REGULAR ARTICLE

How encroaching shrubs and nutrients affect N_2-fixation in the Chihuahuan desert

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Abstract

Background and aims   Organisms that are capable of nitrogen (N) fixation, such as some species of cyanobacteria in biological soil crusts (biocrusts), can be ecologically important especially in low-nutrient dryland ecosystems. In addition, woody plant encroachment is occurring in drylands globally, including many species of N_2-fixing shrubs and trees. We determined the effects of nutrient deposition and shrub encroachment by *Larrea tridentata* on N_2-fixation rates of biocrusts and compared these rates to another encroaching shrub, the N_2-fixer *Prosopis glandulosa* in the northern Chihuahuan Desert.

Methods   We used both the acetylene reduction assay (ARA) and the ^{15}N natural abundance techniques to determine N_2-fixation rates of biocrusts. We also assessed the effects of nutrient additions on biocrust N_2-fixation rates using a long-term N addition experiment started in 2008 that is part of the Nutrient Network (NutNet).

Results   There was no correlation between the ARA and $\delta^{15}N$ methods. We found that *P. glandulosa* likely fixes more N (at least during the spring) than biocrusts based on $\delta^{15}N$ content in roots. There was no significant difference in $\delta^{15}N$ between *P. glandulosa* and our reference plant, *L. tridentata*. $\delta^{15}N$ declined in N-fertilized plots presumably because biological nitrogen fixation (BNF) is costly or because added N has negative effects on microbial communities.

Conclusions   Biocrusts fixed less nitrogen than N_2-fixing legume shrubs during this sampling period. Nonetheless, biocrusts are still a significant contributor to N_2-fixation in these nutrient-poor drylands based on the large proportion of soil surface area occupied by these organisms.

Keywords   Biological N_2 fixation · [15]N natural abundance · Biological soil crusts · Shrub encroachment · Nutrient additions · Desert grassland

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Introduction

Nitrogen (N) and water are frequently co-limiting in arid environments around the world (Xu et al. 2012). Consequently, organisms capable of converting atmospheric N (N_2) into a useable form such as ammonia (NH_3), a process known as biological nitrogen fixation (BNF), are considered ecologically significant and important (e.g. Aranibar et al. 2003). Many N_2-fixing organisms are prokaryotes that include bacteria that form symbiotic relationships with mostly leguminous plants, and free-living soil

bacteria such as cyanobacteria found commonly in biological soil crusts (biocrusts) (Nelson et al. 2021).

Typically, in arid regions where precipitation and nutrients are scarce, vascular plant cover is relatively low but biocrusts are common (Belnap et al. 2016). Biocrusts are surface-soil organisms (including cyanobacteria, lichens and mosses) that contribute significantly to ecosystem services such as increased N (and carbon) availability (Elbert et al. 2012). This combination of micro- and macro-organisms contributes significantly to nutrient concentrations in the interspaces between vascular plants (Liu et al. 2017). In fact, biocrusts are remarkable in terms of their ability to become quickly activated (unlike vascular plants) and potentially fix N_2 even after brief periods of rain (Green and Proctor 2016). Biocrusts occupy large open areas where competition with vascular plants is minimal (Baldarelli et al. 2021).

The encroachment of woody vascular plants is a worldwide trend in arid environments that has significantly altered the landscape (Archer et al. 2017; Van Auken 2000). For example, creosote bush (*Larrea tridentata:* Zygophyllaceae) along with *Prosopis* spp., including honey mesquite (*Prosopis glandulosa:* Fabaceae) and velvet mesquite (*Prosopis velutina*: Fabaceae), are encroaching into the deserts of the North American southwest (Van Auken 2000). A variety of biotic and abiotic drivers are known to promote woody encroachment. Heavy grazing by livestock reduces competition from grasses and creates open space for woody plants to encroach (Mureva and Ward 2016). Other factors include fire suppression that allows woody shrubs to thrive, along with global increases in atmospheric CO_2 that can promote dominance by C_3 shrubs over C_4 grasses at higher CO_2 concentrations (Archer et al. 2017). Regardless of the source or combination of sources, woody encroachment has the ability to significantly alter ecosystem dynamics, especially in arid environments. As the encroachment of woody plants continues to increase, space for biocrusts will become more limited (Baldarelli et al. 2021). Also, the potential biocrust contribution to N_2-fixation and soil fertility will likely decline due to soil enrichment by N_2-fixing shrubs.

Nutrient deposition also has a significant impact on the nutrient availability in arid environments (e.g. Ochoa-Hueso et al. 2016). For example, the southwestern desert areas in North America are experiencing increased N deposition due primarily to continued urbanization and agriculture (Báez et al. 2007). Arid ecosystems in general are more sensitive to increased nutrient levels because most organisms occurring in these environments are adapted to low nutrient conditions (e.g. Reed et al. 2016). Processes such as BNF are typically reduced when additional N is available due to the energetically expensive nature of fixing atmospheric nitrogen (Kambatuku et al. 2013) or because of the community composition effects that added N can have on microbes (e.g. Porras-Alfaro et al. 2007).

The purpose of this study was to compare how two processes (shrub encroachment and added nutrients) affect biocrust (namely cyanobacteria) N_2-fixation from two sites in the Chihuahuan desert. Specifically, we:

1) estimated the rate of N_2-fixation in biocrusts and compared the rates estimated in roots of dominating encroaching shrubs: *P. glandulosa* (a native leguminous species capable of N_2-fixation) and *Larrea tridentata* (also native but not a N_2-fixer), and

2) determined how biocrusts responded to nutrient additions with a particular focus on the light-coloured cyanobacterial species (dominated by *Microcoleus* spp.) that often associate with N_2-fixing cyanobacteria and heterotrophic N_2-fixing bacteria (Nelson et al. 2021). We then could determine how N_2-fixation under these scenarios deviate from the baseline (or controls).

We predicted that biocrusts would fix nitrogen regardless of the time of year as opposed to a particular season or after a monsoon rain event (Massatti and Knowles 2020). We assessed whether rates of N_2-fixing activity by biocrusts would be of the same magnitude as in roots dominated by the range-expanding shrub, *P. glandulosa*, a known N_2-fixing plant. Using an already established fertilization experiment (including additions of N, P, K, and combinations thereof) at Sevilleta in central New Mexico, we also predicted that plots supplemented with N would be less likely to fix N_2 (higher $\delta^{15}N$) compared to the unfertilized plots because BNF is a costly process in terms of carbon loss (Zheng et al. 2019) and/or because nitrogen additions can have negative effects on biocrusts (Dias et al. 2020) and alter the functioning of microbial communities (Porras-Alfaro et al. 2007).

