

# 1 Introduction

Biological soil crusts (BSC) are a microbial mat-like surface layer in arid soil. Millimeters in depth, BSC are found in plant interspaces and cover a wide, global geographic range Garcia-Pichel et al. [2003b]. The ground cover of BSC on the Colorado Plateau has been measured as high as 80% by remote sensing Karnieli et al. [2003]. The global biomass of BSC cyanobacteria alone is estimated at  $54 \times 10^{12}$  g C Garcia-Pichel et al. [2003b]. BSC play important roles in arid ecosystem productivity and are responsible for significant N flux (for review of BSC N-fixation see Belnap [2003]). For example, Evans and Belnap [1999] found approximately five times as many soil crust samples from sites in North America, Africa and Australia had  $\delta^{15}\text{N}$  values indicative of high N-fixation input relative to the number of samples where  $\delta^{15}\text{N}$  values indicated N input was predominantly from atmospheric deposition. Additionally, the presence of BSC is positively correlated with vascular plant survival due in part to BSC ecosystem N contributions (for review of BSC-vascular plant interactions see Belnap et al. [2003]).

Molecular studies of BSC microbial diversity include explorations of vertical BSC microbial diversity with depth Garcia-Pichel et al. [2003a], BSC *nifH* gene content surveys (e.g. Yeager et al. [2004], Yeager et al. [2012], Yeager et al. [2006] and Steppe et al. [1996]), and next-generation-sequencing (NGS) enabled studies of BSC SSU rRNA gene content across wide geographic ranges Garcia-Pichel et al. [2013], Steven et al. [2013]. Garcia-Pichel et al. [2003a] found that BSC microbial diversity is organized vertically, likely as the result of vertically oriented environmental gradients (e.g. light and oxygen). *nifH* surveys have been conducted across BSC development stages Yeager et al. [2004], as well as across seasons, temperatures and precipitation gradients Yeager et al. [2012]. Mature, more fully developed BSC possess greater numbers of heterocystous cyanobacteria (e.g. *Nostoc*, *Scytonema*) than developing BSC but both young and old BSC are dominated by non-heterocystous cyanobacteria (*Microcoleus vaginatus* or *M. steenstrupii*) Yeager et al. [2004], Garcia-Pichel et al. [2013]. Young or recently disturbed BSC are

often described as "light" in appearance relative to "dark" mature BSC Belnap [2002]. Although an early study of Colorado Plateau BSC *nifH* diversity presented *nifH* genes related to *Gammaproteobacteria* as well as a clade that included *nifH* genes from the anaerobes *Clostridium pssteurianum*, *Desulfovibrio gigas* and *Chromatium buderi*, subsequent studies have found heterocystous cyanobacteria to be the numerically dominant BSC diazotrophs Yeager et al. [2006, 2004, 2012]. Specifically, Yeager et al. [2006]—in a study of overall BSC *nifH* diversity—categorized 89% of 693 *nifH* sequences derived from Colorado Plateau and New Mexico BSC samples as heterocystous cyanobacterial (non-cyanobacterial *nifH* sequences were largely attributed to alpha- and beta-*proteobacteria*). The heterocystous cyanobacterial BSC diazotrophs fall into three genera, *Scytonema*, *Spirirestis*, and *Nostoc* Yeager et al. [2006, 2012]. Studies of BSC microbial diversity over broad geographic ranges have elucidated how soil parent material correlates to above and below crust microbial community membership and structure Steven et al. [2013] and that the most abundant BSC cyanobacterial primary producer shifts from *M. vaginatus* to *M. steenstrupii* with increasing mean annual temperature Garcia-Pichel et al. [2013].

BSC N-fixation rate studies (typically employing the acetylene reduction assay (ARA)) have explored BSC diazotroph activity across various ecological gradients. Reported BSC N-fixation rates vary significantly Evans and Lange [2001]. The reasons for this variability are complex and likely include the spatial heterogeneity of BSC Evans and Lange [2001] and the impact of recent environmental conditions on N-fixation rates (see Belnap [2001] for discussion). Moreover, the ARA assay is subject to methodological artifacts that preclude cross-study and possibly intra-study but inter-environment type comparisons (see Belnap [2001] for review). Despite the general BSC N-fixation rate measurement variability, older, dark BSC N-fixation rates have been measured higher than N-fixation rates for younger, light BSC Belnap [2002], Yeager et al. [2004]. This difference may be due to the proliferation of heterocystous cyanobacteria in older mats and is consistent with the theory that heterocystous cyanobacteria are the primary

BSC diazotrophs. Alternatively, the N-fixation rate differences between young and old BSC might be attributable to methodological artifacts. For instance, Johnson et al. [2005] show that N-fixation rates measured from intact cores of developing BSC may be artifactually low due to delayed acetylene/ethylene diffusion through the crust in a typical ARA incubation timeframe. When total N-fixation rates were calculated by integrating N-fixation rates of 1-3 mm depth BSC core slices over the full depth of the BSC (thus mitigating ethene/acetylene flux limitations), N-fixation rate differences between young and old BSCs were not statistically significant Johnson et al. [2005].

The influence of microbial community membership and structure on BSC N-fixation is an ongoing research question Belnap [2013]. While the presence/abundance of heterocystous cyanobacteria has been proposed as the underlying microbial membership influence on increased N-fixation in older BSC, it is unclear if the premise that older BSC fix more N is always correct (see Johnson et al. [2005]). More studies are necessary to elucidate the microbial membership influence on BSC N-fixation and to determine if heterocystous cyanobacteria are the only keystone diazotrophs. To further probe the diversity of diazotrophs in BSC we conducted  $^{15}\text{N}$  DNA stable isotope probing (DNA-SIP) experiments with developing Colorado Plateau BSC. Although molecular characterizations of BSC *nifH* diversity in other studies have yielded predominantly heterocystous cyanobacterial *nifH* genes, microbes from young, developing BSC that incorporated  $^{15}\text{N}$  into DNA as determined by DNA-SIP in this study were not cyanobacteria but members of the *Gammaproteobacteria*, *Clostridiaceae* and *Deltaproteobacteria*. Further, we explore the distribution of putative diazotrophs uncovered in this study as well as by Yeager et al. [2004], Yeager et al. [2006] and Yeager et al. [2012] through collections of SSU rRNA libraries from BSC microbial diversity surveys over a range of spatial scales and soil types Garcia-Pichel et al. [2013], Steven et al. [2013].

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