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Disturbance to biocrusts decreased cyanobacteria, N-fixer abundance, and grass leaf N but increased fungal abundance

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Abstract: Interactions between plants and soil microbes influence plant nutrient transformations, including nitrogen (N) fixation, nutrient mineralization, and resource exchanges through fungal networks. Physical disturbances to soils can disrupt soil microbes and associated processes that support plant and microbial productivity. In low resource drylands, biological soil crusts ("biocrusts") occupy surface soils and house key autotrophic and diazotrophic bacteria, non-vascular plants, or lichens. Interactions among biocrusts, plants, and fungal networks between them are hypothesized to drive carbon and nutrient dynamics; however, comparisons across ecosystems are needed to generalize how soil disturbances alter microbial communities and their contributions to N pools and transformations. To evaluate linkages among plants, fungi, and biocrusts, we disturbed all unvegetated surfaces with human foot trampling twice yearly in dry conditions from 2013-2019 in cyanobacteria-dominated biocrusts in Chihuahuan Desert grassland and shrubland ecosystems. After five years, disturbance decreased the abundances of cyanobacteria (especially *Microcoleus steenstrupii* clade) and N-fixers (*Scytonema* sp., and *Schizothrix* sp.) by >77% and chlorophyll *a* by up to 55%, but conversely, increased soil fungal abundance by 50% compared to controls. Responses of root-associated fungi differed between the two dominant plant species and ecosystem types, with a maximum of 80% more aseptate hyphae in disturbed than control plots. Although disturbance did not affect ^{15}N tracer transfer from biocrusts to the dominant grass, *Bouteloua eriopoda*, disturbance increased available soil N by 65% in the shrubland, and decreased leaf N of *B. eriopoda* up to 16%, suggesting that although rapid N transfer during peak production was not affected by disturbance, over the long term, plant nutrient content was disrupted. Altogether, the shrubland may be more resilient to detrimental changes due to disturbance than grassland, and these results demonstrate that disturbances to soil microbial communities have potential to cause substantial changes in N pools by reducing and reordering biocrust taxa.

Keywords: biological soil crust, *Bouteloua,* disturbance, drylands, fungal loop hypothesis, root fungi, sequencing, Sevilleta LTER, soil nitrogen, stable isotopes

Introduction

Physical disruptions to microbial communities are key components of anthropogenic impacts on biogeochemical cycles and ecosystem functions (Rocca et al. 2019) because soil microbes catalyze reactions that render elements usable for other organisms (Falkowski et al. 2008). Soil microbes that are symbiotic with plants are critical for plant nutrient dynamics (e.g., root nodulating rhizobia or root endophytic fungi; Peterson et al. 2008), but how these interactions change following disturbances is unresolved for most natural, non-agricultural ecosystems. Understanding the impacts of physical disturbance on the interrelationships and nutrient exchanges among plants, symbiotic microbes, and soil microbes is important for predicting changes to ecosystem functions such as productivity and erosion in areas with increasing human impacts from recreation, development, livestock, or military operations.

The impacts of physical soil disruption on soil microbial communities may be particularly pronounced in systems with low resource availability and abundant soil surface microbes, such as drylands. Drylands typically have low levels of N-fixation (under 20 nmol cm^{-1} h^{-1}) and a range of losses from gaseous fluxes (denitrification losses 2-300 ng N m^{-2} s^{-1}) and erosion (0.11-1.17 mg N m^{-2} m^{-1}; Hartley et al. 2007). Drylands support diverse soil surface microbial communities called biological soil crusts ("biocrusts") and composition varies greatly among ecosystem types, from complex biocrusts dominated by autotrophic mosses and lichens that occur in cooler, wetter drylands to less structurally complex biocrusts dominated by cyanobacteria in the genus *Microcoleus* spp. (Weber et al. 2016), which are common in hotter, drier drylands (Garcia-Pichel et al. 2013), all with heterotrophic bacteria, archaea, and fungi. Biocrust taxa such as *Nostoc* spp., *Scytonema* spp., and *Schizothrix* spp. (Weber et al. 2016) fix

atmospheric nitrogen. In moss and lichen-dominated biocrusts, physical disturbance can reduce their cover, alter autotrophic and heterotrophic community composition (Ferrenberg et al. 2015, Steven et al. 2015, Faist et al. 2017, Alfaro et al. 2018, Bao et al. 2019, Bao et al. 2020), and decrease the photosynthetic potential of biocrusts (Belnap et al. 1994, Belnap 2002, Kuske et al. 2012, Chung et al. 2019). The amount of moisture and texture affect the response of biocrusts to disturbance (Belnap and Eldridge 2001). Disturbance can reduce inputs through N-fixation (Belnap et al. 1994, Evans and Belnap 1999, Belnap 2002, Kuske et al. 2012) and increase ammonium availability, but generally has not affected soil nitrate availability (Evans and Belnap 1999, Barger et al. 2006, Alfaro et al. 2018, Bao et al. 2019). Because *Microcoleus*-dominated biocrusts are more tolerant of hot and dry conditions than moss-lichen biocrusts (Ferrenberg et al. 2015), knowledge of how soil disturbance affects these common biocrusts can improve understanding of dryland biogeochemistry with future warming climate**.**