Materials and methods

Study sites

The USDA Jornada Experimental Range (Jornada) is located in southern New Mexico, USA, about 40 km north of Las Cruces in the Chihuahuan Desert (N 32° 36', W 106° 44'). Soils in this area consist of alluvium that form Aridisols. The soil series associated with our study area include the Onite-Pajarito association (pasture 9; N 32° 31', W 106° 48'), the Bluepoint-Caliza-Yturbine complex and the Wink-Harrisburg association (pasture 15; N 32° 34', W 106° 55') (Bulloch and Neher 1980). The mean minimum annual temperature is 13.3°C (January) and the mean maximum annual temperature is 36°C (June) with a mean annual precipitation (MAP) of 247 mm (https://jornada.nmsu.edu/jornada/climate).

The Nutrient Network (NutNet) site at the Sevilleta Long-Term Ecological Research site (Sevilleta) is located between the Rio Grande river to the west and the Los Piños mountains to the east in central New Mexico (N 34° 20', W 106° 50'), about 80 km south of Albuquerque. The experimental plots are located in the northern range of the Chihuahuan desert in a steppe desert grassland. The soil order in this area is Aridisol in the Turney loamy sand series (Bryan-Ricketts, 2015). The climate is comparable to that of the Jornada with a MAP of 242 mm and a MAT of 13.3°C (Shi et al. 2014).

Experimental designs

Jornada

Areas of low and high encroachment (determined by the density of shrubs from the line-intercept method (Etchberger and Krausman 1997; 2–6%=low, 17–24%=high)) were selected along with an adjacent unencroached area to compare biocrust N_2-fixation rates to fixation rates of encroaching shrubs. The remaining percentages were occupied by light cyanobacterial biocrusts and bare soil. We used the line-intercept method with two 100 m-long transects to collect soil samples (bulk soil and biocrusts) and plant roots from both creosote bush (*L. tridentata*) and mesquite (*P. glandulosa*) shrubs during June 2017 before the monsoon season. Root samples

were harvested from 15 *P. glandulosa* shrubs at the two encroachment sites (low and high encroachment; n=30) to estimate rates of N_2-fixation. Roots of 15 *L. tridentata* were also harvested as a control (non-N_2-fixing) at both levels of encroachment. A pickaxe and shovel were used to uproot the shrubs and access the roots. Shallow roots were sampled from the uppermost 15 cm of the soil from a roughly 20 cm×20 cm area within 1 m of the base of the shrub to ensure that both *P. glandulosa* and *L. tridentata* had access to the same pool of N (Virginia et al. 1989). Root samples were collected from Pasture 15 (1,267 ha) (low and high-density of mesquite) and Pasture 9 (279 ha) (low and high-density of creosote) at Jornada. Dominant vegetation in these pastures include bunch grass (*Sporobolus* spp. and *Aristida* spp.), bush muhly (*Muhlenbergia porteri*), black grama (*Bouteloua eriopoda*), and snakeweed (*Gutierrezia* spp.). Pasture 9 also had Lehmann lovegrass (*Eragrostis lehmanniana*). All vegetation was low in cover (John Anderson, pers. comm.).[1]

Biocrusts at this site were dominated by cyanobacteria; there were no visible lichens or mosses. Biocrust samples were collected to a depth of about 3 mm adjacent to all above-mentioned root samples using a metal spoon. All biocrust samples were sampled within 1–2 m from the base of the associated shrub depending on the surrounding plant community. Biocrust samples were collected in interspaces between vascular plants to avoid external N inputs. We collected biocrusts that were undisturbed and visually intact. Although both pastures experience some grazing, disturbance is minor considering the low number of animals: large acreage ratio (~67 ha per animal per year) (Matt McIntosh, pers. comm.). Biocrusts were placed in petri dishes to minimize disturbance and maintain structure. Bulk soil was collected after biocrusts were removed from the surface of the soil using a metal cup (236.6 cm^3).

Sevilleta

The Nutrient Network (NutNet) experiment is a long-term fertilization experiment consisting of eight nutrient treatment combinations (N=5) of nitrogen (N), phosphorus (P), potassium plus micronutrients (K+), and none (control) set up as a fully crossed design

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resulting in 40 experimental units. Ten g m^{-2} N as NH$_4$NO$_3$, P as Ca(H2PO4)2, and K as K2SO4 were applied in June each year prior to the start of the summer monsoon. Each 5 m × 5 m plot is separated by 1 m from other plots. All plots were first fertilized in 2008 and annually thereafter with the exception of the micronutrients which were only applied once at the start of the experiment to avoid potential toxic effects (Biederman et al. 2017). Overall there was a 1:1 ratio of biocrusts and vascular plants (based on mean percent cover). The dominant vascular plants include black grama (*Bouteloua eriopoda*), western tansymustard (*Descurainia pinnata*), thicksepal cryptantha (*Cryptantha crassisepala*) and broom snakeweed (*Gutierrezia sarothrae*).

We used a 1 m × 1 m quadrat (Brun and Box 1963) in each of the four cardinal directions and one in the center of each plot to assess the percent cover of biocrusts and vegetation in May of 2017 before the monsoon season. To avoid edge effects, subsamples in the corners were sampled at least 1 m from the edge. To aid in percent cover estimates, the quadrat was sectioned into 10 cm × 10 cm squares so that every square equaled 1% of the total frame. Five subsamples of biocrusts and bulk soil were collected within each plot also in May of 2017. One subsample was collected from each main cartesian coordinate, plus one from the center of the plot. These subsamples were then mixed to obtain a single sample for each plot. All biocrust samples were dominated by light-coloured cyanobacteria. Biocrusts were scraped off the surface of the soil to a depth of about 3 mm with a metal spoon. Bulk soil samples were collected just below the biocrust samples (within 10 cm from the soil surface) using a metal cup (236.6 cm^3).

