**Fungal loop transfer of N depends on biocrust constituents and N form**

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**Running head:** dark septate fungi translocate ammonium in biocrusts

**Key words:** ammonium, Ascomycota, Colorado Plateau, dark septate endophyte, fungal loop, Indian ricegrass, Pleosporales

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**Abstract.** Besides performing multiple ecosystem services individually and collectively, biocrust constituents may also create biological networks connecting spatially and temporally distinct processes. In the fungal loop hypothesis, rainfall variability allows fungi to act as conduits and reservoirs, translocating resources between soils and host plants. To evaluate the extent biocrust species composition and N form influence loops, we created a minor, localized rainfall event containing $^{15}NH_4^+$ and $^{15}NO_3^-$ and measured the resulting $\delta^{15}N$ in the surrounding dry cyanobacteria- and lichen-dominated crusts and grass, *Achnatherum hymenoides*, after twenty-four hours. We also estimated the biomass of fungal constituents using quantitative PCR and characterized fungal communities by sequencing the 18S rRNA gene. We found evidence for the initiation of fungal loops in cyanobacteria-dominated crusts where $^{15}N$, from $^{15}NH_4^+$, moved 40 mm $h^{-1}$ in biocrust soils with the $\delta^{15}N$ of crusts decreasing as the radial distance from the water addition increased (linear mixed effects model (LMEM): $R^2 = 0.67$, $F_{2,12} = 11$, $P = 0.002$). In cyanobacteria crusts, $\delta^{15}N$, from $^{15}NH_4^+$, was diluted as Ascomycota biomass increased (LMEM: $R^2 = 0.63$, $F_{2,8} = 6.8$, $P = 0.02$), Ascomycota accounted for 82% (±2.8) of all fungal sequences, and one order, Pleosporales, comprised 66% (±6.9) of Ascomycota. The seeming lack of loops in moss-dominated crusts may stem from the relatively large moss biomass effectively absorbing and holding N from our minor wet deposition event. The substantial movement of $^{15}NH_4^+$ may indicate a fungal preference for the reduced N form during amino acid transformation and translocation. We found a marginally significant enrichment of $\delta^{15}N$ in *A. hymenoides*

leaves but only in cyanobacteria biocrusts translocating $^{15}N$, offering evidence of links between biocrust constituents and higher plants. Our results suggest that minor rainfall events may initiate fungal loops potentially allowing constituents, like dark septate Pleosporales, to rapidly translocate N from $NH_4^+$ over $NO_3^-$ through biocrust networks.

## 1 Introduction

Fungi may act as conduits for biological networks connecting belowground ecosystem processes to plants. Soil fungi contribute to all aspects of litter decomposition through the generation of a myriad of extracellular enzymes (Osono 2007, Schneider et al. 2012); altered trophic dynamics, decomposer species diversity, and nutrient turnover rates (Hattenschwiler et al. 2005); and by forming multiple types of endophyte-plant symbioses (Johnson et al. 1997, Saikkonen et al. 2004). Endophytic fungi, in particular, form hubs connecting spatially and temporally distinct microbial-mediated soil processes and plants. For example, the pervasive distribution of mycorrhizae in mesic systems allows common mycorrhizal networks to deliver essential resources, which promotes or hinders seedling growth depending on the network species composition (van der Heijden and Horton 2009), and facilitates the one-way transfer of multiple forms of N and P between two plant species linked by arbuscular mycorrhizae and ectomycorrhizae (He et al. 2003, Walder et al. 2012). In xeric systems, endophytic fungi are also implicated in moving resources within biological networks in a theory known as the fungal loop hypothesis. The hypothesis states that fungi, supported by biotrophic C from plants and terrestrial cyanobacteria, act as intermediate reservoirs transforming and translocating resources between soils and plants (Collins et al. 2008, 2014). Perhaps the most notable example of a fungal loop, albeit from a limited number of studies, occurred in fungal-dominated cyanobacteria biocrusts from a Chihuahuan Desert grassland. Specifically, $^{15}NO_3^-$ applied to a root-free biocrust rapidly moved into the perennial grass,

70 *Bouteloua* sp., up to 1 m away within 24–hours (Green et al. 2008). Furthermore, $^{13}C$-labeled glutamic acid applied to leaf surfaces of *Bouteloua* was found in biocrusts.

72 Despite the intriguing evidence, many aspects of this burgeoning hypothesis remain to be validated (Collins et al. 2014).

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Biocrust composition and soil moisture availability interactions may dictate the

76 movement of resources in fungal loops. Desert fungal-plant interactions occur across spatially discontinuous patches of vegetation interspersed by patches of soils

78 colonized by biocrusts (Belnap et al. 2005). Fungi participating in loops are necessarily associated with a mosaic of other biocrust organisms (i.e., cyanobacteria,

80 green algae, lichens, mosses, and other bacteria). The metabolic activity of biocrust constituents participating in fungal loops, including plants, are moisture-dependent

82 and regulated by the magnitude and seasonality of episodic rainfall events. A pulse-reserve paradigm (Collins et al. 2008) may explain biological activities where minor

84 rainfall pulses stimulate microorganisms, generating reserves of resources to be exploited during subsequent rainfall events (Huxman et al. 2004, Welter et al. 2005).

86 In such loops, minor rainfall events may stimulate $N_2$ fixation by free or lichen-associated cyanobacteria (Belnap et al. 2003), N mineralization by bacteria and fungi

88 (Cable and Huxman 2004, Yahdjian and Sala 2010), and nitrification and possibly denitrification (Wang et al. 2014), potentially altering levels of $NH_4^+$ or $NO_3^-$. Fungal

90 species, including fungal endophytes, may compete with mosses, lichen, cyanobacteria, and other bacteria for newly released N. Once sequestered, the N may

be transformed into amino acids and transported within mycelium (Jin et al. 2012, Behie and Bidochka 2014). Larger rainfall events may then activate plants, allowing the host plant to receive N from the fungi and transfer photosynthate to the fungal endophyte. If fungal endophytes are poor competitors for newly released N, preferentially sequester one inorganic N form over another, or more efficiently transform and transport $NH_4^+$ rather than $NO_3^-$, biocrust constituents and N form may influence the translocation of N in fungal loops.

