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Bacterial, fungal, and plant communities exhibit no biomass or compositional response to two years of simulated nitrogen deposition in a semiarid grassland

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Summary

Nitrogen (N) deposition affects myriad aspects of terrestrial ecosystem structure and function, and microbial communities may be particularly sensitive to anthropogenic N inputs. However, our understanding of N deposition effects on microbial communities is far from complete, especially for drylands where data are comparatively rare. To address the need for an improved understanding of dryland biological responses to N deposition, we conducted a two-year fertilization experiment in a semiarid grassland on the Colorado Plateau in the southwestern United States. We evaluated effects of varied levels of N inputs on archaeal, bacterial, fungal and chlorophyte community composition within three microhabitats: biological soil crusts (biocrusts), soil below biocrusts, and the plant rhizosphere. Surprisingly, N addition did not affect the community composition or diversity of any of these microbial groups; however, microbial community composition varied significantly among sampling microhabitats. Further, while plant richness, diversity, and cover showed no response to N addition, there were strong linkages between plant properties and microbial community structure. Overall, these findings highlight the potential for some dryland communities to have limited biotic ability to retain augmented N inputs, possibly leading to large N losses to the atmosphere and to aquatic systems.

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Introduction

Humans have more than doubled the input of available nitrogen (N) to the Earth's land surface (Galloway *et al.*, 2008; Vitousek *et al.*, 2013), and numerous studies document the significant effects of this change on ecosystem properties (e.g., Baron *et al.*, 2000; Pardo *et al.*, 2011; Ochoa-Hueso *et al.*, 2013; Rao *et al.*, 2014). For example, increased N deposition has been shown to greatly decrease air and water quality, increase greenhouse gas emissions and exotic plant invasion, and alter natural fire regimes (Aber *et al.*, 1989; Townsend *et al.*, 2003; Galloway *et al.*, 2008). Thus, N deposition can affect fundamental aspects of terrestrial ecosystem structure and function. However, not all ecosystems can be expected to respond to N deposition in the same manner, and despite an increase in work in arid ecosystems (reviewed by Sinsabaugh *et al.*, 2015), there is a notably incomplete understanding of how N deposition will affect dryland ecosystems.

Drylands – lands characterized by an overall climatic water deficit ($<$ 0.65 mm/mm threshold) that is calculated using an aridity index: the ratio of precipitation to potential evapotranspiration – account for approximately 40% of the global terrestrial land area (Safriel *et al.*, 2005) and roughly 35% of the United States (Pointing and Belnap, 2012). Drylands include arid, semiarid and dry subhumid areas (Reynolds *et al.*, 2007). Taken together, these diverse ecosystems represent our planet's largest biome (Schimel, 2010) and exhibit some of the greatest observed sensitivity to climatic variability and land use change (Morgan *et al.*, 2011; Poulter *et al.*, 2014; Ahlstrom *et al.*, 2015; Ferrenberg *et al.*, 2015; Wertin *et al.*, 2015; Reed *et al.*, 2016). Based on this sensitivity and the relatively low N stocks typically found in arid and semiarid ecosystems, it is no surprise that several studies have suggested that drylands will be susceptible to increasing anthropogenic N inputs

(Baron *et al.*, 2000; Pardo *et al.*, 2011; Mueller *et al.*, 2015; Sinsabaugh *et al.*, 2015). Indeed, the limited data that do exist suggest North American deserts may maintain lower N deposition critical thresholds – the amount of N deposition beyond which an ecosystem response is observed – compared with more mesic ecoregions (Pardo *et al.*, 2011; Blett *et al.*, 2014; Sinsabaugh *et al.*, 2015). However, dryland ecosystems are diverse, as are the anthropogenic N inputs into these ecosystems (e.g., Reed *et al.*, 2013), and consequently, responses to N deposition may vary. Thus, research is still needed to understand how anthropogenic N inputs affect these varied and important landscapes (Porter *et al.*, 2005; Blett *et al.*, 2014), and to make predictions about the population-, community-, and ecosystem-level effects of N deposition.

In particular, our understanding of dryland microbial community response to N deposition remains notably poor (but see Zeglin *et al.*, 2007; Li *et al.*, 2010, Mueller *et al.*, 2015; Sinsabaugh *et al.*, 2015; Ochoa-Hueso *et al.*, 2016), which significantly constrains our ability to consider and anticipate the effects of N deposition across multiple spatial scales. Microbial community composition is a fundamental control over terrestrial ecosystem functioning, affecting critical ecosystem processes such as litter decomposition (Allison *et al.*, 2013) and plant fitness (Lau and Lennon, 2012). Recent research suggests that N deposition significantly alters soil microbial communities and the functions they perform, and the N fertilization experiments commonly used as a proxy for N deposition have documented N-induced changes to microbial community composition, richness, respiration rates, and enzyme activities across many ecosystem types (e.g., Janssens *et al.*, 2010; Liu and Greaver, 2010; Sinsabaugh *et al.*, 2015). However, data that inform our understanding of dryland microbial reactions to N deposition remain sparse and are unlikely to capture the potential range of dryland responses.

