EFFECTS OF WOODY LEGUME (*PROSOPIS GLANDULOSA)* ENCROACHMENT ON NITROGEN FIXATION, STORAGE AND GAS LOSS IN A SUBTROPICAL, SEMI-ARID SAVANNA

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EFFECTS OF WOODY LEGUME (*PROSOPIS GLANDULOSA)* ENCROACHMENT ON NITROGEN FIXATION, STORAGE AND GAS LOSS IN A SUBTROPICAL, SEMI-ARID SAVANNA

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Woody encroachment, an increase in abundance and density of woody plants, currently affects millions of hectares of grassland, savanna, desert and other dryland systems globally. By altering the structure and function of vegetative communities, the process significantly affects human land use, biodiversity, carbon storage and biogeochemical cycling. In the tropics and subtropics, encroachment is often driven by leguminous trees. These trees support increased rates of nitrogen (N) fixation, significantly altering ecosystem N cycling, accumulation and loss. The genus *Prosopis* is one of the most common encroaching species throughout the tropics, and the N-fixing tree *Prosopis glandulosa* (honey mesquite) affects more than 40 million hectares across the southern US alone.

This thesis investigates the effects of *Prosopis* encroachment on ecosystem N cycling in a subtropical, semi-arid savanna in south Texas. Previous studies have identified increases in soil total N storage, inorganic N concentrations, and soil N cycling rates in *Prosopis*-encroached soils compared with adjacent remnant grasslands. This research characterizes the magnitude and dynamics of three key processes- symbiotic N fixation, N accrual in soil and biomass, and emissions of N trace gases and N_2.

Chapter 1 applies a new methodological approach comparing N isotopic composition ($\delta^{15}N$) of foliage, xylem sap and plant-available soil N to investigate patterns of N fixation along a space-for-time chronosequence of encroachment. Data supports increased rates of N fixation with tree age, and seasonal variability. This approach also demonstrates that the foliar $\delta^{15}N$ method typically applied to estimate rates of N fixation in ecosystems such as this is not reliable. Chapter 2 summarizes two years of field measurements of a complete suite of N trace gases (ammonia, nitrous oxide, nitric oxide and other oxidized N compounds) from encroached and unencroached soils. Measurements revealed no effect of encroachment on upland N trace gas emissions. Rather, abiotic temperature and soil wetting dynamics (including re-wetting interval) were much stronger controllers of total N flux. Chapter 3 used excised-core incubation experiments to determine whether denitrification to N_2 gas might be a significant but unappreciated gas loss pathway. The outcome of these incubations showed that under field-realistic abiotic conditions, N_2 flux is likely to be low or absent.

Chapter 4 uses the chronosequence to infer rates of N fixation over time by constructing a N mass balance for individual *Prosopis* clusters. These calculations combine measured rates of soil N accrual with modeled or estimated rates of biomass N accrual, N deposition and N gas loss to estimate a rate for symbiotic N fixation of 0.15 kg of N per individual tree per year of growth. Scaled to landscape level, this corresponds to fixation inputs of 9.2 kg N per hectare per year.

Taken together, these results suggest that this ecosystem is still in a stage of N accumulation during ongoing *Prosopis* encroachment, experiencing net N storage in soil and biomass that outweighs gaseous losses. Given the large scale of *Prosopis* encroachment in subtropical North America, this finding implies significant and continuing increases in soil N stocks may occur throughout this region.

BIOGRAPHICAL SKETCH

Fiona Soper was born in Harare, Zimbabwe, before moving to the Northwest Territories and Nunavut, Canada, on to Australia and finally to the USA. Fiona attended Stuartholme School in Brisbane, Australia as a boarder and was an active member of the debating team, played the flute in the orchestra and was a member of the athletics team. Fiona completed her Bachelor of Science at the University of Queensland, majoring in Botany, from 2005-2007, and spent one semester abroad at Lund University, Sweden. As a part of the Advanced Study Program in Science, she began research in the lab of Susanne Schmidt and later completed her Honors thesis (2009), focusing on the role of organic nitrogen uptake in invasive species colonization of nutrient poor soils. During her time at UQ, Fiona also spent time working at the Centre For Integrative Legume Research and began to develop an interest in the topic of symbiotic nitrogen fixation.

After graduating, Fiona served as a Research Projects Officer with the Commonwealth Scientific and Industrial Research Organization, (CSIRO, Plant Industry Division), as part of a team investigating the genetic determinants of virulence in plant fungal pathogens. Fiona then returned to the University of Queensland, working as a research technician in the Schmidt lab investigating the role of nitrogen supply on root architecture in sugarcane.

Fiona began her PhD in the Department of Ecology and Evolutionary Biology at Cornell University in 2010 in the lab of Professor Jed Sparks. During this program, she received several honors including an American Australian Association Education Fellowship, a Betty Miller Francis ’47 Fellowship and the Billings Award from the Ecological Society of America. At Cornell, Fiona was very involved in the Biogeochemistry program (serving as President of the

Biogeochemistry Graduate Student Association) and President of the Cornell Chapter of Sigma Xi, as well as serving on several seminar, symposium and grant review committees. During this time, she maintained an ongoing collaboration with the Schmidt lab, publishing a study on woody plant nitrogen relations along a continental rainfall in northern Australia.

During her time in Ithaca, Fiona taught rock climbing for Cornell Outdoor Education, enjoyed hiking, sailing and kayaking Cayuga Lake and took far more physical education classes than academic ones.

For my mother, Val

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opportunities from an early age. She has set an outstanding example of uncomplaining achievement- raising me largely alone across three continents, sacrificing to provide me with an excellent education, completing two degrees in the process and becoming the most well traveled person I know. Her unfailing support and pride have been the largest driving force in my life.

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CHAPTER ONE

INVESTIGATING PATTERNS OF SYMBIOTIC NITROGEN FIXATION DURING VEGETATION CHANGE FROM GRASSLAND TO WOODLAND USING FINE SCALE $\delta^{15}N$ MEASUREMENTS

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Abstract

Biological nitrogen fixation (BNF) in woody plants is often investigated using foliar measurements of $\delta^{15}N$ and is of particular interest in ecosystems experiencing increases in BNF due to woody plant encroachment. We sampled $\delta^{15}N$ along the entire N uptake pathway including soil solution, xylem sap, and foliage to: 1) test assumptions inherent to the use of foliar $\delta^{15}N$ as a proxy for BNF, 2) determine whether seasonal divergences occur between $\delta^{15}N_{xylem\ sap}$ and $\delta^{15}N_{soil\ inorganic\ N}$ that could be used to infer variation in BNF, and 3) assess patterns of $\delta^{15}N$ with tree age as indicators of shifting BNF or N cycling. Measurements of woody N-fixing *Prosopis glandulosa* and paired reference non-fixing *Zanthoxylum fagara* at three seasonal time points showed that $\delta^{15}N_{soil\ inorganic\ N}$ varied temporally and spatially between species. Fractionation between xylem and foliar $\delta^{15}N$ was consistently opposite in direction between species and varied on average by 2.4‰. Accounting for these sources of variation caused percent nitrogen derived from fixation values for *Prosopis* to vary by up to ~70%. Soil-xylem $\delta^{15}N$ separation varied temporally and increased with *Prosopis* age, suggesting seasonal variation in N cycling and BNF and potential long-term increases in BNF not apparent through foliar sampling alone.

Introduction

Biological nitrogen fixation (BNF) in natural ecosystems remains one of the most poorly estimated terms in the global N cycle, primarily because methodological challenges limit accurate quantification (Galloway *et al.* 2004; Cleveland *et al.* 2010). This is especially true for symbiotic N fixation in woody perennials, the dominant source of fixed N in many ecosystems (Boddey *et al.* 2000). This difficulty limits our ability to investigate controls over fixation rates

and their sensitivity to environmental variation (Vitousek *et al.* 2002; Sprent 2005; Vitousek *et al.* 2013), which in turn limits extrapolation of BNF rates to large scales.

N stable isotope ratios have long been recognized as a powerful tool for tracing BNF, because N generated through BNF tends to differ in the ratio of heavy (^{15}N) to light (^{14}N) isotopes ($\delta^{15}N$) compared to other sources (Shearer and Kohl 1986; Boddey *et al.* 2000). One common application in natural systems is the ^{15}N natural abundance method, that compares $\delta^{15}N$ of plant tissue (in practice, usually foliage) of target fixing species and non-fixing reference plants in a common environment (Shearer and Kohl 1986; Boddey *et al.* 2000). Because the non-fixing plant has only one potential N source (soil-derived) while the fixer has two (atmospheric N_2 and soil-derived N), the difference in $\delta^{15}N_{foliage}$ is assumed to be directly proportional to the proportion of N obtained from fixation (Shearer and Kohl 1986; Robinson 2001). This method has proved useful for application in relatively simple cropping or agroforestry systems, but presents several challenges that limit its application in natural environments (Boddey *et al.* 2000). First, the method necessarily assumes that the soil N source is isotopically consistent spatially and through time (Shearer and Kohl 1986; Handley and Scrimgeour 1997). However, localised differences in N cycling, turnover rates between rhizospheres and the accumulation of isotopically-light N from legume litter inputs are likely to alter the isotopic composition of soil solution N both spatially and temporally in a heterogeneous landscape (Shearer and Kohl 1989; Boddey *et al.* 2000; Yoneyama *et al.* 2000; Boutton and Liao 2010; Bai *et al.* 2013). Second, it may be difficult or even impossible to locate multiple suitable reference species in many environments, either because ecologically similar woody species do not co-occur or because fixing and non-fixing species overlap in their $\delta^{15}N$ values (Högberg 1997; Boddey *et al.* 2000). Third, it is necessary to assume that isotopic fractionation during plant uptake, assimilation and

turnover is either small or comparable between unrelated species. This is despite evidence that differential mycorrhizal colonisation affects fractionation during N uptake (Handley and Raven 1992; Evans 2001) and that the site of nitrate assimilation (roots or shoots) is variable between species and affects tissue $\delta^{15}N$ values (Evans 2001). Finally, the temporal resolution of the method is limited; $\delta^{15}N_{foliage}$ represents an integrated isotopic signature of N laid down during leaf formation and subsequently altered by poorly quantified turnover and resorption during leaf lifetime, on scales of months to years (Shearer and Kohl 1986; Gebauer and Schulze 1991; Evans 2001). Leaf lifetime often cannot be readily controlled for between species during sampling, especially in the tropics where many woody species exist as evergreens (Eamus and Prior 2001). This resolution precludes the testing of hypotheses about abiotic or phenological controls over N fixation that might produce variation at sub-annual scales.

We hypothesized that more explicit sampling of $\delta^{15}N$ of N pools along the pathway from the soil solution to leaves may offer a mechanism to test assumptions of the foliar $\delta^{15}N$ method, overcome certain limitations and provide novel information to address hypotheses about N fixation in natural systems. Specifically, we measured the $\delta^{15}N$ of the plant-accessible soil N pool, plant xylem sap and foliage at sub-annual scales. Accurate isotopic measurement of low concentrations of inorganic N ($\delta^{15}N_{soil\ inorganic\ N}$) in soil extracts is now feasible (Stephan and Kavanagh 2009; Lachouani *et al.* 2010; Bell 2012). Xylem sap ($\delta^{15}N_{xylem\ sap}$), a mixture of recently-acquired fixed and soil-derived N (and likely some resorbed N) (Yoneyama 1995; Yoneyama *et al.* 2000; Canton *et al.* 2005; Millard and Grelet 2010), precedes fractionation steps involved in leaf incorporation and turnover. Divergence between soil and xylem $\delta^{15}N$ values ($\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$) could be compared between individuals, or in a single individual across time, as a potential measure of relative fixed N contribution, removing direct comparisons to

reference plants. As both soil inorganic and xylem N pools have turnover times on the order of minutes/hours to perhaps days (Hayashi *et al.* 1997; Stark and Hart 1997; Booth *et al.* 2005), they have the potential to provide much finer temporal resolution than foliage. $\delta^{15}N_{\text{xylem sap}}$ values from woody plants are scarce in the literature, but evidence from N-fixing and non-fixing trees measured along an Australian continental rainfall gradient shows significant differences in the $\delta^{15}N_{\text{xylem sap}}$ between fixers and non-fixers and suggest that $\delta^{15}N_{\text{xylem sap}}$ may provide useful isotopic information not available from foliar sampling (Soper *et al.* 2015).

Encroachment of the N-fixing tree legume *Prosopis glandulosa* (honey mesquite) into grasslands of the Rio Grande Plains offers a useful setting in which to test this extended sampling approach. *Prosopis* is widespread throughout the southern USA and is the dominant N-fixer over more than 38 million hectares (Van Auken 2000). However, its root nodules are difficult to recover in the field and the acetylene reduction method has not been applied successfully (Johnson and Mayeux 1990; Felker 2009), making isotopic approaches particularly appealing. At the site of this study, *Prosopis* seedlings recruit into remnant grasslands of known soil composition and, over time, develop a woody understory forming 'islands' embedded within a relatively homogenous soil matrix. These single-*Prosopis* clusters can be accurately aged (Archer *et al.* 1988; Flinn *et al.* 1994, Stoker, 1997) and the result is a chronosequence of time since symbiotic BNF introduction. Over this encroachment chronosequence, well-characterized shifts in soil parameters such as increases in inorganic N availability and available phosphorus have been observed (Virginia and Jarrell 1983; Hibbard *et al.* 2001; Boutton and Liao 2010; Kantola 2012). These parameters are known to suppress (in the case of inorganic N) or increase (P) nodule formation and N fixation in other woody perennials (Hartwig 1998; Mortier *et al.* 2011; Augusto *et al.* 2013), and variation in this system could be used to test hypotheses about

long term controls over fixation during succession. Finally, *Prosopis* fixation rates may vary on seasonal time scales in response to pronounced changes in temperature and rainfall (Archer *et al.* 2001), supported by observations of seasonal variation in leaf N isotope composition (Bai *et al.* 2009) and root nodule morphology and abundance (Johnson and Mayeux 1990; Zitzer *et al.* 1996).

In this study, we applied expanded isotopic sampling to investigate N fixation dynamics in *Prosopis glandulosa* (honey mesquite) in a subtropical, semi-arid woodland. *Zanthoxylum fagara* (lime prickly ash), an associated woody non-fixer, was investigated as a control for variation that may arise from processes other than fixation. The goals were to: a) test the assumptions of the foliar $\delta^{15}N$ method, specifically that $\delta^{15}N_{soil\ inorganic\ N}$, soil-plant fractionation events and plant internal N fractionation are comparable between plant species and over time, b) to investigate changes in *Prosopis* $\delta^{15}N_{xylem\ sap}$ sub-annually in relation to fluctuations in $\delta^{15}N_{soil\ inorganic\ N}$, and determine whether these differences could be used to infer sub-annual variation in N fixation, and c) use a woody encroachment chronosequence to determine whether measures of $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem}$ show patterns with tree age suggestive of shifting N fixation or N cycling.

Materials and Methods

Study site

Sampling was conducted at the Texas AgriLife La Copita Research Area (27°40'N, 98°12'W), 65 km west of Corpus Christi, Texas, USA on the Rio Grande Plains during 2011-2013. The site consists of savanna parkland with discrete woody patches dominated by one or more *Prosopis glandulosa* (Torr.) var *glandulosa* (honey mesquite, referred to here as *Prosopis*) individuals,

usually embedded in a matrix of C_4 grasses and bare ground. *Prosopis* is a leguminous tree in the family Fabaceae, and generally considered to be capable of substantial rates of BNF (Shearer *et al.* 1983). Though it nodulates readily under both glasshouse and natural conditions (including at this site) its nodules are difficult to recover under field settings, partly because they can occur deep (>1 m) in the soil profile (Virginia *et al.* 1984; Johnson and Mayeux 1990; Zitzer *et al.* 1996). *Prosopis* is winter deciduous and individuals in this study replaced their leaves completely between January and March of both 2012 and 2013. Foliar samplings taken between replacement events are referred to here as 'leaf cohorts'. *Zanthoxylum fagara* (L.) (lime prickly ash, referred to hereafter as *Zanthoxylum*) is in the family Rutaceae and is the most abundant non-fixing woody plant at the site. It is hypothesized that *Zanthoxylum* individuals recruit only in the understory of a *Prosopis* nurse plant, but they may persist long after the *Prosopis* dies and many large free standing individuals exist at this site with no evidence of the original nurse plant present. *Zanthoxylum* is evergreen, with leaf turnover occurring throughout the growing season (March-October) and has an average leaf lifetime of 116 days (Nelson *et al.* 2002).

The climate is subtropical with typically warm, moist winters and hot, dry summers. Mean annual precipitation is 680 mm, occurring year round with maxima in May and September, and minima in July/August. During the study period (August 2011-August 2012) the site was drier that average, experiencing 329 mm annual rainfall. Mean annual temperature is 22.4°C with an average growing season of 289 days. Air temperature and precipitation data were sampled every 10 mins by a weather monitoring system inside the sampling plot (NexSens Technology, Fairborn, OH). Soils at the site are sandy loams (Typic and Pachic Argiustolls) with little to no topography. The sampling plot measured 200 x 100 m and has been fenced to exclude livestock grazing since 1985.

Plant tissue sampling

Nineteen *Prosopis* individuals and ten *Zanthoxylum* individuals were sampled for xylem and foliage in January, May and August of 2012, with a smaller subset of individuals (n=12 and n=6 for *Prosopis* and *Zanthoxylum* respectively) sampled in August 2011. Additional foliage was sampled from the same trees in February, May and August of 2013. *Prosopis* tree age ranged from 25 to 136 years, calculated by basal diameter calibrated for upland clusters at this site (Flinn *et al.* 1994; Stoker 1997). Sampled individuals typically had a diverse woody understory and were selected to have no other potentially N-fixing woody plants within 10 m of the bole. *Zanthoxylum* individuals were located at least 6 m from the drip line of the nearest potentially N-fixing woody plant. *Prosopis* is characteristically the first species to establish in the former C_4 grasslands at the site (Bai *et al.* 2012), so that tree age corresponds to the number of years the soil immediately surrounding each tree has been experiencing symbiotically-fixed N inputs.

Foliage was sampled at the four cardinal locations from each tree, though some or all individuals of *Prosopis* had no attached foliage at the January and February sampling points. In August 2013, additional foliar samples were taken from six individuals of other non-fixing woody species *Condalia hookeri* (M.C. Johnst.), *Diospyros texana* (Scheele), *Celtis pallida* (Torr.), and an N-fixing tree *Acacia farnesiana* (L.) Willd. located in the same plot. Individuals were located at least 6 m from the drip line of the nearest potentially N-fixing woody plant. Samples were dried for three days at 60°C, ground and analyzed for $\delta^{15}N$ using a continuous flow isotope ratio mass spectrometer (IRMS) (Model Delta V Advantage; Thermo-Scientific, Bremen, Germany). All isotope analyses were conducted at the Cornell University Stable Isotope Laboratory (COIL).

Branch xylem sap was collected as in Pate *et al.* (1994), with care taken to fully remove phloem tissue before extraction. Collection occurred between 4:00 and 9:00 am to control for potential diel variation in N metabolism (Peuke *et al.* 2013). Sap was stored on ice and frozen prior to analysis. Sap from 3-6 branches was bulked into a single sample per tree and 25-420 μl of xylem sap was freeze-dried and analyzed for $\delta^{15}N$. A subsample of xylem sap from each time point was assayed for ATP using a Sigma ATP Bioluminescent Assay Kit (Sigma Aldrich, St Louis, USA) to check for phloem sap contamination (Rennenberg *et al.* 1996).

Soil sampling and analysis

At the same time points as xylem samples, two soil cores were taken beneath each tree, within 50 cm of the bole, and divided into two depth increments (0-15 cm and 15-30 cm). The majority of fine roots, responsible for nutrient uptake from soil (Eissenstat 1992), occur in the top 30 cm of soil for *Prosopis* (Ansley *et al.* 2014), and for woody elements overall greatest total root biomass occurs with the top 15 cm (Midwood *et al.* 1998). Soil was passed through a 2 mm screen to remove large organic fragments and was then extracted within 4 h of collection by shaking for 1 h in 1:4 (w/v) 1 M KCl (Fisher Scientific, Pittsburg, PA). The supernatant was filtered through Whatman 41 cellulose filter paper (GE Healthcare Life Sciences, Piscataway, NJ) and frozen prior to analysis. Sub-samples of cores were weighed before and after drying for three days at 105°C to determine percent moisture. NH_4^+ and NO_3^- concentrations in KCl extracts were analyzed colorimetrically using a Lachat flow injection analyzer (Hach Co., Loveland, USA) at the Cary Institute of Ecosystem Studies Rachel L. Carson Analytical Facility (Milbrook, NY).

To determine $\delta^{15}N$ of NO_3^- and NH_4^+, KCl extracts from two soil cores per tree (0-15 cm) were bulked and a subsample of individuals (10 *Prosopis*, 7 *Zanthoxylum*) were analyzed for

each time point. $\delta^{15}N$ of NO_3^- was measured using the microbial denitrifier method at the Facility for Isotope Ratio Mass Spectrometry at University of California, Riverside, controlling for trace NO_3^- contamination in the KCl reagent (Bell 2012). A microdiffusion method was used to determine $\delta^{15}N$ of NH_4^+, as per Stephan and Kavanagh (2009), using a diffusion volume of 40 ml in 90 ml polypropylene containers. Samples were diffused for 7 days at 25 °C and filters were analyzed for $\delta^{15}N$ at COIL. A minimum recovery cut off of >99% of sample N was applied for all samples described here. Filter papers used during extraction were identified as a source of NH_4^+ contamination and controlled for as per Stephan and Kavanagh (2009), using a minimum of five replicate controls matched to each individual batch of filter paper used during extraction. $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ values were weighted by relative concentration in the soil KCl extracts in order to generate a single value for $\delta^{15}N_{\text{soil inorganic N}}$.

Calculation of %Ndfa values

As a theoretical exercise, percent N derived from fixation (%Ndfa) was calculated for *Prosopis* using the $\delta^{15}N$ natural abundance technique (Shearer and Kohl 1986) as commonly applied to foliar measurements. This value was then adjusted to account for observed variation in soil solution $\delta^{15}N$ and $\Delta^{15}N_{\text{xylem-foliage}}$ (both assumed to be constant in the conventional method) between species for two sampling dates. This exercise was intended to indicate the broad sensitivity of %Ndfa to assumptions of the method (rather than absolute values), as application of the technique did not conform to suggested conditions including use of multiple non-fixing reference species (*Zanthoxylum*) and a separation between species of >5 ‰ (Shearer and Kohl 1986; Högberg 1997).

Shearer *et al.* (1983)'s values for *Prosopis glandulosa* leaves grown hydroponically without added N (-1.3 ± 0.5‰, *B*) were used in calculations and were in agreement with values generated using *Prosopis* seed and rhizobial inoculum from the study site (-1.0 ± 0.2‰, Soper, unpublished). To account for the lower baseline soil solution $\delta^{15}N$ in *Zanthoxylum*, the divergence between species $\delta^{15}N_{soil\ inorganic\ N}$ (averaged over the Jan-Aug 2012 sampling period), was subtracted from $\delta^{15}N_{foliage}$. To account for xylem-foliar fractionation, a Δ value of 1.07‰ (taken from field observations for *Prosopis* in this study) was applied to *B* and species- and sampling point-specific $\Delta^{15}N_{xylem\text{-}foliage}$ was subtracted from $\delta^{15}N_{foliage}$. When both corrections were applied together, sampling-date specific $\delta^{15}N_{soil\ inorganic\ N}$ values were used in order to keep temporal scales of N uptake and integration comparable between source and sink. Error propagation was performed as per Shearer *et al.* (1983).

Statistical Analysis

Statistical analyses were performed in R (R Core Team 2012) and JMP Pro v10.0.0 (SAS Institute, Cary, NC, USA). Significance was set at $\alpha=0.05$, unless otherwise indicated.

Pooled t-tests were used to determine significant differences between the two species for $\delta^{15}N_{\ foliage}$, $\delta^{15}N_{soil\ inorganic\ N}$, $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ and $\Delta^{15}N_{xylem\text{-}foliage}$ at each time point, after testing for homogeneity of variance and normality. Paired t-tests were used to determine significance of differences ($\Delta^{15}N$) between N pools.

Simple linear regressions were used to determine relationships between $\delta^{15}N_{xylem\ sap}$ between years for individual plants; $\delta^{15}N_{foliage}$ between years for individual plants, plant age and soil inorganic N concentrations, $\delta^{15}N_{xylem\ sap}$, $\delta^{15}N_{foliage,}$ $\delta^{15}N_{soil\ inorganic\ N}$ and $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (*Prosopis* only); and soil inorganic N concentrations and $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem\ sap}$.

Distribution of residuals was examined to confirm appropriateness of fit. Mixed effects models were used to analyze date effects and generate P values, with tree identity incorporated as a random effect to account for repeated sampling of individuals. Tukey's HSD post-hoc test was used to determine significance of differences between individual time points. For $\delta^{15}N_{xylem\ sap}$ and $\delta^{15}N_{foliage}$, a mixed effect model was created using sampling date and tree age as fixed effects and tree identity as a random effect to generate restricted maximum likelihood variance components estimates. One-way analysis of variance (ANOVA) was used to compare $\delta^{15}N_{foliage}$ between multiple species.

Results

Temporal and interspecies variability in $\delta^{15}N$ and changes between N pools

In both *Prosopis* and *Zanthoxylum*, $\delta^{15}N_{soil\ inorganic\ N}$ was enriched with respect to $\delta^{15}N_{xylem}$ (Fig 1.1, Table 1). In *Zanthoxylum*, both $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem}$ showed variation over time of up to 1.92‰ and 2.23‰, respectively ($P<0.0099$ and $P<0.0001$, Fig 1.1). Separation between the two pools ($\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$) was also variable through time ($P<0.04$), ranging from 1.16‰ in May up to 3.35‰ in August 2012 (Fig 1.1, Table 1.1). In *Prosopis*, $\delta^{15}N_{xylem}$ varied between time points by up to 0.7‰ ($P<0.0001$) but $\delta^{15}N_{soil\ inorganic\ N}$ did not (Fig 1.1, Table 1.1). $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ was greater for *Prosopis* than for *Zanthoxylum* at two of the three time points, from 2.91- 4.42‰ ($P<0.08$, Fig 1.1, Table 1.1 and 1.2). In August 2012, difference in isotopic composition of soil solution inorganic N ($\delta^{15}N_{soil\ inorganic\ N}$) between the species reached 1.17‰ ($P<0.07$, pooled t-test, Fig 1.1), but was otherwise relatively spatially constant.

Within species, both *Zanthoxylum* and *Prosopis* showed significant separation between $\delta^{15}N_{xylem}$ and $\delta^{15}N_{foliage}$ ($\Delta^{15}N_{xylem\text{-}foliage}$) at all time points ($P<0.05$, Table 1.1). However, the

direction of fractionation between these pools was consistently opposite between the two species ($P<0.001$, Fig 1.2, Table 1.1 and 1.2). Averaged across a year, $\Delta^{15}N_{xylem\text{-}foliage}$ was 1.07 ± 0.12‰ (± 1 SE) for *Prosopis*, and -1.35 ± 0.25‰ for *Zanthoxylum*, leading to a difference in fractionation factors of 2.42 ± 0.28‰ between species (Fig 1.2).

%Ndfa values for *Prosopis* were sensitive to variations in $\Delta^{15}N_{xylem\text{-}foliage}$ between species and over time. When foliage-derived %Ndfa values were adjusted for species variation in $\Delta^{15}N_{xylem\text{-}foliage}$, average values dropped from 73% to 35% and 61% to 5% for January and August, respectively (Table 1.3). Accounting for average species differences in $\delta^{15}N_{soil\ inorganic\ N}$ had a smaller effect, reducing foliage-derived %Ndfa by only 2-4%. When the soil correction was applied in a time-point specific manner along with the xylem correction, an effect of sampling date was also apparent.

Table 1.1 Average fractionation ($\Delta^{15}N$) between N pools for *Prosopis glandulosa* and *Zanthoxylum fagara* at three sample dates in 2012.

	Prosopis		*Zanthoxylum*	
	$\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (‰)	$\Delta^{15}N_{xylem\ –foliage}$ (‰)	$\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ (‰)	$\Delta^{15}N_{xylem\ –foliage}$ (‰)
Jan	4.42 ^{a} ***	1.23 ^{a} ***	2.67 ^{ab} ***	-0.65 ^{a} *
May	2.91 ^{a} ***	1.05 ^{a} ***	1.16 ^{b} ^{NS}	-0.91 ^{a} *
Aug	4.37 ^{a} ***	0.51 ^{b} ***	3.35 ^{a} **	-2.64 ^{b} ***

Lowercase letters indicate significant differences between time points determined by mixed effects modeling followed by Tukey's HSD post hoc test.

P values indicate fractionation values significantly different from zero (paired t-test)
*** P<0.001, ** P<0.01, * P<0.05, ^{NS} not significant.

