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Beyond the cellulose: Oxygen isotope composition of plant lipids as a proxy for terrestrial water balance

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Abstract

There is growing interest in using stable isotopes to measure the impact of shifting water regimes on terrestrial ecosystems. The analysis of oxygen isotopes ($\delta^{18}O$) of plant cellulose has been widely used for that purpose, but its application is limited by cellulose's short life in most soils and sediments. Here we compare $\delta^{18}O$ values of cellulose and plant lipids (hexane-extractable compounds) to assess the value of bulk lipids as a proxy for water balance. Using a set of field experiments with three C_3 and three C_4 species, we found significant differences in ^{18}O enrichment in response to irrigation regime, with a strong linear relationship observed between cellulose and lipid signals. Imposed drought increased lipid $\delta^{18}O$ values of all species relative to controls and also affected the carbon isotope composition ($\delta^{13}C$) of cellulose, reflecting increased water-use efficiency in C_3 plants. Lipid extracts did not differ with respect to $\delta^{13}C$ values, but $\delta^{18}O$ signals consistently reflected drought effects in C_3 and C_4 species, regardless of variation in productivity and abundance of oxygen-containing functional groups. These results show that oxygen isotope composition of plant lipids can be used as a proxy for changing water regimes.

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Introduction

The study of shifting water regimes and their impact on the terrestrial ecosystems is a research frontier in which quantitative metrics are still being established. The need to characterise climate variability and to measure the impact of drought on natural and managed ecosystems has led to increasing interest in stable isotope proxies (Silva and Anand, 2013; Maxwell *et al.*, 2014; Silva, 2014). The oxygen isotope composition ($\delta^{18}O$) of plant cellulose provides an integrated measure

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of the balance between water inputs (precipitation and/or irrigation) and losses through evapotranspiration (ET), and has been used to examine the effects of climatic variability on terrestrial systems (Kahmen *et al.*, 2011; Sternberg, 2009). Although extensively used in living plants (*e.g.*, tree rings), the analysis of cellulose is limited by its typically short life after decomposition of plant biomass. Under particular conditions, cellulose extracted from fossil plants have been used to produce climate records that go back millions of years (Jahren and Sternberg, 2003), but such well-preserved samples are rare. In contrast to cellulose, plant-derived lipids can persist in soils and sediments due to their low biodegradability and stabilisation into hydrophobic domains of organic matter (Matsumoto *et al.*, 2007; Assis *et al.*, 2011), providing a potential long-term record of terrestrial water balance.

Plant lipids may be broadly defined as hydrophobic or amphiphilic molecules originating entirely or in part from two types of biochemical subunits, ketoacyl and isoprene groups, which are building blocks for molecules of various structures and polarities, including some with no oxygen at all (*e.g.*, alkanes in leaf surface wax) and others that carry various amount of oxygen (Fahy *et al.*, 2009). The value of lipid hydrogen isotope analysis (δ^2H) has already been recognised for specific compounds recovered from soils and sediments (Sachse *et al.*, 2012). The study of lipid $\delta^{18}O$ signals has the potential to improve δ^2H records by, for example, allowing water loss to be partitioned into evaporation and transpiration (Voelker *et al.*, 2014).

The first step towards the use of lipid $\delta^{18}O$ records is to demonstrate that a signal related to changes in water balance is recorded in such compounds. The second step is then to demonstrate that this signal is preserved in soils and sediments. The present study is concerned with the first step. However, without losing sight of the second step, we imposed the restriction that lipid extracts should contain only the most apolar compounds present in the plant biomass, under the assumption that these are also the most likely to persist after deposition (Matsumoto *et al.*, 2007). To advance the interpretation of lipid $\delta^{18}O$ signals, we compared measurements of cellulose and hexane-extractable compounds of C_3 and C_4 species, grown under contrasting water regimes in replicated field experiments. In addition, we analysed the carbon isotope composition ($\delta^{13}C$) of cellulose and lipids as a way to assess plant water-use efficiency (Farquhar and Von Caemmerer, 1982). Alongside these isotopic measurements, we used spectroscopic analyses to qualitatively describe the molecular composition of lipid extracts and all results are integrated into a consideration of potential applications in ancient and contemporary settings.

