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Stable-carbon isotope composition of Poaceae pollen: an assessment for reconstructing C_3 and C_4 grass abundance

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Abstract: Plants using the C_3 and C_4 photosynthetic pathways differ in carbon-isotope composition, and this difference offers a means to estimate the relative abundance of these two functional groups in the palaeorecord. We report here results of a study aiming to evaluate pollen $\delta^{13}C$ ($\delta^{13}C_p$) of Poaceae (the grass family) as a proxy indicator for palaeoecological studies. On average $\delta^{13}C_p$ differs by $\sim 13‰$ between modern C_3 (-22.6 to $-26.8‰$) and C_4 (-9.2 to $-17.7‰$) grass species. $\delta^{13}C_p$ is 1.2–3.7‰ more negative for modern grass pollen treated with the same protocol as for fossil samples than for untreated modern samples. $\delta^{13}C_p$ ranges from -20.1 to $-25.4‰$ for grass pollen in the middle-Holocene sediments from West Olaf Lake, located near the modern tallgrass prairie–forest ecotone in western Minnesota. We applied a two end-member mixing model to estimate fluctuations in C_3 and C_4 grass abundance around this lake. Both C_3 and C_4 grasses expanded relative to *Ambrosia* and *Artemisia* with the decline of aridity from 8000 to 4000 BP. C_3 grasses were generally more abundant than C_4 grasses throughout the middle Holocene, suggesting the presence of mixed-grass prairie around West Olaf Lake. The grass $\delta^{13}C_p$-based estimates of C_4 plant abundance were lower than charcoal $\delta^{13}C$-based estimates, probably reflecting different source areas of pollen and charcoal. Grass $\delta^{13}C_p$ also revealed greater submillennial-scale variability in C_3 and C_4 abundance than charcoal $\delta^{13}C$. These results suggest that grass $\delta^{13}C_p$ can provide palaeoenvironmental information not available from other proxy indicators.

Key words: Stable-carbon isotope, $\delta^{13}C$, Poaceae, C_3 and C_4 grasses, prairie, palaeoecology, pollen, palynology, modern analogue.

Introduction

Grass-dominated communities occupy a significant portion of Earth's land surface (Saugier and Roy, 2000) and play a key role in global biogeochemical cycles (eg, Knapp *et al.*, 2002). In these communities, C_3 and C_4 plants respond differently to variation in environmental factors, such as temperature, moisture availability and atmospheric CO_2 concentrations. For example, C_4 plants are physiologically more adapted to moisture deficits than C_3 plants, and C_3 plants will probably fare better than C_4 plants as atmospheric CO_2 concentrations rise (Long, 1999; Sage *et al.*, 1999). These two functional groups possess distinct carbon-isotope compositions primarily because they use different enzymes for initial carbon fixation: ribulose-1,5-bisphosphate for C_3 plants and phosphoenolpyruvate for C_4 plants. Ribulose-1,5-bisphosphate discriminates more strongly against ^{13}C than phosphoenolpyruvate (O'Leary, 1981), causing C_3 plants to be more depleted in ^{13}C than C_4 plants (Cerling, 1999). To estimate past fluctuations in the relative abundance of C_3 and C_4 plants, recent studies relied on $\delta^{13}C$ values from a variety of substrates,

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including bulk sediments (eg, Pack *et al.*, 2003), stalagmites (eg, Dorale *et al.*, 1992), charcoal (eg, Clark *et al.*, 2001, Nelson *et al.*, 2004), leafwaxes (eg, Huang *et al.*, 2001), palaeosols (eg, Fox and Koch, 2003) and phytoliths (eg, Smith and White, 2004). These studies provided insights into C_3 and C_4 plant response to past environmental changes, with implications for projecting how plant communities will respond to future environmental changes.

However, the utility of $\delta^{13}C$ analysis of the above substrates in palaeoenvironmental studies is limited by the fact that the C_3 and C_4 photosynthetic pathways are used by plant species other than grasses. For example, a $\delta^{13}C$ signature characteristic of C_3 plants could come from sources as different as mesophilic woody plants and drought-tolerant forbs. Furthermore, these substrates may be biased toward certain plant types. For example, charcoal $\delta^{13}C$ may be predisposed to reflect the $\delta^{13}C$ composition of fire-adapted woody species, because fire-intolerant species rarely burn and because woody taxa possess greater biomass than herbaceous species. Some of these problems may be circumvented by $\delta^{13}C$ analysis of Poaceae (the grass family) pollen, which is extremely resistant to degradation and produced in large quantities (Fægri *et al.*, 1989).

