Non-cyanobacterial diazotrophs dominate dinitrogen fixation in biological soil crusts at the early stage of crust formation.

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1 ABSTRACT

Biological soil crusts (BSC) cover a vast global area and are key components of ecosystem productivity in arid soils. In particular, BSC contribute significantly to the nitrogen (N) budget in arid ecosystems via N_2-fixation. N_2-fixation in mature crusts is largely attributed to heterocystous cyanobacteria, however, early successional crusts possess few N-fixing cyanobacteria and this suggests that microorganisms other than cyanobacteria mediate N_2-fixation during the early stages of BSC development. DNA stable isotope probing (DNA-SIP) with $^{15}N_2$ revealed that *Clostridiaceae* and *Proteobacteria* are the most common microorganisms to assimilate ^{15}N in early successional 'light' crusts. The maximum relative abundance of non-cyanobacterial $^{15}N_2$-assimilating taxa in environmental BSC SSU rRNA gene sequence collections was 0.00225% and 0.00127% for taxa that belong to *Clostridiaceae* and *Proteobacteria*, respectively. Their low abundance may explain why these heterotrophic diazotrophs have not previously been characterized in BSC. Diazotrophs play a critical role in BSC formation and characterization of these organisms represents a crucial step towards understanding how antropogenic change will effect the formation and ecological function of BSC in arid ecosystems.

2 INTRODUCTION

Biological soil crusts (BSC) are specialized microbial mat communitites that form at the soil surface in arid environmets and fill a variety of important ecological functions in arid ecosystems. BSC occupy 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 x 10^{12} g C (Garcia-Pichel et al., 2003b). BSC play important roles in arid ecosystem productivity and are responsible for significant nitrogen (N) flux (for review of BSC N_2-fixation see Belnap (2003)). N_2-fixation represents the dominant source of new ecosystem N in more than 80% of BSC from diverse sites across North America, Africa, and Australia (Evans and Belnap, 1999), while atmospheric N deposition was a dominant source of N in only a minority of sites. 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)). Climate change and disturbance could alter BSC microbial community structure/membership and therefore it is possible that there will also be changes in diazotroph diversity and N_2-fixation and that these changes can alter the BSC N-budget.

29   BSC N_2-fixation rate studies (typically employing the acetylene reduction assay (ARA)) have explored
30   BSC diazotroph activity across various ecological gradients. Reported BSC N_2-fixation rates vary
31   significantly across samples and studies (Evans and Lange, 2001). The reasons for inter-site and inter-
32   study variability are complex and likely include the spatial heterogeneity of BSC (Evans and Lange, 2001)
33   and the impact of recent environmental change on N_2-fixation rates (see Belnap (2001) for discussion).
34   Moreover, the ARA assay is subject to methodological artifacts that can complicate making robust
35   comparisons across sample types that differ in physical and biological characteristics (see Belnap (2001)
36   for review). Nonetheless, N_2-fixation rates are consistently higher in mature BSC than in young, early
37   successional BSC (Belnap, 2002; Yeager et al., 2004). This difference may be due to the proliferation
38   of heterocystous *Cyanobacteria* in older mats and is consistent with the theory that heterocystous
39   *Cyanobacteria* provide the main source of fixed-N in BSC. Alternatively, the N_2-fixation rate differences
40   between young and old BSC might be attributable to methodological artifacts. For instance, Johnson et al.
41   (2005) show that N_2-fixation in mature mats is maximal at the crust surface (coincident with heterocystous
42   cyanobacteria) while it is maximal below the crust surface in early successional BSC. Diffusional
43   limitation can potentially cause ARA to underestimate N_2-fixation which occurs below the crust surface
44   and as a result ARA may systematically underestimate rates of N_2-fixation in early successional BSC.
45   Diffusion would not be an issue when measuring N_2-fixation rates in mature crust as nitrogenase activity
46   peaks near the surface. Differences of N_2 fixation rates between developing and mature BSC were not
47   statistically significant when aerial rates were estimated by integrating across ARA performed on thin
48   (1-3mm) slices across a BSC depth profile Johnson et al. (2005).

49   Molecular studies of BSC microbial diversity include explorations of the BSC microbial community
50   vertical profile (Garcia-Pichel et al., 2003a), BSC *nifH* gene content surveys (e.g. Yeager et al. (2004),
51   Yeager et al. (2012), Yeager et al. (2006) and Steppe et al. (1996)), and next-generation-sequencing
52   (NGS) enabled studies of BSC SSU rRNA gene content across wide geographic ranges (Garcia-Pichel
53   et al., 2013; Steven et al., 2013). Early successional BSC are often described as "light" in appearance
54   relative to "dark" mature BSC (Belnap, 2002; Yeager et al., 2004). Mature BSC possess greater numbers
55   of heterocystous *Cyanobacteria* (i.e *Scytonema*, *Spirirestis*, and *Nostoc* (Yeager et al., 2006, 2012))
56   than developing BSC but both young and old BSC are dominated by non-heterocystous *Cyanobacteria*
57   (*Microcoleus vaginatus* or *M. steenstrupii*) (Yeager et al., 2004; Garcia-Pichel et al., 2013). Heterocystous
58   *Cyanobacteria* are the numerically dominant BSC diazotrophs in *nifH* clone libraries (Yeager et al., 2006,
59   2004, 2012). Eighty-nine perent of 693 *nifH* sequences derived from Colorado Plateau and New Mexico
60   BSC samples as heterocystous cyanobacterial (non-cyanobacterial *nifH* sequences were largely attributed
61   to alpha- and beta- *proteobacteria*) Yeager et al. (2006). However, an early survey of Colorado Plateau
62   BSC *nifH* diversity recovered *nifH* genes related to *Gammaproteobacteria* as well as a clade that included
63   *nifH* genes from the anaerobes *Clostridium pssteurianum*, *Desulfovibrio gigas* and *Chromatium buderi*,

64   The influence of microbial community membership and structure on BSC N_2-fixation is an ongoing
65   research question (Belnap, 2013). While the presence/abundance of heterocystous *Cyanobacteria* has
66   been proposed as the mechanism behind increased N_2-fixation in mature BSC, it is unclear if mature BSC
67   actually fix more N than early successional BSC (see Johnson et al. (2005)). More studies are necessary
68   to elucidate the microbial membership influence on BSC N_2-fixation and to determine if heterocystous
69   *Cyanobacteria* are the only keystone diazotrophs. The first step in defining structure function relationships
70   with respect to N_2-fixation is a full accounting of BSC diazotrophs. Towards this end we conducted $^{15}N_2$
71   DNA stable isotope probing (DNA-SIP) experiments with light, developing Colorado Plateau BSC. DNA-
72   SIP with $^{15}N_2$ has not been attempted with BSC. DNA-SIP provides an accounting of *active* diazotrophs
73   whereas *nifH* clone libraries account for microbes with the genomic potential for N_2-fixation. Further, we
74   investiage the distribution of these active diazotrophs through collections of SSU rRNA gene sequences
75   from BSC NGS microbial diversity surveys over a range of spatial scales and soil types (Garcia-Pichel
76   et al., 2013; Steven et al., 2013).

