Author responses

REPLY TO RC1

We extend our sincere gratitude to both you and Anonymous Referee #2 for taking the time to review our manuscript, "Soil nitrogen response to shrub encroachment in a degrading semiarid grassland". The comments you both provided were constructive, and enormously beneficial to improving the overall quality and accessibility of the paper. Below we address the comments you made in the order in which they were raised.

In the introduction, I strongly suggest the authors to make a correction on page 2/line21. There they say that nitrogen is the nutrient that mostly limits primary productivity in dryland systems, which might be the case for temperate areas, but it is not for tropical areas.

We apologise for any confusion caused. We are explicitly referring to semiarid and arid ecosystems (drylands) – not temperate or tropical areas. The statement that nitrogen is the most significant limiting nutrient of primary productivity in dryland ecosystems is supported by the literature (e.g., Craine et al., 2007, Robertson and Groffman, 2007). For clarity, we will revise the sentence to, "Nitrogen (N), in particular, is considered the most significant limiting nutrient of primary production and decomposition processes in arid and semiarid ecosystems …".

Please correct on page 3 line 16 - asymbiotic instead of asymbioitic.

Thank you for spotting this spelling mistake. It will be corrected in the manuscript.

The quality/resolution of the graphs in the figures can be improved.

We agree. We will increase the point size in each figure to improve their legibility.

REPLY TO RC2

I apologize for the typo (Welfs instead of Jelfs) in the previous comment posted.

Thank you, that is fine.

REPLY TO RC3

We extend our sincere gratitude to both you and Anonymous Referee #1 for taking the time to review our manuscript, "Soil nitrogen response to shrub encroachment in a degrading semiarid grassland". The comments you both provided were constructive, and enormously beneficial to improving the overall quality and accessibility of the paper. Below we address the comments you made in the order in which they were raised.

The appeal of this paper could be broadened by incorporating more literature addressing these ecological phenomena in other biogeographic regions beyond the desert southwest.

This is a fair comment. To highlight the global significance of this phenomenon, we will add the following to the first sentence of the second paragraph on line 27, page 2 in our introduction, "Occurring in Africa (Hudak et al., 2003), Asia (Zhang et al., 2016), Australia (Cookson et al., 2006), Europe (Quero et al., 2013), and the North (Knapp et al., 2008) and South Americas (Cabral et al., 2003), extensive research has…" Further, we will amend the sentence on lines 15-17 on page

16 to, “This is supported by a study for a semiarid grassland in central Spain which showed the availability of inorganic N is largely influenced by the abundance and functional diversity of the microbial community (Delgado-Baquerizo et al., 2015).”.

Page 2, Lines 12-13: This sentence is not a particularly new or novel result. It would be good to summarize and synthesize your findings in a way that adds something new to what we already know about the effects of shrub encroachment on N cycling in drylands.

We will change this sentence to, “Overall, these results provide a greater insight into how grass- to shrubland transitions influence the soil N pool through associated impacts on the soil microbial biomass.”

Page 3, Lines 26-31: I appreciate that the objectives are presented clearly. Are there some hypotheses that could be offered to enhance the objectives?

We agree with the suggestion of supplementing our objectives with hypotheses and thus, we will include the following, starting on line 32, page three: “We expected that reductions in grass biomass resulting from higher levels of shrub encroachment would cause soil N to be progressively redistributed from intershrub zones to areas beneath shrub canopies. As the spatial distribution of soil microorganisms is positively influenced by resource availability (Schlesinger and Pilmanis, 1998), we hypothesised the enhanced fertility and environmental conditions in soils under shrubs would support a higher soil microbial biomass and promote N mineralisation processes leading to an increased availability of inorganic N. Accordingly, we predicted that rates of asymbiotic BNF would decrease in shrub soils as inorganic N increased relative to bioavailable P (Smith, 1992)”.

Page 3, Lines 32-33: This sentence should be moved into the Study Site Description section.

Thank you, we will move this to the Study Site Description.

Page 3, Lines 33-35: This sentence isn’t necessary to your story.

We agree, and we will remove this sentence.

Page 5, Lines 10-11: It would be good to provide a bit more information on the soils across the study areas. Is the taxonomy (i.e. order, series) identical across all of the sites?

Three soil orders have been described by Clemmons & Wheeler (1970) for the SRER. Soils from all of our sampling sites fall in the Combate-Diaspar Complex series (SRER Digital Database, https://cals.arizona.edu/srer/data.html). However, from the available information, we are unable to identify which order(s) our soils belonged to. We will amend the sentence beginning on page 5, line 11 to, “Soils at these sites were formed in alluvium from igneous rock of Holocene and Late Pleistocene origins (Batchily et al., 2003) in the Combate-Diaspar complex (CoB), characterised by excellent drainage, sandy loam textures and 1-8% slopes (SRER Digital Database, https://cals.arizona.edu/srer/data.html).”.

Page 7, Line18: Since ammonium and nitrate are ions, you should show their charges when you abbreviate their chemical formulae (i.e., NH4+-N and NO3–N).

Thank you, we will make the changes as you suggested.

Page 13, Line 9: These C/N ratios for soils seem remarkably low. Although there may be exceptions, C/N ratios in arid and semiarid soils seem to be generally around 10-12. I’m wondering if your very low values are a consequence

of the way that soil was prepared for measurement of organic C concentrations. On page 6 line 13, you indicate that all visible organic matter was removed from the sample using forceps.

Apologies, line 13 of page six was erroneously included in section 2.3. Where necessary, forceps were used to remove any remaining loose plant matter (roots and shoots) from soils used in the acetylene reduction assays only. This was to ensure that plant-*Rhizobium* symbioses did not interfere with the asymbiotic nitrogen fixation aspect of our study. Consequently, we will move this sentence from section 2.3 to section 2.8.

We agree that the soil C:N ratios presented in our study appear low; however, similarly low ratios have been observed in other dryland studies (e.g., Thomas et al. 2018 Land Degradation & Development 29: 1306-1316). As the total nitrogen content of soils in this study are comparable with those from other areas within the SRER (Wheeler et al. 2007), we believe the discrepancies must be due to differences in concentrations of soil organic carbon. To acknowledge this issue, we will amend the sentence on lines nine and 10 of page 15 to, "However, like Wheeler et al. (2007), we found that soil C:N ratios, which are lower than those previously reported for the area (Wheeler et al. 2007), are not affected by shrub encroachment processes in the SRER (Figure 7a).".

Page 15, Line 4: Another good paper to include with your citations on this line would be Schlesinger et al. 1990 (Science 247: 1043-1048).

Thank you, we will include this citation as suggested.

Page 16, Lines 6-19. As another very relevant point of comparison for your work, see Creamer et al. 2016 (J. Geophys. Res. Biogeosci. 121: 1675–1688, doi:10.1002/2016JG003347).

Thank you for making us aware of this study. In order to recognise its findings, we will add the following sentence to the end of the paragraph concluding on page 16, line 19: "As fungi have been shown to preferentially utilise grassland C (Creamer et al., 2016), declines in the shrub soil fungal PLFA content with increasing encroachment may be a response to the progressive depletion of grass-derived SOC."

Page 17, Line 17: Perhaps specify if this value for N deposition is total N, wet deposition only, or dry deposition only. Also, it might be good to check to see if your value derived from a 2006 paper is still valid at the present time.

We will specify that the value reported is for total nitrogen deposition. Further, we will change the citation to the more recent Schwede and Lear 2014 (Atmospheric Environment 92: 207-220) publication, which is used to inform total nitrogen deposition estimates produced by the National Atmospheric Deposition Program.

Page 17, Lines 28-29. The phrase "preferentially selects the development of" makes this phenomenon seem conscious and deliberate. To avoid this, replace that phrase with the word "modifies".

Thank you, we will do as suggested.

Soil nitrogen response to shrub encroachment in a degrading semiarid grassland

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Abstract. Transitions from grass- to shrub-dominated states in drylands by woody plant encroachment represent significant forms of land cover change with the potential to alter the spatial distribution and cycling of soil resources. Yet an understanding of how this phenomenon impacts the soil nitrogen pool, which is essential to primary production in arid and semiarid systems, is poorly resolved. In this study, we quantified how the distribution and speciation of soil nitrogen, as well as rates of free-living biological nitrogen fixation, changed along a gradient of increasing mesquite (*Prosopis velutina* Woot.) cover in a semiarid grassland of the Southwestern US. Our results show that site-level concentrations of total nitrogen remain unchanged with increasing shrub cover as losses from intershrub areas (sum of grass and bare-soil cover) are proportional to increases in soils under shrub canopies. However, despite the similar carbon-to-nitrogen ratio and microbial biomass of soil from intershrub and shrub areas at each site, site-level concentrations of inorganic nitrogen increase with shrub cover due to the accumulation of ammonium and nitrate in soils beneath shrub canopies. Using the acetylene reduction assay technique, we found increasing ratios of inorganic nitrogen-to-bioavailable phosphorus inhibit rates of biological nitrogen fixation by free-living soil bacteria. Overall, these results provide a greater insight into how grass- to shrubland transitions influence the soil N pool through associated impacts on the soil microbial biomass.~~Consequently, we conclude that shrub encroachment has the potential to significantly alter the dynamics of soil nitrogen cycling in dryland systems.~~

1 1 Introduction

Degrading dryland landscapes undergo significant ecological transformations typically associated with gradual and irreversible changes in plant community composition (D'Odorico et al., 2013). Woody plant encroachment is a widespread example of dryland degradation, whereby continuous grasslands are progressively replaced by shrubs interspersed by patches of bare soil (Ravi et al., 2010). These plant community changes result in a structurally, physically and biologically different ecosystem which significantly alter the spatial distribution and fluxes of nutrients and the biogeochemical cycling within dryland landscapes, with implications for the ongoing process of land degradation (Eldridge et al., 2011; Michaelides et al., 2012). Nitrogen (N), in particular, is considered the most significant limiting ~~factor~~ nutrient of primary production and decomposition processes in ~~dryland systems after water~~arid and semiarid ecosystems (Gebauer and Ehleringer, 2000), yet there is currently limited understanding of how ecosystem transformations associated with land degradation changes the distribution, speciation and cycling of N (Browning et al., 2008), as well as the associated soil microbial biomass (Li et al., 2017). As drylands occupy ~40% of the Earth's land surface, changes in the nutrient distributions, patterns and cycling within degrading areas have important implications for biogeochemical cycles at the global scale (Delgado-Baquerizo et al., 2013).

Occurring in Africa (Hudak et al., 2003), Asia (Zhang et al., 2016), Australia (Cookson et al., 2006), Europe (Quero et al., 2013), and the North (Knapp et al., 2008) and South Americas (Cabral et al., 2003), ~~Extensive~~ extensive research has demonstrated that shrub encroachment into grasslands alters the scale of vegetation heterogeneity within the landscape from fine to large scales and changes the microtopography from relatively subdued to high relief as soil mounds develop beneath

shrub canopies (Charley and West, 1975; Wainwright et al., 2000; Schlesinger et al., 1996). These changes in both vegetation heterogeneity and microtopography result in modifications to runoff and erosion patterns (Michaelides et al., 2009; Parsons et al., 1996) which directly affect the redistribution of, and patterns in, nutrients within the landscape in favour of shrub functional types (Cross and Schlesinger, 1999; Michaelides et al., 2012; Schlesinger et al., 1996). In addition, shrubs may further concentrate nutrients beneath their canopies through increased inputs of high-quality above- and belowground detritus (Throop and Archer, 2008). The resulting accumulation of soil organic matter (SOM) in shrub mounds represents an important pool of mineralisable N that provides shrubs with a competitive advantage over herbaceous plants during times of nutrient limitation (Turnbull et al., 2010).

In terrestrial systems, the availability of inorganic N, defined hereafter as the sum of ammonium (NH_4^+-N) and nitrate (NO_3^--N), is largely controlled by microbial decomposition processes (Vitousek et al., 2002). The dominant decomposers in soil are bacteria and fungi, where fungi typically exhibit greater carbon (C):N ratios than bacteria, as well as enhanced C use efficiency and reduced rates of nutrient turnover (Waring et al., 2013). As the amount of N mineralised during decomposition depends on the C and N stoichiometry of decomposer organisms relative to that of organic matter (Schulten and Schnitzer, 1997), differences in the relative abundance of fungal and bacterial biomass can potentially modulate soil N cycling (Waring et al., 2013). Using the phospholipid fatty acid (PLFA) method, Li et al. (2017) demonstrated that the ratio of viable fungi to bacteria for a semiarid grassland was significantly higher in soil under shrubs than for soils in intershrub areas, and that soil microbial biomass was positively correlated with concentrations of total N and available phosphorus (P). However, an understanding of how the fungal and bacterial constituents of the microbial biomass change along a gradient of increasing shrub cover within a semiarid grassland is currently lacking.

Whilst mineralisation may control the availability of N, biological N fixation (BNF) by free-living (asymbiotic) soil bacteria and legume-*Rhizobium* symbioses are the principal inputs of reactive N to semiarid systems (Evans and Ehleringer, 1993), where rates of symbiotic BNF are approximately an order of magnitude higher than inputs through asymbiotic pathways (Cleveland et al., 1999). Many encroaching shrub species, such as mesquite (*Prosopis* spp.), are legumes capable of symbiotic BNF, which reduces their dependence on soil N mineralisation, and offers the potential to increase the availability of N within a system (Blaser et al., 2014). However, since BNF has a high P demand, rates of symbiotic dinitrogen (N_2) fixation by mesquite have been shown to downregulate as N increases relative to concentrations of available P (Geesing et al., 2000). Less-studied are the effects of ecosystem transformations on inputs of asymbiotically-fixed N to semiarid grasslands, which despite being lower than contributions by symbiotic BNF, are still significant due to the small size of the soil N pool in these systems.

The aim of this study was to assess how a transition from a grass- to shrub-dominated state within a dryland ecosystem impacts the soil N status and inputs of N by asymbiotic BNF. Specifically, our objectives were:

1. To examine how pools of total N, NH_4^+-N and NO_3^--N, as well as the microbial biomass, in soil varied across a shrub encroachment gradient; and

2. To determine how the distribution and supply rates of asymbiotically-fixed N varied across a shrub encroachment gradient.

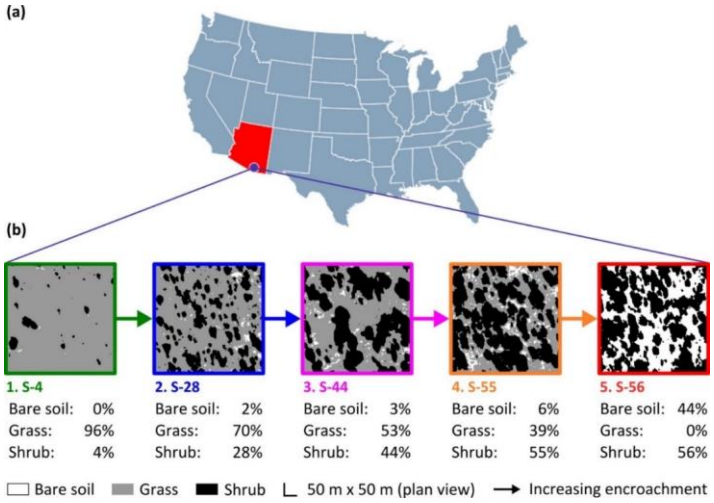
~~The study site was the Santa Rita Experimental Range (SRER), Arizona in the Southwestern US, a region where the density of mesquite has increased by >400% over the past ca. 30 years alone (Resco de Dios et al., 2012). Established in 1903, the SRER is the world's longest operating experimental range intended for advancing knowledge concerning land management (McClaran et al., 2002).~~

We expected that reductions in grass biomass resulting from higher levels of shrub encroachment would cause soil N to be progressively redistributed from intershrub zones to areas beneath shrub canopies. As the spatial distribution of soil microorganisms is positively influenced by resource availability (Schlesinger and Pilmanis, 1998), we hypothesised the enhanced fertility and environmental conditions in soils under shrubs would support a higher soil microbial biomass and promote N mineralisation processes leading to an increased availability of inorganic N. Accordingly, we predicted that rates of asymbiotic BNF would decrease in shrub soils as inorganic N increased relative to bioavailable P (Smith, 1992).

2 Materials and methods

2.1 Study site description

Fieldwork was conducted between March and April 2016 at the ~~SRER~~ Santa Rita Experimental Range (SRER; Figure 1a; 31˚54'N, 110˚53'W), Arizona in the Southwestern US, a region where the density of mesquite has increased by >400% over the past ca. 30 years alone (Resco de Dios et al., 2012).~~ ~40 km south of Tucson, AZ, US, between March and April 2016.~~ Situated on a bajada comprising ~21,000 ha, the SRER ranges in elevation from 900 m asl to 1,400 m asl (McClaran, 2003). Mean daily maximum and minimum air temperatures for the years 2008 to 2015 were 25 ˚C and 13 ˚C, respectively. Mean annual precipitation for the same period was 392 mm, with 61% received during the summer months (July through September). The respective mean daily maximum and minimum summer temperatures between 2008 and 2015 were 31 ˚C and 20 ˚C (Ameriflux, http://ameriflux.lbl.gov/data/download-data/). Vegetation is dominated by native desert grassland species, such as Rothrock grama (*Bouteloua rothrockii* Vasey), sideoats grama (*B. curtipendula* (Michx.) Torr.), Arizona cottontop (*Digitaria californica* (Benth.) Henrard), bush muhly (*Muhlenbergia porteri* Scribn.) and tanglehead (*Heteropogon contortus* (L.) Beauv.), as well as the non-native Lehmann lovegrass (*Eragrostis lehmanniana* Nees.). Cacti, such as cholla cactus species (*Opuntia spp*. Mill.), and shrubs, such as velvet mesquite (*Prosopis velutina* Woot.) and catclaw acacia (*Acacia greggii* A. Gray), were also present (McClaran et al., 2002).