An initial study of pollen $\delta^{13}C$ ($\delta^{13}C_p$) of Poaceae showed that the $\delta^{13}C$ values of untreated pollen samples from different grass species are closely related to the $\delta^{13}C$ composition of the plant from which the pollen was produced (Amundson *et al.*, 1997). However, the standard procedures of pollen extraction altered the pollen–plant $\delta^{13}C$ relationship; in particular, treated pollen samples were 1.3–10.6‰ (5.0‰, on average) more negative than untreated pollen samples (Amundson *et al.*, 1997). This alteration was likely caused by incomplete removal of carbon-containing chemicals used in the acetolysis step of the pollen preparation, a result that has since been verified (Loader and Hemming, 2000). Pollen preparation methods that do not use carbon-containing chemicals and techniques for isolating grass pollen from other pollen and debris are now available (Loader and Hemming, 2004).

We conducted a study to evaluate the potential for using grass $\delta^{13}C_p$ to reconstruct C_3 and C_4 grass abundance. Here we show that the unique $\delta^{13}C$ signal of C_3 and C_4 grasses is retained in modern pollen and demonstrate the feasibility of isolating grass pollen from sediment samples for $\delta^{13}C$ analysis. We apply a mixing model derived from modern grass $\delta^{13}C_p$ data to fossil grass $\delta^{13}C_p$ data from West Olaf Lake, a site located near the ecotone of tallgrass prairie and deciduous forest in western Minnesota, to examine C_3 and C_4 grass response to increased moisture availability from 8000 to 4000 BP (Nelson *et al.*, 2004).

Materials and methods

We obtained modern pollen samples from eight C_3 and eight C_4 grass species (Table 1), many of which are native to tallgrass and mixed-grass prairies. These samples were provided by Dr E. Cushing's pollen laboratory at the University of Minnesota (six species) and the University of Illinois herbarium (eight species). Additionally, pollen from *Sorghum halepense* and *Dactylis glomerata* was purchased from Sigma-Aldrich. Untreated grass pollen from all 16 C_3 and C_4 grass species was suspended in ddH_2O (doubly distilled water: distilled water filtered through a Millipore Milli-Q water purification system) for isolation prior to $\delta^{13}C$ analysis. For the comparison of modern and fossil $\delta^{13}C_p$ data, modern pollen of eight grass species was treated with the protocol for processing sediment samples, as described below.

Sediment subsamples of $\sim$1 cm^3 were obtained from the middle-Holocene portion of a sediment core from West Olaf Lake (46° 37′ N, 96° 11′ W). The methods and results of previous multiproxy analyses at this site, including the charcoal $\delta^{13}C$ ($\delta^{13}C_{CHAR}$) data, have been published elsewhere (Nelson *et al.*, 2004). Several protocols for treating the sediment samples, including heavy-liquid separation, an acid-digestion technique (Loader and Hemming, 2000), and a technique developed to extract pollen for ^{14}C dating (Brown *et al.*, 1989), were explored to isolate grass-pollen grains from the sediment matrix without using carbon-containing chemicals. We employed a modified version of the Brown *et al.* (1989) protocol. Briefly, samples were treated with 10% HCl until effervescence stopped, 10% KOH for 10 min (heated at 90°C), concentrated HF for 40 min (heated at 90°C), and 10% HCl for 8 min (heated at 90°C). The residue was then washed through a 74 µm sieve (to remove particles >74 µm), treated with 50% H_2SO_4 for 2 h, washed onto a 12 µm sieve (to remove particles <12 µm) and treated with 5% Chlorox for 5 min. Following each chemical treatment, the residue was washed thoroughly with ddH_2O. Grass pollen ranges from 20 to 50 µm in size, and was therefore not lost during the sieving steps.

For both modern and fossil samples, we isolated grass-pollen grains by micromanipulation. Approximately 0.2 mL of the sample slurry was transferred to a 25 mm × 75 mm × 1 mm microscope slide divided into three squares with a thick layer of dry nail polish. Grass-pollen grains were visually identified using a Nikon E400 microscope equipped with a 20x objective (20.5 mm working distance). Grains were then suctioned into a borosilicate glass capillary tube with a CellTram Oil manual piston-pump attached to an Eppendorf TransferMan micromanipulator. The capillary tubes were pulled to a diameter of <5 µm using a Flaming/Brown micropipette puller (Sutter Instrument Company, model P-97) and broken back to a diameter of roughly 50 µm using clean forceps. Pollen grains and adhering debris were dispensed from the capillary tube into ddH_2O on an adjacent section of the microscope slide. The grass pollen was then isolated using micromanipulation and transferred to ddH_2O on the third section of the microscope slide. A new capillary tube was used for each sample. On average, 600 modern-grass pollen grains and 60 fossil-grass pollen grains could be isolated per hour with our micromanipulation system.

After isolation using the above procedure, each sample was visually examined and further purified when necessary. The pure grass pollen in ddH_2O was then transferred into preweighed 5 mm × 9 mm tin capsules using a glass pipette. The third section of the microscope slide and pipette were rinsed twice with ddH_2O to recover any grass-pollen grains adhering to the slide or pipette. The ddH_2O was then evaporated from the tin capsules at 70°C. The tin capsules were crushed, reweighed, and analysed for $\delta^{13}C$ in the Boston University Stable Isotope Laboratory.

