus*×*giganteus* across Illinois provided a unique opportunity to test the influence of environmental, rather than genetic control over biomass composition, under US Midwest conditions. Given the interest in *M.* x *giganteus* cv. "Illinois" as a lignocellulosic feedstock, it is valuable to understand the variation in composition of this crop that would need to be dealt with by processors. This study examined the effect of seven sites spanning nearly 5° in latitude and contrasting soil types from sands to clays with land capability classes ranging from 1 to 4. Four levels of nitrogen fertilization (0, 67, 135, and 202 kg N ha^{-1}) were applied on these mature, genetically identical, clonally propagated stands of *M.*×*giganteus* which were harvested both pre- and post-senescence. Despite the wide range of environmental differences, there was minimal variation in the composition across all locations, sampling times, and fertilization treatments. Composition varied from 39–45 % for cellulose, 19–24 % for hemicellulose, to 19–24 % for lignin. Nitrogen fertilization, while having a small effect, decreased the proportion of hemicellulose, acetyl groups, and ash and increased cellulose and lignin at statistically significant levels. Delaying harvest from October to December increased the proportion of cellulose, hemicellulose, and lignin and decreased the proportion of ash and extractables at statistically significant levels. The findings show that in the absence of genetic variation, composition varies minimally with environment or timing of harvest, which has important implications for costs of processing in a given location.

Keywords Bioenergy · Biofuel · Structural carbohydrate · Composition · Nitrogen · Lignocellulose · Cellulose · Hemicellulose · Klason lignin

Electronic supplementary material The online version of this article (doi:10.1007/s12155-015-9613-2) contains supplementary material, which is available to authorized users.

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Introduction

Lignocellulosic biofuel production requires the efficient digestion of large quantities of feedstock. The C4 warm season perennial grasses *Miscanthus*×*giganteus* Greef and Deuter ex Hodkinson and Renvoize [22, 35] is a high-yielding, low-input lignocellulosic bioenergy feedstock which has been grown for many years without fertilization and with limited weed control [11, 2, 3, 47]. The material of this species has become known in the USA as the Illinois clone [20] and has proved particularly productive in the Midwest USA [2, 3, 27]. This is to the extent that by 2012, the US Department of

Agriculture Biomass Crop Assistance Program, which assists owners and operators in the development of commercial biomass operations, stipulated this clone of *Miscanthus* as the sole feedstock for 4 of its 11 Project Areas [19]. Lignocellulose is composed largely of three primary cell wall polymers: cellulose, hemicellulose, and lignin [21, 51]. The relative proportion of these carbohydrates in a feedstock is known to vary significantly and influences ease of conversion to monosaccharides and disaccharides for fermentation and in turn ethanol yield [50, 55, 56, 36]. A constant composition also avoids the need to vary amounts of acid, enzyme, other chemicals, and physical conditions to optimize deconstruction. If the biomass is used for pyrolysis, then the resulting "oil" and optimum temperatures and flows in processing are also affected by feedstock biochemical composition [13, 48, 18]. Consistency in the composition of the harvested biomass is therefore a key factor in realizing economic cellulosic fuels, whether via biochemical or thermochemical routes [21, 31, 14].

With present technologies for biochemical biofuel production, the content of lignin is seen as the major barrier. Lignin is not easily separated from cellulose, impeding separation of cellulose fibrils and slowing access by cellulases. Biomass has to be pretreated in order to make the polysaccharides more accessible to enzymes. A common pretreatment step involves heating biomass in the presence of dilute sulfuric acid. Degradation products formed during this process can inhibit the growth of microorganisms and therefore inhibit fermentation of sugars into biofuels. Lignin can also irreversibly bind enzymes and inhibit the enzymatic de-polymerization of cellulose and hemicellulose into fermentable sugars. In theory, the lower the lignin content in biomass, the better. However, lignin is a cross-linked polymer that is essential in providing structural support, limiting pest damage, and allowing xylem vessels to function under the high tensions that develop during transpiration [21, 25, 43].

Hemicellulose, a heteropolysaccharide composed mainly of C5 sugar residues, is more easily converted to sugars than cellulose, a homopolymer of β-1,4-linked glucose units primarily in crystalline microfibril form [50]. Yeasts have been engineered which can efficiently ferment the C5 sugar xylose with the penultimate product of cellulose degradation, the disaccharide cellobiose [23, 37]. Overall, feedstock composition affects process configurations, reactor designs, and process performance [55]. Variation in feedstock composition would therefore affect processing efficiency since built capacities for different parts of the processing, such as pretreatment, depolymerization, C5 fermentation, and C6 fermentation, can only be optimal for a single feedstock composition.

The hemicelluloses of the cell wall have varying degrees of acetylation [10]. Pretreatment of bioenergy feedstocks therefore results in the release of acetate, which is a partial inhibitor subsequent enzymic saccharification and microbial fermentation [10]. Therefore, it is desirable that acetyl concentration in bioenergy feedstocks is as low as possible. At the time of writing, no studies had been published that examine changes in acetyl contents, which can substantially affect enzymic processing to biofuels.

Inorganic elements such as potassium, phosphorus, calcium, and magnesium in the harvested biomass constitute its ash content after conversion or combustion [52]. Ash is a waste product which can cause slagging in direct combustion and inhibit enzymatic conversion, but in pyrolysis, it can also lead to the valuable co-product biochar [52]. Silica is the largest component of ash affects the use of biomass in combustion and lowers efficiency of production of cellulosic biofuels [4, 8]. Therefore, as a general rule, the lower the ash content, the better the quality for most end uses.