(a)
(b)
1. S-4
Bare soil: 0%
Grass: 96%
Shrub: 4%
2. S-28
Bare soil: 2%
Grass: 70%
Shrub: 28%
3. S-44
Bare soil: 3%
Grass: 53%
Shrub: 44%
4. S-55
Bare soil: 6%
Grass: 39%
Shrub: 55%
5. S-56
Bare soil: 44%
Grass: 0%
Shrub: 56%
Bare soil
Grass
Shrub
50 m x 50 m (plan view)
Increasing encroachment

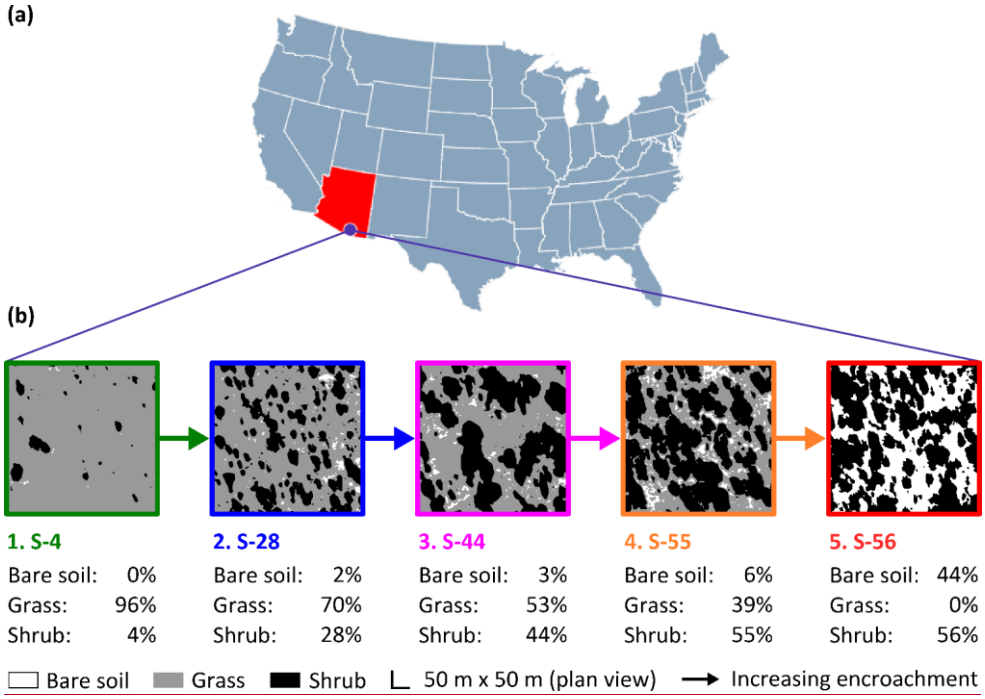


Figure 1. The study site (a) was a semiarid grassland in the Santa Rita Experimental Range, AZ, US (31˚54'N, 110˚53'W; elevation: ~1,250 m asl). Five sampling sites comprised a gradient of shrub encroachment, where grass cover decreased, and bare soil and shrub cover increased between sites 1 and 5 (b). The intershrub area of each site is equal to the sum of the percentage bare soil and grass cover.

Encroachment by mesquite within the SRER has been well documented (McClaran, 2003). Velvet mesquite, which was restricted to drainage arroyos in 1903, currently occupies ~35% of the total grassland area (Figure 2) and represents the dominant shrub species within the elevation band of 990-1,200 m asl (Browning et al., 2008). The following five sampling sites comprise a gradient of increasing shrub cover representing progressive ecosystem degradation: S-4, S-28, S-44, S-55 and S-56 (Figure 1b) where the number signifies the percent shrub cover. The percentage shrub canopy and intershrub area was calculated for the five sites using high-resolution satellite imagery from Google Earth (2016e-e), which were cropped to the same dimensions and segmented using the Trainable Weka Segmentation plugin for Fiji (http://fiji.sc/Fiji). Sobel and Gaussian blur filters were used for edge detection and noise suppression, respectively (Arganda-Carreras et al., 2017). The out-of-bag error for all sites classified was <2%. Soil at these sites was formed in alluvium from igneous rock of Holocene

and Late Pleistocene origins (Batchily et al., 2003) in the Combate-Diaspar complex (CoB), characterised by excellent drainage, sandy loam textures and 1-8% slopes (SRER Digital Database, https://cals.arizona.edu/srer/data.html). All five sites were situated at ~1,250 m asl, exhibited similar topography and had the same land use history.

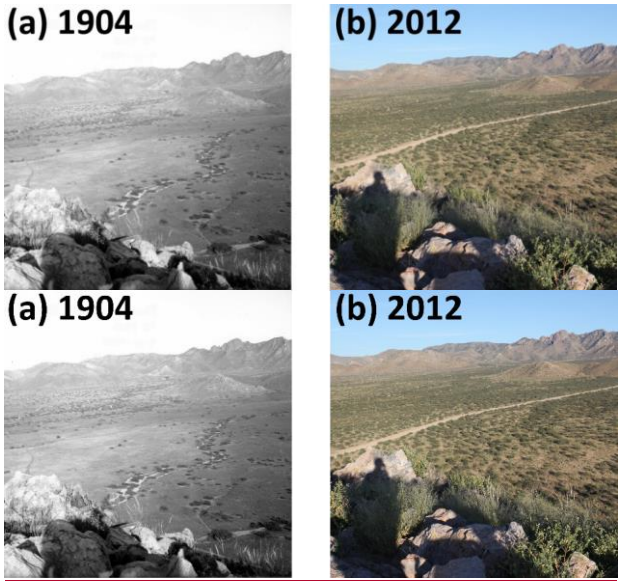
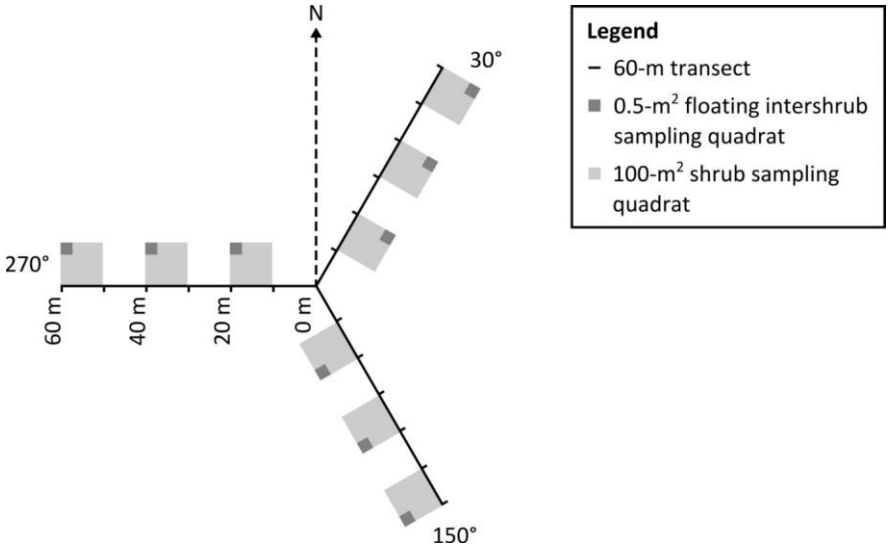


Figure 2. Scattered shrubs replace grasses as dominant vegetation between 1904 (a) and 2012 (b) in the Santa Rita Experimental Range, AZ, US. Facing north northeast in pastures 6B and 6D. Elevation: 1,228 m asl (public domain images available from: https://cals.arizona.edu/srer/photos.html).

2.2 Field measurements and sample collection

To mitigate the effects of directional bias during sampling, a 180-m transect (horizontal distance) was arranged in a Y-configuration at each site, where three 60-m transects radiated out from plot centres at azimuths of 30°, 150° and 270° (Figure 3). Three 100-m^2 quadrats were then installed at 10-m, 30-m and 50-m intervals along each transect for the determination of aboveground shrub biomass. Further, a 0.5-m^2 quadrat was centred within the largest intershrub area of each 100-m^2 quadrat for the determination of aboveground grass biomass. The dry weight of each velvet mesquite plant was estimated from stem basal diameter measurements using methods, and a site- and species-specific allometric equation reported in McClaran et al. (2013). Similarly, the dry weight of individual bunchgrasses were estimated from tussock basal diameter measurements using methods, and a site-specific multispecies allometric equation reported in Nafus et al. (2009). The sums of the individual shrub and herbaceous constituents were calculated and used to estimate the total mesquite and

grass biomass by dry weight for each site, where grass biomass was weighted according to intershrub area (kg m^{-2}). Finally, three intershrub and shrub surface soil (top 10 cm) cores were extracted at equal distances between the stem and drip line on the north side of the mesquite plant with the largest stem basal diameter, and along the centre of the 0.5-m^2 quadrat, respectively. The extracted cores were then homogenised to create single intershrub and shrub samples for each quadrat per site (n = 9) and sealed in sterile bags (Whirl-Pak®, Lactun, Australia).


N
30°
270°
150°
60 m
40 m
20 m
0 m
Legend
60-m transect
0.5-m² floating intershrub sampling quadrat
100-m² shrub sampling quadrat

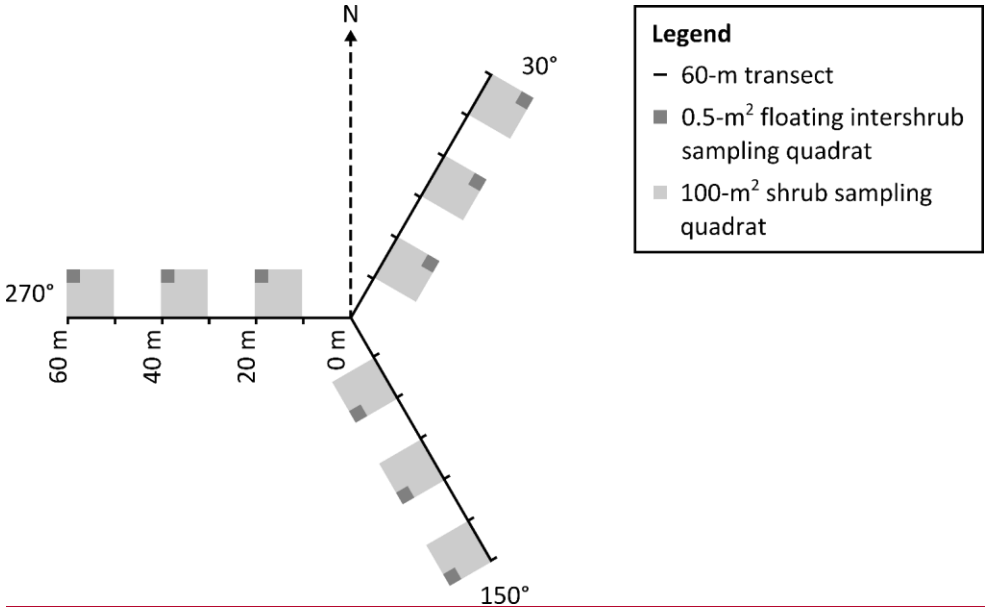


Figure 3. Plan view of sampling design where grass and shrub biomass, as well as intershrub and shrub soil samples, were obtained from 0.5-m^2 and 100-m^2 quadrats, respectively (not to scale). The 0.5-m^2 quadrats were not fixed but were instead centred within the largest intershrub area of each 100-m^2 quadrat.

2.3 Soil preparation

The mechanical sieving method described by Loveland and Whalley (2000) was used to evaluate the coarse fraction (>2 mm, wt%) of each sample. The resulting fine earth fraction (≤2 mm) from ~~For~~ each sample was retained for further analysis.~~, the particle-size distribution of coarse fragments (>2 mm, wt%) was evaluated using the mechanical sieving method described by Loveland and Whalley (2000). Once complete, the coarse material was discarded, and all visible plant and animal matter was removed from the remaining fine earth fraction (≤2 mm) using forceps.~~ For pH, PLFA and elemental analyses, the number of intershrub and shrub samples per site was reduced to three (n = 3) by homogenising the samples collected along each of the three 60-m transects. Samples were then stored in a dark temperature-controlled environment at 4 ˚C. Prior to storage, aliquots of 5 g from each of the n = 9 and n = 3 samples were frozen at -20 ˚C and freeze-dried for elemental and PLFA analyses, respectively.

2.4 Soil characteristics

Soil pH was measured in a 1:5 soil:0.01 M $CaCl_2$ suspension after mixing at 20 rpm for 30 minutes using a Stuart SB3 rotator (Bennett et al., 2003). Particle-size determination for the fine earth fraction (%) was measured using a Malvern Mastersizer 3000 laser particle size analyser with a Hydro EV pump accessory (Malvern Instruments Ltd., Worcestershire, UK), where isopropanol was used as the dispersant (Goossens et al., 2014). Water holding capacity (WHC; %) was estimated using the percentage sand and clay content of each sample (Saxton and Rawls, 2006), which had been defined according to the USDA-SCS (1982) classification scheme. Soil dry matter content (DMC; %) was determined for all samples using the gravimetric method described by Rowell (1994). Lastly, as it was not possible to extract intact soil cores, an average bulk density value of 1.33 g cm^{-3} was assumed for all samples using previously published data for the SRER (Wheeler et al., 2007).

2.5 Elemental analysis

For each sample, an aliquot of ~100 mg of freeze-dried soil was ~~flash-frozen~~embrittled using liquid N_2, and ground to a flour using a pestle and mortar. Concentrations of SOC and total N were then determined on an elemental analyser (CHNS-O EA 1108 Elemental Analyzer; Carlo Erba, Milan, IT) in accordance with methods outlined by Hedges and Stern (1984), where the detection limits were <0.5 µg g^{-1} for both elements measured. The coefficient of variation (six replicate standards) for C and N was ±1.69% and ±1.36%, respectively.

2.6 Soil nutrient analyses

Concentrations of exchangeable ~~ammonium-N (~~NH_4^+-N~~)~~ and ~~nitrate-N (~~NO_3^--N~~)~~ in soil were extracted with 2M KCl (1:10 w/v soil:extractant) and determined colourimetrically using a flow injection analyser (Lachat QuikChem 8500 Series 2 FIA system, Loveland, CO, US), where the coefficient of variation (six replicate standards) for NH_4^+-N and NO_3^--N was ±0.75% and ±5.19%, respectively according to mid-range standards (calibration range: 0-0.5 mg L^{-1}). Thus, for each sample, 1 g of fresh soil was added to 10 mL of 2M KCl, shaken for 30 minutes at 160 rpm, centrifuged for 5 minutes at 4,500 rpm and filtered to 0.45 µm using Whatman WCN plain cellulose nitrate filtrate papers. Extracts were then analysed for NH_4^+-N (QuikChem® Method 31-107-06-1-I) using the Berthelot reaction (Willis et al., 1993) and NO_3^--N (QuikChem® Method 31-107-04-1-K) using the cadmium reduction method (Willis and Gentry, 1987). The respective detection limits for NH_4^+-N and NO_3^--N were 0.04 µg g^{-1} and 0.08 µg g^{-1} for dry sediment. Bioavailable P (sum of loosely-sorbed, and iron- and aluminium-bound P) was sequentially extracted from 0.2 g of soil using a method adapted from Hedley et al. (1982) and Mumford (2003) as described in Michaelides et al. (2012). The concentrations of the resulting extracts were determined photometrically on a Gallery™ Plus Automated Photometric Analyzer (ThermoFisher Scientific, San Jose, CA). The limit of detection for both loosely-sorbed, and iron- and aluminium-bound P was 1.1 µg g^{-1}. The coefficient of variation (six replicate

standards) for the same fractions were ±0.35% and ±0.41%, respectively. All samples were blank corrected where blank concentrations exceeded the detection limits.

2.7 Microbial biomass estimation

PLFAs were extracted from 2 g aliquots of each sample using a modified Bligh and Dyer (1959) method as described by Frostegård et al. (1991). Subsequent fractionation and derivatisation was performed according to methods by Dickson et al. (2009) and Christie (1993), respectively. The methyl ester of nonadecane was added at a concentration of 0.1 mg L^{-1} as an internal standard to quantify the fatty acids. Individual compounds were quantified using a gas chromatograph equipped with an Agilent VF-23ms column (60 m x 0.32 mm x 0.15 µm; He carrier gas, constant 2 mL min^{-1} flow rate), which was operated for 1 min at 50 °C followed by a 10 °C min^{-1} ramp to 100 °C and a 4 °C min^{-1} ramp to a final temperature of 250 °C that was maintained for 15 mins. Compound structures were identified based on comparisons with the retention times and mass spectra for authentic laboratory standards. The mass spectra of individual components were obtained using a ThermoScientific 1300 Series gas chromatograph (column, carrier gas and operation as above) coupled, using a heated transfer line (260 °C), to a ThermoScientific ISQ LT quadrupole mass spectrometer scanning in the range of *m/z* 50-650 with a dwell time of 0.5 s (current was maintained at 50 µA with an ion source temperature of 240 °C and an electron voltage of 70 eV). Fatty acids which were <14 C and >20 C, or which accounted for <0.5% of the total peak area, were excluded from the analysis. Bacterial and fungal biomass were defined according to Bååth and Anderson (2003).

2.8 Acetylene reduction assay

Due to time and cost constraints, rates of asymbiotic BNF (nitrogenase activity) for intershrub and shrub soils ~~-~~were assessed for three sites (S-4, S-44 and S-56) using the acetylene (C_2H_2) reduction assay technique adapted from Telling et al. (2011). Prior to analysis, any remaining loose plant matter (roots and shoots) was removed from samples using forceps. A sample, control and kill control (autoclaved at 126 ˚C for 30 minutes) were created from each homogenised sample by adding aliquots of fresh soil (equivalent to 7.5 g dry mass) to 30 mL serum bottles. Samples were then adjusted to 60% WHC, sealed using grey butyl stoppers and crimped shut. In addition, a blank was created by adding 10 mL of autoclaved Milli-Q water to an empty serum bottle. Serum bottles were placed in an illuminated (0.2 ~~W m^{-2}~~ ±0.005 W m^{-2}) incubator set to 26 ˚C ±0.1 ˚C (mean of maximum and minimum summer temperatures). Following a 72-hour pre-incubation period, 10% of the headspace from the sample, blank and kill control were replaced with 100% ~~acetylene (C_2H_2)~~ gas, which had been produced by adding Milli-Q water to technical grade calcium carbide (Sigma, St. Louis, MO, US), via an air-tight syringe. Serum bottles were sampled at 0-, 3-, 6-, 12-, 20- and 24-hour intervals, where 5 mL of headspace was transferred to pre-evacuated 3.7 mL Exetainers (Labco, Lampeter, UK). A 1 mL aliquot of headspace from each Exetainer was then analysed using a Varian 3800 gas chromatograph (GC; Varian, Inc., Palo Alto, CA, US), where ethylene (C_2H_4) was separated from C_2H_2 using a 6' x 1/8", 80/100 mesh HayeSep T column at 85 ˚C (He carrier gas). Daily standards of 100 ppm C_2H_4 (BOC, Guildford, UK) gave precisions of <5%. The precision for 100 ppm standards that had been stored in 3.7 mL for a period of

one month also gave precisions of <5%. Rates of C_2H_4 production were converted to rates of annual N fixation (mmol N m^{-2} a^{-1}) using a theoretical ratio of 3:1.