The $\delta^{13}C$ samples were combusted in a Fisons NA1500 elemental analyser. The resulting CO_2 was introduced to a Finnigan Delta-S isotope-ratio mass spectrometer (IRMS) through a Finnigan Conflo II continuous flow device. The sensitivity of the elemental analyser was increased by using small-bore combustion/reduction columns and a small (6 mm × 340 mm) water trap. Small-bore columns magnify the chromatogram peaks for measurement by the mass spectrometer, whereas the small water trap increases the uniformity of gas flow. In order to compare samples analysed at different times, the $\delta^{13}C$ and CO_2 area data were blank-corrected

Table 1 $\delta^{13}C$ values of untreated and treated grass pollen exceeding the sample/blank CO_2 ratio threshold of 2.11

Grass species	Photosynthetic pathway	Untreated $\delta^{13}C$	Corrected untreated $\delta^{13}C$	Treated $\delta^{13}C$	Corrected treated $\delta^{13}C$	Untreated $\delta^{13}C$ – treated $\delta^{13}C$ (corrected)
Agropyron repens	C_3	−24.5 (0.3, 3)	−24.1	−27.5 (0.3, 3)	−27.1	3.0
Bromus inermis	C_3	−24.5 (0.6, 3)	−24.1	−25.9 (0.6, 3)	−25.5	1.4
Calamagrostis canadensis	C_3	–	–	NA	NA	
Dactylis glomerata	C_3	−23.8 (0.5, 4)	−22.6	–	–	
Elymus canadensis	C_3	−27.7 (0.6, 2)	−26.8	NA	NA	
Festuca elatior	C_3	−23.4 (0.8, 3)	−23.0	–	–	
Phalaris arundinacea	C_3	−27.6 (0.2, 2)	−26.6	NA	NA	
Stipa comata	C_3	−25.8 (0.5, 3)	−25.2	NA	NA	
C_3 average		−25.3	−24.6	−26.7	−26.3	
Andropogon gerardii	C_4	−12.5 (1.7, 3)	−12.1	NA	NA	
Bouteloua curtipendula	C_4	−11.1 (1.7, 3)	−10.1	NA	NA	
Cynodon dactylon	C_4	−18.1 (0.9, 2)	−17.7	–	–	
Sorghastrum nutans	C_4	−10.4 (2.0, 3)	−9.8	NA	NA	
Sorghum halepense	C_4	−12.7 (0.4, 9)	−11.4	−13.9 (NA, 1)	−12.6	1.2
Sorghum vulgare	C_4	−9.6 (0.3, 3)	−9.2	−13.3 (0.3, 3)	−12.9	3.7
Spartina pectinata	C_4	−11.4 (1.9, 2)	−11.2	NA	NA	
Zea mays	C_4	–	–	−13.5 (1.1, 3)	−13.1	
C_4 average		−12.3	−11.6	−13.6	−12.9	

Raw $\delta^{13}C$ values were corrected to a pre-industrial atmospheric $\delta^{13}C$ value of −6.3‰ based on pollen collection date.
In parentheses are the $\delta^{13}C$ standard deviation and number of replicate samples analysed for each species.
Untreated samples contained 200–420 grains and treated samples contained 600 pollen grains.
NA, not applicable; $\delta^{13}C$ analyses were not performed on these species because of lack of enough pollen from herbarium samples for treatment.
–, sample produced too much CO_2 (untreated *Zea mays*) or too little CO_2 (below the sample/blank CO_2 area threshold of 2.11) for accurate $\delta^{13}C$ analysis.
$\delta^{13}C$ data are expressed in the per mil notation relative to the VPDB standard. The untreated and treated $\delta^{13}C$ data are plotted in Figure 2.

(blanks were tin capsules containing no pollen). The $\delta^{13}C$ data were blank-corrected using a size series of a standard of known $\delta^{13}C$ composition (Fry *et al.*, 1996). The CO_2 area data (in volt-second units) were blank-corrected by converting the CO_2 area of each sample to a sample/blank ratio of CO_2 areas (unitless, hereafter referred to as the S/B CO_2 ratio). Normalizing the data of sample CO_2 area through blank correction was necessary given the small quantity of carbon in our samples and minor differences in blank CO_2 areas between analyses run at different times. The instrumental precision for $\delta^{13}C$ was <0.2‰ based on internal laboratory standards containing similar amounts of carbon as our samples. The precision of grass $\delta^{13}C_p$ is conservatively estimated to be < 1‰ based on the average standard deviations of replicate modern grass-pollen samples. For the comparison of modern and fossil $\delta^{13}C_p$ values, modern $\delta^{13}C_p$ values were corrected for the post-industrial depletion of atmospheric ^{13}C according to the grass pollen or plant collection dates and historical atmospheric $\delta^{13}C$ values (Friedli *et al.*, 1986; Francey *et al.*, 1999). Corrected $\delta^{13}C_p$ values are used for the remainder of this paper.