3 RESULTS

3.1 ORDINATION OF CSCL GRADIENT FRACTION SSU RRNA SEQUENCE COLLECTIONS SHOWS HEAVY FRACTIONS FROM CONTROL AND LABELED CSCL GRADIENTS ARE DIFFERENT

77   BSC were incubated for 4 days in the presence or absence of $^{15}N_2$ and DNA was extracted for DNA-SIP at
78   2 and 4 days. Fractionation of CsCl gradients permitted separation of DNA on the basis of buoyant density.
79   Ordination of Bray-Curtis (Bray and Curtis, 1957) distances between SSU-rRNA amplicon sequence
80   collections from gradient fractions reveals that labeled gradient fraction (i.e. gradient fractions of DNA
81   from $^{15}N_2$ incubations) sequence collections diverge from control (i.e. DNA from incubations without
82   $^{15}N_2$) at the "heavy" of the CsCl gradients (Figure 1 and Figure S2). Differences among label/control
83   groups with heavy fractions are statistically significant by the Adonis test (p-value: 0.001, r^2: 0.18)
84   (Anderson, 2001).

3.2 OTUS RESPONSIVE TO $^{15}N_2$ ARE PRIMARILY *PROTEOBACTERIA* AND *CLOSTRIDIACEAE*

85   A statistically significant increase in OTU abundance in heavy fractions of $^{15}N_2$ labeled samples relative
86   to corresponding control fractions provides evidence for OTUs that have incorporated ^{15}N into their
87   DNA. Specifically, we compared OTU proportion means between labeled and control samples from
88   heavy gradient fractions using statistics developed to find differentially expressed genes with RNASeq
89   data (McMurdie and Holmes, 2014; Love et al., 2014). OTUs that incorporated ^{15}N into DNA and
90   increased in bouyant density were identified by rejecting the the null hypothesis that the labeled versus
91   control proportion mean ratio for an OTU was below a chosen threshold (see methods). p-values were
92   adjusted by the BH method (Benjamini and Hochberg, 1995) and we used a false discovery rate (FDR)
93   cutoff of 0.10 (typical FDR threshold in gene expression data analysis). A total of 2,127 and 2,160
94   OTUs were detected in days 2 and 4, respectively, and interrogated for evidence of $^{15}N_2$-labelling. Of
95   these OTU, only 208 and 233, respectively, passed a sparsity threshold we applied as an independent
96   filtering step to pre-screen out OTUs not likely to produce significant p-values (see Love et al. (2014)
97   for discussion of independent filtering). Of OTUs passing sparsity criteria 38 were found to be enriched
98   significantly in "heavy" fractions relative to control. These OTUs likely incorporated ^{15}N into DNA ($^{15}N_2$
99   "responders"). Of these 38, 26 are annotated as *Firmicutes*, 9 as *Proteobacteria*, 2 as *Acidobacteria* and
100 1 as *Actinobacteria* (Figure 3, Figure 2). If the responder OTUs are ranked by descending, moderated
101 proportion mean labeled:control ratios, the top 10 ratios (i.e. the 10 OTUs that were most enriched in
102 the labeled gradients considering only heavy fractions) are either *Firmicutes* (6 OTUs) or *Proteobacteria*
103 (4 OTUs) (Figure 4). Centroid sequences of strongly responding *Proteobacteria* OTUs all share high
104 sequence identity (>98.48%, Table 1) with cultivars from genera known to possess diazotrophs including
105 *Klebsiella*, *Shigella*, *Acinetobacter*, and *Ideonella*. None of the *Firmicutes* OTUs in the top 10 responders
106 share greater than 97% sequence identity with sequences in the LTP database (release 115) (see Table 1).
107 OTUs that passed the sparsity threshold but were not classified as ^{15}N-responsive were subsequently
108 tested against the null hypothesis that the OTU proportion mean ratio was above the selected threshold.
109 Rejecting the second null would indicate an OTU did *not* incorporate ^{15}N into biomass. There were 58
110 and 70 "non-responders" at days 2 and 4, respectively. OTUs that did not pass sparsity or could not be
111 classified as either a responder or non-responder are simply ambiguous with respect to ^{15}N labelling.

3.3 ^{15}N-RESPONSIVE OTUS IN ENVIRONMENTAL SAMPLES

112 Five of the 6 *Firmicutes* with the strongest response to ^{15}N-labelling (Table X) belong in the *Clostridiacea*.
113 We only observed one of these strongly responding *Clostridiaceae* in the data presented by Garcia-Pichel
114 et al. (2013), "OTU.108" (closest BLAST hit in LTP Release 115 – *Caloramotor proteoclasticus*, BLAST

115 %ID 96.94, Accession X90488). OTU.108 was found in two samples both characterized as "light" crust.
116 One other *Clostridiaceae* OTU with a proportion mean ratio (labeled:control) p-value less than 0.10 but
117 outside the top 10 responders was found in the Garcia-Pichel et al. (2013) data (a "light" crust sample)
118 (Figure 2). None of the strongly responding *Clostridiacea* were found in the sequences provided by Steven
119 et al. (2013). *Clostridiaceae* ^{15}N-responder OTU are not closely related to cultivars. (Table 1, Figure 5).
120 One of the proteobacterial OTUs with the strongest [15]-N response (Table X) was found in Garcia-Pichel
121 et al. (2013) (closest BLAST hit in LTP Release 115, BLAST %ID 100, Accession ZD3440, *Acinetobacter*
122 *johnsonii*). None of the *strongly responding Protebacteria* OTUs were found in the Steven et al. (2013)
123 sequences. Responder OTUs were found in Steven et al. (2013) samples 133 times. 83 were in "below
124 crust" samples, 50 in crust samples (see Figure 2). Two ^{15}N-responsive OTUs were found in an extensive
125 number of environmental samples (61 of 65 samples from the combined data sets of Garcia-Pichel et al.
126 (2013) and Steven et al. (2013)). Both OTUs were annotated as *Acidobacteria* but shared little sequence
127 identity to any cultivar SSU rRNA gene sequences in the LTP (Release 115), with best LTP BLAST hits
128 of 81.91 and 81.32% identity. Additionally, the ^{15}N-response for each OTU was weak relative to other
129 putative responders (3. Of the remaining 36 stable isotope responder OTUs, only 14 were observed in the
130 environmental data (Figure 2, Figure S5).