The fungal endophytes most likely involved in the loop hypothesis are dark septate fungi. Few arbuscular mycorrhizal fungi are found in biocrusts (Porras-Alfaro et al. 2011) or as endophytes in desert plants (Titus et al. 2002), due to mycorrhizae being relatively sensitive to dry soil conditions (Aguilera et al. 2016). In contrast, the majority of biocrust fungi are Ascomycota, with the Pleosporales being widespread and dominant (Bates et al. 2012, Porras-Alfaro et al. 2011). Pleosporales, along with other Ascomycota fungal orders, contain dark septate endophytes (Jumpponen and Trappe 1998). Dark septate endophytes are thermal- and drought-tolerant fungi due to melanin-rich cell walls conferring protection from UV and drought stress (Gostincar et al. 2010). Taken together, the prevalence of dark septate fungi in desert systems, along with their ability to maintain metabolic activity under low water potentials (Barrow 2003), makes these endophytes excellent candidates to translocate resources in loops (Green et al. 2008).

114 Minor rainfall events may allow fungi to act as conduits and reservoirs for N. To investigate the potential for biocrust constituents and N form to influence the

116 movement of N through the putative fungal loops, we created minor, localized rainfall events and measured $\delta^{15}N$, from $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$, within the surrounding dry

118 cyanobacteria- and moss-dominated crusts, and grass, *Achnatherum hymenoides* (Indian ricegrass). In tandem with $^{15}N$ analyses, we estimated the biomass of two

120 major division of fungi (Ascomycota and Basidiomycota) and bacteria, and characterized fungal communities by sequencing the 18S rRNA gene to identify

122 potential links between fungal taxa and $^{15}N$ movement.

## 124 2 Materials and methods

### 2.1 Site description

126 We conducted our study in two cold desert ecosystems of the Colorado Plateau, UT. One site was near Castle Valley (40°05’27.43”N-112°18’18.24”W) and the other was

128 adjacent to the US Geological Survey (USGS) Southwest Biological Science Center Research Station in Moab, UT (40°05’27.43”N-112°18’18.24”W). Rugose crusts

130 consisting of 22% moss *Syntrichia caninervis* , 5-7% cyano-lichens *Collema tenax* and *Collema coccophorum* and 55% cyanobacteria cover the Castle Valley site

132 (Darrouzet-Nardi et al. 2015), while smooth, light algal crusts dominated by one cyanobacterium, *Microcoleus vaginatus*, cover the USGS site. Across both sites,

134 vegetation is dominated by perennial grass *Achnatherum hymenoides* (Roem & Schult) and the native perennial shrub *Atriplex confertifolia* (Torr. & Frém). Mean

136 annual temperature and precipitation at Castle Valley is 13ºC and 269 mm, while the USGS site is slightly warmer (MAT=13.8ºC) and drier (MAP=189 mm; based on 138 1981-2010 data; WRCC 2017). Both soils are Aridisols with Castle Valley classified as a sandy loam, calcareous Rizno series (Darrouzet-Nardi et al. 2015) and USGS as a 140 Bluechief series sandy loam.

142 **2.2 Simulated rainfall events and $^{15}N$ form applications**

We simulated minor, localized rainfall events and measured $\delta^{15}N$, from $^{15}N\text{-}NH_4^+$ and 144 $^{15}N\text{-}NO_3^-$ rainfall events containing two isotopically-labeled, inorganic N forms and tracked the movement of the label through our moss-dominated (Castle Valley) and 146 cyanobacteria-dominated biocrusts (USGS Station), and *A. hymenoides*. First, we randomly selected six circular plots per site with a radius of 1.0 m and at least 10 m 148 apart from each other. Three plots were assigned to be labeled with $K^{15}NO_3$ (99 at.%) and the other three plots to be labeled with $(^{15}NH_4)_2SO_4$ (99 at.%). Second, we 150 randomly selected five biocrust patches and five *A. hymenoides* along eight axes (e.g., N, NE, E, SE, S, SW, W, and NW) radiating from the center of each circular plot and 152 measured the radial distance to biocrusts or grasses. Third, we simulated a 2.5 mm rainfall event by spraying 3 mL of deionized water solution and either isotopic label 154 (0.30 mg $^{15}N$) onto a 5 cm diameter circle in the center of the circular plots (2 biocrust types × 3 circular plots locations × 2 N forms × ≈ 10 samples [4 – 5 biocrusts or 4 – 7 156 *A. hymenoides* depending on grass density in the circular plot] = 137). For the isotopic applications, either 2.60 g of 99 at.% $K^{15}NO_3$ or 1.70 g of 99 at.% $(^{15}NH_4)_2SO_4$ was

158 dissolved in 18 mL of deionized water to create a 1.43 M or 0.72 M solution, respectively. The $^{15}N$ additions wetted the sandy loams (bulk density ≈ 1.5 g $cm^{-3}$) to

160 a depth of 1 cm and added approximately equal $NH_4^+$ (10 μg N g $soil^{-1}$) and double $NO_3^-$ concentrations to surface soils (Sperry et al. 2006). All additions were

162 completed midday in April, as *A. hymenoides* were starting to set seed.

164 **2.3 Sample collection and $^{15}N$ analyses**

Biocrust and foliage samples were collected twenty-four hours after the simulated

166 rainfall event containing our different inorganic $^{15}N$ forms. Biocrusts were removed as three subsamples from each biocrust location with a soil corer (2 cm diameter × 5

168 cm length) to a depth of 2 mm. Crust distances away from the tracer application ranged from 22 – 97 cm. The composited soil samples were kept cold (5°C) in the

170 field, split in the lab, and a portion of the soil was frozen (-20°C) until we performed fungal and bacterial DNA analyses. We randomly selected five leaves from

172 *Achnatherum*, which ranged in distance anywhere from 29 – 120 cm away from the tracer application and in volume from 0.002 – 0.048 $m^3$. The leaves and remaining

174 soils (sieved 2 mm) were air-dried, ground in a reciprocating tissue homogenizer, and analyzed for $^{15}N$ using a PDZ Europa ANCA-GSL elemental analyzer, interfaced

176 with a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the University of California Davis Stable Isotope Facility