2.9 Statistical analysis

Statistical analysis of the data was performed using R version 3.5.0. The Kruskal-Wallis *H* test (KW) was used to determine if significant differences occurred for data within the same cover type along the encroachment gradient. When significance was indicated, Dunn's test of multiple comparisons (DT) was applied. The Mann-Whitney *U* test (MW) was used to make within-site comparisons for data between cover types. Relationships between explanatory and response variables were explored using simple linear regression analysis, where residuals were inspected for evidence of non-normality using the Shapiro-Wilk test. For this study, the alpha level was set to 0.05. All errors reported in the text are one median absolute deviation about the median (Crawley, 2005).

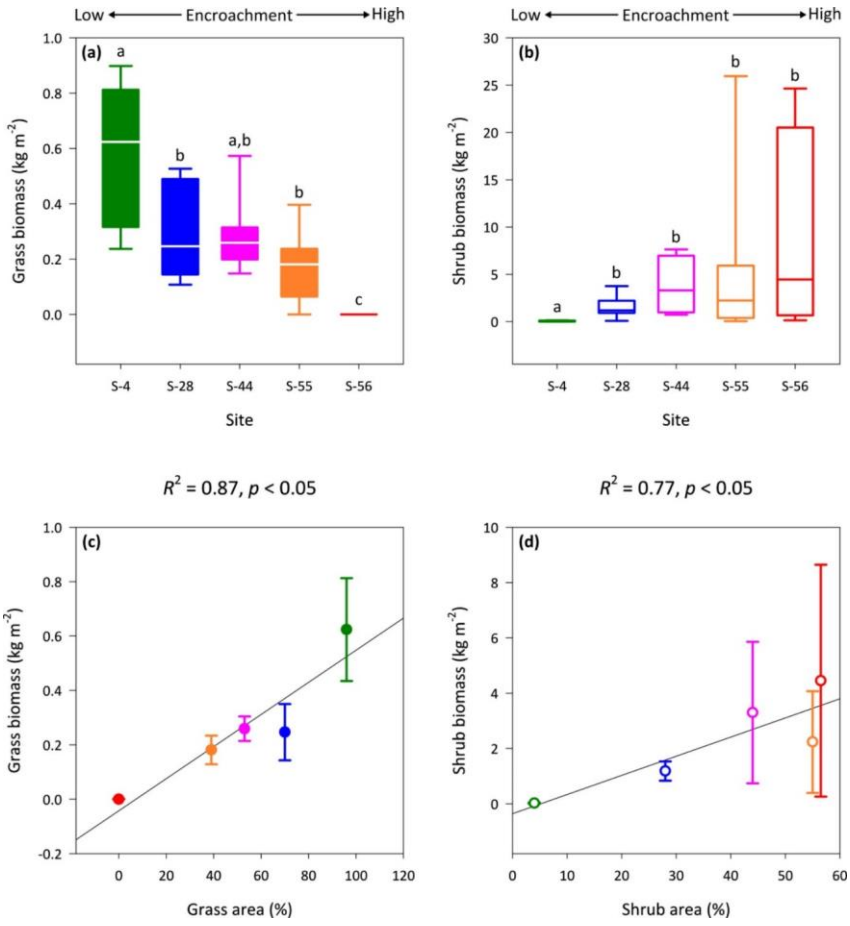
3 Results

3.1 Plant biomass

Vegetation biomass and cover were strongly influenced by the degree of shrub encroachment at each site. Overall, total vegetation (grass and shrub) cover decreased from 100% to 56% between end-member sites, as reductions in grass cover exceeded gains in shrub cover (Figure 1b). The changes in grass and shrub cover were also positively related with grass (R^2 = 0.87; Figure 4c) and shrub (R^2 = 0.77; Figure 4d) biomass, respectively. Thus, grass biomass declined from 0.62 ±0.19 kg m^{-2} at S-4 to 0 kg m^{-2} at S-56 (Figure 4a; DT, $p < 0.001$), whereas shrub biomass increased from 0.02 ±0.02 kg m^{-2} to 4.45 ±4.19 kg m^{-2} between the same sites (DT, $p < 0.001$, Figure 4b). Lastly, shrub biomass was greater than grass biomass at all sites except S-4, where grass biomass surpassed shrub biomass ($p < 0.05$).

3.2 Soil characteristics

Of the soil properties presented in Table 1, only intershrub and shrub soil pH exhibited clear trends with increasing encroachment. Specifically, intershrub soil pH decreased linearly from 6.3 ±0.4 to 4.7 ±0.4 between end-member sites, whereas shrub soil pH increased from 4.9 ±0.1 to 6.0 ±0.2 along the same gradient. Significant differences in the remaining soil properties of Table 1 did not follow clear trends and occurred only in intershrub soils. The sand content of intershrub soil at S-28 was lower in comparison with all other intershrub soils (DT, $p < 0.05$). Further, the silt and clay contents of intershrub soil at S-28 was greater, relative to other sites, excluding sites S-55 and S-56, respectively (DT, $p < 0.05$). Lastly, the coarse fraction of intershrub soil at S-55 was greater than that of other intershrub soils along the encroachment gradient (DT, $p < 0.05$).


Low
Encroachment
High
(a)
Grass biomass (kg m-2)
a
b
a,b
b
c
S-4
S-28
S-44
S-55
S-56
Site
(b)
Shrub biomass (kg m-2)
a
b
b
b
b
Site
R2 = 0.87, p < 0.05
(c)
Grass biomass (kg m-2)
Grass area (%)
R2 = 0.77, p < 0.05
(d)
Shrub biomass (kg m-2)
Shrub area (%)

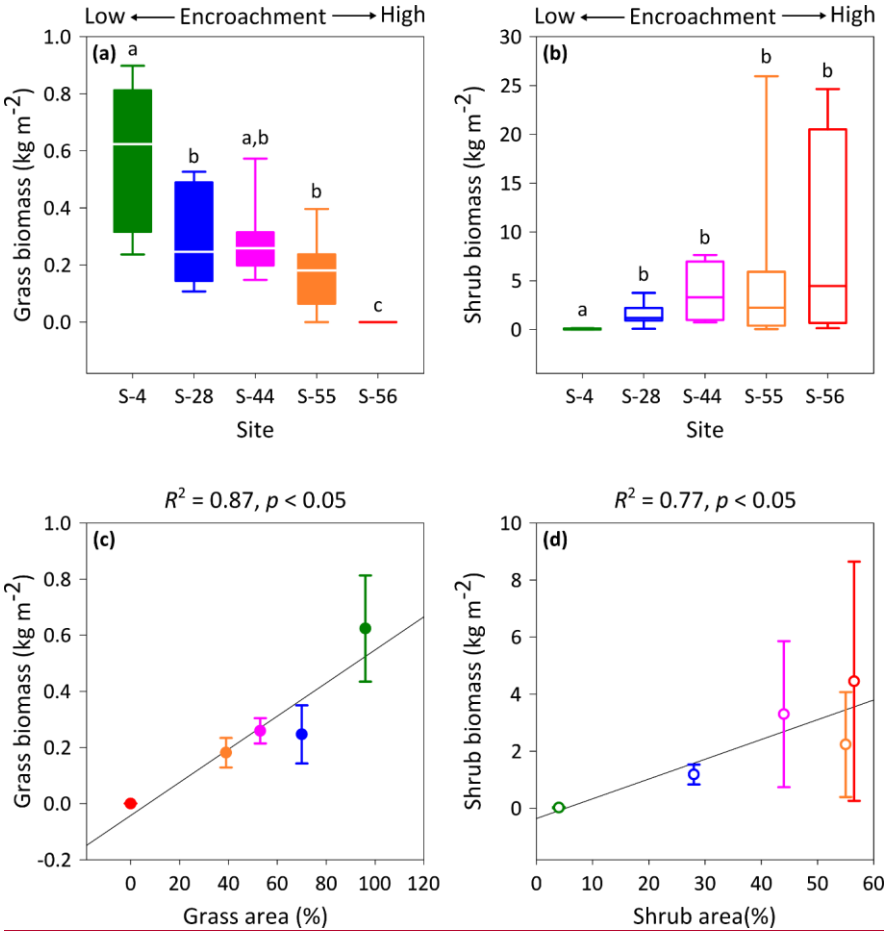


Figure 4. Aboveground multispecies grass (a) and *Prosopis velutina* Woot. (b) biomass by dry weight across a shrub encroachment gradient and according to grass (c) and shrub (d) cover in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with different letters indicate significant differences within biomass classes at $p < 0.05$ (Dunn's test). Error bars represent median absolute deviation about the median.

Soil clay content represented the only consistent physical difference between cover types, where clay content was higher in soils from intershrub areas relative to those from beneath shrub canopies (MW, $p < 0.05$ for sites S-4 and S-56; $p < 0.01$ for sites S-28, S-44, S-55; Table 1). Changes in other physical soil properties did not exhibit a relationship with increasing

encroachment. At S-28, the silt content (MW, $p < 0.001$) and DMC (MW, $p < 0.01$) of intershrub soil were greater than that of the shrub soil; however, the sand content of intershrub soil was lower in comparison with shrub soil (MW, $p < 0.001$). Intershrub soil at S-44 exhibited a higher DMC (MW, $p < 0.05$) and sand content (MW, $p < 0.05$) than shrub soil for the same site; though the silt content was lower than that of the shrub soil (MW, $p < 0.05$). Finally, the percentage of coarse fragments differed between intershrub and shrub soils at S-4 (MW, $p < 0.001$).

Table 1. Median textural characteristics, dry matter content (DMC) and pH (1:5 soil:0.01 M $CaCl_2$) for the surface soil layer (top 10 cm) beneath intershrub (IS) and shrub (S) canopy cover types along a gradient of shrub encroachment in a semiarid grassland of the Santa Rita Experimental Range, AZ, US. Values in parentheses represent one median absolute deviation about the median.

	Site									
	S-4		**S-28**		**S-44**		**S-55**		**S-56**	
	IS	**S**	**IS**	**S**	**IS**	**S**	**IS**	**S**	**IS**	**S**
%coarse[a,b,g]	11.4	11.0	18.0	17.6	18.0	18.4	24.7	24.3	16.5	19.3
	(2.7)	(7.1)	(4.8)	(2.9)	(4.0)	(4.8)	(2.3)	(4.6)	(5.0)	(7.6)
%clay[c,d,g]	1.6	0.8	2.2	0.2	1.5	0.1	1.5	0.2	1.7	0.3
	(0.3)	(0.6)	(0.3)	(0.1)	(0.2)	(0.0)	(0.2)	(0.1)	(0.3)	(0.3)
%silt[c,e,g]	41.9	49.5	53.5	36.2	47.9	53.5	35.7	40.3	39.2	35.4
	(3.6)	(15.4)	(3.5)	(3.3)	(8.9)	(3.5)	(5.1)	(5.6)	(6.0)	(6.1)
%sand[c,f,g]	56.9	49.7	44.4	63.7	44.9	44.4	64.2	59.5	57.6	64.3
	(3.1)	(16.0)	(4.2)	(3.3)	(9.5)	(4.2)	(5.0)	(5.8)	(7.4)	(6.3)
%DMC[c,g]	99.1	99.0	99.1	98.9	99.3	98.8	98.9	98.5	98.7	98.8
	(0.1)	(0.1)	(0.0)	(0.2)	(0.2)	(0.2)	(0.1)	(0.1)	(0.2)	(0.3)
pH[c,h]	6.3	4.9	5.3	5.7	5.2	5.5	5.1	6.0	4.7	6.0
	(0.4)	(0.1)	(0.1)	(0.5)	(0.1)	(0.3)	(0.1)	(0.0)	(0.5)	(0.2)

[a]total soil (>0 µm)
[b]>2,000 µm
[c]fine earth fraction only (≤2,000 µm)
[d]<2 µm
[e]2-50 µm
[f]>50-2,000 µm
[g]n = 9
[h]n = 3

3.3 Distribution and speciation of nitrogen

The total N in shrub soils was more than two times greater than that in intershrub soils (MW, $p < 0.05$) at each site along the degradation gradient (Figure 5a). Further, concentrations of total N in intershrub soils declined proportionally with reductions in grass biomass from 710 ±59 µg g^{-1} at S-4 to 344 ±43 µg g^{-1} at S-56. Specifically, the total N content in

intershrub soil at S-56 was lower in comparison with all other intershrub soils (DT, $p < 0.05$), and concentrations of total N within intershrub soils from sites S-44 and S-55 were lower than the concentrations observed at S-4 (DT, $p < 0.05$). In contrast, a nonlinear trend was observed in the shrub soil total N pool, whereby total N steadily increased from 920 ±198 μg g^{-1} in shrub soil at S-4 to 1,527 ±171 μg g^{-1} at S-55, before declining to ~~at~~ 787 ±398 μg g^{-1} at S-56; however, these differences were not significant (KW, $p > 0.05$).

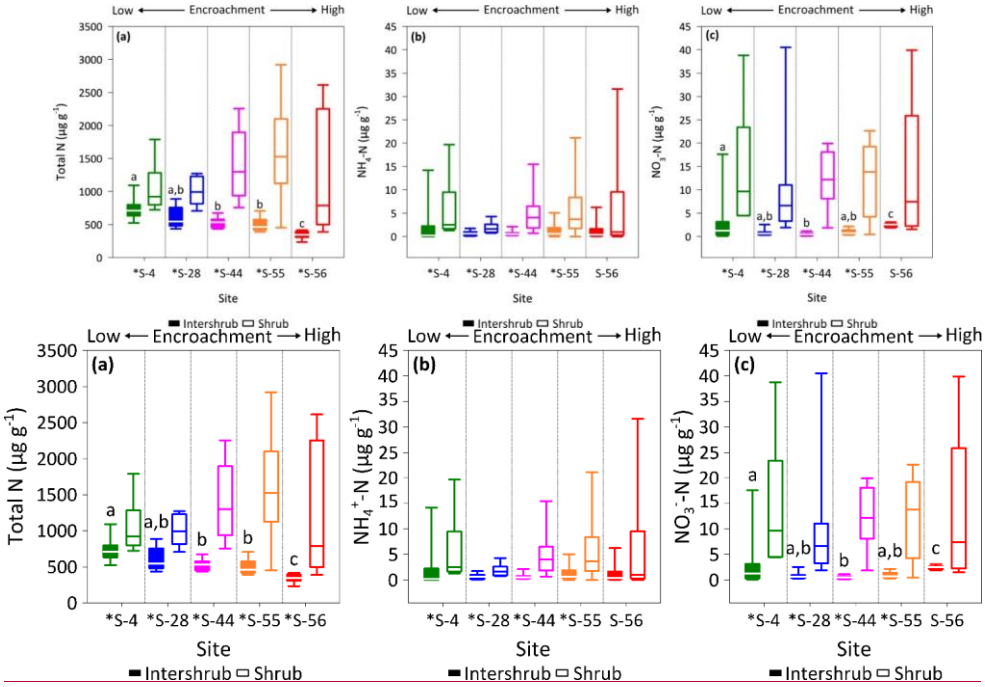


Figure 5. Total N (a), NH_4^+-N (b) and NO_3^--N (c) concentrations for surface soil (top 10 cm) by dry weight under intershrub and shrub cover types along a shrub encroachment gradient in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with different letters indicate significant differences by cover type at $p < 0.05$ (Dunn's test). Intershrub and shrub soils at sites denoted with an asterisk differ significantly from each other at $p < 0.05$ (Mann-Whitney *U* test).

Concentrations of NH_4^+-N did not change with increasing encroachment in either the intershrub (KW, $p > 0.05$) or shrub (KW, $p > 0.05$) soils, where the average of median concentrations in intershrub and shrub soils were 1 μg g^{-1} and 3 μg g^{-1}, respectively (Figure 5b). However, NH_4^+-N concentrations were approximately an order of magnitude higher under shrub canopies compared to intershrub areas for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$). Further, shrub soil NH_4^+-N concentrations exhibited greater variability than that of the intershrub soils.

Like NH_4^+-N, soil NO_3^--N concentrations were greater in soils beneath shrub canopies than in intershrub areas for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$; Figure 5c) and the variability of NO_3^--N in shrub soils was greater than that of their intershrub counterparts. However, unlike NH_4^+-N, intershrub NO_3^--N concentrations varied along the encroachment gradient (KW, $p < 0.001$), where NO_3^--N decreased from 1.2 ±0.8 µg g^{-1} at S-4 to 0.5 ±0.2 µg g^{-1} at S-44, and subsequently rose to 2.5 ±0.2 µg g^{-1} S-56 (Figure 5c). No such trend was identified in the NO_3^--N concentrations of shrub soils, which instead remained constant along the encroachment gradient (KW, $p > 0.05$).

To identify site-level changes in the surface N pools, total area-weighted concentrations of total N, NH_4^+-N and NO_3^--N were calculated for each site according to percentage intershrub and shrub cover (Figure 1b). Overall, site-level total N concentrations (Figure 6a) did not change with increasing shrub encroachment (KW, $p > 0.05$). However, the proportion of total N contained within the inorganic fraction increased in relation to the degree of shrub encroachment. Such increases were largely seen in the form of NO_3^--N (Figure 6c), which increased from 0.2 ±0.1 g m^{-2} to 0.6 ±0.3 g m^{-2} between sites S-4 and S-56 (DT, $p < 0.05$). Concentrations of NH_4^+-N experienced an increase from 0.03 ±0.03 g m^{-2} at S-4 to 0.2 ±0.1 g m^{-2} at S-55 (Figure 6b), but ultimately did not differ between the end-member sites (DT, $p > 0.05$).