The southwestern United States has become a 'hotspot' for N deposition (Fenn *et al.*, 2003; Reed *et al.*, 2013), and population growth and energy development continue to elevate N deposition in North American drylands. Here, we set out to advance our understanding of how soil microbial communities will respond to N deposition using an N fertilization experiment in a semiarid grassland containing common native and exotic plant species and early successional biological soil crust (biocrust) communities on the Colorado Plateau in the southwestern U.S. To search for N deposition critical thresholds, we used a range of N input levels (0, 2, 5 and 8 kg N ha^{-1} $year^{-1}$). These relatively low rates, compared with previous N amendment experiments conducted in forest ecosystems, were designed to assess critical thresholds against the lower background N deposition seen in many North American drylands (N deposition is estimated for this site at $\sim$3 kg N ha^{-1} $year^{-1}$; Fenn *et al.*, 2003), and are more likely to capture biologically meaningful responses (Gomez-Casanovas *et al.*, 2016). Beginning in 2011, fertilizer was applied twice yearly, with half distributed in spring and half in fall. By sampling soils before and after the spring fertilization in 2013, we used high-throughput sequencing and quantitative PCR (qPCR) of ribosomal RNA genes to examine effects of N treatments on soil microbial communities. We also monitored associated nutrient transformations in soil, and the aboveground plant community response to experimental manipulation. Because previous work indicated that dryland soil microbial communities can vary across shallow soil depths and between plant- and biocrust-associated soils (Steven *et al.*, 2013; Steven *et al.*, 2014; Mueller *et al.*, 2015), we sampled discretely among three microhabitats in the ecosystem: within biocrusts, below-biocrusts (3 − 6 cm depth), and in rhizosphere zones. Based on the previous studies and the low N stocks at our site, we had three core hypotheses: (1) increased N availability would be a key driver of microbial community structure and would significantly affect soil microbial community composition and function at all N input levels, (2) the different microhabitats from which soil samples were collected (e.g., biocrust vs. rhizosphere) would harbor distinct microbial communities, and (3) microhabitat would interact with the fertilization treatments to determine the nature and magnitude of the N effect on microbial communities.

Results

Phylogenetic composition and diversity

High-throughput sequencing produced 1,916–49,463 sequences per sample (median: 14,690) of the 16S rRNA gene and 2,101–49,819 sequences per sample (median: 14,917) of the 28S rRNA gene. In an effort to remove sequencing depth heterogeneity, 16S and 28S rRNA gene sequences were randomly subsampled at a depth of 1,916 and 2,101, respectively. In contrast to our predictions that increased N inputs would alter microbial communities, N fertilization did not produce any measureable effects on soil microbial community composition (Fig. 1). This includes finding no differences in soil microbial community after the two years of fertilization (i.e., comparing communities among the treatment plots), and no differences among the community in two sets of soil samples taken within a three week period in spring 2013: one set prior to fertilization and one following fertilization. Because microbial communities from soils collected two weeks prior to fertilization in 2013 were statistically indistinguishable from those in soils collected nine days following fertilization, data for the two time points were combined and analysed concurrently. Specifically, community composition was unaffected by N fertilization for prokaryotes (bacteria plus archaea; PerMANOVA $F = 0.97$, $p = 0.43$), fungi ($F = 0.91$,

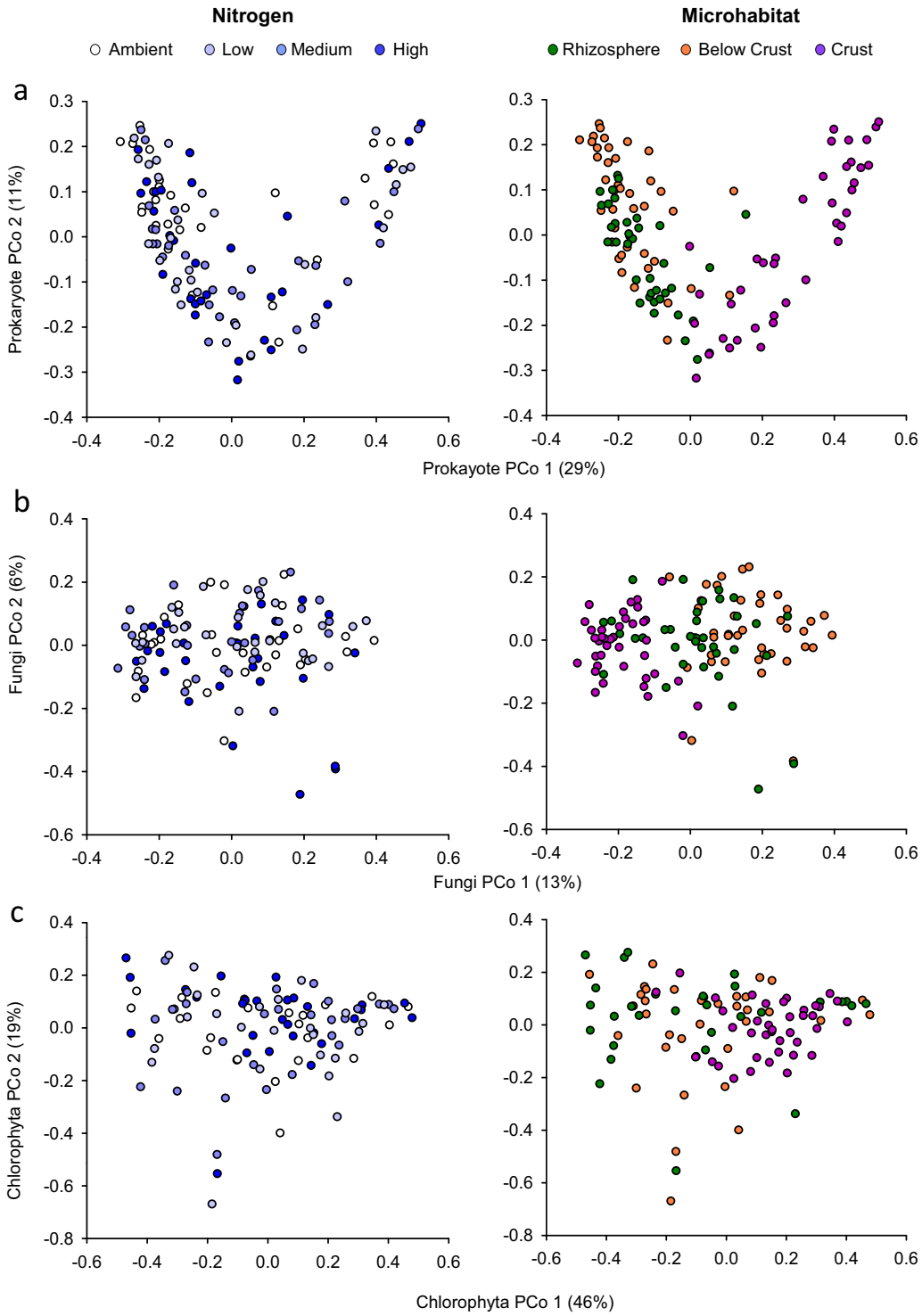

Fig. 1. Community composition of prokaryotes (bacteria plus archaea) (a), fungi (b), and Chlorophyta (c) colored by N treatment (left) and sampling location (right). [Colour figure can be viewed at wileyonlinelibrary.com]