3.4   COMPARING SEQUENCE COLLECTIONS AT "STUDY"-LEVEL

131 We compared the sequences determined in this study to two previous surveys of SSU rRNA amplicons
132 from BSC communities: the Garcia-Pichel et al. (2013) and Steven et al. (2013) study. There were 3,079
133 OTUs (209,354 total sequences after quality control) in the DNA-SIP data, 3,203 OTUs (129,033 total
134 sequences after quality control) in the Garcia-Pichel et al. (2013) study, and 2,481 OTUs (129,358 total
135 sequences after quality control) in the Steven et al. (2013) study. Of the 4,340 OTU centroids established
136 for this study (including sequences from Steven et al. (2013) and Garcia-Pichel et al. (2013)) 445 have
137 matches in the Living Tree Project (LTP) (a collection of 16S gene sequences for all sequenced type
138 strains (Yarza et al., 2008)) at greater or equal than 97% sequence identity (LTP version 115). That is, 445
139 of 4,340 OTUs (~10%) are closely related to cultivars. The DNA-SIP data set shares 56% OTUs with the
140 Steven et al. (2013) data and 46% of OTUs with the Garcia-Pichel et al. (2013) data (where total OTUs
141 are from the combined data for each pairwise comparison). The Steven et al. (2013) and Garcia-Pichel
142 et al. (2013) studies share 46% of OTUs. *Cyanobacteria* and *Proteobacteria* were the top two phylum-
143 level sequence annotations for all three studies of BSC. Only the DNA-SIP data had more *Proteobacteria*
144 annotations than *Cyanobacteria*. *Proteobacteria* represented the 29.8% of sequence annotations in DNA-
145 SIP data as opposed to 17.8% and 19.2% for the Garcia-Pichel et al. (2013) and Steven et al. (2013) data,
146 respectively. There is a stark contrast in the total percentage of sequences annotated as *Firmicutes* between
147 the raw environmental samples and the DNA-SIP data. *Firmicutes* represent only 0.21% and 0.23% of
148 total phylum level sequence annotations in the Steven et al. (2013) and Garcia-Pichel et al. (2013) studies,
149 respectively (Figure S1). In the DNA-SIP sequence collection *Firmicutes* make up 19% of phylum level
150 sequence annotations. SIP places focus upon organisms based on isotope incorporation and has the ability
151 to detect activity by low abundance members of the community. DNA from OTUs that incopororate [15]N
152 into their biomass moves towards the heavy end of the CsCl gradient and therefore OTUs in "labeled"
153 DNA are enriched in the full data pool relative to bulk DNA. Phylum-level taxonomic annotations of
154 ^{15}N-responsive OTUs (i.e. *Firmicutes* and *Proteobacteria*) are enriched in the DNA-SIP data relative to
155 environmental data (Figure S1). Also in sharp contrast for the DNA-SIP versus environmental data is the
156 number of putative heterocystous *Cyanobacteria* sequences. Only 0.29% of *Cyanobacteria* sequences in
157 the DNA-SIP data are annotated as belonging to "Subsection IV" which is the heterocystous order of
158 *Cyanobacteria* in the Silva taxonomic nomenclature (Pruesse et al., 2007). In the Steven et al. (2013) and
159 Garcia-Pichel et al. (2013) studies 15% and 23%, respectively, of *Cyanobacteria* sequences are annotated
160 as belonging to "Subsection IV".

4 DISCUSSION

161 BSC N-fixation has long been attributed to heterocystous *Cyanobacteria* and molecular surveys of BSC
162 *nifH* gene content have been consistent with this hypothesis finding cyanobacterial *nifH* types to be
163 numerically dominant in *nifH* gene libraries from BSC (Yeager et al., 2006, 2004, 2012). However, $^{15}N_2$
164 DNA-SIP revealed non-cyanobacterial microbes fixed N_2 in early successional BSC samples. DNA from
165 early successional BSC samples–incubated for 2 and 4 days in the presence or absence of $^{15}N_2$–was
166 collected and separated by bouyant density in CsCl density gradients. Heavy CsCl gradient fractions
167 from gradients with ^{15}N-labelled DNA were different in phylogenetic membership/structure than heavy
168 fractions with unlabeled DNA (Figure 1 and Figure S2)). Further, heavy gradient fractions clustered
169 by DNA type (labeled or unlabeled) (Figure 1). Therefore, headspace $^{15}N_2$ in early successional BSC
170 microcosms was incorporated into DNA. Further, OTUs enriched in labeled gradient heavy fractions
171 relative to control are the specific taxa that incorporated ^{15}N (from $^{15}N_2$) into biomass. *Proteobacteria* and
172 *Clostridiaceae* represented most OTUs enriched in DNA from labeled gradient heavy fractions relative
173 to control as revealed by a robust statistical framework for quantifying and evaluating differential OTU
174 abundance in microbiome studies. Additionally, *Proteobacteria* and *Clostridiaceae* represented OTUs
175 that most strongly responded to ^{15}N.

176   It is possible that PCR-driven molecular surveys of *nifH* gene content have been biased. We propose
177 three mechanisms that could bias *nifH* clone libraries against heteroptophic diazotrophs. First, polyploidy
178 in *Cyanobacteria* (Griese et al., 2011) would inflate the representation of cyanobacteria in community
179 DNA (beyond a cell ratio). Second, *nifH* PCR primers could be biased against heterotrophic diazotrophs.
180 In general the nifH PCR primers used by Yeager et al. (2006, 2004, 2012) ("19F" and "nifH3") for the first
181 round of nested PCR have broad specificity and display at least 86% *in silico* coverage for *Proteobacteria,*
182 *Cyanobacteria* and "Cluster III" (which includes clostridial *nifH*) reference *nifH* sequences (Gaby and
183 Buckley, 2012). In the second round of the nested PCR protocol used by Yeager et al. (2006, 2004, 2012),
184 primer "nifH11" is' biased against "Cluster III" (50% *in silico* coverage of reference *nifH* sequences),
185 *Proteobacteria* (79% coverage) and *Cyanobacteria* (67% coverage), and, primer "nifH22" matches
186 *Proteobacteria*, *Cyanobacteria* and "Cluster III" reference sequences poorly (16%, 23% and 21% *in*
187 *silico* coverage, respectively) (Gaby and Buckley, 2012). Unfortunately, it is difficult to assess or quantify
188 this bias (in either direction) without knowing the *nifH* gene content *de novo*. Third, heterocysts (the
189 specialized N-fixing cells along the trichome of filamentous heterocystous *Cyanobacteria* such as *Nostoc*
190 and *Scytonema*) may be overrepresented in *nifH* clone libraries. Heterocysts make up a fraction of cells
191 along a trichome and even the non-heterocyst (non-N-fixing) cells in the trichome will possess the *nifH*
192 gene. As a result of polyploidy and the frequency of heterocysts in a cyanobacterial filament, the ratio
193 of cyanobacterial to heterotroph *nifH* gene copies may be 10^2-10^3 higher than the ratio of heterocysts
194 to heterotrophic diazotroph cells. Regardless, our results suggest that BSC N-fixation may include a
195 significant non-cyanobacterial component that requires further assessment across a more comprehensive
196 sampling of BSC types.

