Only a minority of bacteria grow after wetting in both natural and post-mining biocrusts in a hyperarid phosphate mine

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Abstract

Biological soil crusts (biocrusts) are key contributors to desert ecosystem functions; therefore, biocrust restoration following mechanical disturbances is imperative. In the Negev Desert hyperarid regions, phosphate mining has been practiced for over 60 years, destroying soil habitats, and fragmenting the landscape. In this study, we selected one mining site restored in 2007, and used DNA stable isotope probing (DNA-SIP) to identify which bacteria grow in post-mining and adjacent natural biocrusts. Since biocrust communities activate only after wetting, we incubated the biocrusts with $H_2^{18}O$ for 96 hours under ambient conditions. We then evaluated the physicochemical soil properties, chlorophyll a concentrations, activation, and functional potential of the biocrusts. The DNA-SIP assay revealed low bacterial activity in both plot types and no significant differences in the proliferated communities' composition when comparing post-mining and natural biocrusts. We further found no significant differences in the microbial functional potential, photosynthetic rates, or soil properties. Our results suggest that growth of hyperarid biocrust bacteria after wetting is minimal. We hypothesize that due to the harsh climatic conditions, during wetting bacteria devote their meager resources to prepare for the coming drought, by focusing on damage repair, and organic compound synthesis and storage rather than on growth. These low growth rates contribute to the sluggish recovery of desert biocrusts following major disturbances such as mining. Therefore, our findings highlight the need for implementing active restoration practices following mining.

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Keywords

36 Biological soil crust; Biocrust restoration; Stable isotope probing; Hyperarid desert; Mining;

37 Ecological restoration

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1. Introduction

Phosphate mining in the Negev Desert, Israel, has been taking place since the 1960s in large areas. ILC-Rotem mining company leads the phosphate mining activities and has been practicing a reclamation-oriented mining protocol for the past 15 years. The mining protocol entails the excavation of the top 50-70 cm of soil (which they consider to be topsoil) followed by the overburden (the layer covering the phosphate), then storing the two soil layers in separate piles. Following the excavation of the phosphate, the overburden is returned to the mining pit followed by the topsoil. Finally, the terrain is leveled with heavy machinery. The area is then considered a restored, post-mining site.

Open mining activities lead to the destruction of the local vegetation and seed bank, and the fragmentation of the natural landscape (Sengupta, 2021). The consequences include land degradation, erosion, soil and water pollution, and dust dispersion. In addition, mining activity often leads to decreased biodiversity in and around mining sites (Bridge, 2004, Sengupta, 2021). One of the ecosystem components being destroyed by mining activities in the Negev Desert is the biological soil crust layer (biocrust). Biocrust is the topmost layer of many arid soils and comprises primary-producing and heterotrophic microorganisms that bind together soil particles using secreted extracellular polymeric substances (EPS), mainly polysaccharides (Weber et al., 2022). Biocrusts provide many ecosystem services, including fixing nitrogen and carbon, and soil stabilization (Belnap and Lange, 2003). While biocrust microorganisms developed various adaptations to withstand the harsh desert environment

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61   (Makhalanyane et al., 2015), biocrust structures are susceptible to mechanical disturbances.

62   Such a disturbance, especially over large scales (for example, mining activity), breaks and

63   buries biocrust organisms, often resulting in changed biocrust communities (Belnap and

64   Eldridge, 2003).

65

66   In a previous research, we evaluated the biocrust bacterial communities in phosphate

67   mining sites (Gabay et al., 2022). Briefly, we found that natural and post-mining biocrusts

68   differ in community composition and diversity. Following the biocrust community analysis,

69   we sought to identify which bacterial groups are actively growing in the biocrust and

70   whether the composition differs between natural and post-mining sites. To this end, we used

71   DNA-stable isotope probing (DNA-SIP): a culture-free approach that allows the detection of

72   actively growing microorganisms by labeling them with stable isotopes such as ^{15}N, ^{14}C, and

73   ^{18}O (Dumont and Hernández García, 2019). SIP has been widely applied in identifying

74   microbial groups that participate in carbon and nitrogen cycling, such as methanotrophs

75   (Sultana et al., 2019, Zhang et al., 2020), methylotrophs (Macey et al., 2020, Arslan et al.,

76   2022), and nitrogen fixers (Pepe-Ranney et al., 2016, Angel et al., 2018). Likewise, SIP can use

77   the incorporation of heavy water (H$_2$^{18}O) into various biomarkers to study the growth and

78   function of microorganisms that become activated upon wetting (Schwartz et al., 2019).

79   Previous H$_2$^{18}O SIP experiments measured microbial growth rates and dynamics following

80   hydration (Blazewicz et al., 2020). Desert biocrusts make an ideal study system for H$_2$^{18}O SIP

81   experiments, as they become active quickly following hydration (Angel and Conrad, 2013),

82   resuming growth, nutrient cycling, and excretion of extracellular organic materials (Garcia-

83   Pichel and Belnap, 1996, Belnap and Lange, 2003).

84

85   In this research, we investigated the proliferation of bacterial groups in biocrusts taken from

86   reference ('natural') areas and post-mining sites by incubating biocrust samples with

87   isotopically-labeled water ($H_2^{18}O$). We hypothesized that growth patterns and taxonomic

88   identity of bacterial groups would differ significantly when comparing natural and post-

89   mining biocrusts. Specifically, we expected higher bacterial growth rates in natural

90   compared to post-mining biocrusts. Based on our previous findings, we specifically expected

91   higher activity of Cyanobacteria in the natural biocrusts (Gabay et al., 2022).