Biocrusts, fungi, and plants are hypothesized to interact through nutrient transfers along fungal hyphal networks that are regulated by episodic rain events, an idea known as the fungal loop hypothesis (reviewed by Rudgers et al. 2018), and thus disturbance may impact the nutrient cycling among these networks. Dryland plants support root-associated fungi including arbuscular mycorrhizal fungi (Glomeromycotina) and darkly pigmented, septate fungi (phylum Ascomycota) that can supply nutrients to plants (Kiers et al. 2011, Fellbaum et al. 2012). Many fungal taxa are found in both cyanobacterial biocrusts and plant rhizospheres (Green et al. 2008, Steven et al. 2014), suggesting that some root-associated hyphae extend to the biocrust. The fungal loop has largely been investigated using gradients, by isotopically labeling N and/or C; e.g. labeled N added to biocrust is considered the source, fungi are conduits, and plants are sinks due to their N requirements, with sink strength that varies temporally. An alternative method is to disrupt some or all soil surface microbial communities that fix N to investigate how the nutrient

dynamics respond to altered plant-microbe interactions, and this method has practical applications to understanding livestock, vehicles, and other disturbances. Although some disturbances reduced soil fungal biomass (Belnap 1995, Bates and Garcia-Pichel 2009), in one case, soil fungi doubled with intermediate disturbance compared to either none or intense disturbance (Bao et al. 2020). Disturbance was shown to most affect the fungi that depend most strongly on autotrophs (Veresoglou et al. 2012). Disturbance like tillage typically decreases arbuscular mycorrhizae in agricultural contexts (Lekberg and Koide 2005), and thus the effects of disturbance on root-associated fungi may have outsized roles in resource cycling if fungi mediate nutrient transfers between biocrusts and plants.

Given the many roles of microbes in processing C, N, and other resources, disturbance to cyanobacteria and fungi may dramatically affect biogeochemical cycling and productivity, but how disturbance affects pools of soil N appears to be context dependent. Field disturbance experiments showed reduced (Belnap 1995, Barger et al. 2006, Kuske et al. 2012) or no effect on total soil N (Pietrasiak et al. 2011, Alfaro et al. 2018, Bao et al. 2019). Physical disturbances also decreased plant N content (Belnap 1995, Evans and Belnap 1999), but disturbance effects may differ among plant species, causing significant variation in % N by species (Belnap 1995). Thus, experiments that impose the same disturbance treatment to different ecosystem types and plant species are needed to advance a general understanding of the effects on dryland N cycling.

Stable isotope analyses are useful tools to study how disturbance alters dryland resource cycling. First, natural abundance values can suggest the amount of decomposition and cycling of N based on fractionation of ^{15}N. Newly fixed N generally has the same signature as atmospheric $\delta^{15}N$ (0 ‰), whereas N fractionation during decomposition enriches ^{15}N (He et al. 2009). Further work is needed to understand when physical disturbances alter fractionation, because disturbed sites had higher $\delta^{15}N$ in Utah, suggesting that disturbed plots were disconnected from recently-

fixed N (Evans and Belnap 1999), although trampling did not affect $\delta^{15}N$ in Chile (Alfaro et al. 2018). Second, stable isotope tracer experiments can directly investigate the fate of a resource through space and time. Previous researchers used ^{15}N additions to determine rates of N movement from biocrusts (source, N-fixation) to plants (sink, N in tissues) and proposed the fungal loop hypothesis (Green et al. 2008, Rudgers et al. 2018). Surface disturbance may affect biocrust-fungal-plant networks and, in turn, rates of N transfer to plants, and this network function is critical to understand in these low resource environments.

To understand how physical disturbances to cyanobacteria-dominated biocrusts affect microbial abundance and N availability, we added long-term replicated spring and fall soil disturbance treatments to two Chihuahuan Desert ecosystems – grassland and shrubland. We assessed key aspects of the fungal loop (cyanobacterial and fungal abundance and plant N) as well as non-fungal loop N pathways (Appendix S1: Section S1) and we used a ^{15}N tracer to directly assess transfer rates between biocrusts and plants when biocrusts were disturbed compared to intact. We hypothesized that soil surface disturbance would H1) reduce microbial abundance and biomass and H2) reduce microbial diversity because of declines in abundance and the loss of disturbance-susceptible taxa and H3) affect free-living fungi less than root-associated fungi. We predicted that H4) disturbance would reduce N availability in soils through declines in microbes that fix N, and H5) the N present under repeated soil disturbance regimes would be more heavily cycled (larger ^{15}N value) than recently fixed (smaller ^{15}N values), if disturbance reduces N-fixation and the transfer of recently fixed N. H6) Soil disturbance is expected to reduce plant N through declines in N sources (biocrust N fixers) and in fungal conduits in the biocrust-fungi-plant interaction network. Finally, we used a ^{15}N stable isotope tracer experiment in the grassland ecosystem to test directly the hypothesis that H7) disturbance reduced N transfer from biocrusts to plants due to disruption of the biocrust-fungi-plan interaction network.

Methods

We conducted research at the Sevilleta Long Term Ecological Research site in New Mexico, USA at the northern edge of the Chihuahuan Desert. Mean annual temperature and precipitation are 13.2 °C and 234 mm, respectively. Nearly 60% of the precipitation occurs during the summer monsoon from July-September (Báez et al. 2013). Dominant soils are sandy loams with pH >8 and mineralizable N from organic matter is ~7 μg g^{-1} (Kieft et al. 1998).

Soil Disturbance Treatment. Soil disturbance plots were established in May 2013 in two sites. The grassland site (34.33516° N, 106.70551° W, 1580 m) is dominated by black grama (*Bouteloua eriopoda*) and blue grama (*B. gracilis*). The shrubland site (34.34048° N, 106.73406° W, 1580 m) is dominated by creosote bush (*Larrea tridentata*) with *B. eriopoda* as a subdominant, but no *B. gracilis* in the plots. Both sites have biocrusts dominated by *Microcoleus* spp. At each site, we established twenty 6 m × 6 m plots (N = 40 plots). Half were randomly assigned to soil disturbance and half undisturbed controls. Two researchers stomped across all vegetation-free surfaces in the plots twice per year (May and October; 2013-2019) during dry soil conditions using boots with thick treads; plants were not disturbed (Chung et al. 2019). Human footfalls can alter biocrust function and recovery similar to livestock trampling (Barger et al. 2006, Ferrenberg et al. 2015, Faist et al. 2017).