$p = 0.51$) and Chlorophyta (green algae; $F = 0.95$, $p = 0.50$). Microbial diversity was similarly unaffected by N fertilization (Table 1). In sum, there were no observable fertilization effects on microbial community composition with the fertilization event (i.e., communities sampled before and after spring 2013 fertilization were the same), nor with the two years of treatments (i.e., communities compared among $0 - 8$ kg N ha^{-1} $year^{-1}$ plots were the same).

Table 1. Microbial abundance data (assessed by qPCR), microbial diversity, and soil chemistry according to sampling location and N treatment. Values are means and standard errors.

	Microbial abundance			Microbial diversity		Soil chemistry		
Location Treatment	16S rRNA[a]	18S rRNA[a]	F:B ratio	Bacteria (Chao 1)	Fungi (Chao 1)	Total C (%)	Total N (%)	pH
Crust								
Ambient	8.6 ± 0.1	7.7 ± 0.2	0.1	1033 ± 89	168 + 19	0.9 ± 0.1	0.05 ± 0.004	7.8 ± 0.08
Low	8.7 ± 0.1	7.7 ± 0.2	0.1	1007 ± 70	185 + 23	1.0 ± 0.1	0.06 ± 0.004	7.8 ± 0.04
Medium	8.7 ± 0.1	7.9 ± 0.2	0.2	1059 ± 95	175 + 13	1.2 ± 0.1	0.06 ± 0.008	7.8 ± 0.09
High	8.5 ± 0.2	7.7 ± 0.1	0.2	1003 ± 126	186 + 23	1.0 ± 0.1	0.05 ± 0.004	7.8 ± 0.08
Below crust								
Ambient	8.6 ± 0.2	7.1 ± 0.1	0.05	1541 ± 80	199 + 29	0.8 ± 0.1	0.04 ± 0.004	7.8 ± 0.08
Low	8.6 ± 0.1	7.3 ± 0.2	0.1	1512 ± 72	201 + 22	1.0 ± 0.2	0.04 ± 0.004	7.8 ± 0.09
Medium	8.8 ± 0.1	7.4 ± 0.1	0.07	1453 ± 57	184 + 31	0.9 ± 0.1	0.04 ± 0.003	7.8 ± 0.09
High	8.7 ± 0.1	7.1 ± 0.2	0.05	1517 ± 73	177 + 14	0.8 ± 0.1	0.04 ± 0.003	7.8 ± 0.08
Rhizosphere								
Ambient	8.2 ± 0.1	7.3 ± 0.2	0.2	1590 ± 77	200 + 21	0.9 ± 0.1	0.05 ± 0.008	8.0 ± 0.04
Low	8.3 ± 0.1	7.5 ± 0.2	0.3	1510 ± 73	214 + 32	1.0 ± 0.1	0.05 ± 0.004	8.0 ± 0.08
Medium	8.3 ± 0.1	7.7 ± 0.2	0.5	1531 ± 129	197 + 32	0.9 ± 0.1	0.06 ± 0.004	8.0 ± 0.09
High	8.3 ± 0.1	7.7 ± 0.1	0.3	1470 ± 111	176 + 28	0.9 ± 0.1	0.05 ± 0.008	7.9 ± 0.05
ANOVA								
Treatment	0.66	0.20	0.43	0.77	0.59	0.16	0.27	0.95
Location	<0.01	<0.01	<0.01	<0.01	0.38	0.12	<0.01	<0.01
Interaction	0.69	0.60	0.59	0.92	0.90	0.71	0.89	0.96

a. $\log_{10}$ gene copies per g dry soil.

In line with our hypotheses, prokaryotic community composition did differ by microhabitat (PerMANOVA $F = 56.60$, $p < 0.01$), where biocrust communities were compositionally distinct from below-biocrust and rhizosphere communities (Fig. 1a). Biocrust communities were dominated by Cyanobacteria, while below-biocrust and rhizosphere communities had larger proportions of Actinobacteria and Crenarchaeota (Fig. 2a). Fungal communities showed significant compositional differences between biocrust and below-biocrust communities, as well as between biocrust and rhizosphere communities (PerMANOVA $F = 12.92$, $p < 0.01$; Fig. 1b). Fungal communities in all microhabitats were dominated by Ascomycota and Basidiomycota, though below-biocrust and rhizosphere communities exhibited larger proportions of Blastocladiomycota and Chytridiomycota than did biocrust communities (Fig. 2b). Similarly, Chlorophyta showed compositional differences according to microhabitat (PerMANOVA $F = 10.12$, $p < 0.01$), with communities within biocrusts being of distinct composition compared with below-biocrust and rhizosphere communities (Fig. 1c).