Non-structural, solvent-soluble, and non-volatile compounds make up the extractable component of biomass, which is predominantly leaf and stem cuticle waxes [52]. Extractables including fatty acids, starch, resins, chlorophylls, and waxes are typically a minor fraction of total biomass composition, but could comprise a potential source of value-added co-products in large-scale lignocellulosic operations [52] but can also include inhibitors of saccharification of cellulose and hemicellulose and of fermentation of the resulting sugars. It is therefore important to know the concentration of extractables and how environment affects this component.

Large differences between genotypes of individual feedstocks have been shown with more than 20 % variation in lignin, hemicellulose, and cellulose contents [7, 52, 57]. Although much emphasis has been placed on genetic drivers for cell wall composition [21], less emphasis has been given to understanding variation induced by environmental variation, including differences in agronomy. Harvest time and location were recently found to be key drivers of cell wall composition of *Zea mays* (maize) stover [56]. Understanding temporal and spatial variation in feedstock quality in terms of composition will be critical to the performance of lignocellulosic fuel manufacturing facilities. It may also indicate cultural practices, harvest times, soil type, or climates that may favor a higher quality biomass with respect to ease of conversion to biofuels or bioenergy. Since stands of the rhizome-propagated sterile hybrid *M.*×*giganteus* Illinois have been cloned to date from a single hybridization event and therefore lack genetic variation, this species and single clone serves as a unique opportunity to study variation in cell wall components caused solely by environmental factors. Furthermore, given the emergence of *M.*×*giganteus* as a lignocellulosic feedstock, it is important to understand its composition and the factors causing it to vary in designing processing facilities.

Previous studies in W. Europe have examined changes in cell wall composition of *M.*×*giganteus* in response to different fertilizer treatments over the growing season at one location [32], differences between different *M.*×*giganteus* clones

at multiple locations [33], and cultivars of *Miscanthus* spp. at one location [34, 44]. With more mild winters, harvest dates in W. Europe are often later and so composition may be examined in January through March compared to autumn harvests in the Midwest USA [2, 46]. Although these studies provided important information on variation with site and genotype, they did not specifically separate out the effects of location combined with variation in nitrogen fertilization level on *M.*×*giganteus*. Other studies have shown a trend of increasing lignin and cellulose with delayed harvest, but no pattern has emerged for hemicellulose (Table 1). Hodgson et al. [32] showed that with increasing nitrogen fertilization, there was a decrease in the cell wall components' cellulose and hemicellulose in the stem and a decrease in the proportion of cellulose in the leaf material; however, this was only tested at 0 and 50 kg N ha^{-1}. Lemus et al. [38] examined changes in biomass quality in another perennial grass, *Panicum virgatum*, across four nitrogen treatment rates (0, 56, 112, and 224 kg N ha^{-1}) and determined that cellulose and lignin increased with increased rate of nitrogen fertilization, while hemicellulose and ash declined in bulk biomass material. Soil fertility, nitrogen status, and temperature might all be expected to cause significant phenotypic variation in cell wall composition. Further, the first replicated field trials of *M.*×*giganteus* in the Midwestern USA have shown much higher autumn and winter yields than typically observed in Europe; it might therefore be expected that quality, as well as quantity, will be affected by this very different growth environment [2, 27]. Also, and in contrast to the findings of most European studies, a split-plot nitrogen fertilization experiment demonstrated that yield of mature stands of *M.*×*giganteus* was significantly increased by nitrogen fertilization from 23.4 Mg ha^{-1} at 0 kg(N)ha^{-1} to 28.9 Mg ha^{-1} at 202 kg(N)ha^{-1} (+25 %) [3].

Three fully replicated trials were established in 2002 in the Midwestern USA [27], and four further sites were established in 2004 [2]. Starting in 2007, four levels of nitrogen fertilization were added to these established plots using a split-plot design [3]. These seven mature stands of *M.*×*giganteus* in the Midwestern USA, all of similar age, provided a unique opportunity to isolate the effect of location, nitrogen, and harvest time on the quality of harvested feedstock in terms of cell wall components.

Given this unique opportunity, the hypothesis that soil type, nitrogen fertilization, and harvest time will affect cell wall composition, independent of genotype, was tested. This was achieved by quantifying cellulose (as glucan), hemicellulose (as xylan and arabinan), lignin (Klason lignin), acetyl, ash (total ash before extraction), and extractable biomass components at these seven locations, across four nitrogen fertilizer levels, and at two harvest dates on genetically identical clonally propagated and replicated stands of *M.*×*giganteus*.