Table 1.2 Results for paired t-tests testing differences in $\delta^{15}N$ of N pools and fractionation ($\Delta^{15}N$) between pools for *Prosopis glandulosa* and *Zanthoxylum fagara* at three sample dates in 2012. Numbers in bold indicate significant differences ($P < 0.05$).

	Between-species comparisons				
	$\Delta^{15}N_{soil\ inorganic\ N-xylem}$ (‰)	$\Delta^{15}N_{xylem\ -\ foliage}$ (‰)	$\delta^{15}N_{soil}$ (‰)	$\delta^{15}N_{xylem}$ (‰)	$\delta^{15}N_{foliage}$ (‰)
Jan	**0.027**	**<0.001**	0.811	**<0.001**	**<0.001**
May	**0.045**	**<0.001**	0.282	**<0.001**	**<0.001**
Aug	0.291	**<0.001**	0.074	0.315	**<0.001**

Table 1.3 Sensitivity of percent nitrogen derived from fixation (%Ndfa) values for *Prosopis glandulosa* to variation in $\delta^{15}N_{soil\ inorganic\ N}$ and xylem-foliar fractionation ($\Delta^{15}N_{xylem\ -foliage}$) between species and sampling date. Calculated using *Zanthoxylum fagara* as a non-fixing reference. Values are for tissues sampled in January and August 2012, ± 1 SE. Note that these values are intended to provide a general illustration of the effects of accounting for assumptions, rather than absolute values, as application of %Ndfa technique deviates from suggested conditions (Shearer and Kohl 1986; Högberg 1997).

	%Ndfa	
	Jan 2012	Aug 2012
Foliage (conventional)	73 ± 7	61 ± 6
With soil correction [a]	75 ± 7	65 ± 6
With xylem-foliar fractionation correction [b]	35 ± 6	5 ± 14
With soil and xylem-foliar fractionation correction [b]	34 ± 8	27 ± 11

[a] using average soil solution $\delta^{15}N$ (Jan-Aug 2012)
[b] using sampling-point specific soil solution $\delta^{15}N$

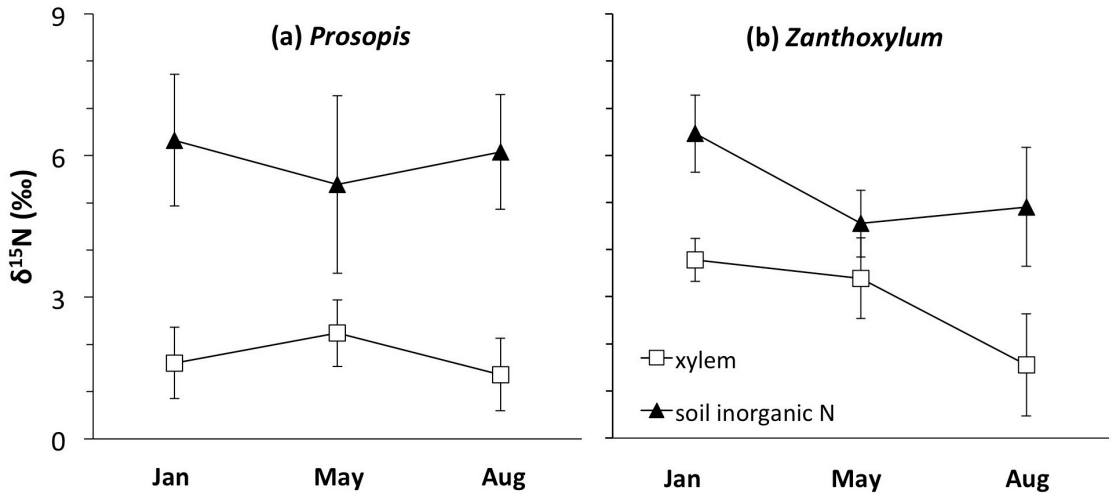

Figure 1.1 $\delta^{15}N_{xylem}$ and $\delta^{15}N_{soil\ inorganic\ N}$ for N-fixing *Prosopis glandulosa* and non-fixing *Zanthoxylum fagara* at three time points in 2012. $\delta^{15}N_{soil\ inorganic\ N}$ is a concentration-weighted value for $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$ in 1M KCl soil extracts from the rooting zone (0-15 cm). Soils were sampled from below each tree (<50 cm from trunk) within 3 h of xylem sampling. Extracts from two soil cores per tree were bulked for analysis. Values represent mean ± 1 SD.

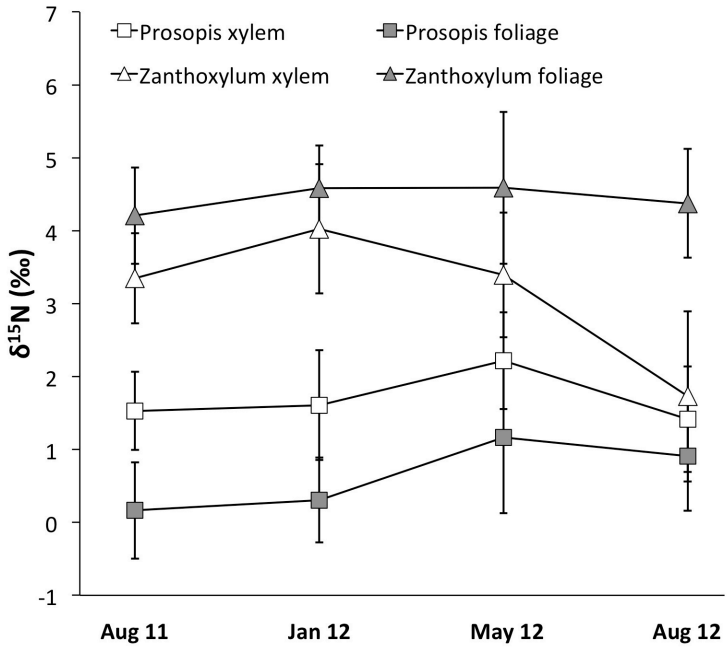

Figure 1.2 $\delta^{15}N_{xylem\ sap}$ and $\delta^{15}N_{foliage}$ for N-fixing *Prosopis glandulosa* and non-fixing *Zanthoxylum fagara* at four time points in 2011 and 2012. Values represent mean ± 1 SD. Statistical relationships are presented in Table 1.

Variability in $\delta^{15}N$ between Prosopis *individuals*

Prosopis individuals were consistently ranked in their relative xylem and foliar $\delta^{15}N$ values across a 12-month period, i.e., the values of individual trees relative to each other remained constant, despite temporal variation over the course of the year (Fig 1.3). $\delta^{15}N_{xylem}$ was similar between August of 2011 and 2012, with linear regression producing a slope close to 1 ($\delta^{15}N_{xylem\ Aug\ 2012}$= 1.0837 *$\delta^{15}N_{xylem\ Aug\ 2011}$ + 0.0016, R^2=0.61, P<0.008, Fig 1.3a). In a mixed effects model, sample date was a significant fixed effect in the model (P<0.0001), though tree age was not (P>0.05), and 64.2% of the residual variation was explained by individual tree identity.

$\delta^{15}N_{foliage}$ values were also correlated across years at the individual plant level, despite being offset between years by ~0.8‰ (linear regression, $^{15}N_{foliage\ Aug\ 2012}$= 0.6715*$\delta^{15}N_{foliage\ Aug\ 2011}$ + 0.8082, R^2=0.68, P<0.0001, Fig 1.3b). Again, sample date was a significant fixed effect in the mixed effects model (P<0.0001) and tree age was not (P>0.05), and 84.1% of the residual variation in $\delta^{15}N_{foliage}$ was explained by individual tree identity.

This same consistency in $\delta^{15}N_{xylem}$ and $\delta^{15}N_{foliage}$ at the individual level over time was not observed in *Zanthoxylum*, and after accounting for date as a fixed effect in a mixed effects model individual tree identity accounted for 16% or less of residual variation.

Effect of Prosopi*s age on plant and soil inorganic $\delta^{15}N$ and soil N concentration*

Prosopis tree age was not a predictor of $\delta^{15}N_{foliage}$ (Fig 1.4a) or $\delta^{15}N_{xylem}$ (Fig 1.4b; P>0.05). However, $\delta^{15}N_{soil\ inorganic\ N}$ in the rooting zone of *Prosopis* increased with tree age (R^2=0.23, P<0.06), by 2.05‰ from 20-100 years (predicted, $\delta^{15}N_{soil\ inorganic\ N}$ = 0.0257*Age + 4.497, Fig 1.4c). As a result of this increasing soil baseline, the $\Delta^{15}N_{soil\ inorganic\ N\text{-}xylem}$ value increased comparably over the same age range (2.10‰ predicted, R^2= 0.12, $\delta^{15}N_{soil\ inorganic\ N}$ = 0.0263*Age + 2.477 P<0.07, Fig 1.4d).

Soil inorganic N concentrations ($NH_4^+ + NO_3^-$, 0-15 cm depth) increased linearly with *Prosopis* age (linear regression, R^2=0.62, P<0.0001, Fig 1.5a). Increasing soil inorganic N also correlated positively with $\delta^{15}N_{soil\ inorganic\ N}$ concentrations in soils under *Prosopis* (linear regression, R^2=0.26, P<0.02, Fig 1.5b), though not in soils under *Zanthoxylum* (data not shown).

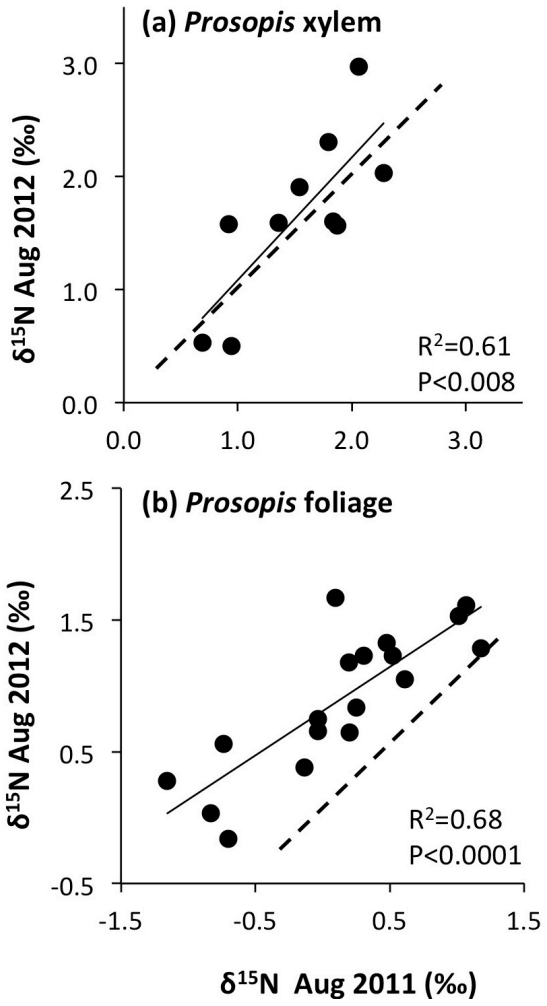

Figure 1.3 Linear relationships between $\delta^{15}N_{xylem\ sap}$ **(a)** and $\delta^{15}N_{foliage}$ **(b)** of individual *Prosopis glandulosa* trees sampled in August 2011 and August 2012. Dashed line represent 1:1 $\delta^{15}N$ relationship. Foliage was replaced completely between the two sampling points. P and R^2 values derived from linear regression.

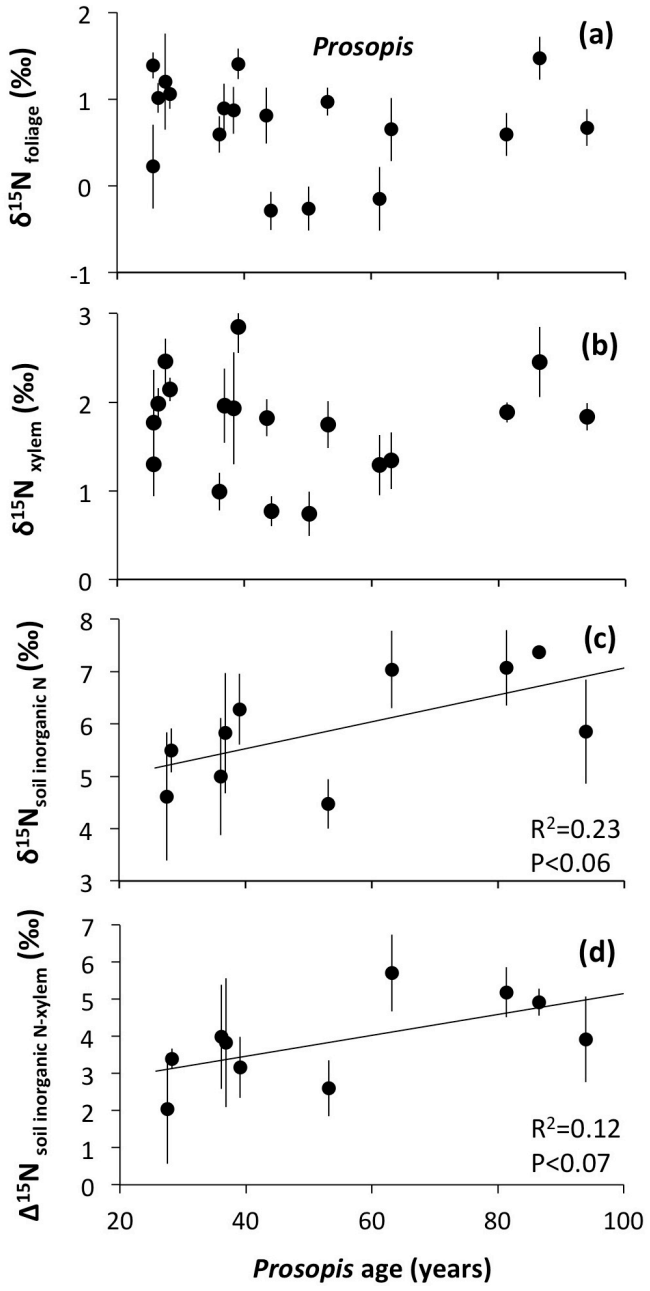

Figure 1.4 (a) $\delta^{15}N_{foliage}$ for *Prosopis glandulosa* trees ranging from 20-100 years in age **(b)** $\delta^{15}N_{xylem}$ sap for *Prosopis* trees ranging from 20-100 years in age. **(c)** Linear relationship between $\delta^{15}N_{soil\ inorganic\ N}$ from soils at 0-15 cm depth in the rooting zone of *Prosopis* and tree age. **(d)** Linear relationship between $\Delta^{15}N$ (difference between $\delta^{15}N_{soil\ inorganic\ N}$ and $\delta^{15}N_{xylem\ sap}$) for *Prosopis* individuals and tree age. All values represent the mean of three time points in 2012 ± 1 SE. P values derived from linear regression.

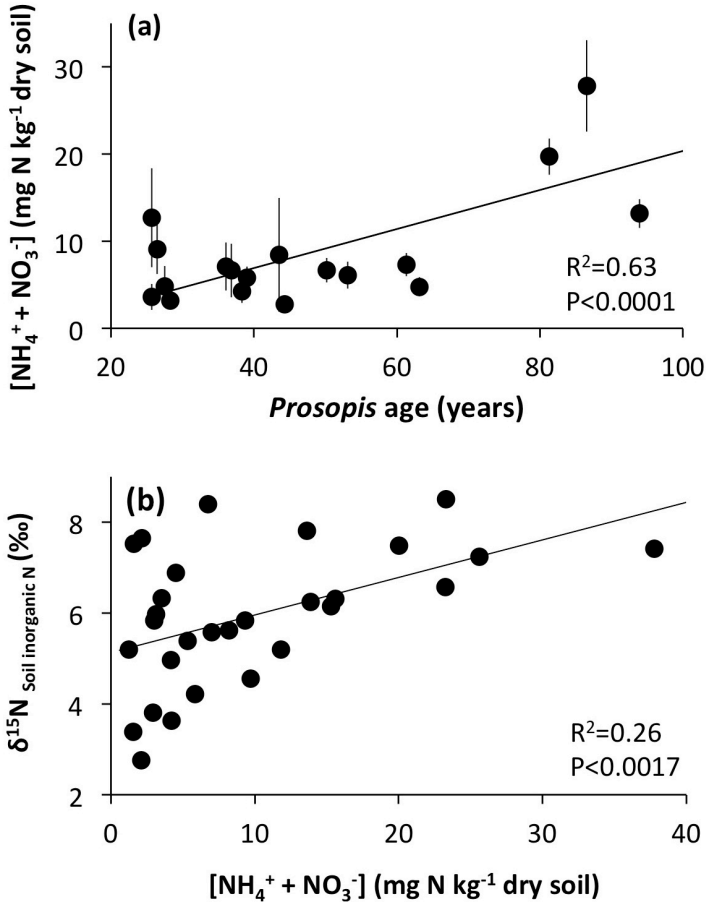

Figure 1.5 (a) Linear relationship between total inorganic N concentration ($NH_4^+ + NO_3^-$) in 1M KCl soil extracts (0-15 cm) and age of *Prosopis glandulosa* under which soil was sampled. Values represent mean of three time points in 2012 ± 1 SE. **(b)** Linear relationship between $\delta^{15}N_{soil\ inorganic\ N}$ and total inorganic N concentration ($NH_4^+ + NO_3^-$) in each sample. P and R^2 values derived from linear regression.

Effect of soil N concentration on xylem $\delta^{15}N$

In *Zanthoxylum*, there was a strong negative relationship between $\delta^{15}N_{xylem}$ and soil NH_4^+, NO_3^- and total inorganic N concentrations when all time points were considered together (linear regression: R^2=0.48, P<0.0001, R^2=0.33, P<0.005, R^2=0.47, P<0.0002, respectively, Fig 1.6a). However, $\delta^{15}N_{xylem}$ was not correlated with $\delta^{15}N_{soil\ inorganic\ N}$. No relationship was observed among any of these parameters in *Prosopis* (P>0.05, Fig 1.6b).

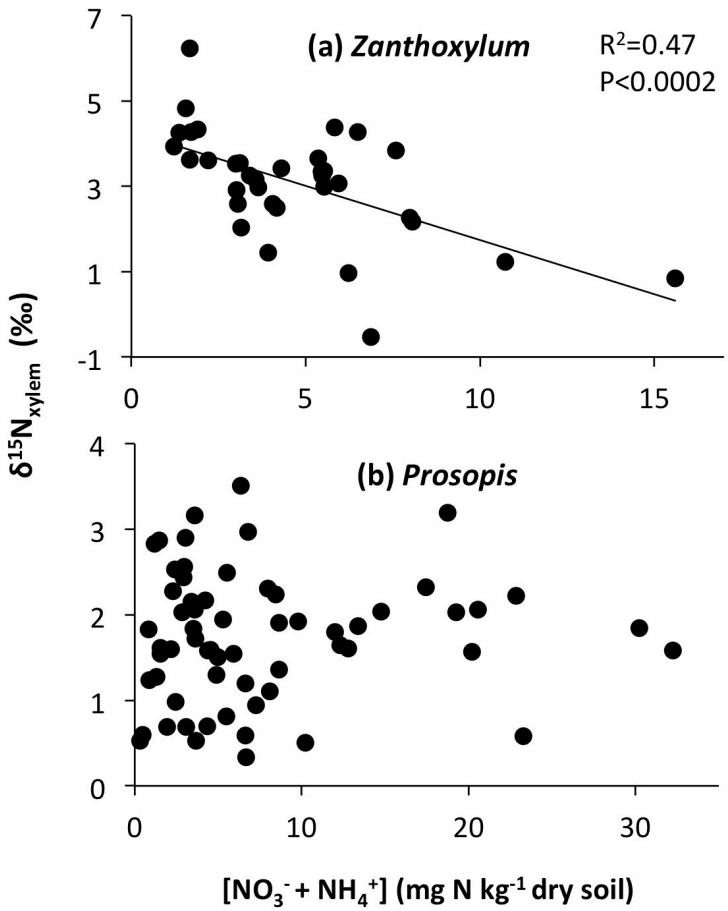

Figure 1.6. (a) Linear relationship between soil nitrate and ammonium concentration (mg N kg^{-1} dry soil, 1M KCl soil extracts, 0-15 cm) and $\delta^{15}N_{xylem}$ in *Zanthoxylum fagara* and **(b)** no significant linear relationship between the two values in *Prosopis glandulosa*. P and R^2 values derived from linear regression.

Inter-annual and between-species variation in foliar $\delta^{15}N$

Over the course of two years, *Prosopis* showed strong temporal variation in $\delta^{15}N_{foliage}$, while non-fixing *Zanthoxylum* did not (Fig 1.7, *Zanthoxylum*: P>0.05: *Prosopis*: P<0.0001). When individual *Prosopis* leaf cohorts were considered, the average $\delta^{15}N_{foliage}$ increased between cohorts 1 (2011) and 2 (2012) by 0.73‰ (P<0.0001, 95 % CI= 0.58-0.89‰) and between cohort 2 and 3 (2013) by 2.12‰ (P<0.0001, 95% CI= 1.94-2.30‰). During February-April of this

study, when new leaf cohorts were produced, total precipitation varied among years (148, 137 and 70 mm in 2011, 2012 and 2013, respectively) and was lower than the average rainfall for this interval over the period 1979 to 2009 (186 mm; National Climatic Data Centre).

When six species at the site were assessed for $\delta^{15}N_{foliage}$ in August of 2013, *Prosopis* was not different from three of the four non-fixing species with the exception of *D. texana*, which was depleted with respect to *Prosopis* (Fig 1.8). Additionally, putatively N fixing *A. farnesiana* was enriched in $\delta^{15}N_{foliage}$ compared to all species except *Zanthoxylum*. At the previous six time points considered in 2011 and 2012, *Prosopis* $\delta^{15}N_{foliage}$ was significantly depleted with respect to *Zanthoxylum* (Fig 1.7).

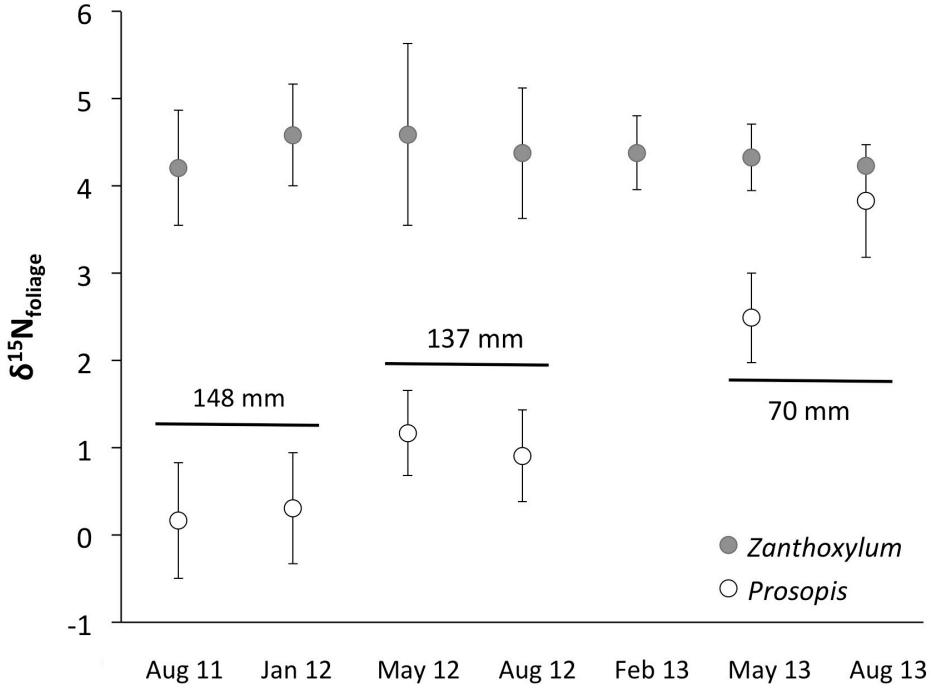

Figure 1.7 Seasonal variation in $\delta^{15}N_{foliage}$ for N-fixing *Prosopis glandulosa* and non-fixing *Zanthoxylum fagara* from August 2011 to August 2013. Black bars indicate different leaf cohorts in *Prosopis*, accompanied by total precipitation (mm) at the site during the 3 month growing period (February to April, inclusive) when each leaf cohort was laid down. Missing value occurs where *Prosopis* trees had no foliage at time of sampling. Values represent mean ± 1 SD.

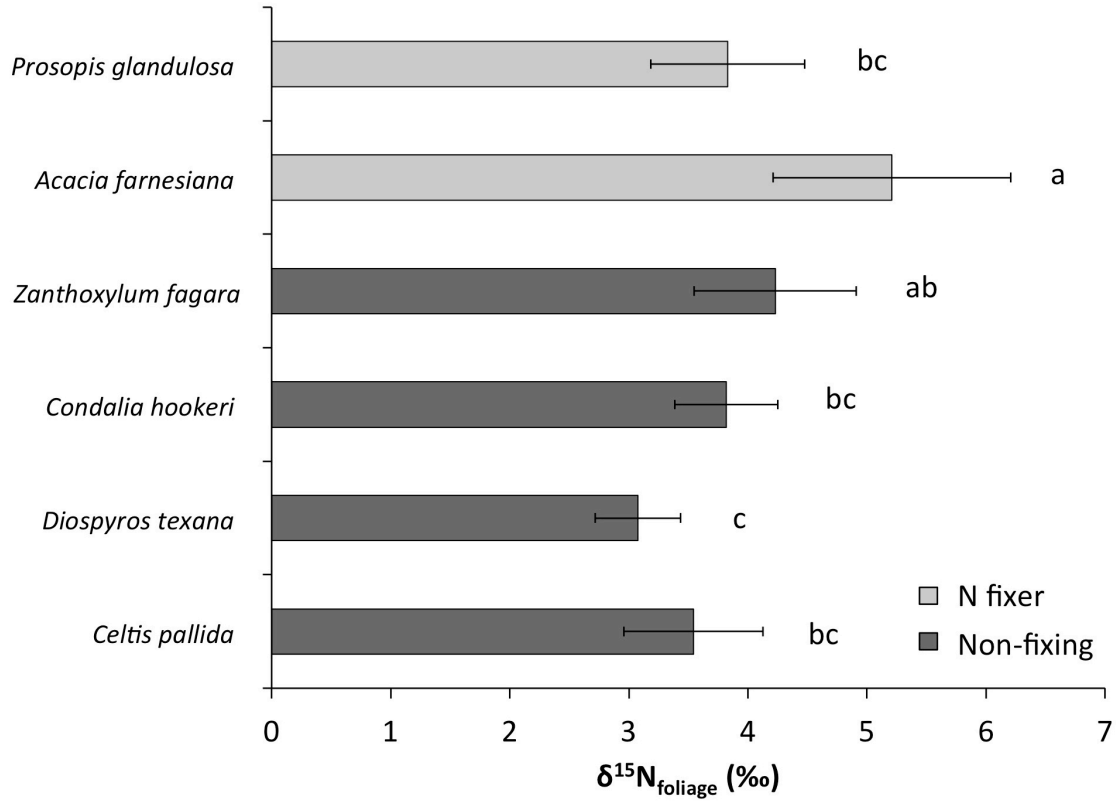

Figure 1.8 $\delta^{15}N_{foliage}$ for four co-occurring non-fixing (dark grey) and two N-fixing (light grey) woody plants, sampled in August 2013. Values represent means ± 1 SD. Letters indicate significant differences between species (Tukey's post hoc test).

Discussion

Explicit isotopic sampling of plant and soil $\delta^{15}N$ pools along the N uptake pathway suggests that applying the foliar $\delta^{15}N$ natural abundance method to estimate BNF would be challenging in this subtropical *Prosopis* savanna woodland. The requirement that the $\delta^{15}N$ of non-BNF-derived N sources be constant among species (Shearer and Kohl 1986) was not met, as the concentration-weighted $\delta^{15}N_{soil\ inorganic\ N}$ was not always the same in the N-fixing *Prosopis* and non-fixing *Zanthoxylum* (Fig 1.1). This parameter also showed significant seasonal variation in *Zanthoxylum*, (up to 1.9‰ over three months) suggesting the soil inorganic $\delta^{15}N$ source is neither temporally nor spatially constant. Additionally, fractionation during N assimilation and

transport were not comparable between the N-fixing and reference species. *Zanthoxylum* and *Prosopis* differed consistently in the magnitude and direction of the separation between xylem and foliar $\delta^{15}N$ ($\Delta^{15}N$), leading to an average $\Delta^{15}N$ difference between species of 2.4‰. Applying this fractionation factor to percent nitrogen derived from fixation calculations for *Prosopis* was sufficient to reduce assumed fixation by 50% or more. These observations demonstrate that applying the natural abundance foliar $\delta^{15}N$ method would be untenable in this and similar ecosystems.