Consistent with the community composition results, qPCR of 16S and 18S rRNA genes suggested that microbial abundance did not vary with N fertilization or time of

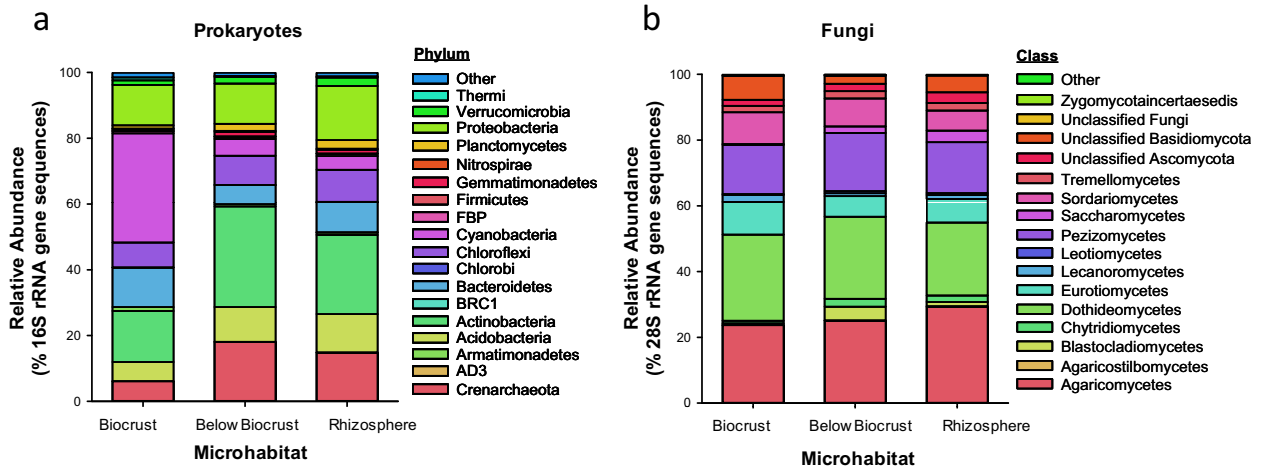

Fig. 2. Relative abundance of prokaryotic phyla (a) and fungal classes (b) as determined by high-throughput sequencing of rRNA genes. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. Plant community metrics associated with each treatment group. Values are means and standard errors.

N treatment	Richness	Diversity[a]	Aerial cover (%)	Basal cover (%)
Ambient	5.3 ± 0.1	0.6 ± 0.01	61.9 ± 0.8	4.5 ± 0.36
Low	5.5 ± 0.2	0.6 ± 0.02	61.6 ± 1.0	5.5 ± 0.4
Medium	6.2 ± 0.1	0.7 ± 0.01	59.6 ± 1.5	3.7 ± 0.2
High	5.2 ± 0.2	0.6 ± 0.1	60 ± 0.76	3.5 ± 0.2
ANOVA (p)	0.37	0.21	0.89	0.21

a. Simpson's Index (1-D).

sampling (before and after the fifth fertilization event), though gene copy numbers and the ratio of fungi to bacteria were significantly different according to microhabitat (biocrust vs. below-biocrust vs. vascular plant rhizosphere; Table 1). Specifically, 16S rRNA gene abundance in the rhizosphere was significantly lower than that of biocrust and below-biocrust microhabitats ($p < 0.01$ in each case). For the 18S rRNA gene, abundance below biocrusts was significantly lower than within biocrusts ($p < 0.01$); abundance in rhizosphere and below-biocrust locations also differed, with fewer gene copies below biocrusts ($p < 0.01$).

Plant response and community linkages

As was the case for soil microbial communities, we saw no impact of N addition on plant communities. Plant richness showed no significant variation among N treatments, with average richness ranging from 5.2 to 6.2 species per plot. Measures of plant diversity, aerial cover and basal cover were statistically indistinguishable among treatment plots (Table 2), and there were no differences in foliar N concentrations among treatment plots for the *Achnatherum hymenoides* (Indian ricegrass) plant centered within each plot ($p = 0.49$ in spring 2013 samples).

We did observe significant relationships among microbial groups and between above- and belowground communities. Prokaryotic community composition covaried with the composition of both fungal (Mantel $r = 0.29$, $p < 0.01$) and chlorophyte communities (Mantel $r = 0.17$, $p < 0.01$). Further variation in prokaryotic community composition was associated with aspects of the plant community, including plant richness (Mantel $r = 0.10$, $p < 0.01$) and aerial cover (Mantel $r = 0.08$, $p < 0.01$). Fungal community composition also covaried with plant aerial cover (Mantel $r = 0.15$, $p < 0.01$). Thus, although treatment effects on the above- and belowground communities were not observed, natural variation among the plots did show significant relationships among factors.

Soil nutrient pools

Total soil carbon (C) and N concentrations were unaffected by N fertilization, though across all treatments combined, total N was significantly higher within biocrusts and in the rhizosphere than below biocrusts (Table 1; $p < 0.01$ in both cases). Soil pH did not respond to N addition, yet varied by microhabitat, with pH in the rhizosphere significantly higher than in biocrust or below-biocrust microhabitats ($p < 0.01$ in each instance). Soil extractable ammonium and nitrate concentrations in the treatment plots were significantly increased on the day immediately following fertilization (21 March 2013; $p < 0.01$ and $p < 0.05$ for ammonium and nitrate, respectively; Fig. 3). Soil extractable ammonium concentrations remained elevated in the N addition plots five days after fertilization (26 March 2013; $p < 0.01$), although extractable nitrate concentrations showed no significant differences at this time. Ammonium and nitrate concentrations returned to pre-fertilization levels 21 days