Methods

Field Experiment. The field experiment was conducted at the University of California Five Points Experimental Station (36°20′10.28″N, 120°6′38.40″W). This site experiences hot summers, cool and dry winters, and average annual

precipitation of 173 mm. Plots of 27 m^2 were established under homogeneous edaphic conditions (Tables S-1 and S-2) in which three C_3 and three C_4 species were grown (Table 1). Following a complete randomised block design with four field replications, monocultures of each species were established using sprinkler irrigation (2008), later converted to flood irrigation (2009), after which two irrigation treatments were imposed through 2011. Daily potential ET was obtained from the on-site meteorological station and multiplied by species-specific coefficients (Allen and Pruitt, 1991). The amount of water to be applied was determined based on 100 % or 70 % of the potential ET, representing full water loss replenishment and deficit irrigation (imposed drought) treatments, respectively. Plots were irrigated when the accumulated ET reached 150 mm, for an average of 11 irrigation events per year. Precipitation water was factored into the calculation for irrigation. The average annual $\delta^{18}O$ of the precipitation is about -7.8 ‰. Irrigation water was a combination of aqueduct source (originating in the Sacramento-San Joaquin Delta region) and a groundwater well at a depth of 150-180 metres. The same irrigation water was used in all plots throughout the experiment.

Table 1 Species description.

Common name	Scientific name	Variety	Metabolism
Miscanthus	*Miscanthus giganteus*	Hybrid giganteus	Warm-season C_4
Switchgrass	*Panicum virgatum*	Alamo	Warm-season C_4
Bermuda grass	*Cynodon dactylon*	Giant NK 37	Warm-season C_4
Alfalfa	*Medicago sativa*	Big Kaw	Warm-season C_3
Tall Fescue	*Festuca arundinacea*	Fawn	Cool-season C_3
Tall wheatgrass	*Agropyron elongatum*	Jose	Cool-season C_3

Sampling and analysis. At the end of the experiment, the aboveground biomass of all plots was harvested and ground to <0.5 mm for analysis. Pure alpha cellulose was isolated (Brendel *et al.*, 2000) and the lipid fraction was obtained by Soxhlet extraction with hexane for 10 hours. Although less efficient at extracting total lipids, as compared to more polar solvent mixtures (*e.g.*, chloroform:methanol), hexane was chosen to minimise extraction of transient polar lipids such as phospholipids and degradation products of chlorophyll.

The isotopic composition of oxygen was determined using an Elementar PyroCube (Elementar Analysensysteme, Hanau, Germany) interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK). Three analytical replicates were used for each plot and working standards of cellulose and hexadecyl palmitate were interspersed throughout each run to determine analytical precision. Carbon isotopic composition of cellulose and lipids was determined using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK). A certified analytical standard of known $\delta^{13}C$ was used to confirm accuracy and precision. Data are expressed as $\delta^{18}O$ and $\delta^{13}C$ (‰) relative to Vienna Standard Mean Ocean Water (VSMOW) and Pee Dee Belemnite (VPDB), respectively.

Elemental analysis of hexane-extracted material showed an oxygen content of 6-11 % and a carbon content of 71-79 % (w/w). Fourier Transform Infrared Spectroscopy (FTIR) was used to describe the molecular composition of hexane extracts, focusing on oxygen-containing groups. Samples were analysed using attenuated total reflectance (ATR) FTIR. The spectra were collected on a Thermo Nicolet 6700 spectrophotometer (Thermo Scientific, Madison, WI) with a diamond single bounce accessory (GladiATR, PIKE Technologies). After collection, the spectra were normalised to the aliphatic C-H peak at approximately 2913 cm^{-1} prior to performing spectral subtraction. All FTIR spectra were collected in triplicate using 4 cm^{-1} resolution and 1.2 kHz scanning speed for a total of 128 co-added scans per analysis (Parikh *et al.*, 2014).

Statistical analysis. Average differences in isotopic composition were compared using paired t-tests. When appropriate, least-square regressions were performed to evaluate the relationship between cellulose and lipid isotopic ratios. Average data points and standard deviations were plotted for each species, blocked by water balance treatment, incorporating 4 field replicates each. Molecular characterisation of lipids was performed separately for each species and treatment.