Results and discussion

Untreated modern-pollen $\delta^{13}C$ of C_3 and C_4 grasses

We estimated that samples containing ~200 untreated grass-pollen grains would approach the IRMS detection limit based on our measured weights of the pollen of various species and on the assumption that untreated grass pollen is ~45% carbon (Amundson *et al.*, 1997). To test this estimate, we prepared samples containing 100–420 untreated pollen grains from *Sorghum halepense* (11 samples) and *Dactylis glomerata* (five samples). The $\delta^{13}C$ values averaged -12.1 ± 2.0 and -23.0 ± 0.9‰ for *Sorghum halepense* and *Dactylis glomerata*, respectively (Figure 1). The $\delta^{13}C$ differences were small (generally <1‰) among the *Sorghum halepense* samples with a S/B CO_2 ratio ⩾2.55. However, the two *Sorghum halepense* samples with the lowest S/B CO_2 ratios (⩽1.90) yielded substantially more negative $\delta^{13}C$ values (−17.6 and −13.6‰). Similarly, the *Dactylis glomerata* sample with the lowest S/B CO_2 ratio (2.11) had the most negative $\delta^{13}C$ value (~1.7‰ lower than the average of the remaining samples), and this $\delta^{13}C$ deviation exceeded the standard deviation of the remaining samples (Table 1). To ensure that the sample size and associated CO_2 yield did not bias our results, we used the S/B CO_2 ratio of 2.11 as our cutoff threshold: samples with a S/B ratio ⩽2.11 were excluded from subsequent data analyses. Samples of *Sorghum halepense* and *Dactylis glomerata* with S/B ratios ⩽2.11 contained only 100 pollen grains, whereas those above this threshold contained ⩾200 grains. Thus for these two species, samples containing at least 200 grains of untreated pollen yielded reproducible $\delta^{13}C$ values.

To further assess $\delta^{13}C_p$ variation within C_3 and C_4 grass taxa, we analysed replicate samples (three per species) containing 210–420 pollen grains for the remaining 14 species. No *Calamagrostis canadensis* samples exceeded the threshold (S/B CO_2 ratio = 0.9–1.8), and the *Zea mays* samples produced too much CO_2 (S/B CO_2 ratio = 49.0–81.0) for accurate $\delta^{13}C_p$ analysis (Table 1). The $\delta^{13}C_p$ values of untreated pollen from the remaining C_3 and C_4 grass species, including *Sorghum halepense* and *Dactylis glomerata*, ranged from −22.6 to −26.8‰ (mean of −24.6‰) and from −9.2 to −17.7‰ (mean of −11.6‰), respectively (Table 1, Figure 2a), consistent with values typical of bulk-tissue samples of C_3 and C_4 grass species (Bender, 1971, Cerling, 1999). The mean $\delta^{13}C$ values differed significantly ($p < 0.00001$, $n = 14$) between untreated modern C_3 and C_4 grass species. Samples outside of the typical range of bulk-tissue samples included the C_4 grass *Cynodon dactylon* whose $\delta^{13}C_p$ value (-17.7 ± 0.9‰) was more negative than typical C_4 plants, and the C_4 grasses

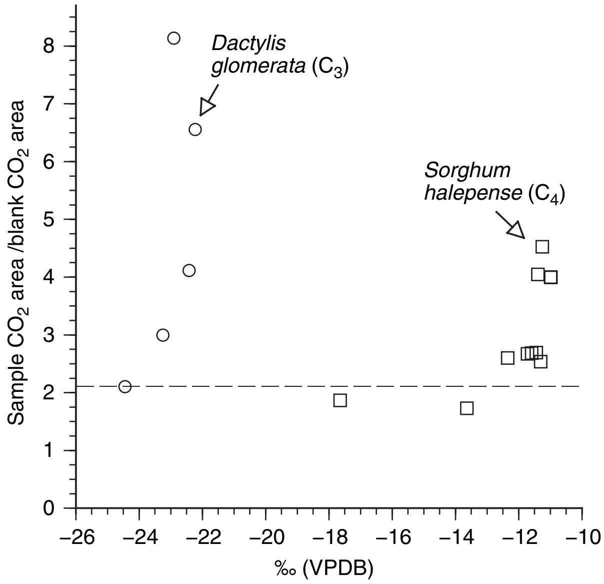

Figure 1 Sample/blank CO_2 area ratio and $\delta^{13}C$ of corrected untreated-pollen from a C_4 grass, *Sorghum halepense* (squares) and a C_3 grass, *Dactylis glomerata* (circles). Each sample contains between 100 and 400 pollen grains. The dashed line represents the sample/blank CO_2 ratio threshold of 2.11

Sorghum vulgare and *Sorghastrum nutans* whose $\delta^{13}C_p$ values (-9.2 ± 0.3‰ and -9.8 ± 2.0‰, respectively) were slightly more positive (Table 1). The reason for these exceptions is unclear.