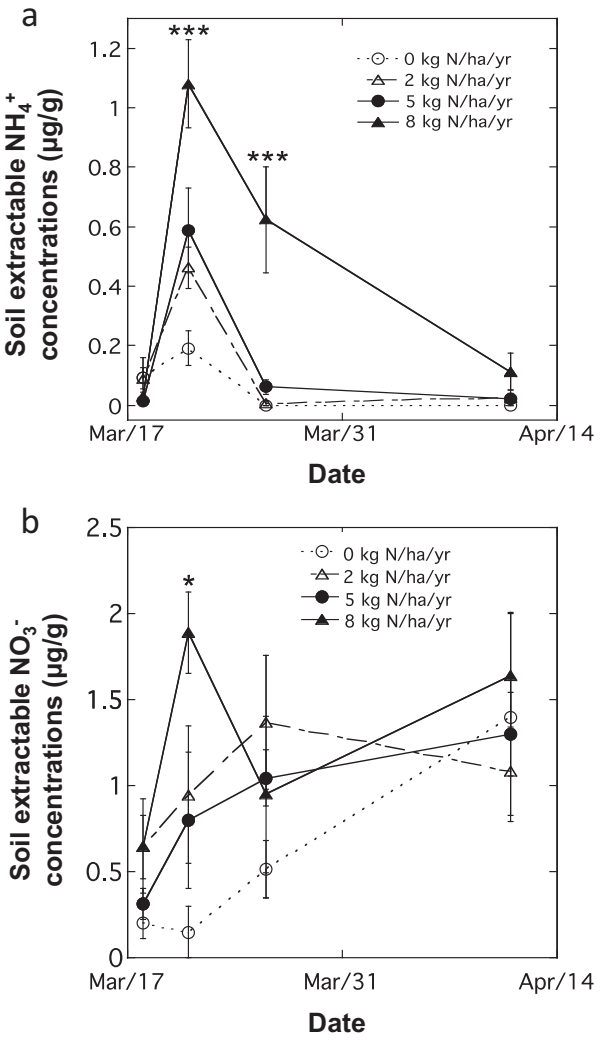

Fig. 3. Soil extractable ammonium (a) and nitrate (b) concentrations for 0-10 cm depth soil collected prior to and following fertilization for the four N fertilization treatment plots. Values are means and standard errors.

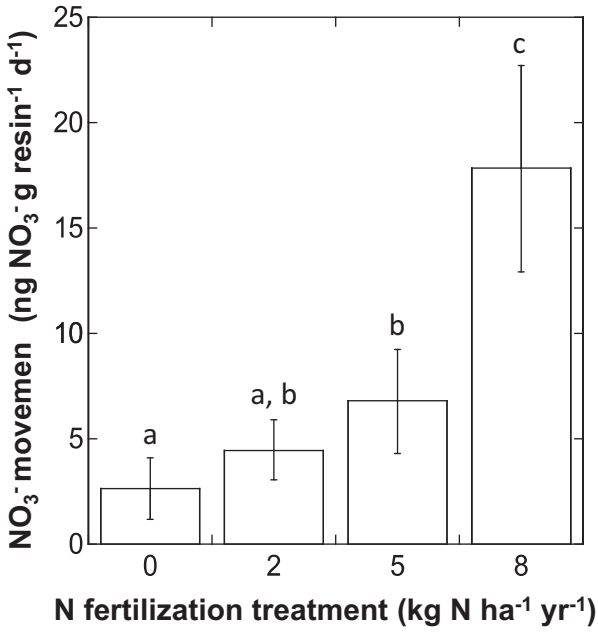

Fig. 4. Nitrate (NO_3^-) flux rates from buried resin caps placed within each plot. Values are means and standard errors, and lowercase letters depict significant differences among treatments ($p < 0.05$).

following fertilization (11 April 2013), suggesting the lack of a sustained N fertilization effect on available soil N pools. Buried resin caps set at 10 cm depth tracked the movement of inorganic N out of surface soil layers, and the flux of nitrate into resin was highest in the 8 kg N ha^{-1} year^{-1} plots, significantly lower in the 5 kg N ha^{-1} year^{-1} plots ($p < 0.05$), and lowest in the 0 kg N ha^{-1} year^{-1} plots ($p < 0.01$), with the 2 kg N ha^{-1} year^{-1} values falling between the 0 and 5 kg N ha^{-1} year^{-1} ($p > 0.1$ for each; Fig. 4). Passive samplers were used to compare NO_x emission rates among treatment plots, and on the day of fertilization the 8 kg N ha^{-1} year^{-1} plots maintained significantly higher outputs of NO_x compared with the plots receiving no additional N ($p < 0.05$). The emissions of NO_x from the 3 and 5 kg N ha^{-1} year^{-1} treatment plots fell between the low and high N input end members.

Discussion

Lack of effect of N addition on soil microbial communities

In contrast to our predictions, no aspect of microbial community composition was affected by two years of N fertilization. For prokaryotes, fungi and chlorophytes, communities from control plots were indistinguishable from those in all treatment plots after two years of N enrichment. Additionally, comparisons among communities from two weeks prior to the fifth fertilizer application and nine days following fertilization showed no effect of N treatment on microbial community composition. We also observed no changes in the abundance of 16S or 18S rRNA genes with N fertilization. Our comprehensive approach examined all measurable aspects of microbial community composition among organisms across the tree of life, and we found no significant effect of any of the N treatments after two years.

Why didn't microbial communities respond to N inputs when other studies have observed responses? There are a number of potential explanations. First, the N inputs used here were based upon levels of N deposition recorded in the study area (~3 kg N ha^{-1} year^{-1}), and while these amounts are more realistic for our area and are in line with many newer studies that use smaller inputs (Gilliam *et al.*, 2016; Kox *et al.*, 2016; Tulloss and Cadenasso, 2016), the inputs are much lower than the levels of enrichment often used in nutrient limitation studies and in areas of exceptional deposition, such as some parts of Europe (Holland *et al.*, 2005). Second, as with most N deposition experiments, we applied the N in pulses that joined smaller daily rates into a compound amount, in our case applied twice yearly. Accordingly, the amounts of N that we added were higher than what would be expected on a short timescale in our area. Yet even with these larger pulse inputs, we did not observe any effect of increasing N. Third, it may be that the availability of another resource, such as water or C, limits the biota's capacity to take advantage of additional N (e.g., Ramirez *et al.*, 2010); thus, the N is not able to be immobilized from the soil environment and is available to be lost from the system even with low levels of N input (Lovett and Goodale, 2011). Fourth, it has been suggested that N deposition can indirectly affect communities via changes to soil pH (e.g., Hallin *et al.*, 2009; Ramirez *et al.*, 2010; Zhalnina *et al.*, 2015), but the buffering capacity of our soils in the form of high calcium carbonate (Table 1) would render these dryland soils resistant to changes in pH. Finally, it is possible that the effect of N deposition as seen from fertilization experiments will take longer to emerge than the two years of this study or, instead, that the thresholds of N deposition effects were so low that they had already been crossed by the low background N deposition occurring in the area. We delve into more detail regarding these concepts in the sections below.