Results and Discussion

Isotopic composition of cellulose and lipids. Irrigation treatments caused clear changes in lipid and cellulose $\delta^{18}O$ values. Despite significant differences among species, plants growing under drought (70 % ET) were always enriched in ^{18}O relative to plants growing under full ET replenishment (Fig. 1a). In cellulose, ^{18}O enrichment in response to drought was clearer in C_3 (> 1 ‰) compared to C_4 (~0.5 ‰) plants, while in lipids, consistent responses were observed (~0.75 ‰) with no statistical difference between C_3 and C_4 plants. We found a strong linear relationship between cellulose and lipid $\delta^{18}O$ values ($P < 0.01$), reflecting consistent responses across species (Fig. 1b).

The effect of drought was also evident in cellulose $\delta^{13}C$, reflecting expected differences between plant metabolism (Pearcy and Ehleringer, 1984), and showing a clear separation in response to water balance treatments in C_3 plants (Fig. 1c). This separation confirms an anticipated increase in water-use efficiency under drought, which was surprisingly not apparent in lipid $\delta^{13}C$ values, and may be explained by the heterogeneous composition of lipid extracts (discussed below). While ^{13}C fractionation in C_3 plants is controlled by the stomata-regulated ratio of intercellular to ambient CO_2 concentrations (c_i/c_a), variation in C_4 bundle-sheath leakiness can either dampen or amplify the effects of c_i/c_a, so that $\delta^{13}C$ measurements cannot be used to assess water-use efficiency in C_4 plants (Cernusak *et al.*,

2013). Nevertheless, we found ^{18}O enrichment of similar magnitude and direction in C_3 and C_4 lipids under drought, demonstrating the value of $\delta^{18}O$ measurements as a general proxy for water balance.

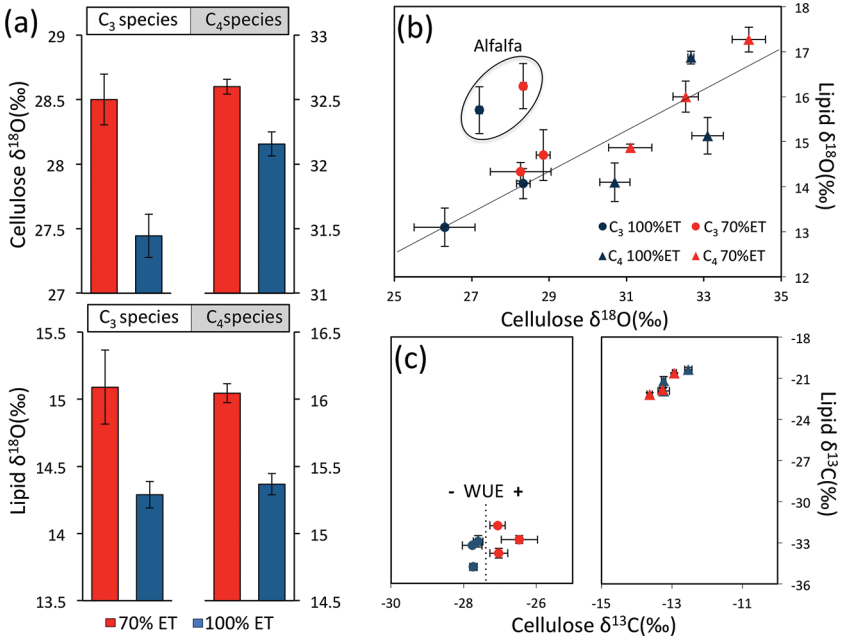

Figure 1 (a) Average $\delta^{18}O$ values of C_3 and C_4 species under full evapotranspiration replenishment (100 % ET) and deficit irrigation (70 % ET). Differences between treatments were significant (t-test; $P < 0.01$) in all cases. **(b)** Relationship between cellulose and lipid $\delta^{18}O$ values excluding alfalfa: $y = 0.4449x + 1.4277$; $R^2 = 0.76$ ($P < 0.01$). **(c)** Cellulose and lipid $\delta^{13}C$ values; significant differences were observed in cellulose of C_3 species (t-test; $P < 0.01$), but not in lipids. Error bars represent one standard deviation.