Techniques for measuring biological nitrogen fixation

Biological nitrogen fixation is commonly estimated using the acetylene reduction assay (ARA) and/or N$_2$ stable isotopes. The ARA is a relatively simple protocol that measures acetylene reduction as a proxy for nitrogenase activity (Hawkes 2001). The amount of sample material used for the ARA (~ 10 g (Hawkes 2001)) is large enough to calculate reliable estimates from small sampling plots. The ARA also serves as an instantaneous measure of N$_2$-fixation (Barger et al. 2016). However, the method is based on an indirect estimate of N$_2$-fixing ability or nitrogenase activity

via the reduction of acetylene to ethylene gases (Bergersen 1980).

An often more reliable way to estimate N$_2$-fixation is by the use of the stable isotope ^{15}N$_2$, which is a direct measure of the N$_2$-fixation process. Furthermore, ^{15}N$_2$ is a measure of long-term N (Barger et al. 2016). Typically, BNF is based on %Ndfa, which is commonly calculated from the raw δ^{15}N values (e.g. Cramer and Bond 2013; Kambatuku et al. 2013) to estimate plant- (and biocrust-) derived nitrogen from the atmosphere. However, we chose to focus on the δ^{15}N values for comparisons as the %Ndfa calculation is considered to be unreliable (Cramer and Bond 2013; Russow et al. 2005). It is now highly recommended to use paired methods (e.g. ARA and ^{15}N) as we have done here when measuring N$_2$-fixation given the shortcomings of both methods when used independently (Bellenger et al. 2020).

Soil/root measurements

Protocols

We used the acetylene reduction assay (ARA) (Hawkes 2001) to determine the relative differences in N$_2$-fixation between biocrust and root samples. Samples were stored at room temperature and the ARA was measured within a few days from the sampling date. Briefly, 2 ml of distilled water was added separately to 10 g of biocrust soil and 0.5 g of roots in air-tight mason jars (236 ml). Immediately upon wetting the biocrusts and roots, acetylene was added to create a 10% acetylene atmosphere. The initial air samples were collected and used as the "time 0" reference (t$_0$) using a gas-tight syringe and stored in an air-tight vial. Samples were incubated for 4 h before subsequent air samples were collected for "time final" (t$_f$) and stored in air-tight vials. All air samples were run on a Shimadzu gas chromatograph fitted with a flame ionization detector to determine the concentration of ethylene produced. All samples were calibrated with 100% pure ethylene. The rate of acetylene reduction to ethylene was calculated as:

$$Rate = \left[\left(E_{total}\right)_{tf} - \left(E_{total}\right)_{t0} \right] / \left(\Delta t \times W_{OD} \right), \qquad (1)$$

where E$_{total}$ = (nmol C$_2$H$_4$ ml^{-1}) x V$_{headspace}$, Δt = t$_f$ – t$_0$ and W_{OD} = the weight of the biocrust soil

(10 g) or roots (0.5 g), following (Hawkes 2001). Final rates were calculated as nmol g^{-1} h^{-1}.

The biocrust and root samples used for the ARA were also analyzed for isotopic analysis of N (δ^{15}N and N amount (µg)) by applying the ^{15}N natural abundance methodology (e.g. Aranibar et al. 2003). This involves differentiating between ^{15}N derived from atmospheric N_2 (%Ndfa) versus plant-available N sources in the soil using a nitrogen standard. All samples were ground and packaged in tin capsules and then sent to the UC Davis Stable Isotope Facility (UC Davis, California). The δ^{15}N values were determined using an elemental analyzer and continuous flow isotope-ratio mass spectrometer (IRMS) and calculated as:

$$\delta^{15}N(\%) = \left(R_{sample}/R_{standard} - 1\right) * 1000, \qquad (2)$$

where R_{sample} is the isotope ratio of the sample (^{15}N/^{14}N) and $R_{standard}$ is the isotope ratio for the nitrogen standard, which is atmospheric N_2 (0.0036765). All values are expressed relative to international standards as VPBD (Vienna Pee Dee Belemnite).

We calculated the percent nitrogen derived from the atmosphere (%Ndfa) as:

$$Ndfa(\%) = \left(\left(\delta^{15}N_{reference} - \delta^{15}N_{fixing}\right)/\delta^{15}N_{reference}\right) * 100, \qquad (3)$$

where $\delta^{15}N_{reference}$ is the mean of N-only plots (10.36‰) and $\delta^{15}N_{fixing}$ is the δ^{15}N value for each plot (Cramer and Bond 2013). It should be noted that this estimate does not represent the total N input, rather a relative value of BNF (Russow et al. 2005).

Bulk soil was air-dried in the respective field laboratories (Jornada and Sevilleta) prior to soil measurements. Bulk density of the soil was determined by weighing the dry soil and dividing it by the volume of the container (236.6 cm^3). After bulk density was recorded, we sieved the soil using 2 mm mesh to exclude rocks or other large material. The < 2 mm fraction was used to measure soil respiration as a measure of microbial activity. Soil respiration was estimated using the Solvita CO_2-Burst protocol (Haney et al. 2008). This method consisted of wetting 40 g of soil with 5 ml of distilled water and incubating samples for 24 h before recording the quantity of CO_2 released using the Solvita CO_2-Burst standard protocol (Haney et al. 2008).

Statistical analysis

The assumptions for parametric tests were not met for the Jornada data so we used the equivalent non-parametric Kruskal–Wallis test to test for significant differences among groups (biocrusts vs. *P. glandulosa* and *L. tridentata* shrubs). We then used a Mann–Whitney test as a post hoc test where differences were significant (P < 0.05).

The Sevilleta data met the assumptions of parametric tests after they were $\log_{10}$-transformed. We ran an ANCOVA with mean vascular plant cover as the covariate to account for the vascular plants present in the nutrient-fertilized plots. Subsequently, we ran an ANOVA and a post hoc Tukey HSD test on the Sevilleta data. We calculated the partial eta-squared ($\eta^2_{partial}$) value as a measure of the effect size of treatment on δ^{15}N (Cohen, 1988). We ran a regression to determine the relationship between soil respiration and δ^{15}N. A multiple regression analysis was used to determine differences in δ^{15}N among treatment groups. We also used an End Member Mixing Analysis (EMMA) to help us better understand the relative importance of the methods (ARA vs. $^{15}N_2$) of a given sample (Barthold et al. 2011). This technique entails using Principal Component Analysis. We differentiated plots by the presence of N, to assess the relative importance of the ARA, N amount (µg) and δ^{15}N. All analyses were done using SPSS version 26 (IBM SPSS, Armonk, NY) and R version 3.6.2 (R Core Team 2013).