Interestingly, a recent fertilization study (Mueller *et al.*, 2015; Sinsabaugh *et al.*, 2015) adds important context to these results, as the study was conducted in another common dryland ecosystem type (Mojave desert; vegetation dominated by *Larrea tridentata* and *Ambrosia dumosa*) and as the fertilization methods also used smaller inputs of N (0, 7 and 15 kg N ha^{-1} year^{-1}). While aspects of soil biogeochemical cycling responded strongly to N fertilization and although there were trends in microbial community data, there were no significant effects of N addition on the abundance of fungi, bacteria, or

cyanobacteria or on fungal:bacterial ratios (Sinsabaugh *et al.*, 2015). In the same plots, significant N impacts were found for bacterial community composition and diversity, but fungal community composition was not significantly affected by N application after two years (Mueller *et al.*, 2015). Thus, while dryland data suggest strong potential for nonlinear biomass and biogeochemical consequences of N deposition (Sinsabaugh *et al.*, 2015), and for components of the community to respond to this enrichment (Mueller *et al.*, 2015), the Mojave desert results alongside the Colorado Plateau data presented here provide novel insight into the potential for aspects of resistance in dryland microbial communities, and for differential dryland community responses to similar N inputs. Further work exploring the mechanisms through which dryland microbial communities are affected by N inputs would represent a dramatic advance in our understanding of how N deposition regulates ecosystem structure and function in our world's largest biome (Schimel, 2010).

A lack of microbial community response has important implications for how N deposition in drylands may affect air quality, water quality, and emissions of the powerful greenhouse gas N_2O. Regardless of the cause (e.g., overwhelming water limitation, high soil buffering capacity, a community that made a rapid initial shift when N deposition began increasing decades ago), the data from this study depict an ecosystem that in many ways behaves like an N saturated ecosystem.

The fate of added N

Taken together, the biogeochemical data suggest that the added N does not persist in soils of this arid grassland, and that plants were not significantly affected by short-term N additions. Fertilization only increased inorganic N concentrations in treatment plots immediately after the application (Fig. 3). Across all levels of N addition, treatment effects on N extractable pools were undetectable within one month of fertilization. Gaseous and dissolved N data suggest N was leaving the system as a gas and in liquid form. For example, NO_x efflux rates were 15% higher in the 8 kg N ha^{-1} $year^{-1}$ plots immediately following fertilization relative to the control plots, and the 3 and 5 kg N ha^{-1} $year^{-1}$ plots fell in between these low and high values. Resin capture results showed N was moving down through the soil profile, potentially into the local hydrological system (Fig. 4). Consistent with these results, foliar N concentration of *A. hymenoides* was not increased by fertilization, and no changes in plant cover or diversity were observed. These data are in agreement with other desert N deposition studies suggesting that plants may only respond to increased N inputs when enough water is available (the relationship between water availability and N sensitivity remains unknown at this site), and some plants do not respond even in wetter years (Hall *et al.*, 2011). Combined, the microbial and biogeochemical data from this Colorado Plateau site illustrate an ecosystem that may not have the capacity to utilize increased inputs of N, perhaps due to water or C limitation. The N is quickly lost as gases and in dissolved forms, and these losses from the system represent potential negative consequences for the region (e.g., NO_x emissions and the formation of O_3) and the planet (e.g., increased N_2O emissions).

Role of plants and soil characteristics in structuring microbial communities

It has been suggested that microbial responses to N deposition may be indirectly mediated by plant C allocation responses to the added N (Ramirez *et al.*, 2010; Leff *et al.*, 2015). In other words, N deposition impacts plants (e.g., by altering plant litter chemistry, root growth, and root exudation; Prober *et al.*, 2015), and it is these plant-driven changes that alter the composition of the microbial community (Ramirez *et al.*, 2010; Leff *et al.*, 2015). In this way, it could be important that neither plant nor microbial communities responded to the experimental addition of N. Clearly more work is needed to elucidate the individual and coupled controls over above- and belowground responses. In arid grasslands, plants and biocrusts are likely to be the main determinants of microbial community composition, as they supply the majority of soil organic C and fixed N that enable a heterotrophic lifestyle (Bardgett and Wardle, 2010). Soil microbial communities also have the capacity to impact plants through a variety of mechanisms including nutrient acquisition, organic matter decomposition, and the promotion or suppression of plant disease (Garbeva *et al.*, 2004; Singh *et al.*, 2004; Berg and Smalla, 2009; Latz *et al.*, 2012). We observed significant correlations between each of the microbial groups we examined, in addition to significant relationships between plant characteristics (richness and aerial cover) and microbial community composition. These results support the growing recognition that the structure and functioning of soil microbial communities result from complex interactions between the plant community, the soil environment, and microbe-microbe associations (Bakker *et al.*, 2014; Schlatter *et al.*, 2015).

Soil microbial communities may also be indirectly affected by chemical changes to soil characteristics (e.g., lowered pH and increased metal solubility; Bowman *et al.*, 2008), and numerous studies have documented substantial changes in pH with N fertilization (e.g., Hallin *et al.*, 2009; Ramirez *et al.*, 2010; Zhalnina *et al.*, 2015). As pH has been shown to be a major predictor of bacterial (Lauber *et al.*, 2009) and fungal (Tedersoo *et al.*, 2014) community composition across the globe, this key factor could also be linked to the changes in soil microbial

community composition and function that have been commonly observed in N fertilization experiments. However, in our study, N addition had no impact on the pH of treatment plots, likely due to the high buffering capacity of our soils (Table 1). With no associated change in pH or in plant foliar chemistry, aerial cover, or plant community composition, perhaps the stability of microbial communities in these alkaline soils should not be surprising.