178 (http://stableisotopefacility.ucdavis.edu). We expressed the resulting isotope ratios in δ notation as parts per thousand (‰) where:

180 $$\delta^{15}N = (R_{sample} / R_{standard}) \times 1000 \quad (1)$$

$R$ is the molar ratio of the heavier to the lighter isotope ($^{15}N/^{14}N$) for the standard or

182 sample. To track the movement of inorganic N forms through our two biocrust types (moss-lichen-dominated and cyanobacteria dominated biocrust) and into grasses, we

184 analyzed the relationships between $\delta^{15}N$ present in crust and leaf tissue to the distance of the crust and *Achnatherum* by site by creating linear mixed effects models for the

186 biocrust type and N form combinations. In all models, distances of biocrusts or grasses was nested within individual circular plots, and the three plots were treated as

188 a random factor to control for the impact of plot differences on our response variable. All models were created with the *lm* function in R (R Development Core Team 2017).

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**2.4 Biomass estimations of major fungal components**

192 To investigate the potential for fungi to translocate our $^{15}N$ forms, we estimated the biomass of two major divisions of fungi (Ascomycota and Basidiomycota) and

194 bacteria in biocrusts using quantitative PCR. From the frozen biocrust samples, we extracted genomic DNA using a DNeasy PowerLyzer PowerSoil Kit (Qiagen, MD,

196 USA) and quantified the gene copy numbers of Ascomycota and Basidiomycota on a Mastercycler EP Realplex qPCR (Eppendorf, Hamburg, Germany) with SYBR Green.

198 We amplified division-specific regions of the internal transcribed spacer (ITS) with primer pair ITS5 (forward) and ITS4A (reverse) for Ascomycota (Larena et al. 1999)

200 and ITS4B (forward) and 5.8sr (reverse) for Basidiomycota (Fierer et al. 2005). We selected the universal bacterial 16S rRNA primer set EUB338, forward, and Eub518,

202 reverse, to estimate the biomass of bacteria (Aanderud et al. 2013). In 12.5 µl
reactions, using KAPA2G Robust PCR Kits (KAPA Biosystems, Wilmington, MA,
204 USA), we amplified targeted genes using the following thermocycler condition: an
initial denaturation step at 94ºC for 3 min followed by 35 cycles of denaturation at
206 94ºC for 45 s, annealing at either at 55 ºC (Ascomycota), 64ºC (Basidiomycota), or
60ºC (bacteria) for 30 s, and extension at 72ºC for 90 sec. We generated qPCR
208 standards for Basidiomycota, Ascomycota, and bacteria from biocrusts using the
TOPO TA Cloning® Kit (ThermoFisher Scientific, MA, USA) as outlined by
210 Aanderud et al. (2013). The coefficients of determination ($R^2$) for our assays ranged
from 0.90 to 0.99, and amplification efficiencies fell between 0.99 and 1.92. We
212 analyzed the relationships between biocrust $\delta^{15}N$ and the gene copy number of
Ascomycota, Basidiomycota, and bacteria by creating linear mixed effects models for
214 each biocrust type and N form combination (2 biocrust types × 3 circular plots
locations × 2 N forms × ≈ 1 – 5 biocrusts = 48). DNA from eight moss-dominated and
216 four cyano-dominated biocrusts were difficult to amplify, especially one moss
biocrust circular plot reducing the number of replicants from five to one. We used the
218 *lm* function in R and treated plot as a random factor and gene copy number nested
within plot. We further tested for differences in our biomass estimates between the
220 crust types using multiple t-tests and a Benjamini-Hochberg correction to control for
the false discovery rate associated with multiple comparisons (Benjamini and
222 Hochberg 1995).

224 **2.5 Biocrust fungal communities**

To identify the fungal taxa participating in N translocation, we characterized fungal

226 communities in biocrusts using bar-coded sequencing. We PCR amplified the V9 region of the 18S rRNA gene using a universal eukaryote primer set, 1391F and

228 EukBr, with a unique 12-bp Golay barcode fused to EukBr (Amaral-Zettler et al. 2009, Hamady et al. 2008). Thermocycler parameters were similar to qPCR analyses

230 and consisted of a denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, an annealing step at 57°C for 60 s, elongation at 72°C

232 for 90 s, and a final extension at 72°C for 10 min. We then purified and pooled PCR amplicon libraries to near equimolar concentrations using SequalPrep™

234 Normalization Plate Kits (Invitrogen, Carlsbad, CA, USA) and quantified the amplicon libraries by real-time qPCR using a KAPA Library Quantification Kit (Kapa

236 Biosystems, Wilmington, MA, USA). All samples were sequenced at the Brigham Young University DNA Sequencing Center (http://dnasc.byu.edu/) using the Illumina

238 HiSeq 2500 platform (Illumina Biotechnology, San Diego, CA, USA), generating 2 × 250 paired-end reads. Illumina sequence reads were analyzed within QIIME (v.

240 1.9.1), an open-source software pipeline suitable for microbial community analysis (Caporaso et al. 2010). We removed barcodes and primers with a custom, in-house

242 script previous to joining paired-end reads by using fastq-join under default parameters (Aronesty 2011). Joined reads were then de-multiplexed and checked for

244 chimeras (Haas et al. 2011). We then clustered the de-multiplexed reads into OTUs, applying a similarity threshold of 97%, using QIIME's default OTU clustering tool-

246 uclust (Edgar et al. 2011). Taxonomies of representative OTUs were assigned using uclust and the 18S rRNA gene SILVA 128 database which was clustered into OTUs

248 at 97% similarity (Quast et al. 2013). To evaluate if biocrust type supported similar fungal composition, we calculated the relative recovery of 27 fungal orders, including

250 dark septate lineages. We tested for differences between biocrust types using t-tests and a Benjamini-Hochberg correction (2 biocrust types × 6 randomly selected

252 biocrusts = 12).

