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1 **Bacterial, fungal, and plant communities exhibit no biomass or compositional response to**
2 **two years of simulated nitrogen deposition in a semiarid grassland**
3

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24 **Originality-Significance Statement:** Human activities have increased the amount of reactive
25 nitrogen entering ecosystems across the globe. The consequences of this excess nitrogen remain
26 unclear for many ecosystems, including drylands, which account for over a third of terrestrial
27 land area. Here we show that two years of nitrogen enrichment has no impact on bacterial,
28 archaeal, fungal, or chlorophyte community composition or biomass in a dryland ecosystem. In
29 contrast to results commonly seen in more mesic ecosystems, these drylands showed an
30 exceptional lack of response to increased nitrogen inputs across multiple temporal scales. Our
31 results suggest that excess nitrogen is not assimilated into biomass. Instead, nitrogen is exiting
32 the ecosystem through processes such as leaching, which could lead to the eutrophication of
33 associated aquatic ecosystems. These unique findings are supported by an uncommonly robust
34 experimental design and community assessment, making this work highly significant and of
35 broad interest to the readership of *Environmental Microbiology*.
36

37 **Summary**

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

53

54 **Key words**: drylands, global change, nitrogen enrichment, ribosomal RNA genes, soil

55 microorganisms

56

57 **Introduction**

58 Humans have more than doubled the input of available nitrogen (N) to the Earth's land

59 surface (Galloway *et al*., 2008; Vitousek *et al*., 2013), and numerous studies document the

60 significant effects of this change on ecosystem properties (*e.g*., Baron *et al*., 2000; Pardo *et al*.,

61 2011; Ochoa-Hueso *et al*., 2013; Rao *et al*., 2014). For example, increased N deposition has been

62 shown to greatly decrease air and water quality, increase greenhouse gas emissions and exotic

63 plant invasion, and alter natural fire regimes (Aber *et al*., 1989; Townsend *et al*., 2003; Galloway

64 *et al*., 2008). Thus, N deposition can affect fundamental aspects of terrestrial ecosystem structure

65 and function. However, not all ecosystems can be expected to respond to N deposition in the

66 same manner, and despite an increase in work in arid ecosystems (reviewed by Sinsabaugh *et al*.,

67 2015), there is a notably incomplete understanding of how N deposition will affect dryland

68 ecosystems.

69 Drylands – lands characterized by an overall climatic water deficit (< 0.65 mm/mm

70 threshold) that is calculated using an aridity index: the ratio of precipitation to potential

71 evapotranspiration – account for approximately 40% of the global terrestrial land area (Safriel *et*

72 *al*., 2005) and roughly 35% of the United States (Pointing and Belnap, 2012). Drylands include

73 arid, semiarid, and dry subhumid areas (Reynolds *et al*., 2007). Taken together, these diverse

74 ecosystems represent our planet's largest biome (Schimel, 2010) and exhibit some of the greatest

75 observed sensitivity to climatic variability and land use change (Morgan *et al*., 2011; Poulter *et*

76 *al*., 2014; Ahlstrom *et al*., 2015; Ferrenberg *et al*., 2015; Wertin *et al*., 2015; Reed *et al*., 2016).
77 Based on this sensitivity and the relatively low N stocks typically found in arid and semiarid
78 ecosystems, it is no surprise that several studies have suggested that drylands will be susceptible
79 to increasing anthropogenic N inputs (Baron *et al*., 2000; Pardo *et al*., 2011; Mueller *et al*., 2015;
80 Sinsabaugh *et al*., 2015). Indeed, the limited data that do exist suggest North American deserts
81 may maintain lower N deposition critical thresholds – the amount of N deposition beyond which
82 an ecosystem response is observed – compared with more mesic ecoregions (Pardo *et al*., 2011;
83 Blett *et al*., 2014; Sinsabaugh *et al*., 2015). However, dryland ecosystems are diverse, as are the
84 anthropogenic N inputs into these ecosystems (*e.g*., Reed *et al.,* 2013), and consequently,
85 responses to N deposition may vary. Thus, research is still needed to understand how
86 anthropogenic N inputs affect these varied and important landscapes (Porter *et al*., 2005; Blett *et*
87 *al*., 2014), and to make predictions about the population-, community-, and ecosystem-level
88 effects of N deposition.

89 In particular, our understanding of dryland microbial community response to N
90 deposition remains notably poor (but see Zeglin *et al*., 2006; Li *et al*., 2010, Mueller *et al*., 2015;
91 Sinsabaugh *et al*., 2015; Ochoa-Hueso *et al*., 2016), which significantly constrains our ability to
92 consider and anticipate the effects of N deposition across multiple spatial scales. Microbial
93 community composition is a fundamental control over terrestrial ecosystem functioning,
94 affecting critical ecosystem processes such as litter decomposition (Allison *et al.,* 2013) and
95 plant fitness (Lau *et al.,* 2012). Recent research suggests that N deposition significantly alters
96 soil microbial communities and the functions they perform, and the N fertilization experiments
97 commonly used as a proxy for N deposition have documented N-induced changes to microbial
98 community composition, richness, respiration rates, and enzyme activities across many

