**The effects of harvest regime, irrigation, and salinity on stem
lignocellulose concentrations in alfalfa (*Medicago sativa* L.)**

Adam H. Warnke and Christopher T. Ruhland*

Department of Biological Sciences, Minnesota State University, Mankato, MN 56001 USA

*Corresponding author: Christopher T. Ruhland, Department of Biological Sciences, TS-
242 Trafton Sciences Center, Minnesota State University,
Mankato, MN 56001, USA

Email: christopher.ruhland@mnsu.edu

Phone: +1 507 389-1323

Fax: +1 507 389-2788

ABSTRACT

Alfalfa (*Medicago sativa* L.) is a potential candidate for cellulosic ethanol production due to its high biomass, perennial-habit, relationship with nitrogen-fixing bacteria, and other co-products. We examined the effects of harvest regime, irrigation, and salinity on stem lignocellulose concentrations in alfalfa during the 2010 and 2011 growing seasons in Southern Minnesota. Stem cellulose, hemicellulose and lignin concentrations, and theoretical ethanol yields were examined in eight alfalfa cultivars with full-bud and 50%-flower harvest regimes, irrigation, and salinity as applied treatments. Plants received weekly applications of (1) 1.27 cm of well water ("fresh water," 0.75 dS m⁻¹), (2) 1.27 cm of saline water (NaCl; "brackish water"; 5.0 dS m⁻¹) or (3) ambient precipitation ("rainfed"). Holocellulose concentrations reached the highest values during the full bud (2010) and 50% flower (2011) harvest regimes with concentrations averaging 45%. Theoretical ethanol yields were generally higher for the 50%-flower harvest regime, suggesting the longer growth period increased holocellulose concentrations while not being hindered by more lignin in older stems. Alfalfa growing under brackish-water treatments had 1.3 to 6.1% more holocellulose than those receiving irrigation or ambient precipitation over two growing seasons. Lignin concentrations across all treatments were almost 23% lower during the second growing season. Interestingly, plants growing under brackish-water treatments had higher holocellulose to lignin ratios and higher theoretical ethanol yields during both field seasons suggesting that moderate levels of salt may stimulate holocellulose concentrations.

40 keywords: alfalfa, biofuel, cellulose, feedstocks, hemicelluloses, holocellulose, lignin

41

42 abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; ANOVA, analysis of
43 variance; DRI, disease and pest resistance index; FD, fall dormancy; NDF, neutral detergent
44 fiber; WSI, winter survival index

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1. Introduction

At the current rate of consumption, world crude oil reserves are predicted to deplete in approximately 40 years, hence, it has become essential to find methods of processing renewable and sustainable raw materials for conversion into fuel (Maheshwari, 2008). Increasing global population has further magnified this necessity. Shifting society's reliance away from petroleum to renewable biomass resources is viewed as an important contribution to the development of a sustainable industrial society and to an effective management of greenhouse gas emissions (Rugauska et al., 2006). Biofuels have been recognized as a renewable, cost-effective alternative to petroleum-based liquid fuels. The starch-based ethanol industry has grown very rapidly in the United States, however, most experts see the need for the development of a lignocellulose-based biofuels industry to meet the current Federal Biofuels Mandate for displacing 30% of petroleum consumption by 2030 (McCaslin and Miller, 2007).

A major source for biofuel comes from polysaccharides created by the photosynthetic process. These polysaccharides can be divided into two major groups: starch, a storage polymer, consisting of glucose monomers with α (1 $\rightarrow$ 4) and α (1 $\rightarrow$ 6) glycoside linkages, and cellulose, a structural polymer, consisting of glucose monomers with β (1 $\rightarrow$ 4) glycoside linkages. In addition to cellulose, plant secondary cell walls also contain appreciable amounts of lignin. Lignin is a complex phenolic polymer that is closely linked to polysaccharides in the cell wall and hinders the degradation of these polysaccharides to simple sugars, which is required for fermentation to ethanol (Chapple et al., 2007).

Ethanol production from plant-produced polysaccharides has been commercialized using starch from corn grains. The starch in corn kernels is much easier to break down than cellulose

and hemicelluloses (“holocellulose”) in the cell wall of biomass material. In this sense corn starch is converted to glucose and fermented to produce ethanol. However, there are several economic problems associated with the production of ethanol from corn grain. The increased demand for corn may impact the world’s food stock and possibly drive up the prices of corn-based products. In addition, the large amounts of fossil fuels used to process starch-based ethanol are expensive and release greenhouse gases. The vision of a future bio-based industry includes the simultaneous production of biofuels, bioelectricity, and bioproducts using not only corn grain and soybean oil, but also a host of renewable lignocellulose-based feedstock (Walsh et al., 2007). Lignocellulose-based ethanol is particularly promising because it can take advantage of biotechnology to dramatically reduce costs, is derived from low-cost and abundant feedstocks, can achieve high yields, and is typically environmentally friendly (Wyman, 2007). However, there are problems with commercializing lignocellulose-based ethanol due to the high initial capital cost. Separating cellulose from lignin during processing is costly and produces potentially harmful bi-products.