254 **3 Results**

**3.1 Translocation of $^{15}NH_4^+$ in cyanobacteria biocrusts**

256 The movement of $^{15}N$ was clearly apparent in cyanobacteria-dominated biocrusts following the addition of $^{15}NH_4^+$, with $\delta^{15}N$ decreasing as the radial distance from the

258 central application point of $^{15}NH_4^+$ increased. In the overall model:

260 $$\delta^{15}N \text{ biocrust} = 51 - 0.71 \text{ (distance)} + 0.06 \text{ (distance} \times \text{plot)} \quad \text{(eq. 1)}$$

262 ($R^2 = 0.67$, $F_{2,12} = 11$, $P = 0.002$, figure 1A), distance was significant ($P = 0.003$) and possessed a negative slope. Surrounding the tracer application, $\delta^{15}N$ was enriched

264 upwards of 40‰ more than 20 cm away and continued to be enriched to approximately 10‰ almost 100 cm away with enrichment ranging from 9.1 – 49‰.

266 There was no such relationship between $\delta^{15}N$ and the radial distance from any other crust type or N form as demonstrated by the mixed effect models (cyanobacteria

268 biocrust $^{15}NO_3^-$ addition: $R^2 = 0.17$, $F_{2,12} = 1.2$, $P = 0.32$, figure 1B; moss biocrust

$^{15}NH_4^+$ addition: $R^2 = 0.01$, $F_{2,12} = 0.07$, $P = 0.94$, figure 1A; and moss biocrust

270 $^{15}NO_3^-$ addition: $R^2 = 0.16$, $F_{2,12} = 1.2$, $P = 0.33$, figure 1B). The location of the three circular plots was not a significant factor in any linear mixed effects models for

272 biocrusts.

274 **3.2 Potential movement of $^{15}NH_4^+$ into grass leaves in cyanobacteria biocrusts**

None of the label appeared to reach *A. hymenoides* leaves in either of the two types of

276 biocrusts. However, in cyanobacteria-dominated biocrusts where $^{15}N$ from $^{15}NH_4^+$ was translocated, the mixed effects model relating $\delta^{15}N$ from $^{15}NH_4^+$ into *A.*

278 *hymenoides* leaves was significant ($R^2 = 0.37$, $F_{2,17} = 5.1$, $P = 0.02$):

280 $$\delta^{15}N \text{ leaves} = 3.1 - 0.03 \text{ (distance)} + 0.01 \text{ (distance} \times \text{plot)} \quad \text{(eq. 2)}$$

282 and the radial distance was marginally significant ($P = 0.08$, data not shown). The $\delta^{15}N$ in leaves declined the further away the grass was from the $^{15}N$ application point,

284 with leaf enrichment between 1.0 – 6.7‰. In the other three models, the $R^2$, F values, and *P*-values of all other mixed effects models ranged from 0.06 – 0.10, 0.46 – 0.67,

286 and 0.54 – 0.64 respectively (data not shown). The $\delta^{15}N$ found in *A. hymenoides* leaves was 2.6‰ ±0.45 (means ±SEM) in cyanobacteria biocrusts exposed to $^{15}NO_3^-$

288 and 0.85‰ ±0.47 in moss biocrusts.

290 **3.3 $^{15}NH_4^+$ movement in cyanobacteria biocrusts related to Ascomycota**

The biocrust that translocated N also exhibited a robust relationship between the

292 proxy for Ascomycota biomass and biocrust $\delta^{15}N$. In cyanobacteria biocrusts, the greater the gene copy number of Ascomycota the lower the $\delta^{15}N$ from $^{15}NH_4^+$. In the

294 overall model:

296 $\delta^{15}N = 280 - 37$ (gene copy number Ascomycota) + 1.1 (distance × plot) (eq. 3)

298 ($R^2 = 0.63$, $F_{2,8} = 6.8$, $P = 0.02$, figure 2A), the proxy for Ascomycota biomass was significant ($P = 0.01$) and negatively related to $\delta^{15}N$. In cyanobacteria crusts exposed

300 to $^{15}NO_3^-$, and in the moss-dominated crusts, however, there was no such relationship between $\delta^{15}N$ from $^{15}NO_3^-$ and Ascomycota gene copy number (cyanobacteria crust

302 model: $R^2 = 0.08$, $F_{2,12} = 0.6$, $P = 0.58$, figure 2B; moss crust model: $R^2 = 0.16$, $F_{2,12} = 1.2$, $P = 0.33$, figure 2B) or in moss crusts exposed to $^{15}NH_4^+$ additions ($R^2 = 0.01$,

304 $F_{2,12} = 0.07$, $P = 0.94$, figure 2A). Basidiomycota and bacteria biomass in both crust types was not related to either N form (data not shown). The biomass estimates of all

306 measured biocrust components were consistently higher in moss- than cyanobacteria-dominated crusts. Basidiomycota biomass was $1.5\times10^9 \pm 5.5\times10^8$ in cyanobacteria and

308 $5.8\times10^9 \pm 7.2\times10^8$ in moss biocrusts (t-test, t = 4.5, $P < 0.0001$, $df = 1$, data not shown). Ascomycota biomass was $2.6\times10^7 \pm 4.5\times10^6$ in cyanobacteria and $1.1\times10^8$

310 $\pm 2.4\times10^7$ in moss biocrusts (t-test, t = 3.3, $P = 0.003$, $df = 1$). Bacterial biomass was at least two orders of magnitude lower in cyanobacteria biocrusts

312 (cyanobacteria=$5.5\times10^6$ $\pm8.9\times10^5$ and moss crusts=$2.7\times10^7$ $\pm4.8\times10^6$, (t-test, t = 4.5, $P < 0.0001$, $df = 1$).