Interpreting differences in isotopic composition. Leaf and xylem water are both sources of oxygen incorporated into organic compounds during biosynthesis, but each of these sources contributes a distinct isotopic signal (Sternberg, 2009). Reductions in plant water supply are expected to increases the contribution of isotopically enriched leaf water, while decreasing the flux of relatively unenriched xylem water to the leaf (Ogée *et al.*, 2007). This process is influenced by the distribution of leaf veins, which caused the greatest ^{18}O enrichment in lipids relative to cellulose in alfalfa, the only dicot species. This observation is consistent with previous work on leaf δ^2H values showing significant differences in ^2H enrichment of the *n*-C_{29} alkane biomarker among plant growth forms, in which dicot species were the most and graminoids the least enriched in ^2H, respectively (Sachse *et al.*, 2012).

Fatty acid transfer reactions within the plastid yield a significant reduction in ^{18}O content of water in relation to the cytosol (Pollard and Ohlrogge, 1999) contributing to differences between the isotopic composition of lipids and cellulose. It is important to note that lipids contain far less oxygen than cellulose and do not undergo the postsynthetic isotopic exchange that happens during the hydration of carbonyl groups of the intermediates of cellulose synthesis (Sternberg and DeNiro, 1983). Therefore, intrinsic species traits and biosynthetic differences explain the offset between lipid and cellulose $\delta^{18}O$ values across species (Fig. 1b).

Our observations show that the balance between water input and evapotranspiration can be directly interpreted from lipid oxygen isotopes in monocultures of C_3 and C_4 species, providing a powerful tool for selecting traits that would optimise water-use efficiency under field conditions. However, the large variation in ^{18}O enrichment between species indicates that the effect of a diverse vegetation cover could confound water balance signals. Therefore, analyses of lipids in natural ecosystems are more likely to successfully reconstruct climate variability where vegetation composition is known to be stable. This could be accomplished by appropriate evaluation of vegetation composition in contemporary settings, and palaeoecological reconstructions in ancient settings based on companion proxies for vegetation change (*e.g.*, fossil pollen and $\delta^{13}C$ of organic matter; Silva and Anand, 2013; Silva, 2014).

Characterisation of lipid extracts. The FTIR spectra of lipid extracts show that substantial differences in molecular composition exist among species and in response to growing conditions. The difference spectra (Fig. 2), obtained by subtracting results of water-limited from non-limited fields, show that all common oxygen-containing functional groups found in lipids are more abundant under drought. These groups include long-chain aliphatic esters, ketones, carboxylic acids, ethers, alcohols, as well as aromatic compounds (Table S-3 and Fig. S-1). This observation is consistent with general variations in plant lipid composition (Fahy *et al.*, 2009), as drought stress tends to decrease the abundance of more polar lipids (*e.g.*, phospholipids) while increasing the relative abundance of nonpolar compounds (Harwood, 1998).

Since the 1970s, when the use of $\delta^{18}O$ values obtained from tree rings was first established, cellulose $\delta^{18}O$ records have been used as a proxy for drought stress. Classic conceptual models (Scheidegger *et al.*, 2000) have coupled $\delta^{18}O$ and $\delta^{13}C$ values to distinguish differences in stomatal response from photosynthetic capacity. In the present dataset, this link was apparent in cellulose but not in lipids, probably due to the heterogeneity of bulk lipid extracts. Nevertheless, lipid extracts of C_3 and C_4 plants showed clear isotopic signals in response to drought. This result demonstrates that $\delta^{18}O$ values of bulk plant lipids serve as a more general proxy for water balance than $\delta^{13}C$ records, which are limited to C_3 plants and therefore necessarily exclude many important crops and tropical ecosystems. This is particularly important because shifts in $\delta^{18}O$ signals in response to treatments were independent of variation in plant productivity (Fig. S-2) and large interspecific differences in molecular composition in response to drought (Fig. S-1).