Our study included microbial communities and N pools sampled at different time points in the disturbance treatments at one or both sites. We began our sampling after observations in April 2018 that the chlorophyll *a* content was at least double in control than disturbed plots in both ecosystems (Chung et al. 2019). Stomping occurred in May, and we collected soil and plant samples in June 2018 for N pools and soil and root fungal abundance (see details below). We collected additional soil samples in September 2018 and conducted the ^{15}N tracer experiment to observe rates of N transfer from biocrust to plants before the fall stomp treatment in October. We

collected chlorophyll *a* samples and soils for sequencing bacteria in September of 2019, also before the fall stomp treatment (see details below). Statistical analyses were performed in R (version 3.6.1, 2019-07-05; R Core Team 2019) and are described for each hypothesis test below. Post-hoc analyses used t-tests with false discovery rate adjustment for *P* values within each site (Benjamini and Hochberg 1995).

Disturbance effects on biocrust chlorophyll a *as measure of photosynthetic capacity.* In October 2018 and 2019, we collected ~0.5 cm depth × 0.82 cm diameter biocrust samples from the interspace between plants following Chung et al. (2019). In 2018, we incubated ~1 g of biocrust with 1 mL of dimethyl sulfoxide in the dark for 72 h at 23 °C and recorded absorbance at 750 and 665 nm (Castle et al. 2011). In 2019, we ground ~2 g of biocrust with acetone for 3 min and incubated in the dark for 12 h at 23 °C. Because methods differed, we analyzed each year's chlorophyll *a* (log transformed) with linear models with disturbance × site.

Disturbance effects on cyanobacteria community composition. We used qPCR paired with next generation amplicon sequencing of the 16S rRNA region following Fernandes et al. (2018; details in Appendix S1: Section S2). In September 2019, 10 biocrust subsamples per plot were collected using a soil corer (diameter = 1 cm, depth = 1 cm), then aggregated, homogenized, and sieved (2 mm). All representative sequences assigned to the phylum cyanobacteria or plastids were subject to individual, full phylogenetic scrutiny against the cyanobacterial database, Cydrasil, to produce accurate assignments at high level of resolution, following the default workflow (Garcia-Pichel et al. 2020). We calculated total cyanobacterial and potentially N-fixing taxon abundances (summed *Scytonema* sp. and *Schizothrix* sp.) as the total qPCR gene copies per sample, calculated Shannon diversity using the *vegan* package (Oksanen et al. 2019), and used linear models with disturbance × site. A randomization wrapper with 10000 permutations

("perm.") was used for total abundance and N-fixing taxa due to non-normal distribution of model residuals (Cassell 2002); sites were analyzed separately due to their large differences in community abundance. We used perMANOVA and permdisp (Anderson 2001; Bray-Curtis distances and 10000 permutations to create pseudo-*F* values) in Primer (version 6) with disturbance × site for the biocrust cyanobacteria community matrix consisting of absolute taxon abundances adjusted with qPCR. We identified Indicator Values ("IV") for all taxa using the "multpatt" function (De Cáceres et al. 2010; indicspecies package, 10000 perm.) that designate which taxa are bioindicators of each site × treatment group.

Disturbance effects on fungal abundance: soil ergosterol and root colonization. We aggregated two shallow biocrust samples (5 × 5 cm surface area, 0-2 cm depth) and aggregated two soil cores (2.5 cm diameter, 2-10 cm depth) from each plot in June 2018. We sieved soils (2 mm), removed roots, and subsampled for ergosterol, available N (below), and soil moisture. Soils were stored at 20 °C for < 3 d, extracted in 5 mL 0.8% KOH in MeOH, and heated at 80 °C for 30 m before filtration (Wallander et al. 2001). Ergosterol content was determined using high-performance liquid chromatography (HPLC; Thermo Scientific Ultimate 3000, Waltham, MA, USA) with a C18 reversed phase column and standards of 10 mg L^{-1} and 1 mg L^{-1} for calibration. Ergosterol content is ~5.70 μg mg^{-1} fungal biomass (Antibus and Sinsabaugh 1993) but arbuscular mycorrhizal fungi do not produce ergosterol (Olsson et al. 2003). Ergosterol content per g dry soil was log-transformed for linear mixed effects models ("lmer" function; lme4 package, Bates et al. 2015) with disturbance × depth (0-2 cm vs. 2-10 cm) × site as fixed effects, and plot as a random effect to account for non-independence of samples per plot.

Abundance of fungal morphotypes in the roots of the dominant grasses (*B. eriopoda* and *B. gracilis* in the grassland, *B. eriopoda* in the shrubland) were determined using microscopy. In

June 2018, we aggregated ~10, 3 cm segments of roots from the top ~10 cm of soil from three individual plants of each species. Roots were cleared in 10% KOH at room temperature, acidified with 1N HCl overnight, then stained with 5% ink (Parker Quink, Parker, Atlanta, GA, USA) and vinegar (Vierheilig et al. 1998). We examined roots at 200× and counted hyphal morphotypes as aseptate (potentially Glomeromycotina or other basal fungi) or septate (Ascomycota, Basidiomycota) in 100 fields of view following McGonigle et al. (1990). Data were log-transformed for linear mixed effects models with fixed effects of disturbance × morphotype (aseptate vs. septate) × site (for *B. eriopoda* roots) or disturbance × morphotype (*B. gracilis* roots), with plot as a random effect. We also cultured fungal endophytes from grassland *B. eriopoda* roots in June 2018 using Sanger Sequencing (Appendix S1: Section S3).

Disturbance effects on soil N availability. In June 2018, we analyzed the concentrations of biologically available forms of N, ammonium (NH_4^+) and nitrate (NO_3^-) at 0-2 cm and 2-10 cm depths. Values can vary considerably through time (Cregger et al. 2014), and this was a single snapshot. We used 0.5M K_2SO_4 extracts and conducted colorimetric assays (Rhine et al. 1998, Doane and Horwáth 2003) using a Synergy HT microplate reader (BioTek Instruments Inc., Winooski, VT, USA). We log-transformed ammonium or nitrate for linear mixed effects models that included disturbance × depth × site as fixed effects and plot as a random effect.