314

**3.4 Dark septate fungi as major components of biocrusts**

316 Four of the nine fungal orders, known to contain dark septate endophyte members, were present in both biocrust types, with the Pleosporales and Pezizales being

318 dominant taxa. In biocrusts, fungi comprised much of eukaryotic community (cyanobacteria = 30% ±4.7 and moss = 33% ±4.0), Ascomycota was the most

320 common fungal division (cyanobacteria = 82% ±2.8 and moss = 87% ±2.9), and orders with known dark septate members accounted for at least 67% of the

322 Ascomycota (cyanobacteria = 83% ±4.8 and moss = 67% ±8.6, figure 3). In cyanobacteria biocrusts, Pleosporales accounted for 66% (±6.9) of all dark septates

324 and the recovery of this taxa was twice as high in cyanobacteria- than moss-dominated crusts (t-test, t = 03.0, $P = 0.01$, $df = 1$). Even though the relative

326 abundance of Pleosporales differed, the number of gene copies of Pleosporales was similar between the two biocrusts (cyanobacteria = $1.7\times10^7$ $\pm6.3\times10^6$ and

328 moss=$2.9\times10^7$ $\pm1.3\times10^7$, t-test, t = 0.99, $P = 0.35$, $df = 1$) as determined by an extrapolation of qPCR values in conjunction with percent recovery of taxa for

330 Ascomycota. The *Pezizales* comprised a relatively large percentage of the biocrust community in moss-dominated biocrust with a recovery of 15% (± 8.3) and 28% (±

332 9.0) in cyanobacteria- and moss-dominated crusts, respectively (t-test, t = 1.1, $P =$

0.32, *df* = 1). Eukaryotic community data was based on the recovery of 1,232,312 quality sequences and 5,176 unique OTUs.

## 4 Discussion

In biological networks, the magnitude and direction of resource transfer in fungi is predominantly thought to be influenced by the physiological source-sink gradients created by individual plants (Fellbaum et al. 2014) or between plants (Weremijewicz et al. 2016). However, fungi may be more than just passive conduits by exerting control over resources due to their own sink-source resource needs (Simard and Durall 2004). Our findings suggest that a minor, localized rainfall event may initiate fungal loops by allowing the rapid translocation of N in biocrusts at a rate of 40 mm $h^{-1}$ in twenty-four hours. The movement of N was only apparent in the cyanobacteria-dominated crusts, where $\delta^{15}N$ decreased as the distance from the addition of the $^{15}NH_4^+$ label increased. Further, the presence of Ascomycota was related to the amount of biocrust $\delta^{15}N$ from $^{15}NH_4^+$, with the isotope being diluted in soils with higher levels of Ascomycota biomass. Eighty-three percent of the Ascomycota were from four fungal orders containing known dark septate endophytes and 66% of these taxa were from one order, the Pleosporales. Taken together, our results suggest that fungal loops are potentially structured by fungal constituents, especially Pleosporales, translocating N from $NH_4^+$ over $NO_3^-$.

### 4.1 Fungal loops only in cyanobacteria-dominated crusts

Our findings suggest that fungal loops do occur in cyanobacteria-dominated biocrusts. Biocrust components, predominantly cyanobacteria, are known to fix and secrete up to 50% of their newly fixed C and 88% of N into surrounding soils within minutes to days of fixation, depending on precipitation characteristics (Belnap et al. 2003). Thus, active crust constituents may exude resources for other biocrust constituents, such as fungi, to acquire and initiate fungal loops. Our results are similar to Green et al. (2008) whose previous work identified loops in cyanobacteria biocrusts moving $^{15}N$ from isotopically labeled nitrate in the Chihuahuan Desert. Although we only found movement of $^{15}N$ as labeled ammonium, our label moved at a comparable rate to Green et al. (2008; 40 mm $h^{-1}$ versus 44 mm $h^{-1}$, respectively). The lack of $^{15}N$ movement from labeled nitrate in our study may stem from the short time period that we employed to measure translocation. When Green et al. (2008) evaluated $^{15}N$ movement at two time points, 24 hours and four days after tracer application, they found that biocrust enrichment consistently increased over time. Therefore, if we had continued to assess biocrusts isotopic signatures after 24 hours, we potentially could have captured the movement of labeled nitrate as well.

**4.2 Addressing the lack of loops in moss-dominated crusts**

The seeming lack of loops in moss-dominated crusts may stem from the relatively large moss biomass of *S. caninervis* effectively absorbing and holding N from our minor wet deposition event. We measured elevated levels of biomass of all biocrust constituents (i.e., mosses, fungi, and bacteria) in moss-dominated relative to

cyanobacteria-dominated biocrusts. If most biocrust constituents were N limited,
378 including mosses, then the $^{15}N$ was possibly retained in place to meet N deficiencies
before being disseminated to other crust constituents outside the application zone. The
380 ability of mosses to scavenge atmospheric deposited N is well recognized in other
systems (Liu et al. 2013, Fritz et al. 2014). Most mosses acquire N from either
382 wet/dry atmospheric deposition (Yaunming et al. 2016) or as biogenic sources from
cyanobacterial associations on their leaves (Rousk et al. 2013). Mosses do alter N
384 dynamics in arid environments. When *S. caninervis* was lost from our system, a
dramatic increase in nitrification rates were observed (Reed et al. 2012). The higher
386 levels of nitrification were most likely supported by the decomposition of dead moss
biomass and subsequent release of new $NH_4^+$; however, after the moss mortality,
388 inorganic N pooled as $NO_3^-$, in the remaining cyanobacteria-dominated biocrust.
Thus, the presence or absence of mosses has the potential to alter N pools and fluxes
390 in biocrusts.

The lack of $^{15}N$ movement in moss-dominated crusts may reside in the nature of
392 our minor rainfall event. Our moss, *S. caninervis*, became photosynthetically active
following the 2 mm rainfall event, changing in color from brown to green, but only in
394 the discrete biocrust patches that we watered. Mosses, including *S. caninervis*, are
stimulated by minor rainfall events (Wu et al. 2014), with events as small as 1 mm
396 activating moss photosynthesis (Coe et al. 2012). Our rainfall event was intended to
wet a small circle of biocrust to a depth of 1 cm. However, the additional
398 aboveground biomass of mosses and the rugose topography of moss-dominated crusts

relative to the smooth cyanobacteria-dominated crusts may have limited the depth our minor rainfall event penetrated the soil and, in turn, activated other biocrust components. Also, water from our event might have evaporated more quickly from the mossy biocrust surface, limiting the activity time of all constituents involved. To more conclusively determine the potential for fungal loops to exist in moss-dominated biocrusts, more information is needed to determine the importance of effective rainfall size in initiating fungal loops.