Treated modern-pollen $\delta^{13}C$ of C_3 and C_4 grasses

Based upon the sample weights required to exceed the S/B CO_2 ratio of 2.11 for untreated modern grass-pollen grains and our measured weights of treated modern grass-pollen grains, we estimated that ~600 treated grass-pollen grains per sample would be sufficient for $\delta^{13}C$ analysis. However, only five out of eight treated pollen samples with 600 grains exceeded the S/B CO_2 ratio threshold. No samples of *Dactylis glomerata*, *Festuca elatior* or *Cynodon dactylon* exceeded this threshold (Table 1), even though their pollen grains did not appear smaller than those of the species above the threshold. For the remaining samples, $\delta^{13}C_p$ values fell within the range typical of bulk-tissue samples of C_3 and C_4 grass species (Figure 2b). The $\delta^{13}C_p$ means were -26.3 and -12.9‰ for treated C_3 and C_4 pollen samples, respectively, and these values differed significantly ($p < 0.05$, $n = 5$).

$\delta^{13}C_p$ values were lower for treated than untreated samples of the same species by 1.2–3.7‰ (Table 1). The differences were statistically significant for *Agropyron repens* ($p < 0.001$, $n = 6$), *Bromus inermis* ($p < 0.05$, $n = 6$) and *Sorghum vulgare* ($p < 0.0001$, $n = 6$). No statistical analysis could be performed for *Sorghum halepense* because only one treated sample exceeded the S/B CO_2 ratio threshold. Loader and Hemming (2000) reported a similar range of $\delta^{13}C$ reduction (1.8–3.6‰) in H_2SO_4-treated pollen samples of three plant species. Such an isotopic shift can be explained by the preferential removal of pollen-intine cellulose during treatment. Cellulose in the intine is more easily degraded and isotopically heavier than the extremely recalcitrant sporopollenin exine (Loader and Hemming, 2000).

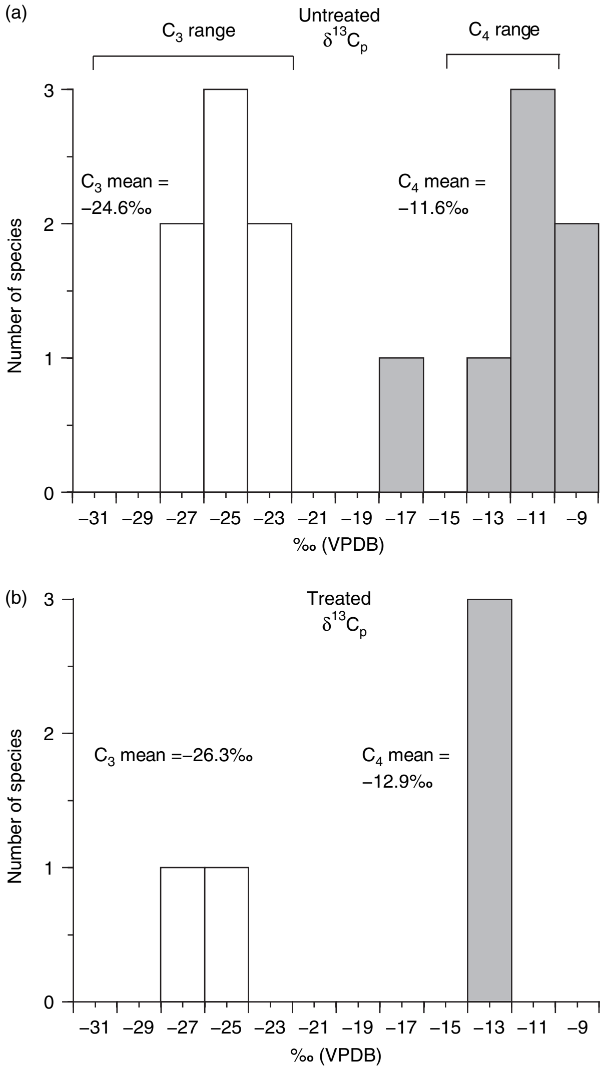

Figure 2 Mean $\delta^{13}C$ values (corrected for the post-industrial depletion of atmospheric ^{13}C) of untreated (a) and treated (b) modern pollen from C_3 and C_4 grass species listed in Table 1. The typical ranges of $\delta^{13}C$ values from C_3 and C_4 plants are indicated

Sediment grass-pollen $\delta^{13}C$

For the samples containing 600 grass pollen grains isolated from the sediments of West Olaf Lake, the S/B CO_2 ratio ranged from 2.08 to 8.58 with a mean of 3.59. $\delta^{13}C_p$ ranged from -20.1 to -25.4‰ for these samples (Figure 3). One sample did not exceed the S/B CO_2 ratio of 2.11, and it had a $\delta^{13}C_p$ value of -21.0‰.