Within-plant fractionation has the potential to occlude or even mislead the interpretation of foliar $\delta^{15}N$ as a proxy for BNF. Foliage is usually observed to be ^{15}N-depleted with respect to xylem sap (Pate *et al.* 1993; Schmidt and Stewart 2003; Peuke *et al.* 2013; Soper *et al.* 2015), as we saw in *Prosopis* (Fig 1.2). Our observation of enriched foliar tissue in *Zanthoxylum* is rare, with only one observation found in the literature (Yoneyama *et al.* 2000). Foliar isotope ratios are influenced by several processes, many of which are likely to differ between taxa [e.g. (Britto and Kronzucker 2013)], including preferences for N forms with different isotopic ratios (Yoneyama 1996), distribution of nitrate reduction between roots and shoots (Andrews 1986; Comstock 2001; Werner and Schmidt 2002; Tcherkez and Hodges 2008), relative leaf turnover/export of N compounds with differing $\delta^{15}N$ signatures (Tcherkez 2011; Gauthier *et al.* 2012), and leaf age and lifetime (Kolb and Evans 2002; Peuke *et al.* 2013). Regardless of the mechanisms of fractionation, the opposing patterns observed in *Prosopis* and *Zanthoxylum* create a separation between foliar isotopic values that significantly overemphasize the apparent contribution of BNF (Table 1.3).

We observed variable relationships between soil source $\delta^{15}N$ and xylem $\delta^{15}N$

in the two species. In *Zanthoxylum*, but not *Prosopis*, the N isotopic composition of xylem was strongly negatively related to the concentration of soil inorganic N (both ammonium and nitrate), but not to its isotopic composition, i.e. $\delta^{15}N_{xylem}$ became more depleted at higher soil inorganic N concentrations (Fig 1.6). To our knowledge, this relationship has not previously been investigated in natural ecosystems under ambient soil N concentrations, and is counter to general predictions of concentration/fractionation relationships for nitrate (Evans 2001). The $\delta^{15}N$ of xylem sap reflects the relative content of nitrate and organic N compounds and integrates a complex set of fluxes (Handley and Scrimgeour 1997; Peuke *et al.* 2013). Nitrate taken up from soil may be partly reduced in root tissue. During reduction, this nitrate is fractionated by nitrate reductase (Robinson *et al.* 1998) producing ^{15}N-depleted organic N compounds that are either incorporated directly into root tissue or exported to the xylem. The remaining, unreduced, ^{15}N-enriched nitrate pool is exported to the xylem and reduced in the shoot (Robinson *et al.* 1998). Ammonium, both soil-derived and as the product of N fixation, is immediately incorporated into organic compounds prior to xylem transport (Evans 2001) and does not show the same shift in isotopic distribution within the plant (Werner and Schmidt 2002).

We propose two potential mechanisms that may account for the inverse relationship between soil N concentration and xylem $\delta^{15}N$ relationship that we observed in *Zanthoxylum*. The relative degree of nitrate reduction in roots versus shoots may have decreased with external nitrate concentration, resulting in a smaller proportion of the increased nitrate pool being reduced in roots and reducing enrichment of the remaining nitrate pool exported in the xylem. This is however the opposite of what Kolb and Evans (2003) observed in barley (*Hordeum vulgare*) grown with increasing nitrate concentrations, where the root to shoot ratio of nitrate reductase activity increased and exported nitrate was more enriched. Alternatively, shifts in N source

preference with concentration have been also observed in woody species (Kronzucker *et al.* 1997). Here, *Zanthoxylum* may have increased uptake of ammonium relative to nitrate as external ammonium concentrations increased, leading to a decreased proportion of enriched nitrate in the xylem sap. However, the ratio of the two compounds did not change with concentration.

Substantial increases in foliar $\delta^{15}N$ occurred across three *Prosopis* leaf cohorts over two years (>2.8‰), but no comparable variation was seen in non-fixing *Zanthoxylum* (Fig 1.7), raising an important point for general interpretation of foliar $\delta^{15}N$ data. As a result of this temporal variation, foliar $\delta^{15}N$ did not differ between *Prosopis* (or N-fixing *A. farnesiana*) and non-fixing plants at the single time point when we sampled multiple species (Fig 1.8), though it likely would have two years previously. Few studies that rely on the foliar $\delta^{15}N$ method consider sampling time as an important consideration, or conduct multiple staggered samplings, despite the fact that sample date has been recognized as an important determinant of the relative isotopic rankings of different species for some time [e.g. (Handley and Scrimgeour 1997)]. Given our observation of variable relationships between soil solution, xylem and foliar $\delta^{15}N$, it would be invalid to directly compare isotopic values between species to interpret this shift as directly proportional to decreasing BNF.

The increase in $\delta^{15}N_{foliage}$ observed through time across all *Prosopis* (Fig 1.7) studied suggests external environmental change is potentially affecting the isotopic composition of soil solution N, internal plant N assimilation and partitioning, rates of BNF, or all three. Precipitation was notably variable during this time, declining over 50% between 2012 and 2013 in the February-April period when *Prosopis* leaves were flushed, coincident with an increase in $\delta^{15}N_{foliage}$ of 2.1‰. Low water availability has been linked to decreased nodule abundance and

acetylene reduction rates (a proxy for nitrogenase activity) in *Prosopis* and other woody legumes (Zitzer *et al.* 1996; Zahran 1999) and could account for our observation here. However, both drought and rates of BNF have been shown to alter internal ^{15}N distribution in herbaceous plants (Handley *et al.* 1999; Wanek and Arndt 2002), making it difficult to attribute the increasing foliar isotope values solely to decreasing BNF.

We observed seasonal variation in *Prosopis* xylem $\delta^{15}N$ that was largely independent of shifts in the isotopic composition of the soil inorganic N pool (Fig 1.1, Table 1.1). Controls on N fixation, especially the effect of abiotic conditions (in the context of global climate change for example), are of significant interest to the scientific community (Vitousek *et al.* 2013), and existing seasonal variation may provide an opportunity to investigate these controls on manageable time scales in natural environments. However, it is difficult to attribute our observations to only changes in BNF because of similar observed shifts in the xylem-soil relationship of non-fixing *Zanthoxylum.* Most likely, these shifts integrate changes in both fractionation events during uptake and BNF effects over time. Previous experiments with soybean have shown that a nodulating strain had lower xylem $\delta^{15}N$ values than the non-nodulating strain grown under the same field conditions, indicating that the isotopic signature of fixation is likely detectable in N transported from the roots (Yoneyama *et al.* 2000). Both that study and Schmidt and Stewart (2003) looking at Australian *Acacias* observed variation in xylem sap $\delta^{15}N$ that was apparently influenced by abiotic conditions. While it may prove challenging to parse out the effects of abiotic (temperature, water availability) and biotic (plant phenology) influences, the present study does demonstrate that measurable isotopic variation occurs and could be used to investigate the effect of these processes on N cycling in a natural ecosystem. Potential also exists to compare xylem data to that derived from other methods, e.g. nodule

abundance and acetylene reduction activity, to begin to address questions about *in situ* N fixation.

Individual *Prosopis* trees showed a high degree of consistency in tissue $\delta^{15}N$ relative to each other over time (Fig 1.3) and non-fixing *Zanthoxylum* did not, potentially indicating sustained differences in BNF rates between *Prosopis* individuals. This consistency was not explained by age or any soil N variable tested. Instead, individual *Prosopis* tree identity accounted for 60-80% of the observed variation after accounting for sample date. While heterogeneous availability of other soil nutrients necessary for BNF or degree of mycorrhizal colonization (Hobbie and Högberg 2012) could account for this observation, its restriction to *Prosopis* suggests that genotypic factors affecting N fixation rates [for example genotype-mediated plant-rhizobial interactions, (Mytton *et al.* 1977)] may be a probable explanation.

Prosopis encroachment into grassland and subsequent growth greatly increases soil inorganic N concentrations (McCulley *et al.* 2004, Fig. 1.5), a factor that might be expected to suppress rates of BNF as trees age (Hartwig 1998). In this study, *Prosopis* $\delta^{15}N_{foliage}$ was constant among trees ranging in age from 20 to 100 years (Fig. 1.4) suggesting constant rates of BNF. However, over this same chronosequence, soil solution inorganic $\delta^{15}N$ increased linearly by >2‰, leading to an increased separation ($\Delta^{15}N$) between tissue and soil isotopic values with time since establishment. This suggests increasing rates of BNF by *Prosopis* over time. Assuming that fractionation of soil N upon uptake does not change with plant age, in order for $\delta^{15}N_{xylem}$ to remain stable while $\delta^{15}N_{soil\ inorganic\ N}$ falls there must be a corresponding increasing input of isotopically depleted N, assumed to come from BNF. This implies either that a) there is increased survival of trees that have a greater BNF capacity (for example, as a result of plant-*Rhizobium* genotype interactions [Mytton *et al*. 1977]) leading to an age distribution in which

older trees are likely to be inherently higher N fixers, or b) that fixation capacity increases with plant age. The latter could result from increasing access to resources (such as phosphorus or soil moisture) that might limit fixation (Hartwig 1998; Augusto *et al.* 2013). At this site, available P in the rooting zone increases significantly with *Prosopis* age (Kantola 2012), and larger root systems increase access to soil moisture, shown to correlate with nodule abundance (Zitzer *et al.* 1996).

Alternatively, increasing fractionation of soil solution N upon uptake with plant age could explain our observation, but seems mechanistically less likely. Increasing $\delta^{15}N_{\text{soil inorganic N}}$ values with plant age also correlated strongly with an increase in the concentration of inorganic N in the rooting zone (Fig 1.5). Lab experiments have demonstrated that isotopic fractionation of N upon uptake increases dependent on external N availability in certain herbaceous species (Evans 2001; Yoneyama *et al.* 2001; Kolb and Evans 2003), though the concentrations used are typically very high. In natural systems it is assumed that N availability is too low relative to plant N demand for such a fractionation to be realized (Evans 2001; Hobbie and Högberg 2012). Even if uptake discrimination did occur at field concentrations, the contribution of fractionation during this step would need to be great enough to counter the increase in the $\delta^{15}N$ of the total soil inorganic N pool that we observed with increasing concentration.

In conclusion, the explicit sampling of $\delta^{15}N$ in xylem, foliar and soil pools can provide useful information on seasonal and long term variation describing N fixation, fractionation, N use physiology in woody perennials and ecosystem-scale N cycling. For example, explicit sampling of the isotopic composition of plant available soil N sources, and comparison with simultaneously-sampled xylem sap, can be used to infer patterns of increasing contribution of BNF to plant N nutrition over time not apparent via tissue sampling alone. Measuring and

subsequently being able to control for the composition of the soil pool identified other sources of variability in plant isotopic composition, such as individual tree identity. In addition, seasonal variation identified here as well as long-term, potentially climate-driven shifts in foliar isotopic values introduce variation that significantly affects interpretation of potential BNF, emphasizing the importance of repeated sampling over time. Finally, applying this method to test the assumptions of the foliar ^{15}N technique demonstrates violation of assumptions that preclude its use in many systems, further highlighting the need for alternative approaches to investigating BNF in natural ecosystems.

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CHAPTER TWO

NITROGEN TRACE GAS FLUX FROM A SEMI-ARID SAVANNA EXPERIENCING WOODY LEGUME ENCROACHMENT

Abstract

Savanna ecosystems are a major source of nitrogen (N) trace gases that influence air quality and climate. These systems are experiencing widespread encroachment by woody plants, frequently associated with large increases in soil N, with no consensus on implications for trace gas emissions. We investigated the impact of encroachment by N-fixing tree *Prosopis glandulosa* on total reactive N gas flux ($N_t = NO+N_2O+NO_y+NH_3$) from south Texas savanna soils over two years. Contrary to expectations, upland *Prosopis* groves did not have greater N_t fluxes than adjacent unencroached grasslands. However, abiotic conditions (temperature, rainfall, and topography) were strong drivers. Emissions from moist, low-lying *Prosopis* playas were up to three times higher than from *Prosopis* uplands. Though NO dominated emissions, NH_3 and NO_y (non-NO oxidized N) comprised 12-16% of the total summer N flux (up to 7.9 μg N m^{-2} h^{-1}). Flux responses to soil wetting were temperature-dependent for NO, NH_3 and NO_y: a 15 mm rainfall event increased flux 3-22 fold after 24 hours in summer, but had no effect in winter. Repeated soil wetting reduced N flux responses, indicating substrate depletion as a likely control. Rapid (<1 min) increases in NO emissions following wetting of dry soils suggested that abiotic chemodenitrification contributes to pulse emissions. We conclude that temperature and wetting dynamics, rather than encroachment, are primary drivers of N flux from these upland savannas, with implications for future emission patterns under altered precipitation regimes.

Introduction

Savanna and grassland ecosystems occupy ~40% of the terrestrial surface and may contribute up to 40% of the total global flux of nitric oxide (NO) (Davidson and Kingerlee 1997; Yan *et al.* 2005). Many savannas worldwide are undergoing significant changes in vegetation structure and function due to the encroachment of woody plants (Eldridge et al. 2011). In subtropical, semi-

arid savannas, encroachment is frequently driven by nitrogen (N)-fixing trees (Archer 1995; Roques *et al.* 2001; Moleele *et al.* 2002; Cabral *et al.* 2003; Asner and Martin 2004) and contributes to large increases in quantity and transformation rates of soil N (Archer *et al.* 2001; Boutton and Liao 2010; Blaser *et al.* 2014). A key question is whether these changes translate into increased rates of N trace gas loss from soils, and if so, how climate and soil conditions mediate these changes. Though encroachment is widespread throughout the subtropics, few studies have explicitly investigated associated changes in trace gas emissions and there is currently no consensus on expected effects.

Woody encroachment can have variable effects on soil properties (Eldridge et al. 2011), but in subtropical/tropical semi-arid systems, changes often include increased soil N and organic C, decreased bulk density, increased mineralization and respiration rates and increased microbial biomass (Stock *et al.* 1995; Archer *et al.* 2001; Hibbard *et al.* 2001; McCulley *et al.* 2004; Liao and Boutton 2008; Throop and Archer 2008; Pellegrini *et al.* 2013). *Prosopis glandulosa* (honey mesquite) encroachment in the southern United States has been shown to increase soil total N storage by two-fold or greater and increase NH_4^+ and NO_3^- concentrations and net mineralization and nitrification rates (Hibbard *et al.* 2001; McCulley *et al.* 2004; Throop and Archer 2008; Bai *et al.* 2009). Prior studies have reported higher rates of NO or N_2O flux from beneath *Prosopis* or other woody legume canopies, compared with between-canopy interspaces, likely an effect of the 'islands of fertility' that form around encroaching trees (Virginia *et al.* 1982; Hartley and Schlesinger 2000; Feig *et al.* 2008a; McLain *et al.* 2008). However, studies of trace gas flux responses to encroachment at the landscape scale have produced variable results. Martin et al. (2003) found a strong correlation between *Prosopis* biomass and NO production, but both

Hartley and Schlesinger (2000) and Feig *et al.* (2008a) found higher emissions from grasslands than from N-fixing shrublands.

NO is typically the most abundant N trace gas emitted by tropical savanna ecosystems (Bustamante et al. 2006). NO affects the oxidative capacity of the atmosphere by catalyzing the formation of tropospheric ozone, a regionally-important greenhouse gas and pervasive air pollutant (Denman 2007). The long-lived global greenhouse gas N_2O is also produced in savannas and encroached soils but typically at rates much lower than NO, particularly in well-drained soils (Scholes *et al.* 1997; Martin *et al.* 2003; Werner *et al.* 2014). Nitrogen gas (N_2) was found to constitute 82-99% of total $NO+N_2O+N_2$ flux in savannas of northern Australia under a broad range of incubation conditions (Werner et al. 2014), but measures from North American *Prosopis* savanna-woodland soils suggest that N_2 makes up a much smaller proportion (31-77% or less; Soper *et al.*, in review). Other gaseous N compounds including ammonia (NH_3) and non-NO reactive N oxides (NO_y, including NO_2, HONO, HNO_3 and organic oxides) can form particulates and influence the oxidative capacity of the atmosphere. Several studies report fluxes of NH_3 from desert soils (Schlesinger and Peterjohn 1991; McCalley and Sparks 2008), and of HONO (a component of NO_y) from desert biocrusts (Weber *et. al.* 2015), but neither NH_3 nor NO_y are routinely measured in natural ecosystems and their fluxes remain poorly defined.

Biogenic production of N_2O and NO in soils occurs during the microbial processes of nitrification and denitrification, and additional NO production also possible during abiotic chemodenitrification (Pilegaard, 2013). Flux rates are linked to rates of these processes and the availability of NH_4^+, NO_2^- and NO_3^- substrates (Firestone and Davidson 1989; Medinets *et al.* 2015). N_2O and NO emissions from arid and semi-arid soils are strongly moderated by abiotic conditions, most significantly soil moisture (which also affects O_2 availability), with secondary

roles for temperature and pH (Davidson *et al.* 1993; Ludwig *et al.* 2001; Meixner and Yang 2006; Pilegaard 2013; Werner *et al.* 2014). Seasonal variability in plant N uptake may also influence fluxes in some arid systems (Homyak and Sickman, 2014). Labile C and N availability and microbial N demand are additional controls (Ludwig *et al.* 2001; Pilegaard 2013) that may be affected by soil type, vegetation cover and disturbance history (Parsons *et al.* 1996; Hartley and Schlesinger 2000; Erickson and Davidson 2002).

Pulse dynamics, where large fluxes are associated with infrequent rainfall events, can contribute substantially to total N emissions from seasonally dry soils (Davidson *et al.* 1991; Austin *et al.* 2004). During soil drying, labile N compounds accumulate in soil due to reduced diffusivity and atmospheric dry deposition. These compounds, along solutes released during cell lysis, create a substrate pool for rapid mineralization and nitrification/denitrification upon soil wetting (Davidson *et al.* 1993; Austin *et al.* 2004; Borken and Matzner 2009; Kim *et al.* 2012). Large increases in NO flux are often observed in dry subtropical soils at the beginning of the monsoon (Johansson *et al.* 1988; Davidson *et al.* 1991; Butterbach-Bahl *et al.* 2004). However, subsequent fluxes in response to wetting are reduced, presumably because repeated watering reduces the accumulated substrate pool (Johansson *et al.* 1988; Davidson *et al.* 1991; Hartley and Schlesinger 2000). The effect of abiotic conditions on fluxes of NH_3 and NO_y are poorly understood in natural ecosystems. Ammonia emissions, produced by dissociation of NH_4^+ ions at low pH, appear to increase with rates of mineralization and NH_4^+ availability (Schlesinger and Peterjohn 1991; Kim *et al.* 2012). A few studies report increased NH_3 emissions in response to wetting in desert soils (Schlesinger and Peterjohn 1991; McCalley and Sparks 2008). However, the mechanistic understanding of this responses is limited (Kim et al. 2012).

The primary goal of this study was to determine whether changes in N cycling associated with woody legume encroachment in a subtropical, semi-arid savanna translate into increased fluxes of N trace gases from soils. We also sought to determine the contribution of different N compounds to the total surface N flux, and to quantify the importance of abiotic conditions as a determinant of flux rates in this system. We measured a complete suite of reactive N trace gas (N_t, the sum of N_2O, NH_3, NO_y and NO) emissions across a spectrum of un-encroached grassland to *Prosopis*-dominated vegetation types in south Texas. We sampled fluxes across three seasons, replicated over two years from a range of landscape positions. Fluxes were measured before and after the addition of water to simulate single or repeated rainfall events, and responses considered in the context of broader rainfall patterns.

Materials and Methods

Study site

Sampling was conducted at the Texas A&M AgriLife La Copita Research Area (27°40'N, 98°12'W), 65 km west of Corpus Christi, Texas, USA on the eastern Rio Grande Plains. The climate is subtropical with typically warm, moist winters (December-February) and hot, dry summers (June-August). Mean annual temperature is 22.4°C with an average growing season of 289 days (March-November). Mean annual precipitation is 680 mm, falling year round but with maxima in May and September. During the study period, the site experienced 510, 690 and 750 mm annual rainfall in 2012-2014, respectively. Air temperature and precipitation data were measured every 10 minutes by a weather monitoring system on site (Nexsens Technology, Alpha, OH).

The study sites lie along a 1.5 m change in elevation, from an upland matrix of remnant grasslands, woody clusters and N-fixing *Prosopis glandulosa* (honey mesquite) groves through *Prosopis*-dominated drainage woodlands into *Prosopis*-dominated lowland playa (Fig 2.1). Upland remnant grasslands are characterized by native C4 grasses (genera *Paspalum, Bouteloua, Chloris* and *Egragrostis*), interspersed with herbaceous cover and bare ground. Clusters are 2-4 m diameter discrete assemblages of mixed woody species, usually dominated by *Zanthoxylum fagara* and commonly including *Condalia hookeri and Berberis trifoliolata*. Clusters are hypothesized to have originally contained a *Prosopis* individual at the center (Archer et al. 1988), though often no evidence of these original trees remain. Upland groves are defined by two or more proximate *Prosopis* trees with a diverse woody understory. Upland soils are primarily Typic Argiustolls, usually with a subsurface argillic horizon, interspersed with patches of Typic Ustochrepts lacking an argillic horizon (Archer 1995). Drainage woodlands (Pachic Argiustolls) have a similar vegetation composition to groves, but with a closed canopy. Low-lying closed-basin depressions (playas) consist of a continuous grass layer (*Paspalum pubiflorum* and *Bothriochloa ischaemum*) with an overstory of *Prosopis* and *Acacia farnesiana*. Playa soils (Ustic Epiaquerts and Vertic Argiaquolls) are capable of supporting standing water after heavy rainfall. Soil characteristics of the five vegetation types (all sampled from the same site used in this study) are summarized in Table 2.1.

The site has been grazed rotationally by cattle at a low stocking rate for ~30 years, with livestock exclusion occurring for at least two years prior to these measurements (Bai *et al.* 2013). There is no record of fire since at least 1950, and prior to that heavy grazing likely kept fire risk low (Bai *et al.* 2013). Additional description of soils, climate and vegetation elements can be found in Archer (1995) and Boutton *et al.* (1998).

Table 2.1. Soil characteristics of five vegetation cover types at the study site, the Texas A&M AgriLife La Copita Research Area. VWC, volumetric water content; WHC, water holding capacity; SMB, soil microbial biomass.

Characteristic	Depth (cm)	Error unit	Grassland	Woody cluster	Upland *Prosopis* grove	*Prosopis* drainage woodland	*Prosopis* playa	References
Landscape position			Upland	Upland	Upland	Sloping	Lowland basin	
Soil type			Sandy loam	Sandy loam	Sandy loam	Clay loam	Clay loam	Liu *et. al.* [2013]; Hibbard *et. al.,* [2001]
Sand/silt/clay (%)			79/9/12	78/10/12	78/9/13	70/13/17	48/20/32	Liu *et. al.* [2013]
pH	0-15	SE	7.1 ± 0.1^{a}	7.1 ± 0.2^{a}	7.2 ± 0.1^{a}	7.2 ± 0.1^{a}	6.0 ± 0.1^{b}	Liu *et. al.* [2013]
Bulk density (g/cm^3)	0-15	SE	1.4 ± 0.01^{a}	1.3 ± 0.02^{ab}	1.2 ± 0.02^{b}	1.2 ± 0.01^{b}	1.8 ± 0.03^{b}	Liu *et. al.* [2013]
VWC (%)	0-15	SE	5.1 ± 0.1^{a}	4.6 ± 0.4^{a}	4.2 ± 0.01^{a}	7.4 ± 0.2^{b}	16.4 ± 0.5^{c}	Liu *et. al.* [2013]
Potential WHC -0.3 MPa (% volumetric)	0-10	SE	12 ± 1^{a}	13 ± 2^{a}	13 ± 2^{a}		20 ± 2^{b}	Hibbard *et. al.,* [2001]
Total N ($g\ m^{-2}$)	0-15	SE	123 ± 2^{a}	143 ± 5^{ab}	182 ± 8^{b}	288 ± 10^{c}	253 ± 14^{c}	Liu *et. al.* [2013]
SMB N ($mg\ N\ kg^{-1}$)	0-20	SD	39 ± 13^{c}	51 ± 26^{b}	59 ± 32^{abc}	90 ± 40^{a}		McCulley *et. al.,* [2004]
N Mineralization ($mg\ N\ kg^{-1}\ d^{-1}$)	0-20	SD	0.5 ± 0.5^{b}	1.0 ± 0.9^{a}	1.0 ± 0.8^{a}	1.0 ± 0.8^{a}		McCulley *et. al.,* [2004]

Table 2.1 (continued)

N mineralization (mg N kg^{-1} d^{-1})	0-10	SE	42 ± 5^{a}	200 ± 18^{b}	137 ± 16^{bc}		127 ± 29^{c}	Hibbard *et. al.*, [2001]
Nitrification (mg N kg^{-1} d^{-1})	0-20	SD	0.4 ± 0.4^{b}	0.8 ± 0.8^{ab}	1.0 ± 0.8^{a}	1.0 ± 0.7^{a}		McCulley *et. al.*, [2004]
[NO_3^-] (mg N kg^{-1})	0-20	SD	6.3 ± 4.4^{b}	9.1 ± 5.7^{ab}	21.9 ± 17.1 ^{a}	19.7 ± 15.5 ^{ab}		McCulley *et. al.*, [2004]
[NH_4^+] (mg N kg^{-1})	0-20	SD	4.1 ± 5.5^{a}	5.5 ± 6.1^{a}	8.6 ± 8.8^{a}	7.3 ± 6.4^{a}		McCulley *et. al.*, [2004]
Organic C ($g\ m^{-2}$)	0-15	SE	1233 ± 22^{a}	1482 ± 69^{ab}	1925 ± 87^{b}	3035 ± 99^{c}	3142 ± 155^{c}	Liu *et. al.* [2013]
SMB C (mg C kg^{-1})	0-20	SD	328 ± 130^{b}	458 ± 166^{a}	435 ± 183^{a}	592 ± 212^{a}		McCulley *et. al.*, [2004]
C mineralization (mg C kg^{-1} d^{-1})	0-20	SD	8 ± 4.7^{b}	13.8 ± 7.2^{a}	14.9 ± 7.6^{a}	16.9 ± 8.8^{a}		McCulley *et. al.*, [2004]
Total soil respiration ($g\ C\ m^{-2}\ d^{-1}$)		SD	611 ± 83^{b}	683 ± 51^{ab}	780 ± 69^{a}	771 ± 85^{a}		McCulley *et. al.*, [2004]

Lowercase values indicate significant statistical differences between vegetation types within a row. Analyses by original authors.

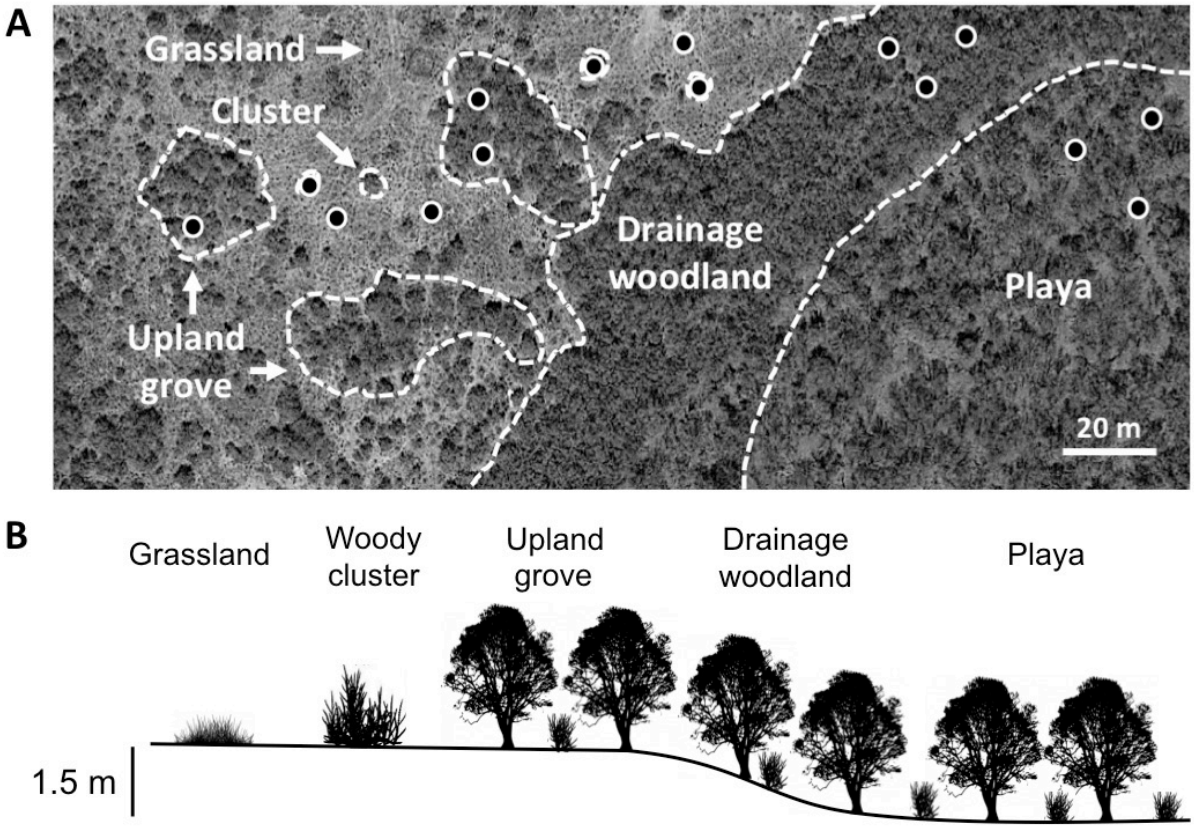


Figure 2.1 **A** Aerial view of study site near Ben Bolt, TX, with vegetation types delimited-remnant grassland, woody clusters and *Prosopis glandulosa*-dominated upland groves, drainage woodland and playa. Sampling sites are indicated by white circles (each site equals one group of three collars. N = 45). **B** Site topography.