N may move differently in moss-dominated biocrusts compared to other biocrust types. If desert mosses have fungal associations, then fungi have the potential to move sequestered N to the mosses in a new kind of loop. Rhizoids and thalli of bryophytes may contain fungal associations (Pressel et al. 2010). Dark septate endophytes colonize mosses (Day and Currah 2011). Taken together, the exchange of photosynthate and N may occur in a tighter, more localized loop between mosses and dark septate fungi, even following a minor rainfall event.

### 4.3 Loops may preferentially move $NH_4^+$ over $NO_3^-$

We found that $NH_4^+$, but not $NO_3^-$, was rapidly translocated within cyanobacterial crusts. The enrichment of $\delta^{15}N$ from $^{15}NH_4^+$ in cyanobacteria biocrusts was related to our proxy for Ascomycota biomass and potentially dark septate fungi due to their dominance in our sequencing effort. We explain the negative relationship between Ascomycota gene copy number and $\delta^{15}N$ signal as a simple dilution—the higher our biomass estimates of Ascomycota, the more spread in the $^{15}N$ signal. Although the

physiology of dark septate fungi remains relatively unexplored, if our desert fungi are like most other fungi, then the preferential movement of $NH_4^+$ is understandable. Generally, fungi prefer $NH_4^+$ over $NO_3^-$ (Eltrop and Marschner 1996), as $NH_4^+$ is readily acquired by fungi and assimilated into amino acids. After $NH_4^+$ uptake and assimilation via the glutamate synthase or GS/GOGAT cycle, N is incorporated into arginine through the urea cycle (Jin et al. 2012) due to the direct assimilation of $NH_4^+$ into the GS/GOGAT pathway (Courtly et al., 2015). Thus, $NH_4^+$ is most likely transformed into arginine and moved within mycelium by amino acid transporters (Govindarajulu et al. 2005, Garcia et al. 2016). There is some evidence for the importance of amino acid transporters in fungal endophytes, as arbuscular colonization leads to an increase in the uptake of arginine and multiple other amino acids (e.g., phenylalanine, lysine, asparagine, histidine, methionine, tryptophan, and cysteine) by their host plants (Whiteside et al. 2012). However, to verify the movement of N through Ascomycota and the role of biomass in translocation, a more direct approach is needed. For example, quantum dots (fluorescent nanoscale semiconductors) have tracked the flow of organically derived N into arbuscular mycorrhizae and into *Poa annua* in less than 24 hours (Whiteside et al. 2009). The N form $NO_3^-$ did move into our cyanobacteria crusts but not nearly to the extent reported by Green et al. (2008). Besides fungal preferences, other factors may play a role in the uptake of N, such as the increase in mobility of $NO_3^-$ in soils, differences in soil cation exchange capacity due to clay content, or fungi capitalizing on the more abundant N form specific to a soil. Unfortunately, based on our design, we were

unable to distinguish the form of N captured or translocated by biocrust constituents. More information is needed to identify the importance of N form and the movement of organic N within fungal loops.

**4.4 Dark septate and Pleosporales as conduits**

Our results support the idea that Pleosporales are the most likely conduits for N. Four of the nine fungal orders we identified contained known dark septate endophytes with one order, Pleosporales, being the most abundant. The Pleosporales accounted for 66% of the Ascomycota taxa in cyanobacteria crusts. Based on the relationship between $\delta^{15}N$ and Ascomycota biomass, the overwhelming abundance of Pleosporales, and the universal occurrence of Ascomycota in biocrusts, the Pleosporales assumedly play a role in fungal loops. We are not the first to reach this conclusion. Green et al. (2008) also identified Pleosporales as being the primary candidate involved in fungal loops. In their semi-arid grassland, Pleosporales were the most common taxa on *Bouteloua* roots, in the rhizosphere, and in biocrusts. We found 799 operational taxonomic units, based on 97% similarity, with all of the identifiable sequences, belonging to three genera: *Leptosphaeria* (1.6% of Pleosporales sequences), *Morosphaeria* (3.8% of Pleosporales sequences), and *Ophiosphaerella* (8.1% of Pleosporales sequences; data not shown). *Leptosphaeria* and *Ophiosphaerella* may be pathogenic endophytes on grass species (Martin et al. 2001, Yuan et al. 2017), but may also be beneficial by delaying and reducing the symptoms of other fungal pathogens (Yuan et al. 2017). However, 86% of our Pleosporales taxa

were unidentifiable based on our target genetic region and are potentially novel,
466 suggesting that much remains unknown about dark septates in deserts. Further
research is needed to address the theory of Pleosporales conduits in biocrusts and the
468 ecological importance of the dark septate endophytes in desert systems.

470 **4.5 Little N translocation to grass**

Due to the discrete nature of our minor, localized rainfall event, we were not surprised
472 that little of the label entered the leaves of *A. hymenoides*. In the fungal loop
hypothesis, a larger rainfall event triggers the plant to become a sink for the N
474 building up in fungi over previous minor rainfall events. We conducted our
experiment absent of a larger rainfall event and our 2.5 mm rainfall event was applied
476 over a 5 cm diameter circle of soil in early summer. When a similar precipitation
event size (2 mm) was applied across a much larger area (4 x 4 $m^2$ plot) on Colorado
478 Plateau soils during spring or summer, the predawn water potential of *A. hymenoides*
was similar one day after and one day prior to watering (Schwinning et al. 2003).
480 Thus, our minor rainfall event most likely failed to alter the water status of the grass
or cause grasses to become a sink for N. However, we did find some evidence of $^{15}N$
482 in *A. hymenoides* leaves following our $^{15}NH_4^+$ application in cyanobacteria-dominated
crusts. The pattern of the enrichment, a dilution with increasing distance from the
484 application point, was similar to the pattern found in biocrusts, but the most enriched
leaves (6.7‰) were less than the least enriched crust (9.1‰). Even though the
486 enrichment was minor, the presence of $^{15}N$ in *A. hymenoides* provides some evidence

of biocrust constituents being linked to plants and the translocation of N occurring even in the absence of a larger rainfall event to enhance grass activity. In April, at the time of the experiment, *A. hymenoides* was photosynthetically active. If we had added more label or evaluated the isotope signature of roots, we may have detected more $^{15}N$ label in grass tissue.