To estimate C_4 grass abundance from $\delta^{13}C_p$ we used a simple mixing model with the equation (Clark *et al.*, 2001):

$$C_4 \text{ grass } \% = 100^* \, (\delta_s - \delta_3)/(\delta_4 - \delta_3),$$

where δ_s is the sample $\delta^{13}C_p$ value, and δ_3 and δ_4 are the $\delta^{13}C_p$ end members for C_3 and C_4 grasses, respectively. This mixing model assumes that treated pollen grains of C_3 and C_4 grasses contained the same amount of carbon. This assumption appears valid because the amount of CO_2 produced did not differ between treated C_3 and C_4 grass-pollen samples containing 600 grains ($p = 0.95$, $n = 10$; excluding *Zea mays*, which is known to have much larger pollen grains than other grass species).

Because of the $\delta^{13}C$ difference between treated and untreated samples, $\delta^{13}C_p$ values of treated grass-pollen samples should ideally be used as C_3 and C_4 end members. Given our low number of treated samples, we averaged the mean $\delta^{13}C_p$ values of (1) our treated grass pollen samples (Table 1, -26.3 and -12.9‰ for C_3 and C_4 grasses,

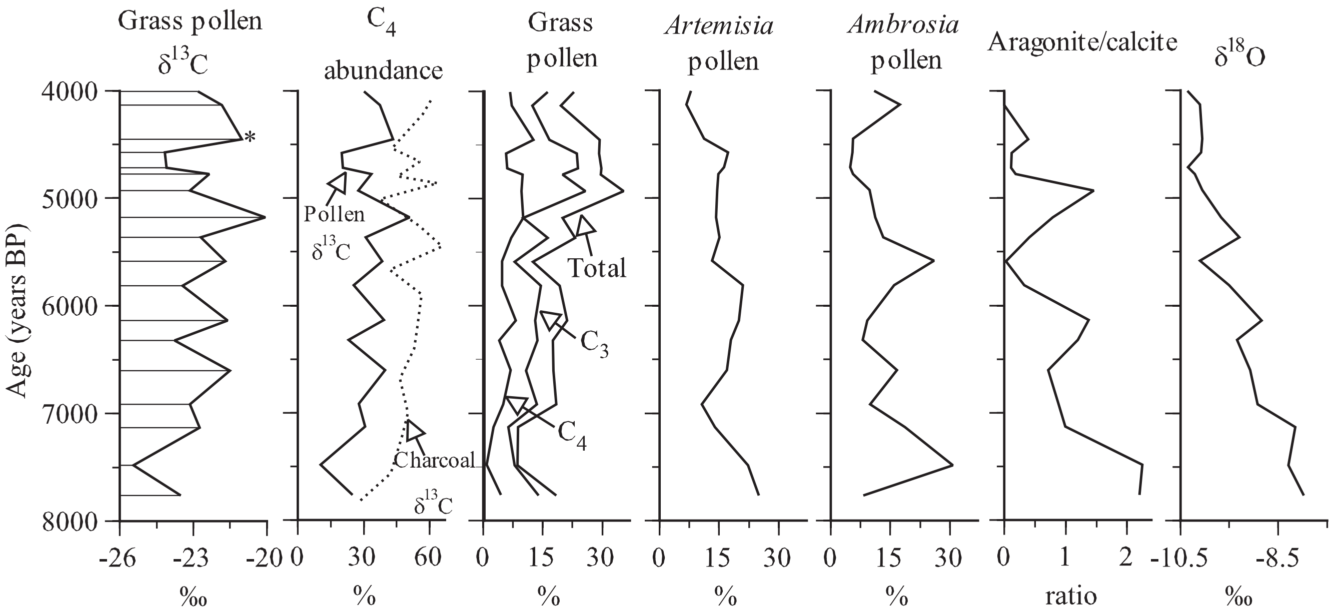

Figure 3 Multiproxy data from the sediments of West Olaf Lake and calcite δ^{18}O data from Steel Lake, ~ 120 km east of West Olaf Lake (Nelson *et al.*, 2004). The grass-pollen δ^{13}C data point labelled with * is below the sample/blank CO_2 area ratio threshold

respectively) and (2) our untreated pollen samples (Table 1, −24.6 and −11.6‰, respectively) after subtracting the mean treatment effect of 2.4‰ (based on our results and those of Loader and Hemming, 2000). Together these data yielded end member $\delta^{13}C_p$ values of −26.7 and −13.5‰ for C_3 and C_4 grasses, respectively. Estimates of C_4 grass abundance using end member values from (1) or (2) differ from the average of (1) and (2) by only ~ 3% C_4 grass abundance, suggesting that our low number of treated samples had minimal effect on mixing model results.