Disturbance effects on plant N, isotopic signatures. We compared N content and $\delta^{15}N$ in grasses from both grassland and shrubland in early June (pre-monsoon) and late July 2018 (monsoon). We aggregated 5-10 green leaves from each of three individual plants from *B. eriopoda* at both sites and from *B. gracilis* in the grassland. We dried the leaves at 60 ˚C for 3 d, ground them with a metal bead (0.2 mm chrome steel ball bearings; BioSpec Products Cat. No. 11079132c) in a bead beater (Qiagen Tissuelyser II, Germantown, MD, USA), and packed

approximately 5 mg of ground leaf tissue into tin capsules (Analytical Technologies Inc., Valencia, CA, USA). Samples were submitted to the Center for Stable Isotopes (University of New Mexico, USA) and analyzed on an ECS 4010 Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA) and a Delta V Isotope Ratio Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). We used linear mixed effects models with disturbance × site × month (for *B. eriopoda*) or disturbance × month (*B. gracilis*) and plot as a random effect.

Disturbance effects on N transfers from biocrusts to plant. Within the disturbed and control grassland plots, we added a 25 × 50 cm quadrat around an individual plant that had ~5 cm diameter root crown in preparation for the ^{15}N tracer experiment. In February 2018, metal flashing was added vertically to a depth of 8-10 cm in the quadrat with the target plant at one end to isolate surface roots and microbes of the target plant from the rest of the plot. In September 2018, we added 450 mL DI water to the quadrat to activate the plant and microbial community. The following morning, we collected 0.5 cm depth × 0.82 cm diameter biocrust soil samples and 5-10 green leaf samples (0 d; natural abundance) from each target individual and collected samples from 0-2cm and 2-10cm for gravimetric water content. We then added 11.6 mg of ^{15}N as NH_4NO_3 in 2.5 mL DI water to biocrusts 35 cm away from the base of the grass tussock. We harvested leaf samples 1 and 7 d after ^{15}N tracer addition.

We dried, ground, and weighed 5 mg of leaves into tin capsules. We packed ~50 mg of dried biocrust in silver capsules (Costech Analytical Technologies Inc., Valencia, CA, USA), acid-fumigated them for 36 h to remove carbonates (Harris et al. 2001), then repacked them in tin capsules to improve combustion during stable isotope analysis as above. $\delta^{15}N$ of biocrust was analyzed with a linear model with disturbance, and leaves with a linear mixed effects model with disturbance × time as fixed factors and plot as a random effect to account for repeated measures, within a randomization wrapper with 10000 perm.

Results

Surface soil disturbance reduced the photosynthetic capacity, abundance, and diversity of the biocrust autotrophic community. In 2018, grassland disturbed plots had 42% less chlorophyll *a* than controls (post hoc $P = 0.029$), the shrubland disturbed plot chlorophyll *a* content did not significantly differ from controls (Treatment × Site $P = 0.080$), but the shrubland biocrusts had 45% more chlorophyll *a* than the grassland ($P = 0.027$); In 2019, disturbed plots had 55% less chlorophyll *a* than controls across both sites ($P < 0.001$), and the shrubland had 45% less chlorophyll *a* than grassland ($P = 0.021$; Appendix S1: Table S1; Fig. 1a-b). The total abundance of cyanobacteria in shrubland was at least two orders of magnitude higher than the grassland. In the grassland, total abundance of cyanobacteria was 75% lower (perm. $P < 0.001$) in disturbed than control, and the shrubland difference was 95% lower (perm. $P < 0.001$, Fig. 2). Both sites had many plots with no detectable N-fixer sequences (9/20 in grassland, 14/20 in shrubland), but N-fixer abundance was overall much higher in the shrubland (5.23 ± 2.52 SE $\times 10^7$) than grassland (5.18 ± 1.62 SE $\times 10^4$). Disturbance decreased the abundance of potentially N-fixing taxa by 77% in grassland (perm. $P = 0.041$) and 95% in shrubland (perm. $P = 0.028$ Cyanobacterial diversity was 60% lower in disturbed plots than control (Disturbance $F = 16.15$, $P < 0.001$), and overall cyanobacterial diversity did not differ between sites ($F = 1.46$, $P = 0.235$; Disturbance × Site $F = 1.40$, $P = 0.231$, $R^2_{adj} = 0.36$; Fig. 2). The effects of disturbance on biocrust cyanobacterial community composition diverged between the sites (Site *pseudo-F* = 44.78, perm. $P = 0.001$; Disturbance × Site *pseudo-F* = 17.61, perm. $P = 0.001$), and though disturbance affected composition in both communities (Disturbance *pseudo-F* = 14.64, perm. $P = 0.001$; Appendix S1 Figure S2a), disturbance increased dissimilarity among plots in the shrubland more than in the grassland. In both sites, disturbance nearly doubled the heterogeneity

in biocrust community composition relative to controls (*pseudo-F* = 9.95, perm. *P* = 0.001). *Microcoleus vaginatus* was the most abundant cyanobacteria taxon and remained relatively abundant in disturbed plots. In contrast, *M. steenstrupii* taxa were 95% less abundant in disturbed than control. Control plot indicators included *Coleofasciculus cthnoplastes* (IV = 0.71) in the grassland site, PCC7113 (IV = 0.98), HS024 (IV = 0.94), SEV30 clades and *M. glaciei* (IV = 0.71), and *Schizothrix* sp. (IV = 0.69) in the shrubland site, and CYAN3 (IV = 0.89), SON57 (IV = 0.76), Undefined clades (IV = 0.89), and *Pycnacronema* sp. (IV = 0.84) in both sites (all $P < 0.020$). Disturbance did not affect total bacterial abundance (Appendix S1: Section S2).