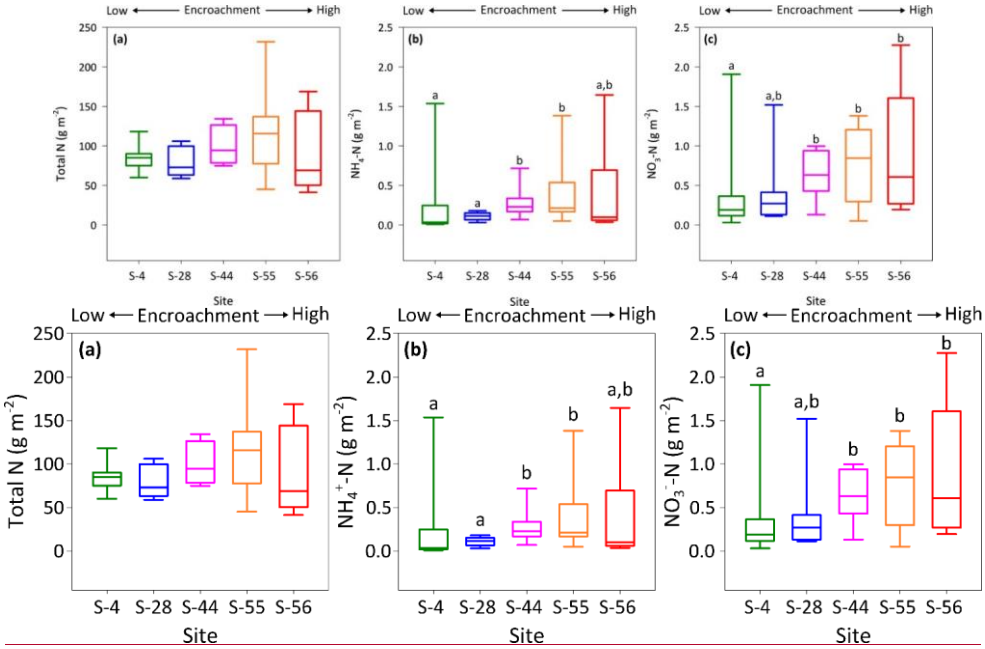


Figure 6. Site-level total N (a), NH_4^+-N (b) and NO_3^--N (c) concentrations (dry matter) weighted by relative area of intershrub to shrub cover for surface soil (top 10 cm) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with alternative letters differ significantly from one another at $p < 0.05$ (Dunn's test).

3.4 Nutrient ratios

Ratios of SOC:total N (C:N) did not differ across the encroachment gradient for either cover type (KW, $p > 0.05$). The average C:N ratios for intershrub and shrub soils were 7.0 and 7.7, respectively (Figure 7a) and these did not differ between cover types at any of the five sites (MW, $p > 0.05$). Similarly, inorganic N:bioavailable P (N:P) ratios (Figure 7b) for intershrub ($p > 0.05$) and shrub (KW, $p > 0.05$) soils did not differ significantly between sites, where the respective mean ratios for intershrub and shrub soils were 0.1 and 1.2. However, shrub soil N:P ratios exhibited a high degree of variability and were greater than those of intershrub soils for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$).

3.5 Microbial biomass

No trends were observed in bacterial PLFA biomass across the shrub encroachment gradient (Figure 8a). The greatest (3.6 ± 0.1 nmol g^{-1}) bacterial PLFA concentrations were found in the intershrub soils at S-44 and the lowest (0.6 ± 0.1 nmol g^{-1}) in intershrub soils at S-56. The fungal PLFA biomass of intershrub soils was less variable in comparison with the bacterial PLFA biomass and decreased from a maximum concentration of 0.2 ± 0.1 nmol g^{-1} at S-4 across the

encroachment gradient, where concentrations were below the limits of detection at both S-28 and S-56 (Figure 8b). Similarly, the fungal PLFA content of shrub soils, which were typically greater, but more variable than that of intershrub soils, decreased steadily from 0.3 ±0.1 nmol g^{-1} to 0.05 ±0.05 nmol g^{-1} between end-member sites, where fungal PLFA concentration were below the limits of detection at S-55. Consequently, the fungal-to-bacterial ratio decreased under both shrub and intershrub soils with increasing shrub encroachment.

3.6 Acetylene reduction assay

Rates of C_2H_2 ~~acetylene~~ reduction were only above the limits of detection in intershrub soils at sites S-4 and S-44, where rates of ~~ethylene~~ C_2H_4 production were linear over the 24-hour incubation period (R^2 = 0.97 for S-4, and R^2 = 0.84 for S-44, slopes of regression lines were significantly different from zero for both S-4 ($p < 0.001$) and S-44 ($p < 0.01$); data not shown here). When converted to annual rates of N fixation per unit area (Figure 9a), inputs of N by asymbiotic BNF were similar between sites S-4 (2.4 g N m^{-2} a^{-1}) and S-44 (2.5 g N m^{-2} a^{-1}). However, when adjusted for percentage cover (Figure 9b), rates of asymbiotic BNF declined in relation to reductions in intershrub area from 2.3 g N m^{-2} a^{-1} at S-4 to at 1.4 g N m^{-2} a^{-1} S-44.

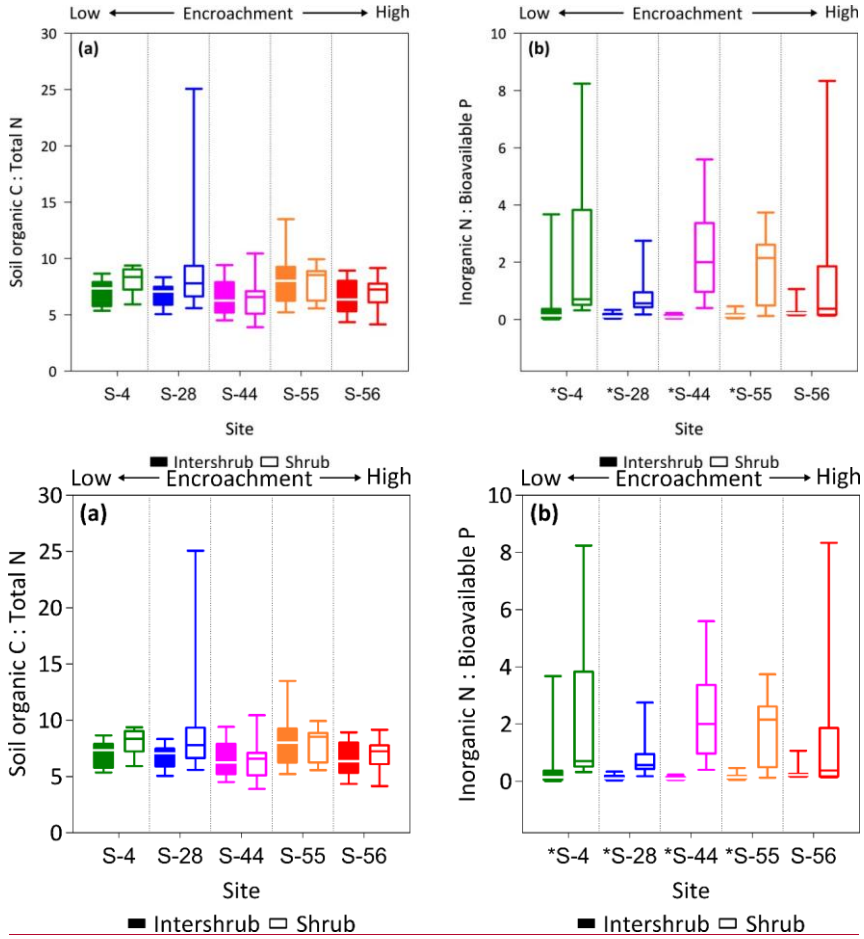


Figure 7. SOC : total N (a) and inorganic N : bioavailable P (b) ratios for surface soil (top 10 cm) under intershrub and shrub cover types along a shrub encroachment gradient in the Santa Rita Experimental Range, AZ, US (n = 9). Intershrub and shrub soils at sites denoted with an asterisk differ significantly from each other at $p < 0.05$ (Mann-Whitney *U* test).

4 Discussion

Our study revealed the distribution and speciation of soil N, as well as the extent of nitrogenase activity, is strongly influenced by the degree of shrub encroachment in a semiarid grassland of the SRER. Yet overall, the soil N pool incurred no net change with the transition from a grass- to shrub-dominated state. Below we discuss specifically how the soil N pool

responds to increasing shrub cover, and how changes in the soil microbial biomass and nutrient ratios associated with shrub encroachment influence N inputs by asymbiotic BNF in a semiarid grassland.

4.1 Distribution and speciation of soil nitrogen

Like other studies for the Southwestern US (e.g., Wheeler et al., 2007; Turnbull et al., 2010; Schlesinger et al., 1996; Michaelides et al., 2012), we determined that concentrations of total N are greater in surface soils under shrubs relative to intershrub areas for all sites along the encroachment gradient (Figure 5a-c). Further, whilst concentrations of total N in shrub soils are not statistically separable, we found the total N content of intershrub soils declined between end-member sites coincident with reductions in herbaceous biomass (Figure 4a) and cover (Figure 1b). At site-level, concentrations of total N remained constant with increasing shrub cover (Figure 6a), and the accumulation of N beneath shrub canopies can be considered a direct consequence of N losses from intershrub soils. These findings contrast with studies which identified net increases (e.g., Boutton and Liao, 2010) and declines (e.g., Jackson et al., 2002) in the total N pool with shrub encroachment, but support the widely held perspective that transitions from continuous grass- to shrub-dominated states increase the spatial heterogeneity of soil resources (D'Odorico et al., 2013; Peters et al., 2006; Schlesinger et al., 1990).

Organic N (total N minus inorganic N) comprises >98% of total N in all intershrub and shrub soils (Figure 5a-c). Thus, the elevated concentrations of total N in shrub soils, which are ≥1.5 times those of intershrub soils, represent larger stores of mineralisable N. Rates of N mineralisation are inversely related to soil C:N ratios (Finzi et al., 1998), which in turn have been shown to decrease under mesquite canopies in response to inputs of higher quality litter (Geesing et al., 2000; Throop and Archer, 2007). However, like Wheeler et al. (2007), we found that soil C:N ratios, which are lower than those previously reported for the area (Wheeler et al., 2007), are not affected by shrub encroachment processes in the SRER (Figure 7a). Whilst this could be due in part to depressed subcanopy soil-litter mixing (Throop and Archer, 2008), the accumulation (and flux) of N has been shown to share a positive relationship with the soil clay content (Virginia and Jarrell, 1983; Michaelides et al., 2012), which was lower in soils beneath shrub canopies relative to those from intershrub areas (Table 1). Nevertheless, shrub soils contain greater concentrations of NH_4^+-N and NO_3^--N than intershrub soils at all sites except S-56 (Figure 5b,c), which suggests the microbial community beneath shrub canopies can mineralise organic forms of N more readily. This is supported by a study for a semiarid grassland in central Spain ~~from Delgado-Baquerizo et al. (2015)~~ which showed the availability of inorganic N is largely influenced by the abundance and functional diversity of the microbial community (Delgado-Baquerizo et al., 2015).

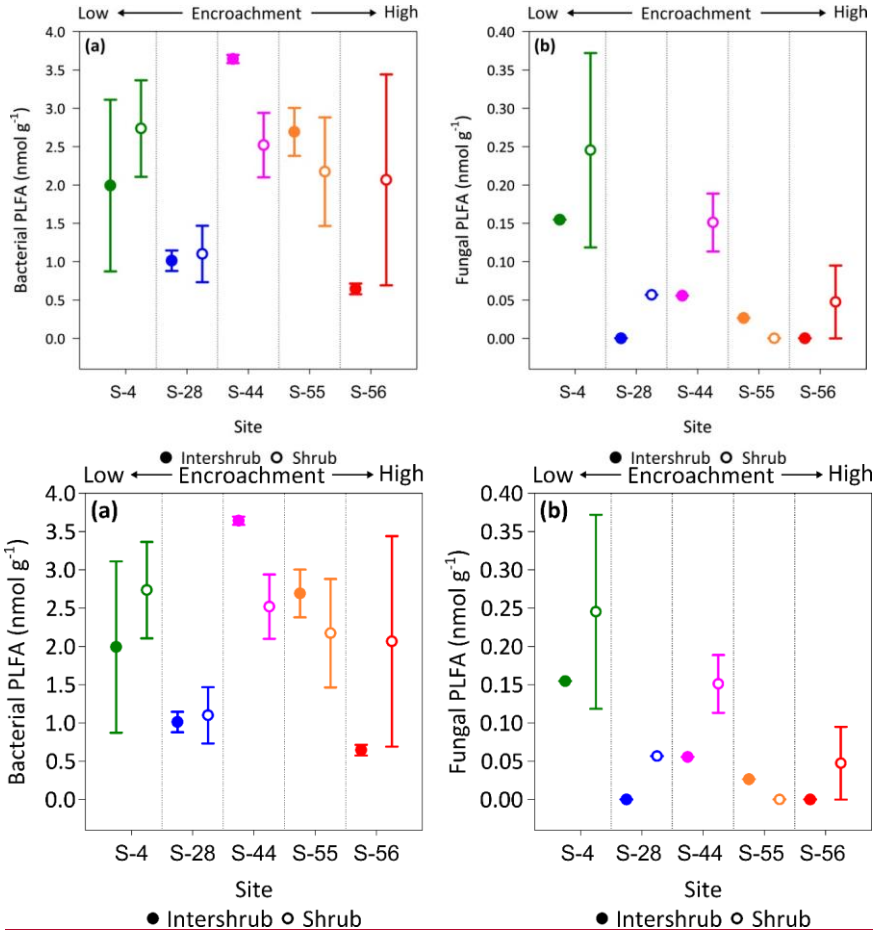


Figure 8. Median bacterial (a) and fungal (b) phospholipid fatty acid (PLFA) concentrations for surface soil (top 10 cm) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 3). Error bars represent median absolute deviation about the median. All results reported on dry matter basis.

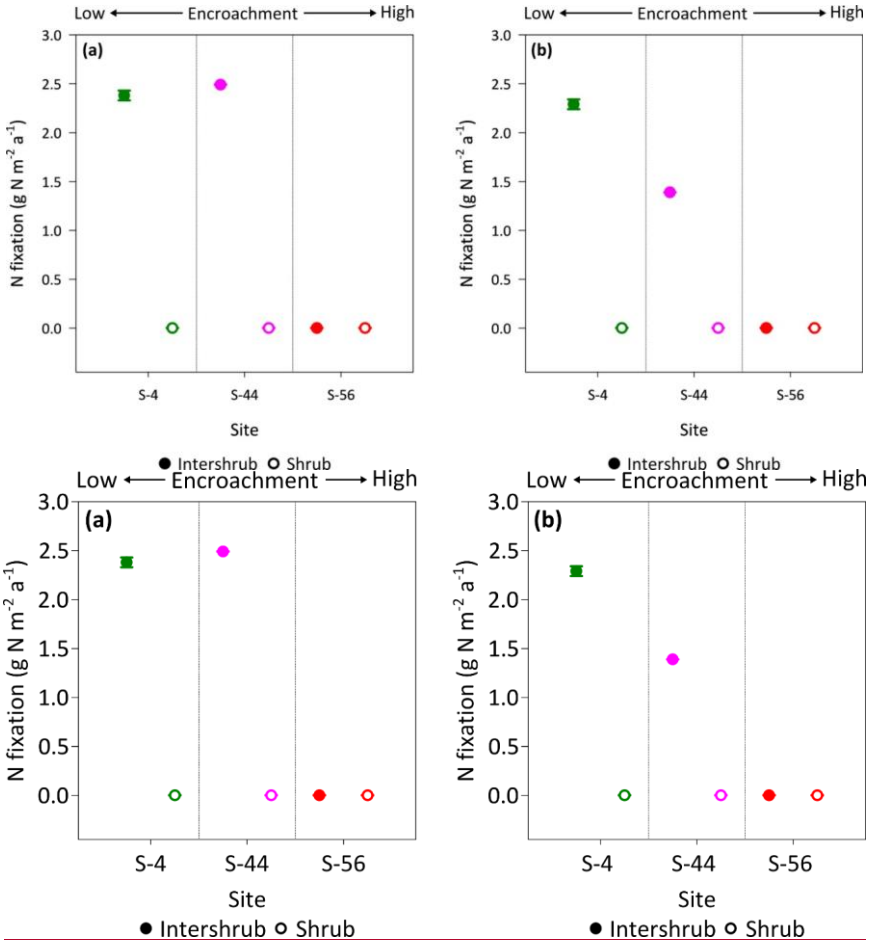


Figure 9. Median rates of asymbiotic nitrogen (N) fixation for intershrub and shrub surface (top 10 cm) soil (a) and intershrub and shrub surface soil weighted by area cover (b) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 3). Error bars represent median absolute deviation about the median. N fixation rates were determined using the acetylene reduction assay method where ethylene was converted to N using a 3:1 ratio (Telling et al., 2011). Results reported on dry matter basis.

In this study, bacterial PLFA concentrations did not follow a clear trend with increasing shrub encroachment (Figure 8a), whereas the fungal PLFA content of both intershrub and shrub soils decreased linearly between end-member sites (Figure 8b). These results contrast with previous studies which found the microbial biomass to be greater in soils beneath

shrub canopies than adjacent grasses due to reduced environmental stress and greater stores of SOM (Gallardo and Schlesinger, 1992; Li et al., 2017; Ewing et al., 2007). As ratios between the fungal and bacterial constituents of the microbial community share a negative relationship with soil pH (Bååth and Anderson, 2003), a decline in the fungal PLFA content of shrub soils can be attributed to changes in shrub soil pH which increased with gains in shrub cover (Table 1). However, the same cannot be ascribed to changes in the fungal PLFA content of intershrub soils, as declines between end-member sites occurred with decreases in soil pH. Instead, if total N is used as a proxy for SOC (intershrub soil C:N ratios do not differ between sites), reductions in intershrub fungal PLFA concentrations with increasing encroachment may be indicative of C limitation for growth, which would be expected to reduce the microbial biomass and release inorganic N (Cookson et al., 2006). Accordingly, in addition to an absence of fungal PLFAs, intershrub soil at S-56 exhibits the lowest bacterial PLFA content in comparison with all other soils along the encroachment gradient, as well as the highest concentration NO_3^--N within the intershrub soils (Figure 5c). As fungi have been shown to preferentially utilise grassland C (Creamer et al., 2016), declines in the shrub soil fungal PLFA content with increasing encroachment may also be a response to the progressive depletion of grass-derived SOC.