The value of a new lipid proxy. The present study provides empirical evidence to support the use of lipid oxygen isotopic composition as a proxy for water balance in contemporary settings, which also has the potential to improve reconstructions of past climates. Measurements of hydrogen isotopes in plant lipids preserved in soils and sediments have been previously used to reconstruct

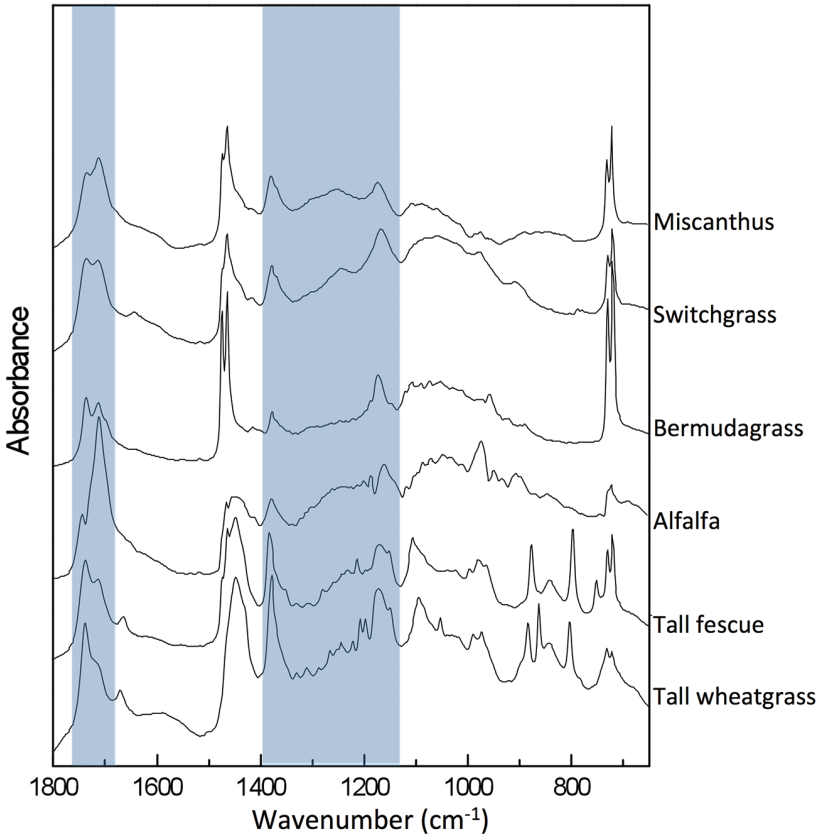

Figure 2 Difference spectra obtained by subtracting the spectra of lipid extracts of plants growing under deficit irrigation (70 % ET) from those growing under full water replenishment. Highlighted areas correspond to oxygen-containing groups (*e.g.*, vibration from aromatic C-O, aliphatic esters and ketones). The spectra have been shifted vertically for clarity. A complete spectral characterisation is presented in Table S-3 and Figure S-1.

climate variability, but these isotopic signals are sensitive to biochemical, physiological, and environmental influences (Sachse *et al.*, 2012), which can be better constrained by dual isotope analyses (Scheidegger *et al.*, 2000; Maxwell *et al.*, 2014; Voelker *et al.*, 2014). The much lower abundance of oxygen relative to

hydrogen atoms in plant lipids has so far limited the production of $\delta^{18}O$ records from specific compounds. However, persistent oxygen-containing lipids, such as long-chain *n*-alkanoic acids, are commonly recovered in sedimentary profiles and analysed for $\delta^{2}H$ (Feakins *et al.*, 2007), and could lead to water balance reconstructions with improved precision. In the case of hydrogen, the key to success has been the study of specific compounds in which the effects of hydrological shifts and biosynthesis can be isolated. Similarly, the analysis of oxygen isotopes of hemicellulose degradation products in soil has been recently shown to reflect the balance between precipitation and evapotranspiration across broad latitudinal gradients (Tuthorn *et al.*, 2014; Zech *et al.*, 2014). We expect bulk lipid samples to serve as a basis from which compound-specific oxygen and hydrogen records can be developed to better understand present and decipher past impacts of climatic change on terrestrial systems.

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Additional Information

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Beyond the cellulose: Oxygen isotope composition of plant lipids as a proxy for terrestrial water balance

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Supplementary Information

The Supplementary Information includes:

- Tables S-1 to S-3
- Figures S-1 and S-2

Table S-1 Climate at the study location.