Gas sampling and analysis

Trace gas flux sampling was conducted at seven time points: August 2012, January, May and August 2013, and January, May and August 2014. Fluxes were measured from nine collars in each of the five vegetation types (grassland, cluster, *Prosopis* grove, *Prosopis* drainage woodland and *Prosopis* playa, n = 45), distributed across 1.5 ha (Fig 2.1). Collars were constructed from PVC, measuring 25.5 cm in diameter, 15 cm in height and installed 7.5 cm into the soil six months prior to measurement.

For the six sampling campaigns between August 2012 and May 2014, fluxes were measured twice within a 2-3 day period: once before and once 24 h after the addition of a 15.3 mm artificial precipitation event. Events of this magnitude or greater occurred 10-12 times per year from 2011-2014, represent 20% of total rainfall events and can occur all year round.

To investigate short-term flux dynamics during a wetting event, one collar in *Prosopis* playa and one in grassland was measured every 10 seconds before and after the addition of a 3 mm artificial precipitation event in August 2013. In August 2014, six collars (three in *Prosopis* playa and three in the grassland) were measured repeatedly to determine flux response to wetting and rewetting over a period of seven days. Soil fluxes were measured before and after application of a 15.3 mm artificial precipitation event, allowed to dry down for three days and then measured again after the application of a second event of the same magnitude. Average interval between rainfall events at the site for 2011-2014 was 5.3 days, with median interval of 2 days. Soil moisture was determined gravimetrically by taking a 10 cm depth soil core from replicate wetted collars at each measurement time point. Cores were weighed before and after drying at 105°C for 3 days.

Instantaneous flux of NO, NO_y (all other oxidized forms of N, including HONO, HNO_3, NO_2 and organic oxides) and NH_3 was measured using a chemiluminescent NO analyzer (Thermo Scientific, Waltham, MA) after selective chemical and thermal decomposition to reduce or oxidize all reactive N trace gases to NO. A detailed description of the system can be found in (McCalley et al. 2011). Collar lids were covered in foil to reduce temperature increase during measurement. The instrument was calibrated by sequential dilution of an NO standard (Scott-Marrin, Inc. Riverside, California).

Static chamber samples were taken from the same collars as instantaneous fluxes to quantify CO_2 and N_2O flux. Chamber lids were 8 cm tall and were covered in foil to reduce temperature increase during measurement. Chamber lids contained a septa-sealed sampling port and a vent of path length 60 cm to allow pressure equalization with the atmosphere. Using a needle syringe, 50 ml of headspace was withdrawn immediately after closing the chambers and at 30 and 60 minutes and placed into pre-evacuated glass vials fitted with rubber septa (Geo-Microbial Technologies Inc., Ochleata, OK). Samples were analyzed within one month of collection at Cornell University, using a gas chromatograph fitted with electron capture and thermal conductivity detectors (Shimadzu GC-2014, Shimadzu Corp. Kyoto, Japan). Chamber volume was calculated using the average of four depth measurements inside of the collar. Fluxes were calculated based on the rate of increase in concentration over time. In cases where this increase was not linear, fluxes were calculated using the slope of the 0-1 hour interval.

Statistical Analyses

Statistical analyses were performed in R (R Core Team, 2012) and JMP Pro v10.0.0 (SAS Institute, Cary, NC) with a significance value of $p<0.05$, unless otherwise indicated. Johnson SI transformations were applied to normalize gas flux data prior to analysis and model building (Moulin et al. 2014). In cases where rainfall occurred less than 48 h prior to measurement, data were excluded from 'pre-wetting' analyses (25 values excluded out of 506 measurements). For the August sampling seasons five very large N_t flux measurements (defined as greater than two standard deviations above the mean for that sampling season and wetting treatment) were not included in the analysis but are discussed in section 3.1. Repeated-measures two-way ANOVA was used to assess the effects of vegetation type and sampling month, followed by Tukey's HSD

post-hoc test to isolate differences between specific areas and months. Regression analysis was used to determine the relationship between flux and temperature. A mixed effects model was fitted to investigate the effects of environmental parameters on soil trace gas flux after artificial wetting. Vegetation type, temperature, time since previous rainfall and quantity of previous rainfall were tested as fixed effects and collar identity and year were incorporated as random effects to generate restricted maximum likelihood variance component estimates. Repeated measures t-tests were used to compare pre- and post-wetting fluxes.

Results

Effect of vegetation cover and landscape position on N gas emissions

Under pre-wetting soil moisture conditions, total N gas flux ($N_t = N_2O+NO+NH_3+NO_y$) did not differ significantly between vegetation cover types on upland soils (Table 2.2, Fig 2.2). Specifically, total N emissions did not differ significantly between remnant grassland (8.2 ± 3.2 μg N m^{-2} h^{-1}, annual average ± SE) and adjacent upland *Prosopis* groves (5.5 ± 2.3 μg N m^{-2} h^{-1}, Table 2.2, Fig 2.2). Within cover types dominated by *Prosopis*, there was a significant effect of landscape position/soil type- low-lying, clay-rich *Prosopis* playa soils had the largest average annual emissions (14.0 ± 3.1 μg N m^{-2} h^{-1}), significantly greater than sandier upland *Prosopis* groves (Table 2.2, Fig 2.2). Elevated playa emissions were pronounced in summer, where average emissions were two-fold (May, 18.5 ± 3.4 μg N m^{-2} h^{-1}) to three-fold (August, 17.0 ± 3.6 μg N m^{-2} h^{-1}) greater than for other vegetation types. Under post-wetting conditions (24 h after the addition of a 15.3 mm artificial precipitation event), there was no significant difference in flux between vegetation types.

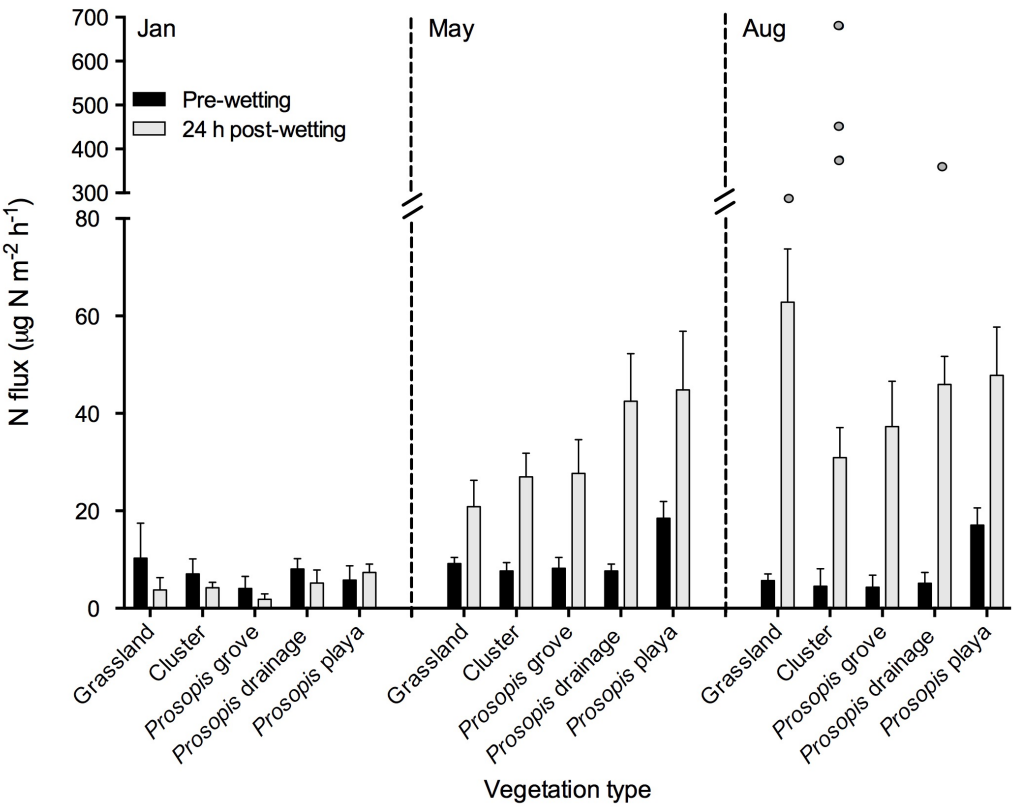


Figure 2.2 Reactive nitrogen trace gas flux (NH_3 + N_2O + NO + NO_y, μg N m^{-2} h^{-1}) from five vegetation types before and after a 24 h following the addition of a 15.3 mm artificial precipitation event. Values are the mean of nine collars per vegetation type sampled across two years (n = 18) ± 1 SE. Outliers not included in average calculations are indicated by grey circles. Statistical analysis is presented in Table 2.2.

Flux composition

NO was the single largest component of the total N flux for all sampling dates (data not shown), vegetation types, and moisture conditions (Fig 2.3). During summer months, precipitation caused an increase in NO flux for all vegetation types ($p<0.001$, Fig 2.3). NH_3 and NO_y also made up a significant portion of the total surface N flux during summer (Fig 2.3). For NH_3, wetting induced an increase in flux magnitude in three of the five vegetation types (grassland, cluster and *Prosopis* grove). Overall, NH_3 made up 8.3% of the total flux measured under pre-wetting conditions and 9.3% post-wetting, equivalent to a flux rate of 0.67-6.12 μg N m^{-2} h^{-1} (Fig 2.3).

The absolute magnitude of the NO_y flux decreased with wetting only in the *Prosopis* drainage woodland, though overall NO_y dropped from 7.7% to 2.3% as a percentage of total emissions upon wetting (0.12-1.77 μg N m^{-2} h^{-1}). By comparison, N_2O comprised 9.8 and 6.6% (-0.33-4.02 μg N m^{-2} h^{-1}) of pre- and post-wetting emissions, respectively.

Temperature response

Temperature had a positive effect on N_t flux, particularly following soil wetting (Fig 2.4, Table 2.3). Under post-wetting conditions, there was a positive relationship between temperature and flux rate for NH_3 and NO_y (linear) and NO (log linear, Fig 2.4d, f, h), but not for N_2O (Fig 2.4b). NO was the only compound that also showed a substantial positive flux response to increasing temperature under pre-wetting conditions (linear, Fig 2.4e). For NO, this corresponded to an increase from 1.61 μg N m^{-2} h^{-1} to 8.51 μg N m^{-2} h^{-1} (pre-wetting) or 3.34 μg N m^{-2} h^{-1} to 53.70 μg N m^{-2} h^{-1} (post-wetting) moving from 5°C to 35°C. For individual vegetation types, average post-wetting N_t fluxes were 7-26 fold greater in August than in January (Fig 2.2, Table 2.2). N_2O, NO_y and NH_3 all experienced instances of both net emission and net deposition to the soil surface, with consistent deposition occurring at lower temperatures (<18 °C) for NO_y (Fig 2.4).

Table 2.2. Two-way ANOVA of vegetation type by sampling month for total reactive nitrogen trace gas flux (NH_3 + NO_y + NO + N_2O, µg N m^{-2} h^{-1}), before soil wetting or 24 h after the addition of a 15.3 mm artificial precipitation event (n = 206 and 217, respectively). Lower case letters denote statistically significant differences between groups. Flux values are displayed in Fig 2.2. Interactions were not statistically significant (p<0.359 and p<0.205, respectively).

		Vegetation type	Month
Pre-wetting		Grassland [ab]	Jan [b]
		Cluster [b]	May [a]
		Prosopis grove [b]	Aug [b]
		Prosopis drainage [ab]	
		Prosopis playa [a]	
	F	3.229	5.586
	p	**0.022**	**0.002**
24 h post wetting		Grassland [ns]	Jan [c]
		Cluster [ns]	May [b]
		Prosopis grove [ns]	Aug [a]
		Prosopis drainage [ns]	
		Prosopis playa [ns]	
	F	2.12	130.65
	p	0.96	**<0.001**

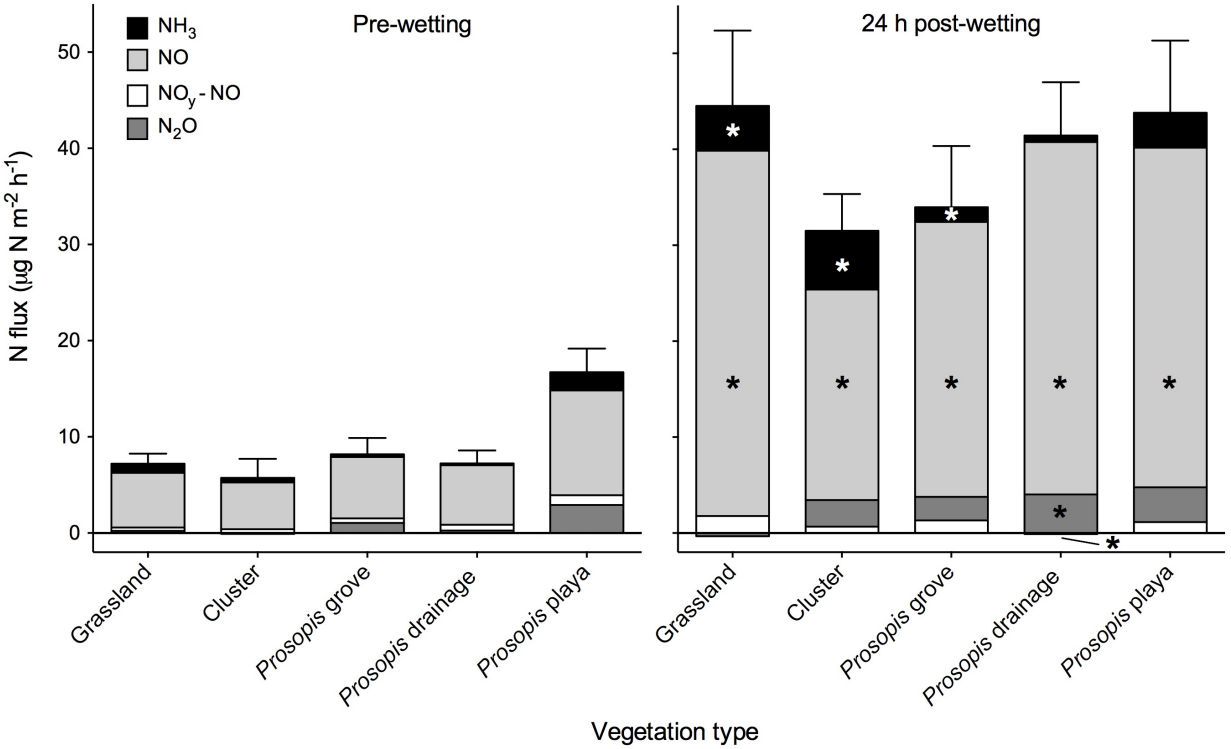
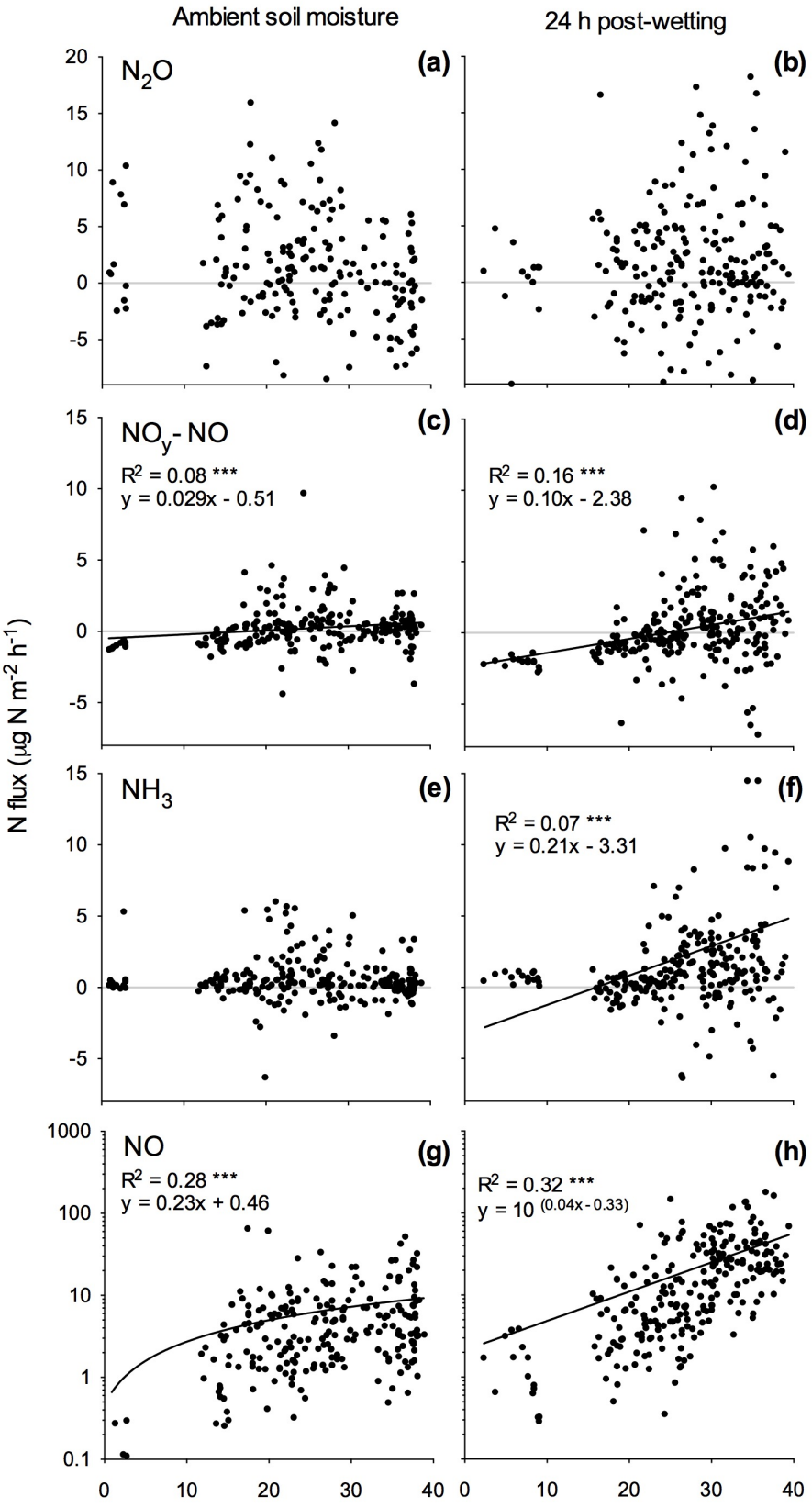


Figure 2.3 Flux of N_2O, NH_3, NO_y (not including NO) and NO (µg N m^{-2} h^{-1}) before and 24 h after the addition of a 15.3 mm artificial precipitation event for five vegetation types. Values are the mean for summer months (May and August) ± 1 SE. Asterisks indicate where the flux rate of a compound changed significantly with soil wetting ($p<0.05$).

Figure 2.4 Relationship between reactive nitrogen trace gas flux (**a**, **b** N_2O; **c, d** NO_y minus NO; **e**, **f** NH_3; **g**,**h** NO, µg N m^{-2} h^{-1}) and temperature (**a, c, e, g**) before soil wetting or (**b, d, f, h**) 24 h after the addition of a 15.3 mm artificial precipitation event (n = 233 and 248, respectively). R^2 and p-values are for normalized data. Note log scale for NO.


Ambient soil moisture
24 h post-wetting
N2O
(a)
(b)
NOy- NO
R2 = 0.08 ***
y = 0.029x - 0.51
(c)
R2 = 0.16 ***
y = 0.10x - 2.38
(d)
NH3
(e)
R2 = 0.07 ***
y = 0.21x - 3.31
(f)
NO
R2 = 0.28 ***
y = 0.23x + 0.46
(g)
R2 = 0.32 ***
y = 10 (0.04x - 0.33)
(h)
N flux (μg N m-2 h-1)
Temperature (°C)

Variation in response to soil wetting and rainfall patterns

Several 'super pulses' (anomalously large fluxes, defined here as more than two standard deviations above the mean for that sampling season and wetting treatment) were observed 24 h after wetting of soils under hot, dry August conditions (Fig 2.2). Five of these super pulses were observed in grassland, cluster and *Prosopis* drainage woodland sites across both measurement years and ranged in magnitude from 337-698 μg N m^{-2} h^{-1}. These super pulses exceeded average emissions for these vegetation types by between 4 and 20 fold, and occurred five times out of 90 total post-wetting flux measurements taken during August. These super pulses did not correlate with any physical factors or disturbances we observed (such as evidence of animal activity), and came from collars that did not display consistently elevated flux values at any other time points.

Total N_t fluxes showed a positive response to soil wetting during summer months (repeated measures t-test, $P<0.001$ for all vegetation types, Fig 2.2), but no response in winter. In August, water addition increased baseline N_t flux by 3 fold in *Prosopis* playa, and between 11-22 fold for other vegetation types after 24 hours.

To investigate the effect of rainfall timing on soil N fluxes, a field manipulation wetting experiment was conducted in August 2014 where two artificial precipitation events of 15.3 mm were applied 3 days apart. For both grassland and *Prosopis* playa, the maximum flux response to wetting of dry soil was lower for the second wetting than for the first, although soil moisture had returned to pre-wetting levels before the second addition (Fig 2.5). For playa, gravimetric water content was 3.7% prior to wetting, increased to 14.4% after the first water addition and declined to 4.8% before the second addition. For grassland, these values were 3.2%, 10.1% and 4.0%, respectively. The maximum flux rate for the second wetting was only 42 ± 10% (grassland, mean ± 1 SD) or 53 ± 25% (playa) as great as the first. As with previous measurements, there was no

significant difference between playa and grassland fluxes 24 h post-wetting (43 ± 4 and 49 ± 5 μg N m^{-2} h^{-1}, respectively), however the peak flux rate immediately following wetting was markedly higher and occurred earlier for playa (322 ± 39 μg N m^{-2} h^{-1}, 1 h post-wetting) than for grassland (106 ± 17 μg N m^{-2} h^{-1}, 7 h post-wetting, Fig 2.5). When NO flux was monitored continuously following a 3 mm water addition, fluxes increased above pre-wetting levels within 50-80 seconds for both vegetation types, though the rate of increase was greater for playa (Fig 2.6).

A mixed effects model identified time since previous wetting as a significant predictor of total flux response to a wetting event, along with vegetation type, air temperature and a significant interaction between the two (Table 2.3). Amount of prior wetting was identified as a borderline significant predictor ($P<0.054$).

Table 2.3 Significant fixed effects in a linear mixed effects model for total N flux measured 24 h post-wetting. Flux data was transformed for normality using a Johnson SI transformation prior to analysis. Collar identity and year were included as random effects to account for repeated sampling. n = 243.

Variable	F ratio	Prob>F
Air temperature	3490.67	**<0.001**
Vegetation type	8.96	**<0.001**
Time since previous wetting	16.37	**<0.001**
Amount of previous wetting	3.76	0.054
Air temperature * vegetation type	7.80	**<0.001**

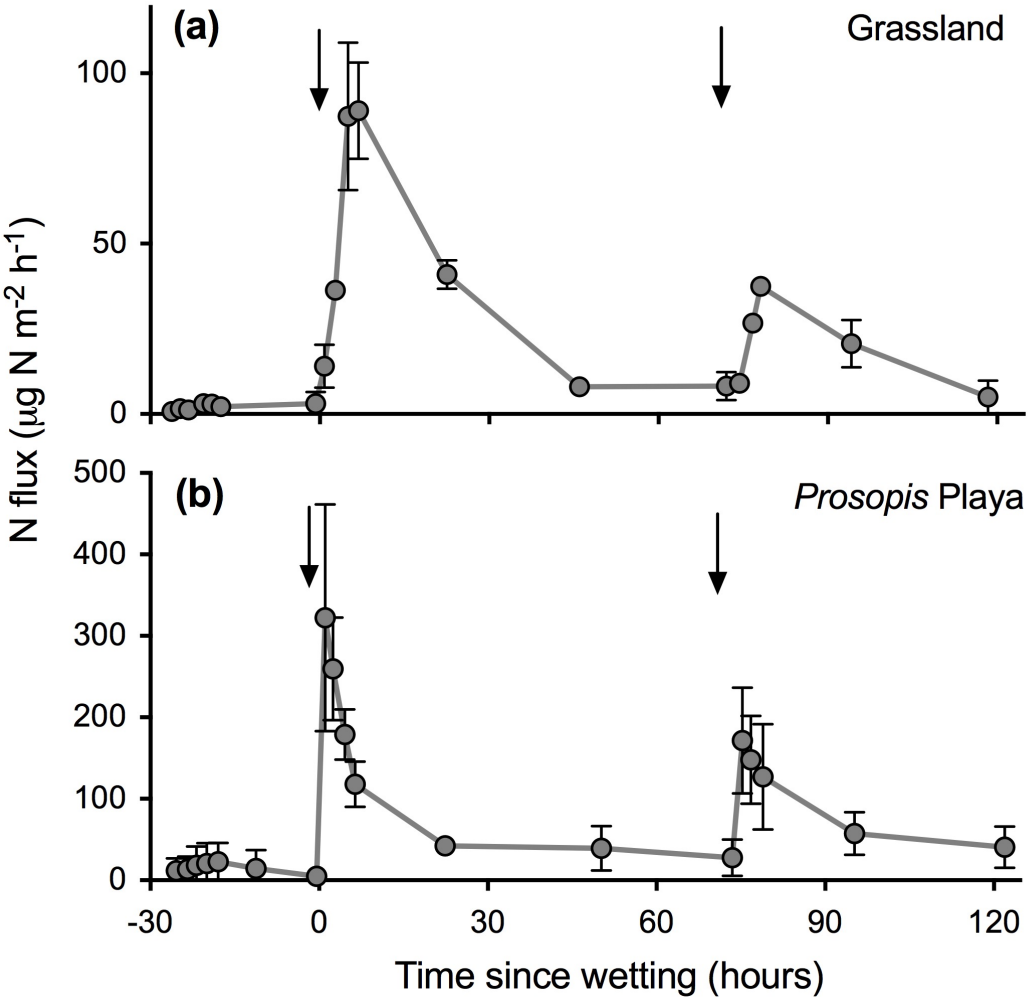


Figure 2.5 Reactive nitrogen trace gas flux (NH_3 + NO_y + NO, μg N m^{-2} h^{-1}) from **a** grassland and **b** *Prosopis* playa in response to sequential soil wettings of event size 15.3 mm, indicated by arrows. Values are means of three collars per vegetation type (n = 3) ± 1 SD, sampled in August 2014.