99 ecosystem types (*e.g*., Janssens *et al*., 2010; Liu and Greaver, 2010; Sinsabaugh *et al*., 2015).
100 However, data that inform our understanding of dryland microbial reactions to N deposition
101 remain sparse and are unlikely to capture the potential range of dryland responses.
102 The southwestern United States has become a 'hotspot' for N deposition (Fenn *et al*.,
103 2003; Reed *et al*., 2013), and population growth and energy development continue to elevate N
104 deposition in North American drylands. Here, we set out to advance our understanding of how
105 soil microbial communities will respond to N deposition using an N fertilization experiment in a
106 semiarid grassland containing common native and exotic plant species and early successional
107 biological soil crust (biocrust) communities on the Colorado Plateau in the southwestern U.S. To
108 search for N deposition critical thresholds, we used a range of N input levels (0, 2, 5, and 8 kg N
109 ha^{-1} yr^{-1}). These relatively low rates, compared to previous N amendment experiments conducted
110 in forest ecosystems, were designed to assess critical thresholds against the lower background N
111 deposition seen in many North American drylands (N deposition is estimated for this site at ~3
112 kg N ha^{-1} yr^{-1}; Fenn *et al*., 2003), and are more likely to capture biologically meaningful
113 responses (Gomez-Casanovas *et al*., 2016). Beginning in 2011, fertilizer was applied twice
114 yearly, with half distributed in spring and half in fall. By sampling soils before and after the
115 spring fertilization in 2013, we used high-throughput sequencing and quantitative PCR (qPCR)
116 of ribosomal RNA genes to examine effects of N treatments on soil microbial communities. We
117 also monitored associated nutrient transformations in soil, and the aboveground plant community
118 response to experimental manipulation. Because previous work indicated that dryland soil
119 microbial communities can vary across shallow soil depths and between plant- and biocrust-
120 associated soils (Steven *et al*., 2013; Steven *et al*., 2014; Mueller *et al*., 2015), we sampled
121 discretely among three microhabitats in the ecosystem: within biocrusts, below-biocrusts (3–6

122 cm depth), and in rhizosphere zones. Based on the previous studies and the low N stocks at our
123 site, we had three core hypotheses: (1) increased N availability would be a key driver of
124 microbial community structure and would significantly affect soil microbial community
125 composition and function at all N input levels, (2) the different microhabitats from which soil
126 samples were collected (*e.g.*, biocrust vs. rhizosphere) would harbor distinct microbial
127 communities, and (3) microhabitat would interact with the fertilization treatments to determine
128 the nature and magnitude of the N effect on microbial communities.
129

130 **Results**

131 *Phylogenetic composition and diversity*

132 High-throughput sequencing produced 1,916–49,463 sequences per sample (median:
133 14,690) of the 16S rRNA gene and 2,101–49,819 sequences per sample (median: 14,917) of the
134 28S rRNA gene. In an effort to remove sequencing depth heterogeneity, 16S and 28S rRNA gene
135 sequences were randomly subsampled at a depth of 1,916 and 2,101, respectively. In contrast to
136 our predictions that increased N inputs would alter microbial communities, N fertilization did not
137 produce any measureable effects on soil microbial community composition (Figure 1). This
138 includes finding no differences in soil microbial community after the two years of fertilization
139 (*i.e.*, comparing communities among the treatment plots), and no differences among the
140 community in two sets of soil samples taken within a three week period in spring 2013: one set
141 prior to fertilization and one following fertilization. Because microbial communities from soils
142 collected two weeks prior to fertilization in 2013 were statistically indistinguishable from those
143 in soils collected nine days following fertilization, data for the two time points were combined
144 and analyzed concurrently. Specifically, community composition was unaffected by N

145 fertilization for prokaryotes (bacteria plus archaea; PerMANOVA $F = 0.97$, $p = 0.43$), fungi ($F =$
146 0.91, $p = 0.51$) and Chlorophyta (green algae; $F = 0.95$, $p = 0.50$). Microbial diversity was
147 similarly unaffected by N fertilization (Table 1). In sum, there were no observable fertilization
148 effects on microbial community composition with the fertilization event (*i.e.*, communities
149 sampled before and after spring 2013 fertilization were the same), nor with the two years of
150 treatments (*i.e.*, communities compared among 0–8 kg N ha^{-1} yr^{-1} plots were the same).

151 In line with our hypotheses, prokaryotic community composition did differ by
152 microhabitat (PerMANOVA $F = 56.60$, $p < 0.01$), where biocrust communities were
153 compositionally distinct from below-biocrust and rhizosphere communities (Figure 1a). Biocrust
154 communities were dominated by Cyanobacteria, while below-biocrust and rhizosphere
155 communities had larger proportions of Actinobacteria and Crenarchaeota (Figure 2a). Fungal
156 communities showed significant compositional differences between biocrust and below-biocrust
157 communities, as well as between biocrust and rhizosphere communities (PerMANOVA $F =$
158 12.92, $p < 0.01$; Figure 1b). Fungal communities in all microhabitats were dominated by
159 Ascomycota and Basidiomycota, though below-biocrust and rhizosphere communities exhibited
160 larger proportions of Blastocladiomycota and Chytridiomycota than did biocrust communities
161 (Figure 2b). Similarly, Chlorophyta showed compositional differences according to microhabitat
162 (PerMANOVA $F = 10.12$, $p < 0.01$), with communities within biocrusts being of distinct
163 composition compared to below-biocrust and rhizosphere communities (Figure 1c).

164 Consistent with the community composition results, qPCR of 16S and 18S rRNA genes
165 suggested that microbial abundance did not vary with N fertilization or time of sampling (before
166 and after the fifth fertilization event), though gene copy numbers and the ratio of fungi to
167 bacteria were significantly different according to microhabitat (biocrust vs. below-biocrust vs.

168 vascular plant rhizosphere; Table 1). Specifically, 16S rRNA gene abundance in the rhizosphere
169 was significantly lower than that of biocrust and below-biocrust microhabitats ($p < 0.01$ in each
170 case). For the 18S rRNA gene, abundance below biocrusts was significantly lower than within
171 biocrusts ($p < 0.01$); abundance in rhizosphere and below-biocrust locations also differed, with
172 fewer gene copies below biocrusts ($p < 0.01$).

173 *Plant response and community linkages*

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

180 We did observe significant relationships among microbial groups and between above-
181 and belowground communities. Prokaryotic community composition covaried with the
182 composition of both fungal (Mantel $r = 0.29$, $p < 0.01$) and chlorophyte communities (Mantel $r =$
183 0.17, $p < 0.01$). Further variation in prokaryotic community composition was associated with
184 aspects of the plant community, including plant richness (Mantel $r = 0.10$, $p < 0.01$) and aerial
185 cover (Mantel $r = 0.08$, $p < 0.01$). Fungal community composition also covaried with plant aerial
186 cover (Mantel $r = 0.15$, $p < 0.01$). Thus, although treatment effects on the above- and
187 belowground communities were not observed, natural variation among the plots did show
188 significant relationships among factors.