Soil fungal abundance generally increased with soil disturbance, but the response of root colonization varied by ecosystem type. Ergosterol was 50% greater in disturbed than control across both sites and soil depths ($P = 0.065$), although ergosterol content overall was 60% lower in the shrubland than the grassland ($P < 0.001$, Appendix S1: Table S2; Fig. 1c-d). For *B. eriopoda* in grassland, aseptate root colonization was 45% higher in disturbed than control (post hoc $P = 0.105$), but in shrubland, aseptate root colonization was 85% lower in disturbed than control plots (post hoc $P = 0.010$; Appendix S1: Table S3; Fig. 1e-f). Septate hyphae were over an order of magnitude more abundant than aseptate hyphae, and average colonization (27 ± 2 %) was similar across sites and disturbance treatments (post hoc $P > 0.050$). For *B. gracilis* in grassland, aseptate root colonization was 80% greater (post hoc $P = 0.105$) in disturbed (2 ± 0.5%) than control plots (1% ± 0.4; Appendix S1: Table S3), and septate colonization averaged 59 ± 6% across both disturbance treatments. We cultured a total of 165 morphotypes of endophytic root fungi from grassland *B. eriopoda* representing 21 operational taxonomic units (OTU; Appendix S1: Section S3). The most common were in phylum Ascomycota (Pleosporales: control = 75 isolates, disturbed = 76; Xylariales including *Monosporascus* sp.: control = 27, disturbed = 13; Hypocreales including *Fusarium* sp.: control = 13, disturbed = 21). One OTU

was in phylum Basidiomycota (Agaricales; control = 5 isolates, disturbed = 15).

Available N was dominated by ammonium over nitrate, especially in the grassland. In the shrubland, at 0-2 cm depth, disturbed plots had 65% more ammonium than control plots, and ammonium was >300% more plentiful at 0-2 cm depth than 2-10 cm (Appendix S1: Table S2; Fig. 1g-h). In the grassland, nitrate was reduced 80% in disturbed plots (0.003 ± 0.003SE μg g^{-1} dry soil) compared to controls (0.018 ± 0.014 μg g^{-1} dry soil; $P = 0.077$, Appendix S1: Table S2). In the shrubland, disturbance did not reduce nitrate availability (average 0.367 ± 0.211 μg g^{-1} dry soil), which was >450% greater than in grassland soils (Appendix S1: Table S2). Biocrust natural abundance $\delta^{15}N$ averaged 4.2 ‰ ± 0.05SE and disturbance caused no significant change ($F_{1,19} = 1.95$, $P = 0.179$, Fig. 3a).

Disturbance decreased *B. eriopoda* leaf %N by a maximum of 16% compared to control, but this occurred only in the grassland during June sampling (post hoc $P = 0.003$, Appendix S1: Table S4). Disturbance did not affect *B. eriopoda* leaf %N in shrubland or in late July at either site (Fig. 1i-j) and *B. gracilis* leaf %N was unaffected by disturbance (Appendix S1: Table S4). *B. eriopoda* had 22% higher leaf %N ($P < 0.001$) in July than June, and *B. gracilis* leaf %N was 37% higher in July (2.08% ± 0.06) than in June (1.52% ± 0.06; $P < 0.001$, Appendix S1: Table S4). Natural abundance stable isotope values reflected seasonal differences but not site or disturbance differences (Appendix S1: Table S4).

In the stable isotope tracer experiment addressing the fungal loop, disturbance caused no significant change in grassland ^{15}N tracer retention in leaves of *B. eriopoda*. Soil gravimetric water content (GWC) at the time that the ^{15}N solution was added at 0-2cm averaged 2.1± 0.16SE and 2-10cm averaged 4.15 ± 0.35SE, which corresponds to moisture potential >-1.5MPa (data not shown), indicating that plants with leaves in this shallow soil layer, including *B. eriopoda* could be active. The soils dried over 3d but GWC at 2-10cm was still 2.57 ± 0.43SE

corresponding to moisture potential ~-9Mpa, still within the activity threshold of fungi (Marusenko et al. 2013). Leaf $\delta^{15}N$ increased through time (perm. $P < 0.001$), from an average natural abundance value of -0.51 ‰ (0 d) to 7.1 ‰ after 7 d, but disturbance did not affect $\delta^{15}N$ (perm. $P = 0.294$; Disturbance × Time perm. $P = 0.237$; Fig. 3b-e).

Discussion

Our results demonstrated that repeated footfall disturbances to dryland grass and shrub ecosystems reduced the abundance of cyanobacteria involved in N fixation (H1) and reduced microbial diversity (H2; Appendix S1 Section S1). This finding is consistent with previous results that physical soil disturbance harms biocrusts (Bates and Garcia-Pichel 2009, Steven et al. 2015, Alfaro et al. 2018, Bao et al. 2019, Bao et al. 2020). Soil disturbance of *Microcoleus*-dominated biocrusts reduced the abundances of the N-fixers and *M. steenstrupii* species complex, which dominate hot deserts (Garcia-Pichel et al 2013) more than *M. vaginatus*. These consequences were especially large in the grassland where soil disturbance also reduced N in leaves of the dominant grass. Our data matched previous observations of Ascomycota dominance in the Chihuahuan Desert (Green et al. 2008, Steven et al. 2014). However, although soil N availability (H4) and soil fungal abundance (H3) were in some cases increased by disturbance, the dominant, ascomycetous root endophytes (H3) and N transport to plants via movement of isotopic tracers (H7) were unaffected. Thus, although N availability significantly varied through time (indicating our study had the statistical power to detect changes), and despite the reduced plant leaf N caused by soil disturbance, we did not detect increased N cycling in soil ^{15}N values (H5) and did not support biocrust-fungal-plant networks as key conduits for N between biocrusts and plants in Chihuahuan Desert ecosystems (H6).