197   We did not observe evidence for N-fixation by heterocystous *Cyanobacteria* in the early successional
198 BSC samples used in this study. One possible explanation for our results is that the early successional
199 BSC samples used in this study possessed too few heterocystous *Cyanobacteria* to statistically evaluate
200 their ^{15}N-incorporation. Indeed, only 0.29% of sequences from this study's DNA-SIP 16S rRNA gene
201 sequence collections were from heterocystous *Cyanobacteria* (see results) as opposed to 15% and 23% of
202 total sequences in the Steven et al. (2013) and Garcia-Pichel et al. (2013) data, respectively. Nonetheless,
203 we would still expect even low abundance diazotrophs to show evidence for ^{15}N-incorporation, provided
204 sequence counts were not too sparse in heavy fractions. The OTUs defined by selected heterocystous
205 *Cyanobacteria* sequences presented in Yeager et al. (2006), however, all fall below the sparsity threshold
206 used in our analysis (see methods). Given the sparsity of heterocystous *Cyanobacteria* sequences in
207 the DNA-SIP data set, it is not possible to assess whether heterocystous *Cyanobacteria* incorporated
208 ^{15}N during the incubation. It should be noted that "light" and in particular "sub-biocrust" samples

209 possess much less heterocystous *Cyanobacteria* in general (Figure S3) so the samples used in this study
210 are not necessarily unrepresentative of typical poorly developed BSC simply because they are lacking
211 heterocystous *Cyanobacteria*.

212 The OTUs that did appear to incorporate ^{15}N during the incubation were predominantly *Proteobacteria*
213 and *Firmicutes*. The *Proteobacteria* OTUs for which ^{15}N-incorporation signal was strongest all shared
214 high sequence identity ($>=$98.48%) with 16S sequences from cultivars in genera with known diazotrophs
215 (Table 1). The *Firmicutes* that displayed signal for ^{15}N-incorporation (predominantly *Clostridiaceae*)
216 were not closely related to any cultivars (Table 1, Figure 5). These BSC *Clostrodiaceae* diazotrophs
217 represent a gap in culture collections. As culture-based ecophysiological studies have proven useful
218 towards explaining ecological phenomena in BSC 16S rRNA gene sequence surveys (Garcia-Pichel
219 et al., 2013), it would seem that these putative *Clostridiaceae* diazotrophs would be prime candidates
220 for targeted culturing efforts. Assessing the physiological response of these diazotrophic *Clostridiaceae*
221 to temperature would be useful for predicting how climate change will affect the BSC nitrogen budget.

222 Although too undersampled in the environmental data sets to reach statistical conclusions, ^{15}N-
223 responsive OTUs were found more often in sub-crust or or early successional BSC samples (Figure 2
224 and Figure S5). This result generates some hypotheses that are counter to prior discussions regarding
225 BSC diazotroph temporal dynamics. Specifically, the succession of BSC may not be marked by the
226 *emergence* of diazotrophs in BSC but rather the *transition* of the diazotroph community from heterotroph
227 to cyanobacteria dominance. Additionally, sub-crust soil may contribute significantly to the N budget in
228 arid ecosystems.

229 We propose that fast-growing heterotrophic diazotrophs such as *Clostridiaceae* are likely BSC
230 ecosystem pioneers. *M. vaginatus* accumulates compatible solutes such as trehalose and sucrose as
231 osmoprotectants during dessication (Rajeev et al., 2013). Additionally, although not demonstrated
232 specifically with *M. vaginatus*, microorganisms can rapidly excrete compatible solutes upon wetting
233 (Poolman and Glaasker, 1998). Many *Clostridiaceae* have a saccharolytic metabolism (Wiegel et al.,
234 2006) and *Clostridiaceae* isolates have been shown to utilize trehalose and/or sucrose (summarized in
235 Wiegel et al. (2006)). Further, *Clostridiaceae* isolates are fast-growing (doubling times typically between
236 30 min and 3 hr when grown on monosaccharides in culture (Wiegel et al., 2006)). Upon wetting, the early
237 successional BSC environment may become rapidly rich in compatible solutes excreted by *M. vaginatus*.
238 This boom-bust cycle would favor fast-growing microorganisms such as *Clostridiaceae* that can double
239 rapidly and also fix N.

240 Rarefaction curves of all samples from Steven et al. (2013) and Garcia-Pichel et al. (2013) are still
241 sharply increasing especially for sub-crust samples (Figure S4). Parametric richness estimates of BSC
242 diversity indicate the Steven et al. (2013) and Garcia-Pichel et al. (2013) sequencing efforts recovered on
243 average 40.5% (sd. 9.99%) and 45.5% (sd. 11.6%) of existing 16S OTUs from samples (inset Figure S4),
244 respectively. Therefore, is not alarming that few of the ^{15}N-responsive OTUS were found by Garcia-Pichel
245 et al. (2013) and Steven et al. (2013). Even next-generation sequencing efforts of BSC 16S rRNA genes
246 have only shallowly sampled the full diversity of BSC microbes.