189 *Soil nutrient pools*

190 Total soil carbon (C) and N concentrations were unaffected by N fertilization, though
191 across all treatments combined, total N was significantly higher within biocrusts and in the
192 rhizosphere than below biocrusts (Table 1; $p < 0.01$ in both cases). Soil pH did not respond to N
193 addition, yet varied by microhabitat, with pH in the rhizosphere significantly higher than in
194 biocrust or below-biocrust microhabitats ($p < 0.01$ in each instance). Soil extractable ammonium
195 and nitrate concentrations in the treatment plots were significantly increased on the day
196 immediately following fertilization (21 March 2013; $p < 0.01$ and $p < 0.05$ for ammonium and
197 nitrate, respectively; Figure 3). Soil extractable ammonium concentrations remained elevated in
198 the N addition plots five days after fertilization (26 March 2013; $p < 0.01$), although extractable
199 nitrate concentrations showed no significant differences at this time. Ammonium and nitrate
200 concentrations returned to pre-fertilization levels 21 days following fertilization (11 April 2013),
201 suggesting the lack of a sustained N fertilization effect on available soil N pools. Buried resin
202 caps set at 10 cm depth tracked the movement of inorganic N out of surface soil layers, and the
203 flux of nitrate into resin was highest in the 8 kg N ha^{-1} yr^{-1} plots, significantly lower in the 5 kg N
204 ha^{-1} yr^{-1} plots ($p < 0.05$), and lowest in the 0 kg N ha^{-1} yr^{-1} plots ($p < 0.01$), with the 2 kg N ha^{-1}
205 yr^{-1} values falling between the 0 and 5 kg N ha^{-1} yr^{-1} ($p > 0.1$ for each; Figure 4). Passive
206 samplers were used to compare NO_x emission rates among treatment plots, and on the day of
207 fertilization the 8 kg N ha^{-1} yr^{-1} plots maintained significantly higher outputs of NO_x compared
208 with the plots receiving no additional N ($p < 0.05$). The emissions of NO_x from the 3 and 5 kg N
209 ha^{-1} yr^{-1} treatment plots fell between the low and high N input end members.
210
211 **Discussion**
212 *Lack of effect of N addition on soil microbial communities*

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

222 Why didn't microbial communities respond to N inputs when other studies have observed
223 responses? There are a number of potential explanations. First, the N inputs used here were
224 based upon levels of N deposition recorded in the study area (~3 kg N ha^{-1} yr^{-1}), and while these
225 amounts are more realistic for our area and are in line with many newer studies that use smaller
226 inputs (Gilliam *et al*., 2016; Kox *et al*.,2016; Tulloss and Cadenasso, 2016), the inputs are much
227 lower than the levels of enrichment often used in nutrient limitation studies and in areas of
228 exceptional deposition, such as some parts of Europe (Holland *et al*., 2005). Second, as with
229 most N deposition experiments, we applied the N in pulses that joined smaller daily rates into a
230 compound amount, in our case applied twice yearly. Accordingly, the amounts of N that we
231 added were higher than what would be expected on a short timescale in our area. Yet even with
232 these larger pulse inputs, we did not observe any effect of increasing N. Third, it may be that the
233 availability of another resource, such as water or C, limits the biota's capacity to take advantage
234 of additional N (*e.g.*, Ramirez *et al*., 2010); thus, the N is not able to be immobilized from the
235 soil environment and is available to be lost from the system even with low levels of N input

236 (Lovett and Goodale, 2011). Fourth, it has been suggested that N deposition can indirectly affect
237 communities via changes to soil pH (*e.g.*, Hallin *et al*., 2009; Ramirez *et al*., 2010; Zhalnina *et*
238 *al*., 2015), but the buffering capacity of our soils in the form of high calcium carbonate (Table 1)
239 would render these dryland soils resistant to changes in pH. Finally, it is possible that the effect
240 of N deposition as seen from fertilization experiments will take longer to emerge than the two
241 years of this study or, instead, that the thresholds of N deposition effects were so low that they
242 had already been crossed by the low background N deposition occurring in the area. We delve
243 into more detail regarding these concepts in the sections below.

244 Interestingly, a recent fertilization study (Mueller *et al*., 2015; Sinsabaugh *et al*., 2015)
245 adds important context to these results, as the study was conducted in another common dryland
246 ecosystem type (Mojave desert; vegetation dominated by *Larrea tridentata* and *Ambrosia*
247 *dumosa*) and as the fertilization methods also used smaller inputs of N (0, 7, and 15 kg N ha^{-1} yr^{-}
248 ^{1}). While aspects of soil biogeochemical cycling responded strongly to N fertilization and
249 although there were trends in microbial community data, there were no significant effects of N
250 addition on the abundance of fungi, bacteria, or cyanobacteria or on fungal:bacterial ratios
251 (Sinsabaugh *et al*., 2015). In the same plots, significant N impacts were found for bacterial
252 community composition and diversity, but fungal community composition was not significantly
253 affected by N application after two years (Mueller *et al*., 2015). Thus, while dryland data suggest
254 strong potential for non-linear biomass and biogeochemical consequences of N deposition
255 (Sinsabaugh *et al*., 2015), and for components of the community to respond to this enrichment
256 (Mueller *et al*., 2015), the Mojave desert results alongside the Colorado Plateau data presented
257 here provide novel insight into the potential for aspects of resistance in dryland microbial
258 communities, and for differential dryland community responses to similar N inputs. Further work

259 exploring the mechanisms through which dryland microbial communities are affected by N
260 inputs would represent a dramatic advance in our understanding of how N deposition regulates
261 ecosystem structure and function in our world's largest biome (Schimel, 2010).