Using the mixing model with end member $\delta^{13}C_p$ values of −26.7 and −13.5‰ for C_3 and C_4 grasses, respectively, we estimated that C_4 grasses fluctuated between 10 and 51% of the total grass abundance. Thus C_3 grasses were generally more abundant than C_4 grasses during the middle Holocene at West Olaf Lake. Immediately prior to European settlement, C_4 grass abundance was highest in the tallgrass prairie near West Olaf Lake, with C_3 grasses becoming more important to the west in the mixed-grass prairie (Sage *et al.*, 1999). Therefore, during the middle Holocene when the regional climate was warmer and drier (Wright, 1992; Nelson *et al.*, 2004), the grasses around West Olaf Lake may have been those typical of the mixed-grass prairie. This interpretation is consistent with the relatively high abundance of *Artemisia* pollen in the middle-Holocene sediments of West Olaf Lake (Figure 3), as *Artemisia* herbs and shrubs are abundant in mixed-grass prairie (Coupland, 1950).

Within the middle Holocene, the abundance of C_4 grasses increases from a low of ~10% around 7500 BP to ~40% around 6600 BP (Figure 3). Between 6500 BP and 5400 BP C_4 grasses exhibit relatively small submillennial-scale fluctuations. Most of these fluctuations exceed 1‰, suggesting that they represent changes in the proportion of C_3 and C_4 grasses, rather than analytical noise. C_4 grass abundance reaches a peak of ~51% at 5200 BP before declining to a well-defined trough of ~20% around 4600 BP. After ~4400 BP C_4 grass abundance increases, followed by a decrease to 30% ~4000 BP.

These mixing-model results, in conjunction with pollen percentage data (Nelson *et al.*, 2004), allowed for estimation of the abundance of C_3 and C_4 grasses relative to other plants on the landscape around West Olaf Lake. The ability to estimate the relative abundance of C_3 and C_4 grasses is a significant advantage of grass $\delta^{13}C_p$. Such estimation cannot be made with δ^{13}C data from substrates such as charcoal, which may be produced by trees and forbs, in addition to grasses. An increase in grass pollen percentages occurred from 8000 to 4000 BP (Figure 3), and $\delta^{13}C_{CHAR}$ data suggest that this increase was primarily caused by the expansion of C_4 grasses from 8000 to 4000 BP (Nelson *et al.*, 2004). In contrast, our grass $\delta^{13}C_p$ data indicate that the increase was driven by both C_3 and C_4 grasses.

The expansion of both C_3 and C_4 grasses occurred in association with diminished abundance of *Artemisia* and *Ambrosia* (Figure 3). These changes together offer information on climatic conditions during the middle Holocene. *Artemisia* thrives under overall dry climatic conditions, and *Ambrosia* requires high interannual moisture variability during the summer, with dry summers creating bare ground for establishment when moisture becomes available (Grimm, 2001). In contrast, C_3 grasses require abundant cool-season (spring) moisture, and C_4 grasses require warm season (summer) moisture (Knapp and Medina, 1999). Thus the decreased abundance of *Artemisia* and *Ambrosia* in favour of both C_3 and C_4 grasses probably resulted from increased availability of moisture in both spring and summer and decreased moisture variability. This interpretation is supported by sediment mineralogy and oxygen-isotope data from the region (Figure 3; Nelson *et al.*, 2004). Together these data suggest that drought-like conditions and high interannual moisture variability (Grimm, 2001) prevented grasses from being abundant during the early portion of the middle Holocene.

C_4 estimates from grass $\delta^{13}C_p$ are generally lower than those from $\delta^{13}C_{CHAR}$ at West Olaf Lake (Figure 3). This discrepancy is too large to be caused entirely by the small effect (1‰ enrichment) of charring on δ^{13}C (eg, Beuning and Scott, 2002). Higher $\delta^{13}C_{CHAR}$-based C_4 estimates contradict the fact that charcoal sources include C_3 forbs and woody species, in addition to C_3 and C_4 grasses, which should result in lower $\delta^{13}C_{CHAR}$-based C_4 estimates. Several factors may account for this pattern. For example, lower C_4 estimates from grass $\delta^{13}C_p$ than from $\delta^{13}C_{CHAR}$ could result from a large contribution of charcoal from C_4 sedges (Cyperaceae family). However, sedge pollen averages only 5% of the middle-Holocene pollen spectra at West Olaf Lake (D.M. Nelson, unpublished data, 2004), and thus it is unlikely that C_4 sedges affect $\delta^{13}C_{CHAR}$. Alternatively, if aquatic C_3 grasses, such as *Zizania aquatica*, which are unlikely to burn, contributed a significant amount of pollen to West Olaf Lake, $\delta^{13}C_p$-based C_4 estimates could be lower than $\delta^{13}C_{CHAR}$-based estimates. However, this explanation is also unlikely given the facts that the relatively steep bathymetry of West Olaf Lake (Nelson *et al.*, 2004) restricts the potential size of the littoral zone, and that this relatively large lake (~58 ha) should have a large source area for grass pollen (Sugita, 1994). For these reasons, most of the middle-Holocene grass pollen was produced by terrestrial grasses, rather than by aquatic grasses living in the littoral zone.