Results

Shrub encroachment

There was no significant difference in N_2-fixation rates (per hour) for the type of sample (biocrust or shrub) when analyzing the Acetylene Reduction Assay (ARA) data from the Jornada (Kruskal–Wallis H test, equivalent $\chi^2 = 3.982$, df = 3, p = 0.263) (Appendix 2). The mean ± S.E. ARA rate was $0.557 \pm [0.160]$ nmol g^{-1} h^{-1}.

There was a significant difference (Kruskal–Wallis H test, equivalent $\chi^2 = 91.054$, df = 3, p < 0.001) between the δ^{15}N values of biocrusts (biocrustc and
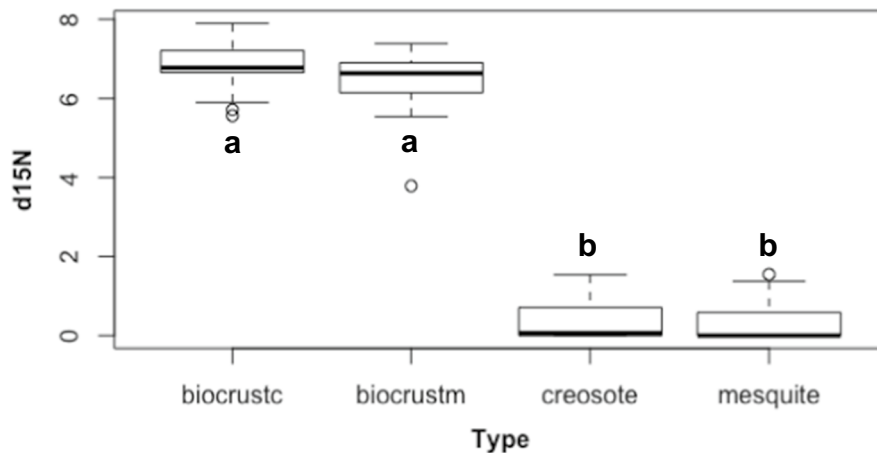
Fig. 1 Box plots indicating median (bold line), quartiles and ranges of stable nitrogen isotope values ($\delta^{15}N$ values, ‰) for both biocrusts and shrubs (roots) at the Jornada Experimental Range during the sampling period in June of 2017. Biocrusts are categorized as either adjacent to creosote bush (biocrust-c) or adjacent to mesquite (biocrust-m). Open circles indicate outliers. The different letters above and below the error bars indicate significant differences among treatments

biocrustm) and the shrubs (creosote and mesquite) (Fig. 1). However, there was no significant difference between $\delta^{15}N$ values of mesquite (*P. glandulosa*, N_2-fixing target plant) and creosote (*L. tridentata*, non-N_2-fixing reference plant) (Mann–Whitney test: U=437, p=0.848) (Fig. 1). The mean±S.E. $\delta^{15}N$ for creosote was -0.031±0.176 and for mesquite was -0.053±0.158, indicating that both species were high in biologically fixed N_2 because their $\delta^{15}N$ values overlapped with 0 (Cramer et al. 2007). We found no significant correlation (r=0.07; F=0.505, p=0.48) between the ARA rate and $\delta^{15}N$. Additionally, there was no significant relationship between the area of the shrubs and the $\delta^{15}N$ values (r=0.02; F=0.024, p=0.88).

Nutrient additions

The mean±S.E. ARA rate for the Sevilleta study site was 0.013±[0.004] nmol g^{-1} h^{-1}. No significant differences were detected among treatments (p>0.31) (Appendix 3). For most plots, no N_2-fixation was recorded from biocrust samples.

Vascular plant cover did not significantly affect biocrust $\delta^{15}N$ values in the fertilized plots (ANCOVA; F=2.66, p=0.11). The effect of treatment (nutrient combination) was significant (ANOVA; F=33.87, p<0.001) (Fig. 2). There was a significant effect of treatment on $\delta^{15}N$ (ANOVA; F=33.87, p<0.001,

$\eta^2_{partial}=0.864$). The N and NK-supplemented plots had the highest $\delta^{15}N$ values, corresponding with the lowest presumed N_2-fixation rates. However, NP and NPK did not have significantly higher values than the control or other N-containing treatments (Fig. 2). All of the treatments other than N and NK (Control, K, NP, NPK, P and PK) had significantly higher %Ndfa values, indicating more atmospheric N_2-fixation (Appendix 1). The lower %Ndfa in the N-fertilized plots suggests little or no atmospheric N_2-fixation (Fig. 3). Note that values lower than 0%Ndfa are possible (Cramer et al. 2010). There was a significant correlation between soil respiration and $\delta^{15}N$ (r=0.382, F=6.481, p=0.01). There was no significant correlation between ARA rate and $\delta^{15}N$ (r=-0.069, F=0.185, p=0.67).

We tested the effect of the different treatments (N, P, K) on $\delta^{15}N$ using an ANOVA. The interaction of N*P was significant (F=48.16, p<0.001) (Table 1, Fig. 4). The main effects of both N (F=78.85, p<0.001) and P (F=72.66, p<0.001) were also significant. The mean and ±S.E. of $\delta^{15}N$ for N was 10.11±0.29 and of $\delta^{15}N$ for P-fertilized plots was (5.80±0.13). The mean and ±S.E. of $\delta^{15}N$ for the control was 6.20±0.16.

To test the effect of N on biocrust cover, we grouped non-N fertilized plots and N-fertilized plots. We found a significant difference between these two groups (ANOVA: F=19.33, p<0.001). The