The influence of soil disturbance differed between grassland and shrubland and it yields insight on the consequences of long-term conversion of grassland to shrubland, a global

phenomenon over the last 150 years (D'Odorico et al. 2012). Greater shrub cover compared to grass cover favors biocrust cover, perhaps due to plot-level differences in temperature, relatively humidity, litter accumulation patterns, and surface morphology (Turnbull et al. 2010, Soliveres and Eldridge 2020), and we found higher microbial abundance (qPCR) in shrubland than grassland. The strong declines in cyanobacteria abundance with soil disturbance in both sites can point to additional roles of surface disturbance in addition to herbivory in grassland to shrubland conversion (D'Odorico et al. 2012), because surface disturbance in grasslands can lead to conditions of increased erosion and reduced organic input in interspaces. At the Sevilleta, the grassland had fewer pebbles than the shrubland (Turnbull et al. 2010) which may have made the surface more vulnerable to the compressive forces of footfalls, increasing the impact of the treatment on microbes in grassland compared to shrubland. With soil disturbance, soil surface stability and water infiltration declined more in the grassland than shrubland (Chung et al. 2019), potentially increasing wind and water erosion. Thus, soil disturbance may decrease grassland productivity more than shrubland productivity but how this difference contributes to shifts in vegetation community remains to be investigated.

The variation in climate context and thus phenology of the plants and microbes likely influenced the magnitude and direction of biotic and abiotic responses to soil disturbance. In June 2018, disturbed plots in both ecosystems had 50% higher soil fungal abundance estimated by ergosterol than control plots, a maximum of 65% greater soil ammonium, and grassland had 16% lower %N in *B. eriopoda* leaves. There had only been 22 mm of precipitation February-May, so newly released N from the physical rupture of cells was likely unavailable for plant uptake because plants require larger rain events to activate growth (Thomey et al. 2011). However, soil fungi can maintain activity at lower soil moisture thresholds than plants (Marusenko et al. 2013) so could capitalize on this N flush associated with disturbance,

explaining their increased abundance. In summer, monsoon precipitation (156 mm, June-September) had event sizes sufficiently large to activate plant growth. Seasonality of the N cycle driven by rain pulses may explain the lack of soil disturbance effects on *B. eriopoda* leaf N in July compared to June. Similarly, seasonal lags in plant response to N addition occurred in a Chinese grassland, with plant responses activated by rains (Zhou et al. 2018). Additionally, if N responds to soil disturbance more strongly earlier in the growing season than during the summer monsoon, then phenology of N may explain the absence of a response in ^{15}N transfer during September 2018. In September 2018, chlorophyll *a* in the shrubland disturbed plots recovered to the level of control plots, but in the grassland, disturbed plots still had 20% less chlorophyll *a* than controls. In 2019, the spring was 63% wetter (60mm) than in 2018 but the monsoon season was 44% drier (87mm), and chlorophyll *a* and cyanobacterial abundance declined (>50%, >80% respectively) with disturbance in both sites, and control plots were generally warmer by 0.76℃ than disturbed plots ($P = 0.065$, Appendix S1: Section S8). Altogether, our results combined with the increasing variability in dryland climates (Maurer et al. 2020) indicate that microbes and N dynamics can be severely disrupted by soil disturbance but that quantifying the net impacts of these disruptions will require detailed phenological data on coupled N, plant, and microbe responses over multiple seasons, years, and ecosystem types.

Repeated disturbance disrupted dryland soil and plant N pools. Consistent with strong results of reduction in N-fixation from studies on complex biocrusts (Belnap et al. 1994, Evans and Belnap 1999, Belnap 2002, Kuske et al. 2012), N-fixing cyanobacteria (*Scytonema* and *Schizothrix* spp.) abundances decreased by at least 77%, but we found no effect on N fixation in a pilot project (Appendix S1: Section S9). Additionally, disturbance reduced the diversity of cyanobacteria, and thus may affect other biogeochemical processes besides just N-fixation (Delgado-Baquerizo et al. 2013). However, given that soil disturbance did not affect natural

abundance values of biocrust or leaf $\delta^{15}N$ (Fig. 3, Appendix S1: Section S7), there was no indication of strong differences in microbial processing of the N source material incorporated into plant leaves. The result of 65% higher ammonium in disturbed than control in shrubland may be due to the remaining microbes decomposing ruptured cells. Interestingly, inorganic ^{15}N was retained in plants and biocrusts up to 1y (Appendix S1: Section S7), showing that labile N is retained at the annual scale rather than rapidly denitrified (Hartley et al. 2007).

We manipulated the proposed source of N in the fungal loop hypothesis by reducing the abundance of biocrust cyanobacteria, including N-fixers. The grassland experienced larger effects of soil disturbance on plant nitrogen than the shrubland, for which disturbance effects were non-significant. In the grassland we used an isotopic tracer approach that revealed no reduction in rapid N transfer from biocrust to the plant, *B. eriopoda* (the proposed sink) when biocrusts were disrupted. Direct measures of N content in the grassland plant *B. eriopoda* suggested that leaf N was transiently suppressed by disturbance in late spring, indicating temporal context dependency in the influence on soil disruption on N pools in plants. However, there was no supporting evidence that the reductions in leaf N caused by soil disruption of the biocrust-fungal-plant network were mediated by fungi. Specifically, we did not observe a proliferation of fungi in the shallow soil layer where the biggest shifts in available N occurred, and disturbance did not reduce fungal abundances (Fig. 1c-g). Previous research showed that inhibiting fungal connections between *B. gracilis* plants and cyano-lichen biocrusts reduced ^{15}N transfer from biocrusts to plants by 20% (Dettweiler-Robinson et al. 2020), but that case used a tracer approach with a fungal hyphal barrier to prevent fungal connections, and all organisms were undisturbed after initial experimental set-up. Thus, results could differ from ours because our target species was *B. eriopoda* rather than *B. gracilis*, because the biocrust communities differed, or because the focal manipulation to the loop differed (soil disturbance vs. fungal

barrier). Prior work supports the hypothesis that plant species may differ in the N sources and dynamics. For example, labeled N was not transferred from biocrust patches to plants in a prior study of *B. eriopoda* (Kwiecinski et al. 2019), suggesting that *B. eriopoda* is not reliant on the fungal loop for N nutrition. In the shrubland, there was no evidence that soil disturbance altered N pools in plants. Although aseptate fungal colonization of roots decreased with disturbance, and soil fungi increased, these changes did not correlate with altered N dynamics in plants. Recent evidence indicated rapid N transfer in creosote shrublands of the Sonoran Desert (Janke and Coe 2021), but the effect of disturbance has yet to be investigated in shrublands with tracer experiments. Thus, although disturbance disrupted one or more components of the proposed fungal loop in our study, this disruption was not strong enough to alter rates of N transfer from biocrusts to plants or to change fungal networks in ways that correlated with N dynamics and thus there is not strong support for the fungal loop hypothesis in the dominant ecosystems of the Chihuahuan Desert.