It must be noted that we recommend caution when interpreting the vegetation cover and biomass data presented here. The classification accuracy of cover estimates, which are subject to image spatial resolution, as well as land-cover spatial and spectral heterogeneity (Hu et al., 2013), were not formally assessed. Likewise, woody and herbaceous biomass estimates were derived from allometric relationships that had not been ground-truthed and which could potentially vary by soil type, surface morphology and disturbance history (Nafus et al., 2009; McClaran et al., 2013). However, as relative changes in percentage grass and shrub cover can explain concurrent changes in grass and shrub biomass (Figure 4c,d), these estimates were useful for capturing the extent of shrub encroachment at each site.

4.2 Inputs of asymbiotically-fixed nitrogen

In this study we explored a gap in the literature concerning the impacts of increasing shrub encroachment (at sites S-4, S-44 and S-56 only) on the rates and distribution of asymbiotic BNF in a semiarid grassland. We found that asymbiotic BNF was only detectable in intershrub soils at sites S-4 and S-44 (Figure 9a), which exhibited significantly lower N:P ratios than adjacent shrub soils (Figure 7b). These findings reinforce the theory that increases of inorganic N relative to available P inhibit nitrogenase activity, the enzyme responsible for mediating the adenosine triphosphate-reduction of N_2 to ammonia in BNF (Smith, 1992). However, despite intershrub soil N:P ratios remaining constant with increasing encroachment, there was an absence of asymbiotic BNF at S-56. As asymbiotic diazotrophs (N fixing organisms) are largely contained within the bacterial fraction of the microbial biomass (Poly et al., 2001), the undetectable rates of asymbiotic BNF at S-56 could be attributed to the low bacterial PLFA concentration at this site (Figure 8a). This conclusion is supported by increases in asymbiotic BNF rates with gains in the bacterial PLFA content of intershrub soils between sites S-4 and S-44.

When weighted by intershrub area, rates of asymbiotic BNF declined from 2.3 g N m^{-2} a^{-1} at S-4 to 1.4 g N m^{-2} a^{-1} at S-44 (Figure 9b). As levels of ~~atmospheric~~ total N deposition (0.1-0.2 g N m^{-2} a^{-1}) in the Southwestern US are generally low (Schwede and Lear, 2014), the decreasing rates of BNF associated with increasing site-level concentrations of inorganic N

(Figure 6b,c) with shrub encroachment represent the loss of a significant N input pathway to these already N-limited systems, which Belnap (1995) argues may not be readily recovered. However, the rates of asymbiotic BNF reported here, which exceed previous estimates for dryland systems by an order of magnitude (Cleveland et al., 1999), represent an absolute upper limit as soil moisture, temperature and light were maintained during the incubation experiments. Constant incubation parameters were used to achieve linear rates of C_2H_2 ~~acetylene~~ reduction over a 24-hour period, where parameters were chosen to reflect mean summer conditions when microbial activity is greatest due to increased soil water availability (Loik et al., 2004).

A study by Geesing et al. (2000) showed the development of the soil N pool beneath mesquite canopies is attributable to inputs of symbiotically-fixed N. As site-level concentrations of total N do not differ between end-member sites, we argue the loss of intershrub soil N and asymbiotic BNF with shrub encroachment seen here are balanced by inputs of symbiotically-fixed N by mesquite. However, as the redistribution of soil resources with shrub encroachment ~~preferentially selects~~modifies the development of shrub over intershrub soils, inputs of N by mesquite-*Rhizobium* symbioses are ultimately unavailable to sustain grass production in intershrub areas. Consequently, transitions from grass- to shrub-dominated semiarid systems may be viewed as a shift between bistable states from which intershrub areas may not readily recover (D'Odorico et al., 2013).

5 Conclusions

Over the past century, the semiarid grasslands of the southwestern US have been degraded by the encroachment of mesquite, an N-fixing shrub. From this comprehensive study, we found the site-level soil N pool remained constant along a gradient of increasing shrub cover within a semiarid grassland of the SRER, but that the distribution of nitrogen within the ecosystem changed as declines in the intershrub soil N pool were proportional to increases in soils under shrub canopies. Yet the inorganic fraction of the site-level soil N pool increased with shrub area and biomass due to the long-term accumulation of NH_4^+-N and NO_3^--N in soils beneath shrub canopies. Considering soil C:N ratios did not differ between sites or cover type, increases in concentrations of inorganic N in shrub soils were attributed to the capacity of the soil microbial community to mineralise organic forms of N. Finally, as the ratio of inorganic N to bioavailable P has been shown to influence nitrogenase activity, we explored the effects of shrub encroachment on the distribution and rates of asymbiotic BNF in the grassland soil. We found that BNF was inhibited by higher concentrations of inorganic N to available P and, when weighted by intershrub area, provided a lower input of new N to the system with increasing shrub cover. The loss of this ecosystem process, as well as declines in the intershrub soil N pool, were potentially balanced by inputs of symbiotically-fixed N by mesquite. Consequently, we conclude the long-term accumulation of N beneath shrub canopies combined with increases in shrub canopy area have the potential to significantly change the dynamics of soil N cycling in dryland systems.

Data availability

All data presented in figures 4-9 are available online at DOI: 10.5523/bris.28ck0lsp9c48e1zosa7h2c538u.

Author contributions

TTJ led the design of the study, assisted by KM and AMA. All sample collection and analyses were performed by TTJ. TTJ wrote the manuscript with contributions from KM, JAB and AMA.

Competing interests

The authors declare that they have no conflict of interest.

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Google Earth: Shrub-Dominant Grass Mix 31°47'57.39"N, 110°49'33.13"W, elevation 1,250 m, 2016c.

Google Earth: Shrub-Dominant with Bare Intershrub Areas 31°48'9.75"N, 110°50'15.46"W, elevation 1,250 m, 2016d.
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Soil nitrogen response to shrub encroachment in a degrading semiarid grassland

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Abstract. Transitions from grass- to shrub-dominated states in drylands by woody plant encroachment represent significant forms of land cover change with the potential to alter the spatial distribution and cycling of soil resources. Yet an understanding of how this phenomenon impacts the soil nitrogen pool, which is essential to primary production in arid and semiarid systems, is poorly resolved. In this study, we quantified how the distribution and speciation of soil nitrogen, as well as rates of free-living biological nitrogen fixation, changed along a gradient of increasing mesquite (*Prosopis velutina* Woot.) cover in a semiarid grassland of the Southwestern US. Our results show that site-level concentrations of total nitrogen remain unchanged with increasing shrub cover as losses from intershrub areas (sum of grass and bare-soil cover) are proportional to increases in soils under shrub canopies. However, despite the similar carbon-to-nitrogen ratio and microbial biomass of soil from intershrub and shrub areas at each site, site-level concentrations of inorganic nitrogen increase with shrub cover due to the accumulation of ammonium and nitrate in soils beneath shrub canopies. Using the acetylene reduction assay technique, we found increasing ratios of inorganic nitrogen-to-bioavailable phosphorus inhibit rates of biological nitrogen fixation by free-living soil bacteria. Overall, these results provide a greater insight into how grass- to shrubland transitions influence the soil N pool through associated impacts on the soil microbial biomass.

1 1 Introduction

Degrading dryland landscapes undergo significant ecological transformations typically associated with gradual and irreversible changes in plant community composition (D'Odorico et al., 2013). Woody plant encroachment is a widespread example of dryland degradation, whereby continuous grasslands are progressively replaced by shrubs interspersed by patches of bare soil (Ravi et al., 2010). These plant community changes result in a structurally, physically and biologically different ecosystem which significantly alter the spatial distribution and fluxes of nutrients and the biogeochemical cycling within dryland landscapes, with implications for the ongoing process of land degradation (Eldridge et al., 2011; Michaelides et al., 2012). Nitrogen (N), in particular, is considered the most significant limiting nutrient of primary production and decomposition processes in arid and semiarid ecosystems (Gebauer and Ehleringer, 2000), yet there is currently limited understanding of how ecosystem transformations associated with land degradation changes the distribution, speciation and cycling of N (Browning et al., 2008), as well as the associated soil microbial biomass (Li et al., 2017). As drylands occupy ~40% of Earth's land surface, changes in the nutrient distributions, patterns and cycling within degrading areas have important implications for biogeochemical cycles at the global scale (Delgado-Baquerizo et al., 2013).

Occurring in Africa (Hudak et al., 2003), Asia (Zhang et al., 2016), Australia (Cookson et al., 2006), Europe (Quero et al., 2013), and the North (Knapp et al., 2008) and South Americas (Cabral et al., 2003), extensive research has demonstrated that shrub encroachment into grasslands alters the scale of vegetation heterogeneity within the landscape from fine to large scales and changes the microtopography from relatively subdued to high relief as soil mounds develop beneath shrub canopies (Charley and West, 1975; Wainwright et al., 2000; Schlesinger et al., 1996). These changes in both vegetation heterogeneity and microtopography result in modifications to runoff and erosion patterns (Michaelides et al., 2009; Parsons et al., 1996)

which directly affect the redistribution of, and patterns in, nutrients within the landscape in favour of shrub functional types (Cross and Schlesinger, 1999; Michaelides et al., 2012; Schlesinger et al., 1996). In addition, shrubs may further concentrate nutrients beneath their canopies through increased inputs of high-quality above- and belowground detritus (Throop and Archer, 2008). The resulting accumulation of soil organic matter (SOM) in shrub mounds represents an important pool of mineralisable N that provides shrubs with a competitive advantage over herbaceous plants during times of nutrient limitation (Turnbull et al., 2010).

In terrestrial systems, the availability of inorganic N, defined hereafter as the sum of ammonium (NH_4^+-N) and nitrate (NO_3^--N), is largely controlled by microbial decomposition processes (Vitousek et al., 2002). The dominant decomposers in soil are bacteria and fungi, where fungi typically exhibit greater carbon (C):N ratios than bacteria, as well as enhanced C use efficiency and reduced rates of nutrient turnover (Waring et al., 2013). As the amount of N mineralised during decomposition depends on the C and N stoichiometry of decomposer organisms relative to that of organic matter (Schulten and Schnitzer, 1997), differences in the relative abundance of fungal and bacterial biomass can potentially modulate soil N cycling (Waring et al., 2013). Using the phospholipid fatty acid (PLFA) method, Li et al. (2017) demonstrated that the ratio of viable fungi to bacteria for a semiarid grassland was significantly higher in soil under shrubs than for soils in intershrub areas, and that soil microbial biomass was positively correlated with concentrations of total N and available phosphorus (P). However, an understanding of how the fungal and bacterial constituents of the microbial biomass change along a gradient of increasing shrub cover within a semiarid grassland is currently lacking.

Whilst mineralisation may control the availability of N, biological N fixation (BNF) by free-living (asymbiotic) soil bacteria and legume-*Rhizobium* symbioses are the principal inputs of reactive N to semiarid systems (Evans and Ehleringer, 1993), where rates of symbiotic BNF are approximately an order of magnitude higher than inputs through asymbiotic pathways (Cleveland et al., 1999). Many encroaching shrub species, such as mesquite (*Prosopis* spp.), are legumes capable of symbiotic BNF, which reduces their dependence on soil N mineralisation, and offers the potential to increase the availability of N within a system (Blaser et al., 2014). However, since BNF has a high P demand, rates of symbiotic dinitrogen (N_2) fixation by mesquite have been shown to downregulate as N increases relative to concentrations of available P (Geesing et al., 2000). Less-studied are the effects of ecosystem transformations on inputs of asymbiotically-fixed N to semiarid grasslands, which despite being lower than contributions by symbiotic BNF, are still significant due to the small size of the soil N pool in these systems.

The aim of this study was to assess how a transition from a grass- to shrub-dominated state within a dryland ecosystem impacts the soil N status and inputs of N by asymbiotic BNF. Specifically, our objectives were:

1. To examine how pools of total N, NH_4^+-N and NO_3^--N, as well as the microbial biomass, in soil varied across a shrub encroachment gradient; and
2. To determine how the distribution and supply rates of asymbiotically-fixed N varied across a shrub encroachment gradient.

We expected that reductions in grass biomass resulting from higher levels of shrub encroachment would cause soil N to be progressively redistributed from intershrub zones to areas beneath shrub canopies. As the spatial distribution of soil microorganisms is positively influenced by resource availability (Schlesinger and Pilmanis, 1998), we hypothesised the enhanced fertility and environmental conditions in soils under shrubs would support a higher soil microbial biomass and promote N mineralisation processes leading to an increased availability of inorganic N. Accordingly, we predicted that rates of asymbiotic BNF would decrease in shrub soils as inorganic N increased relative to bioavailable P (Smith, 1992).

2 Materials and methods

2.1 Study site description

Fieldwork was conducted between March and April 2016 at the Santa Rita Experimental Range (SRER; Figure 1a; 31˚54'N, 110˚53'W), Arizona in the Southwestern US, a region where the density of mesquite has increased by >400% over the past ca. 30 years alone (Resco de Dios et al., 2012). Situated on a bajada comprising ~21,000 ha, the SRER ranges in elevation from 900 m asl to 1,400 m asl (McClaran, 2003). Mean daily maximum and minimum air temperatures for the years 2008 to 2015 were 25 ˚C and 13 ˚C, respectively. Mean annual precipitation for the same period was 392 mm, with 61% received during the summer months (July through September). The respective mean daily maximum and minimum summer temperatures between 2008 and 2015 were 31 ˚C and 20 ˚C (Ameriflux, http://ameriflux.lbl.gov/data/download-data/). Vegetation is dominated by native desert grassland species, such as Rothrock grama (*Bouteloua rothrockii* Vasey), sideoats grama (*B. curtipendula* (Michx.) Torr.), Arizona cottontop (*Digitaria californica* (Benth.) Henrard), bush muhly (*Muhlenbergia porteri* Scribn.) and tanglehead (*Heteropogon contortus* (L.) Beauv.), as well as the non-native Lehmann lovegrass (*Eragrostis lehmanniana* Nees.). Cacti, such as cholla cactus species (*Opuntia spp*. Mill.), and shrubs, such as velvet mesquite (*Prosopis velutina* Woot.) and catclaw acacia (*Acacia greggii* A. Gray), were also present (McClaran et al., 2002).

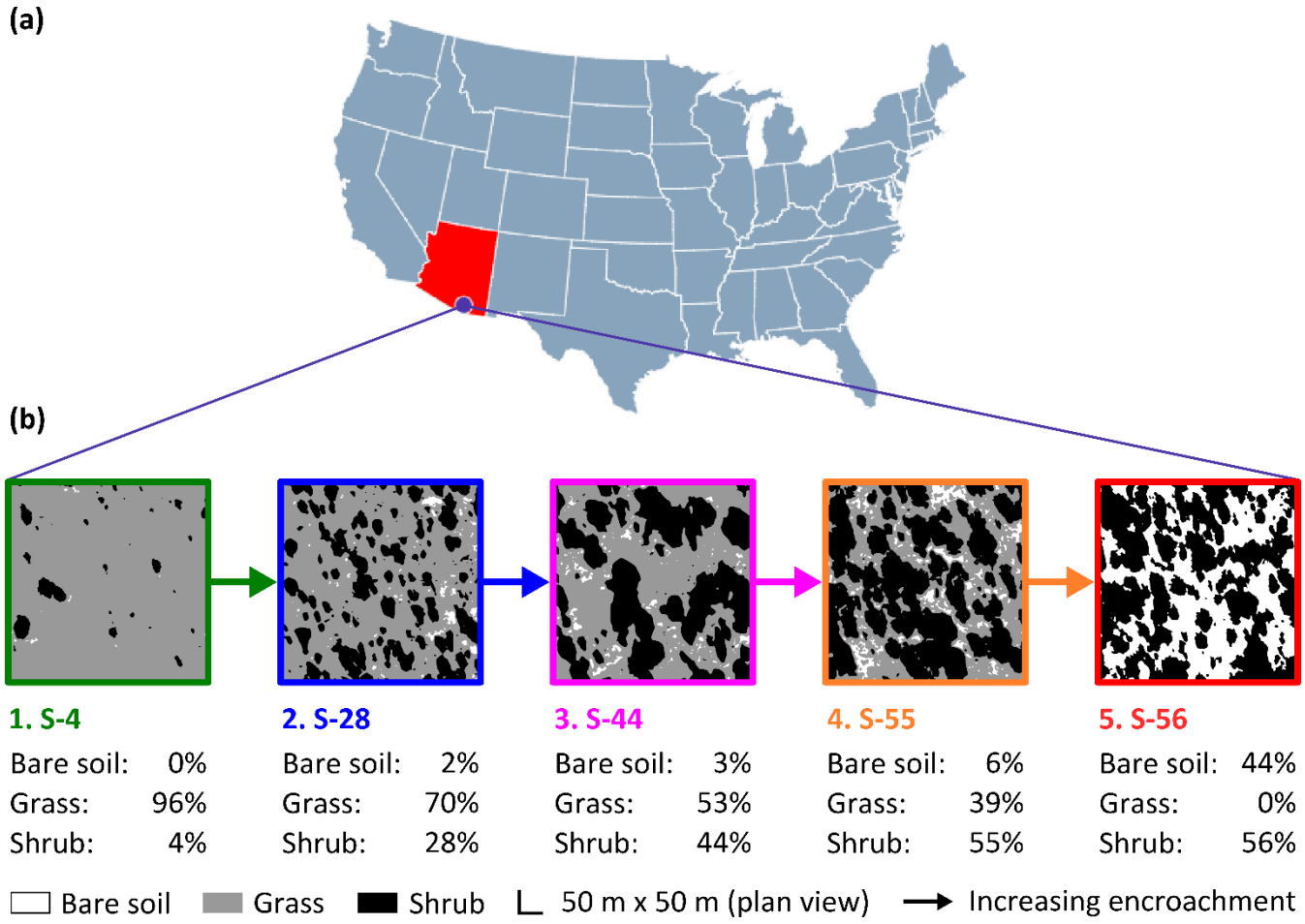

Figure 1. The study site (a) was a semiarid grassland in the Santa Rita Experimental Range, AZ, US (31˚54'N, 110˚53'W; elevation: ~1,250 m asl). Five sampling sites comprised a gradient of shrub encroachment, where grass cover decreased, and bare soil and shrub cover increased between sites 1 and 5 (b). The intershrub area of each site is equal to the sum of the percentage bare soil and grass cover.