Corn stover and cobs, as well as wheat straw are obvious annual crop residue feedstocks for lignocellulose ethanol production. Switchgrass (*Panicum virgatum*), a native C4 perennial forage grass, is often mentioned as a leading perennial energy crop candidate. Drought tolerance, low fertility requirements, and the ability to grow on marginal soils will likely make switchgrass an important component in a biofuel cropping system in some regions (McCaslin and Miller, 2007). Ultimately, identifying plants that have high holocellulose to lignin ratio is an essential step when determining what species are best suited for ethanol production. This

study will focus on the use of alfalfa (*Medicago sativa*) as a potential crop in a biofuels production system.

Alfalfa has promise as a feedstock for production of ethanol and other industrial materials because of its high biomass yields, perennial-habit, relationship with nitrogen-fixing bacteria and other valuable co-products (Jung and Engels, 2002). It grows on soils with a pH level of 6.5-7.0 and adequate levels of P (60 to 100 kg ha⁻¹) and K (180 to 250 kg ha⁻¹) are optimal for subsequent years of production (McKenzie, 2005). Varietal selection of alfalfa is typically based upon the winter survival index (WSI), fall dormancy (FD), and disease and pest resistance index (DRI). Alfalfa cultivars with lower WSI ratings will have the ability to survive potentially harsh winters. Fall dormancy is the measure of how tall an alfalfa plant grows after the last cutting and before going dormant for the season. The DRI is based upon selecting cultivars with superior disease resistance to ensure a long productive stand. The WSI rating is very important in Minnesota due to potentially harsh winters. A cultivar that can withstand severe low temperatures is crucial when selecting alfalfa.

Alfalfa can be harvested for biomass in the year of planting and provides N to the soil for use by subsequent cereal crops in rotation (Sheaffer et al., 2000). The growth stages of alfalfa are well known in this sense, harvest schedules for leaf protein are determined upon them for ruminant livestock feed. Typical harvest schedules produce three to four cutting per growing season. An advantage of using alfalfa for lignocellulose biofuel production, compared to other crops, is the ability to easily separate leaves and stems to produce co-products (Samac et al., 2006). Alfalfa leaves typically have two to three times the crude protein of the stems while stems typically have two to three times the crude fiber of the leaves (Shinners et al., 2007). The

high protein leaf portion could be utilized as an animal feed, while the high lignocellulose stem portion could be used as a biofuel feedstock (McCaslin and Miller, 2007).

Using alfalfa for biofuels would require research to determine the optimal holocellulose to lignin ratio for ethanol production. Recommended harvest schedules for modern alfalfa cultivars in a lignocellulose biofuel system are unknown because the comparative value of leaf and stem components is likely to vary with energy consumption and livestock feed prices (Sheaffer et al., 2000). Based on previous research (Lamb et al., 2007), mature alfalfa stems had higher concentrations of lignocellulose on a seasonal, yield adjusted basis under the biomass management system than the hay system. Typically as alfalfa ages, the stems become more lignified and have lower cellulose concentrations (Sanderson and Wedin, 1988). Previous research has focused on plant density along with harvest intervals. In this study, we focused on harvest intervals across the same plant density. Determining the optimal harvest schedule for protein and lignocellulose concentrations will be a vital step for the future of alfalfa as a biofuel feedstock. However, to achieve maximum biomass yields of alfalfa, irrigation may be needed in several productive regions of the world.

Crop yield depends on the amount of irrigation water and its distribution (Montazar and Sadeghi, 2008). Alfalfa has a high water requirement compared to other commonly grown crops because it has a long growing season, a deep root system, and high biomass yields (Krogman and Hobbs, 1965; Bauder et al., 1992). Drought stress on alfalfa can inhibit cell elongation, reduce photosynthesis, interfere with nutrient uptake, and alter plant regulators (Saeed and El-Nadi, 1997). Saeed and El-Nadi (1997) observed that alfalfa stem density, stem height and leaf size decreased when soil water deficits occurred.

Root and shoot growth in alfalfa is restricted by increased salinity (Esechie et al., 2002), presumably due to changes in water uptake (Munns et al., 2006) and salt toxicity (Munns, 2002) resulting in reductions in growth and yield (Allakhverdiev et al., 2000). For long-term productivity, perennial crops such as alfalfa must be able to adapt to increasing heterogeneous root zone salinity (Vaughan et al., 2002). The relationship between alfalfa growth and water use, under an irrigated system, is very important in determining the effects of salinity on stem lignocellulose concentrations.

The purpose of this study was to analyze the effects of harvest regime, irrigation, and salinity on stem lignocellulose concentrations in alfalfa. Irrigation and salinity are both factors that affect plant growth and there is little data on how they affect stem lignocellulose concentrations. In combination with a harvest schedule these two factors should provide valuable information for alfalfa's potential as a biofuel feedstock.

2. Materials and methods

2.1 Plot establishment and cultivar selection

The field experiment was conducted during three growing seasons (2009 - 2011) on an agricultural field located 2.5 miles west of Geneva, Minnesota (43°81'N × 93°32'W). The soil at this location is a Webster Clay Loam-113 (Carlson et al., 1980) and has a pH of 6.5. Phosphorous and potassium concentrations averaged ≥ 65 and ≥ 190 kg ha⁻¹, respectively (data not shown). Precipitation was collected by a rain gauge at the field site. The site was tilled using a 3.05-m field cultivator at a depth of 0.10 to 0.15 m. Alfalfa cultivars were planted using a 2.0-m Tye drill with 0.2-m row spacing.