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

268 *The fate of added N*

269 Taken together, the biogeochemical data suggest that the added N does not persist in soils
270 of this arid grassland, and that plants were not significantly affected by short-term N additions.
271 Fertilization only increased inorganic N concentrations in treatment plots immediately after the
272 application (Figure 3). Across all levels of N addition, treatment effects on N extractable pools
273 were undetectable within one month of fertilization. Gaseous and dissolved N data suggest N
274 was leaving the system as a gas and in liquid form. For example, NO_x efflux rates were 15%
275 higher in the 8 kg N ha^{-1} yr^{-1} plots immediately following fertilization relative to the control
276 plots, and the 3 and 5 kg N ha^{-1} yr^{-1} plots fell in between these low and high values. Resin
277 capture results showed N was moving down through the soil profile, potentially into the local
278 hydrological system (Figure 4). Consistent with these results, foliar N concentration of *A.*
279 *hymenoides* was not increased by fertilization, and no changes in plant cover or diversity were
280 observed. These data are in agreement with other desert N deposition studies suggesting that
281 plants may only respond to increased N inputs when enough water is available (the relationship

282 between water availability and N sensitivity remains unknown at this site), and some plants do
283 not respond even in wetter years (Hall *et al*., 2011). Combined, the microbial and
284 biogeochemical data from this Colorado Plateau site illustrate an ecosystem that may not have
285 the capacity to utilize increased inputs of N, perhaps due to water or C limitation. The N is
286 quickly lost as gases and in dissolved forms, and these losses from the system represent potential
287 negative consequences for the region (*e.g.*, NO_x emissions and the formation of O_3) and the
288 planet (*e.g.*, increased N_2O emissions).

289 *Role of plants and soil characteristics in structuring microbial communities*

290 It has been suggested that microbial responses to N deposition may be indirectly
291 mediated by plant C allocation responses to the added N (Ramirez *et al*., 2010; Leff *et al*., 2015).
292 In other words, N deposition impacts plants (*e.g*., by altering plant litter chemistry, root growth,
293 and root exudation; Prober *et al*., 2015), and it is these plant-driven changes that alter the
294 composition of the microbial community (Ramirez *et al*., 2010; Leff *et al*., 2015). In this way, it
295 could be important that neither plant nor microbial communities responded to the experimental
296 addition of N. Clearly more work is needed to elucidate the individual and coupled controls over
297 above- and belowground responses. In arid grasslands, plants and biocrusts are likely to be the
298 main determinants of microbial community composition, as they supply the majority of soil
299 organic C and fixed N that enable a heterotrophic lifestyle (Bardgett and Wardle, 2010). Soil
300 microbial communities also have the capacity to impact plants through a variety of mechanisms
301 including nutrient acquisition, organic matter decomposition, and the promotion or suppression
302 of plant disease (Garbeva *et al*., 2004; Singh *et al*., 2004; Berg and Smalla, 2009; Latz *et al*.,
303 2012). We observed significant correlations between each of the microbial groups we examined,
304 in addition to significant relationships between plant characteristics (richness and aerial cover)

305 and microbial community composition. These results support the growing recognition that the
306 structure and functioning of soil microbial communities result from complex interactions
307 between the plant community, the soil environment, and microbe-microbe associations (Bakker
308 *et al*., 2014; Schlatter *et al*., 2015).

309 Soil microbial communities may also be indirectly affected by chemical changes to soil
310 characteristics (*e.g*., lowered pH and increased metal solubility; Bowman *et al*., 2008), and
311 numerous studies have documented substantial changes in pH with N fertilization (*e.g*., Hallin *et*
312 *al*., 2009; Ramirez *et al*., 2010; Zhalnina *et al*., 2015). As pH has been shown to be a major
313 predictor of bacterial (Lauber *et al*., 2009) and fungal (Tedersoo *et al*., 2014) community
314 composition across the globe, this key factor could also be linked to the changes in soil microbial
315 community composition and function that have been commonly observed in N fertilization
316 experiments. However, in our study, N addition had no impact on the pH of treatment plots,
317 likely due to the high buffering capacity of our soils (Table 1). With no associated change in pH
318 or in plant foliar chemistry, aerial cover, or plant community composition, perhaps the stability
319 of microbial communities in these alkaline soils should not be surprising.

320 Microhabitat was a stronger driver of microbial community composition than N
321 amendment in our study, with distinct communities present in soils derived from biocrust,
322 below-biocrust, and rhizosphere zones. These finding are consistent with prior studies in arid
323 grasslands of this region (Steven *et al*., 2013, 2014). The qPCR assays showed that bacterial and
324 fungal abundances were higher within biocrusts than below biocrusts, which is in accordance
325 with other dryland studies that have documented a concentration of microbial biomass in the top
326 few centimeters of soil (Dunbar *et al*., 2012; Pointing and Belnap, 2012; Steven *et al*., 2013;
327 Sinsabaugh *et al*., 2015). Plants have been shown to have a strong influence on soil microbial

328 communities in dryland systems, promoting islands of fertility (Schade and Hobbie, 2005;
329 Perroni-Ventura *et al*., 2009). Accordingly, rhizosphere communities are often compositionally
330 dissimilar from those in unvegetated soils (Andrew *et al*., 2012; Steven *et al*., 2012; Ramond *et*
331 *al*., 2014). Thus, while simulated N deposition had no effect on any aspect of microbial
332 community, our data are in line with other dryland results of distinct microbial communities
333 among microhabitats separated by mere centimeters.

334 *Conclusion*

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

344

345 **Experimental Procedures**

346 *Site description*

347 The experimental site is within Arches National Park (38° 47’ N, 109° 39’ W) near the
348 Park’s northwest boundary. Arches National Park is located on the Colorado Plateau and is near
349 the town of Moab, UT (38.5725° N, 109.5497° W). On average, the area receives 219 mm of
350 precipitation annually in three distinct seasons: winter snow, spring rain, and summer monsoons.