4.1 CONCLUSION

247 Heterocystous *Cyanobacteria* are key contributors to the BSC N-budget, but, the ^{15}N-responsive OTUs
248 found in this study and the *nifH* gene sequences from Steppe et al. (1996) in addition to the N-fixation
249 rate data presented by Johnson et al. (2005) suggest there may be significant non-cyanobacterial BSC
250 diazotrophs specifically within the *Clostrideaceae* and *Proteobacteria*. It seems clear that heterocystous
251 *Cyanobacteria* increase in abundance with BSC age (Yeager et al., 2004). It is less clear if this transition
252 marks the emergence of diazotrophs versus a re-structuring of the BSC diazotroph community from one
253 dominated by *Clostridiaceae* and *Proteobacteria* to one predominantly heterocystous *Cyanobacteria*.
254 DNA-SIP is a valuable tool in the molecular microbial ecologist's toolbox for identifying members of
255 microbial community functional guilds (Neufeld et al., 2007). PCR-based surveys of diagnostic marker

256 genes and DNA-SIP are both used to connect microbial phylogenetic types to microbial activities, but
257 they occupy a non-overlapping set of strengths and weaknesses. DNA-SIP does not focus on a specific
258 diagnostic marker but does identify *active* players in the studied process (i.e. N-fixation). Combined these
259 tools can powerfully reveal connections between ecosystem membership/structure and function. Here we
260 supplement previous surveys of BSC *nifH* diversity, a diagnostic marker PCR-driven approach, with $^{15}N_2$
261 DNA-SIP, While we do not confirm previous results, we expand knowledge of BSC diazotroph diversity.
262 Predicting BSC N-fixation with respect to climate change, althered precipitation regimes and physical
263 disturbance requires a careful accounting of diazotrophs including non-cyanobacterial types.

5 MATERIALS AND METHODS

5.1 BSC SAMPLING AND INCUBATION CONDITIONS

264 Light crust samples (37.5 cm^2, average mass 35 g) were incubated in sealed chambers under controlled
265 atmosphere and in the light for 4 days. Crusts were dry prior to time zero and were wetted at initiation of
266 experiment. Treatments included control air (unenriched headspace) and enriched air (>98% atom $^{15}N_2$)
267 headspace. Samples were taken at 2 days and 4 days incubation. Acetylene reduction rates were measured
268 daily. DNA was extracted from 1 g of crust. Samples were taken from Green Butte, Arizona as previously
269 described (site CP3, Beraldi-Campesi et al. (2009)). All samples were from light crusts as described by
270 Johnson et al. (2005).

5.2 DNA EXTRACTION

271 DNA from each sample was extracted using a MoBio PowerSoil DNA Isolation Kit (following
272 manufacturers protocol, but substituting a 2 minute bead beating for the vortexing step), and then gel
273 purified. Extracts were quantified using PicoGreen nucleic acid quantification dyes (Molecular Probes).

5.3 DNA-SIP

274 Gradient density centrifugation of DNA was undertaken in 4.7 mL polyallomer centrifuge tubes in a
275 TLA-110 fixed angle rotor (both Beckman Coulter) in CsCl gradients with an average density of 1.725
276 g/mL. Average density for all prepared gradients was checked with an AR200 refractometer before runs.
277 Between 2.5-5 μg of DNA extract was added to the CsCl solution (15mM Tris-HCl, pH 8; 15mM EDTA;
278 15mM KCl), and gradients were run under conditions of 20C for 67 hours at 55,000 rpm (Buckley et al.,
279 2007). Centrifuged gradients were fractionated from bottom to top in 36 equal fractions of 100 μL, using a
280 by syringe pump as described Manefield et al. (2002). The density of each fraction was determined using
281 using an AR200 refractometer modified to accomodate 5ul samples (Buckley et al., 2007). DNA in each
282 fraction was desalted on a filter plate (PALL, AcroPrep Advance 96 Filter Plate, Product Number 8035),
283 using four washes with 300μL TE per fraction. After each wash, the filter plate was spun at 500 g for 10
284 minutes, with a final spin of 20 minutes. Fractions were resuspended in 50 uL of TE buffer.

5.4 PCR, LIBRARY NORMALIZATION AND DNA SEQUENCING

285 Barcoded PCR of bacterial and archaeal 16S rRNA genes, in preparation for 454 Pyrosequencing, was
286 carried out using primer set 515F/806R (Walters et al., 2011) (primers purchased from Integrated DNA
287 Technologies). The primer 806R contained an 8 bp barcode sequence, a "TC" linker, and a Roche 454
288 B sequencing adaptor, while the primer 515F contained the Roche 454 A sequencing adapter. Each
289 25 μL reaction contained 1x PCR Gold Buffer (Roche), 2.5 mM MgCl$_2$, 200 μM of each of the four
290 dNTPs (Promega), 0.5 mg/mL BSA (New England Biolabs), 0.3 μM of each primers, 1.25 U of Amplitaq
291 Gold (Roche), and 8 μL of template. Each sample was amplified in triplicate. Thermal cycling occurred
292 with an initial denaturation step of 5 minutes at 95C, followed by 40 cycles of amplification (20s at

293 95C, 20s at 53C, 30s at 72C), and a final extension step of 5 min at 72C. Triplicate amplicons were
294 pooled and purified using Agencourt AMPure PCR purification beads, following manufacturers protocol.
295 Once cleaned, amplicons were quantified using PicoGreen nucleic acid quantification dyes (Molecular
296 Probes) and pooled together in equimolar amounts. Samples were sent to the Environmental Genomics
297 Core Facility at the University of South Carolina (now Selah Genomics) to be run on a Roche FLX 454
298 pyrosequencing machine.

5.5 DATA ANALYSIS

299 All code to take raw sequencing data through the presented figures can be found at:

300   `http://nbviewer.ipython.org/github/chuckpr/NSIP_data_analysis`

301 *5.5.1 Sequence quality control* Sequences were initially screened by maximum expected errors at a
302 specific read length threshold (Edgar, 2013) which has been shown to be as effective as denoising 454
303 reads with respect to removing pyrosequencing errors. Specifically, reads were first truncated to 230
304 nucleotides (nt) (all reads shorter than 230 nt were discarded) and any read that exceeded a maximum
305 expected error threshold of 1.0 was removed. After truncation and max expected error trimming, 91% of
306 original reads remained. The first 30 nt representing the forward primer and barcode on high quality,
307 truncated reads were trimmed. Remaining reads were taxonomically annotated using the "UClust"
308 taxonomic annotation framework in the QIIME software package (Caporaso et al., 2010; Edgar, 2010)
309 with cluster seeds from Silva SSU rRNA database (Pruesse et al., 2007) 97% sequence identity OTUs as
310 reference (release 111Ref). Reads annotated as "Chloroplast", "Eukaryota", "Archaea", "Unassigned" or
311 "mitochondria" were culled from the dataset. Finally, reads were aligned to the Silva reference alignment
312 provided by the Mothur software package (Schloss et al., 2009) using the Mothur NAST aligner (DeSantis
313 et al., 2006). All reads that did not appear to align to the expected amplicon region of the SSU rRNA gene
314 were discarded. Quality control parameters removed 34,716 of 258,763 raw reads.