Encroachment by mesquite within the SRER has been well documented (McClaran, 2003). Velvet mesquite, which was restricted to drainage arroyos in 1903, currently occupies ~35% of the total grassland area (Figure 2) and represents the dominant shrub species within the elevation band of 990-1,200 m asl (Browning et al., 2008). The following five sampling sites comprise a gradient of increasing shrub cover representing progressive ecosystem degradation: S-4, S-28, S-44, S-55 and S-56 (Figure 1b) where the number signifies the percent shrub cover. The percentage shrub canopy and intershrub area was calculated for the five sites using high-resolution satellite imagery from Google Earth (2016a-e), which were cropped to the same dimensions and segmented using the Trainable Weka Segmentation plugin for Fiji (http://fiji.sc/Fiji). Sobel and Gaussian blur filters were used for edge detection and noise suppression, respectively (Arganda-Carreras et al., 2017). The out-of-bag error for all sites classified was <2%. Soils at these sites were formed in alluvium from igneous rock of Holocene and Late Pleistocene origins (Batchily et al., 2003) in the Combate-Diaspar complex (CoB), characterised by excellent

drainage, sandy loam textures and 1-8% slopes (SRER Digital Database, https://cals.arizona.edu/srer/data.html). All five sites were situated at ~1,250 m asl, exhibited similar topography and had the same land use history.

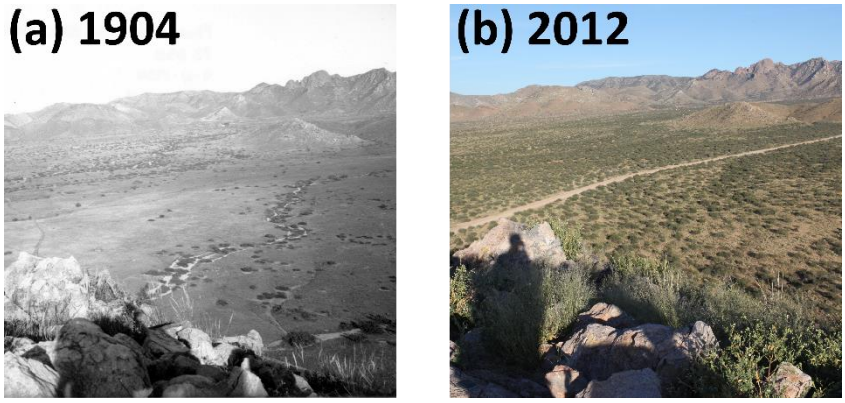

Figure 2. Scattered shrubs replace grasses as dominant vegetation between 1904 (a) and 2012 (b) in the Santa Rita Experimental Range, AZ, US. Facing north northeast in pastures 6B and 6D. Elevation: 1,228 m asl (public domain images available from: https://cals.arizona.edu/srer/photos.html).

2.2 Field measurements and sample collection

To mitigate the effects of directional bias during sampling, a 180-m transect (horizontal distance) was arranged in a Y-configuration at each site, where three 60-m transects radiated out from plot centres at azimuths of 30°, 150° and 270° (Figure 3). Three 100-m^2 quadrats were then installed at 10-m, 30-m and 50-m intervals along each transect for the determination of aboveground shrub biomass. Further, a 0.5-m^2 quadrat was centred within the largest intershrub area of each 100-m^2 quadrat for the determination of aboveground grass biomass. The dry weight of each velvet mesquite plant was estimated from stem basal diameter measurements using methods, and a site- and species-specific allometric equation reported in McClaran et al. (2013). Similarly, the dry weight of individual bunchgrasses were estimated from tussock basal diameter measurements using methods, and a site-specific multispecies allometric equation reported in Nafus et al. (2009). The sums of the individual shrub and herbaceous constituents were calculated and used to estimate the total mesquite and grass biomass by dry weight for each site, where grass biomass was weighted according to intershrub area (kg m^{-2}). Finally, three intershrub and shrub surface soil (top 10 cm) cores were extracted at equal distances between the stem and drip line on the north side of the mesquite plant with the largest stem basal diameter, and along the centre of the 0.5-m^2 quadrat, respectively. The extracted cores were then homogenised to create single intershrub and shrub samples for each quadrat per site (n = 9) and sealed in sterile bags (Whirl-Pak®, Lactun, Australia).

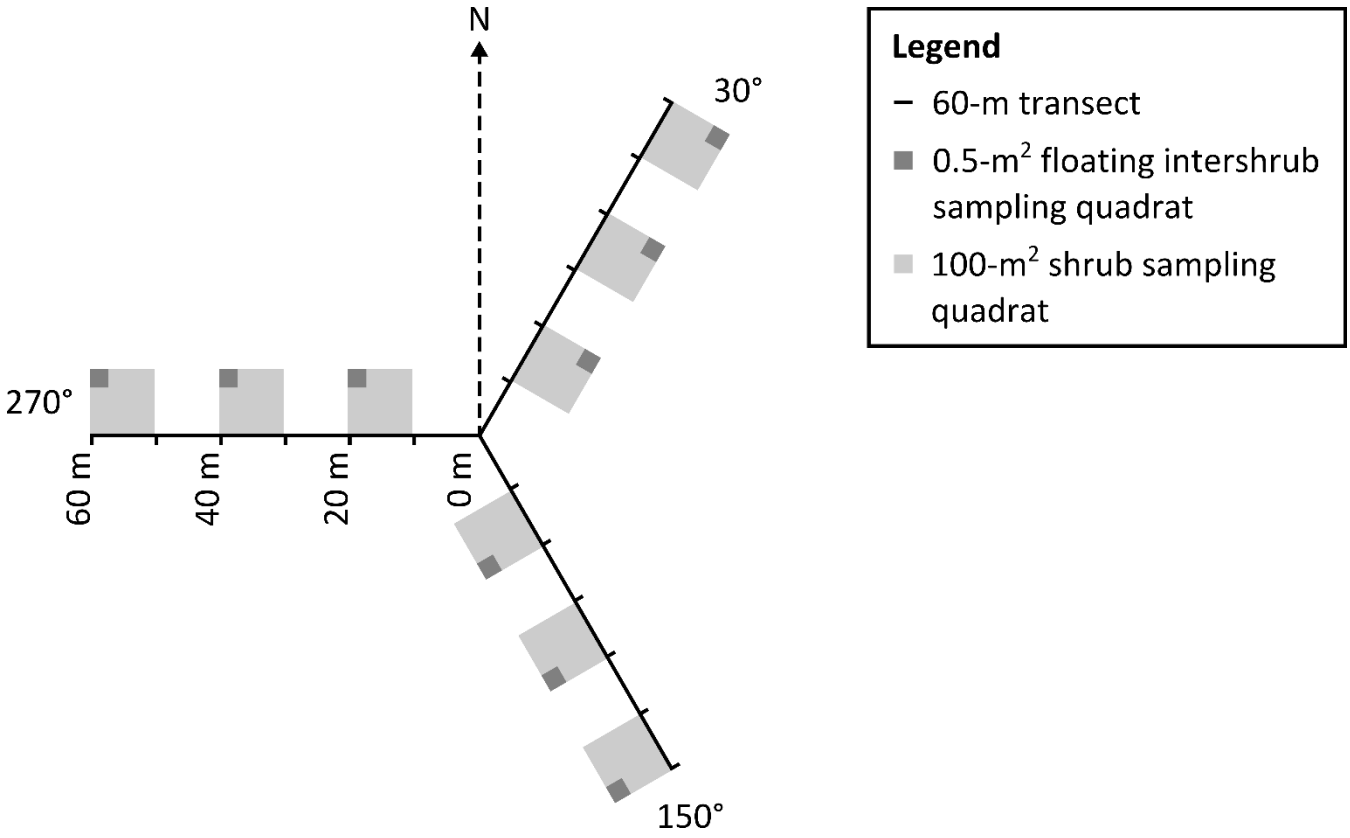

Figure 3. Plan view of sampling design where grass and shrub biomass, as well as intershrub and shrub soil samples, were obtained from 0.5-m^2 and 100-m^2 quadrats, respectively (not to scale). The 0.5-m^2 quadrats were not fixed but were instead centred within the largest intershrub area of each 100-m^2 quadrat.

2.3 Soil preparation

The mechanical sieving method described by Loveland and Whalley (2000) was used to evaluate the coarse fraction (>2 mm, wt%) of each sample. The resulting fine earth fraction (≤2 mm) from each sample was retained for all further analyses. For pH, PLFA and elemental analyses, the number of intershrub and shrub samples per site was reduced to three (n = 3) by homogenising the samples collected along each of the three 60-m transects. Samples were then stored in a dark temperature-controlled environment at 4 ˚C. Prior to storage, aliquots of 5 g from each of the n = 9 and n = 3 samples were frozen at -20 ˚C and freeze-dried for elemental and PLFA analyses, respectively.

2.4 Soil characteristics

Soil pH was measured in a 1:5 soil:0.01 M $CaCl_2$ suspension after mixing at 20 rpm for 30 minutes using a Stuart SB3 rotator (Bennett et al., 2003). Particle-size determination for the fine earth fraction (%) was measured using a Malvern Mastersizer 3000 laser particle size analyser with a Hydro EV pump accessory (Malvern Instruments Ltd., Worcestershire, UK), where isopropanol was used as the dispersant (Goossens et al., 2014). Water holding capacity (WHC; %) was

estimated using the percentage sand and clay content of each sample (Saxton and Rawls, 2006), which had been defined according to the USDA-SCS (1982) classification scheme. Soil dry matter content (DMC; %) was determined for all samples using the gravimetric method described by Rowell (1994). Lastly, as it was not possible to extract intact soil cores, an average bulk density value of 1.33 g cm^{-3} was assumed for all samples using previously published data for the SRER (Wheeler et al., 2007).

2.5 Elemental analysis

For each sample, an aliquot of ~100 mg of freeze-dried soil was embrittled using liquid N_2, and ground to a flour using a pestle and mortar. Concentrations of SOC and total N were then determined on an elemental analyser (CHNS-O EA 1108 Elemental Analyzer; Carlo Erba, Milan, IT) in accordance with methods outlined by Hedges and Stern (1984), where the detection limits were <0.5 µg g^{-1} for both elements measured. The coefficient of variation (six replicate standards) for C and N was ±1.69% and ±1.36%, respectively.

2.6 Soil nutrient analyses

Concentrations of exchangeable NH_4^+-N and NO_3^--N in soil were extracted with 2M KCl (1:10 w/v soil:extractant) and determined colourimetrically using a flow injection analyser (Lachat QuikChem 8500 Series 2 FIA system, Loveland, CO, US), where the coefficient of variation (six replicate standards) for NH_4^+-N and NO_3^--N was ±0.75% and ±5.19%, respectively according to mid-range standards (calibration range: 0-0.5 mg L^{-1}). Thus, for each sample, 1 g of fresh soil was added to 10 mL of 2M KCl, shaken for 30 minutes at 160 rpm, centrifuged for 5 minutes at 4,500 rpm and filtered to 0.45 µm using Whatman WCN plain cellulose nitrate filtrate papers. Extracts were then analysed for NH_4^+-N (QuikChem® Method 31-107-06-1-I) using the Berthelot reaction (Willis et al., 1993) and NO_3^--N (QuikChem® Method 31-107-04-1-K) using the cadmium reduction method (Willis and Gentry, 1987). The respective detection limits for NH_4^+-N and NO_3^--N were 0.04 µg g^{-1} and 0.08 µg g^{-1} for dry sediment. Bioavailable P (sum of loosely-sorbed, and iron- and aluminium-bound P) was sequentially extracted from 0.2 g of soil using a method adapted from Hedley et al. (1982) and Mumford (2003) as described in Michaelides et al. (2012). The concentrations of the resulting extracts were determined photometrically on a Gallery™ Plus Automated Photometric Analyzer (ThermoFisher Scientific, San Jose, CA). The limit of detection for both loosely-sorbed, and iron- and aluminium-bound P was 1.1 µg g^{-1}. The coefficient of variation (six replicate standards) for the same fractions were ±0.35% and ±0.41%, respectively. All samples were blank corrected where blank concentrations exceeded the detection limits.

2.7 Microbial biomass estimation

PLFAs were extracted from 2 g aliquots of each sample using a modified Bligh and Dyer (1959) method as described by Frostegård et al. (1991). Subsequent fractionation and derivatisation was performed according to methods by Dickson et al. (2009) and Christie (1993), respectively. The methyl ester of nonadecane was added at a concentration of 0.1 mg L^{-1} as an

internal standard to quantify the fatty acids. Individual compounds were quantified using a gas chromatograph equipped with an Agilent VF-23ms column (60 m x 0.32 mm x 0.15 μm; He carrier gas, constant 2 mL min^{-1} flow rate), which was operated for 1 min at 50 °C followed by a 10 °C min^{-1} ramp to 100 °C and a 4 °C min^{-1} ramp to a final temperature of 250 °C that was maintained for 15 mins. Compound structures were identified based on comparisons with the retention times and mass spectra for authentic laboratory standards. The mass spectra of individual components were obtained using a ThermoScientific 1300 Series gas chromatograph (column, carrier gas and operation as above) coupled, using a heated transfer line (260 °C), to a ThermoScientific ISQ LT quadrupole mass spectrometer scanning in the range of *m/z* 50-650 with a dwell time of 0.5 s (current was maintained at 50 μA with an ion source temperature of 240 °C and an electron voltage of 70 eV). Fatty acids which were <14 C and >20 C, or which accounted for <0.5% of the total peak area, were excluded from the analysis. Bacterial and fungal biomass were defined according to Bååth and Anderson (2003).

2.8 Acetylene reduction assay

Due to time and cost constraints, rates of asymbiotic BNF (nitrogenase activity) for intershrub and shrub soils were assessed for three sites (S-4, S-44 and S-56) using the acetylene (C_2H_2) reduction assay technique adapted from Telling et al. (2011). Prior to analysis, any remaining loose plant matter (roots and shoots) was removed from samples using forceps. A sample, control and kill control (autoclaved at 126 ˚C for 30 minutes) were created from each homogenised sample by adding aliquots of fresh soil (equivalent to 7.5 g dry mass) to 30 mL serum bottles. Samples were then adjusted to 60% WHC, sealed using grey butyl stoppers and crimped shut. In addition, a blank was created by adding 10 mL of autoclaved Milli-Q water to an empty serum bottle. Serum bottles were placed in an illuminated (0.2 ±0.005 W m^{-2}) incubator set to 26 ˚C ±0.1 ˚C (mean of maximum and minimum summer temperatures). Following a 72-hour pre-incubation period, 10% of the headspace from the sample, blank and kill control were replaced with 100% C_2H_2 gas, which had been produced by adding Milli-Q water to technical grade calcium carbide (Sigma, St. Louis, MO, US), via an air-tight syringe. Serum bottles were sampled at 0-, 3-, 6-, 12-, 20- and 24-hour intervals, where 5 mL of headspace was transferred to pre-evacuated 3.7 mL Exetainers (Labco, Lampeter, UK). A 1 mL aliquot of headspace from each Exetainer was then analysed using a Varian 3800 gas chromatograph (GC; Varian, Inc., Palo Alto, CA, US), where ethylene (C_2H_4) was separated from C_2H_2 using a 6’ x 1/8”, 80/100 mesh HayeSep T column at 85 ˚C (He carrier gas). Daily standards of 100 ppm C_2H_4 (BOC, Guildford, UK) gave precisions of <5%. The precision for 100 ppm standards that had been stored in 3.7 mL for a period of one month also gave precisions of <5%. Rates of C_2H_4 production were converted to rates of annual N fixation (mmol N m^{-2} a^{-1}) using a theoretical ratio of 3:1.

2.9 Statistical analysis

Statistical analysis of the data was performed using R version 3.5.0. The Kruskal-Wallis *H* test (KW) was used to determine if significant differences occurred for data within the same cover type along the encroachment gradient. When significance was indicated, Dunn’s test of multiple comparisons (DT) was applied. The Mann-Whitney *U* test (MW) was used to make

within-site comparisons for data between cover types. Relationships between explanatory and response variables were explored using simple linear regression analysis, where residuals were inspected for evidence of non-normality using the Shapiro-Wilk test. For this study, the alpha level was set to 0.05. All errors reported in the text are one median absolute deviation about the median (Crawley, 2005).

3 Results

3.1 Plant biomass

Vegetation biomass and cover were strongly influenced by the degree of shrub encroachment at each site. Overall, total vegetation (grass and shrub) cover decreased from 100% to 56% between end-member sites, as reductions in grass cover exceeded gains in shrub cover (Figure 1b). The changes in grass and shrub cover were also positively related with grass ($R^2 = 0.87$; Figure 4c) and shrub ($R^2 = 0.77$; Figure 4d) biomass, respectively. Thus, grass biomass declined from 0.62 ±0.19 kg m^{-2} at S-4 to 0 kg m^{-2} at S-56 (Figure 4a; DT, $p < 0.001$), whereas shrub biomass increased from 0.02 ±0.02 kg m^{-2} to 4.45 ±4.19 kg m^{-2} between the same sites (DT, $p < 0.001$, Figure 4b). Lastly, shrub biomass was greater than grass biomass at all sites except S-4, where grass biomass surpassed shrub biomass ($p < 0.05$).

3.2 Soil characteristics

Of the soil properties presented in Table 1, only intershrub and shrub soil pH exhibited clear trends with increasing encroachment. Specifically, intershrub soil pH decreased linearly from 6.3 ±0.4 to 4.7 ±0.4 between end-member sites, whereas shrub soil pH increased from 4.9 ±0.1 to 6.0 ±0.2 along the same gradient. Significant differences in the remaining soil properties of Table 1 did not follow clear trends and occurred only in intershrub soils. The sand content of intershrub soil at S-28 was lower in comparison with all other intershrub soils (DT, $p < 0.05$). Further, the silt and clay contents of intershrub soil at S-28 was greater, relative to other sites, excluding sites S-55 and S-56, respectively (DT, $p < 0.05$). Lastly, the coarse fraction of intershrub soil at S-55 was greater than that of other intershrub soils along the encroachment gradient (DT, $p < 0.05$).