Eight cultivars, two harvest regimes (full-bud and 50%-flower), and irrigation/salinity regimes (irrigated with fresh water, brackish water and rainfed) were compared. We selected cultivars of alfalfa based upon the winter survival index (WSI), fall dormancy (FD), and disease and pest resistance (DRI). The eight cultivars used in this study included: 1-WL 363HQ: *Waterman-Loomis Seed Company* (WSI-1.6 and FD-4.8), 2-Viking 357: *Viking Seeds* (WSI-2.5 and FD-3.4), 3-L447HD: *Wolf River Valley Seeds* (WSI-2.0 and FD-3.7), 4-Enforcer: *Allied Seed* (WSI-2.2 and FD-3.5), 5-Viking 3100: *Viking Seeds* (WSI-2.6 and FD-3.0), 6-Fontanelle Hybrid – Ovation 2: *Fontanelle Hybrids* (WSI-2.3 and FD-3.4), 7-Gold Country 24/7: *Gold Country Seed* (WSI-2.5 and FD-3.8), and 8-Iroquois: *Iroquois Seed* (WSI and FD-unknown). All cultivars had sufficient disease and pest resistance ratings. Cultivars 1-5 were obtained from Albert Lea Seed in Albert Lea, Minnesota. Cultivars 6 to 8 were obtained from a local dairy farmer. These cultivars are all well established in Minnesota and are suitable for this research.

A complete random block in a split-plot arrangement design was applied with two or three harvest regimes as whole plots and eight alfalfa cultivars, irrigation, and salinity treatments as subplots. There were two replicates at the experimental location (Supplementary Information A). Plots were 3.66 by 5.49 m (cultivar) and subplots were 1.83 by 3.66 m (treatment). A seeding rate of 14.6 kg ha⁻¹ resulted in stand densities for all plots $\approx$ 450 plants m⁻². Weeds were controlled with a post-emergence application of 292 mL ha⁻¹ of ammonium salt of imazethapyr (Pursuit) {(±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid}. Plots were sprayed as needed with S-Cyano (Mustang Max) [(3-phenoxyphenyl) methyl (+) cis/trans 3-(2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate] for potato leafhopper [*Empoasca fabae* (Harris)] control.

2.2 Irrigation and salinity application

Irrigation was applied using a 492-L water tank located in the back of a pickup truck. Water was supplied from a local well (pH 6.9). Applications were applied with an 18.9 L per minute pump attached by rubber hoses to a hand held sprinkler. The rain feature on the attached sprinkler was used in order to deliver a consistent application. Each subplot had 5 rain gauges (one in each corner and one in the center of the plot) to ensure accurate treatment applications. For irrigation treatments, each cultivar subplot was irrigated with either 1.27 cm (83.3 L) application of well water (“fresh water,” 0.75 dS m^{-1}) or brackish water (5.0 dS m^{-1}) every 7 to 10 days, depending on local weather patterns. Salinity measurements were performed with a conductivity meter (model: Field Scout Direct Soil EC Meter; Spectrum Technologies Inc., Aurora IL, USA) capable of measuring salts in both soil and liquid samples. In order to simulate water with high salinity, NaCl (Fisher Scientific, Pittsburgh, PA, USA) was added until an electrical conductivity of 5.0 dS m^{-1} was obtained for the brackish water treatments. Fresh- and brackish-water treatments were applied to individual subplots a total of eight and seven times for a total application of 666 and 583 L subplot⁻¹ for the 2010 and 2011 growing season, respectively. Soil salinity was also measured directly at these times using the soil conductivity meter approximately 45-60 min after treatment applications. During each survey, five replicate electrical conductivity measurements were made at random locations in each of the subplots at a depth of 5 cm directly in the rooting zone. Direct soil electrical conductivity measurements were then extrapolated to saturated-paste extract values following manufacturer guidelines.

2.3 Forage sampling and sample preparation

Forage was harvested in 2009 prior to treatment application using a sickle-bar mower (Jari Mowers, Mankato, MN, USA) at 0.5 cm above the soil line. Subplots were harvested in the second and third years of production (2010 and 2011) when alfalfa reached full-bud (>50% of stems having one or more buds) and 50%-flower (66-100% of stems having one or more flower). The full-bud regime was harvested three times per season and the 50%-flower regime was harvested twice per season. Each subplot had ten plant samples collected at each growth stage.

Herbage yields were measured by harvesting a 0.91-by-3.66 m strip of forage to a 5-cm height from the center of each plot with a hand operated sickle bar mower. At harvest, ten random subsamples were collected for analysis. Samples were placed in labeled paper bags and oven dried at 60°C. The remaining non-sampled plants were cut at 5-cm above ground level and then baled and removed from the plots. Each subsample was manually separated into leaf and stem fractions. The remaining portions of the stems were ground with a Wiley mill through a 1-mm screen in preparation for constituent analysis.