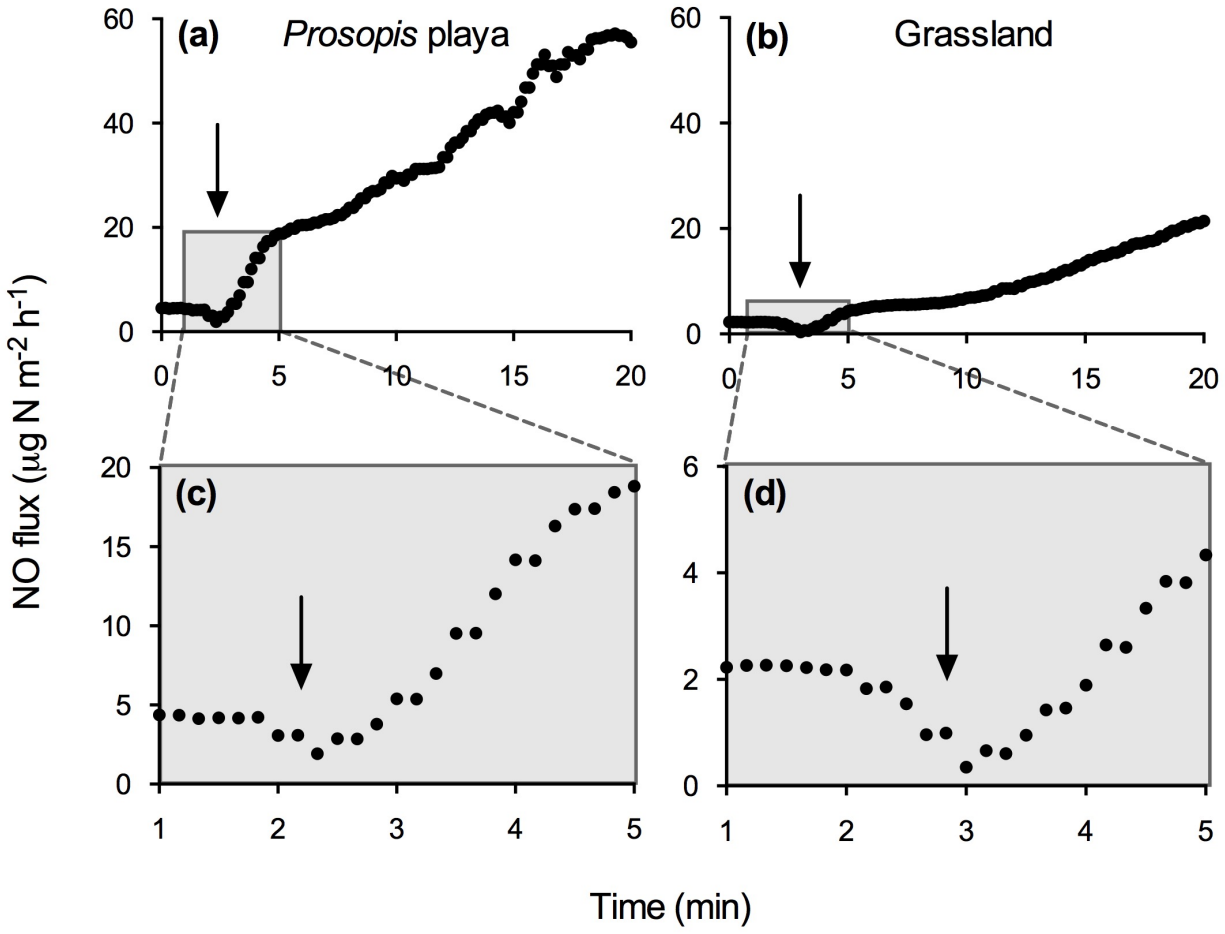


Figure 2.6 Nitric oxide flux (NO, μg N m^{-2} h^{-1}) from **a**, **c** *Prosopis* playa and **b**, **d** grassland soil in response to 3 mm water addition, indicated by arrow. Sampled in August 2013. Decreasing flux at ~2-3 min reflects brief removal of collar lid for water addition.

Discussion

We found no evidence that *Prosopis* encroachment increased total reactive N gas (N_t) emissions from upland soils when comparing established *Prosopis* groves with adjacent remnant grasslands (Fig 2.2, Table 2.2). This observation is surprising given that *Prosopis* groves at this site have two-fold or greater mineralization and nitrification rates, ammonium, and nitrate concentrations and 50% greater total N and microbial biomass N than adjacent grasslands (Table 2.1). NO (which dominates emissions at this site) is produced during microbial nitrification and denitrification. Increases in the substrate pools for these processes (NH_4^+ and NO_3^-, respectively)

and the rates at which these processes occur would be expected to increase gaseous N losses (Davidson *et al.* 2000), especially because other influencing variables such as pH and temperature are held relatively constant (Table 2.1). Correlations between inorganic N concentrations (particularly NH_4^+) and NO flux have been observed in similar ecosystems (Scholes *et al.* 1997; Martin *et al.* 2003). However, other arid-land studies have shown that inorganic N pools and cycling rates are not always good predictors of NO flux (Hartley and Schlesinger 2000). Within a *Prosopis*-dominated shrubland, (Hartley and Schlesinger 2000) observed that short-term variation in mineralization and nitrification rates and inorganic N concentrations of up to an order of magnitude did not correspond to significant increases in NO production and vise versa. It is possible that increases in N demand during encroachment may reduce the potential for 'leakiness' of NO from encroached soils (Davidson et al. 2000), even in the face of measurable changes in N cycling. In *Prosopis* groves, a greater proportion of soil N occurs as microbially recalcitrant compounds and N-acquiring enzyme production increases at a greater rate than total N (Creamer et al. 2013). This can be interpreted as evidence of continued N limitation to the microbial community even in the face of greater total pools (Creamer et al. 2013). In addition, the large increase in plant biomass with encroachment almost certainly drives increased root inorganic N demand.

Several other studies have also found that woody legume encroachment does not have predictable effects on N trace gas fluxes. The range of observed responses are variable, from apparently strong relationships between *Prosopis* biomass and NO emissions (Martin et al. 2003) to equivalent or greater NO flux from grasslands than legume-encroached shrublands (Hartley and Schlesinger 2000; Feig, *et al.* 2008a). With few examples to work with, and the complication of confounding factors within studies (particularly different soil types), it is difficult to parse out

which features of a system determine response. (Jackson et al.) 2002 determined that precipitation was a major determinant of C and N storage or loss during shrub encroachment. We note that in the examples above, the temperate system showed a positive NO response to encroachment while the two arid systems both showed no response. The temperate *Prosopis* system in north Texas (Martin et al. 2003) has comparable rainfall to the system studied here but was somewhat cooler, with lower *Prosopis* density and a wider range of summer soil NO fluxes (1.67 to 32.5 μg N m^{-2} h^{-1}) than we observed. In practice, changes in N emissions are likely dependent on the specific attributes of a system, such as degree of C and N increase, precipitation and temperature, time since encroachment and plant density, and soil physical characteristics.

Results from this study point to temperature and wetting conditions, rather than encroachment, as the major controls over total reactive N trace gas emissions in this ecosystem. The highest N_t emissions in the landscape occurred in *Prosopis* playa soils (Fig 2.2) and were likely driven by soil type and topographic effects on soil moisture. Compared with upland soils (which represent >80% of the landscape), low-lying, clay-rich playa soils have elevated water holding capacity and gravimetric soil water content and receive runoff from uplands (Bai *et al.* 2008; 2010). Strong positive relationships between soil moisture and NO emissions and soil moisture (below field capacity) are common in savanna, grassland and arid ecosystems (Cárdenas *et al.* 1993; Hartley and Schlesinger 2000; Martin *et al.* 2003). Thus, increased gas emissions from this soil type may be driven by more favorable ambient moisture conditions for NO production (Pilegaard 2013) under ambient conditions between rainfall events. Following a large (15 mm) simulated rainfall event, playa fluxes were comparable to those from other vegetation types, suggesting that water availability is the primary difference influencing flux

under ambient conditions. While we cannot rule out an interaction between *Prosopis* encroachment and soil type/topography as a contributor to increased playa N_t fluxes, the lack of flux increase seen in upland vegetation (between remnant grasslands and encroached *Prosopis* groves) indicates that encroachment alone does not drive increases.

Temperature drives strong seasonal variability in flux response to wetting in this ecosystem (Fig 2.2, 2.4). Water addition increased flux by 11-22 fold in August (average monthly temperature 29°C), but had no effect in January (14°C). Positive relationships between temperature and NO, NH_3 and NO_y flux were stronger after wetting. More pronounced temperature dependence is frequently recognized for NO production in wet soils (Smart *et al.* 1999; Hartley and Schlesinger 2000; Meixner and Yang 2006; McCalley and Sparks 2008), probably driven by greater microbial activity when water availability is not a limiting factor. It is also possible that plant phenology plays a distal role in mediating seasonal flux-wetting responses, i.e. capacity for plant N uptake to respond to wetting events may be lower in August when soils are very dry and plants less physiologically active. However, hydraulic lift (which may facilitate ongoing N uptake) occurs throughout the summer in *Prosopis* (Zou *et. al.* 2005), potentially countering this effect.

NH_3 fluxes in this study increased in response to wetting (by one order of magnitude on average, Fig 2.4f), and were positively correlated with temperature. This relationship may be mediated by increasing NH_4^+ supply due to increased mineralization with temperature (Dawson 1977; McCalley and Sparks 2008; Kim *et al.* 2012), or by decreased hydrologic connectivity under hotter conditions that promoted NH_4^+ accumulation (Davidson *et al.* 1990; Parker and Schimel, 2011; Hoymak *et al.* 2014). Unlike most N_2O and NO production (excluding chemodenitrification), NH_3 volatilization is an abiotic process, depending partly on the

availability of NH_4^+ ions in soil solution. Though this response to wetting seems intuitive, this effect has been illustrated primarily in deserts and there are few or no reports from other ecosystems (Kim et al. 2012). This result is significant given the appreciable fluxes of NH_3 in this system, equal to or greater than N_2O (average rates up to 6.12 μg N m^{-2} h^{-1} 24 h post wetting). NH_3 volatilization is not routinely measured in natural (unfertilized) soils because this process is considered to occur primarily at alkaline pH (Dawson 1977; Schlesinger and Peterjohn 1991). Fluxes here overlapped with those observed by Schlesinger and Peterjohn (1991) in Chihuahua shrubland at similar pH (6.7-7.4) (McCulley et al. 2004), but were an order of magnitude lower than those observed by McCalley and Sparks (2008) in the highly alkaline (pH 9-11) Mojave Desert. Our results support the idea that NH_3 volatilization can be a significant flux even in non-fertilized soils with close to neutral pH.

Rapid NO increases in response to soil wetting, even for superficial surface events, suggest that abiotic production may play a role in NO flux. For *Prosopis* playa soils, maximum rates of gas flux occurred ~1 hour following initial wetting and NO emissions began to increase within less than a minute when only the surface/litter was wetted (3 mm event, Fig 2.6a). These rapid rates of response, also seen in chaparral soils (Homyak and Sickman 2014), have been attributed to chemodenitrification (abiotic conversion of nitrite to NO) (Pilegaard 2013; Medinets *et al.* 2015). Microbial nitrifier response times after long periods of stasis have been reported in the order of minutes to hours (Davidson 1992; Borken and Matzner 2009), but not seconds. Generally, NO_2^- occurs as an intermediate product during nitrification and is metabolized by microbes, but it can also be isolated and concentrated in thin water films during soil drying (Davidson 1992; Pilegaard 2013). While this abiotic NO process is more favorable at lower pH, it can occur in slightly acidic, neutral or even alkaline soils, or in acidic microsites

(Van Cleemput and Samater 1995; Venterea *et al.* 2005). Though both *Prosopis* playa and grassland soils saw a flux increase within 50-80 seconds of wetting, the rate of increase was much slower in the grassland. This may reflect the fact that playa soils have higher C, inorganic N and clay concentrations and lower pH (6.15), conditions more favorable to chemodenitrification (Venterea *et al.* 2005; Liu *et al.* 2010). Small events (3 mm or less, sufficient to induce apparent abiotic NO fluxes but perhaps enough not substantively influence plant uptake) make up more than half of all rainfall events in this region, but their contribution to annual fluxes remains unknown.

Soil wetting legacy also impacted flux magnitude, and this effect was evident both in experimental wetting manipulations and in response to natural rainfall variation. N_t flux declined with subsequent water additions under dry summer conditions, even when soil moisture returned to pre-wetting levels between events (Fig 2.5). This provides support for the hypothesis that large flux responses to initial soil wetting are driven by rapid abiotic and biotic depletion of accumulated labile N, and that subsequent fluxes are lower as a result of reduced substrate availability (Johansson *et al.* 1988; Hartley and Schlesinger 2000; Butterbach-Bahl *et al.* 2004; Kim *et al.* 2012; Homyak and Sickman 2014). This accumulated N may be derived from atmospheric dry deposition, microbial cell lysis and exposure of previously protected soil surfaces, all of which increase to at least some extent with the duration or severity of a dry period (Borken and Matzner 2009). In the Chihuahua Desert, repeated wettings of creosote bush soils were shown to deplete the soil NH_4^+ pool and reduce associated NO fluxes (Hartley and Schlesinger 2000).

Analysis of our complete field data set (243 measurements across two years) identified the duration of antecedent dry period as a significant predictor of N_t flux in response to an

artificial precipitation event. Recognizing the importance of this process in arid or seasonally dry systems is important for a number of reasons. Predicted decreases in average soil moisture over the next century (particularly in the subtropics) and a likelihood of shifting precipitation patterns (Meehl et al. 2007), suggest that the influence of dry-wetting dynamics in controlling N trace gas flux may increase. However, many process-based biogeochemical models represent flux as a function of soil moisture, without incorporating an effect of wetting history or complex shifts in microbial substrate utilization (Groffman *et al.* 2009; Kim *et al.* 2012). Drying-wetting pulse responses have been developed for the DAYCENT and NOE models and tested with lab data (e.g. Li *et. al.* 2010; Rabot *et. al.* 2014), but DAYCENT still fails to predict seasonal NO fluxes well in arid landscapes (Homyak *et. al*. 2014). Collecting more data on the magnitude and mechanistic relationships behind flux responses to repeated wetting in dry ecosystems is a necessary step towards modeling and parameterizing this process.

A number of very large NO fluxes ('super pulses', more than 2 S.D. above the mean) were measured sporadically 24 h after wetting of dry summer soils and may point to additional biotic factors affecting local gas flux (Fig 2.2). Though hot moments are commonly associated with substrate accumulation and soil wetting (Groffman et al. 2009), it is unclear why some collars experienced hot moments while adjacent collars of the same vegetation type that were indistinguishable in all other regards did not and why repeated measurements from the same collar produced super pulses only occasionally. These super pulses were distributed across years and vegetation types and accounted for <3% of the total number of summertime measurements. Subsurface termite activity, common in this system, could be a possible cause of occasional highly elevated fluxes. Termite activity is spatially and temporally variable and has been observed to drive elevated NO emissions in southern hemisphere savannas (Rondon et al. 1993).

Annual NO emissions estimates for this ecosystem were generated using 'back of the envelope' calculations incorporating two years of January, May and August sampling data, proportional vegetation cover and rainfall records (Appendix 2.1). The resulting NO flux, 0.52 - 0.57 kg NO-N ha^{-1} y^{-1}. is comparable to estimates for savannas globally. Other studies report NO flux rates ranging from 0.18 kg NO-N ha^{-1} y^{-1} (Chihuahuan desert *Prosopis* shrubland, Hartley and Schlesinger, 2000), to 0.23 kg NO-N ha^{-1} y^{-1} (southern US shrubland/bare soil, Davidson et al., 1998) to 0.36-0.61 kg NO-N ha^{-1} y^{-1} (semi-arid South African savanna, Davidson *et al.* 1998; Hartley and Schlesinger 2000; Feig *et al.* 2008b). For a north Texas *Prosopis* savanna (Martin and Asner 2005) estimated an NO flux of 0.9 kg NO-N ha^{-1} y^{-1} from field-based measurements, or 1.6 kg NO-N ha^{-1} y^{-1} incorporating remotely-sensed vegetation cover distribution and pulse responses to rainfall. Though we incorporated these factors, we found no effect of encroachment on NO or total N emissions, while Martin et al. (2003) identified a relationship between flux and *Prosopis* biomass, a key difference in an ecosystem where *Prosopis* is the dominant vegetation cover. . Including other N trace gas compounds in addition to NO increased estimated emissions by 0.06 kg N ha^{-1} y^{-1}, an increase of ~11% over NO alone on an annual basis (Appendix Table 2.1). Weber *et. al.* (2014) recently reported maximum fluxes of HONO (a component of NO_y) from bare desert soils that fell within the same order of magnitude as many of the NOy fluxes measured in this study, as well as substantially higher emissions from soils with developed biocrusts.

We find no evidence that encroachment of a widespread woody legume *Prosopis* increases N trace gas fluxes from an upland semi-arid subtropical savanna, despite myriad changes in soil N cycling. This contrasts with findings of some previous encroachment studies and highlights the lack of consensus on the expected effects of a globally widespread land cover change. Instead,

temperature, topography, soil properties and the occurrence and frequency of precipitation are more important determinants of flux in this ecosystem. Rapidity of flux responses to wetting provide evidence that abiotic NO production (chemodenitrification) may contribute to pulse emissions on soil wetting. Because rainfall history has an important effect independent of soil moisture, modeling efforts and supporting data collection should consider incorporating temporal distribution and dry period severity/duration. This may become increasingly relevant in the future, when precipitation patterns (quantity, temporal distribution) in arid lands of the southern US and elsewhere are expected to shift as a result of global change (Archer and Predick, 2008). We also found that less routinely measured N compounds (NH_3 and NO_y) can comprise a significant proportion, up to 16%, of total N emissions. This adds to evidence that these compounds are an important component of savanna and arid land N fluxes and should be measured more routinely, especially when the goal is to generate estimates of total surface N flux or to create N budgets for arid lands.

APPENDIX 2.1

DESCRIPTION OF TRACE GAS SCALING PARAMETERS

Methods for scaling seasonal trace gas fluxes to annual emissions

Annual N efflux estimates for this ecosystem for the ten-year period 2004-2013 were generated using a model of baseline NO and N_t flux as a function of temperature under pre-wetting (dry soil) moisture conditions, a response function for wetting events, and vegetation type cover.

Annual pre-wetting NO or N_t emissions were calculated as a function of average monthly temperature (National Ocean and Atmospheric Administration National Climatic Data Centre, NOAA-NCDC, www.ncdc.noaa.gov) fitted to vegetation type-specific linear temperature-flux regressions derived from the two-year field data set (n = 54 per vegetation type). Flux response to precipitation was determined for a single class of event size ≥ 15.3 mm, as there was insufficient data to determine the response to smaller events. The least squares method was used to fit gamma distribution curves to field measurements of NO or N_t flux for 72 h following a precipitation event of 15.3 mm applied in August. Separate curves were fit for *Prosopis* playa and grassland, and the grassland response was used for upland cluster, *Prosopis* grove and *Prosopis* drainage woodland vegetation types. Flux response to precipitation was set to zero in January, as field measurements indicated that this response was small or absent, and to then scale linearly with temperature according to vegetation type-specific temperature-flux response regressions. Instantaneous post-wetting flux values (24 h after the application of an artificial 15.3 mm event) were calculated as above, using temperature regressions derived from post-wetting field measurements (n = 54 per vegetation type). Instantaneous flux values were mapped onto gamma distribution curves and the area under the curve was calculated to determine total flux for

72 hours after each rainfall event. Precipitation data for the period 2004-2013 was obtained from the NOAA-NCDC. Fluxes were calculated separately for each vegetation type and then weighted based on percentage cover (constant over the study period), which was determined using manual classification of Google Earth satellite images (50 cm resolution) in ArcGIS 10.2.2 (ESRI INC, Redlands, USA) for an 18 ha area surrounding the study site.

Results

Seasonal patterns of N gas emissions, wetting-induced response functions, vegetation cover proportions and precipitation records for the period 2004-2013 were combined to produce annual flux estimates for this ecosystem (Appendix 2.1, Table 1). For NO alone, this flux was 0.52-0.57 kg NO-N ha^{-1} yr^{-1}, and for all traces gases combined (N_t) was 0.59-0.64 kg N ha^{-1} yr^{-1}. Flux responses to precipitation contributed 0.04 – 0.7 g NO-N ha^{-1} yr^{-1} (for NO) or 0.02 – 0.10 g N ha^{-1} yr^{-1} (for N_t) above baseline dry fluxes, depending on number and timing of precipitation events (≥ 15.3 mm) across years.

Table 2.4 Estimated annual NO and N_t (NH_3 + NO_y + NO + N_2O) gas flux (kg N ha^{-1} y^{-1}) for a subtropical savanna ecosystem experiencing encroachment by *Prosopis glandulosa* for the years 2004-2013. Annual flux estimates include precipitation response function.

Year	Number of precipitation events ≥15.3 mm	Dry soil flux (NO)	Annual flux estimate (NO)	Dry soil flux (N_t)	Annual flux estimate (N_t)
2004	13	0.47	0.54	0.53	0.60
2005	11	0.48	0.54	0.54	0.61
2006	11	0.50	0.57	0.56	0.64
2007	14	0.47	0.54	0.53	0.61
2008	9	0.48	0.55	0.54	0.62
2009	5	0.48	0.53	0.57	0.59
2010	16	0.51	0.55	0.52	0.62
2011	6	0.46	0.52	0.55	0.59
2012	12	0.49	0.56	0.57	0.63
2013	14	0.51	0.57	0.54	0.64
Mean (± 1 S.D.)		**0.48 ± 0.02**	**0.55 ± 0.02**	**0.55 ±0.02**	**0.61 ± 0.02**

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CHAPTER THREE

DENITRIFICATION IN A SUBTROPICAL, SEMI-ARID NORTH AMERICAN SAVANNA: FIELD MEASUREENTS AND INTACT SOIL CORE INCUBATIONS

Abstract

Information on denitrification (particularly N_2) losses from dry ecosystems is limited despite their large area. Here, we present the first direct denitrification measurements for a northern hemisphere savanna, a *Prosopis*-dominated grassland/grove matrix in south Texas. We used the gas-flow intact soil core method to quantify N_2, N_2O and CO_2 losses and compared these with field measurements of N_2O, NO_y, NH_3 and CO_2. Under field-realistic soil moisture and O_2 conditions (average 17.5 - 20% O_2, minimum 15%) incubated soils produced no measurable N_2 flux (detection limit 52.2 μg N m^{-2} h^{-1}). Only in a subset of grove soils were fluxes of 70-75 μg N_2-N m^{-2} h^{-1} recorded after 102 h of incubation at 5-10% O_2 following wetting of very dry soils. Making the assumption that potential N_2 production falls just below the detection limit (likely an overestimate given the conditions needed to generate measurable fluxes), N_2 flux rates would fall on the low end of that recorded for a tropical Australian savanna (45-110 μg N m^{-2} h^{-1}) under comparable abiotic conditions. Assuming maximum possible production rates, N_2 could comprise <32-76% of total soil N gas flux following soil wetting in summer. Lack of flux response to soil wetting in winter suggests that cold-season N_2 fluxes are negligible. N_2O fluxes for core incubations were significantly higher than for field chambers; thus it is likely that incubations may overestimate N_2O flux by reducing soil column consumption. Overall, results indicate that soil N_2 fluxes are less dominant in this savanna than in other ecosystems investigated.

Introduction

The production of N_2 by denitrification is one of the largest uncertainties in the global nitrogen (N) budget (Pinder *et al.* 2012). Because the process is challenging to measure, estimates are

missing for many ecosystems (Kulkarni *et al.* 2008; Pinder *et al*. 2012). Denitrification in arid and semi-arid systems is usually assumed to be low but few field measurements corroborate this assumption, especially for the production of N_2 (Werner *et al.* 2014). This is problematic given the large global area of arid lands, which make up ~45% of the terrestrial surface (Galbally *et al.* 2008; Schimel 2010). Recently, the first estimates for a tropical savanna (derived using the He gas flow soil core technique) indicate that N_2 may comprise the majority (82-99%) of the soil N flux in northern Australia (Werner *et al.* 2014), but no corresponding estimates exist for subtropical or northern hemisphere savannas.

Microbially-mediated denitrification reduces nitrite (NO_2^-) and nitrate (NO_3^-) to nitric oxide (NO), nitrous oxide (N_2O) and di-nitrogen (N_2), with N_2O and NO also produced during the preceding nitrification step in the N cycle. The process of denitrification is notoriously patchy across space and time (Groffman *et al*. 2000). High variability is especially common in arid ecosystems, where microbial activity and N cycling are highly correlated with pulses of water availability following rainfall (Austin *et al.* 2004; Hartley *et al.* 2007) and the distribution of carbon (C) and N varies with vegetation cover and landscape position (Austin *et al*. 2004). Denitrification rates are controlled by a complex interaction between soil organisms, soil properties and climatic conditions (Saggar *et al.* 2013). Observed drivers include the availability of substrates (mineral N and labile C), temperature, pH, soil moisture and O_2 concentrations; the proportional flux of different products depends on these drivers as well as depth of production in the soil profile (Firestone 1982; Weier *et al.* 1993; Butterbach-Bahl *et al.* 2002; Simek *et al.* 2002; Clough *et al.* 2005; Saggar *et al.* 2013). N_2:N_2O ratio can vary by orders of magnitude between ecosystems (Wang *et al.* 2011). Limited observations for arid systems suggests that N_2 can dominate the flux in semi-arid Mediterranean shrublands (Dannenmann *et al*. 2011) as well

as monsoonal Australian savannas (Werner *et al.* 2014).

In arid and semi-arid systems, water addition is the major control over denitrification rate (Peterjohn and Schlesinger 1991; Davidson *et al.* 1993; Galbally *et al.* 2008). Upon wetting, anaerobic microsites considered necessary to facilitate denitrification have been observed (Garcia-Pichel and Belnap 1996; Hartley *et al.* 2007) as a result of increased respiration. During dry down in arid soils, substantial NO_2^- and NO_3^- accumulation can occur in surface soils once organisms are no longer capable of N uptake and this may provide a ready substrate pool for denitrification upon rewetting (Davidson *et al.* 1993; Austin *et al.* 2004). Plant uptake of N may also be water-limited or temporally offset from availability after rainfall (Schwinning and Sala 2004), making this pool more available for loss than in many other ecosystems.

Measuring *in situ* fluxes of N_2 directly is not currently possible in the field without disruption of the soil physical and chemical system, e.g. by the addition of tracers, because high background concentrations of N_2 in the atmosphere exceed fluxes by orders of magnitude (Groffman *et al.* 2006; Kulkarni *et al.* 2008). However, development of enclosed incubation methods that replace the atmosphere with an N-free headspace can quantify this flux using intact soil cores without the need to add extra N (Scholefield *et al.* 1997; Butterbach-Bahl *et al.* 2002; Wang *et al.* 2011). These systems do present some limitations, including the removal of plant effects on rhizospheres processes, difficulty in identifying very low fluxes, some changes to soil physical structure, and effects on residence time and potential reduction of N_2O in the soil matrix. The atmosphere replacement step of the incubation method allows for manipulation of headspace/macropore O_2 concentrations, which can be matched to macropore O_2 concentrations measured in the field. The method assumes that gas exchange between macro- and micropores is comparable under field and lab conditions (one rationale for prioritizing maintenance of soil

structure), and that biological consumption of O_2 by respiration re-establishes field-comparable levels of micropore anoxia which drive denitrification fairly rapidly after replacement. However, these systems do allow for reasonable replication of abiotic field conditions (such as water filled pore space, WFPS, or O_2 concentration) necessary to make meaningful extrapolations of fluxes.

As part of an ongoing campaign to quantify seasonal gas fluxes from a subtropical, semi-arid *Prosopis* savanna in the Rio Grande Plains, we quantified respiration (CO_2) and denitrification (N_2, N_2O) using the Nitrogen-Free-Atmospheric Recirculation Method on excised soil cores (Butterbach-Bahl *et al.* 2002; Burgin *et al.* 2010; Burgin and Groffman 2012). For N_2O and CO_2, we compared these incubation fluxes with those derived from field-based static chambers. To assess the importance of N_2 production as a loss pathway in this system, we also compared lab N_2 fluxes with field measures of other N species (ammonia; NH_3, NO, and other non-NO NO_y) made using a real time dynamic chamber system (McCalley *et al.* 2011). We compared samples representative of spatial soil heterogeneity common to savannas- N-poor remnant grasslands and N- and C-enriched soils from beneath established *Prosopis* groves (Hibbard *et al.* 2001). To explore the climatic heterogeneity also characteristic of arid and savanna biomes (Hutley and Setterfield 2008), we compared lab and field measurements taken under both winter (moist) and summer (dry, sporadically wetted) conditions following simulated precipitation events.

Materials and Methods

Study site

Sampling was conducted at the Texas A&M AgriLife La Copita Research Area (27°40'N, 98°12'W), 65 km west of Corpus Christi, Texas, USA on the Rio Grande Plains. The upland

savanna parkland site contains groves dominated by the N-fixing tree *Prosopis glandulosa* (Torr.) var *glandulosa* with a mixed woody understory and remnant grasslands made up of C_4 grasses, perennial herbs and bare ground. Upland soils at the site are sandy loams (Typic and Pachic Argiustolls) with little to no topography. The climate is subtropical with typically warm, moist winters and hot, dry summers (Archer *et al.* 2001). Mean annual temperature is 22.4°C with an average growing season of 289 d. Mean annual rainfall is 680 mm, falling predominantly in May and September. The site is a working ranch and has been grazed at a low stocking rate for ~30 years, with livestock exclusion occurring for >3 y prior to this study.

Soil collection and processing

Soils cores were collected in January and August 2014 using custom 4 cm diameter PVC spilt cores inserted to a depth of 10 cm. Four sets of five spatially clustered cores (within ~0.5 m^2) were sampled from each vegetation type (upland *Prosopis* groves and remnant grasslands). Grove cores were sampled from within the drip line of a *Prosopis* tree, >0.5 m from the bole. Grassland cores were sampled from the unvegetated matrix between grass and herbs. Cores were left inside the PVC sleeves to maintain structure, carefully padded and wrapped in plastic, and transported to the Cary Institute of Ecosystem Studies (Millbrook, NY) at ambient temperature to begin analysis within 4-11 d.