Materials and Methods

Plant Material

Biomass samples were collected in 2009 from trials of *M.*×*giganteus* established at seven sites spanning a variety of soil and weather conditions within Illinois (Supplementary Table 1, Fig. 1). Planted in 2002, the three field trials included in this study are the oldest replicated research trials of *M.*×*giganteus* in the USA and are located in DeKalb (88.15 W, 41.85 N) and Urbana (88.23 W, 40.08 N), Dixon Springs (88.67 W, 37.45 N), Illinois; establishment and maintenance of these trials have been previously described by Heaton et al. [27] and Arundale et al. [2]. Four additional field sites were established in 2004 following the same procedures and experimental design in Havana (89.84 W, 40.30 N), Orr (90.82 W, 39.81 N), Brownstown (88.96 W, 38.95 N), and Fairfield (88.39 W, 38.38 N), Illinois. Briefly, these field trials were established in a completely randomized design with plots measuring 10 m×10 m (n=4) planted with rhizome-propagated *M.*×*giganteus* stock of the genotype which has become known as the Illinois clone [20, 19]. Trials were established from greenhouse-grown potted plants planted on 1-m centers. A split-plot N fertility treatment was initiated in DeKalb and Dixon Springs in 2007 and at the remaining five sites in 2008 [3]. Briefly, each whole 10 m×10 m *M.*×*giganteus* plot was sub-divided into four 5 m×5 m sub-plots that were randomly assigned a nitrogen treatment of 0, 67, 134, or 202 kg N ha^{-1}. Nitrogen was applied in the form of either granular ammonium nitrate (34 % N; Dixon Springs and DeKalb) or granular ammonium sulfate (21 % N; all other locations) in the spring, as described previously [3]. In summary, the design was four whole plots at each site, with each of these whole plots that was split into 5 m×5 m sub-plots representing the four nitrogen rates.

In 2009, 5–7 years after planting, above-ground biomass samples were collected from all seven locations prior to senescence on October 23–26 and again after completion of senescence and dry-down of the crop on December 4–7. Sampling followed the methods outlined by Heaton et al. [27]. Briefly, all standing biomass (i.e., stems and leaves) were harvested as a bulk biomass subsample from a quadrat of 0.19 m^2 to a 5-cm stubble height in each treatment sub-plot (i.e., 5 by 5 meter nitrogen sub-plot within each *M.*×*giganteus* whole plot). This was to mimic commercial harvesting which is based on hay cutting and harvesting which could not separate dead stem and leaf material [58]. Hence, analysis of the resulting bulk material as would be provided to a processing facility. There were four independent biological replications for each nitrogen treatment at each location (n=4). Samples were dried to constant mass at 74 °C. Dry tissue was then ground in a cutting mill (Cutting Mill SM 200, Retsch, Inc., Haan, Germany) fitted with a 2-mm sieve. From each

Table 1 Mixed model analysis of variance associated with *Miscanthus×giganteus* across four nitrogen fertilization rates (0, 67, 134, or 202 kg N ha^{-1}), seven locations, and two harvest dates (October and December)

		Location (L)	Nitrogen treatment (N)	Harvest month (M)	L*N	L*M	N*M	L*N*M
Lignin	Numerator DF	6	3	1	18	6	3	18
Cellulose (glucan)	Den DF	21.4	141	141	141	141	141	141
	F value	5.98	9.69	239.93	2.64	1.02	0.22	0.78
	P value	*0.0009*	*<.0001*	*<.0001*	*0.0008*	0.4148	0.8842	0.7245
Hemicellulose (arabinan+xylan)	Den DF	21	141	140	141	140	141	141
	F value	2.25	2.13	341.38	1.1	0.66	1.55	0.85
	P value	*0.0782*	*0.0989*	*<.0001*	0.3604	0.683	0.2042	0.6358
Holocellulose/lignin	Den DF	20.8	141	140	140	140	141	140
	F value	13.85	16.21	33.51	1.3	5.38	0.76	0.78
	P value	*<.0001*	*<.0001*	*<.0001*	*0.1957*	*<.0001*	0.5182	0.7212
Acetyl	Den DF	21.2	141	141	141	141	141	141
	F value	12.47	18.83	24.48	2.88	2.17	0.3	0.75
	P value	*<.0001*	*<.0001*	*<.0001*	*0.0002*	*0.0496*	0.8284	0.7506
Ash	Den DF	21.5	142	141	142	141	142	142
	F value	12.34	3.37	483.57	1.23	7.93	2.74	1.09
	P value	*<.0001*	*0.0203*	*<.0001*	0.2418	*<.0001*	*0.0459*	0.3648
Extractables	Den DF	21.9	140	140	140	140	140	140
	F value	7.75	7.24	131.44	1.3	2.64	2.34	0.58
	P value	*0.0002*	*0.0002*	*<.0001*	0.1995	*0.0187*	*0.0761*	0.9074
Theoretical ethanol conversion factor	Den DF	21.6	142	141	142	141	142	142
	F value	12.48	1.05	1284.98	1.72	6.22	1.43	0.41
	P value	*<.0001*	0.3728	*<.0001*	*0.0422*	*<.0001*	0.2356	0.9839
Biomass yield	Den DF	20.7	141	141	141	141	141	141
	F value	13.39	3.35	881.66	881.66	8.56	1.13	0.45
	P value	*<.0001*	*0.0209*	*<.0001*	*<.0001*	*<.0001*	0.3391	0.9726
Ethanol yield	Den DF	21	63	–	63	–	–	–
	F value	7.47	2.38	–	1.08	–	–	–
	P value	*0.0002*	*0.0783*	–	0.3876	–	–	–
	Den DF	20.9	62.3	–	62.3	–	–	–
	F value	7.23	2.3	–	1.11	–	–	–
	P value	*0.0003*	*0.0861*	–	0.363	–	–	–