2.4 Constituent analyses

A fiber analyzer (model A200; ANKOM Technology, Macedon NY, USA) was used to estimate concentrations of cellulose, hemicellulose and lignin in dried samples following Van Soest (1991). Dried samples (0.5 g) were placed into pre-weighed filter bags and analyzed with acid detergent fiber (ADF) solution (20 g cetyl trimethylammonium bromide to 1 L 1.00N H₂SO₄) at 100°C for 60 min. Samples were rinsed with hot dH₂O and acetone, dried, and placed in a drying oven (102°C) overnight. Samples were then cooled, weighed and %ADF (cellulose + lignin) was calculated.

The second sub-sample for each treatment was then used to estimate neutral detergent fiber (NDF). Dried samples (0.5 g) were placed into pre-weighed filter bags and analyzed with NDF solution (sodium lauryl sulfate, ethylenediamine-tetraacetic disodium salt dihydrate, sodium tetraborate decahydrate, sodium phosphate dibasic, anhydrous and triethylene glycol). Heat-stable bacterial alpha amylase and sodium sulfite was added to the analyzer, afterwards samples were incubated at 100°C for 75 min. Samples were then rinsed twice with alpha amylase solution, then once in acetone and dried overnight (102°C). Samples were then cooled, weighed and %NDF (cellulose, hemicellulose + lignin) was calculated.

Acid detergent lignin (ADL) was estimated on samples used for ADF analysis. Samples were immersed in 72% H₂SO₄ for 3 h and agitated every 30 min. Samples were then rinsed in dH₂O and acetone, dried overnight (102°C) and weighed. Samples were then ashed in a muffle furnace (525°C) for 3 h, cooled and weighed. Cellulose concentrations were calculated as %ADF - %ADL, and hemicellulose concentration were calculated as %NDF - %ADF.

Theoretical ethanol yields were determined using assumed cellulose and hemicellulose conversion and fermentation efficiencies following Badger (2002). Fermentation assumptions were based on 1000 kg of dried biomass. Ethanol yields from glucose were calculated for alfalfa stems using the average cellulose concentrations and ethanol yields from xylose were calculated for alfalfa stems using the average hemicellulose concentrations.

2.5 Statistical analyses

A general linear model procedure was used with a one-way analysis of variance (ANOVA; SigmaPlot, 2008) to examine differences in cellulose, hemicellulose, lignin, and

theoretical ethanol yields between harvest regimes and treatments (rainfed, brackish and fresh water) during the 2010 and 2011 growing seasons. The least significant difference (LSD) post-hoc test was used to compare individual means. A two-way ANOVA was used to analyze differences in cellulose, hemicellulose, lignin, and theoretical ethanol yields between the harvest regime + treatment (rainfed, brackish and fresh water) and the cultivar differences during the 2010 and 2011 growing seasons, followed by post-hoc LSD test (SigmaPlot, 2008). Differences were considered significant at the $P < 0.05$ level unless otherwise noted.

3. Results

3.1 *Precipitation patterns and soil salinity*

During the 2010 growing season, the field site received 91.9 cm of precipitation which is 17.4 cm more than the historical average from 2001-2009 (Supplementary Information B). The months of June and September received 60.0 and 57.3% more precipitation than the historical averages for these months, respectively. During the 2011 growing season the field site received 48.8 cm of precipitation which is 25.7 cm less than the historical average from the same period. Precipitation during August, September, and October was 84.0, 88.4, and 87.9% lower than the historical averages from 2001-2009 for those months, respectively (Supplementary Information B).

At the start of the experiment (2009), soil salinity measurements at our site ranged between 0.97 to 1.61 dS m⁻¹. In 2010 and 2011, soil salinity measurements ranged from 0.99 to 5.69 dS m⁻¹ and 0.94 to 7.15 dS m⁻¹, respectively (Supplementary Information B). Due to the

large variance in soil salinity measurements, we did not detect any consistent differences between treatments over the course of the experiment.

3.2 Harvest regimes and irrigation treatments

In 2010, cellulose concentrations in alfalfa averaged 36.7% and did not differ between the full-bud and 50%-flower harvest regimes. However, in 2011 average cellulose concentrations were 5.4% higher in plants at the 50%-flower harvest than those at full-bud ($p<0.001$; Figure 1A). Interestingly, cellulose concentrations decreased 4.6% at the full-bud harvest and increased 1.6% at the 50%-flower harvest after two seasons ($p<0.01$; Figure 1A). Cellulose concentrations were highest in alfalfa plants in brackish-water treatments for both years ($p<0.05$; Figure 2A). These plants had 2.1 -3.5% (2010) and 4.1 – 2.4% (2011) more cellulose than those in the rainfed and fresh-water treatments, respectively. Alfalfa growing in brackish-water treatments generally had higher cellulose concentrations in both full-bud and 50%-flower harvest in both 2010 and 2011 (Figure 1A).

Hemicellulose concentrations at the full-bud harvest were 5.8 and 4.8% higher than those at the 50%-flower harvest for 2010 and 2011, respectively ($p<0.05$; Figure 1B). Alfalfa plants growing under brackish and fresh-water treatments had 9.2 and 8.1% (2010) and 14.1 and 18.0% (2011) higher hemicellulose concentrations than those in rainfed treatments, respectively ($p<0.01$; Figure 2B). These differences were typically more pronounced during the full-bud harvest during both years (Figure 1B).