351 The area received 211 and 261 mm per year for 2011 and 2012, respectively (data from Arches
352 National Park Headquarters weather station). Mean annual temperature for the area is 14.4 °C.
353 The site's soils are classified as Aridisols (U.S. Department of Agriculture Natural Resources
354 Conservation Service), and soil texture is a sandy loam. The dominant vegetation structure is a
355 mix of C_3 and C_4 bunch grasses and annual grasses and forbs. Dominant perennial grasses
356 include *Achnatherum hymenoides* and *Pleuraphis jamesii*. Common annuals include *Bromus*
357 *tectorum* (an exotic invasive), *Vulpia octoflora*, and *Salsola tragus* (an exotic invasive).
358 Biological soil crusts are also present within each plot, and the community is dominated by
359 cyanobacteria, *Microcoleus spp*. Site characteristics were assessed immediately prior to the first
360 fertilization event, which was in March of 2011 (see Supplemental Material for methodological
361 details). At this time, soil texture was characterized as 71.5% sand, 15.1% silt, and 13.4% clay.
362 Soil pH was determined to be 7.99 ± 0.02 (SE). Percentages of soil total C and N were also
363 determined as organic C: 0.40 ± 0.06 (SE), inorganic C: 0.48 ± 0.04 (SE), and N: 0.04 ± 0.00
364 (SE).
365 *Experimental design*
366 In twenty plots with a randomized block design, we established four levels of N
367 fertilization with five replicate plots per treatment. Each plot was 1 m x 1 m with an additional
368 0.25 m buffer along each edge that received treatment but that was not sampled. An adult,
369 healthy *A. hymenoides* was centered within each plot to explore the effects of N deposition on a
370 common native perennial grass.
371 Fertilization began in spring 2011, and the four N fertilization treatment levels were 0, 2,
372 5, and 8 kg N ha^{-1} yr^{-1}. This amount of N fertilization is an order of magnitude lower than many
373 other N deposition fertilization studies, and the inputs used here were selected with the goal of

374 exploring regionally relevant effects and thresholds. In particular, N addition treatments were
375 selected using N deposition inputs as a guide: National Atmospheric Deposition Program
376 (NADP) and Interagency Monitoring of Protected Visual Environments (IMPROVE) stations in
377 Canyonlands National Park (approximately 40 km from our study site) suggest regional wet and
378 dry deposition to total 2–3 kg N ha^{-1} yr^{-1}. We aimed to: (1) fertilize with similar annual inputs
379 and (2) use a regression approach to fertilization (*i.e.*, using multiple application amounts). For
380 each fertilization event, we applied the treatments in liquid form (NH_4NO_3 dissolved in
381 deionized water) in a volume of solution equivalent to a 3 mm rainfall event over the plot.
382 Fertilizer was applied twice per year: half the annual addition was applied in spring (March) and
383 half in fall (September).
384 *Soil collection for analysis of microbial communities*
385 Soil samples for molecular analyses were collected at two time points: on 7 March 2013,
386 after four prior N deposition treatments and two weeks prior to the fifth fertilization event, and
387 on 30 March 2013, nine days after the fifth fertilization event. Three types of samples were
388 collected: (1) biocrust, (2) below-biocrust, and (3) plant rhizosphere. For sampling of biocrusts,
389 which represent the biological soil crust community in the plots, the top 0–1 cm of soil was
390 collected from the spaces between plant canopies within each plot. The below-biocrust sample
391 was collected immediately beneath each biocrust sampling location at a depth of approximately
392 3–6 cm. For the plant rhizosphere, soil was also collected approximately 3–6 cm below the soil
393 surface; however, these samples were collected directly beneath plant stems. Soil samples were
394 stored at -40 °C until processing.
395 *DNA extraction, qPCR, and sequencing of ribosomal RNA genes*

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

405 *Assessment of soil nutrients*

406 Soil biogeochemical sampling occurred four times relative to the timing of the
407 fertilization event: three days prior to fertilization, immediately following fertilization (the same
408 day), and 5 and 21 days following fertilization. At each sampling, we used a 2.54 cm diameter
409 soil corer to collect soil from 0–10 cm depth at multiple locations within the plot. For each plot,
410 we composited soils in the field to create a single plot sample. Samples were immediately
411 returned to the laboratory, sieved to 4 mm, homogenized, and divided for separate analyses. Soil
412 extractable inorganic N concentrations (NH_4^+ and NO_3^-) were assessed by extracting ~10 g of
413 fresh soil (analyzed within 5 hours of collection) with a 2 M KCl solution. Samples were shaken
414 for an hour and left to sit for 18 hours prior to filtration using Whatman #42 filter paper.
415 Extractable ammonium (NH_4^+) and nitrate (NO_3^-) concentrations of the extracts were determined
416 using a Westco Scientific auto analyzer (Brookfield, CT, USA). The first date of soil inorganic N
417 assessment was 18 March 2013, which was three days prior to the spring fertilization event in
418 2013. The plots had been fertilized for two years in advance of this collection, with the most

419 recent fertilization occurring 6 months prior. The second round of extractable inorganic N

420 analysis happened on 21 March 2013, immediately following fertilization, and the final sampling

421 dates for N analysis were 26 March 2013 and 11 April 2013.

422 Nitrate moving down the soil profile was assessed using buried resin caps (Unibest Inc.,

423 Walla Walla, WA, USA) at 10 cm depth. Briefly, resin caps were buried at 10 cm depth using a

424 flat trowel and an angled insertion to keep the upper soil column intact. Resin caps were placed

425 in each plot ten days prior to fertilization and were removed from the plots three months

426 following fertilization. We focused on nitrate because of its mobility in soil and its known

427 consequences for water quality. After removal from the plots, resin caps were immediately

428 extracted using 2 N HCl (per the manufacturer's instructions) and assessed for nitrate using an

429 autoanalyzer (Westco International, Inc., Pittsburgh, PA, USA). Rates on N access by the resin

430 were determined by standardizing for the weight of the resin and the number of days in the plots

431 (Crews et al. 1995). Plant foliar samples were collected in April 2013, more than a month

432 following fertilization and at a time of year when plants were at their maximum activity.