Values in italics are significant at $\alpha=0.1$

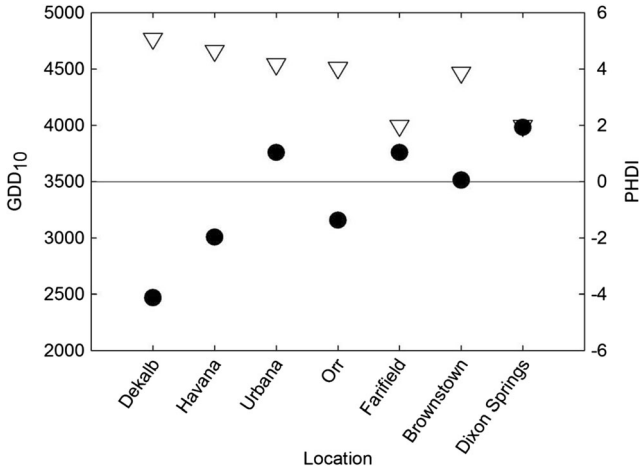

Fig. 1 Cumulative annual (Jan 1–Dec 31) GDD base 10 °C (GDD_{10}) (*filled circle*) and Palmer Hydrological Drought Index (PHDI) (*inverted triangle*) in 2009 from seven monitoring stations of the Midwestern Regional Climate Center (http://mrcc.isws.illinois.edu) near field stations planted in 2002 located at **a)** DeKalb, **b)** Urbana, **c)** Dixon Springs, and planted in 2004 **d)** Havana, **e)** Orr, **f)** Brownstown, and **g)** Fairfield. *Solid line* indicates "0" value of PHDI

biological replicate, approximately 50 g of ground material was subsampled for compositional analysis.

Growing Conditions

Soil descriptions, previous cropping history, and previous plot maintenance were described previously ([2, 17, 27], Supplemental Table 1). Weather data were obtained from NOAA's Midwestern Regional Climate Center's Applied Climate System (http://mrcc.isws.illinois.edu; Fig. 1) and reported from the nearest weather station to each field trial. These were all within 1 km, except for Dixon Springs, where the nearest weather station with data for >90 % of the relevant period covered was 34 km away (Supplementary Table 2). The Palmer Hydrological Drought Index (PHDI) provides an integrated measure of water availability reflecting soil moisture throughout the entire year [30, 49] and is therefore more closely related to crop growth than precipitation. This measure ranges from −6 for the driest conditions to +6 for the wettest, with 0 as optimal for crop growth in a given climate zone. It is reported for most crop-producing areas of the USA [49] and so was readily available for each site. The PHDI was therefore used in this study to account for variation in moisture availability between sites in the given year. Growing degree days provide an integration of heat sufficient to support crop production (GDD_1); this was calculated for each site as:

$$GDD_{10} = \sum \left(T_{avg} - 10\,^{\circ}C\right)$$

where T_{avg} is mean daily temperature and GDD_{10} is 0 on days where $T_{avg} \leq 10$ °C.

Compositional Analysis

Seven locations with four whole plots per location and four nitrogen sub-plots per whole plot and sampled on the two dates given above resulted in the 224 biomass samples analyzed. From each biomass sample, a subsample of 50 g was taken to provide material for two technical replicates, giving a total of 448 measurements. Compositional analysis was performed via wet chemistry following the methods described by Sluiter et al. [53] and Haffner et al. [24].

M.×giganteus was ground using a high-speed rotor mill (Ultra Centrifugal Mill ZM 200, Retsch) passing a 2-mm sieve and then oven-dried. In an extraction cell, 1 g of biomass and a preweighed microfiber filter (Dionex, Sunnyvale, CA, USA) were extracted with water and ethanol in an accelerated solvent extractor (ASE350, Dionex). The extraction conditions were 100 °C temperature, 5 min holding time, 3 cycles per solvent, and a 60-s nitrogen purge. The biomass after removal of soluble components was transferred into preweighed aluminum pans and dried and cooled in a desiccator. The difference of starting mass and mass after extraction provided the mass of extractables, i.e., that dissolved by the solvent.

The remaining biomass was pulverized in a canister ball-mill (model 8200 tissue pulverizer, Kinetic Laboratory Equipment Company, Visalia, CA, USA) and oven-dried. Fifty milligram was incubated at room temperature with 0.5 cm^3 of 72 % (*w*/*w*) sulfuric acid in a modified Hungate vial capped with a rubber stopper; the mixture was vortexed every 15 min. After 1 h, deionized water was added, and the mixture was autoclaved for 60 min. A sugar recovery standard containing glucose, xylose, and arabinose with the same sulfuric acid concentration was prepared in parallel and co-autoclaved with the samples. After cooling to room temperature, the suspension was vigorously shaken, kept at 4 °C for 12 h to precipitate the solids. Two cubic centimeters of the clear supernatant was then removed and filtered and used for HPLC analysis.

The precipitated solids were re-suspended by vortexing, and the suspension was filtered through a glass microfilter. Both the vial and filter were repeatedly rinsed with deionized water and then dried; the residual mass (m_1) was determined after cooling in a desiccator. The filter and solids were then heated to 575 °C, and the residual mass or ash (m_2) was determined after cooling in a desiccator for 30 min. The difference $m_1 - m_2$ represented the amount of Klason lignin corrected for ash content.