Holocellulose (cellulose + hemicellulose) concentrations were mainly a function of cellulose content and mirrored these values across seasons, harvest regimes and irrigation treatments. Holocellulose concentrations averaged 45.1% and did not differ between the full-bud and 50% flower harvests in 2010. However in 2011, plants sampled at the 50%-flower harvest had 3.5% more holocellulose than those at the full-bud harvest ($p<0.01$; Figure 1C). Similar to cellulose, holocellulose concentrations at the full-bud harvest decreased 4.7% from 2010 to 2011 (Figure 1C) and were highest in the brackish-water treatments both growing years ($p<0.01$; Figure 2C).

Lignin concentrations were 13.9% higher in plants harvested at full bud compared to those at the 50%-flower stage in 2010 ($p<0.01$), but there were no differences observed between these two harvest regimes in 2011 (Figure 1D). Lignin concentrations decreased 28.7 and 15.5% in plants sampled at the full-bud and 50%-flower regimes, respectively, from the 2010 to 2011 ($p<0.01$; Figure 1D). There were no differences in lignin content between the irrigation treatments in 2010, but in 2011 alfalfa in the brackish-water treatments had 2.3% less lignin in their stems (Figure 2D).

Surprisingly, the overall holocellulose to lignin ratio (holocellulose:lignin) was greater in 2011 than in 2010 ($p<0.01$; Figure 1E). There were no differences in this ratio between harvest regimes in 2011, but in 2010 plants in the 50%-flower regime had a 12.8% higher holocellulose:lignin than those harvested at full bud ($p<0.01$; Figure 1E). The ratio tended to be higher in plants under brackish-water treatments during both 2010 and 2011 ($p=0.09$ and $p<0.05$, respectively; Figure 2E).

3.3 *Cultivar selection*

The average cellulose concentrations of the eight cultivars during the 2010 ranged from 35.1 to 38.0%. In 2011 concentrations of cellulose ranged from 35.5 to 37.2% (Figure 3A). The Gold Country cultivar had the highest cellulose concentration for both years. Hemicellulose concentrations for all cultivars ranged from 7.8 to 9.2% over the course of two years. The Iroquois and WL363HQ consistently had high concentrations of hemicellulose in both 2010 and 2011 ($p < 0.05$; Figure 3B). Holocellulose concentrations of the eight cultivars ranged from 43.4 to 46.4% during both seasons (Figure 3C). Holocellulose concentrations were consistently high in the Gold Country cultivar during both seasons, mainly due to increased cellulose content.

Lignin concentrations in alfalfa stems of the eight cultivars ranged from 14.0 to 18.4% over the course of the experiment (Figure 3D). The Enforcer cultivar consistently had low concentrations of lignin during both years, and all eight cultivars experienced significant reductions in overall lignin content from the 2010 to 2011 ($p < 0.01$; Figure 3D). There were few differences in the holocellulose:lignin ratio in either year (Figure 3E), but the ratio was once again reduced from 2010 to 2011 in all eight cultivars ($p < 0.01$), mainly due to the reduction in lignin content.

3.4 *Theoretical ethanol yields.*

Total theoretical ethanol yields were mainly a function of cellulose concentrations. Cellulose ethanol yields comprised 84.2 to 85.5% of total holocellulose yields. Total theoretical ethanol yields across all treatments averaged 146.3 and 143.0 in 2010 and 141.6 and 145.8 L t⁻¹ in 2011 for plants at the full-bud and 50% flower stage, respectively (Table 1). Total theoretical

ethanol yields decreased 4.3% for plants sampled at the full-bud harvest regime from 2010 to 2011 ($p<0.01$), but increased 1.2% for plants sampled at the 50%-flower harvest ($p<0.01$; Table 1). In a manner similar to cellulose concentrations, total theoretical ethanol yields were highest under brackish-water treatments during both years ($p<0.01$; Figure 4; Table 1).

The Iroquois cultivar had the highest theoretical ethanol yield averaging 149.1 L in 2010 ($p<0.05$; Table 2) while the Viking 357 had the highest yields in 2011 and averaged 146.7 L ($p<0.05$; Table 2). Both Iroquois (2010) and Viking 357 (2011) cultivars had high hemicellulose concentrations (Figure 3B) and the higher conversion efficiencies of hemicellulose compared to that of cellulose contributed to their higher total theoretical ethanol yields.

4. Discussion

Growing season, harvest regime, irrigation, and salinity caused effects on stem lignocellulose concentrations in alfalfa. Harvest regime affected stem lignocellulose concentrations but results varied significantly between growing seasons. It appears that precipitation patterns had a major influence on the effects of irrigation and salinity on stem lignocellulose concentrations. Plants under supplemental irrigation had higher lignocellulose concentrations and this effect was more pronounced during the dry period (August-October) in the 2011 growing season. However, over the duration of this study no signs or symptoms of drought stress occurred.