433 A passive gas collection system was used to compare the emission of nitrogen oxides

434 (NO and NO_2) among treatment plots. We installed a 20-cm diameter PVC collar into each plot

435 (collar and lid were lined with Teflon). Fertilizer was evenly applied within the collar at the same

436 volume:area ratio as what was added to the rest of the plots. Following fertilization, we waited

437 three minutes and then capped the collar. Triethenolamine-coated NO_2 filters and

438 triethenolamine and PTIO-coated NO_x filters were placed inside the collar lid (PS-124, Ogawa

439 and Company, Pompano Beach, FL). These filters were housed within a manufacturer-provided

440 container that was attached to the top of the gas collar lid (filters were ~15 cm from the soil

441 surface). The use of passive filters represents an emerging technology for assessing NO_x and,

442 while it cannot yet be used to perfectly quantify fluxes, the method is functional in the field
443 without the measurement difficulties associated with temperature and humidity fluctuation for
444 the more commonly used, luminol-based 'NO_x box' method (Hall *et al*., 2008). This method was
445 particularly appropriate for our research questions, as we were not trying to determine the exact
446 rates of gas efflux, but instead to compare NO_x production and efflux among treatments. The
447 filters were removed after the collar had been capped for an hour, based on Barger *et al*. (2005).
448 Briefly, per the manufacturer's instructions (available at http://ogawausa.com/protocols/), NO
449 and NO_2 pads were extracted in deionized water. We used sulfanilamide color reagent before
450 reading on a 96-well plate reader (Biotek, Synergy HT, Winooski, VT), using a sodium nitrate
451 standard.

452 *Plant community metrics*

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

462 *Data analysis*

463 All statistical analyses were conducted with the R statistical platform (r-project.org) using
464 the vegan and ecodist packages. The significance levels for differences in microbial abundance

465 and soil chemistry according to sampling time, N addition, and microhabitat were determined
466 with analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test. Plant
467 richness, diversity, and cover were also assessed with ANOVA. To examine the effects of
468 sampling time, N treatment, and microhabitat on the composition of soil microbial communities,
469 Bray-Curtis dissimilarities were compared with permutational multivariate analysis of variance
470 (PerMANOVA), and communities were visualized with Principle Coordinate Analysis (PCoA).
471 Correlations between pairwise distances from the various bioassays were determined using
472 Mantel tests. Differences among biogeochemical pools and fluxes were determined using general
473 linear model and repeated measures analyses of data, with treatment and block identification as
474 factors and using Tukey's Honestly Significant Difference test to assess significant differences
475 with multiple comparisons. All data were tested for normality and homoscedasticity (using
476 Levene's test for the equality of variances); if either assumption was violated, data were ln
477 transformed before analysis. Significance was determined at $\alpha \leq 0.05$.
478

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791 **Table and Figure Legends**
792
793 **Table 1**. Microbial abundance data (assessed by qPCR), microbial diversity, and soil chemistry
794 according to sampling location and N treatment. Values are means and standard errors.
795
796 **Table 2**. Plant community metrics associated with each treatment group. Values are means and
797 standard errors.
798

799 **Figure 1**. Community composition of prokaryotes (bacteria plus archaea) (a), fungi (b), and
800 Chlorophyta (c) colored by N treatment (left) and sampling location (right).
801
802 **Figure 2**. Relative abundance of prokaryotic phyla (a) and fungal classes (b) as determined by
803 high-throughput sequencing of rRNA genes.
804
805 **Figure 3**. Soil extractable ammonium (a) and nitrate (b) concentrations for 0-10 cm depth soil
806 collected prior to and following fertilization for the four N fertilization treatment plots. Values
807 are means and standard errors.
808
809 **Figure 4**. Nitrate (NO_3^-) flux rates from buried resin caps placed within each plot. Values are
810 means and standard errors, and lowercase letters depict significant differences among treatments
811 ($p < 0.05$).
812
813
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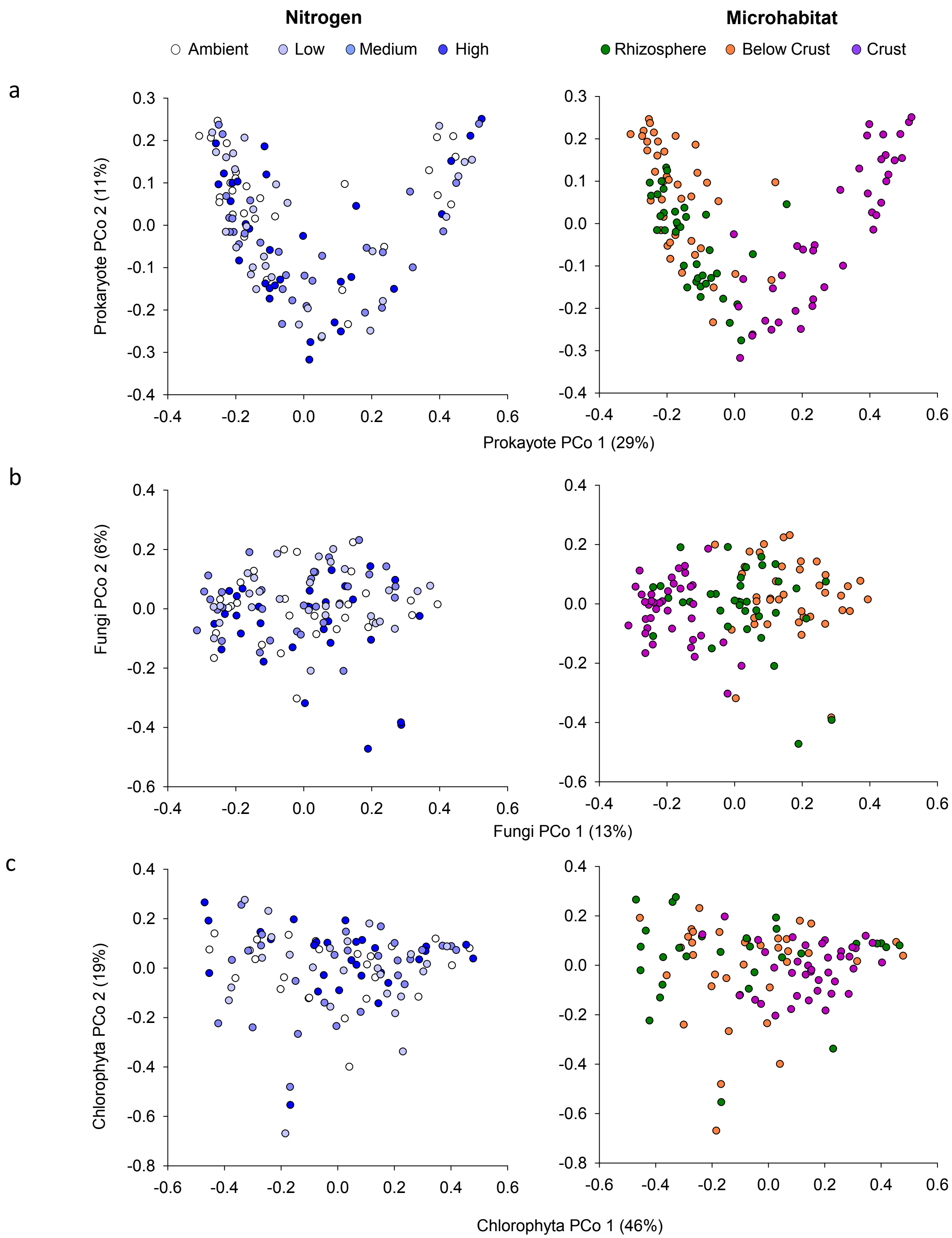
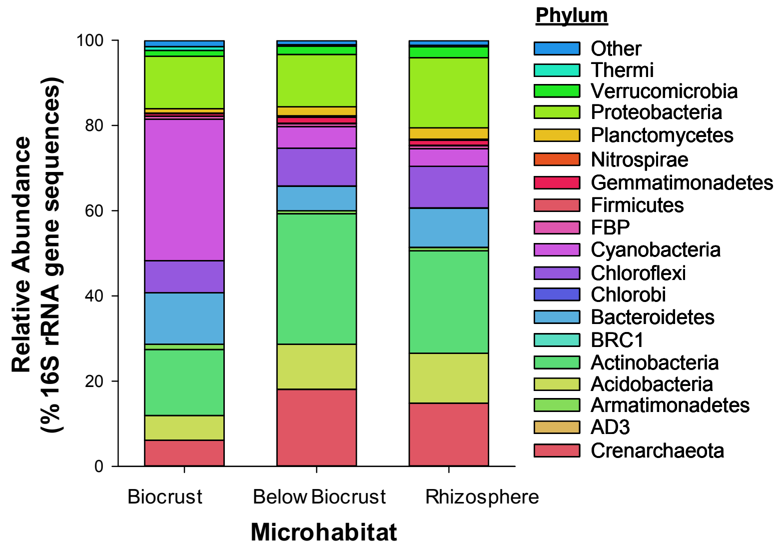