Cellulose (as glucan), hemicellulose (as xylan and arabinan), and acetyl contents were determined at 50 °C by HPLC on an organic acid separation column (Aminex HPX-87H, 300×7.8 mm, Bio Rad, Richmond, CA on a 1200 series HPLC) with a refractive index detector (Agilent Technologies, Santa Clara, CA). Elution was performed with 0.005 M sulfuric acid at a flow rate of 0.6 cm^3 min^{-1}. For calibration,

solutions of reference compounds in the range of 0.01−10 mg were prepared.

Calculation of Theoretical Ethanol Production

The US Department of Energy, Energy Efficiency and Renewable Energy "Theoretical Ethanol Yield Calculator" [15] was used to calculate theoretical ethanol production based on measured glucan, xylan, and arabinan concentrations of the biomass samples. The theoretical ethanol conversion factor was calculated as:

$$\frac{1\,\text{Ethanol}}{\text{Mg dry biomass}} = \frac{1.11\,\text{kg of C6 sugar}}{1\,\text{kg of C6 polymeric sugar}} \times (\text{glucan}\,\%) + \frac{1.136\,\text{kg of C5 sugar}}{1\,\text{kg of C5 polymeric sugar}} \times (\text{xylan}\,\% + \text{arabinan}\,\%) \times \frac{0.51\,\text{kg ethanol}}{1\,\text{kg sugar}} \times \frac{1000\,\text{kg ethanol}}{\text{Mg C6 or C5 polymeric sugar}} \times \frac{3.785\,\text{l ethanol}}{2.971\,\text{kg of ethanol}}$$

The product of the theoretical ethanol conversion factor (l Mg^{-1}) and biomass yield (Mg ha^{-1}) gives the overall ethanol yield per unit land area.

Statistical Analysis

The mean value of the two technical replications of each biological replication was treated as a single experimental sample for statistical analysis (n=4). Each factor was evaluated individually via split-plot mixed model analysis of variance (ANOVA) in the SAS statistical software package (PROC MIXED, SAS Institute Inc., Cary, NC, USA [42]). That is, a separate analysis was performed for each of cellulose, hemicellulose, lignin, H/L, acetyl, ash, total extractables, ethanol conversion factor, biomass yield, and ethanol yield, each in turn represented as y. Location (L) and harvest month (M) were considered categorical fixed effects and nitrogen treatment (N), a continuous fixed effect, while plot effect at each location was considered random, as follows.

Model I:

$$y = L + M + N + L*M + L*N + M*N + L*M*N + L(P)$$

Given the relatively small sample size and given that this experiment was performed only over 1 year, statistical significance is reported at α<0.1 to minimize type II errors, i.e., risk of accepting the null hypothesis when it is in fact false. In all displayed figures, arithmetic means with 1 standard error of the mean are shown.

Results

Site Conditions

Relative to the average, 2009 was a wet and cool year for Illinois [1]. It was the 11th coolest and 8th wettest summer in Illinois since weather records began in 1895 [1]. However, the Annual Palmer hydrological drought index (PHDI), a measure of drought impact on crops, ranged from −4 in DeKalb at the northern end of Illinois to 2 in Dixon Springs at the southern end, while growing degree days (GDD_{10}) ranged from 4000 to 4800 across the seven sites, representing very considerable variation, 20 % variation in heat and 30 % in available moisture between sites (Fig. 1).

Cell Wall Composition

Although many statistically significant differences were detected, their magnitude is small due to the low variance. Response to nitrogen was location dependent for lignin, extractables, H/L, and ethanol yield (Table 1). While this interaction was statistically significant, Table 2 shows the very small magnitude of the effects due to this interaction. Nitrogen fertilization led to statistically significant variation in the proportion of all components except extractables (Tables 1 and 2). However, all changes were small and almost all were under 2 % (Tables 1 and 2). When pooled across locations, increasing the nitrogen fertilization rate decreased the proportion of acetyl, ash, hemicellulose, and theoretical ethanol conversion factor and increased lignin, biomass, and theoretical ethanol yield (Table 2). Response to nitrogen was also dependent on harvest month for the theoretical ethanol conversion factor and proportions of acetyl and ash (Tables 1 and 2).

Harvest date had the largest individual impact on composition, as indicated by the higher F values (Table 1, Fig. 2). However, the overall changes while significant were again small. Between October and December changes, these were just +3 % in cellulose, +1.5 % in lignin, with decreases in hemicellulose, and a halving of ash and extractable contents (Table 2, Fig. 2). There was a statistically significant interaction of time of harvest with location for hemicellulose, ash, acetyl, and extractables and a statistically significant interaction of time of harvest with nitrogen treatment for ash and acetyl contents (Table 1, Fig. 2). Location had a small, yet statistically significant, impact on the proportion of lignin, cellulose (glucan), total hemicellulose (xylan+arabinan), hollocellulose/lignin ratio (H:L), acetyl, ash, and extractables, as well as the ethanol conversion factor, biomass yield, and overall ethanol yield (Table 1).