## 5. Conclusion

Cyanobacterial biocrusts are potentially interconnected in extensive biological networks. In light of the absence of N movement in moss-dominated crusts, mosses may hinder fungal loops. The abundant moss biomass in some biocrust may absorb and retain the applied N label. Due to the dominance of dark septate endophytes in biocrusts, fungi may act as conduits within the network. Our results add to the indirect evidence of fungal loops, but more information is needed to quantify the environmental conditions and biocrust constituents controlling the magnitude and directionality of the translocation of N to vascular plants.

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**Author contributions** ZTA and JB designed the study. ZTA, TBS, NW, AST, and JB conducted the experiments. ZTA, TBS, NW, AST, YZ, and JB analyzed and interpreted the data. ZTA, TBS, NW, AST, YZ, and JB helped write and review the manuscript. ZTA agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of interest** The authors declare no conflict of interest.

520 **Figure legend**

**Figure 1 Cyanobacteria biocrusts facilitated the translocation of N from $^{15}NH_4^+$**

522 **in twenty-four hours.** Based on linear mixed effects models, $\delta^{15}N$, from $^{15}NH_4^+$, ($R^2$ = 0.67, $F_{2,12}$ = 11, *P* = 0.002, A) decreased as the radial distance from the isotopic

524 application increased. There was no such relationship between $\delta^{15}N$ and the radial distance from any other crust type or N form (A, B) Values are $\delta^{15}N$ (‰) from two

526 biocrust types, cyanobacteria- and moss-dominated crusts, across circular plots (radius of 1.0 m) with a central application (5 cm diameter circle) of $^{15}NH_4^+$ or $^{15}NO_3^-$

528 in a simulated, minor rainfall event.

530 **Figure 2 Ascomycota biomass influenced the distance N traveled.** In cyanobacteria crusts, $\delta^{15}N$, from $^{15}NH_4^+$, was diluted as Ascomycota gene copy

532 number increased ($R^2$ = 0.63, $F_{2,8}$ = 6.8, *P* = 0.02, A). A similar pattern was not apparent in any other crust type or N form (A, B). Values are $\delta^{15}N$ (‰) from biocrusts

534 and Ascomycota gene copy numbers, an approximation of biomass, from qPCR of the ITS region with primer pair ITS5 and ITS4A.

536

**Figure 3 Pleosporales were the dominant Ascomycota order and contained**

538 **dark septate species.** Pie chart values are means (*n*=6) of the relative recovery from nine fungal orders, four of which contain dark septate endophytic taxa. Recovery was

540 based on OTUs from eukaryotic community libraries of the 18S rRNA gene (97% similarity cutoff).

542

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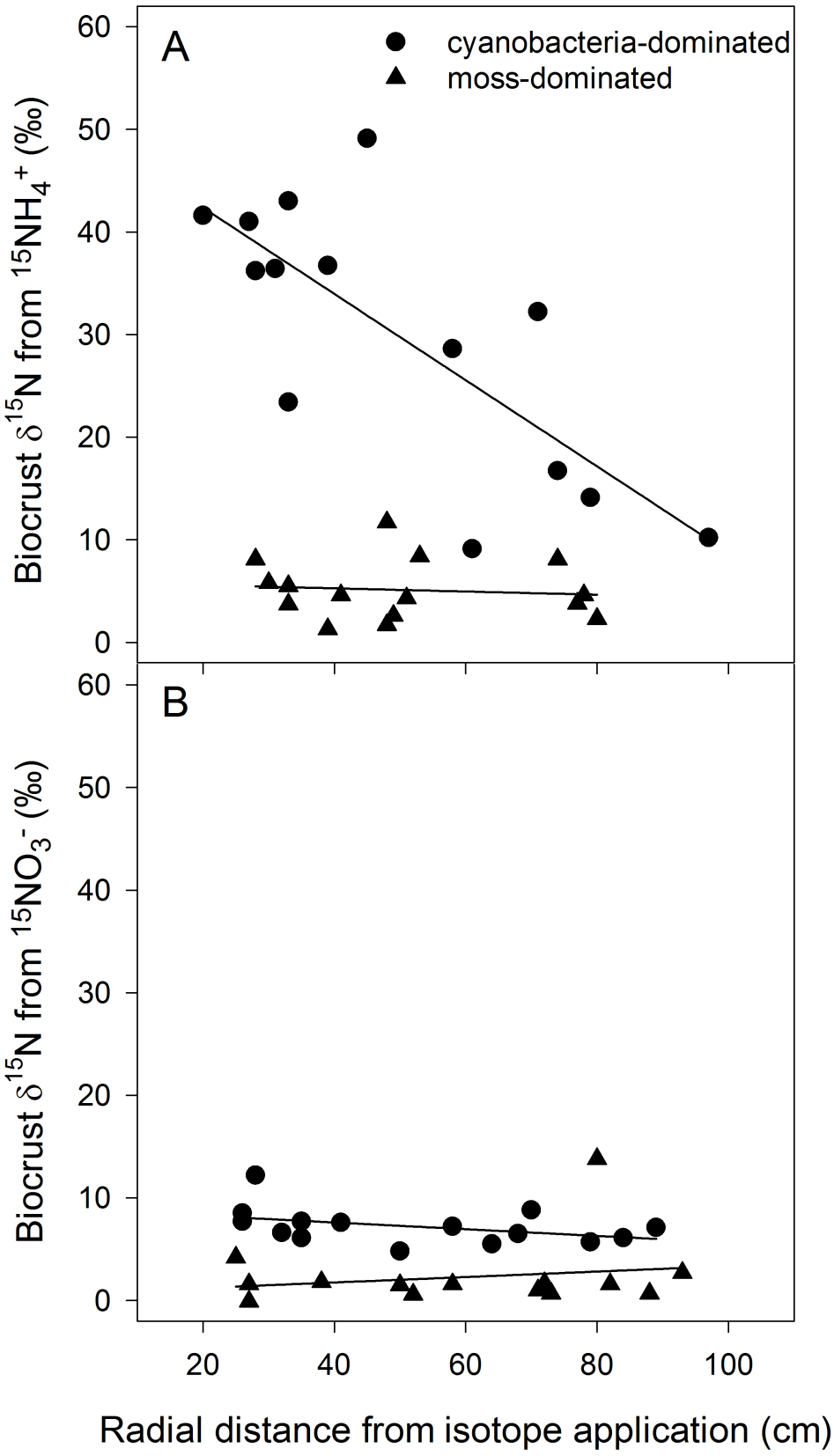
**Figure 1**