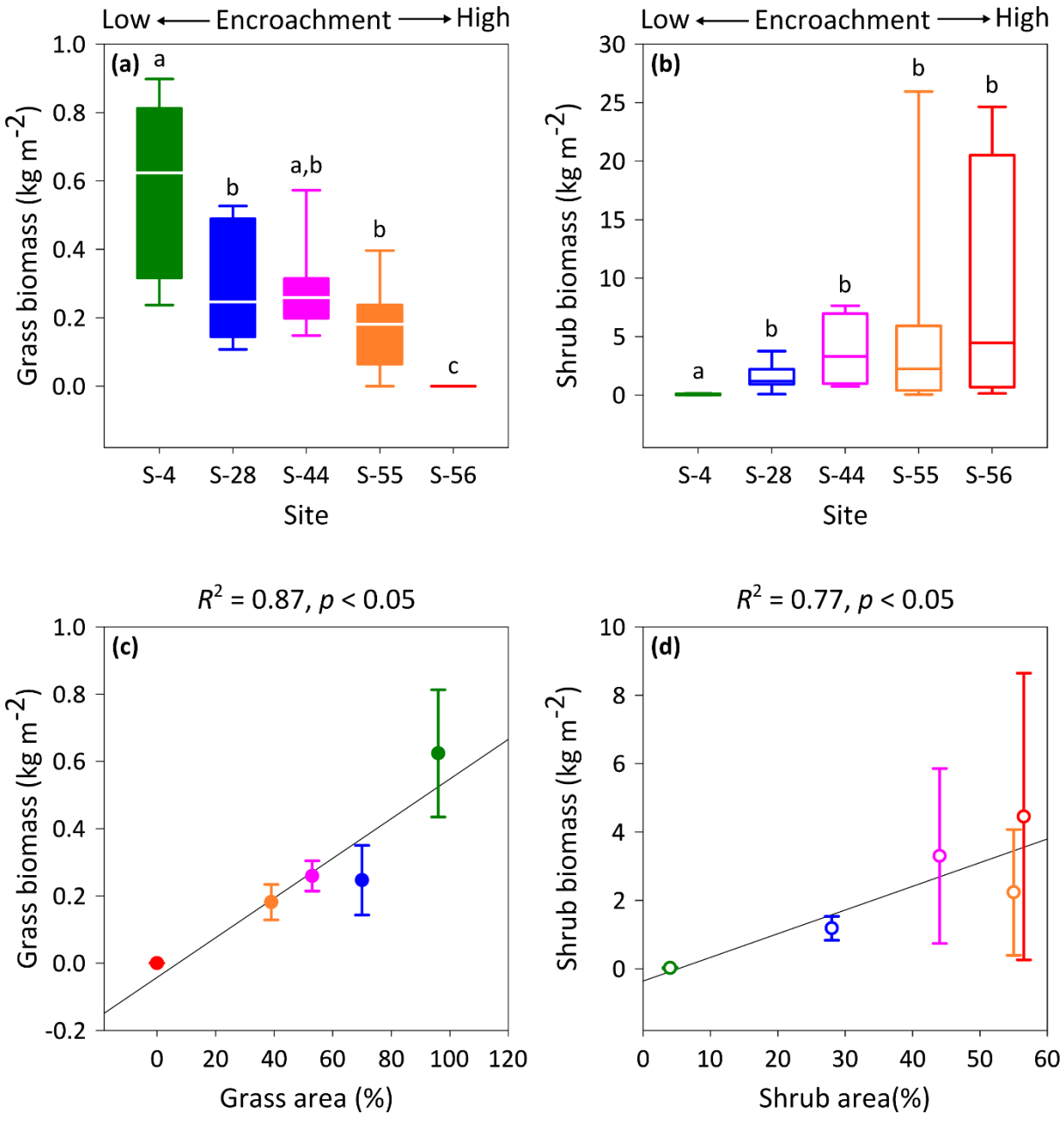

Figure 4. Aboveground multispecies grass (a) and *Prosopis velutina* Woot. (b) biomass by dry weight across a shrub encroachment gradient and according to grass (c) and shrub (d) cover in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with different letters indicate significant differences within biomass classes at $p < 0.05$ (Dunn's test). Error bars represent median absolute deviation about the median.

Soil clay content represented the only consistent physical difference between cover types, where clay content was higher in soils from intershrub areas relative to those from beneath shrub canopies (MW, $p < 0.05$ for sites S-4 and S-56; $p < 0.01$ for sites S-28, S-44, S-55; Table 1). Changes in other physical soil properties did not exhibit a relationship with increasing

encroachment. At S-28, the silt content (MW, $p < 0.001$) and DMC (MW, $p < 0.01$) of intershrub soil were greater than that of the shrub soil; however, the sand content of intershrub soil was lower in comparison with shrub soil (MW, $p < 0.001$). Intershrub soil at S-44 exhibited a higher DMC (MW, $p < 0.05$) and sand content (MW, $p < 0.05$) than shrub soil for the same site; though the silt content was lower than that of the shrub soil (MW, $p < 0.05$). Finally, the percentage of coarse fragments differed between intershrub and shrub soils at S-4 (MW, $p < 0.001$).

Table 1. Median textural characteristics, dry matter content (DMC) and pH (1:5 soil:0.01 M $CaCl_2$) for the surface soil layer (top 10 cm) beneath intershrub (IS) and shrub (S) canopy cover types along a gradient of shrub encroachment in a semiarid grassland of the Santa Rita Experimental Range, AZ, US. Values in parentheses represent one median absolute deviation about the median.

	Site									
	S-4		**S-28**		**S-44**		**S-55**		**S-56**	
	IS	**S**	**IS**	**S**	**IS**	**S**	**IS**	**S**	**IS**	**S**
%coarse[a,b,g]	11.4	11.0	18.0	17.6	18.0	18.4	24.7	24.3	16.5	19.3
	(2.7)	(7.1)	(4.8)	(2.9)	(4.0)	(4.8)	(2.3)	(4.6)	(5.0)	(7.6)
%clay[c,d,g]	1.6	0.8	2.2	0.2	1.5	0.1	1.5	0.2	1.7	0.3
	(0.3)	(0.6)	(0.3)	(0.1)	(0.2)	(0.0)	(0.2)	(0.1)	(0.3)	(0.3)
%silt[c,e,g]	41.9	49.5	53.5	36.2	47.9	53.5	35.7	40.3	39.2	35.4
	(3.6)	(15.4)	(3.5)	(3.3)	(8.9)	(3.5)	(5.1)	(5.6)	(6.0)	(6.1)
%sand[c,f,g]	56.9	49.7	44.4	63.7	44.9	44.4	64.2	59.5	57.6	64.3
	(3.1)	(16.0)	(4.2)	(3.3)	(9.5)	(4.2)	(5.0)	(5.8)	(7.4)	(6.3)
%DMC[c,g]	99.1	99.0	99.1	98.9	99.3	98.8	98.9	98.5	98.7	98.8
	(0.1)	(0.1)	(0.0)	(0.2)	(0.2)	(0.2)	(0.1)	(0.1)	(0.2)	(0.3)
pH[c,h]	6.3	4.9	5.3	5.7	5.2	5.5	5.1	6.0	4.7	6.0
	(0.4)	(0.1)	(0.1)	(0.5)	(0.1)	(0.3)	(0.1)	(0.0)	(0.5)	(0.2)

[a]total soil (>0 µm)
[b]>2,000 µm
[c]fine earth fraction only (≤2,000 µm)
[d]<2 µm
[e]2-50 µm
[f]>50-2,000 µm
[g]n = 9
[h]n = 3

3.3 Distribution and speciation of nitrogen

The total N in shrub soils was more than two times greater than that in intershrub soils (MW, $p < 0.05$) at each site along the degradation gradient (Figure 5a). Further, concentrations of total N in intershrub soils declined proportionally with reductions in grass biomass from 710 ±59 µg g^{-1} at S-4 to 344 ±43 µg g^{-1} at S-56. Specifically, the total N content in

intershrub soil at S-56 was lower in comparison with all other intershrub soils (DT, $p < 0.05$), and concentrations of total N within intershrub soils from sites S-44 and S-55 were lower than the concentrations observed at S-4 (DT, $p < 0.05$). In contrast, a nonlinear trend was observed in the shrub soil total N pool, whereby total N steadily increased from 920 ±198 μg g^{-1} in shrub soil at S-4 to 1,527 ±171 μg g^{-1} at S-55, before declining to 787 ±398 μg g^{-1} at S-56; however, these differences were not significant (KW, $p > 0.05$).

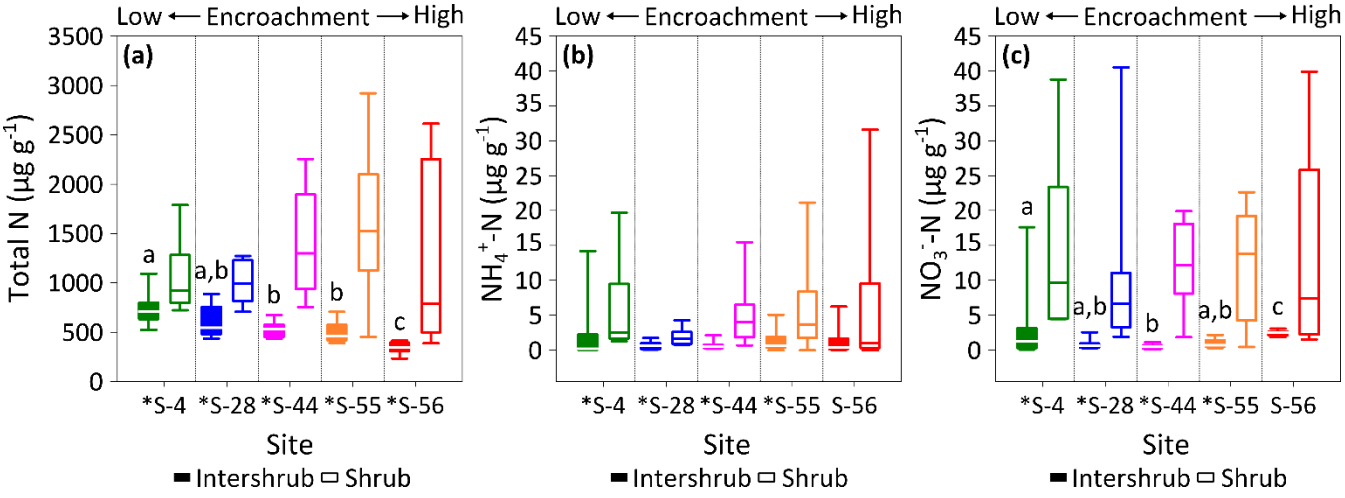

Figure 5. Total N (a), NH_4^+-N (b) and NO_3^--N (c) concentrations for surface soil (top 10 cm) by dry weight under intershrub and shrub cover types along a shrub encroachment gradient in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with different letters indicate significant differences by cover type at $p < 0.05$ (Dunn's test). Intershrub and shrub soils at sites denoted with an asterisk differ significantly from each other at $p < 0.05$ (Mann-Whitney *U* test).

Concentrations of NH_4^+-N did not change with increasing encroachment in either the intershrub (KW, $p > 0.05$) or shrub (KW, $p > 0.05$) soils, where the average of median concentrations in intershrub and shrub soils were 1 μg g^{-1} and 3 μg g^{-1}, respectively (Figure 5b). However, NH_4^+-N concentrations were approximately an order of magnitude higher under shrub canopies compared to intershrub areas for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$). Further, shrub soil NH_4^+-N concentrations exhibited greater variability than that of the intershrub soils.

Like NH_4^+-N, soil NO_3^--N concentrations were greater in soils beneath shrub canopies than in intershrub areas for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$; Figure 5c) and the variability of NO_3^--N in shrub soils was greater than that of their intershrub counterparts. However, unlike NH_4^+-N, intershrub NO_3^--N concentrations varied along the encroachment gradient (KW, $p < 0.001$), where NO_3^--N decreased from 1.2 ±0.8 μg g^{-1} at S-4 to 0.5 ±0.2 μg g^{-1} at S-44, and subsequently rose to 2.5 ±0.2 μg g^{-1} S-56 (Figure 5c). No such trend was identified in the NO_3^--N concentrations of shrub soils, which instead remained constant along the encroachment gradient (KW, $p > 0.05$).

To identify site-level changes in the surface N pools, total area-weighted concentrations of total N, NH_4^+-N and NO_3^--N were calculated for each site according to percentage intershrub and shrub cover (Figure 1b). Overall, site-level total N concentrations (Figure 6a) did not change with increasing shrub encroachment (KW, $p > 0.05$). However, the proportion of total N contained within the inorganic fraction increased in relation to the degree of shrub encroachment. Such increases

were largely seen in the form of NO_3^--N (Figure 6c), which increased from 0.2 ±0.1 g m^{-2} to 0.6 ±0.3 g m^{-2} between sites S-4 and S-56 (DT, $p < 0.05$). Concentrations of NH_4^+-N experienced an increase from 0.03 ±0.03 g m^{-2} at S-4 to 0.2 ±0.1 g m^{-2} at S-55 (Figure 6b), but ultimately did not differ between the end-member sites (DT, $p > 0.05$).

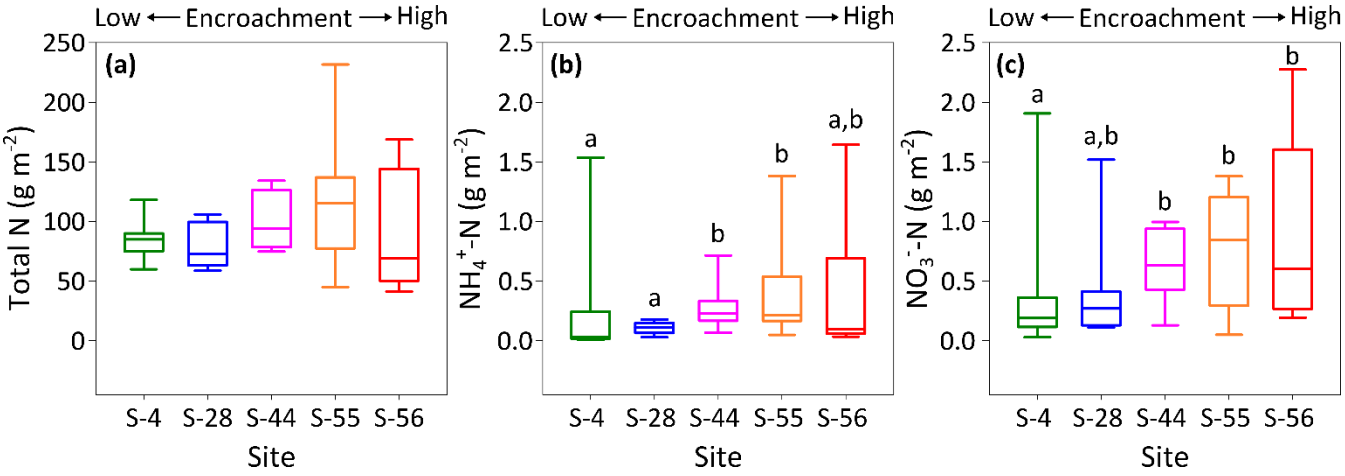

Figure 6. Site-level total N (a), NH_4^+-N (b) and NO_3^--N (c) concentrations (dry matter) weighted by relative area of intershrub to shrub cover for surface soil (top 10 cm) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 9). Boxes denoted with alternative letters differ significantly from one another at $p < 0.05$ (Dunn's test).

3.4 Nutrient ratios

Ratios of SOC:total N (C:N) did not differ across the encroachment gradient for either cover type (KW, $p > 0.05$). The average C:N ratios for intershrub and shrub soils were 7.0 and 7.7, respectively (Figure 7a) and these did not differ between cover types at any of the five sites (MW, $p > 0.05$). Similarly, inorganic N:bioavailable P (N:P) ratios (Figure 7b) for intershrub ($p > 0.05$) and shrub (KW, $p > 0.05$) soils did not differ significantly between sites, where the respective mean ratios for intershrub and shrub soils were 0.1 and 1.2. However, shrub soil N:P ratios exhibited a high degree of variability and were greater than those of intershrub soils for all sites (MW, $p < 0.05$) except S-56 (MW, $p > 0.05$).

3.5 Microbial biomass

No trends were observed in bacterial PLFA biomass across the shrub encroachment gradient (Figure 8a). The greatest (3.6 ±0.1 nmol g^{-1}) bacterial PLFA concentrations were found in the intershrub soils at S-44 and the lowest (0.6 ±0.1 nmol g^{-1}) in intershrub soils at S-56. The fungal PLFA biomass of intershrub soils was less variable in comparison with the bacterial PLFA biomass and decreased from a maximum concentration of 0.2 ±0.1 nmol g^{-1} at S-4 across the encroachment gradient, where concentrations were below the limits of detection at both S-28 and S-56 (Figure 8b). Similarly, the fungal PLFA content of shrub soils, which were typically greater, but more variable than that of intershrub soils, decreased steadily from 0.3 ±0.1 nmol g^{-1} to 0.05 ±0.05 nmol g^{-1} between end-member sites, where fungal PLFA concentration were below the limits of detection at S-55. Consequently, the fungal-to-bacterial ratio decreased under both shrub and intershrub soils with increasing shrub encroachment.

3.6 Acetylene reduction assay

Rates of C_2H_2 reduction were only above the limits of detection in intershrub soils at sites S-4 and S-44, where rates of C_2H_4 production were linear over the 24-hour incubation period ($R^2 = 0.97$ for S-4, and $R^2 = 0.84$ for S-44, slopes of regression lines were significantly different from zero for both S-4 ($p < 0.001$) and S-44 ($p < 0.01$); data not shown here). When converted to annual rates of N fixation per unit area (Figure 9a), inputs of N by asymbiotic BNF were similar between sites S-4 (2.4 g N m^{-2} a^{-1}) and S-44 (2.5 g N m^{-2} a^{-1}). However, when adjusted for percentage cover (Figure 9b), rates of asymbiotic BNF declined in relation to reductions in intershrub area from 2.3 g N m^{-2} a^{-1} at S-4 to at 1.4 g N m^{-2} a^{-1} S-44.