Fig. 2 Mean stable nitrogen isotope (δ^{15}N, ‰) values of the biocrusts at the Sevilleta Nutrient Network plots. High δ^{15}N values (indicated by treatments N and NK) suggest reduced N_2-fixation. The control plots are biocrust plots with no added nutrients. Different letters above the error bars indicate significant differences among treatments from post hoc tests. The bars indicate 1 S.E
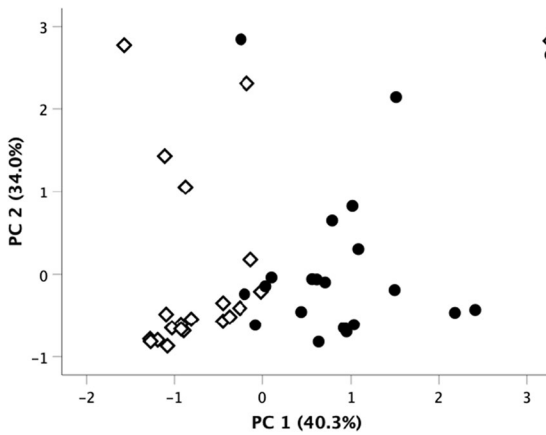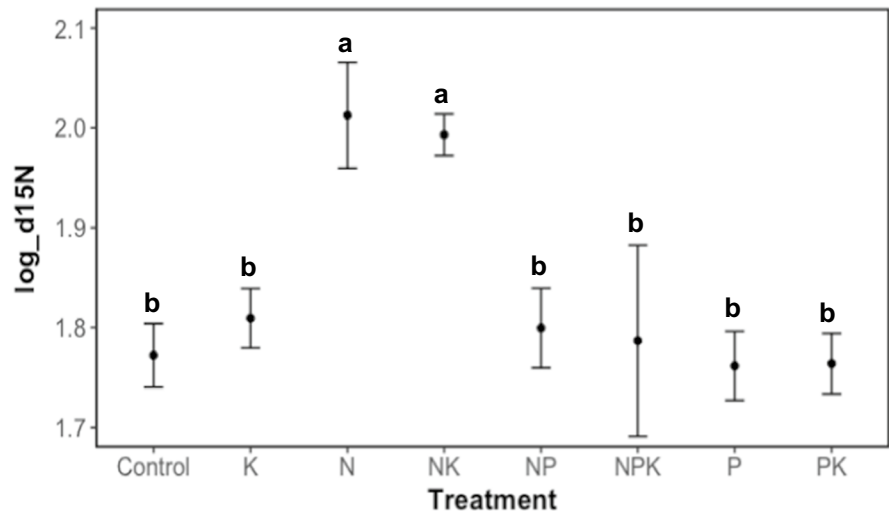
Fig. 3 A principal component analysis of ARA, N amount (µg) and δ^{15}N differentiated by the presence of N in the plots at the Sevilleta. Open diamonds indicate plots not receiving N (N=0) fertilization whereas filled circles represent plots receiving fertilizer N (N=1). Note the general separation between N-fertilized plots (circles) and plots without N (diamonds)

Table. 1 ANOVA of the effects of fertilizer treatment on δ^{15}N. Note the significant interaction effect of N*P. Both N and P also had significant main effects on δ^{15}N. Bold font indicates significant effects

| Source	df	Mean Square	F	P
N	**1**	**48.182**	**78.847**	**< 0.001**
P	**1**	**44.401**	**72.660**	**< 0.001**
K	1	0.001	0.001	0.976
N * P	**1**	**29.430**	**48.161**	**< 0.001**
N * K	1	0.812	1.329	0.258
P * K	1	0.004	0.007	0.935
N * P * K	1	0.533	0.871	0.358
Error	32	0.611		

important variables describing PC1 whereas ARA was the most important variable describing PC2 (PC component weighting=0.953). We differentiated these plots by the presence of N fertilizer and found a general separation between the groups (N or no N) (Fig. 3).

mean ± S.E. %Ndfa for the non-N plots was about twice that for the N plots (42.08 ± 1.05 (non-N plots); 20.89 ± 4.70 (N plots)).

The first principal component axis of the EMMA, using Principal Components Analysis explained 40.3% of the variance (eigenvalue=1.209) and the second principal component axis explained a further 34.0% of the variance (eigenvalue=1.020). δ^{15}N (PC component weighting=0.789) and N amount (PC component weighting=0.754) were the most

Discussion

We used two sites in the Chihuahuan desert to investigate the effects of shrub encroachment (Jornada) and added nutrients (Sevilleta) on the ability of biocrusts to fix N_2. The N_2-fixation values indicated lower N_2-fixation by the biocrusts compared to the values of the known N_2-fixer (*P. glandulosa*). Moreover, we

Fig. 4 The effects of N*P fertilizer on mean $\delta^{15}N$. There were significant interaction effects between N and P on $\delta^{15}N$. Note that whenever N is added (N fertilizer, blue bar on the left), $\delta^{15}N$ is higher, possibly indicating reduced need to fix N_2 (see *Discussion*). Error bars represent 95% confidence intervals (CI)
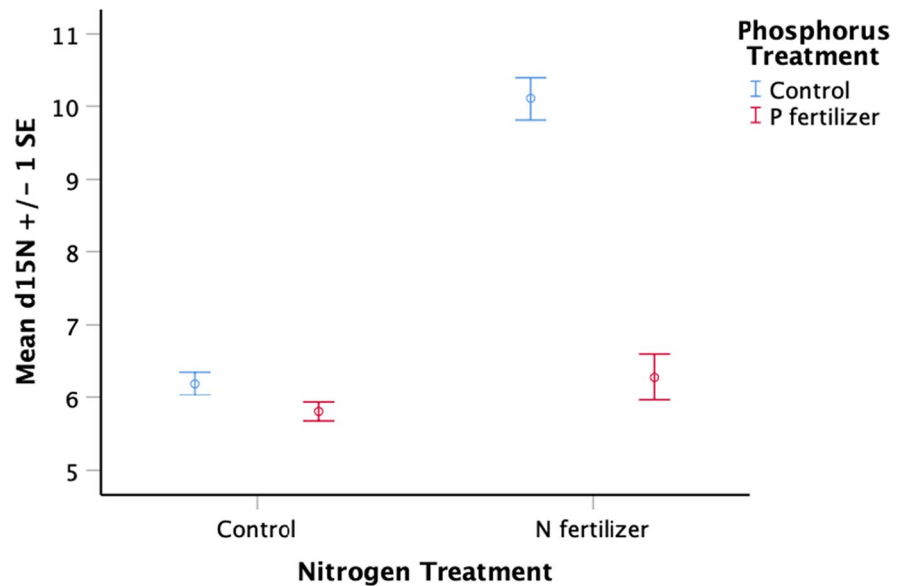
found support for added nutrients (specifically N and NK) suppressing biocrust N_2-fixation. This could be a direct negative effect of nitrogen-based compounds on biocrust N_2-fixation due to the carbon cost to the biocrusts (Dias et al. 2020; Zheng et al. 2019). Both shrub encroachment and excess nutrients (via nutrient deposition) are relevant topics to understand N_2-fixation, especially regarding arid environments and their associated biocrusts.