Replicate cores were dried for 3 d at 105°C and weighed to determine bulk density and soil moisture. Additional replicate cores were sieved with 2 mm mesh, extracted in 1:4 (w/v) 2M KCl (Fisher Scientific, Pittsburg, PA) for 2 h and analyzed to determine NH_4^+ and NO_3^- concentrations colorimetrically using a Lachat flow injection analyzer (Hach Co., Loveland, USA). These analyses were replicated three times for each soil core (n = 4 biological replicates

by 3 extraction replicates for each vegetation type). Soils were homogenized in a ball mill (Retsch, MM-2, Haan, Germany) and analyzed for %N and %C using a continuous flow isotope ratio mass spectrometer interfaced with an elemental analyzer (Model Delta V Advantage; Thermo-Scientific, Bremen, Germany) at the Cornell University Stable Isotope Laboratory.

Field O_2 measurements

In August 2013, Apogee diffusion head soil O_2 sensors (SO-100 series, Apogee Instruments, Logan, UT) were installed at the site, one each in adjacent grassland and grove areas. Diffusion heads (3 cm diameter) were placed at ~7 cm depth and connected to a CR23X datalogger (Campbell Scientific, Logan, UT) programmed to record macropore O_2 concentration and soil temperature every hour.

Direct measurement of N_2, N_2O and CO_2 from intact soil cores

N_2, N_2O and CO_2 fluxes from intact soil cores were measured using the Nitrogen-Free Atmospheric Recirculation Method (N-FARM), as described in Burgin and Groffman (2012), Raciti *et al.* (2011) and in Appendix 3.1. Four cores from each vegetation type (grassland and grove) were incubated at two O_2 concentrations (10% and 20% O_2 in He) with headspace samples taken at 0, 8, 24 and 48 h in January and 0, 8, 24, 48, 72, and 102 h in August, 2014, following an 18 h headspace replacement and pressure equalization step. Cores were removed from the PVC sleeves with minimal disruption to core structure and fresh cores were used for each incubation/O_2 treatment. Immediately prior to sealing incubation vessels, cores were weighed and wet with 19.2 ml ultrapure H_2O, equivalent to a rain event of 15.3 mm. Rain events of this magnitude or greater occurred 10-12 times per year at La Copita during 2011-2014.

Incubation temperature was 21-22°C in January and 23-25°C in August. To test for outgassing of N_2 from soil pore spaces (i.e. any residual N_2 not removed by the headspace replacement step), cores were sterilized to remove most microbial activity and re-incubated. After each initial incubation, cores were allowed to re-equilibrate with the atmosphere under refrigeration for 5 days, autoclaved twice at 121°C for 1 h to sterilize, cooled for 24 h and incubated as above. No measurable N_2 production occurred in autoclaved cores.

Flux rates were calculated by regression of chemical species concentration versus time of incubation corrected for headspace dilution during sampling. The linear portion of the time-concentration curve was used to calculate flux rates; generally the first 0-48 h. Calculations of the instrument detection limit are described in Appendix 3.1. Flux rates were expressed in areal terms by dividing by the top surface area of the core.

Field flux measurements for CO_2, N_2O, NO, NO_y and NH_3.

Field fluxes were measured in August of 2012 and 2013 and January of 2013 and 2014 from nine soil collars per vegetation type, after application of a 15.3 mm rainfall event. NO_y and NH_3 were measured with a flow-through chamber, using selective thermal and chemical decomposition to reduce or oxidize all reactive N trace gases (NH_3 and other non-NO NO_y but not N_2O) to NO followed by measurement with a chemilluminescent NO detector (Thermal Electron Corporation 42i-TL) as described in McCalley *et al.* (2011). CO_2 and N_2O fluxes were measured using the static chamber method (McCalley *et al.* 2011). Chamber fluxes were calculated based on the rate of concentration increase over time. In cases where the concentration increase was not linear, the slope of the 0-1 h interval was used.

Statistical analysis

Statistical analyses were performed in R (R Core Team, 2012) and JMP Pro v10.0.0 (SAS Institute, Cary, NC, USA). Significance was set at α=0.05. Johnson SI transformations were applied to account for non-normal data distribution prior to analysis (Moulin *et al.* 2014). Two-way analysis of variance (ANOVA) was used to compare CO_2 and N_2O fluxes between sites, with vegetation type and sampling date as main effects. One-way ANOVA followed by Tukey's post-hoc test was used to compare CO_2 and N_2O fluxes between N-FARM incubations and field measurements within seasons, and t-tests were used to compare field and N-FARM fluxes separately between seasons, and soil physical and chemical characteristics.

Results and Discussion

N_2 and N_2O fluxes

We measured N_2 production in from subtropical, semi-arid grassland/woodland soils under two sets of incubation conditions. Incubations that mimicked field conditions (14-20% O_2, recently-wetted winter- and summer-sampled soils) produced no measurable N_2 fluxes above the instrument detection limit of 52.2 μg N m^{-2} h^{-1}. In incubations in which O_2 concentration was well below that measured in the field (4-10%), we observed N_2 production in a small subset of cores- 2 out of 8 samples of the more C- and N-enriched *Prosopis* grove soils, after the addition of a large rainfall event to very dry summer (August)-sampled soils (Table 3.1, 3.2). These fluxes (70.1 and 75.9 μg N m^{-2} h^{-1}) were observed after 102 hours of incubation at 31% WFPS and 23-25°C, when the initial 10% O_2 headspace concentration had declined to 3.8-5.6% O_2 as a result of respiration (Table 3.1, 3.2). Field measurements of these sandy, well-drained grassland and grove soils average 17.5-20% O_2 in summer, dropping once to ~15.5% for <5 hours

following a large, infrequent rain event (Fig 3.1). These measurements also spanned the period beginning 24 hours after wetting of very dry (6% WFPS) summer soils when highest pulse emissions of N trace gases are observed under field conditions (Soper *et al*. in review).

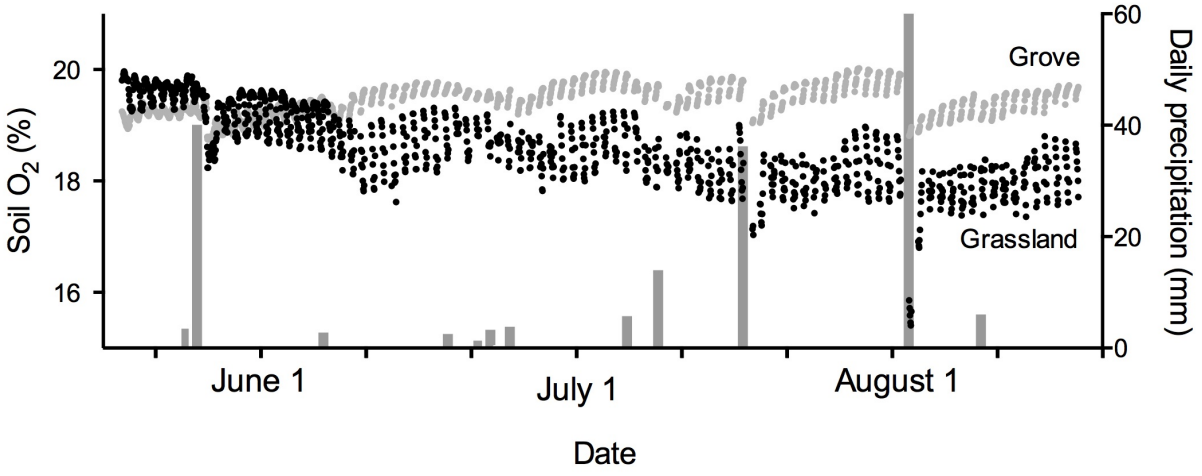

Figure 3.1 Soil O_2 concentrations (%) at ~7 cm depth for *Prosopis* grove (light grey points) and remnant grassland (black points) and daily rainfall (mm, grey bars) for summer 2014

In the context of upland soil N_2 fluxes in the core-incubation literature, these measurements fall on the low end of observed values (Butterbach-Bahl *et al.* 2002; Dannenmann *et al.* 2008; Kulkarni *et al.* 2013; Eickenscheidt *et al.* 2014; Morse *et al.* 2014, Werner *et al.* 2014). Given the conditions needed to exceed instrument detection limit in incubations, it is likely that actual fluxes fall somewhere well below 52.2 μg N m^{-2} h^{-1} under realistic O_2 conditions. In contrast, Werner *et al.* (2014) measured minimum average N_2 fluxes of 45 ± 33 μg N m^{-2} h^{-1} in Australian savanna soils at 25% WFPS, 30°C and 20% headspace O_2. Further incubations of these soils at 25-50% WFPS and 20-30°C reached 108 ± 82 μg N m^{-2} h^{-1}. These N_2 measurements were performed when soils had been continuously wet for many days and pulse effects would likely have subsided. Comparing the 24-hour post-wetting pulse period and

subsequent days between the two studies, we observed markedly higher summer N_2O flux rates (15-65 μg N_2O-N m^{-2} h^{-1}, Table 3.2) than the Werner study (<5 μg N_2O-N m^{-2} h^{-1}). Over the course of four days of incubation at either 10 or 20% initial O_2, total N_2O emissions exceeded total N_2 emission by three fold or greater in *Prosopis* grove soils (Table 3.2). We conclude that even under comparable rates of soil respiration (CO_2 fluxes overlapped between studies), North American savanna soils studied here may experience less complete denitrification or higher rates of nitrifier denitrification (Wrage *et al.* 2001) than savannas elsewhere, at least under incubation conditions. We did not test the effect of rainfall events larger than 15 mm (which constitute on average ~12% of total events at the site). Though these events are not sufficient to significantly reduce bulk soil O_2 (Fig 3.1), they may reduce diffusivity in soil and thus increase complete emissions during the brief periods when they occur. Direct flux comparisons with other ecosystem types are challenging because incubations are typically conducted at much higher soil water contents characteristic of temperate ecosystems. However, even within temperate forests relative fluxes can be highly variable, dominated by either N_2 or N_2O under comparable incubation conditions both within and between sites (Butterbach-Bahl *et al.* 2002; Dannenmann *et al.* 2008; Eickenscheidt *et al.* 2014).

What features of this system might support low N_2 flux rates? Soils have a high sand content (77-78%; Liu *et al.* 2010) that likely facilitates consistently high field soil O_2 concentrations (Fig 3.1). In general, the availability of inorganic N was very low compared with temperate forest sites (Dannenmann *et al.* 2008; Eickenscheidt *et al.* 2014), but similar or higher than Australian savanna (Werner *et al.* 2014). While we did not quantify labile C, this may serve as an additional limiting resource for microbial denitrifying activity (Weier *et al.* 1993). *Prosopis* grove soils with higher total C and N produced higher N_2O and N_2 fluxes than the more

depauperate grassland (Table 3.2). Though basic pH ranges are generally considered to promote complete denitrification (Saggar et al. 2013), both Dannemann et al. (2008) and Werner et al. (2014) recorded the greatest absolute N_2 fluxes at lower pH values. pH values in this study fell between 6.3-6.5 (Table 3.1).

Table 1. Soil physical and chemical properties of remnant grassland and *Prosopis* groves. For ambient soil moisture, bulk density, %C, %N and $\delta^{15}N$, values represent means (1 SD in brackets) for four soil cores (0-10 cm depth, n = 4) of each land cover type at each time point. For other parameters, values represent mean + 1 SD of three technical replicates for each of the four cores (n = 12).

	Bulk density ($g\ cm^{-3}$)	pH†	C concentration ($mg\ g^{-1}$ dry soil)	N concentration ($mg\ g^{-1}$ dry soil)	$[NH_4^+]$ ($mg\ N\ kg^{-1}$ dry soil)	$[NO_3^-]$ ($mg\ N\ kg^{-1}$ dry soil)	Ambient WFPS (%)	WFPS after simulated rainfall event
JANUARY								
Grassland	1.33 (0.02)[a]	6.5 (0.1)	0.54 (0.05)[a]	0.07 (0.01)[a]	0.36 (0.15) [a] *	1.42 (0.29)[a] *	22 (3)[a] *	51 (3)[a]*
Grove	0.98 (0.04)[b]	6.3 (0.1)	2.04 (0.58)[b]	0.22 (0.05)[b]	1.14 (0.53) [b]	1.97 (0.53)[b] *	13 (4)[b] *	36 (2)[b]*
AUGUST								
Grassland					0.67 (0.18)[a]	2.62 (0.88) [a]	6 (1)	38 (5)
Grove					1.49 (0.31)[b]	6.31 (3.01) [b]	6 (1)	31 (3)

† Taken from Boutton and Liao (2010). 0-15 cm depth value for the same sampling sites as this study, as measured in a 1:2 soil/water mixture

[a,b] Within a time point, indicates whether two vegetation types differ significantly from each other ($P<0.05$, student's t-test)

* Indicates whether values within a vegetation type differ significantly between time points ($P<0.05$, student's t-test)

Table 3.2. Cumulative N_2O and N_2 emission (μg N m^{-2}) and actual headspace $\%O_2$ concentration for lab incubations with grassland and *Prosopis* grove cores in August (summer) 2014. Values are average of four cores + SD.

	10% O_2						20% O_2					
	Grassland			Grove			Grassland			Grove		
Incubation time	**N_2†**	**N_2O**	**O_2**	**N_2†**	**N_2O**	**O_2**	**N_2†**	**N_2O**	**O_2**	**N_2†**	**N_2O**	**O_2**
8 h	n.d.	96 ± 51	10 ± 0.1	n.d.	606 ± 374	10 ± 0.1	n.d.	100 ± 81	19 ± 0.1	n.d.	219 ± 189	19 ± 0.1
24 h	n.d.	333 ± 152	10 ± 0.1	n.d.	2340 ± 1149	9 ± 0.4	n.d.	369 ± 332	19 ± 0.4	n.d.	1531 ± 746	18 ± 0.4
48 h	n.d.	543 ± 284	10 ± 0.2	n.d.	7038 ± 3267	9 ± 0.7	n.d.	497 ± 369	18 ± 0.6	n.d.	4286 ± 1607	17 ± 0.6
68 h	n.d.	568 ± 212	10 ± 0.3	n.d.	11634 ± 5559	8 ± 0.9	n.d.	531 ± 374	17 ± 0.7	n.d.	6108 ± 2085	16 ± 0.8
102 h	n.d.	836 ± 360	9 ± 0.4	7942*	27130 ± 12976	5 ± 1.3	n.d.	824 + 552	16 ± 1.2	n.d.	16699 ± 11770	14 ± 1.2

* Average for two out of four cores for which flux exceeded minimum detection limit.

† N_2 detection limit is 5324 μg N m^{-2}. For a 102 h incubation this is equivalent to 52.2 μg N m^{-2} h^{-1}.

Bounding of percentage flux composition

If we assume that actual N_2 flux rates fell just below the instrument detection limit (likely an overestimate) and place them in the context of other measured gaseous N fluxes (NH_3, NO, non-NO NO_y and N_2O), N_2 could comprise a maximum of 32-48% (grassland) or 59-76% (*Prosopis* grove) of soil N gas flux, depending on sampling year (Fig 3.2). These values are specifically for 24 hours after the addition of a large rainfall event (15.3 mm, a magnitude occurring ~10 times per year at this site) under summer conditions.

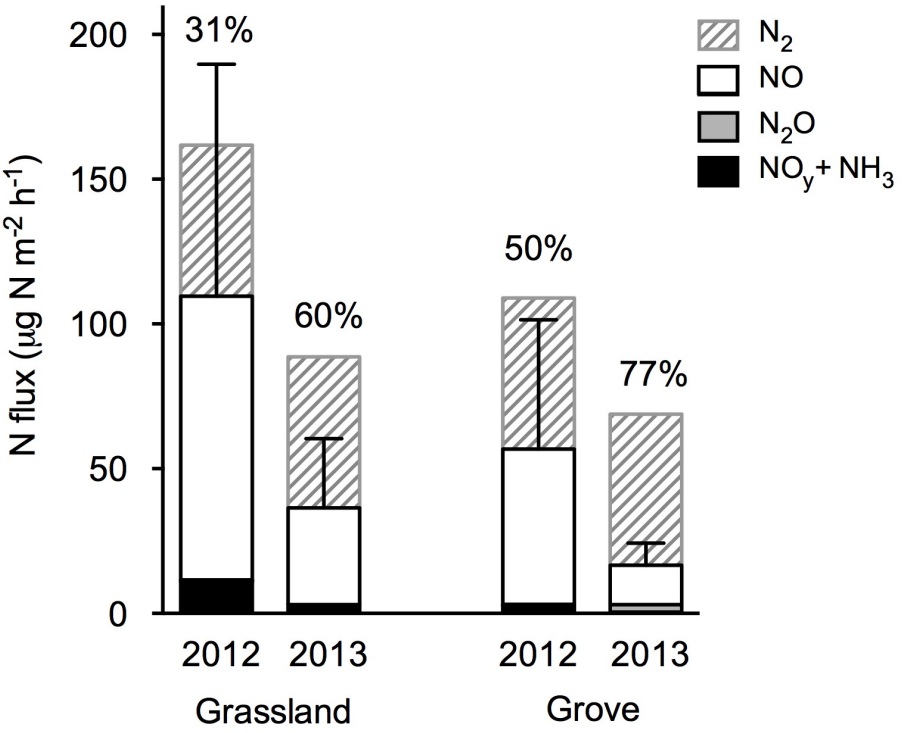

Figure 3.2 Field fluxes of and NO, N_2O, NH_3 and non-NO NO_y (μg N m^{-2} h^{-1}) in August of 2012 and 2013 for grassland and *Prosopis* groves, plus maximum potential N_2 flux derived from lab incubations at 20% O_2 in August 2014 (equal to the minimum detection limit for a 96 h incubation, no fluxes above detection limit were measured). Upper potential percent of total flux comprised of N_2 is indicated above bars. Both field and lab flux measurements were begun 24 h following the addition of a 15.3 mm simulated rainfall event. Field values represent a mean of nine collars per vegetation type + 1 SD

The alternative scenario is that fluxes fall well below the detection limit, in which case actual N_2 emissions from this site may be closer to zero. In contrast, N_2 flux from excised Australian savanna soil cores comprised 82-94% of N_2+N_2O+NO flux under comparable incubation conditions (Werner *et al.* 2014). In practice, the high soil O_2 concentrations, rarity of large rainfall events and seasonal nature of this savanna likely mean that even sporadic relatively high (for this site) N_2 fluxes would likely to constitute a small percentage of total N gas emissions.

Comparisons between N-FARM incubation and field flux rates

We compared N-FARM-derived N_2O and CO_2 fluxes with field-based static chamber fluxes (Fig 3.3) to assess how representative lab-measured fluxes might be. Comparisons were made for the same season (January or August), under comparable wetting conditions (24 hours following the addition of a 15.3 mm simulated rainfall event) and under comparable O_2 concentrations (N-FARM incubations began at 20% O_2 and reached 17-18% after 48 hours, summer field soil O_2 concentrations average 17-20%, Table 3.2). During the summer time points, N_2O fluxes observed in the lab were much greater than in the field; 15 ± 13 and 65 ± 31 μg N m^{-2} hr^{-1} for grassland and *Prosopis* grove, respectively, compared with field rates of -0.6 and 2.5 μg N m^{-2} hr^{-1} (Fig 3.3). This is likely driven in part to a shorter path length between microsites of N_2O production and the headspace/atmosphere due to higher surface area of incubated cores (Morse *et al.* 2015). There is an inherent trade off however, between reducing core surface area to counteract this issue and still achieving effective replacement of the soil atmosphere. In the field, N_2O experiences a longer path length and residence time between the site of production and the emission surface, and thus a greater fraction may be reduced to N_2 prior to emission

(Schlesinger 2013, Morse *et al.* 2014). If we repeat calculations for total soil N gas flux and assume that N-FARM incubations underestimate complete denitrification (i.e. all N_2O produced in soil would be reduced to N_2 under field conditions), the maximum possible N_2 flux percentage increases to 39-66 and 68-88% (grassland and *Prosopis* grove, respectively). It is also possible that the difference between field and laboratory N_2O fluxes is a result of denaturation of N_2O reductase by oxygenation of pore spaces during headspace replacement. However, we assume that the residence time differences between cores in the lab and field chambers were the dominant effect, as re-synthesis of N_2O reductase should be rapid once anoxic microsites are reestablished following replacement.

Unlike N_2O, CO_2 does not typically undergo chemical changes between microsite production and emission from the soil surface and thus CO_2 emissions can be used as a coarse proxy to asses the relative availability of microbial substrates for denitrification (Werner *et al.* 2014). CO_2 fluxes were similar between N-FARM incubations and field chamber fluxes (Fig 3.3), indicating that the core incubations supported a realistic level of microbial activity (Morse *et al.* 2015). The disconnect between CO_2 and N_2O fluxes as measured using N-FARM versus field methods has implications for extrapolating incubation-derived N_2O flux rates and particularly N_2:N_2O ratios to field scales. While experimental configurations that direct gas flow from the bottom to the top of a soil core (e.g., Dannenmann *et al.* 2010) may compensate somewhat for this effect, soil residence time is still likely to be greater under ambient diffusion conditions and thus flow-through systems may still underestimate N_2O reduction.

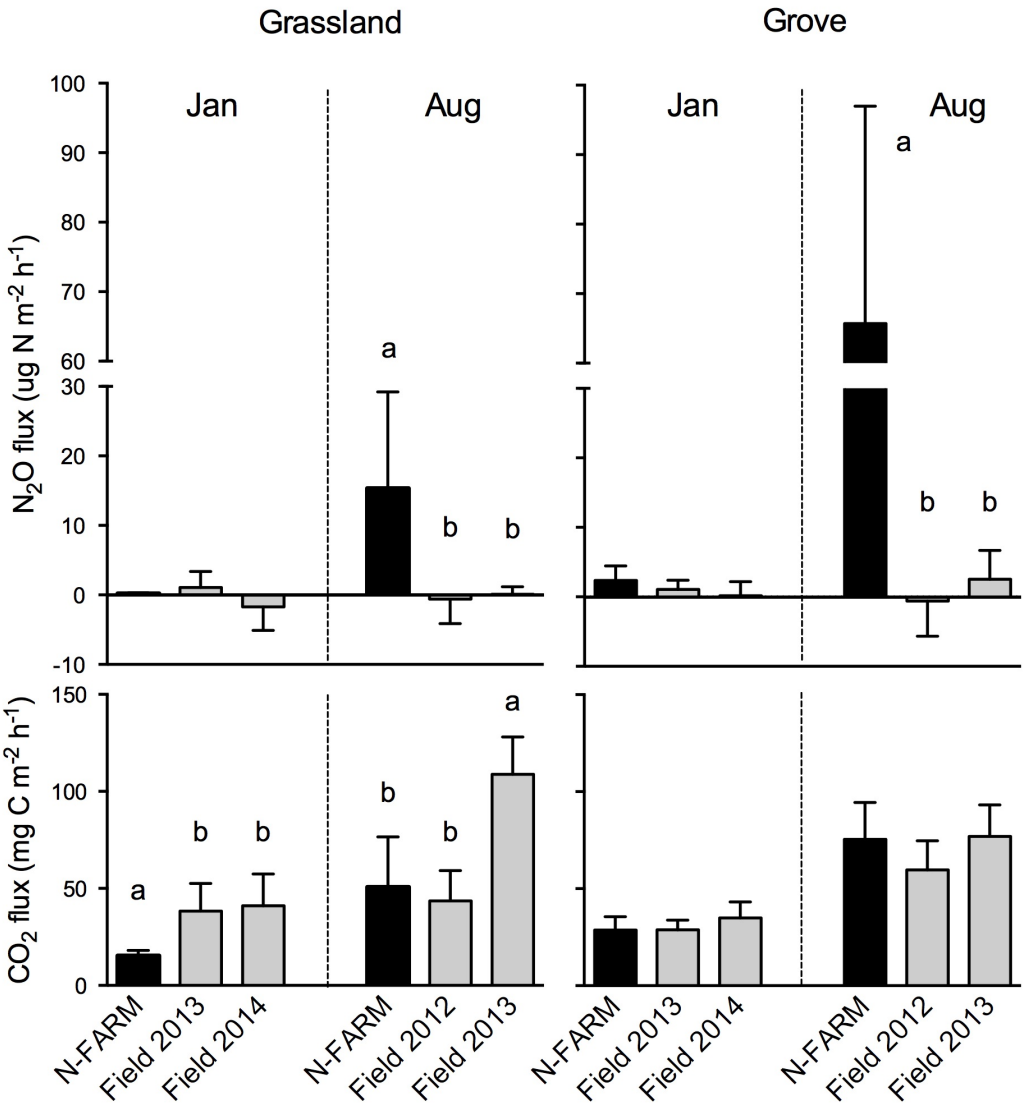

Figure 3.3 N_2O (μg N m^{-2} h^{-1}) and CO_2 (mg C m^{-2} h^{-1}) flux for lab (N-FARM) incubations (48 h incubation, 20% O_2) versus field static chamber fluxes (1 h incubation). Lab values represent mean of four soil cores ± 1 SD. Field values represent a mean of nine collars + 1 SD. Lowercase letters indicate significant (P<0.05) differences between fluxes within a season and vegetation type

Seasonal patterns of trace gas flux

Gas fluxes following soil wetting were significantly higher in summer than in winter for N_2O (N-FARM incubations), CO_2 (in both the field and N-FARM, Fig 3.3) and NH_3/NO/non-NO NO_y (field, Soper *et al.* in review). Summer soils had a very low ambient WFPS (~6%) and showed

two-fold greater accumulation of inorganic N (Table 1), consistent with substrate accumulation during soil drying (Austin *et al.* 2004). Pulse emissions of N_2O and NO are often reported after initial wetting following a dry period in tropical and subtropical soils (Davidson *et al.* 1991; Davidson *et al.* 1993; Butterbach-Bahl *et al.* 2004; Groffman *et al.* 2009) and are consistently observed for NO under field conditions at this site (Soper *et al.* in review). In winter, consistently moister soils preclude substrate accumulation and low temperatures (January average 17˚C) fall below the optimum range for denitrification (Stanford *et al.* 1975; Billings and Tiemann 2014) and wetting responses are not observed (Soper *et al.* in review). In N-FARM incubations, N_2O fluxes were lower from winter-sampled soils even at summer incubations temperatures (Fig 3.3), indicating that substrate depletion explains at least some proportion of flux variation.

Methodological considerations

Finer resolution measurements of N_2 flux should be an important aspect of the study of arid and semi-arid ecosystem N budgets (Werner *et al.* 2014). There are several strategies available to deal with measuring very low N_2 fluxes. We found that extending incubation times beyond 48 hours had the effect of reducing headspace O_2 concentrations (Table 1) due to high rates of respiration, making it challenging to make comparisons over time as O_2 decreases. Instead, more specialized instrumentation systems with lower detection limits and shorter incubations (e.g. Wang *et al.* 2011) would be a better approach to investigate low N_2 fluxes, though unfortunately these systems are not widely accessible at this time. The addition of isotopic tracers can circumvent some of the issues of headspace replacement, though these methods require disruption of the soil physical structure (and thus likely gas exchange dynamics) in order to achieve homogenous label application (Yang et al. 2014). In addition, effect of incubation on the

rate of reduction of N_2O to N_2 is an important methodological consideration when using incubations to draw conclusions about real world N fluxes.

We conclude that N_2 losses from sub-tropical North American savanna soils are low compared with other sampled ecosystems that have been studied with similar methodology. N_2 emissions also comprise a smaller proportion of total N gas flux than in savannas soils elsewhere. Including maximum potential N_2 fluxes in soil N flux budgets has the potential to double total N loss estimates for this ecosystem under recently-wetted summer conditions. However, this estimate largely reflects the difficulty of measuring very low N_2 fluxes, and in reality the proportion of N_2 may be much lower under realistic field O_2 concentrations. Very low fluxes and lack of N flux response to wetting in winter suggest that N_2 production during cold months is also insignificant. By comparing lab and field N_2O fluxes under comparable abiotic conditions, we find support for the suggestion that incubation systems may underestimate reduction of N_2O to N_2, or overestimate N_2O production. More broadly, N_2 estimates from a wider variety of ecosystem types are needed before general conclusions about the role of denitrification in the global N cycle can be confidently drawn.

APPENDIX 3.1

DESCRIPTION OF NITROGEN-FREE ATMOSPHERIC RECIRCULATION METHOD

EXPERIMENTAL SET UP AND FLUX CALCULATIONS

N-FARM set up

The N-FARM system consisted of soil incubation vessels connected inline with two Shimadzu GC8A gas chromatograph (GC; Shimadzu, Kyoto, Japan), one with a thermal conductivity detector (TCD, to measure O_2, N_2 and CO_2) and one with an electron capture device (ECD, to measure N_2O). The air-tight flow injection system was built from Swagelok connections (Swagelok, Crawford Fitting Co., Solon, OH) and stainless steel tubing.

Incubation vessels consisted of 600 ml glass jars with screw-on gas tight lids (fitted with rubber O-rings and treated with a small amount of vacuum grease) and quick-connect fittings. Stainless steel switch valves above the quick-connects were used to either close off the vessels or place them inline with the GC system. The vessels were were submerged in water in a plexiglass box, with individual plastic sheaths covering the quick-connect fitting, valve and lid and extending below the water surface. The plexiglass box was continuously flushed with He headspace to reduce background N_2 levels. The system held 10 incubation vessels, eight of which contained soil cores during incubations and two of which were left empty to check for leaks in the system.