Climatic characteristics	Five Points
Köppen climate classification	(BSk) Semi-arid steppe
Average annual temp. (°C)	17.1
Average max. annual temp. (°C)	25
Average min. annual temp. (°C)	9.2
Average annual precipitation (mm)	173
Frost free days	335
Altitude (m)	70

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Table S-2 Soil properties at the study location.

Soil Properties	
Clay (g kg^{-1})	310
Silt (g kg^{-1})	340
Sand (g kg^{-1})	350
pH (saturated paste extract)	7.6
CEC ($cmol_c$ kg^{-1})	30.7
Olsen-P (mg kg^{-1})	7.4
NO_3-N (mg kg^{-1})	10.6
Extractable K (mg kg^{-1})	439
Organic matter (g kg^{-1})	9.5
Total N (g kg^{-1})	0.7

Table S-3 Peaks associated with O-containing groups in the infrared spectra.

Wavenumber (cm^{-1})	Assignment	Reference
3360	ν(O-H) vibrations in OH	Dubis *et al.*, 1999
2950	ν(C-H) asymmetric vibrations in lipid CH_3	Wallach *et al.*, 1979; Casal and Mantsch, 1984
2915	ν(C-H) asymmetric vibrations in lipid CH_2	Wallach *et al.*, 1979; Casal and Mantsch, 1984
2850	ν(C-H) symmetric vibrations in lipid CH_2	Wallach *et al.*, 1979; Casal and Mantsch, 1984
1735	ν(C=O) vibration from aliphatic esters	Wallach *et al.*, 1979; Casal and Mantsch, 1984; Dubis *et al.*, 1999
1712	ν(C=O) vibration from aliphatic ketones	Wallach *et al.*, 1979; Casal and Mantsch, 1984; Dubis *et al.*, 1999
1471 and 1461	δ(C-H) mode in lipid CH_2	Wallach *et al.*, 1979; Casal and Mantsch, 1984; Merk *et al.*, 1997
1446	δ(C-H) mode adjacent to C=O	Spiker Jr *et al.*, 1976; Wallach *et al.*, 1979
1380	ν(C-O) vibration from aromatic C-O, δ(C-H) symmetric in lipid CH_3	Wallach *et al.*, 1979; Casal and Mantsch, 1984
1174	δ(C-H) mode in CH_2, ν(C-O) asymmetric vibration for esters	Spiker Jr *et al.*, 1976; Wallach *et al.*, 1979; Casal and Mantsch, 1984
977	ν(C-N) asymmetric vibration in lipid $N(CH_3)_3$	Wallach *et al.*, 1979; Casal and Mantsch, 1984
729 and 719	ρ(C-H) vibration in lipid CH_2	Wallach *et al.*, 1979; Casal and Mantsch, 1984; Merk *et al.*, 1997

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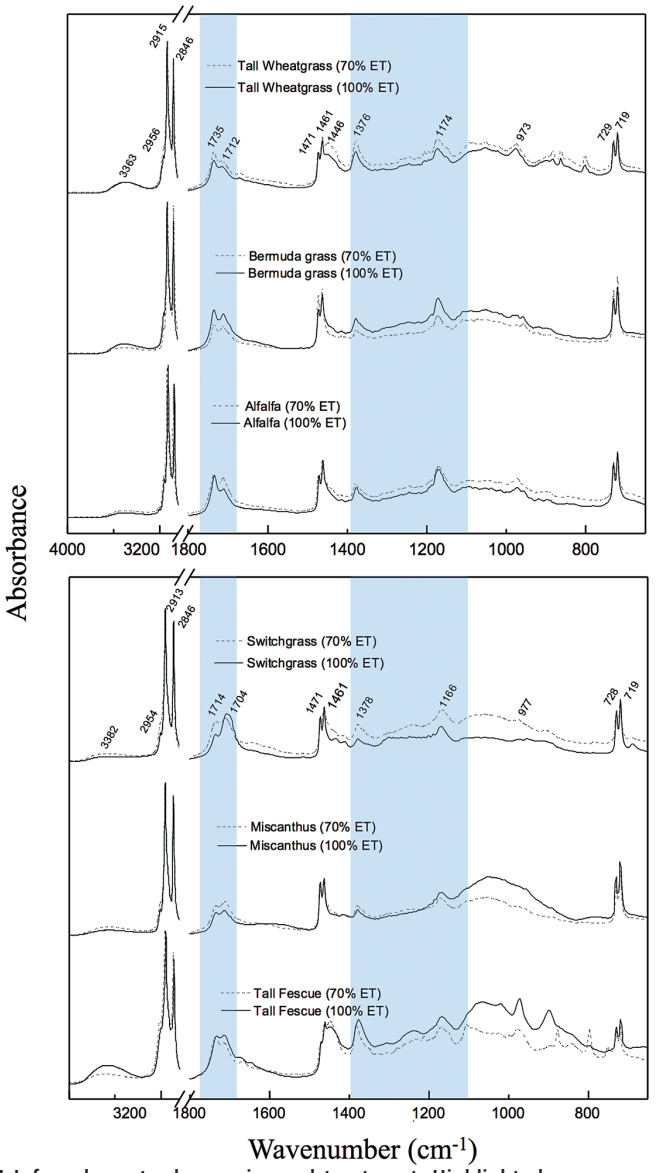