315 *5.5.2 Sequence clustering* Sequences were distributed into OTUs using the UParse methodology
316 (Edgar, 2013). Specifically, cluster seeds were identified using USearch with a collection of non-redundant
317 reads sorted by count as input. The sequence identity threshold for establishing a new OTU centroid
318 was 97%. After initial cluster centroid selection, select 16S rRNA gene sequences trimmed to the same
319 alignment positions as the other centroids from Yeager et al. (2006) were added to the centroid collection.
320 Specifically, Yeager et al. (2006) Colorado Plateau or Moab, Utah sequences were added which included
321 the 16S rRNA gene sequences for *Calothrix* MCC-3A (accession DQ531700.1), *Nostoc commune* MCT-1
322 (accession DQ531903), *Nostoc commune* MFG-1 (accession DQ531699.1), *Scytonema hyalinum* DC-A
323 (accession DQ531701.1), *Scytonema hyalinum* FGP-7A (accession DQ531697.1), *Spirirestis rafaelensis*
324 LQ-10 (accession DQ531696.1). Centroid sequences that matched selected Yeager et al. (2006) sequences
325 with greater than to 97% sequence identity were subsequently removed from the centroid collection. With
326 USearch/UParse, potential chimeras are identified during OTU centroid selection and are not allowed to
327 become cluster centroids effectively removing chimeras from the read pool. All quality controlled reads
328 were then mapped to cluster centroids at an identity threshold of 97% again using USearch. 95.6% of
329 quality controlled reads could be mapped to centroids. Unmapped reads do not count towards sample
330 counts and are essentially removed from downstream analyses. The USearch software version for cluster
331 generation was 7.0.1090.

332 *5.5.3 Merging data from this study, Garcia-Pichel et al. (2013), and Steven et al. (2013)* As only
333 sequences without corresponding quality scores were publicly available from Garcia-Pichel et al. (2013)
334 and Steven et al. (2013), these data sets were only quality screened by determining if they covered the
335 expected region of the 16S rRNA gene (described above). All data (this study, Garcia-Pichel et al. (2013)
336 and Steven et al. (2013)) were included as input to USearch for OTU centroid selection and subsequent
337 mapping to OTU centroids.

338 *5.5.4 Phylogenetic tree*   The alignment for the "*Clostridiaceae*" phylogeny was created using SSU-
339 Align which is based on Infernal (Nawrocki and Eddy, 2013; Nawrocki et al., 2009). Columns in
340 the alignment that were not included in the SSU-Align covariance models or were aligned with poor
341 confidence (less than 95% of characters in a position had posterior probability alignment scores of
342 at least 95%) were masked for phylogenetic reconstruction. Additionally, the alignment was trimmed
343 to coordinates such that all sequences in the alignment began and ended at the same positions. The
344 "*Clostridiaceae*" tree included all top BLAST hits (parameters below) for ^{15}N *Clostridiaceae* responders
345 in the Living Tree Project database (Yarza et al., 2008) in addition to BLAST hits within a sequence
346 identity threshold of 97% to ^{15}N responders from the Silva SSURef_NR SSU rRNA database (Pruesse
347 et al., 2007). Only one SSURef_NR115 hit per study per OTU ("study" was determined by "title" field)
348 was selected for the tree. FastTree (Price et al., 2010) was used to build the tree and support values are
349 SH-like scores reported by FastTree.

350 *Placement of short sequences into backbone phylogeny* Short sequences were mapped to the reference
351 backbone using pplacer (Matsen et al., 2010) (default parameters). pplacer finds the edge placements that
352 maximize phylogenetic likelihood. Prior to being mapped to the reference tree, short sequences were
353 aligned to the reference alignment using Infernal (Nawrocki et al., 2009) against the same SSU-Align
354 covariance model used to align reference sequences.

355 *5.5.5 BLAST searches*   BLAST searches were done with the "blastn" program from BLAST+ toolkit
356 (Camacho et al., 2009) version 2.2.29+. Default parameters were always employed and the BioPython
357 (Cock et al., 2009) BLAST+ wrapper was used to invoke the blastn program. Pandas (McKinney, 2012)
358 and dplyr (Wickham and Francois, 2014) were used to parse and munge BLAST output tables.

359 *5.5.6 Identifying OTUs that incorporated ^{15}N into their DNA*   SIP is a culture-independent approach
360 towards defining identity-function connections in microbial communities (Buckley, 2011; Neufeld et al.,
361 2007). Microbes incubated in the presence of ^{13}C or ^{15}N labeled substrates can incorporate the stable
362 heavy isotope into biomass if they participate in the substrate's transformation. Stable isotope labeled
363 nucleic acids can then be separated from unlabeled by buoyant density in a CsCl gradient. As the buoyant
364 density of a macromolecule is dependent on many factors in addition to stable isotope incorporation
365 (e.g. GC-content in nucleic acids (Youngblut and Buckley, 2014)), labeled nucleic acids from one
366 microbial population may have the same buoyant density of unlabeled nucleic acids from another (i.e.
367 each population's nucleic acids would be found at the same point along a density gradient although
368 only one population's nucleic acids are labeled). Therefore it is imperative to compare density gradients
369 with nucleic acids from heavy stable isotope incubations to gradients from "control" incubations where
370 everything mimics the experimental conditions except that unlabeled substrates are used (and all DNA
371 would be unlabeled). By contrasting "heavy" density gradient fractions in experimental density gradients
372 (hereafter referred to as "labeled" gradients) against heavy fractions in control gradients, the identities of
373 microbes with labeled nucleic acids can be determined

374 We used an RNA-Seq differential expression statistical framework (Love et al., 2014) to find OTUs
375 enriched in heavy fractions of labeled gradients relative to corresponding density fractions in control
376 gradients (for review of RNA-Seq differential expression statistics applied to microbiome OTU count data
377 see McMurdie and Holmes (2014)). We use the term differential abundance (coined by McMurdie and
378 Holmes (2014)) to denote OTUs that have different proportion means across sample classes (in this case
379 the only sample class is labeled/control). CsCl gradient fractions were categorized as "heavy" or "light".
380 The heavy category denotes fractions with density values above 1.725 g/mL. Since we are only interested
381 in enriched OTUs (labeled versus control), we used a one-sided z-test for differential abundance (the null
382 hypothesis is the labeled:control proportion mean ratio for an OTU is less than a selected threshold). P-
383 values were corrected with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). We
384 selected a $\log_2$ fold change null threshold of 0.25 (or a labeled:control proportion mean ratio of 1.19).
385 DESeq2 was used to calculate the moderated $\log_2$ fold change of labeled:control proportion mean ratios

386 and corresponding standard errors. Mean ratio moderation allows for reliable ratio ranking such that
387 high variance and likely statistically insignificant mean ratios are appropriately shrunk and subsequently
388 ranked lower than they would be as raw ratios. To summarize, OTUs with high moderated labeled:control
389 proportion mean ratios have higher proportion means in heavy fractions of labeled gradients relative to
390 heavy fractions of control gradients, and therefore have likely incorporated ^{15}N into their DNA during the
391 incubation.