Nitrogen
Ambient
Low
Medium
High
Microhabitat
Rhizosphere
Below Crust
Crust
a
Prokaryote PCo 2 (11%)
Prokayote PCo 1 (29%)
b
Fungi PCo 2 (6%)
Fungi PCo 1 (13%)
c
Chlorophyta PCo 2 (19%)
Chlorophyta PCo 1 (46%)

a



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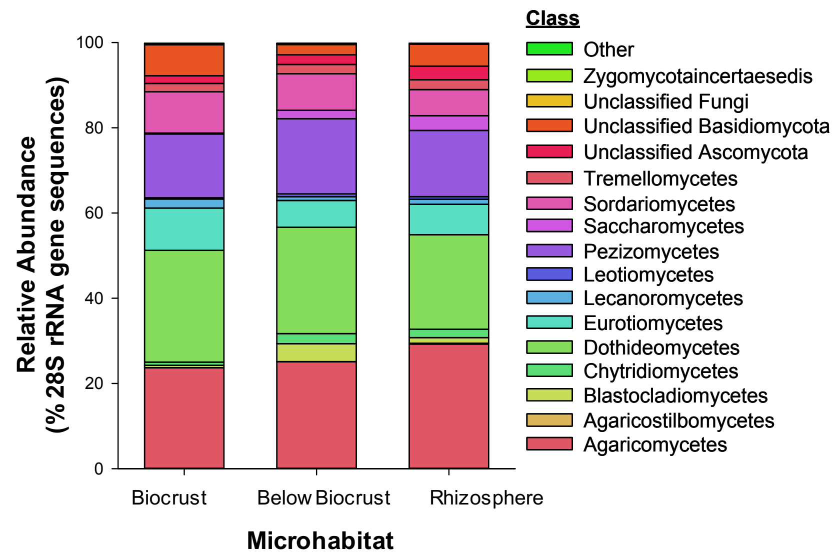
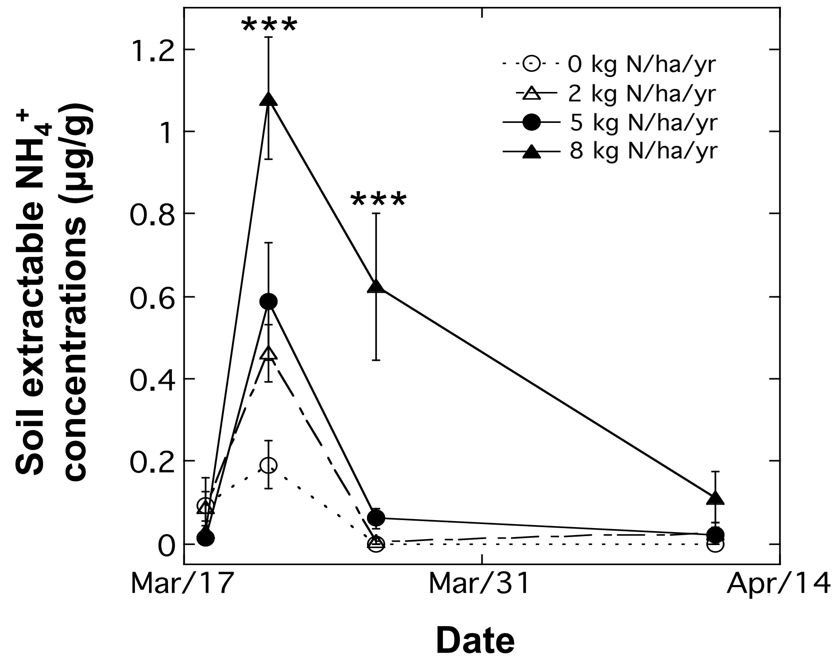