Microhabitat was a stronger driver of microbial community composition than N amendment in our study, with distinct communities present in soils derived from biocrust, below-biocrust, and rhizosphere zones. These finding are consistent with prior studies in arid grasslands of this region (Steven *et al.*, 2013; 2014). The qPCR assays showed that bacterial and fungal abundances were higher within biocrusts than below biocrusts, which is in accordance with other dryland studies that have documented a concentration of microbial biomass in the top few centimeters of soil (Dunbar *et al.*, 2012; Pointing and Belnap, 2012; Steven *et al.*, 2013; Sinsabaugh *et al.*, 2015). Plants have been shown to have a strong influence on soil microbial communities in dryland systems, promoting islands of fertility (Schade and Hobbie, 2005; Perroni-Ventura *et al.*, 2010). Accordingly, rhizosphere communities are often compositionally dissimilar from those in unvegetated soils (Andrew *et al.*, 2012; Steven *et al.*, 2012; Ramond *et al.*, 2014). Thus, while simulated N deposition had no effect on any aspect of microbial community, our data are in line with other dryland results of distinct microbial communities among microhabitats separated by mere centimeters.

Conclusion

In sum, this work suggests a lack of response for native bunchgrass and soil microbial communities of the Colorado Plateau after two years of N amendment. In contrast to our predictions, the data highlight that the dryland soil microbial communities studied here were compositionally unresponsive to increased inputs of N. The implications of insensitivity to N deposition are considerable, as an unresponsive belowground community may result in heightened gaseous and dissolved N losses. Leaching of dissolved N can contribute to reduced water quality and eutrophication in aquatic ecosystems (Bergström and Jansson, 2006; Howarth and Marino, 2006; Turner *et al.*, 2006). Enhanced gaseous N losses can be similarly detrimental, resulting in increased emissions of the powerful greenhouse gas N_2O.

Experimental procedures

Site description

The experimental site is within Arches National Park (38° 47′N, 109° 39′W) near the Park's northwest boundary. Arches National Park is located on the Colorado Plateau and is near the town of Moab, UT (38.5725° N, 109.5497° W). On average, the area receives 219 mm of precipitation annually in three distinct seasons: winter snow, spring rain and summer monsoons. The area received 211 and 261 mm per year for 2011 and 2012, respectively (data from Arches National Park Headquarters weather station). Mean annual temperature for the area is 14.4°C. The site's soils are classified as Aridisols (U.S. Department of Agriculture Natural Resources Conservation Service), and soil texture is a sandy loam. The dominant vegetation structure is a mix of C_3 and C_4 bunch grasses and annual grasses and forbs. Dominant perennial grasses include *Achnatherum hymenoides* and *Pleuraphis jamesii*. Common annuals include *Bromus tectorum* (an exotic invasive), *Vulpia octoflora* and *Salsola tragus* (an exotic invasive). Biological soil crusts are also present within each plot, and the community is dominated by cyanobacteria, *Microcoleus* spp. Site characteristics were assessed immediately prior to the first fertilization event, which was in March of 2011 (see Supporting Information for methodological details). At this time, soil texture was characterized as 71.5% sand, 15.1% silt, and 13.4% clay. Soil pH was determined to be 7.99 ± 0.02 (SE). Percentages of soil total C and N were also determined as organic C: 0.40 ± 0.06 (SE), inorganic C: 0.48 ± 0.04 (SE), and N: 0.04 ± 0.00 (SE).

Experimental design

In 20 plots with a randomized block design, we established four levels of N fertilization with five replicate plots per treatment. Each plot was 1 m x 1 m with an additional 0.25 m buffer along each edge that received treatment but that was not sampled. An adult, healthy *A. hymenoides* was centered within each plot to explore the effects of N deposition on a common native perennial grass.

Fertilization began in spring 2011, and the four N fertilization treatment levels were 0, 2, 5 and 8 kg N ha^{-1} $year^{-1}$. This amount of N fertilization is an order of magnitude lower than many other N deposition fertilization studies, and the inputs used here were selected with the goal of exploring regionally relevant effects and thresholds. In particular, N addition treatments were selected using N deposition inputs as a guide: National Atmospheric Deposition Program (NADP) and Interagency Monitoring of Protected Visual Environments (IMPROVE) stations in Canyonlands National Park (approximately 40 km from our study site) suggest regional wet and dry deposition to total $2-3$ kg N ha^{-1} $year^{-1}$. We aimed to: (1) fertilize with similar annual inputs and (2) use a regression approach to fertilization (i.e., using multiple application amounts). For each fertilization event, we applied the treatments in liquid form (NH_4NO_3 dissolved in deionized water) in a volume of solution equivalent to a 3 mm rainfall event over the plot. Fertilizer was applied twice per year: half the annual addition was applied in spring (March) and half in fall (September).

Soil collection for analysis of microbial communities

Soil samples for molecular analyses were collected at two time points: on 7 March 2013, after four prior N deposition

treatments and two weeks prior to the fifth fertilization event, and on 30 March 2013, nine days after the fifth fertilization event. Three types of samples were collected: (1) biocrust, (2) below-biocrust, and (3) plant rhizosphere. For sampling of biocrusts, which represent the biological soil crust community in the plots, the top 0–1 cm of soil was collected from the spaces between plant canopies within each plot. The below-biocrust sample was collected immediately beneath each biocrust sampling location at a depth of approximately 3–6 cm. For the plant rhizosphere, soil was also collected approximately 3–6 cm below the soil surface; however, these samples were collected directly beneath plant stems. Soil samples were stored at −40°C until processing.