Harvest regime did not significantly affect cellulose concentrations during the 2010 growing season (Figure 1A). However, during the 2011 there was an increase in cellulose concentration between the plants sampled at full-bud and 50%-flower harvest regimes. Plants

355 harvested after the 2010 and 2011 growing seasons followed similar trends for hemicellulose
356 concentrations. Hemicellulose concentrations of the plants sampled at the 50%-flower harvest
357 regime in both growing seasons decreased from the full-harvest regime. Plants harvested at the
358 full-bud harvest regime had significantly higher lignin concentrations than those harvested at the
359 50%-flower harvest regime in 2010. However, there were no significant differences in lignin
360 concentrations in plants sampled during the 2011 growing season. While maturity is the single
361 most important factor impacting stem lignocellulose concentrations in alfalfa, growth
362 environment causes some additional shifts in stem lignocellulose allocation concentrations.
363 Unfortunately these environmental impacts are complex and difficult to predict (Samac et al.,
364 2006). Sanderson and Wedin (1988) found substantially higher lignocellulose concentrations in
365 alfalfa stems during one year, however, the same plots harvested the following year showed a
366 small difference between harvest regimes. In this study temperature and moisture were not
367 independently evaluated. In studies that have evaluated temperature and moisture separately, the
368 major effect of moisture stress alone appeared to be on the amount of cell wall accumulated by
369 alfalfa plants as opposed to changes in cell wall composition (Samac et al., 2006). We found
370 similar results in the holocellulose to lignin ratios between harvest regimes and growing seasons.
371 During the 2010 growing season, there was an increase in the holocellulose to lignin ratio
372 between the full-bud to the 50%-flower harvest regimes. However, in 2011, there was no
373 significant difference between harvest regimes, but the holocellulose: lignin ratio in this case
374 increased 24.8 and 14.5% for plants sampled at the full-bud and 50%-flower harvest regimes,
375 respectively, from the 2010 to the 2011 growing season (Figure 1A). Total estimated ethanol
376 yields also varied by harvest regime and growing season. Lamb et al. (2007) found that alfalfa

grown under a biomass-type management system (50%-flower harvest regime) increased lignocellulose concentrations by 4% and could increase hypothetical ethanol yields by 6.5%. During the 2011 growing season the plants sampled at the 50%-flower harvest regime had a 3.2% increased lignocellulose concentrations over the full-bud harvest regime which increased theoretical ethanol yields by 4.0% (Table 1). However, during the 2010 growing season there was a decrease in theoretical ethanol yield from the plants sampled at the full-bud to the 50%-flower harvest regime. Potential ethanol yields associated with stem-maturity harvests appear to be quite variable. Lamb et al. (2014) found that potential yields were higher at late-flower harvests, while Rock et al. (2009) observed similar patterns similar to ours; where stem lignocellulose concentrations in alfalfa exhibited year by harvest interactions with no clear pattern. Both studies concluded that industries that wish to utilize alfalfa for lignocellulose ethanol production must be prepared to deal with significant feedstock quality variation due to macro-environment fluctuations such as location, harvest date, irrigation regime and cultivar selection.

This study was conducted in a natural field setting with the rainfed plots receiving ambient amounts of precipitation. During the 2010 growing season the field site received 23% more precipitation than the historical average (Supplementary Information B). The fresh- and brackish-water treatments received an additional 5.0 cm of well water per harvest regime depending on local precipitation patterns. Irrigation did not have a significant effect on stem lignocellulose concentrations during the 2010 growing season although the hemicellulose concentrations showed a significant increase as compared to rainfed plots. However, plants growing in brackish-water treatments had significantly higher holocellulose concentrations

399 (cellulose and hemicellulose), holocellulose to lignin ratios, and the theoretical ethanol yields
400 (Figures 2; 4). In 2011, which was a drier year (34% less precipitation than the historical
401 monthly average), the addition of fresh and brackish water appear to have contributed to plants
402 with higher holocellulose concentrations (cellulose and hemicellulose) over the rainfed treatment
403 (Figures 2; 4). Deetz et al. (1994) found that alfalfa plants that grew under water-deficit
404 conditions had reduced stem lignocellulose concentrations, reduction which was most likely the
405 result of delayed maturity and decreased cell wall concentrations. Interestingly, alfalfa growing
406 under brackish-water treatments had higher holocellulose to lignin ratios (and higher theoretical
407 yields) during both growing season suggesting that moderate levels of salt may stimulate
408 holocellulose concentrations. Moderate levels of salinity can reduce root and shoot growth
409 (Esechie et al., 2002), alter photosynthesis (Khan et al. 1994) and modify overall plant water
410 relations (Serraj and Drevon, 1998). However, the effects of salt on cell wall composition in
411 alfalfa has only recently been examined. In other agronomic species such as tomato, moderate
412 levels of salt increased lignin deposition and the number of lignified xylem cells (Quiroga et al.,
413 2000; Sanchez-Aguayo et al., 2004). In short term growth chamber studies examining alfalfa,
414 the application of salt increased the upregulation of several groups of cellulose synthase genes
415 (Guerriero et al., 2014) and genes involved in lignin biosynthesis (Behr et al., 2015). Our results
416 agree with these findings and it appears that strengthening of the cell wall is a common response
417 to salinity and other abiotic stresses (Tenhaken, 2015). Alfalfa growing under our brackish-
418 water treatments had higher holocellulose concentrations but lower lignin concentrations during
419 the 2011 growing season. These findings could be significant because selecting species with

high holocellulose to lignin ratios will be an important characteristic when selecting feedstocks for ethanol production.