a



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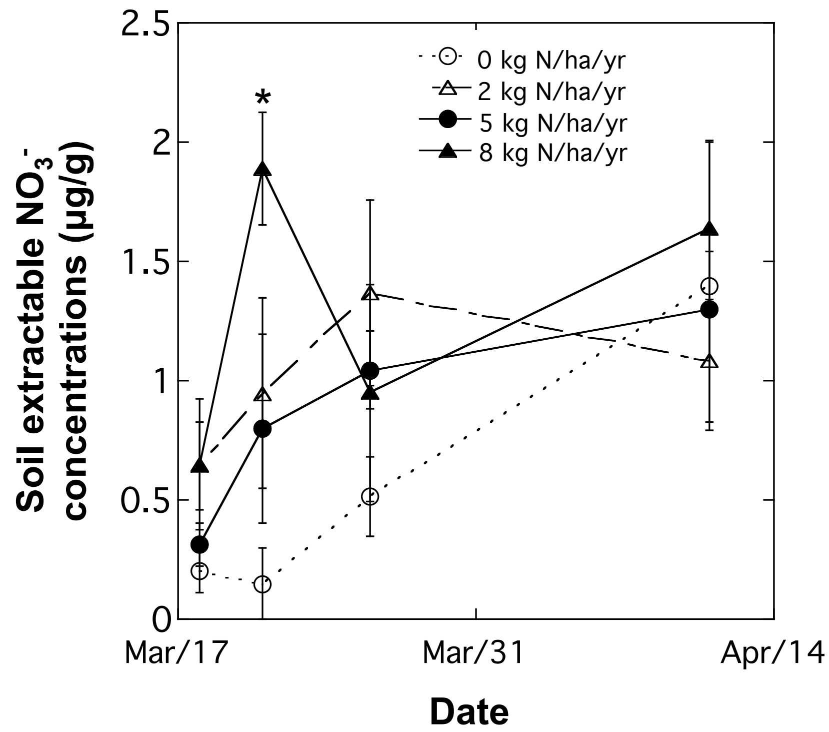

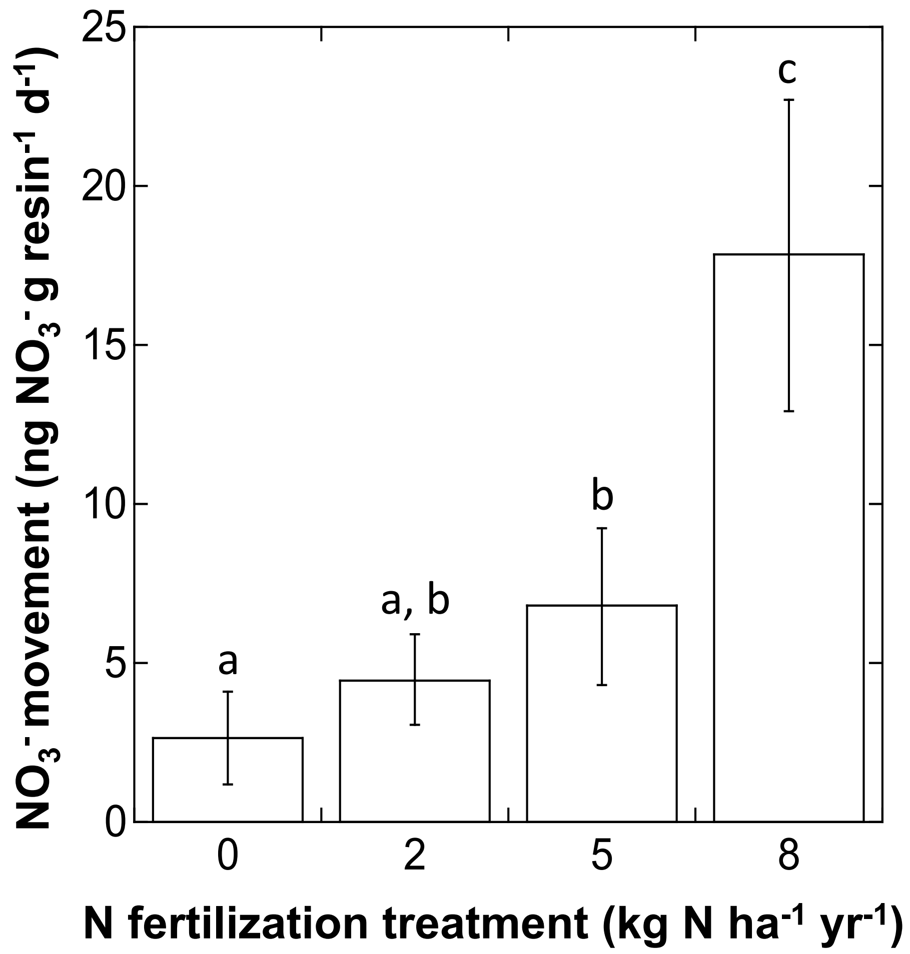
25
20
15
10
5
0
c
b
a, b
a
0
2
5
8
NO_3^- movement (ng NO_3^- g resin^{-1} d^{-1})
N fertilization treatment (kg N ha^{-1} yr^{-1})

Location / Treatment	Microbial Abundance			Microbial Diversity		Soil Chemistry		
	16S rRNA*	18S rRNA*	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

* log_{10} gene copies per g dry soil

N treatment	Richness	Diversity*	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

* Simpson's Index (1-D)