In many drylands, biocrusts provide critical ecosystem services through resource cycling and interactions with plants (Havrilla et al. 2019) and disturbance may disrupt these services. In our experiment, disturbance substantially affected the plant community, with a ~30% decline in *B. eriopoda*, ~25% increase in *B. gracilis* (the dominant grasses), and notable increases in the invasive forb *Salsola kali* compared to control plots (*unpublished*). Biocrust disturbances favored invasive plants in other systems (Havrilla et al. 2019), and introduced plants can alter resource cycling (Parker et al. 1999). For example, litter from *S. kali* can produce oxalates and other compounds that alter phosphorus availability (Cannon et al. 1995). Thus small changes in N content of the dominant plants and compositional shifts among plants and microbes may affect N and other resources at the ecosystem level, but these remain to be investigated. Overall, repeated disturbance to biocrusts from human activities can have prolonged impacts on the C and N cycles

in dry grasslands and shrublands dominated by *Microcoleus* spp. Physical disruption can decrease biocrust productivity and N-fixation, cause microbial communities to re-order in species relative abundances, and decrease leaf N to support photosynthesis.

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Figure 1. Means ± SE of (a., b.) chlorophyll *a* concentration in October by year (light grey = 2018, olive green = 2019), (c., d.) ergosterol content in June 2018, (e., f.) colonization of aseptate fungal hyphae in *B. eriopoda* roots in August 2018, (g., h.) ammonium concentration in June 2018 by soil depth (light grey = 0-2 cm, white = 2-10 cm), (i., j.) leaf %N by month in 2018 (dark grey = early June, dark green = late July) in *B. eriopoda* from grassland and shrubland in disturbance treatments. Bold letters indicate $P < 0.050$, italicized letters indicate $P < 0.100$, and no letters indicate $P > 0.100$. In a., b.: 2018: Different lowercase letters above bar indicate post hoc disturbance effects within site; Grassland < Shrubland, $F = 5.3$, $P = 0.027$. 2019: Uppercase letters above bars indicate disturbance effects; Grassland > Shrubland, $F = 6.1$, $P = 0.021$. In c.-f.: Different letters above bars indicate post hoc disturbance effects within site. Ergosterol: Grassland > Shrubland ($F = 16.9$, $P < 0.001$). In g.-j.: different letters indicate post hoc tests for disturbance v. control within site and depth or within site and month, respectively. Ammonium: 0-2 cm > 2-10 cm ($F = 192.6$, $P < 0.001$). $n = 10$ each for treatment combination.

Figure 2. Cyanobacteria abundance and community structure (a., b.) of biocrusts from grassland and shrubland in disturbance treatments. Cyanobacteria abundance was determined by high-throughput 16S rRNA gene analyses coupled to qPCR. Each bar represents an independent plot (numbered 1-40) in each disturbance treatment ($n = 10$). Phylogenetic assignments for each OTU were based on blast to a biocrust cyanobacteria database Cydrasil.

Figure 3. Natural abundance (a.) biocrust and (c.) leaf $\delta^{15}N$, (b.) target plant with the aluminum flashing around the plant and biocrust, and (d., e.) leaf enrichment by day since ^{15}N tracer addition to biocrust in the grassland site disturbance treatments. Leaf enrichment increased significantly with each time point (all post hoc $P < 0.050$) but equally across both disturbance treatments ($P = 0.968$). $n = 10$ each for disturbed and control at grassland and shrubland.