Once cores were placed in incubation vessels and sealed, the headspace in each vessel was replaced with a UHP He-O_2 mix (Airgas, Berwyn, PA). Percent O_2 in the mix varied by incubation (either 10% or 20% O_2). This was achieved by alternating a low vacuum (67 kPa for 90 seconds) with an over-pressurization (115 kPa for 90 seconds). This treatment was applied for 16 hours, followed by a two-hour flushing cycle to equalize the incubation vessels and soil

matrix with ambient atmospheric pressure in preparation for measurement. In this step, cores were alternately flushed with He-O_2 for 30 seconds and then vented into a water trap for 80 seconds.

Once cores had completed flushing, the incubation vessels were sealed so that any gases produced by the soil accumulated in the vessel headspace. Headspace samples were withdrawn at ~8, 24, 48, 68, and 102 hours by over-pressurizing the vessels with 40 ml He-O_2 and then opening them in-line with the GC. Excess headspace sample was used to flush the lines and then fill the sample loops (total volume ~10 ml) before injection into the GC, with excess gas escaping via water traps. Lines leading to and from the cores were flushed extensively with He-O_2 before and after each sample. UHP Ar was used to dry the sample stream (across a Nafion membrane; Perma-Pure, Toms River, NJ) prior to sample loop filling. The first measurements taken at the end of the flushing period (incubation time = 0) did not detect N_2 presence above the detection limit (defined by the lowest standard, 10.2 ppm N_2), nor did any subsequent measurements from the blank (empty) vessels, suggesting system leaks were not present.

Instrument calibration

The TCD and ECD were calibrated prior to the start of each set of measurements. For N_2O, CO_2, and O_2, calibrations were performed via manual injection by transferring the calibration sample from the standard tank to the GC port using syringes. Standards concentrations used were as follows: 0.021, 0.21, 0.41, 1.43, 5.08, 28.2, 74.1 ppm N_2O, 180, 360, 1017, 5090, 9900, 24900 ppm CO_2 and 0, 5, 10, 20% O_2 in UHP He balance (Airgas, Berwyn, PA). Three to five replicates were run for each standard. N_2 calibration was performed using standard tanks (10.2, 27.2, 52, 109, and 156 ppm N_2) plumbed in-line with the GC using stainless steel tubing and

connections as described above. Before each sample was injected, the tank regulator space was purged 10 times, the stainless steel tubing connecting the tank to the GC inlet was flushed with UHP He at 20 psi for at least 5 minutes, and then with standard gas at 10 psi for 5 minutes, then again with standard gas at 2 psi for an additional 5 minutes. 10 replicates were run for each N_2 standard.

Calculation of instrument detection limit for N_2

Flux rates for N_2O, CO_2 and O_2 were calculated by regression of sampled headspace gas concentration (calibrated for headspace volume) vs. time of incubation. These were expressed in areal terms by dividing by the top surface area of the core (12.56 cm^2). Concentration calculations took into account dilution caused by sequential removal and replacement (with He-O_2) of 40 mls of headspace at each sampling time point. The detection limit for N_2 production for this study (52.2 μg N_2-N m^{-2} h^{-1}) was defined using several parameters. Specifically, the N_2 concentration was bounded by the detection limit of the TCD instrument (the lowest standard that was reliably detectable was 10.2 ppm N_2, replicate SD= 12.3%), the time variable was set by the maximum length of incubation tested (102 h), the area was given as the top surface area of the core and the headspace volume was given as the jar volume minus the average core volume (adjusted for bulk density). This calculation assumed that N_2 production was linear with respect to time.

It is useful to note that the same volume of soil sampled with a wider, shorter core (with greater aerial surface area) would generate a lower calculated detection limit. Sample volume, dimensions and units are therefore a factor to be considered in the design and interpretation of studies of this nature. Fluxes expressed in this study can be converted from an aerial basis to an

approximate per kg dry soil basis by dividing by 148.9 for grassland samples (based on average core weight) and 120.2 for grove samples.

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CHAPTER FOUR

ESTIMATING ECOSYSTEM NITROGEN ADDITION BY A LEGUMINOUS TREE: A MASS BALANCE APPROACH USING A WOODY ENCROACHMENT CHRONOSEQUENCE

Abstract

Difficulty in estimating and partitioning nitrogen (N) additions to ecosystems, particularly over long time periods, is a challenge to understanding changes in nitrogen dynamics during ecosystem perturbations. As a case study, we applied an N mass balance approach to a well-characterized chronosequence of woody legume encroachment into subtropical grasslands to estimate N additions from fixation. We measured soil N accrual to 30 cm beneath spatially discrete *Prosopis glandulosa* trees ranging in age from 28-99 years. We combined these data with measurements and modeled estimates of above- and below-ground biomass N accrual, wet and dry atmospheric N deposition, N_2 and N trace gas loss, and baseline grassland soil N concentrations at time of establishment to calculate fixed N inputs as the residual. For an upland area with 12% *Prosopis* canopy cover and average tree age of 47 years, contemporary fixation rates are estimated to be 9.2 kg N ha^{-1} yr^{-1}. Accounting for changes in tree density due to encroachment, this is equal to total N fixation of 235 kg N ha^{-1} over the last ~130 years. These values are lower than previous per-area fixation estimates for *P. glandulosa*, but are consistent with lower plant density and with environmental conditions (especially low water availability) at this site. We also explored whether higher rates of dry N deposition to *Prosopis* canopies versus open grasslands may contribute to N accretion beneath trees. To investigate this effect, we adjusted the mass balance to weight N dry deposition by *Prosopis* leaf area and doubled total deposition rates to *Prosopis* foliage. This had the effect of reducing total N inputs from fixation to 210 kg N ha^{-1}, with modern rates of 8.1 kg N ha^{-1} yr^{-1}. We also compared the $\delta^{15}N$ of soil under *Prosopis* canopies to remnant grasslands to examine the isotopic changes in soil N with encroachment. Bulk soil $\delta^{15}N$ adjacent to *Prosopis* trees was depleted by up to 2 ‰ compared with surrounding grassland (mean 7.1 ‰). As tree age and canopy size increased, so did the

distance over which trees influenced soil N, with isotopic depletion evident up to 3 m from the tree bole. These observations suggest trees are a point source of fixed N. In this relatively well-constrained system, a mass balance approach provides a reasonable method of estimating N fixation and offers an opportunity to cross-calibrate alternative estimation approaches.

Introduction

Quantifying N inputs from biological N fixation is complicated by difficulty in measuring this process directly, particularly in long-lived woody plants in heterogeneous environments (Boddey *et al.* 2000). Combining estimates of nodule density with measures of nitrogenase activity is a common way to calculate fixation rates, but this approach often cannot be applied in dryland systems where nodule recovery is difficult (Hartley *et al.* 2007). Estimates based on foliar N isotopic composition (Shearer and Kohl 1986) cannot be applied in many ecosystems where the assumptions of the method are frequently violated (Soper *et al.* 2015). Measuring accumulation of N in plant tissue and soil over time is one way to get at fixation indirectly, but this approach is complicated by the presence of other N addition and removal processes acting simultaneously. These processes include loss of N trace gases or N_2 to the atmosphere, leaching through the soil profile, and atmospheric deposition of dissolved N or particulates, that are themselves often poorly quantified. Mass balance approaches are also complicated by the effect of high variance in each of the input and output terms on derived balance estimates (Likens 2013).

Many savannas and grasslands worldwide are experiencing increases in abundance or density of woody plants, termed woody encroachment. In the US alone, this process has already affected or has potential to affect 220-330 million ha of land (Houghton *et al.* 1999, Pacala *et al.*

2001). In the tropics and subtropics, woody encroachment is often facilitated by nitrogen (N)-fixing trees and shrubs in the family Fabaceae (Asner and Martin 2004). The large inputs of fixed N that often accompany woody legume encroachment are associated with significant changes in storage, gas flux, biochemical composition and spatial distribution of carbon and N within ecosystems (Stock *et al.* 1995, Archer *et al.* 2001, Martin *et al.* 2003, Boutton and Liao 2010, Barger *et al.* 2011, Creamer *et al.* 2013).

Encroachment of the leguminous tree *Prosopis glandulosa* (honey mesquite) into grasslands of south Texas offers a tractable system in which to apply a mass balance approach to estimate inputs from N fixation. Encroachment in the region has been ongoing for the last 100-200 years (Archer 1995), and is associated with significant increases in ecosystem N storage (Archer *et al.* 2001). *Prosopis* recruits only into open grassland areas and not beneath existing canopies (Archer *et al.* 1988) and can develop into spatially discrete clusters made up of a single *Prosopis* individual with a diverse woody understory and allometric equations have been developed for *Prosopis* at this site to estimate N storage in above ground plant tissue (Northup *et al.* 2005)., *Prosopis* encroachment offers a unique space-for-time chronosequence to investigate changes in N storage over time.

Other N balance processes have also been quantified at this site. N gas loss rates, both for N_2 and N trace gases, have been recently estimated for upland grassland and *Prosopis* grove cover (Soper *et al*. in review). A long-term measurement record for wet N deposition rates exists nearby and modeled dry deposition rates are available (National Atmospheric Deposition Program). Allometric equations have been developed for *Prosopis* at this site to estimate N storage in above ground plant tissue (Northup *et al.* 2005). Other input and loss pathways, including leaching, N fixation by free-living bacteria, uplift of N from deep soil, plant biomass

removal by herbivore grazing, and fire are likely to be insignificant in this system (Boutton and Liao 2010). Thus, by developing a mass balance calculation of N distribution in soil and plant biomass, in addition to N deposition and gas loss over time, N fixation can be inferred as the difference between these values and regressed against tree age.

Increases in soil N are sometimes observed beneath encroaching woody canopies even in the absence of N-fixing species (Eldridge *et al.* 2011). This 'island of fertility' effect has been attributed, at least in part, to greater rates of dry N deposition to tree canopies compared to adjoining grasslands or bare soil (Archer *et al.* 2001, Boutton and Liao 2010). This occurs because certain N-containing compounds, especially ammonia, have greater deposition velocities to tall, aerodynamically rough woody canopies than to relatively-low statured grassy or herbaceous vegetation (Fowler *et al.* 1989, Bobbink *et al.* 2010). However, the relative influence of differential dry deposition versus fixation on soil N accrual during encroachment of *Prosopis* or other woody legumes has not been quantitatively explored.

The isotope ratio of N ($\delta^{15}N$) can also be used as a tracer of N fixation, because fixed N typically has a $\delta^{15}N$ close to zero while soil N is often several per mil more enriched (Shearer and Kohl 1986, Boddey *et al.* 2000). Previous studies at this site have characterized a decrease in surface soil $\delta^{15}N$ (0-15 cm depth) associated with *Prosopis* cover, from 7.5 ‰ under remnant grasslands to 6.1 ‰ under upland groves (Boutton and Liao 2010), with the lowest $\delta^{15}N$ values measured directly next to *Prosopis* trunks (Bai *et al.* 2013), consistent with inputs of isotopically light N (Boutton and Liao 2010).

In this study, we use a well-characterized encroachment chronosequence of developing *Prosopis glandulosa* clusters in a grassland matrix to infer fixed N inputs over the last 100 years. We measured soil N accumulation associated with *Prosopis* clusters of known ages and

developed a mass balance calculation using soil and biomass N accrual, N gas loss and N deposition values to constrain potential fixation rates. We also varied rates of dry N deposition to *Prosopis* canopies to assess the effect of differential deposition on N accumulation around encroaching trees (Archer *et al*. 2001). Finally, we assessed the distribution of soil $\delta^{15}N$ patterns to determine their utility in independently supporting mass balance approaches

Materials and Methods

Study site

Sampling was conducted at the Texas A&M AgriLife La Copita Research Area (27°40'N, 98°12'W) in the eastern Rio Grande Plains during 2012, 2013 and 2014. The site consists of savanna parkland with discrete woody patches dominated by one or more *Prosopis glandulosa* (Torr.) var *glandulosa* (honey mesquite, referred to here as *Prosopis*) individuals with a mixed woody understory (including *Zanthoxylum fagara, Celtis pallida*, *Condalia hookeri* and *Diospyros texana*) embedded in a matrix of C_4 grasses, forbs and bare ground. At the time of sampling (2012 and 2014), forb cover in remnant grasslands was limited or absent. *Prosopis* is a leguminous tree in the family Fabaceae, and generally considered to be capable of substantial rates of biological N fixation (BNF; Shearer *et al.* 1983). Though it nodulates readily under both glasshouse and natural conditions (including at this site) its nodules are difficult to recover under field settings, partly because they occur deep (>1 m) in the soil profile (Virginia *et al.* 1984; Johnson and Mayeux 1990; Zitzer *et al.* 1996). Putatively N-fixing *Acacia farnesiana* occurs at the site but is limited to a few scattered individuals.

The climate at this site is subtropical with typically warm, moist winters and hot, dry summers. Mean annual precipitation is 680 mm, occurring year round with maxima in May and

September. Mean annual temperature is 22.4°C with an average growing season of 289 days. Soils at the site are sandy loams (Typic and Pachic Argiustolls) with little to no topography. The sampling plot measured 260 x 215 m and has been fenced to exclude livestock grazing since 1985. Fire history for the site is not formally known but documentation (beginning ~1950) does not record any burning for this plot, and heavy grazing prior to that likely kept fire probability low (Bai *et al.* 2013).

Plant density and demography measurements

Basal diameter (BD) was measured for all *Prosopis* individuals within a 200 x 100 m (2 ha) area within the sampling plot. For single-bole individuals, tree ages were calculated from basal diameter according to Stoker (1997), using equations developed specifically for upland single-bole *Prosopis* at this site. For plants with multiple or split boles, these equations were applied to the largest of the stems only, likely underestimating the age of some trees within the plot. Approximately 2.5 trees per hectare had basal diameters ≥ 40 cm (up to 52 cm), exceeding the range for which the size-age equations were developed.

Soil sampling and analysis

All samples were taken from discrete *Prosopis* clusters, defined as individual, single-stemmed trees with a non-fixing woody understory, surrounded completely by remnant grassland and at least 6 m from the dripline of another *Prosopis* individual. All remnant grassland samples were taken at least 2 m from the drip line of the nearest woody plant and at least 4 m from the drip line of a *Prosopis*. Two sets of soil cores were sampled - one to determine N concentration radially

around *Prosopis* trees and another to determine N concentration with soil depth in finer resolution.

Twenty-four soil cores (0-15 cm depth) were sampled from beneath *Prosopis* individuals ranging in age from 20 to 144 years, 50 cm from the bole. An additional 10 cores were sampled in remnant grassland. To characterize N distribution around trees, a subset of 8 individuals were sampled intensively with cores taken every 30 cm outward from the bole in an easterly direction to ~1 m past the drip line for *Prosopis* individuals ranging in age from 28-99 years (7-14 cores). This age range was selected based on the availability of trees at the site that matched the selection parameters (discrete single-*Prosopis* cluster spatially surrounded by grassland). *Prosopis* at this site displays no directional pattern of understory plant density around individuals (Franco-Pizaña *et al.* 1995). Cores were divided into two depth increments (0-15 cm and 15-30 cm).

To determine N distribution in soil profiles, cores were sampled from five sites in remnant grassland, five under *Zanthoxylum*, and five under *Prosopis* ranging in age from 44-107 years. For woody plants, cores were sampled ~50 cm from the bole. Cores were divided into 3 cm increments down to 30 cm depth.

All soil samples were passed through a 2 mm screen to remove large organic fragments and dried for 3 days at 105°C. Un-sieved replicate cores were used to determine bulk density, using a core of 4 cm diameter. No obvious soil compaction occurred in these sandy soils and no rocky material was present in the samples. Soils were homogenized using a ball mill (Retsch, MM-2, Haan, Germany) and analyzed for $\delta^{15}N$ and %N using a continuous flow isotope ratio mass spectrometer (Model Delta V Advantage; Thermo-Scientific, Bremen, Germany). All isotope analyses were conducted at the Cornell University Stable Isotope Laboratory (COIL).

Plant biomass, leaf area and N content calculations

Above-ground biomass (AGB_P) N accrual in *Prosopis* tissue (large stems, small stems, standing dead wood and foliage) was calculated according using equations developed by Northup *et al.* (2005) specifically for *Prosopis glandulosa* at this site. Total below ground biomass (BGB_P) was calculated as a percentage of AGB_P (45%, BGB fraction for *Prosopis caldenia* in semi arid Argentinian savannas; Risio *et al.* 2014). Proportion of BGB_P composed of coarse versus fine roots was taken as the average of twelve monthly measurements for the top 10 cm of soil in *Prosopis* groves at this site (45% coarse, 55% fine; Hibbard *et al.* 2001). N content for *Prosopis glandulosa* coarse roots was assumed to be the same as stem wood (0.97% N; Northup *et al*. 2005) and for fine roots was taken as 1.99% N (value for *Prosopis juliflora*; Jha and Prasad Mohapatra 2009). Proportion BGB biomass and root size distribution was held constant with tree age.

To determine N content in understory shrubs, the relationship between woody understory canopy area (for understory shrubs with BD ≥ 4 cm) and *Prosopis* BD was derived from Archer *et al*. (1988). Canopy area was then converted to N content (AGB_U) using N concentration equations from Northup *et al.* (2005). Insufficient data was available to calculate root biomass N for these species.

Grassland-specific total root biomass and above ground biomass values (AGB_G and BGB_G) were taken from Liu *et al.* (2010) and N concentrations in common grassland species from Creamer *et al.* (2013).

Other N inputs and losses

Atmospheric wet N deposition (NH_4^+ and NO_3^-) rates were obtained from a National Atmospheric Deposition Program (NADP) monitoring station at Beeville, TX (~90 km north east of the study site) for the years 1984-2013 (www.nadp.sws.uiuc.edu). Modeled dry N deposition rates for the La Copita site for the years 2000-2013 came from the NADP Total Deposition maps (www.nadp.sws.uiuc.edu/committees/tdep/tdepmaps/). Average values for each of these fluxes were calculated and assumed to be constant over the last century.

N trace gas flux rates (sum of NO, NO_y, NH_3 and N_2O) were measured for upland *Prosopis* groves and grasslands at this site and scaled to annual rates using temperature and rainfall (Soper *et al*., a, b, in review). Rates used are averages for the years 2000-2010. Proportional N_2 flux rates were taken from Soper *et al.* (b, in review). Maximum potential N_2 emissions following soil wetting events of 15 mm or greater were applied to annual estimates of post-wetting N fluxes from Soper *et al.* (a, in review). N_2 production under ambient soil moisture was considered to be negligible.

Estimating N fixation by difference

A mass balance framework was developed to represent gas loss, wet and dry atmospheric N deposition, soil and biomass N accrual and N fixation over time for two landscape entities-remnant grasslands and seven individual *Prosopis* trees of known ages. Variables are summarized in Table 4.1.

Total contemporary soil N beneath individual *Prosopis* trees was calculated from radial soil core samples, adjusted for bulk density, to 30 cm depth. Total soil N was integrated over a circular area, the radius of which was represented by the distance from the tree bole to the point

at which N concentration (per unit soil volume) coincided with mean grassland levels (termed area of influence, AOI).

Separate annual N gas loss rates (N trace gases plus N_2) were used for grassland and beneath *Prosopis* canopies (gaseous N flux, GF_G and GF_P, respectively). Wet and dry deposition (total N deposition, TD) rates were initially held equal for both grassland and *Prosopis* canopies. Age-specific *Prosopis* biomass N and woody understory biomass N (AGB_P, BGB_P and AGB_U) were summed to generate a value for total biomass N (TB_P).

N concentration in the grassland at the time of *Prosopis* establishment was subtracted from contemporary soil N to calculate accrual of soil N under *Prosopis* canopies (S_P). These historical grassland N concentrations ($S_{G\ n=0}$) were calculated by taking average contemporary grassland N (S_G) plus grassland biomass N (TB_G), subtracting rates of total N deposition (TD) and adding gas loss (GF_G) for *n* years, where *n*= tree age.

$$S_{G\ n=0} = S_G - TB_G + GF_G * n - TD * n$$

For individual *Prosopis* trees at current age *n*, N inputs derived from fixation (BNF) for the AOI were calculated as:

$$S_P + TB_P + TB_U = S_{G\ n=0} + (GF_P * n * AOI) + (TD * n * AOI) + BNF$$

Derived linear relationships between tree age and N_F were multiplied by *Prosopis* age and density data to extrapolate fixation estimates to plot scale.

Differential dry N deposition scenarios

Sensitivity analyses were conducted to determine the effect of varying N dry deposition parameters on calculated fixed N inputs, in order to test whether differential deposition is an important driver of N accumulation between trees. It is challenging to develop a simple

representation of N deposition to individual *Prosopis* canopies versus grassland, due to the myriad factors that affect deposition to vegetative surfaces (including N species, concentration, boundary layer, canopy structure, stomatal conductance, surface wetness etc; Wesely and Hicks 2000). Therefore, three simplified deposition scenarios were considered: even dry N deposition across the landscape, dry deposition weighted by surface area (calculated leaf area for *Prosopis* individuals, with grassland considered to be a flat plane equal to ground surface area) and dry deposition weighted by surface area plus two times greater deposition velocity for *Prosopis* leaf surfaces. Total dry deposition was held constant at 3.88 kg N ha^{-1} yr^{-1} and background deposition rates to grassland were reduced to compensate for increased canopy deposition.

Prosopis canopy area was calculated according allometric equations determined by Stoker (1997). *Prosopis* leaf area was found by calculating foliar biomass as a function of basal diameter (Northup *et al.* 2005), and multiplying by average specific leaf area values for *Prosopis glandulosa* from Bai *et al*. (2008). As *Prosopis* is winter deciduous, it was assumed that trees had no standing foliage for 3 months per year (Nelson *et al.* 2002). Tree age distribution was used to determine percentage canopy area over the last century.

Statistical analysis

Statistical analyses were performed in R (R Core Team 2015) and GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA, USA). Best fit regressions for tree age versus other parameters (N accumulation in soil and biomass, mass-balance fixation estimates, distance of influence on soil N) were selected using Akaike's Information Criteria (AIC). For N content and $\delta^{15}N$ in soil profiles, a mixed effects model used to determine the significance of cover and sampling depth, and student t-tests were used to compare between cover types within depths. %N

values were first subject to Johnson Su transformation to improve normality. ANCOVA was used to test for significant differences in slopes between differential N deposition scenarios.

Results

N mass balance along the Prosopis *age chronosequence*

If N deposition additions and N gas losses have remained constant in this ecosystem over time, the balance of the two would have led to a net annual N addition of 5.93 kg N ha^{-1} yr^{-1} (grassland values, Table 4.1). This is equivalent to an increase in grassland N stocks (at 0-30 cm depth) of ~17% N over the last 50 years, and is consistent with soil N values measured at this site between 1990-1995 (Archer 1995). Because of this imbalance in inputs and losses, the baseline grassland soil N content (kg N ha^{-1}, 0- 30 cm) changes through time and is described by the equation N = -5.934*YBP + 2037, where YBP = years before present (2014). This equation was used to estimate the grassland N content value at the time of establishment for a given *Prosopis* cluster and we refer to this value as the 'grassland baseline'.

Measured accrual of N in soil since *Prosopis* cluster establishment (above the grassland baseline) increases linearly with *Prosopis* age from 28 to 99 years (N (kg) =0.066*age-1.844, r^2 = 0.89, P<0.002, Fig 4.1). N accrual in *Prosopis* and understory biomass also increases with tree age (Fig 4.1, N (kg) = 0.0006*age^2 + 0.0387*age - 1.2836, P<0.004, Fig 4.1). Data from Archer *et al.* (1988) and observations made from this study indicate that understory biomass does not begin to accrue until *Prosopis* plants are 30 years old. For clusters older than 30 years, N accrued in *Prosopis* biomass more quickly than in the understory. *Prosopis* thus comprised 49% of total biomass N accrued for a 36-year-old cluster and increased to 85% by age of 99 (data not shown).

Table 4.1 Mass balance input variables. Rate values are in units of kg N ha^{-1} yr^{-1} unless indicated. Standing stock values are in in units of kg N ha^{-1}, adjusted for bulk density. BD = basal diameter in cm.

	Variable	Grassland value	*Prosopis* value	Source
Addition/loss rates	N trace gas loss	0.65	0.56	Soper *et al.* (b, in review)
	N_2 loss	0.07	0.10	Soper *et al.* (a, in review)
	Wet N deposition ($NH_4^+ + NO_3^-$)	2.77	2.77	National Atmospheric Deposition Program (measured at Beeville, TX, 1984-2013).
	Dry N deposition	3.88	3.88	National Atmospheric Deposition Program (modeled, 2000-2013)
Standing stock	Above ground biomass N	8.36	Varies with tree age (0.1 - 5.0 kg per tree)	Grassland: Liu et al (2011), Creamer *et al.* (2012) *Prosopis*: Northup *et al.* (2005) Woody understory: Archer (1988), Northup *et al.* (2005)
	Below-ground biomass N	10.14	Varies with tree age (0.1 - 3.1 kg per tree)	Grassland: Liu *et al.* (2011), Creamer *et al.* (2012) *Prosopis:* Proportion BGB from Risio *et al.* (2014). Proportion coarse/fine roots from Hibbard *et al.* (2001). Root N content from Jha & Prasad Mohapatra (2009) and Northup *et al.* (2005).
	Bulk soil N (0-30 cm depth)	1965	Varies with tree age (0.1- 10.1 kg per tree)	Calculated from field measurements (see Methods)

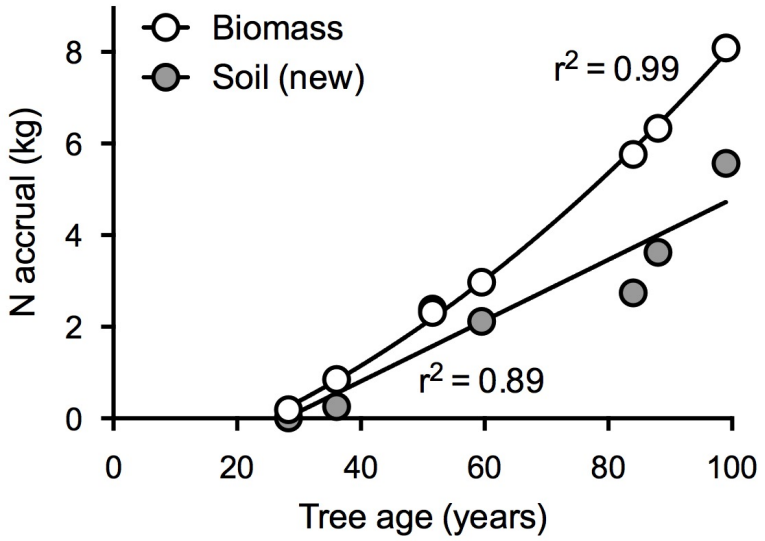

Figure 4.1 N accrual in plant biomass (*Prosopis* and understory) and soil N (new N, above grassland baseline at time of establishment) for *Prosopis* clusters of varying ages. n = 7. Soil volume considered varied by tree, and was calculated using the distance between the tree bole and the point at which soil %N returned to average background grassland concentrations (range 0.1-14.5 m^{-3}).

When grassland baseline N, gas loss, wet and dry N deposition estimates (for a given cluster area and age) are subtracted from biomass and soil accrual N values, the remainder is attributed to inputs of fixed N from *Prosopis* (Fig 4.2). This estimate of fixed N is positive, and increases linearly with tree age (Fig 4.2, 4.3, 4.4) at rate of 0.145 kg N $tree^{-1}$ yr^{-1}, with net N accrual from fixation in a cluster beginning at 28 years since *Prosopis* establishment. Variation in the magnitude of deposition inputs and baseline soil N in Fig 4.2 reflects variation in the relationship between *Prosopis* age and cluster area.