A
cyanobacteria-dominated
moss-dominated
Biocrust δ15N from 15NH4+ (‰)
B
Biocrust δ15N from 15NO3- (‰)
0
10
20
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40
60
80
100
Radial distance from isotope application (cm)

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716 **Figure 2**

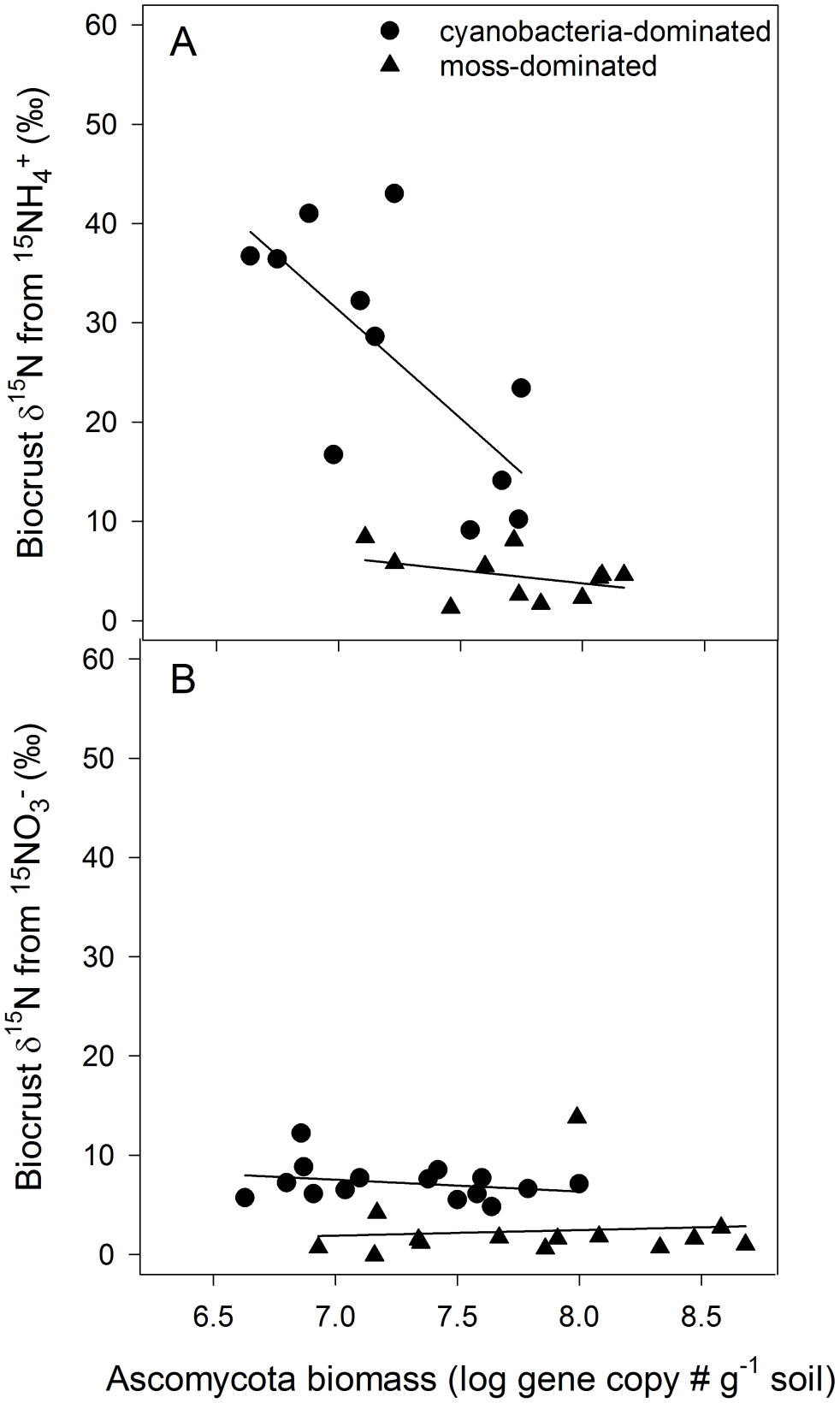


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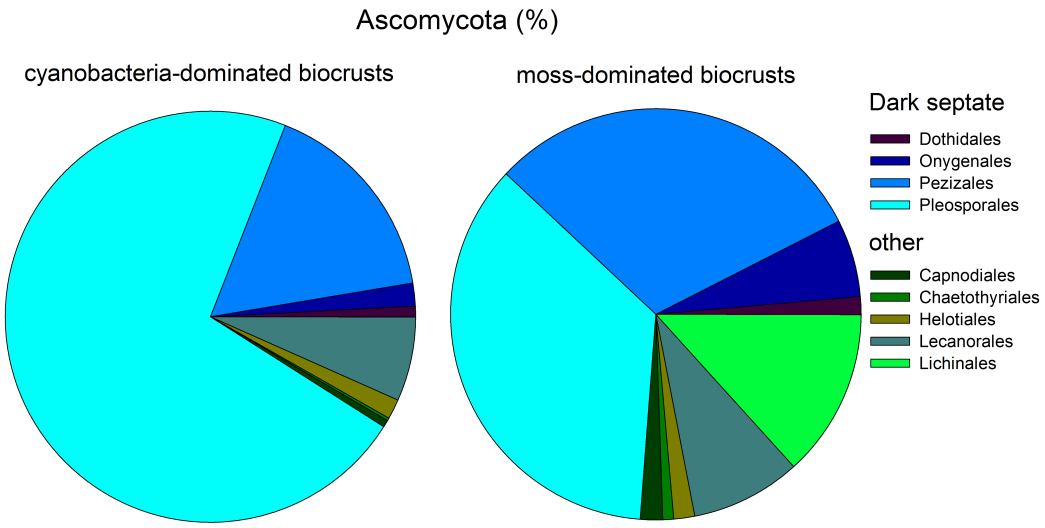
**Figure 3**

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