Table 2 Proportion (% total mass) of (a) lignin, (b) cellulose, and (c) hemicellulose of unfertilized *Miscanthus×giganteus* across seven locations in Illinois, four nitrogen fertilization rates (0, 67, 134, and 202 kg N ha^{-1}), and two harvest dates

Date - kg/N ha^{-1}	DeKalb		Havana		Urbana		Orr		Fairfield		Browns town		Dixon Springs		Pooled	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
a) Lignin																
Oct - 0	18.50	0.70	20.35	0.61	20.08	0.43	19.30	0.39	19.52	0.76	18.76	0.50	18.76	0.08	19.29	0.22
Dec - 0	19.50	0.35	21.56	0.23	22.57	0.11	21.20	0.42	21.60	0.29	20.15	0.54	20.21	0.27	20.97	0.22
Oct - 67	20.09	0.24	19.21	0.27	21.06	0.37	19.66	0.35	19.86	0.52	19.31	0.49	19.35	0.18	19.69	0.16
Dec - 67	20.93	0.29	20.80	0.42	22.68	0.25	21.22	0.29	21.83	0.25	21.57	0.34	20.33	0.24	21.32	0.17
Oct - 134	19.81	0.58	20.12	0.55	21.06	0.66	20.54	0.48	19.98	0.59	19.79	0.31	19.10	0.61	20.03	0.22
Dec - 134	21.48	0.33	21.50	0.17	22.53	0.10	21.58	0.33	21.34	0.32	21.10	0.30	21.18	0.26	21.53	0.12
Oct - 202	20.63	0.35	19.80	0.41	20.56	0.35	20.26	0.26	19.93	0.40	19.80	0.52	19.64	0.46	20.10	0.15
Dec - 202	21.17	0.14	21.36	0.34	21.63	0.60	21.69	0.18	21.79	0.17	21.30	0.32	21.65	0.11	21.51	0.11
Oct - Pooled	19.76	0.30	19.84	0.24	20.60	0.24	19.94	0.21	19.82	0.26	19.39	0.24	19.21	0.19	19.77	0.10
Dec - Pooled	20.77	0.23	21.30	0.16	22.35	0.18	21.42	0.15	21.62	0.13	21.03	0.22	20.84	0.19	21.33	0.08
b) Cellulose																
Oct - 0	38.89	0.98	41.58	0.93	40.37	0.77	40.44	0.71	40.43	1.67	39.59	0.94	40.35	0.36	40.19	0.36
Dec - 0	41.57	0.78	43.55	0.34	44.41	0.22	43.45	0.38	44.36	0.60	42.07	0.75	44.27	0.39	43.38	0.27
Oct - 67	40.13	0.40	39.90	0.32	41.91	0.04	41.24	0.58	39.97	0.78	39.79	1.06	41.46	0.46	40.53	0.26
Dec - 67	43.60	0.47	43.48	0.48	44.16	0.36	43.43	0.19	43.83	0.25	43.62	0.35	44.28	0.18	43.77	0.13
Oct - 134	40.04	0.82	40.50	0.87	41.49	0.81	41.42	0.56	40.89	0.66	40.97	0.65	40.77	1.13	40.84	0.29
Dec - 134	43.43	0.43	43.26	0.11	43.19	0.54	43.16	0.42	43.18	0.60	42.59	0.38	44.08	0.18	43.27	0.16
Oct - 202	41.32	0.46	39.95	0.79	41.59	0.73	41.24	0.28	41.15	0.56	40.63	0.57	41.57	0.71	41.11	0.22
Dec - 202	42.72	0.39	43.40	0.28	43.78	0.23	43.81	0.28	43.92	0.35	43.03	0.59	44.46	0.16	43.59	0.16
Oct - Pooled	40.09	0.39	40.44	0.37	41.24	0.38	41.08	0.27	40.61	0.47	40.20	0.41	41.03	0.35	40.66	0.15
Dec - Pooled	42.83	0.32	43.42	0.15	43.88	0.20	43.46	0.16	43.82	0.25	42.83	0.28	44.27	0.12	43.50	0.09
c) Hemicellulose																
Oct - 0	22.13	0.48	21.75	0.40	20.66	0.64	22.60	0.62	21.04	0.71	23.31	0.50	21.76	0.07	21.90	0.24
Dec - 0	23.17	0.44	22.02	0.24	20.52	0.42	21.90	0.31	21.43	0.37	23.70	0.78	22.88	0.34	22.23	0.24
Oct - 67	21.05	0.25	22.08	0.35	19.83	0.25	21.82	0.46	20.86	0.28	22.70	0.43	20.71	0.09	21.40	0.20
Dec - 67	21.77	0.38	22.00	0.27	20.48	0.30	21.91	0.31	21.84	0.32	22.42	0.37	22.42	0.30	21.83	0.16
Oct - 134	21.00	0.63	21.91	0.23	19.74	0.34	21.36	0.26	20.35	0.21	22.33	0.37	21.07	0.59	21.11	0.21
Dec - 134	21.62	0.41	22.23	0.20	20.60	0.37	21.64	0.23	21.90	0.39	23.01	0.53	22.00	0.25	21.86	0.18
Oct - 202	19.97	0.48	22.03	0.03	18.97	0.48	21.75	0.47	20.12	0.65	22.39	0.50	20.07	0.27	20.71	0.28
Dec - 202	21.77	0.13	21.79	0.22	20.12	0.27	21.17	0.07	21.56	0.32	22.23	0.61	21.49	0.16	21.45	0.16
Oct - Pooled	21.04	0.29	21.95	0.14	19.80	0.30	21.88	0.24	20.59	0.25	22.71	0.23	20.90	0.22	21.28	0.12
Dec - Pooled	22.08	0.23	22.01	0.11	20.43	0.16	21.65	0.14	21.67	0.17	22.84	0.30	22.20	0.18	21.84	0.10