Different source areas of pollen and charcoal are the most likely explanation for lower C_4 estimates from grass $\delta^{13}C_p$ than from $\delta^{13}C_{CHAR}$. In contrast to the wide dispersal ranges of grass pollen, the macroscopic charcoal particles (>180 μm) analysed for $\delta^{13}C$ are generally dispersed within ~100 m of the lakeshore (Lynch *et al.*, 2004). Thus if C_4 plant abundance was greater in the immediate vicinity of the lake than in the broader region, the estimates of C_4 plant abundance should be greater from $\delta^{13}C_{CHAR}$ than from grass $\delta^{13}C_p$. This explanation is counter-intuitive because woody C_3 species, such as *Quercus*, generally prefer relatively moist soils around lakes and streams. However, woody species were likely rare around West Olaf Lake during the middle Holocene because of high aridity (Nelson *et al.*, 2004), and the landscape around the lake is relatively flat and conducive to burning. Thus C_4 tallgrasses, which are drought-intolerant (Knapp and Medina, 1999) but burn easily, may have been more abundant on the relatively moist soils near West Olaf Lake than in the broader region where soils were likely drier. This interpretation is consistent with evidence for greater C_4 grass abundance on relatively moist sites in North American grassland communities (Paruelo and Lauenroth, 1996; Epstein *et al.*, 1998).

The temporal patterns in C_4 abundance also differ between $\delta^{13}C_p$-based and $\delta^{13}C_{CHAR}$-based estimates. For example, the broad trough in grass $\delta^{13}C_p$-based C_4 abundance around 4600 BP does not occur in $\delta^{13}C_{CHAR}$-based C_4 abundance. In addition, the submillennial-scale variability is generally greater in grass $\delta^{13}C_p$ than $\delta^{13}C_{CHAR}$ C_4 abundance estimates, particularly prior to ~5500 BP (Figure 3). This pattern may indicate C_3 and C_4 grass fluctuations in response to climatic variations within the middle Holocene. However, we do not attempt to decipher the palaeoenvironmental significance of these submillennial-scale grass $\delta^{13}C_p$ fluctuations, because the temporal resolution of our $\delta^{13}C_p$ record remains coarse and the fluctuations are mostly represented by single data points. Nevertheless, the submillennial-scale differences between $\delta^{13}C_p$ and $\delta^{13}C_{CHAR}$ C_4 estimates suggest that grass $\delta^{13}C_p$ holds potential for improving palaeovegetational and palaeoclimatic reconstructions.

Conclusions

Grass pollen is commonly a major constituent of pollen records, exceeding 25% of the total pollen abundance in those from the grassland biome (eg, Laird *et al.*, 1996; Wooller *et al.*, 2003). With few exceptions (eg, Wooller *et al.*, 2000, 2003; Beuning *et al.*, 2003), little is known about the variations of grass functional types in the palaeorecord because pollen of most grass taxa is morphologically indistinguishable, making it difficult to resolve grass-pollen grains beyond the family level (Fægri *et al.*, 1989). Our results demonstrate that it is feasible to estimate the relative abundance of C_3 and C_4 grass species from grass $\delta^{13}C_p$ and that paleoenvironmental reconstructions of C_3 and C_4 grass dynamics can be enhanced with fossil-pollen $\delta^{13}C_p$ data. Grass $\delta^{13}C_p$ improves upon $\delta^{13}C$ data from other substrates (eg, charcoal, soils), which cannot unequivocally detect fluctuations in the relative abundance of C_3 and C_4 grasses.

The limiting factor in grass $\delta^{13}C_p$ analysis is the time required to manually isolate fossil grass-pollen grains (~10 h per sample). However, emerging techniques are expected to expedite sample preparation. In particular, modifying versions of laser extraction and combustion (eg, Wieser and Brand, 1999) or moving-wire (eg, Sessions *et al.*, 2005) techniques could greatly reduce the amount of carbon and thus the pollen isolation time required per sample (Loader and Hemming, 2004). For routine applications of grass $\delta^{13}C_p$ analysis in palaeoenvironmental reconstruction, it is also important to further constrain the C_3 and C_4 grass $\delta^{13}C_p$ end members. A thorough evaluation of end members should include an expanded data set of treated $\delta^{13}C_p$ values from modern C_3 and C_4 grass species, as well as $\delta^{13}C$ values of grass pollen in surface lake-sediment samples from regions spanning gradients of C_3 and C_4 grass abundance.

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