Figure S-1 Infrared spectra by species and treatment. Highlighted areas correspond to oxygen-containing groups depicted in Table S-3. Differences between full water replenished (solid lines) and deficit irrigation (dashed lines) were used to generate the drought response spectra shown in Figure 2.

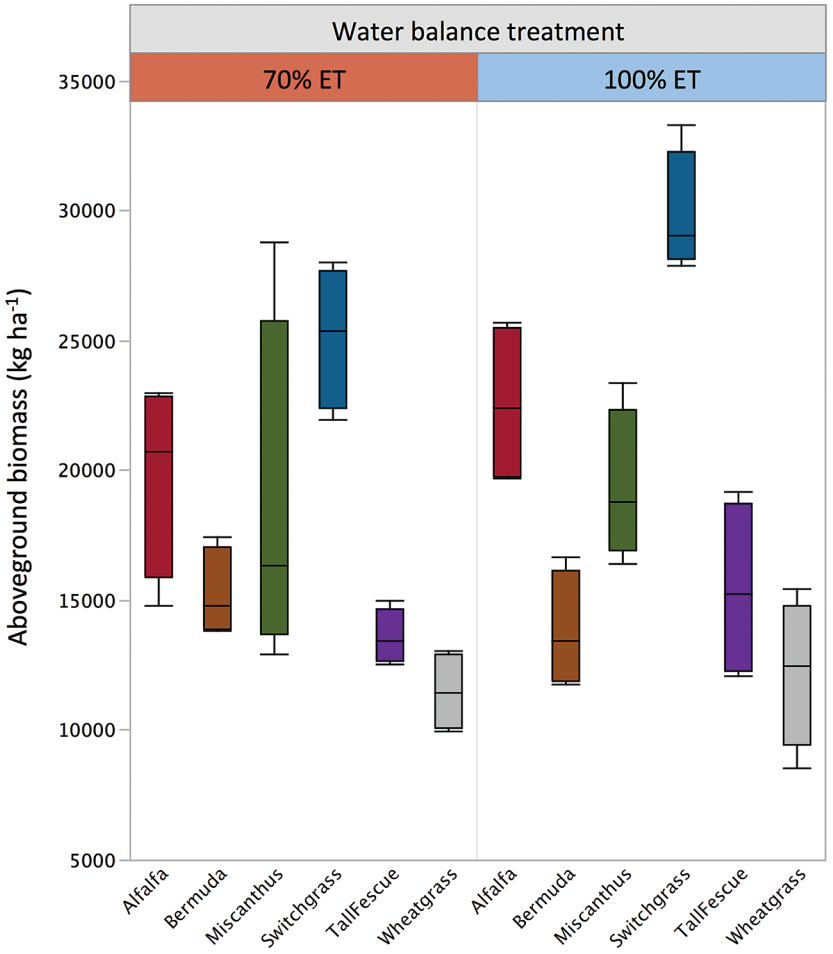

Figure S-2 Total aboveground biomass by species and treatment. Horizontal lines within the boxes represent median values. The ends of the boxes represent the 75th and 25th quantiles and whiskers span the entire data set including outliers.