Discussion

The purpose of this study was to determine if there would be variation in the composition of biomass fed to a processing plant from the same genotype grown at different locations, with different soils, weather, nitrogen fertilization levels, and dates of harvest within a supply region. The findings suggest surprisingly little variation, indicating with respect to genotype–environment interactions (G×E); the environmental component is very small. These trials used a single, but very widely used, clone of *M.×giganteus*. Nitrogen fertilization rate, time of harvest, and location led to statistically significant, but very small, changes in cell wall composition. Based on the measured cellulose and hemicellulose content, it was possible to calculate variation in the potential ethanol yield. However, when this chemical conversion factor is considered in conjunction with overall biomass yield ($tDMha^{-1}$), it is clear that the influence of nitrogen application rate, time of harvest, and location on ethanol yield is predominantly through the effect on the quantity of biomass produced with very little effect on quality.

The lack of change in the proportion of hemicellulose with autumn versus winter harvest in the present study is in disagreement with previous studies which reported a decline in hemicellulose ([32–34]; Table 3). This apparent disagreement

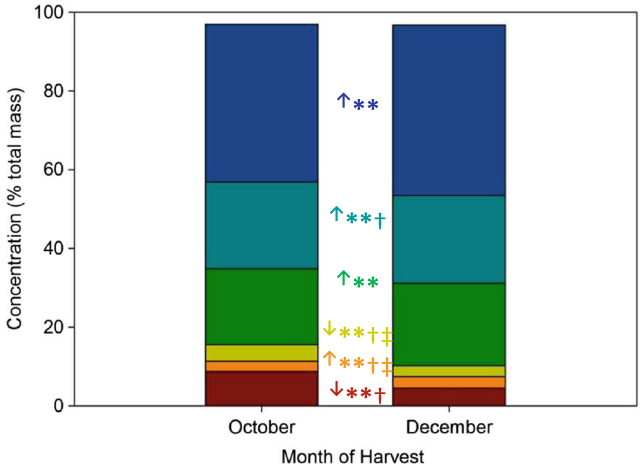

Fig. 2 Changes in proportion (% of total mass) of extractables (●), acetyl (●), ash (●), lignin (●), hemicellulose (●), and cellulose (●) by month of harvest (October and December) in unfertilized *Miscanthus×giganteus* pooled across seven locations in Illinois in 2009. *Arrows* indicate direction of change in concentration of component from October to December. *Double asterisks* significant difference between months at $\alpha < 0.05$. *Dagger* significant interaction effect of month of harvest with location. *Double dagger* significant interaction effect of month of harvest with nitrogen fertilization rate

regarding changes in hemicellulose content is likely driven by differences in the proportion of leaf loss in this timeframe and earlier dates of harvest necessitated by climatic conditions in the US Midwest. The present study simulated mechanical harvesting with a hay cutter set at about 5 cm to avoid collecting any surface litter which may be contaminated with soil, as recommended to US growers [58]. This method at farm level could not practically separate stems and leaves, and our analysis was limited to the bulk that would be collected regardless of the proportion of stems and leaves. The observed increase in cellulose and lignin concentrations from October to December parallels previous findings ([26, 32–34], Table 1). The major change from October to December was an approximate halving of contents of extractables and ash (Fig. 2). This is most likely due to leaf drop and, therefore, a smaller proportion of the harvested mass being leaf material [28, 26]. Cuticle waxes are the major component of extractables, and with a much larger surface area to total mass, cuticle waxes are a larger proportion of the mass of leaves. Leaves similarly have higher ash content [7]. Decreases in inorganic content from October to December agree with the results of previous trials that found decreases in ash throughout the growing season [26, 34, 41]. The decline observed here in the autumn could result from re-translocation and from physical leaching of soluble ash components, as well as a decreased proportion of leaves in the harvested material [6, 28, 54]. These results furthermore suggest that a later harvest would result in yet further reductions in ash content. Current agronomic advice in Europe is to delay biomass harvest until late winter or early spring in order to reduce concentrations of K, Cl, S, N, and P

Table 3 Composition of *Miscanthus×giganteus* biomass harvested prior to senescence and after completion of senescence in unfertilized plots and reported in the peer-reviewed literature and the present study

	Lignin		Hemicellulose		Cellulose		Ash	
	Pre-senescence	Post-senescence	Pre-senescence	Post-senescence	Pre-senescence	Post-senescence	Pre-senescence	Post-senescence
de Vrije et al. 2002	n/a	25.0	n/a	24.3	n/a	38.2	n/a	2.0
Magdid et al. 2004	13.3	n/a	26.6	n/a	41.9	n/a	3.1	n/a
Hodgson et al. [32, 33]	8.89	10.59	25.76	24.93	42.74	48.4	n/a	n/a
Hodgson et al. [32, 33]	11.40	14.09	26.17	23.98	46.88	54.02	n/a	n/a
Hodgson et al. [34]	12.02	12.58	24.83	25.76	50.34	52.13	n/a	n/a
Lygin et al. [44]	n/a	27.68	n/a	34.84	n/a	44.04	n/a	n/a
Present study	19.29	20.97	21.9	22.23	40.19	43.38	3.65	2.53

which can negatively impact combustion of the feedstock [39, 40]. However, under US Midwest conditions, large losses in harvestable biomass occur in the winter, which were not apparent under western European conditions. This might be explained by fragmentation of the standing biomass under harsher freezing conditions [5, 6, 28]).