The Gold Country cultivar had the highest percent cellulose concentrations (Figure 3A); this is a high yielding cultivar with WSI and FD rankings of 2.5 and 3.8, respectively. Although having the highest percent cellulose concentrations during both growing seasons, the Gold Country cultivar also had some of the highest lignin concentrations. This resulted in lower holocellulose to lignin ratios compared to other cultivars (Figure 3E). The Iroquois cultivar had the highest total theoretical ethanol yield during the 2010 growing season (Table 2); it was obtained from a local farmer and the WSI and FD rankings were unknown. Typically, Iroquois alfalfa is a non-genetically modified cultivar with average yields (Manske and Goetz, 1982); during the 2011 growing season it produced the lowest total theoretical ethanol yield (Table 2). The Gold Country cultivar also had very high total theoretical ethanol yields during both growing seasons. Cultivar selection did not seem to have a pronounced effect on stem lignocellulose concentrations in alfalfa. High yielding cultivars (Gold Country) did not have a significantly greater holocellulose to lignin ratio or total theoretical ethanol yields compared to typical non-genetically modified alfalfa (Iroquois). Research on biomass yields and forage nutrition quality could prove beneficial for cultivar selection in the future if alfalfa is used as a feedstock for lignocellulose ethanol production.

Feedstocks for the sole purpose of biofuel production must be sustainable, carbon neutral and have little environmental impact. Alfalfa has a reduced requirement for nitrogen fertilizer, increased environmental protection and a well-known cropping system which gives it an advantage over other comparable feedstocks. In many scenarios alfalfa leaves and stems would

be separated for lignocellulose ethanol production. Generally, management systems have emphasized harvesting alfalfa forage at immature growth stages to maximize the leaf component and crude protein concentrations; although a biomass production system would make the stem component as valuable as the leaf yield (Lamb et al., 2003; 2014). Separating the leaves from the stems in the field would create a more viable system than separation facilities that have been proposed by other researchers (Arinze et al., 2003; Downing et al., 2004). Another improvement on the alfalfa cropping system would be to seed the alfalfa in the fall after the current crop has been harvested, which has the potential to increase the first year alfalfa yields. Genetic improvements could also increase alfalfa's value as biomass feedstock. Genetically decreasing the concentration of lignin in alfalfa stems would decrease fermentation costs and in turn increase ethanol yields.

Crops such as *Miscanthus spp.*, *Populus spp.*, and switchgrass (*Panicum virgatum*) could be used for lignocellulose ethanol production as an alternative to corn-based feedstocks. Unlike alfalfa, many of the proposed crops do not have well-established cropping systems and farmers may be reluctant to invest in these systems. While corn stover has a potentially high ethanol yield (227 L t^{-1} ; Badger 2002), it also has high energy inputs and is associated with large amounts of erosion and N leaching (Vadas et al., 2008). Alfalfa has a high N use efficiency but also has a high water requirement (Krogman and Hobbs, 1965), especially when used as hay, which has contributed to the increase in irrigation in the United States in recent years (Howell, 2001). In our study, theoretical ethanol yields ranged from $134\text{-}150 \text{ L t}^{-1}$, which is comparable to findings from natural wetland (136 L t^{-1}) and prairie (150 L t^{-1}) systems (Ruhland et al. 2011). A recent cost-benefit analysis by Vadas et al., (2008) has demonstrated that a corn-alfalfa system

increases energy production efficiency and decreases energy inputs with only a slight reduction in net energy yield per hectare over a continuous corn scenario. However, in their analysis they assumed only two alfalfa cuttings per season, and in many scenarios three to four cuttings are possible. Alfalfa rotations would decrease the need for N inputs and help reduce soil erosion. In the future, the sustainability of a cellulosic-based biofuel industry will likely depend on a wide range of factors that will need to account for reductions in product yield based on plant stress, the selection of cultivars that are best suited to local agronomic conditions and the protection of natural resources and environmental quality.

5. Conclusions

Alfalfa shows high potential as a biomass feedstock for lignocellulose ethanol production. Growing season, harvest regime, irrigation and salinity influenced cell-wall constituents in the stems of alfalfa. However there were few consistent effects of harvest regime on stem lignocellulosic concentrations between the two years suggesting that differences in growth environment between the two seasons may exert a strong influence on cell-wall composition in alfalfa. Interestingly, irrigation with brackish water (5.0 dS m^{-1}) increased theoretical ethanol yields during both field seasons mainly due to a 2.1-4.1% increase in stem cellulose concentrations. It appears that the eight cultivars of alfalfa we examined in this study may be tolerant to moderate levels of salinity. However, further studies on the effects of salinity on total stem yield and density are still required.

Cellulose concentrations were consistently highest in the Gold Country cultivar, but this variety also had high lignin content which may constrain ethanol yields. The Iroquois and Viking cultivars had the highest theoretical ethanol yields, mainly due to the high hemicellulose content of their stems. While hemicellulose is only a minor component of total stem holocellulose concentrations (7.8-9.2%), it may be important for large-scale ethanol production due to a higher conversion efficiency than cellulose. Therefore selection of alfalfa cultivars for the sole purpose of feedstock for biofuels should focus on those that have high holocellulose to lignin ratios.