Shrub encroachment

We predicted that biocrust $\delta^{15}N$ values would be similar to *P. glandulosa* $\delta^{15}N$ values due to the ability of cyanobacterial biocrusts to fix N (like most legumes). In contrast to this prediction, we found that biocrusts had greater $\delta^{15}N$ values (~6–8‰) (suggesting lower N_2-fixation) than roots of the known N_2-fixing shrub, *P. glandulosa*. $\delta^{15}N$ values close to 0‰ suggest that there is biological nitrogen fixation (BNF) because atmospheric N_2 is mostly made up of ^{14}N and contains very little ^{15}N (Unkovich and Pate 2001). Therefore, N_2-fixing leguminous shrubs often have a $\delta^{15}N$ signature of ~0‰ (e.g. Kambatuku et al. 2013).

P. glandulosa $\delta^{15}N$ values did not significantly differ in $\delta^{15}N$ values from those of our supposedly non-N_2 fixing reference plant, *L. tridentata* at the Jornada. Busse et al. (2007) also found no significant difference between $\delta^{15}N$ values of their target N_2-fixing plant (mahala mat; *Ceanothus prostratus*) and their

selected reference plant (manzanita; *Arctostaphylos patula*). It is possible that shrubs in close proximity to a N_2-fixer (such as *P. glandulosa*) that have a $\delta^{15}N$ signature similar to zero could be subject to a similar signature (Unkovich and Pate 2001). However, this may not be the case for our study as our reference transects were separated from our N_2-fixing transects in very large but adjacent pastures (pasture 9: 279 ha; pasture 15: 1,267 ha) at the Jornada.

Another reasonable explanation for the nonsignificant difference between $\delta^{15}N$ signatures of our target and reference plants is that the reference plant (*L. tridentata*) did not actually serve as a reference (Busse et al. 2007). In fact, the ^{15}N natural abundance method is only useful when differences between the reference plant (in our study, *L. tridentata*) and target species (in our study, *P. glandulosa*) are large (Cramer et al. 2007). Although *L. tridentata* is not known to be a N_2-fixer, there are some actinorhizal species (that form a symbiosis with N_2-fixing actinobacteria) which could be contributing to the low $\delta^{15}N$, a factor not studied in this work (e.g. Roley et al. 2018). This type of N_2-fixation, often referred to as *associative N_2-fixation* (ANF), differs from BNF in that the relationship between the plant and the bacterium is not necessarily symbiotic, but rather that each species is only partially dependent on the other (Postgate 1998). Furthermore, the reference plant used in this study (*L. tridentata*), is known to have very high leaf N possibly due to the accumulation of nitrate and

considerably low rates of denitrification (Vince Guts-chick, pers. comm.).[2]

Our average $\delta^{15}N$ values for the biocrust samples (6.7‰) are just slightly greater than the high end of the range reported by Billings et al. (2003) for biocrust N_2-fixation. The high-end values in Billings et al. (2003) correspond to biocrusts under non-N_2-fixing shrubs, *Lycium* spp., while low-end values (closer to 0‰) correspond with biocrusts in the interspaces in their study. Incomplete nitrification and denitrification that result in a loss of N in cyanobacterial biocrusts have been linked to higher $\delta^{15}N$ values (Barger et al. 2016). Other researchers have also suggested that high positive $\delta^{15}N$ values (as seen with our biocrust samples) could possibly be explained by N_2-fixation of fungal mycorrhizae or through nitrogen mineralization (Craine et al. 2015). Even though it was not monsoon season, biocrusts still fixed N_2, although fixation by biocrusts was less than by *P. glandulosa* (the N_2-fixer) and *L. tridentata* (the non-N_2-fixer).

The $\delta^{15}N$ of biocrusts was greater than we anticipated but aligns well with what others have found. Moreira-Grez et al. (2019) reported biocrust $\delta^{15}N$ values ranging from ~2‰ (foliose lichens) to ~8‰ (bare soil) with cyanobacterial biocrust signature slightly less than bare soil but not significantly different. Furthermore, light-coloured cyanobacterial biocrusts are reported elsewhere to be less likely to fix N_2 (Pietrasiak et al. 2013), supporting our values for the Jornada. It is noteworthy to also mention that the less stable structure of cyanobacteria biocrusts (as opposed to lichen or moss dominated biocrusts) are likely to include bare soil that could ultimately affect the $\delta^{15}N$ (Moreira-Grez et al. 2019). Soil texture and pH have also been suggested to affect the ability of microbes to fix N. In particular, clay soils and high pH soils tend to have more optimal N_2-fixing conditions due to oxygen availability and microbial community dynamics (Smercina et al. 2019). We note that the soil differences between sites were not great enough to be considered explanatory and perhaps supports why significant differences were not found.

Nutrient additions

The results of the fertilization portion of the study indicate that biocrusts can fix N_2 even in dry periods

before the monsoon season (Fig. 2). The $\delta^{15}N$ values of the Sevilleta partially align with our second prediction that plots supplemented with N (including P and K treatments) would have lower N_2-fixing activity (higher $\delta^{15}N$ values) compared to unfertilized plots. Others have also found that N_2-fixation (measured by ARA) was suppressed with the addition of N (Dynarski and Houlton, 2018). This could be because N_2-fixation is costly; when N is readily available, there is no need to engage in this process (Dias et al. 2020; Zheng et al. 2019) or because added N has a detrimental effect on biocrusts (e.g. Dias et al. 2020) and alters microbial composition and functioning (Porras-Alfaro et al. 2007). This is supported by our finding that N and NK-fertilized plots had higher $\delta^{15}N$ values compared to the control plots (Fig. 2).

The addition of P had the opposite effect; N_2-fixation rates were higher (lower $\delta^{15}N$ values) than plots supplemented with N (plots N and NK) and non-significantly different compared to *control* plots. Note that all treatments including P do not significantly differ from the control plots. This finding that P additions increased N_2-fixation rates (measured by ARA) has previously been shown (Reed et al. 2007). Our results support the notion of P limitation and the importance and connectivity of the N and P cycles (Baldarelli et al. 2021; Reed et al. 2007).

Conclusions and implications

We found no significant difference in N_2-fixation between shrubs and biocrusts (Jornada) nor between nutrient treatments (Sevilleta) when using the ARA technique. However we found more consistency using the $\delta^{15}N$ method; at both sites, there were significant differences in N_2-fixation between shrubs and biocrusts (Jornada) and between some of the N treatments (Sevilleta). Although *P. glandulosa* likely contributes more fixed N_2 (compared to biocrusts) during the springtime, biocrusts are still significant diazotrophs in arid environments. We show reduced $\delta^{15}N$ values in N-fertilized plots suggesting that either N is plentiful in these plots or that added N has negative effects on microbial communities.