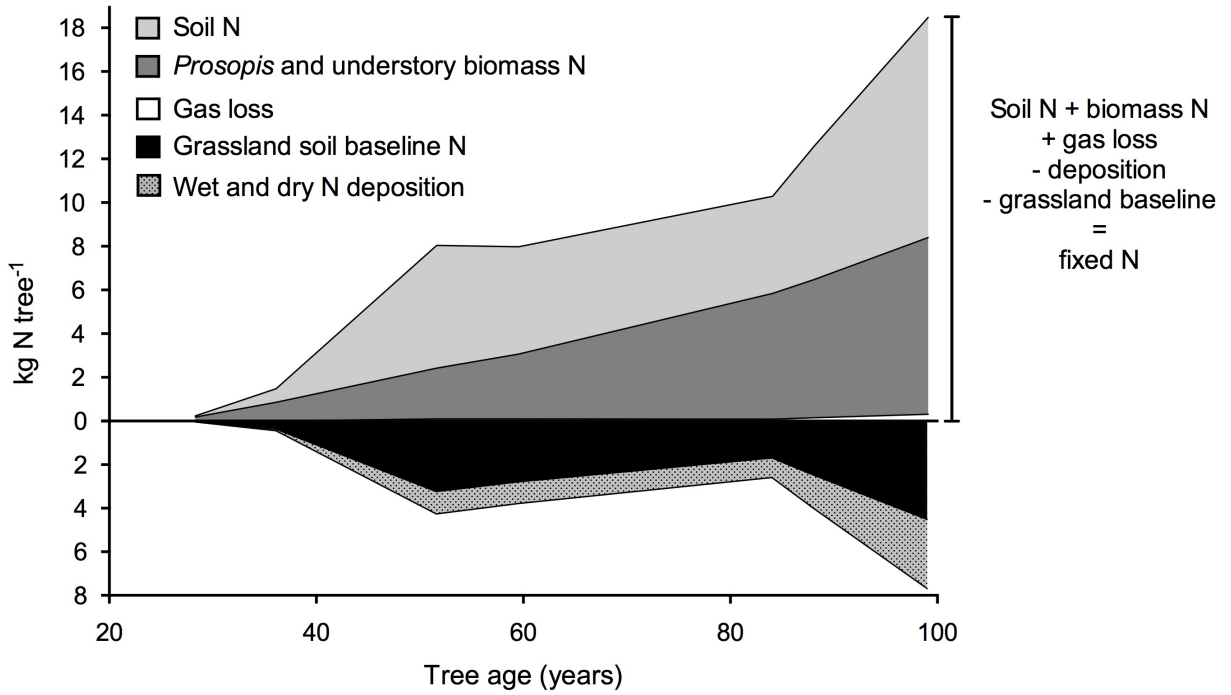

Figure 4.2 Total N inputs and losses for individual *Prosopis* trees of varying ages (kg N tree^{-1} over tree lifetime). n = 7 trees. Assumes no differential N dry deposition to *Prosopis* canopies. Fixed N is calculated as the sum of N in soil and *Prosopis* tissue (above and belowground, plus aboveground understory tissue), plus gaseous N losses, minus N derived from deposition, minus baseline N concentration in pre-encroachment grassland at the time of establishment (adjusted for grassland atmospheric deposition and gas loss).

Variable deposition scenarios

Estimates of fixed N inputs are relatively insensitive to adjustments in rates of dry deposition to *Prosopis* canopies. Assuming dry deposition rates of 3.88 kg N ha^{-1} yr^{-1} (constant over time) and weighting deposition by *Prosopis* leaf area decreases the lifetime fixation estimate for a 99 year old *Prosopis* tree by 9.1% (1 kg, Fig 4.3, Table 4.2) compared with assuming even deposition to the ground surface area. The slope of the two linear fits for N-fixation versus tree age did not differ significantly between these two scenarios ($P<0.12$). Further adjusting this rate by assuming both leaf area-weighted deposition and a doubling of deposition rate to *Prosopis* leaf surfaces

reduces the 99-year-old tree lifetime fixation estimate by 16.8% (1.8 kg) compared with even deposition (Fig 4.3, Table 4.2. P<0.019).

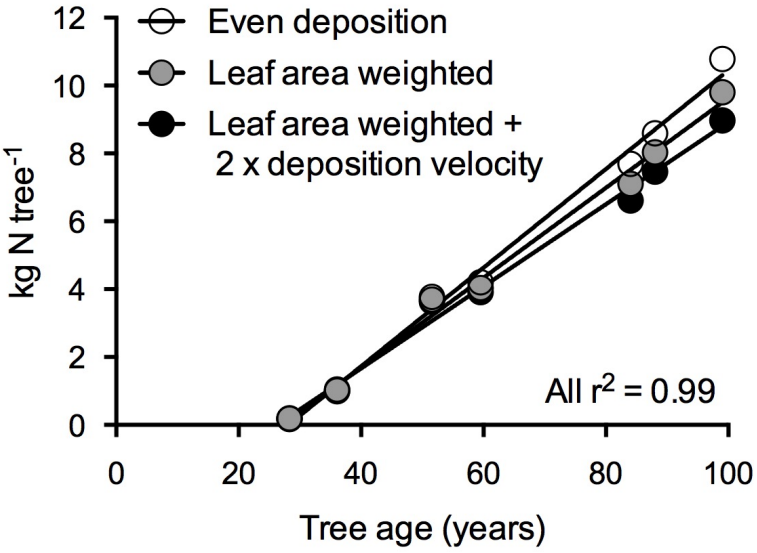

Figure 4.3 Mass balance output for total N derived from fixation (kg) versus tree age under three N dry deposition scenarios. Even deposition = dry deposition velocity is constant across the land surface, leaf area weighted = dry deposition weighted by average annual leaf area (where leaf area is proportional to tree age for *Prosopis* and grassland considered to be 1 m^2/m^2), leaf area weighted + 2 x deposition velocity = dry deposition weighted by surface area plus two times greater deposition velocity to *Prosopis* leaf surfaces than to grassland. Total dry N deposition per hectare is held constant. Lines represent linear regression.

Table 4.2. Estimates of N derived from *Prosopis* fixation under three N dry deposition scenarios. Total values are summed for the period 1883-2014, using tree demography density (Fig 4.4). Equations are for the *Prosopis* age-fixation relationships (Fig 4.3). Even deposition = dry deposition velocity is constant across the land surface, leaf area weighted = dry deposition weighted by average annual leaf area (where leaf area is proportional to tree age for *Prosopis* and grassland considered to be 1 m^2/m^2), leaf area weighted + 2 x deposition velocity = dry deposition weighted by surface area plus two times greater deposition velocity to *Prosopis* leaf surfaces than to grassland. Total dry N deposition per hectare is held constant.

N dry deposition scenario	Total N derived from fixation (kg N ha^{-1})	Current annual fixation (kg N ha^{-1} yr^{-1})	Equation
Even	235	9.2	y = 0.1454*age – 4.087
Leaf area weighted	221	8.5	y = 0.1324*age – 3.607
Leaf area weighted + 2 x deposition velocity	210	8.1	y = 0.1209*age – 3.173

Prosopis *density and demography*

Average *Prosopis* density in a 2 ha upland plot at the La Copita site in 2014 was 81.4 trees ha^{-1} (Fig 4.4A), corresponding to canopy area cover of 11.6%. Of these, 20% (16.6 trees ha^{-1}) had more than one stem arising from the base and could not be accurately aged and that only the largest stem was used to calculate tree age. Single-stemmed *Prosopis* ranged in age from 13-131 years, with a mean age of 46.7 years (Fig 4.4B).

Scaling to landscape

Extrapolating the *Prosopis* N fixation-age relationship derived above to the landscape scale (using measured tree density and age, Fig 4.4) generates a contemporary annual fixation estimate of 9.2 kg N ha^{-1} yr^{-1} for this upland semi-arid, subtropical savanna-woodland (Table 4.3). Accounting for increasing *Prosopis* density and maturity in the plot over time (Fig 4.4), this is

equal to 235 kg ha^{-1} of fixed N inputs from *Prosopis* since encroachment began ~130 years ago. Under the most conservative N dry deposition scenario described above, this estimate is reduced by ~10%, to 210 kg N ha^{-1} (Table 4.2). Two possible sources of error in scaling are related to tree size and age. First, extrapolating *Prosopis* basal diameter-age equations beyond the 100 years they were originally calibrated for had the effect of increasing total fixation values by 1.6-3.8%, depending on the deposition scenario. Secondly, for ~20% of *Prosopis* trees with multiple stems, age was underestimated by considering only the largest stem (conversely, counting all stems would almost certainly overestimate tree age). Treating split boles as individuals trees increased areal fixation estimates to 11.6 kg N ha^{-1} yr^{-1}, or 269 kg ha^{-1} of total fixation.

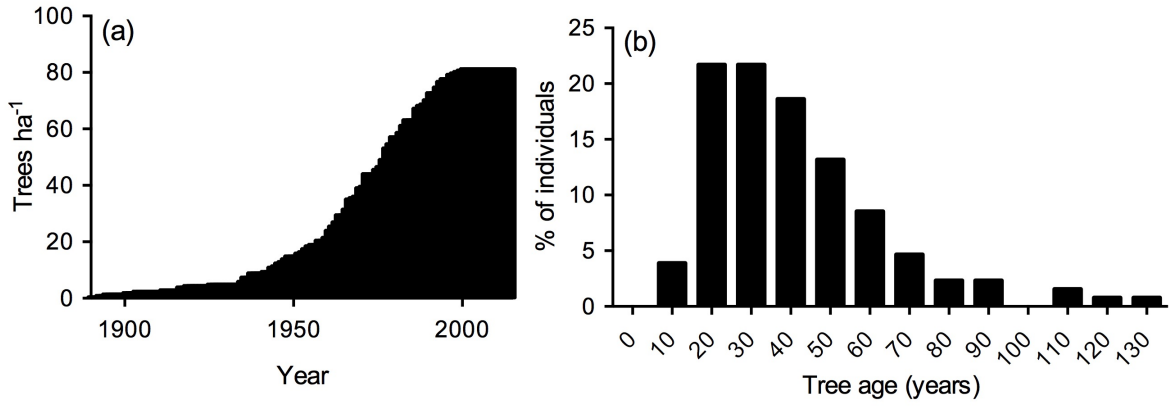

Figure 4.4 (a) Upland *Prosopis glandulosa* density (trees ha^{-1}) from 1890-2010 in a 2 ha upland area of the La Copita Research Area and **(b)** Age distribution of trees in 2014. Ages calculated from basal diameter according to Stoker (1997) (n = 132).

N and $\delta^{15}N$ distribution in soil

There was a positive relationship between *Prosopis* age and distance to which the tree influenced soil N content and $\delta^{15}N$ in the top 15 cm of soil (Fig 4.5A, B), where N concentrations were highest and $\delta^{15}N$ values lowest closer to the tree bole. For N content, this relationship was

described by D = 3.726*age - 5.3151 (D= distance in cm from the center of the *Prosopis* bole, r^2 = 0.65, P>0.03). For $\delta^{15}N$, this relationship was described by D = 2.3164*age + 60.949 (r^2 = 0.40, P>0.13). For $\delta^{15}N$, values increased significantly with distance from the bole for trees older than 28 years (P<0.05). For %N, values decreased significantly moving away from the bole for trees older than 36 years (P<0.05).

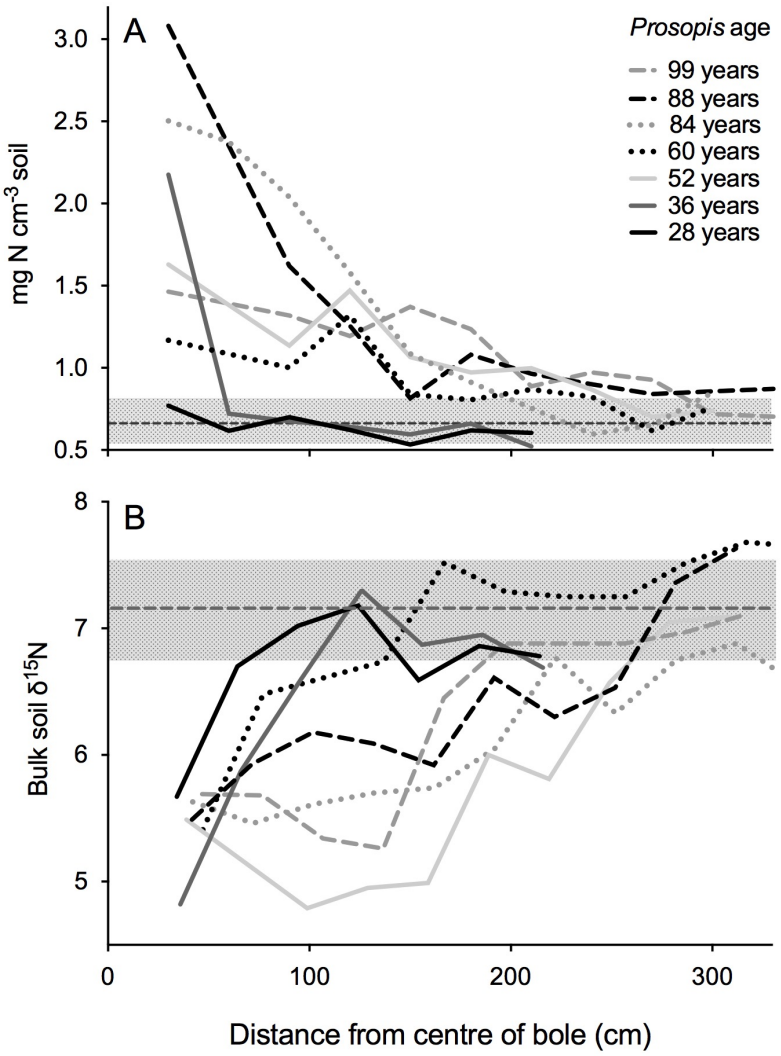

Figure 4.5 (a) Soil N concentration (mg N cm^3 soil) and **(b)** $\delta^{15}N$ with distance from bole for *Prosopis glandulosa* trees of varying ages (0-15 cm depth). Grey dashed line and shaded area represents average grassland $\delta^{15}N \pm 1$ SD (n = 10).

For *Prosopis* trees aged 47-131 years, average soil $\delta^{15}N$ at 60 cm from the bole was 0.3-1.2 ‰ lower than adjacent grassland through the soil profile (Fig 4.6B, $P<0.02$). Profile $\delta^{15}N$ also increased significantly with depth under both cover types ($P<0.0001$, Fig 4.6B). For %N, values decreased significantly with depth beneath *Prosopis* ($P<0.0001$, predominantly from the surface to 12 cm depth), but remained constant with depth in grassland soils (Fig 4.6A).

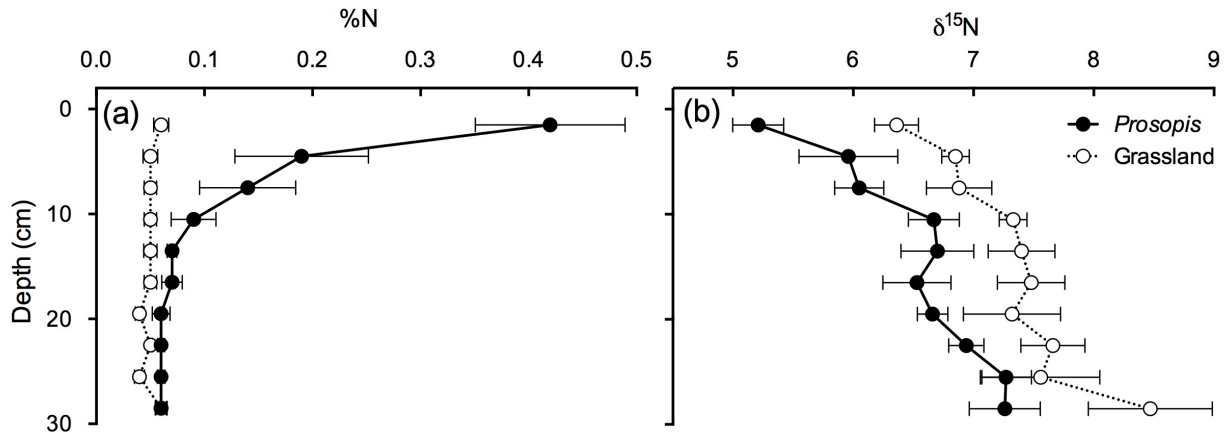

Figure 4.6 (a) %N and **(b)** $\delta^{15}N$ distribution in soil profiles under remnant grassland (white symbols, n = 3), or *Prosopis glandulosa* (black symbols, n = 5, 43-131 years old). Values represent mean values ± 1 S.E.

Discussion

Applying a mass balance approach to N inputs and losses in developing woody clusters in a subtropical savanna over ~100 years, we find that N fixation by *Prosopis glandulosa* represents an input of 0.15 kg N $tree^{-1}$ yr^{-1} for trees aged 28 years and older. This is equivalent to 9.3 kg N ha^{-1} yr^{-1} at current *Prosopis* densities. N fixation thus represents a large input term, and is primarily responsible for the observed increases in ecosystem N storage during *Prosopis* encroachment. Though the calculated fixation rate is less than estimated for other *Prosopis* stands (discussed below), it is consistent with lower plant density and with environmental

conditions (especially low water availability) at this site. The mass balance is relatively insensitive to changing distribution of dry atmospheric N deposition, which has been previously identified as a potential driver of N accumulation in this system (Boutton and Liao 2010). $\delta^{15}N$ patterns in soil also support the notion that *Prosopis* individuals are point sources of N isotope ratios indicative of N fixation.

N accrual and fixation in time and space

The balance between N accrual, gas loss and deposition inputs since establishment was negative for all *Prosopis* clusters measured in this study, indicating a missing source of N that is likely *Prosopis* fixation. Total N attributed to fixation increased linearly with tree age from 28 to 99 years. This suggests that after trees begin accruing N, fixed N inputs remain constant even as trees increase in size and soil N content increases. However, because tree size increases with age, stable fixation rates on a per tree basis are equivalent to declining fixation on a per unit biomass basis over time. Net N accrual in 28 year old trees, the youngest we measured, was very small (less than 0.2 kg) and regression equations fitted to all trees indicated that 28 years is the approximate threshold at which N begins to accrue. The lack of younger trees identified at the site may be a legacy of recent drought-induced mortality of smaller individuals, or suppression of establishment by increased grass growth after grazing was ceased at the site ~30 years ago.

There is conflicting evidence regarding the relationship between tree age, size and fixation rates. Increasing soil inorganic N concentrations associated with tree development would usually be expected to suppress fixation in older *Prosopis* trees (Hartwig 1998). However, previous isotopic measurements at this site found indirect evidence that fixation rates increase with age (Soper *et al.* 2015). Alternatively, fixation may remain relatively stable through time as

a result of shifting limitations at different growth or establishment phases. For example, low N availability may favor fixation during early establishment when soil N concentrations are low, while ability to obtain additional resources necessary to support fixation (such as phosphorus or soil moisture) may increase as root systems expand with age (Soper *et al.* 2015). Resource-limitation early in life may also explain why *Prosopis* didn't appear to begin fixing N for some time. Trees grow slowly at this site, and a 28 year old *Prosopis* still has a basal diameter of only ~8 cm (Stoker 1997) and height of less than 2 m.

Though encroachment has occurred at this site for the past 100-200 years, this process has not yet gone to completion on many upland portions of the landscape (Archer 1995). We found that more than half of the trees surveyed in an upland plot were 40 years or younger, with a fifth 28 years or younger. As we did not measure net N accrual from fixation until this age, it is likely that fixation rates in this ecosystem will continue to increase as these trees mature. Assuming no recruitment or mortality, maturation of existing trees would be expected to increase fixation rates to ~12 kg N ha^{-1} yr^{-1} within the next 15 years.

Sensitivity to atmospheric deposition rates

N deposition represents a significant input of N to this system, accounting for ~23% of total N accrual observed in *Prosopis* clusters after 100 years. Some authors have suggested that differential dry N deposition during encroachment may explain in part the N soil enrichment frequently observed beneath woody invaders (Boutton and Liao 2010, Eldridge *et al.* 2011). We examined this by varying rates of deposition to *Prosopis* canopies in our calculations. Though other woody biomass is present in the woody clusters we sampled, the central *Prosopis* individuals represented the greatest height and leaf area (Archer *et al*. 1988; Soper, personal

observation). While it is challenging to calculate actual deposition rates, scenarios in which we weighted dry deposition by *Prosopis* leaf area or leaf area plus two times deposition velocity reduced total N attributed to fixation by ~10% over the last 100 years. This suggests that while uneven deposition could play a role in N accrual beneath woody species, it is not the primary driver of N enrichment in this ecosystem.

We made the assumption that total wet and dry deposition rates in this region remained constant over time, and have no reason to believe that this scenario isn't broadly true. While the proximity of the site to coastal oil refineries may contribute to N deposition, these refineries have been active since the 1930s, comprising most of the time period considered in thus study. Additionally, total deposition rates (averaging 6.5 kg N ha^{-1} yr^{-1} for 2000-2013, NADP) for this area are relatively low compared with more developed parts of North America and so likely do not reflect strong temporal increases associated with anthropogenic development seen elsewhere.

Comparison to other fixation estimates

Estimates of N fixation by woody legumes vary widely, though our values were generally consistent with other natural *Prosopis*-dominated systems. Rundel *et al.* (1982) used a mass balance approach to estimate fixation rates of 23-40 kg N ha^{-1} yr^{-1} in a non-water-limited stand with 34% *Prosopis glandulosa* cover in the Sonoran desert. In a water-limited stand in Texas, Johnson and Mayeux (1990) used nodule abundance to estimate rates of 45-150 kg N ha^{-1} yr^{-1} at 50% canopy cover. By contrast, we estimated contemporary fixation rates (for ~12% *Prosopis* canopy cover) of 9.2 kg N ha^{-1} yr^{-1}, increasing to 12 kg N ha^{-1} yr^{-1} when all existing individuals reach maturity. Adjusting for canopy cover would bring our estimate into the lower end of these other reported ranges. In a 10-year-old irrigated planting of another sub-species of *P. glandulosa,*

Abrams *et al.* (1990) used a simple mass balance to calculate fixation rates of ~100 kg N ha^{-1} yr^{-1}. Given the strong relationship between water availability and potential symbiotic fixation in *Prosopis* and other legumes (Zitzer *et al.* 1996, Zahran 1999), it is likely that the much higher fixation rates calculated for that system reflect physiological than methodological differences. By contrast, López Villagra and Felker (1997) found much lower rates (2-2.8 kg N ha^{-1} yr^{-1}) in another planted stand, this time using a foliar $\delta^{15}N$ approach. Given that our previous work in *Prosopis* has identified significant issues with application of the foliar $\delta^{15}N$ method (Soper *et al.* 2015), we conclude that mass balance provide a more robust approach to estimating fixation rates in this *Prosopis*.

What is the significance of N additions during woody legume encroachment to N budgets at a larger scale? Though continental-scale aerial estimates of *Prosopis* cover (and of encroachment and N-fixing plant cover more generally) are scarce (Sobota *et al*. 2013), first-order estimates suggest that contributions may be significant. Van Auken (2000) estimates that the genus *Prosopis* (species *glandulosa, velutina, toreyana and justiflora*) is the dominant woody plant across 38 million ha of semi-arid grasslands of southwestern North America. Assuming *Prosopis* canopy cover of 10-20% over this area (Johnson and Mayeux 1990), and fixation rates from 9.2 - 12.0 kg N ha^{-1} yr^{-1} (current fixation rate and projected rate at tree maturity for this site), this generates total *Prosopis* N inputs across North America of 0.3-0.8 Tg N yr^{-1}. Estimates for total non-cultivated BNF in the USA vary widely (0.5-12.2 Tg N yr^{-1} Sobota *et al*. 2013), with best recent estimates in the range of 6.4 Tg N yr^{-1} (SAB, 2011). Thus, *Prosopis* encroachment may account for between 5 and 13% of total BNF. Though this estimate does not account for variation in fixation in response to precipitation across the range (approx. 200-1000

mm yr^{-1}; Johnson and Mayeux 1990), our fixation rate falls within the range estimated by other authors across a rage of water availability.

Robustness of mass balance approach

We considered wet and dry atmospheric deposition, symbiotic fixation and N trace gas/N_2 loss as the primary fluxes of N in and out of spatially-discrete *Prosopis* clusters, and measured/simulated 0-30 cm soil, *Prosopis* live and dead tissue and woody understory plant tissue as the primary N accrual reservoirs. Furthermore, we assumed that that baseline level of soil N at the time of *Prosopis* establishment was represented by current grassland soil N concentrations, adjusted to account for historical N deposition and gas loss. How realistic is this approach? Where certain processes and pools were not considered, their inclusion would tend to increase, rather than decrease, fixation estimates or have negligible effects.

We did not consider accrual in small understory shrubs (basal diameter < 4 cm) or understory root biomass because estimates were not available for these pools. Assuming similar N content and root distribution to *Prosopis,* suggest that adding this pool would increase total fixation estimates by less than 10%. Lateral movement of *Prosopis* litter outside the cluster area was also not accounted for, as the magnitude of this redistribution is unknown. Estimates of leaching N losses from this system (beyond 30 cm) are not available. However, leakage of water beyond the rooting zone and runoff below *Prosopis* at this site is 'negligible' under typical low rainfall conditions due to high evapotranspiration rates (Illescas and Porporato 2001). Weltz and Blackburn (1995) also found no deep soil water drainage beneath shrub clusters, suggesting that leaching is unlikely to be a significant process at this site. However, it is possible that leaching does occur sporadically in response to large rainfall events. Patches of biological soil crust are

present in some remnant grasslands at the site, though not in the areas we sampled. $\delta^{15}N$ of the crusts at this site (1.7‰) is depleted with respect to the top 2 cm of soil (6.1‰) suggesting that active fixation does occur (Soper, unpublished). However given the small area of cover, crust fixation occurring at even the highest reported rates (9 kg ha^{-1} $yr^{-}1$ for undisturbed crusts on the Colorado plateau; Belnap 2002), would be still be relatively insignificant. Redistribution of N between grassland and *Prosopis* rhizospheres is considered unlikely because *Prosopis* roots do not typically extend beyond the reach of the canopy (Watts 1993). Uplift of N from deeper soil layers (beyond 30 cm) by *Prosopis* roots is possible, but isotopic data indicates that it cannot be a major source of surface soil N under *Prosopis* canopies (Bai *et al.* 2013). Bulk soil $\delta^{15}N$ decreases with encroachment, while $\delta^{15}N$ at depth is isotopically enriched with respect to surface grassland soil (Boutton and Liao 2010). Finally, significant grazing and fire removal of biomass N are unlikely, given that grazing has been restricted for at least 30 years (stocking rates were likely low prior to that due to low rainfall) and site records bear no evidence of fire for at least 60 years and likely longer (Bai *et al.* 2013).

$\delta^{15}N$ patterns in soil

If unsuitable for application in inferring fixation rates directly, what can N isotopes tell us in this system? We found gross correlation between patterns of N accrual and $\delta^{15}N$ in soils. The distance from the bole over which *Prosopis* influenced both surrounding soil total N and $\delta^{15}N$ increased with tree age and canopy size. Total soil N was highest and $\delta^{15}N$ lowest closest to the bole, where N inputs from litter and roots have been occurring the longest and atmospheric deposition may have been concentrated by canopy interception and stemflow (Virginia 1986, Whitford *et al.* 1997). Fixation-derived N is expected to be close to ~0 ‰ (compared with

grassland soil values of ~7 ‰), while values for deposition are much more variable, challenging to predict and not widely available. $\delta^{15}N$ values for precipitation nitrate have been reported as -3 to -5 ‰ for this part of the US (Kendall *et al.* 2007), while dry deposition (which makes up a greater fraction of total deposition at this site) tends to be more enriched (Kendall *et al*. 2007). On average, mean deposition values of less than 7 ‰ should tend to move soil $\delta^{15}N$ in the same direction as fixation. However, grassland soils have also been experiencing N deposition, so any effect on reducing soil $\delta^{15}N$ should only be apparent if N deposition is indeed greater to *Prosopis* canopies than to surrounding areas. Finally, bulk soil $\delta^{15}N$ integrates not only N additions but also removals. As seen in the mass balance, more N accrues in biomass than in 0-30 cm soil during cluster formation (at least over 100 years) and $\delta^{15}N$ of foliage ranges from 0-4 ‰ in *Prosopis* and 1-6 ‰ in other upland understory shrubs (Bai *et al.* 2009). This suggests that any isotopic mass balance approach to determining fixation and/or deposition inputs would need to consider both the soil pool and allocation of light N to biomass, as well as attempt to constrain deposition $\delta^{15}N$ values at the site.

Soil $\delta^{15}N$ represents a balance of the quantity and $\delta^{15}N$ of N inputs with the quantity and $\delta^{15}N$ of existing soil N, as well as the effect of fractionating processes such as denitrification that tend to enrich the remaining N pool (Robinson 2001). Soil profiles taken 60 cm from tree boles show isotopic depletion beneath *Prosopis* to at least 30 cm down, compared with grassland. The offset between grassland and *Prosopis* $\delta^{15}N$ remains relatively constant down the profile (though both tend toward enrichment with depth), while N concentrations are much higher in the surface soil under *Prosopis*. This is consistent with a scenario in which new N inputs come primarily from above (e.g. from relatively isotopically-depleted leaf litter or deposition), but are exposed to greater enriching fractionation at the surface, for example by denitrification. Depletion of $\delta^{15}N$

deeper in the profile under *Prosopis* (compared with grassland values) may represent a combination of smaller N inputs derived from roots at depth and/or downward movement of N inputs from above.

Conclusions

We conclude that in this system, where N inputs and outputs can be well constrained, mass balance approaches offer a useful approach to the challenging task of estimating N fixation in woody perennials. This is especially true for systems such as *Prosopis*, where N inputs from fixation may represent a significant and ongoing source of N over large areas, and have important implications for the magnitude of ecosystem carbon storage and future vegetative cover (Archer *et al.* 2001). Future work will explore whether a $\delta^{15}N$ mass balance, including both soil and vegetation pools, could be developed to complement this N mass balance model by independently estimating fixation inputs and fractionating losses.

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