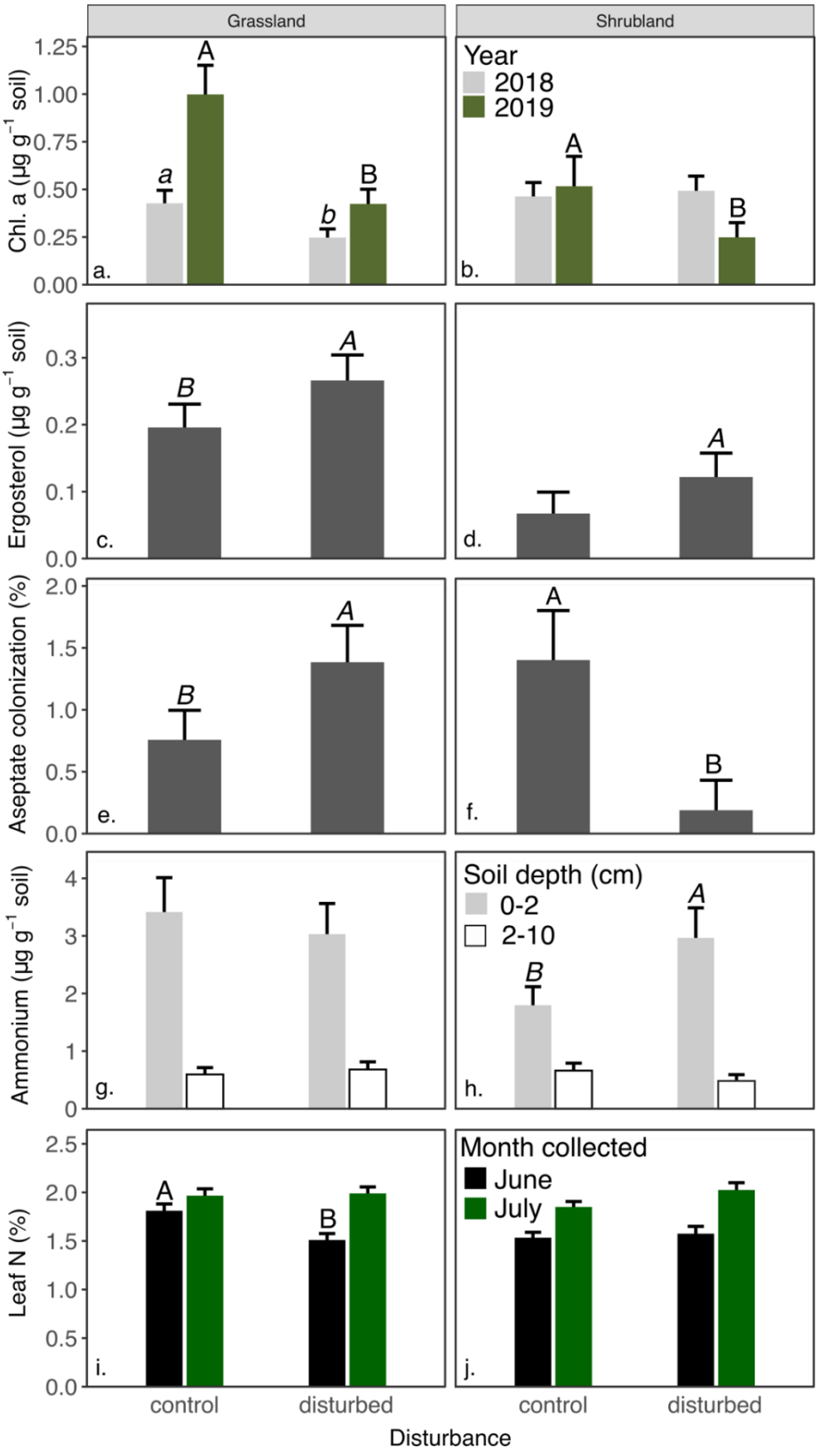

Figure 1.

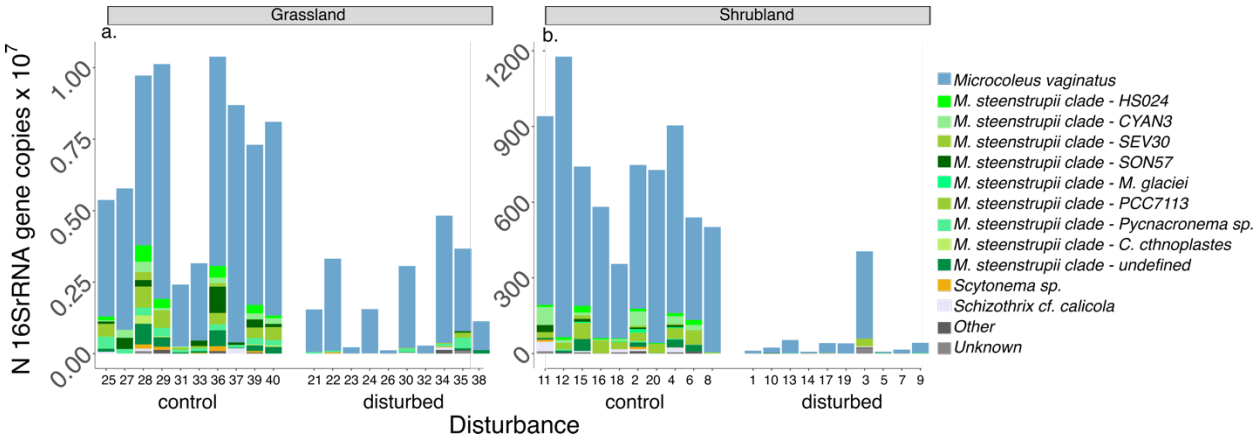

Figure 2

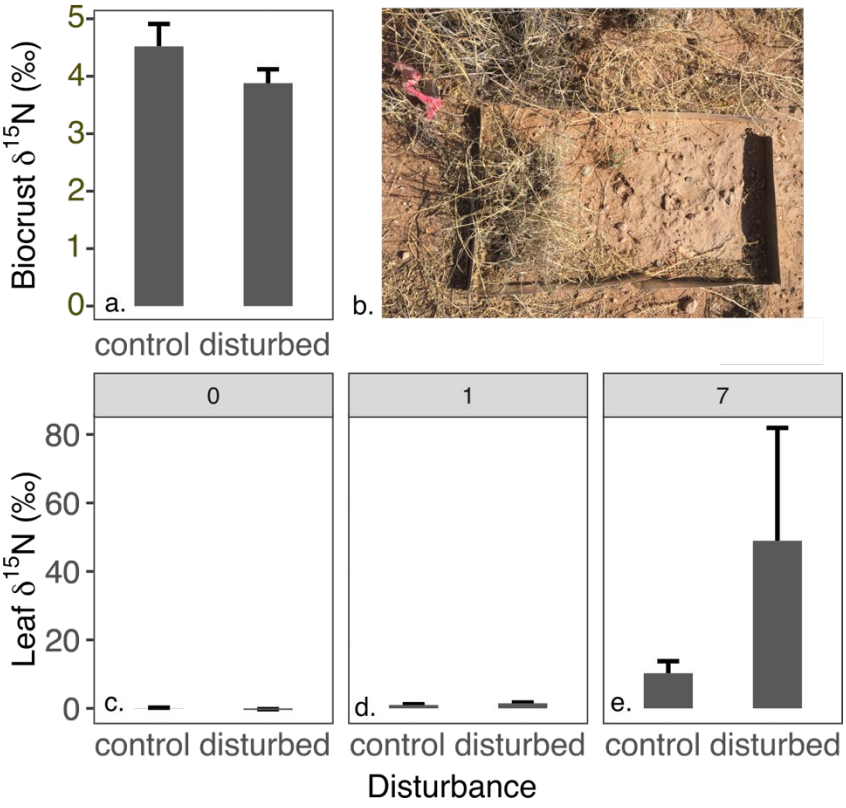

Figure 3