392   Although DNA-SIP is a powerful technique, analysis of DNA-SIP data is not without ambiguities. One
393 limitation is the discrete, selected boundary in the form of a adjusted p-value threshold (or false discovery
394 rate) that marks which OTUs we consider to be enriched in the heavy fractions of labeled CsCl gradients
395 (and thus have likely incorporated ^{15}N into their DNA during the incubation). In reality the metric we
396 use to quantify the magnitude of an OTU's response to a stable isotope is continuous, and there is only
397 an artificial boundary between which OTUs appear to have "responded" and which OTUs have unknown
398 response. For this reason, we have presented all the OTUs that satisfy our "response" criteria but focused
399 on the most strongly responding OTUs. As with any hypothesis-based statistical test, care should be taken
400 when interpreting the significance of results where p-values are near the selected threshold for rejecting
401 the null hypothesis.

402 *5.5.7 Ordination*   Principal coordinate ordinations depict the relationship between samples at each time
403 point (day 2 and 4). Bray-Curtis distances were used as the sample distance metric for ordination. The
404 Phyloseq (McMurdie and Holmes, 2014) wrapper for Vegan (Oksanen et al., 2013) (both R packages) was
405 used to compute sample values along principal coordinate axes. GGplot2 (Wickham, 2009) was used to
406 display sample points along the first and second principal axes. Adonis tests Anderson (2001) were done
407 with default number of permutations (1000).

5.6 RICHNESS ANALYSES

408 Rarefaction curves were created using bioinformatics modules in the PyCogent Python package (Knight
409 et al., 2007). Parametric richness estimates were made with CatchAll using only the best model for total
410 OTU estimates (Bunge, 2010).

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6   FIGURES AND LONG TABLES

578   **Table 1.**[15]N responders BLAST search against Living Tree Project

OTU ID	Species Names	BLAST %ID	Phylum
OTU.78	*Desulfocella, Bryobacter*	80.31	*Acidobacteria*
OTU.55	*Desulfocella, Bryobacter*	81.03	*Acidobacteria*
OTU.116	*Streptomyces*	100.0	*Actinobacteria*
OTU.140	*Bacillus*	100.0	*Firmicutes*
OTU.3	*Bacillus*	100.0	*Firmicutes*
OTU.1747	*Clostridium*	94.36	*Firmicutes*
OTU.327	*Clostridium*	94.92	*Firmicutes*
OTU.17	*Clostridium*	95.45	*Firmicutes*
OTU.61	*Clostridium*	95.92	*Firmicutes*
OTU.11	*Clostridium*	95.94	*Firmicutes*
OTU.2175	*Clostridium*	95.96	*Firmicutes*
OTU.1673	*Clostridium*	95.9	*Firmicutes*
OTU.330	*Clostridium*	96.94	*Firmicutes*
OTU.75	*Clostridium*	96.97	*Firmicutes*
OTU.643	*Clostridium*	97.45	*Firmicutes*
OTU.3712	*Clostridium, Eubacterium*	96.43	*Firmicutes*
OTU.37	*Clostridium, Ilyobacter*	96.43	*Firmicutes*
OTU.2404	*Domibacillus*	99.49	*Firmicutes*
OTU.4167	*Fonticella*	93.43	*Firmicutes*
OTU.4037	*Fonticella*	93.85	*Firmicutes*
OTU.57	*Fonticella, Caloramator*	93.88	*Firmicutes*
OTU.575	*Gracilibacter*	94.42	*Firmicutes*
OTU.259	*Parasporobacterium*	98.47	*Firmicutes*
OTU.278	*Symbiobacterium*	90.62	*Firmicutes*
OTU.88	*Symbiobacterium*	92.86	*Firmicutes*
OTU.342	*Acinetobacter*	100.0	*Proteobacteria*
OTU.263	*Azospirillum*	98.48	*Proteobacteria*
OTU.137	*Azospirillum*	98.98	*Proteobacteria*
OTU.176	*Delftia*	100.0	*Proteobacteria*
OTU.14	*Klebsiella, Pantoea, Erwinia, Enterobacter, Kluyvera, Buttiauxella*	99.49	*Proteobacteria*
OTU.586	*Ottowia, Diaphorobacter, Ideonella, Vitreoscilla, Comamonas*	98.48	*Proteobacteria*

Table 1 – continued from previous page

OTU ID	*Species Names*	BLAST %ID	*Phylum*
OTU.321	*Pseudomonas*	100.0	*Proteobacteria*
OTU.54	*Shigella, Escherichia*	100.0	*Proteobacteria*

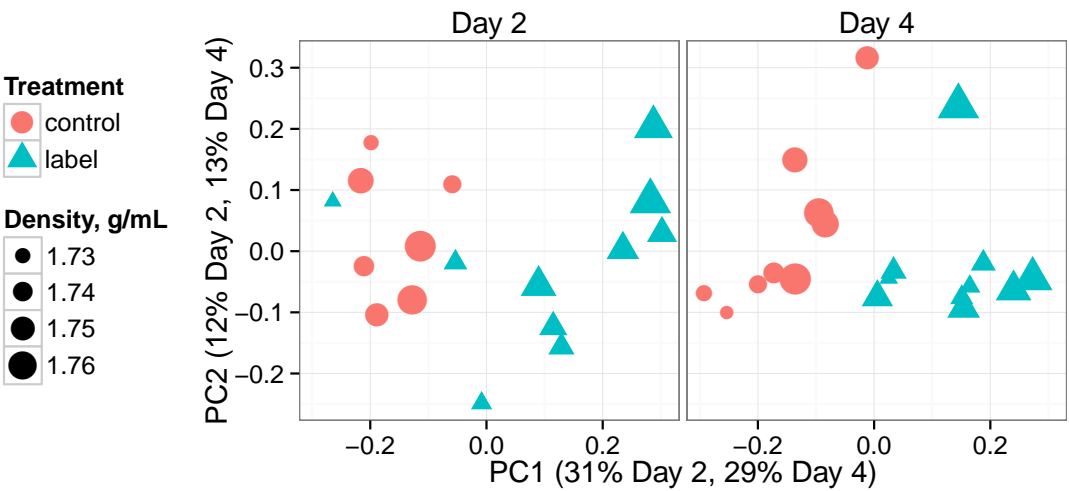**Figure 1.** Ordination of heavy gradient fraction sequence collections by Bray-Curtis distances.