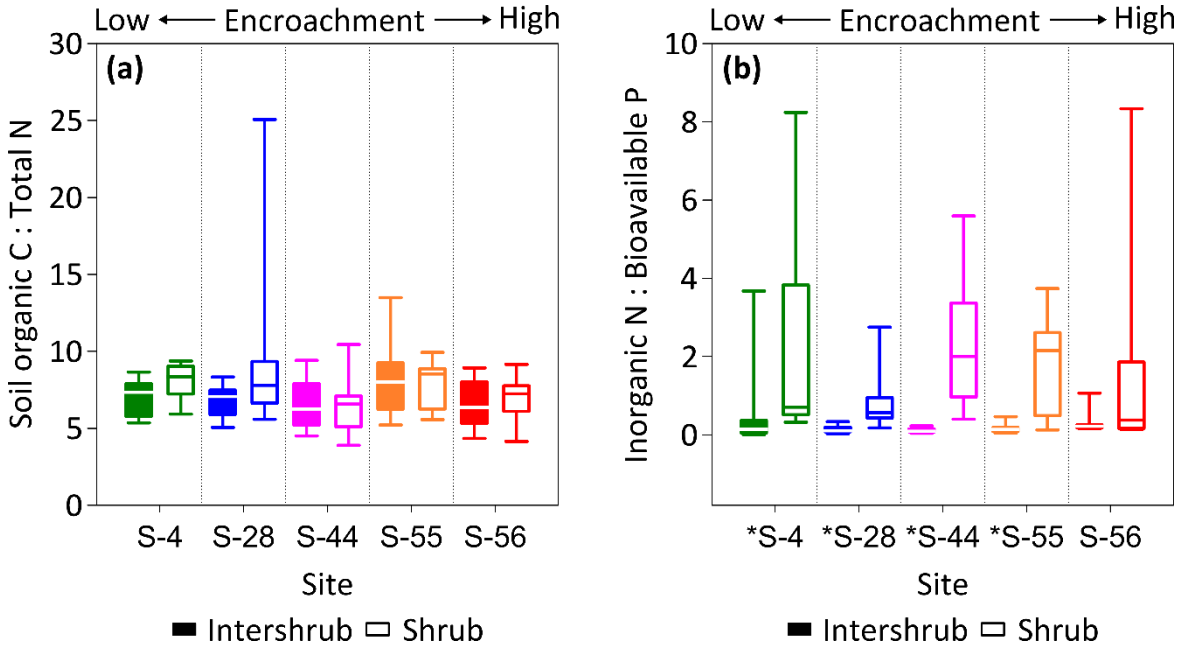

Figure 7. SOC : total N (a) and inorganic N : bioavailable P (b) ratios for surface soil (top 10 cm) under intershrub and shrub cover types along a shrub encroachment gradient in the Santa Rita Experimental Range, AZ, US (n = 9). Intershrub and shrub soils at sites denoted with an asterisk differ significantly from each other at $p < 0.05$ (Mann-Whitney *U* test).

4 Discussion

Our study revealed the distribution and speciation of soil N, as well as the extent of nitrogenase activity, is strongly influenced by the degree of shrub encroachment in a semiarid grassland of the SRER. Yet overall, the soil N pool incurred no net change with the transition from a grass- to shrub-dominated state. Below we discuss specifically how the soil N pool responds to increasing shrub cover, and how changes in the soil microbial biomass and nutrient ratios associated with shrub encroachment influence N inputs by asymbiotic BNF in a semiarid grassland.

4.1 Distribution and speciation of soil nitrogen

Like other studies for the Southwestern US (e.g., Wheeler et al., 2007; Turnbull et al., 2010; Schlesinger et al., 1996; Michaelides et al., 2012), we determined that concentrations of total N are greater in surface soils under shrubs relative to intershrub areas for all sites along the encroachment gradient (Figure 5a-c). Further, whilst concentrations of total N in shrub

soils are not statistically separable, we found the total N content of intershrub soils declined between end-member sites coincident with reductions in herbaceous biomass (Figure 4a) and cover (Figure 1b). At site-level, concentrations of total N remained constant with increasing shrub cover (Figure 6a), and the accumulation of N beneath shrub canopies can be considered a direct consequence of N losses from intershrub soils. These findings contrast with studies which identified net increases (e.g., Boutton and Liao, 2010) and declines (e.g., Jackson et al., 2002) in the total N pool with shrub encroachment, but support the widely held perspective that transitions from continuous grass- to shrub-dominated states increase the spatial heterogeneity of soil resources (D'Odorico et al., 2013; Peters et al., 2006; Schlesinger et al., 1990).

Organic N (total N minus inorganic N) comprises >98% of total N in all intershrub and shrub soils (Figure 5a-c). Thus, the elevated concentrations of total N in shrub soils, which are ≥1.5 times those of intershrub soils, represent larger stores of mineralisable N. Rates of N mineralisation are inversely related to soil C:N ratios (Finzi et al., 1998), which in turn have been shown to decrease under mesquite canopies in response to inputs of higher quality litter (Geesing et al., 2000; Throop and Archer, 2007). However, like Wheeler et al. (2007), we found that soil C:N ratios, which are lower than those previously reported for the area (Wheeler et al., 2007), are not affected by shrub encroachment processes in the SRER (Figure 7a). Whilst this could be due in part to depressed subcanopy soil-litter mixing (Throop and Archer, 2008), the accumulation (and flux) of N has been shown to share a positive relationship with the soil clay content (Virginia and Jarrell, 1983; Michaelides et al., 2012), which was lower in soils beneath shrub canopies relative to those from intershrub areas (Table 1). Nevertheless, shrub soils contain greater concentrations of NH_4^+-N and NO_3^--N than intershrub soils at all sites except S-56 (Figure 5b,c), which suggests the microbial community beneath shrub canopies can mineralise organic forms of N more readily. This is supported by a study for a semiarid grassland in central Spain which showed the availability of inorganic N is largely influenced by the abundance and functional diversity of the microbial community (Delgado-Baquerizo et al., 2015).

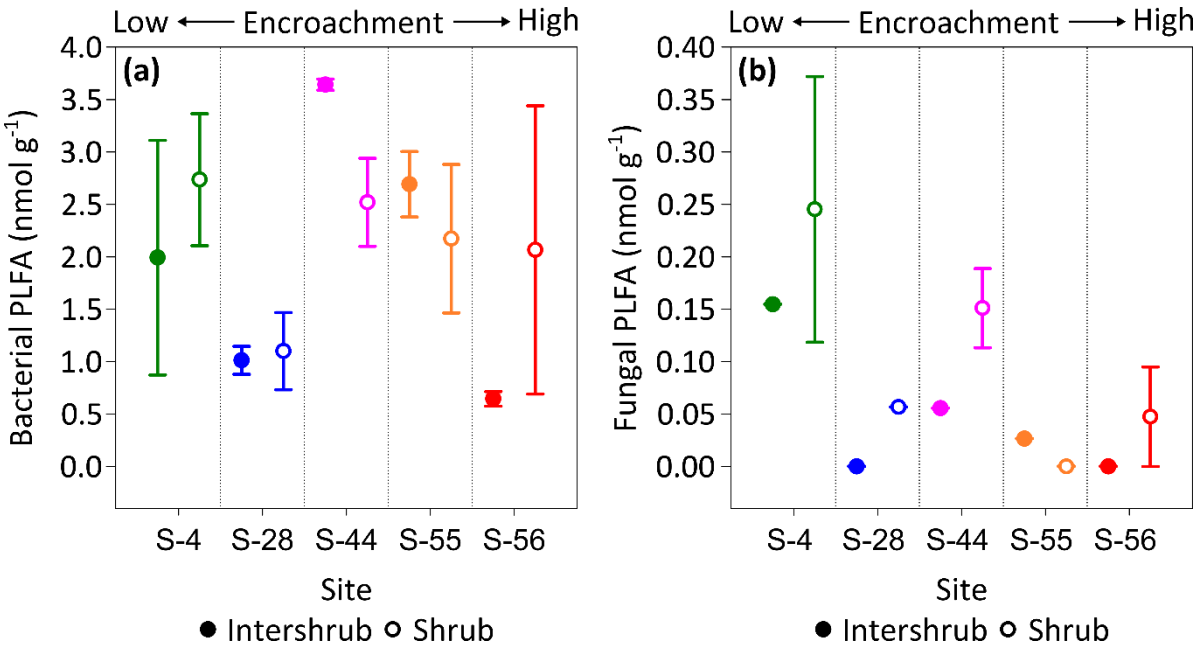

Figure 8. Median bacterial (a) and fungal (b) phospholipid fatty acid (PLFA) concentrations for surface soil (top 10 cm) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 3). Error bars represent median absolute deviation about the median. All results reported on dry matter basis.

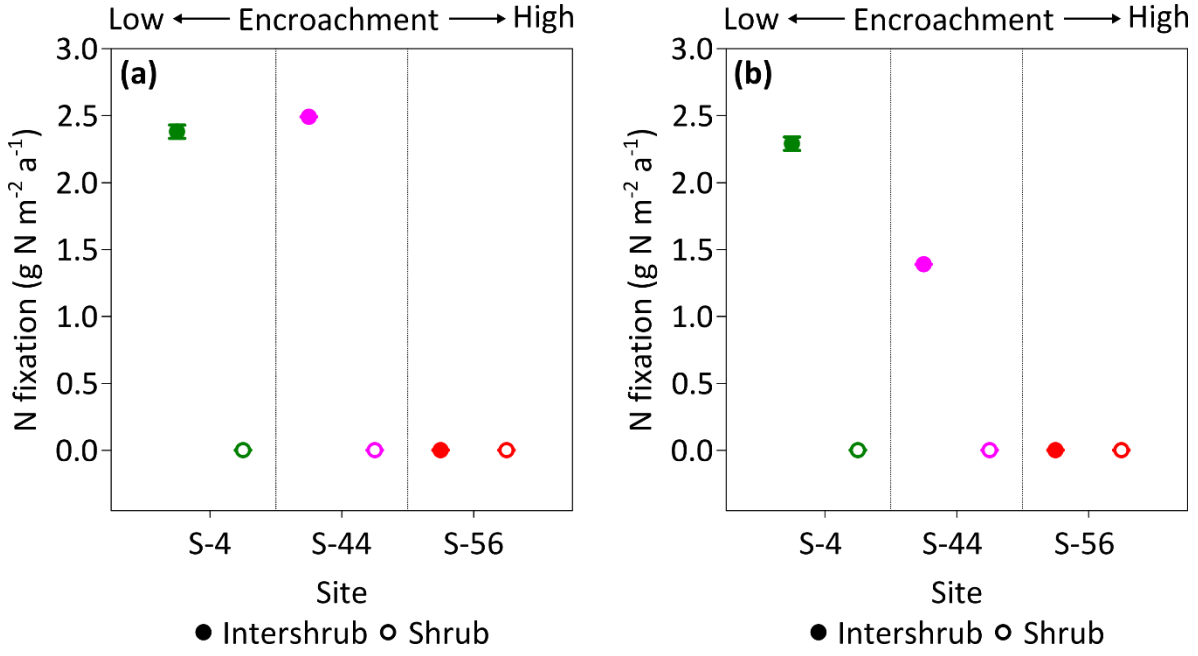

Figure 9. Median rates of asymbiotic nitrogen (N) fixation for intershrub and shrub surface (top 10 cm) soil (a) and intershrub and shrub surface soil weighted by area cover (b) along a gradient of shrub encroachment in the Santa Rita Experimental Range, AZ, US (n = 3). Error bars represent median absolute deviation about the median. N fixation rates were determined using the acetylene reduction assay method where ethylene was converted to N using a 3:1 ratio (Telling et al., 2011). Results reported on dry matter basis.

In this study, bacterial PLFA concentrations did not follow a clear trend with increasing shrub encroachment (Figure 8a), whereas the fungal PLFA content of both intershrub and shrub soils decreased linearly between end-member sites (Figure 8b). These results contrast with previous studies which found the microbial biomass to be greater in soils beneath shrub canopies than adjacent grasses due to reduced environmental stress and greater stores of SOM (Gallardo and Schlesinger, 1992; Li et al., 2017; Ewing et al., 2007). As ratios between the fungal and bacterial constituents of the microbial community share a negative relationship with soil pH (Bååth and Anderson, 2003), a decline in the fungal PLFA content of shrub soils can be attributed to changes in shrub soil pH which increased with gains in shrub cover (Table 1). However, the same cannot be ascribed to changes in the fungal PLFA content of intershrub soils, as declines between end-member sites occurred with decreases in soil pH. Instead, if total N is used as a proxy for SOC (intershrub soil C:N ratios do not differ between sites), reductions in intershrub fungal PLFA concentrations with increasing encroachment may be indicative of C limitation for growth, which would be expected to reduce the microbial biomass and release inorganic N (Cookson et al., 2006). Accordingly, in addition to an absence of fungal PLFAs, intershrub soil at S-56 exhibits the lowest bacterial PLFA content in comparison with all other soils along the encroachment gradient, as well as the highest concentration NO_3^--N within the intershrub soils (Figure 5c). As fungi have been shown to preferentially utilise grassland C (Creamer et al., 2016), declines in the shrub soil fungal PLFA content with increasing encroachment may also be a response to the progressive depletion of grass-derived SOC.

It must be noted that we recommend caution when interpreting the vegetation cover and biomass data presented here. The classification accuracy of cover estimates, which are subject to image spatial resolution, as well as land-cover spatial and spectral heterogeneity (Hu et al., 2013), were not formally assessed. Likewise, woody and herbaceous biomass estimates were derived from allometric relationships that had not been ground-truthed and which could potentially vary by soil type, surface morphology and disturbance history (Nafus et al., 2009; McClaran et al., 2013). However, as relative changes in percentage grass and shrub cover can explain concurrent changes in grass and shrub biomass (Figure 4c,d), these estimates were useful for capturing the extent of shrub encroachment at each site.

4.2 Inputs of asymbiotically-fixed nitrogen

In this study we explored a gap in the literature concerning the impacts of increasing shrub encroachment (at sites S-4, S-44 and S-56 only) on the rates and distribution of asymbiotic BNF in a semiarid grassland. We found that asymbiotic BNF was only detectable in intershrub soils at sites S-4 and S-44 (Figure 9a), which exhibited significantly lower N:P ratios than adjacent shrub soils (Figure 7b). These findings reinforce the theory that increases of inorganic N relative to available P inhibit nitrogenase activity, the enzyme responsible for mediating the adenosine triphosphate-reduction of N_2 to ammonia in BNF (Smith, 1992). However, despite intershrub soil N:P ratios remaining constant with increasing encroachment, there was an absence of asymbiotic BNF at S-56. As asymbiotic diazotrophs (N fixing organisms) are largely contained within the bacterial fraction of the microbial biomass (Poly et al., 2001), the undetectable rates of asymbiotic BNF at S-56 could be attributed to the low bacterial PLFA concentration at this site (Figure 8a). This conclusion is supported by increases in asymbiotic BNF rates with gains in the bacterial PLFA content of intershrub soils between sites S-4 and S-44.

When weighted by intershrub area, rates of asymbiotic BNF declined from 2.3 g N m^{-2} a^{-1} at S-4 to 1.4 g N m^{-2} a^{-1} at S-44 (Figure 9b). As levels of total N deposition (0.1-0.2 g N m^{-2} a^{-1}) in the Southwestern US are generally low (Schwede and Lear, 2014), the decreasing rates of BNF associated with increasing site-level concentrations of inorganic N (Figure 6b,c) with shrub encroachment represent the loss of a significant N input pathway to these already N-limited systems, which Belnap (1995) argues may not be readily recovered. However, the rates of asymbiotic BNF reported here, which exceed previous estimates for dryland systems by an order of magnitude (Cleveland et al., 1999), represent an absolute upper limit as soil moisture, temperature and light were maintained during the incubation experiments. Constant incubation parameters were used to achieve linear rates of C_2H_2 reduction over a 24-hour period, where parameters were chosen to reflect mean summer conditions when microbial activity is greatest due to increased soil water availability (Loik et al., 2004).

A study by Geesing et al. (2000) showed the development of the soil N pool beneath mesquite canopies is attributable to inputs of symbiotically-fixed N. As site-level concentrations of total N do not differ between end-member sites, we argue the loss of intershrub soil N and asymbiotic BNF with shrub encroachment seen here are balanced by inputs of symbiotically-fixed N by mesquite. However, as the redistribution of soil resources with shrub encroachment modifies the development of shrub over intershrub soils, inputs of N by mesquite-*Rhizobium* symbioses are ultimately unavailable to sustain grass production in intershrub areas. Consequently, transitions from grass- to shrub-dominated semiarid systems may be viewed as a shift between bistable states from which intershrub areas may not readily recover (D'Odorico et al., 2013).

5 Conclusions

Over the past century, the semiarid grasslands of the southwestern US have been degraded by the encroachment of mesquite, an N-fixing shrub. From this comprehensive study, we found the site-level soil N pool remained constant along a gradient of increasing shrub cover within a semiarid grassland of the SRER, but that the distribution of nitrogen within the ecosystem changed as declines in the intershrub soil N pool were proportional to increases in soils under shrub canopies. Yet the inorganic fraction of the site-level soil N pool increased with shrub area and biomass due to the long-term accumulation of NH_4^+-N and NO_3^--N in soils beneath shrub canopies. Considering soil C:N ratios did not differ between sites or cover type, increases in concentrations of inorganic N in shrub soils were attributed to the capacity of the soil microbial community to mineralise organic forms of N. Finally, as the ratio of inorganic N to bioavailable P has been shown to influence nitrogenase activity, we explored the effects of shrub encroachment on the distribution and rates of asymbiotic BNF in the grassland soil. We found that BNF was inhibited by higher concentrations of inorganic N to available P and, when weighted by intershrub area, provided a lower input of new N to the system with increasing shrub cover. The loss of this ecosystem process, as well as declines in the intershrub soil N pool, were potentially balanced by inputs of symbiotically-fixed N by mesquite. Consequently, we conclude the long-term accumulation of N beneath shrub canopies combined with increases in shrub canopy area have the potential to significantly change the dynamics of soil N cycling in dryland systems.

Data availability

All data presented in figures 4-9 are available online at DOI: 10.5523/bris.28ck0lsp9c48e1zosa7h2c538u.

Author contributions

TTJ led the design of the study, assisted by KM and AMA. All sample collection and analyses were performed by TTJ. TTJ wrote the manuscript with contributions from KM, JAB and AMA.

Competing interests

The authors declare that they have no conflict of interest.

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Google Earth: Grass-Dominant Shrub Mix, 31°47'16.12"N, 110°50'34.84"W, elevation 1,250 m, 2016c.
Google Earth: Shrub-Dominant Grass Mix 31°47'57.39"N, 110°49'33.13"W, elevation 1,250 m, 2016d.
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