DNA extraction, qPCR, and sequencing of ribosomal RNA genes

Total nucleic acids were extracted from soils using the Fast DNA for Soils kit (MP Biomedical, Santa Ana, CA, USA). Extracted DNA was quantified using the Quant-it PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA), measured using a BioTech Synergy H1 plate reader and normalized to 1 ng/μl for subsequent qPCR and sequencing. qPCR amplifications targeted the 16S and 18S rRNA genes. High-throughput sequencing on an Illumina MiSeq platform targeted the V3-V4 region of the bacterial 16S rRNA gene, which also amplifies some archaeal sequences, and the D2 hypervariable region of the fungal and chlorophyte large subunit rRNA gene (see Supporting Information for additional methodological details).

Assessment of soil nutrients

Soil biogeochemical sampling occurred four times relative to the timing of the fertilization event: three days prior to fertilization, immediately following fertilization (the same day), and 5 and 21 days following fertilization. At each sampling, we used a 2.54 cm diameter soil corer to collect soil from $0-10$ cm depth at multiple locations within the plot. For each plot, we composited soils in the field to create a single plot sample. Samples were immediately returned to the laboratory, sieved to 4 mm, homogenized, and divided for separate analyses. Soil extractable inorganic N concentrations (NH_4^+ and NO_3^-) were assessed by extracting ~10 g of fresh soil (analysed within 5 h of collection) with a 2 M KCl solution. Samples were shaken for an hour and left to sit for 18 h prior to filtration using Whatman 42 filter paper. Extractable ammonium (NH_4^+) and nitrate (NO_3^-) concentrations of the extracts were determined using a Westco Scientific auto analyzer (Brookfield, CT, USA). The first date of soil inorganic N assessment was 18 March 2013, which was three days prior to the spring fertilization event in 2013. The plots had been fertilized for two years in advance of this collection, with the most recent fertilization occurring six months prior. The second round of extractable inorganic N analysis happened on 21 March 2013, immediately following fertilization, and the final sampling dates for N analysis were 26 March 2013 and 11 April 2013.

Nitrate moving down the soil profile was assessed using buried resin caps (Unibest, Walla Walla, WA, USA) at 10 cm depth. Briefly, resin caps were buried at 10 cm depth using a flat trowel and an angled insertion to keep the upper soil column intact. Resin caps were placed in each plot 10 days prior to fertilization and were removed from the plots three months following fertilization. We focused on nitrate because of its mobility in soil and its known consequences for water quality. After removal from the plots, resin caps were immediately extracted using 2 N HCl (per the manufacturer's instructions) and assessed for nitrate using an autoanalyzer (Westco International, Pittsburgh, PA, USA). Rates on N access by the resin were determined by standardizing for the weight of the resin and the number of days in the plots (Crews *et al*. 1995). Plant foliar samples were collected in April 2013, more than a month following fertilization and at a time of year when plants were at their maximum activity.

A passive gas collection system was used to compare the emission of nitrogen oxides (NO and NO_2) among treatment plots. We installed a 20-cm diameter PVC collar into each plot (collar and lid were lined with Teflon). Fertilizer was evenly applied within the collar at the same volume:area ratio as what was added to the rest of the plots. Following fertilization, we waited three minutes and then capped the collar. Triethenolamine-coated NO_2 filters and triethenolamine and PTIO-coated NO_x filters were placed inside the collar lid (PS-124, Ogawa and Company, Pompano Beach, FL). These filters were housed within a manufacturer-provided container that was attached to the top of the gas collar lid (filters were ~15 cm from the soil surface). The use of passive filters represents an emerging technology for assessing NO_x and, while it cannot yet be used to perfectly quantify fluxes, the method is functional in the field without the measurement difficulties associated with temperature and humidity fluctuation for the more commonly used, luminol-based 'NO_x box' method (Hall *et al*., 2008). This method was particularly appropriate for our research questions, as we were not trying to determine the exact rates of gas efflux, but instead to compare NO_x production and efflux among treatments. The filters were removed after the collar had been capped for an hour, based on Barger *et al*. (2005). Briefly, per the manufacturer's instructions (available at http://ogawausa.com/protocols/), NO and NO_2 pads were extracted in deionized water. We used sulfanilamide color reagent before reading on a 96-well plate reader (Biotek, Synergy HT, Winooski, VT), using a sodium nitrate standard.

Plant community metrics

Vascular plant cover was assessed by placing a point frame over each sample plot and lowering a pin through 50 different locations within the frame. For each interception above the soil surface, the plant species contacted were reported. Percent cover was then calculated by dividing the total number of species hits above the soil surface per point frame by 50. Plant richness was calculated as the total number of species hits per plot, and diversity was calculated as the weighted arithmetic mean of the proportional abundance of each species using Simpson's Index. Values are reported as 1-Simpson's Index, such that higher values indicate higher diversity. In this case, the value is the probability that two entities, taken at random, are different species.

Data analysis

All statistical analyses were conducted with the R statistical platform (r-project.org) using the vegan and ecodist packages. The significance levels for differences in microbial abundance and soil chemistry according to sampling time, N addition, and microhabitat were determined with analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test. Plant richness, diversity, and cover were also assessed with ANOVA. To examine the effects of sampling time, N treatment, and microhabitat on the composition of soil microbial communities, Bray-Curtis dissimilarities were compared with permutational multivariate analysis of variance (PerMANOVA), and communities were visualized with Principle Coordinate Analysis (PCoA). Correlations between pairwise distances from the various bioassays were determined using Mantel tests. Differences among biogeochemical pools and fluxes were determined using general linear model and repeated measures analyses of data, with treatment and block identification as factors and using Tukey's Honestly Significant Difference test to assess significant differences with multiple comparisons. All data were tested for normality and homoscedasticity (using Levene's test for the equality of variances); if either assumption was violated, data were ln transformed before analysis. Significance was determined at $\alpha \leq 0.05$.

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Supporting information

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