Location had a strong statistical effect, but small absolute effect, on overall cell wall composition (Table 1). Given that harvest occurred following the seventh growing season at three of the locations and following the fifth growing season at the remaining four locations, one aspect that could be driving location-dependent changes is stand age. However, no differences in composition were apparent between stands in their fifth and seventh year. Previous studies in Europe have similarly shown that cell wall composition is little affected by growing seasons or age of stand [34]. Harvest method also affects end-use value [46]. Only one harvest method was used here, and as explained earlier, this was designed to mimic the cutting of the whole stem, as in hay collection, which is the method used predominantly in the USA [29, 58].

Effects of location are more likely to be due to differences in baseline soil fertility, as well as precipitation and temperature patterns (Fig. 1). The lignin content of material from the driest site, Fairfield, was less than 1 % more than the wettest site, DeKalb (Table 2). Other site factors similarly appeared to have little effect (Table 2). Nitrogen fertilization had remarkably little effect on cell wall constituents; neither lignin, cellulose, nor hemicellulose showed a more than 1 % variation between the four treatments within either of the two harvest dates (Table 2). The average increase in lignin content with increasing N-fertilization rate was 0.75 %. Despite this small change, the low variance meant that this was a statistically significant increase. Similarly, acetyl and ash contents showed a significant decrease with increased nitrogen fertilization rate, but again, the changes were very small. When comparing locations to each other, the location with the highest land capability class, Urbana, which is a class "1" soil ([59, 60] Supplementary Table 2) and by definition is well-supplied with plant nutrients, showed the highest concentrations of lignin and the lowest concentrations of hemicellulose relative to the other locations (Table 2). However, DeKalb, which is a land capability class "2w" soil and has only minor limitations as compared to Havana (4 s) or Orr (3e, Supplementary Table 2), showed the lowest observed concentrations of lignin. This suggests that it is not only nitrogen availability which is driving these small differences.

The ethanol conversion factor is calculated as a theoretical maximum and does not take into account the inhibitory effects of lignin, ash, and acetyl, nor does it include possible additional ethanol produced from non-structural carbohydrates in the extractables which may also include precursors for value-added co-products. Specifically, lignin contributes to the recalcitrance of a feedstock and increasing lignin concentrations increase the cost of conversion of cellulose and hemicellulose by reducing the efficiency of the process [9]. The (cellulose+hemicellulose)/lignin ratio across locations ranged significantly from 2.88 (±0.03) to 3.32 (±0.04), a 15 % change, in unfertilized plots harvested in December. This ratio was seen to decline with increasing nitrogen fertilization rates, suggesting that as nitrogen fertilization increases, it will likely become more difficult to digest the feedstock (Tables 1 and 2), so while nitrogen fertilization increased biomass yield, this may be slightly offset by the small decline in quality with respect to ethanol production. Likewise, while yield was greater at the December harvest, this was partially offset by a decline in quality with respect to ethanol production. Biomass harvested in October has a 2.5 % higher (cellulose+hemicellulose)/lignin ratio 3.22 (±0.03) than biomass harvested in December 3.14 (±0.04). However, biomass harvested in October would be of a higher percent moisture and nitrogen concentration than when harvested in December [28], which would need to be adjusted for in the conversion process. An October harvest may also affect nitrogen use since the N offtake at this time of year is very much higher than in December [28, 16]. Moisture content was not considered in the present study since it was highly dependent on the conditions on the day of collection and would not provide a realistic average for the two harvest periods. The ethanol conversion factor does not take account of the adverse effect of ash content [8]. Ash content was halved by the later harvest which could offset the effect of the increased lignin content (Fig. 2).

In total, this study analyzed the chemical composition of almost 500 samples of a single genotype grown at seven contrasting sites under four nitrogen treatments and on two harvest dates. The results are most notable for showing that while environment strongly affects the quantity of biomass, it has little numerical effect on quality. This is a positive finding from the viewpoint of processing to biofuels or use in combustion. Large changes in feedstock composition would, for example, in the case of cellulosic ethanol production, require continual modifications to processing operations such as resetting of flows, adjustment of pretreatment conditions, and quantities of digestion enzymes. Any necessary changes would cause not only increased operational complexity but would also drive increased capital costs and operational expenditures. Although several statistically significant differences in composition were found between locations, nitrogen treatment, and harvest date, none of these changes were large in magnitude. The remarkable consistency of *M.*×*giganteus* composition across different soil types, nitrogen fertilization regimes, and harvest times means accommodating for variation within only a few percentage points rather than larger changes. This suggests that if the same clone of *M.*×*giganteus* is used at diverse sites and harvest dates within a region supplying a processing operation, a stable composition will be delivered.

Acknowledgments This work was funded by the Energy Biosciences Institute. Special thanks to Noel Piatek, Ben Lewis, Ethan Kessler, Mike Hradek, Ariel Raneiri, Liz Vonderhaar, Neal Makela, John Jurveis, and Keith Chan for their help in collecting and processing samples.

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