Overall, we found that biocrusts are fixing less N than the known N-fixer *P. glandulosa* and the non-N-fixer *L. tridentata* during early summer (June) in the Chihuahuan desert. However, we did find

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support that biocrusts are able to fix N during this time before the monsoon season. For the most part, biocrust N_2-fixation was suppressed when N was added, suggesting that either N_2-fixation is energetically costly for biocrusts or that biocrusts were negatively affected by added N.

Understanding the spatially heterogeneous distribution of nutrients and the coexistence of biocrust and vascular plant communities (typically in N_2-fixing encroaching shrubs) is useful for understanding the impacts of encroachment in desert landscapes. While the source of N_2-fixation by our reference shrub *L. tridentata* is unclear, future research should investigate the source of N_2-fixation in this species, such as by associative fixation. Future studies should also include a pilot study to test the appropriateness of potential reference plants prior to embarking on examination of N_2-fixation. If possible, multiple $\delta^{15}N$ values for different parts of the shrubs could be compared to increase the accuracy of the method (Cramer et al. 2007).

To fully understand N deposition in a system, baseline N should be measured, and it should be determined if the N input(s) are beyond organismal stoichiometry or the optimal levels needed for ordinary functioning. Excess nutrients have the ability to be toxic, especially for organisms adapted for low nutrient (particularly N) environments (e.g. Dias et al. 2020). As N continues to rise due to an ever-growing and expanding populace, understanding how biocrusts and vascular plants will respond will enable us to increase our knowledge about general N cycling in arid ecosystems.

References

Aranibar JN, Anderson IC, Ringrose S, Macko SA (2003) Importance of nitrogen fixation in soil crusts of southern African arid ecosystems: acetylene reduction and stable isotope studies. J Arid Environ 54:345–358

Archer SR, Andersen EM, Predick KI, Schwinning S, Steidl RJ, Woods SR (2017) Woody plant encroachment: causes and consequences. In: Systems R (ed) Briske DD. Springer, Cham, pp 25–84

Baldarelli LM, Throop HL, Collins SL, Ward D (2021) Nutrient additions have direct and indirect effects on biocrust biomass in a long-term Chihuahuan Desert grassland experiment. J Arid Environ 184:04317

Barger NN, Weber B, Garcia-Pichel F, Zaady E, Belnap J (2016) Patterns and controls on nitrogen cycling of biological soil crusts. In: Weber B, Büdel B, Belnap J (eds) Biological Soil Crusts: An Organizing Principle in Drylands. Springer, Cham, pp 257–285

Barthold FK, Tyralla C, Schneider K, Vaché KB, Frede HG, Breuer L (2011) How many tracers do we need for end member mixing analysis (EMMA)? A sensitivity analysis. Water Resour Res 47:1–14. https://doi.org/10.1029/2011WR010604

Báez S, Fargione J, Moore DI, Collins SL, Gosz JR (2007) Atmospheric nitrogen deposition in the northern Chihuahuan desert: temporal trends and potential consequences. J Arid Environ 68:640–651

Bellenger JP, Darnajoux R, Zhang X, Kraepiel AML (2020) Biological nitrogen fixation by alternative nitrogenases in terrestrial ecosystems: a review. Biogeochemistry 149:53–73

Belnap J, Weber B, Büdel B (2016) Biological soil crusts as an organizing principle in drylands. In: Weber B, Büdel B, Belnap J (eds) Biological Soil Crusts: An Organizing Principle in Drylands. Springer, Cham, pp 3–13

Bergersen FJ (1980) Measurement of nitrogen fixation by direct means. Bergersen FJ Methods for Evaluating Biological Nitrogen Fixation. John Wiley and Sons, Chichester, pp 65–110

Biederman L, Mortensen B, Fay P, Hagenah N, Knops J, La Pierre K, Laungani R, Lind E, McCulley R, Power S,

Seabloom E, Tognetti P (2017) Nutrient addition shifts plant community composition towards earlier flowering species in some prairie ecoregions in the US Central Plains. PloS One 12:e0178440

Billings SA, Schaeffer SM, Evans RD (2003) Nitrogen fixation by biological soil crusts and heterotrophic bacteria in an intact Mojave Desert ecosystem with elevated CO2 and added soil carbon. Soil Biology and Biochemistry 35:643–649

Brun JM, Box TW (1963) A comparison of line intercepts and random point frames for sampling desert shrub vegetation. J Range Manag 16:21–25

Bryan-Ricketts D (2015) Soil Geomorphology of the Eastern Sevilleta Long Term Ecological Research Site. University of Albuquerque, Albuquerque, NM, PhD thesis

Bulloch HE, Neher, RE (1980). Soil Survey of Dona Ana County Area, New Mexico. Department of Agriculture, Soil Conservation Service, NM

Busse MD, Jurgensen MF, Page-Dumroese DS, Powers RF (2007) Contribution of actinorhizal shrubs to site fertility in a Northern California mixed pine forest. Forest Ecol Manag 244:68–75

Cohen J (1988) Statistical power analysis for the behavioral sciences, 2nd ed. Lawrence Erlbaum Associates, Hillsdale, New Jersey, pp 18–74

Craine JM, Elmore AJ, Wang L, Augusto L, Baisden WT, Brookshire EJ, Cramer MD, Hasselquist NJ, Hobbie EA, Kahmen A, Koba K (2015) Convergence of soil nitrogen isotopes across global climate gradients. Sci Rep 5:e8280

Cramer MD, Chimphango SB, Van Cauter A, Waldram MS, Bond WJ (2007) Grass competition induces N_2 fixation in some species of African *Acacia*. J Ecol 95:1123–1133

Cramer MD, Van Cauter A, Bond WJ (2010) Growth of N_2-fixing African savanna *Acacia* species is constrained by below-ground competition with grass. J Ecol 98:156–167

Cramer MD, Bond WJ (2013) N-fertilization does not alleviate grass competition induced reduction of growth of African savanna species. Plant and Soil 366:563–574

Dias T, Crous CJ, Ochoa-Hueso R, Manrique E, Martins-Loução MA, Cruz C (2020) Nitrogen inputs may improve soil biocrusts multifunctionality in dryland ecosystems. Soil Biol Biochem 149:107947