Figure 2. Phylogenetic trees of OTUs passing sparsity threshold for selected phyla. *A)* Point denotes OTU is classified as a [15]N "responder". *B)* Heatmap of moderated $\log_2$ proportion mean ratios (labeled:control gradients) for each OTU at each incubation day. High values indicate ^{15}N incorporation. *C)* Presence/absence of OTUs (black indicates presence) in lichen, light, or dark environmental samples (Garcia-Pichel et al., 2013). *D)* Presence/absence of OTUs (black indicates presence) in crust and below crust samples (Steven et al., 2013).
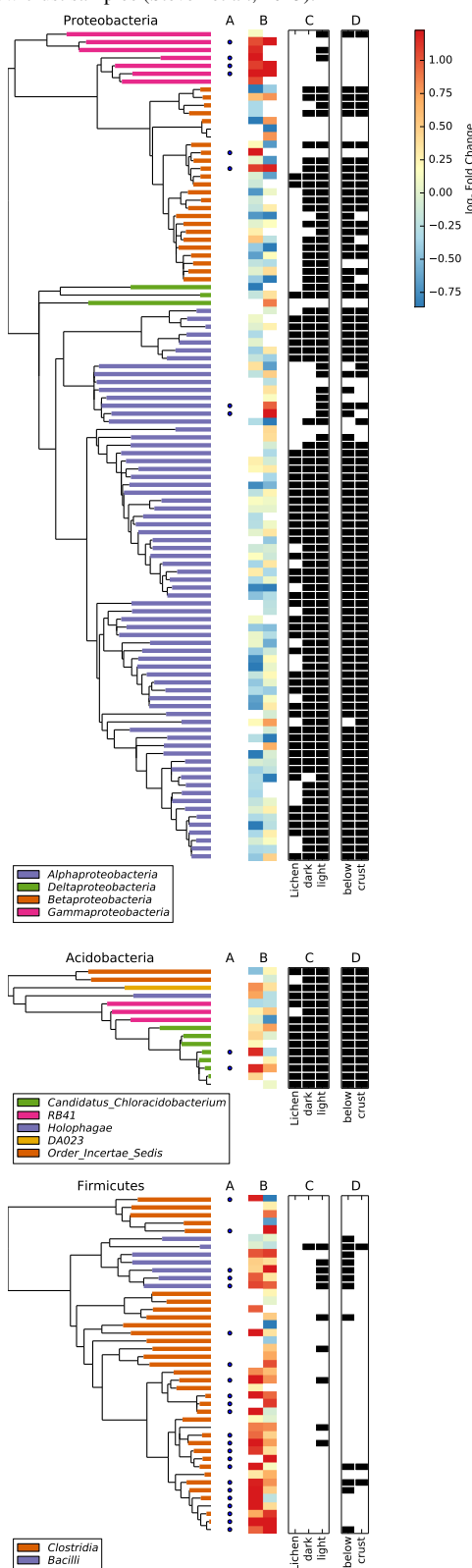
Figure 3. Moderated log₂ of proportion mean ratios for labeled versus control gradients (heavy fractions only, densities ¿1.725 g/mL). All OTUs found in at least 62.5% of heavy fractions at a specific incubation day are shown. Red color denotes a proportion mean ratio that has a corresponding adjusted p-value below a false discovery rate of 10% (the null model is that the proportion mean is ratio is below 0.25). The horizontal line is the proportion mean threshold for the null model, 0.25. The inset figure summarizes the taxonomy of OTUs that with proportion mean ratio p-vaules under 0.10 for at least one time point.

Figure 4. Relative abundance values in heavy fractions (density greater or equal to 1.725 g/mL) for the top 10 ^{15}N "responders" (putative diazotrophs, see results for selection criteria of top 10) at each incubation day. See Table 1 for BLAST results of top 10 responders against the LTP database (release 115). Point area is proportional to CsCl gradient fraction density, and color signifies control (red) or labeled (blue) treatment.

Figure 5. See methods for selection criteria for sequences in backbone tree. Edge width is proportional to number of short putative *Clostridiaceae* diazotroph sequences placed at that position. Placement of short sequences can be spread across multiple edges Matsen et al. (2010). Reference sequences from cultivars have boxes at tips and full species names. Tips with only accession annotations are from environmental reference sequences.

7 SUPPLEMENTAL FIGURES

Figure S1. Distribution of sequences into top 9 phyla (phyla ranked by sum of all sequence annotations).

Figure S2. Ordination of Bray-Curtis sample pairwise distances for each incubation time. Point area is proportional to the density of the CsCl gradient fraction for each sequence library, and color/shape reflects control (red triangles) or labeled (blue circles) treatment. Inset shows Bray-Curtis distances for paired control versus labeled CsCl gradient fractions (i.e. fractions from the same incubation day and same density) against the density of the pair (p-value: $4.526e^{-5}$, r^2: 0.434).

Figure S3. Relative abundance of selected heterocystous cyanobacterial OTUs with centroids from sequences described in Yeager et al. (2006) (see methods for selection criteria) in Steven et al. (2013) data set.

Figure S4. Rarefaction curves for all samples presented by Garcia-Pichel et al. (2013) and Steven et al. (2013). Inset is boxplot of estimated sampling effort for all samples in Garcia-Pichel et al. (2013) and Steven et al. (2013) (number of observed OTUs divided by number of CatchAll Bunge (2010) estimated total OTUs)

Figure S5. Counts of "responder" OTU occurrences in samples from Steven et al. (2013) and Garcia-Pichel et al. (2013). Steven et al. (2013) collected BSC samples (25 samples total) and samples from soil beneath BSC (17 samples total, "below" column in figure). Garcia-Pichel et al. (2013) collected samples from "dark" (9 samples total) and "light" (12 samples total) crusts in addition to "lichen" (2 samples total) dominated crusts.