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FIGURE LEGENDS

Figure 1. The percent cellulose (A), hemicellulose (B), holocellulose (C), and lignin (D) concentrations and holocellulose to lignin ratio (E) for plants sampled at the full-bud and 50%-flower regimes with the corresponding treatment (rainfed, brackish water, or fresh water) during 2010 and 2011. Values are means of the harvest regime and the corresponding treatment during the 2010 (n=80, white shading) and 2011 (n=160, grey shading) growing seasons. Vertical error bars represent $\pm$ 1SE. Letters (a+b, 2010) and (r-u, 2011) denote significant differences among treatments and a * signifies differences between years ($p < 0.05$).

Figure 2. Constituent analyses for plants growing under the rainfed, brackish-water and fresh-water treatments during the 2010 and 2011 growing seasons. Values are means of the treatments during each growing season (n=160, 2010 and n=320, 2011). Letters (a-e) denote significant differences among treatments ($p < 0.05$).

Figure 3. Constituent analyses for each cultivar during the 2010 and 2011 growing seasons. Values are means of each cultivar during 2010 (n=90, white shading) and 2011 (n=140, grey shading). Letters (a-d, 2010) and (r-t, 2011) denote significant differences among cultivars and a * signifies differences between years ($p < 0.05$).

Figure 4. The theoretical ethanol yield ($L\ t^{-1}\ DW$) for the rainfed, brackish-water, and fresh-water treatments during the 2010 and 2011 growing seasons. Values are means of the treatments during each season (n=160, 2010 and n=320, 2011). Letters (a-d) denote significant differences among treatments ($p < 0.05$).

Table 1. Cellulose, hemicellulose, and total theoretical ethanol yields¹ following Boyer (2002) for the full-bud control, saline, and irrigated treatments and the 50%-flower control, saline, and irrigated treatments for the 2010 and 2011 growing seasons. Values represent treatment means (n=80) and letters designate differences between treatments (LSD; p < 0.05) and * indicates differences between years (p<0.05).

Growth Stage and Treatment	Cellulose Yield 2010	Cellulose Yield 2011	Hemicellulose Yield 2010	Hemicellulose Yield 2011	Total Yield 2010	Total Yield 2011
Full-bud Control	123.8 ^{abc}	114.8 ^e	19.2 ^d	19.0 ^c	143.0 ^{bc}	133.8 ^d
Full-bud Saline	125.0 ^{ab}	121.7 ^c	24.8 ^a	22.8 ^b	149.8 ^a	144.5 ^b
Full-bud Irrigated	121.6 ^{abc}	117.1 ^d	24.4 ^a	24.5 ^a	146.0 ^{ab}	141.6 ^c
Full-bud Average					146.3 [*]	139.9
50%-flower Control	121.3 ^{bc}	122.9 ^c	22.6 ^c	19.3 ^c	143.9 ^b	142.2 ^c
50%-flower Saline	125.8 ^a	126.2 ^a	21.2 ^a	22.2 ^b	147.0 ^{ab}	148.4 ^a
50%-flower Irrigated	120.1 ^c	124.7 ^b	20.8 ^b	22.2 ^b	140.9 ^c	146.9 ^a
50%-flower Average					143.9	145.8 [*]

¹Ethanol yields are expressed in liters of ethanol per 1000 kg of dried biomass.

Table 2. Cellulosic, hemicellulosic, and total theoretical ethanol yields¹ following Boyer (2002) for the eight varieties we examined during the 2010 and 2011 growing seasons. Values represent treatment means (n=90) and letters designate differences between cultivars (LSD; $p < 0.05$).

Cultivar	Cellulosic Yield 2010	Cellulosic Yield 2011	Hemicellulosic Yield 2010	Hemicellulosic Yield 2011	Total Yield 2010	Total Yield 2011
Fontanelle	123.2 ^{bc}	124.2 ^{ab}	20.6 ^c	20.6 ^d	143.8 ^{bc}	144.8 ^{ab}
Gold Country	127.2 ^a	124.5 ^a	21.3 ^{bc}	20.8 ^{cd}	148.5 ^a	145.3 ^{ab}
Iroquois	124.9 ^{ab}	118.9 ^d	24.2 ^a	21.9 ^{abc}	149.1 ^a	140.8 ^c
Viking 3100	126.1 ^{ab}	121.9 ^c	21.3 ^{bc}	21.3 ^{bcd}	147.4 ^{ab}	143.2 ^b
Viking 357	122.4 ^{bc}	123.9 ^{abc}	21.4 ^{bc}	22.8 ^a	143.8 ^{bc}	146.7 ^a
L447HD	120.3 ^{bc}	122.3 ^{bc}	22.2 ^{bc}	22.2 ^{ab}	142.5 ^c	144.5 ^{ab}
WL363HQ	117.7 ^d	123.2 ^{abc}	24.3 ^a	22.5 ^{ab}	142.0 ^c	145.7 ^a
Enforcer	117.8 ^d	119.1 ^d	22.4 ^b	21.4 ^{bcd}	140.2 ^c	140.5 ^c

¹Ethanol yields are expressed in liters of ethanol per t of dried biomass.

Figure 1

Figure 2

Figure 3

Figure 4