Dynarski KA, Houlton BZ (2018) Nutrient limitation of terrestrial free-living nitrogen fixation. New Phytologist 217:1050–1061

Elbert W, Weber B, Burrows S, Steinkamp J, Büdel B, Andreae MO, Pöschl U (2012) Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. Nat Geosci 5:459–462

Etchberger RC, Krausman PR (1997) Evaluation of five methods for measuring desert vegetation. Wildl Soc Bull 25:604–609

Green TA, Proctor MC (2016) Physiology of photosynthetic organisms within biological soil crusts: their adaptation, flexibility, and plasticity. In: Weber B, Büdel B, Belnap J (eds) Biological Soil Crusts: An Organizing Principle in Drylands. Springer, Cham, pp 347–381

Haney RL, Brinton WF, Evans E (2008) Soil CO_2 respiration: comparison of chemical titration, CO_2 IRGA analysis and the Solvita gel system. Renew Agric Food Syst 23:171–176

Hawkes CV (2001) Methods for studying biological soil crusts. Methodology Paper of the 4th International Conference on ILTER East Asian and Pacific Region. Ulaanbaatar-Hatgal, Mongolia, pp 36–45

IBM Corp. 2019. IBM SPSS Statistics for Mac, Version 26.0. Armonk, NY: IBM Corp

Kambatuku JR, Cramer MD, Ward D (2013) Nitrogen fertilisation reduces grass-induced N_2 fixation of tree seedlings from semi-arid savannas. Plant Soil 365:307–320

Liu YR, Delgado-Baquerizo M, Trivedi P, He JZ, Wang JT, Singh BK (2017) Identity of biocrust species and microbial communities drive the response of soil multifunctionality to simulated global change. Soil Biol Biochem 107:208–217

Massatti R, Knowles LL (2020) The historical context of contemporary climatic adaptation: a case study in the climatically dynamic and environmentally complex southwestern United States. Ecography 43:735–746

Moreira-Grez B, Tam K, Cross AT, Yong JW, Kumaresan D, Farrell M, Whiteley AS (2019) The bacterial microbiome associated with arid biocrusts and the biogeochemical influence of biocrusts upon the underlying soil. Front Microbiol 10:2143

Mureva A, Ward D (2016) Spatial patterns of encroaching shrub species under different grazing regimes in a semi-arid savanna, eastern Karoo, South Africa. Afr J Range Forage Sci 33:77–89

Nelson C, Giraldo-Silva A, Garcia-Pichel F (2021) A symbiotic nutrient exchange within the cyanosphere microbiome of the biocrust cyanobacterium, *Microcoleus vaginatus*. The ISME Journal 15:282–292

Ochoa-Hueso R, Delgado-Baquerizo M, Gallardo A, Bowker MA, Maestre FT (2016) Climatic conditions, soil fertility and atmospheric nitrogen deposition largely determine the structure and functioning of microbial communities in biocrust-dominated Mediterranean drylands. Plant Soil 399:271–282

Pietrasiak N, Regus JU, Johansen JR, Lam D, Sachs JL, Santiago LS (2013) Biological soil crust community types differ in key ecological functions. Soil Biol Biochem 65:168–171

Porras-Alfaro A, Herrera J, Natvig DO, Sinsabaugh RL (2007) Effect of long-term nitrogen fertilization on mycorrhizal fungi associated with a dominant grass in a semiarid grassland. Plant and Soil 296:65–75

Postgate JR (1998) Nitrogen Fixation, 3rd edn. University Press, Cambridge, p 112

R Core Team. (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/

Reed SC, Maestre FT, Ochoa-Hueso R, Kuske CR, Darrouzet-Nardi A, Oliver M, Darby B, Sancho LG, Sinsabaugh RL, Belnap J (2016) Biocrusts in the context of global change. In: Weber B, Büdel B, Belnap J (eds) Biological Soil Crusts: An Organizing Principle in Drylands. Springer, Cham, pp 451–476

Reed SC, Seastedt TR, Mann CM, Suding KN, Townsend AR, Cherwin KL (2007) Phosphorus fertilization stimulates nitrogen fixation and increases inorganic

nitrogen concentrations in a restored prairie. Appl Soil Ecol 36:238–242

Roley SS, Duncan DS, Liang D, Garoutte A, Jackson RD, Tiedje JM, Robertson GP (2018) Associative nitrogen fixation (ANF) in switchgrass (*Panicumvirgatum*) across a nitrogen input gradient. PloS One 13:e0197320

Russow R, Veste M, Böhme F (2005) A natural ^{15}N approach to determine the biological fixation of atmospheric nitrogen by biological soil crusts of the Negev Desert. Rapid Commun Mass Spectrom 19:3451–3456

Shi Z, Thomey ML, Mowll W, Litvak ME, Brunsell NA, Collins SL, Pockman WT, Smith MD, Knapp AK, Luo Y (2014) Differential effects of extreme drought on production and respiration: synthesis and modeling analysis. Biogeosciences 11:621–633

Smercina DN, Evans SE, Friesen ML, Tiemann LK (2019) To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. Appl Environ Microbiol 85:e02546-e2618

Unkovich M, Pate JS (2001) Assessing N$_2$ fixation in annual legumes using ^{15}N natural abundance. In: Unkovich M, Pate J, McNeill A, Gibbs DJ (eds) Stable Isotope Techniques in the Study of Biological Processes and Functioning of Ecosystems. Springer, Dordrecht, pp 103–118

Van Auken OW (2000) Shrub invasions of North American semiarid grasslands. Annu Rev Ecol Syst 31:197–215

Virginia RA, Jarrell WM, Rundel PW, Shearer G, Kohl DH (1989) The use of variation in the natural abundance of ^{15}N to assess symbiotic nitrogen fixation by woody plants. In: Rundel PW, Ehleringer JR, Nagy KA (eds) Stable Isotopes in Ecological Research. Springer, New York, pp 375–394

Xu Z, Wan S, Ren H, Han X, Li MH, Cheng W, Jiang Y (2012) Effects of water and nitrogen addition on species turnover in temperate grasslands in northern China. PloS One 7:e39762

Zheng M, Zhou Z, Luo Y, Zhao P, Mo J (2019) Global pattern and controls of biological nitrogen fixation under nutrient enrichment: A meta-analysis. Glob Chang Biol 25:3018–3030