2. Materials and Methods

2.1. Study site and sample collection

Sampling was conducted during June 2020 at the Gov Mining Site, located in the Zin Valley (30.84 °N, 35.09 °E, 98 m above sea level), where restoration was completed in 2007. The study area was previously described in Gabay et al., (2022). Briefly, Zin Valley is a hyperarid region of the Negev Desert, with 50 mm average annual rainfall (Zin factory meteorological data) and highly saline soils (average EC = 24 dS/m). The main soil cover types in Zin Valley are biocrusts and desert pavement, with scarce vegetation of mainly annual species. The soil composition in the post-mining site and natural area is similar with 70% sand, 18% silt and 12% clay, and 68% sand, 20% silt and 12% clay for natural and post-mining respectively (Gabay et al., 2022). The soils in Zin Valley are classified as Solonchaks according to the World Reference Based soil classification system.

Biocrusts were sampled either from the post-mining site or the adjacent natural area. The biocrusts in Gov are thin (between 1.5 - 2 .5 mm deep), and smooth. The site is characterized by areas covered in biocrusts or desert pavement. In each sampling site, we sampled along a 100 m strip at approximately 10 m intervals (Fig. 1). In total, we sampled 20 biocrust samples (10 from each site). We collected the biocrusts using a spatula, at an average depth of 2 mm. Biocrusts were placed in 100 mm x 15 mm petri dishes lined with cotton. For the SIP assay, we

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112   chose 5 of the 10 samples from each site containing the highest chlorophyll *a* concentrations

113   as estimated in preliminary experiments (Table S1).
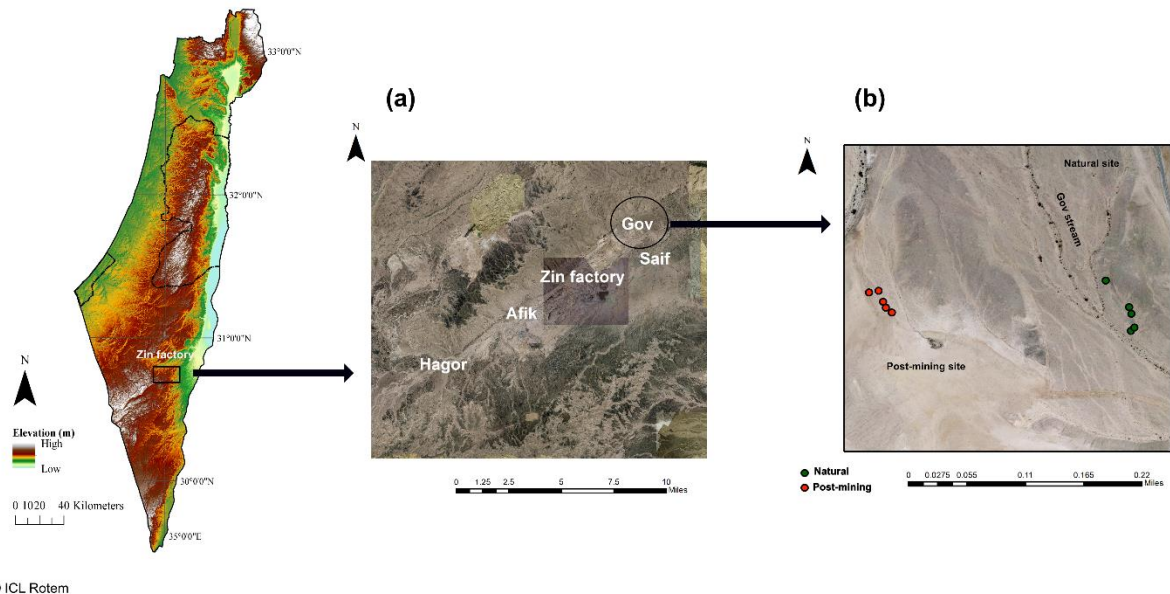

114

115   Figure 1: map of the research area. Map a shows the different post-mining sites around the Zin

116   factory. Map b shows the biocrust sampling points in gov mining site used for this research.

117   Green dots represent the natural biocrusts, and red dots represent the post-mining biocrusts.

2.2. Soil properties

119   Five biocrust samples from each plot type (post-mining and natural) were sent for analysis of

120   soil properties (pH, EC, NO_3^- concentrations, and soil organic matter). The analysis was

121   performed at the Gilat Soil Laboratory (Gilat Research Center, Gilat, Israel).

122

123   2.3. chlorophyll *a* extraction

124   Chlorophyll *a* was extracted from biocrust samples using a protocol previously described in

125   Gabay et al., (2022). Briefly, chlorophyll *a* was extracted from 3 g soil of each biocrust sample

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126   was diluted in 9 mL of methanol for 15 min at 65 °C. The soil solution was centrifuged at 2000

127   rpm for 5 minutes, supernatant was collected, and absorbance was measured in a

128   spectrophotometer at 665 nm. Concentrations were calculated according to (Ritchie, 2006)

129   and normalized to 1 g of soil. Extractions of the biocrusts were performed before (dry

130   biocrusts) and after 96 hr incubation with distilled water (DW) under identical conditions to

131   the incubation with $H_2{}^{18}O$.

132

2.4. Stable isotope probing

2.4.1. Soil incubation

135   To test the incorporation of ^{18}O into biocrust samples, a microcosm was designed to control

136   for the incubation conditions. Each microcosm consisted of a 10 mL glass vial in which 1 g of

137   biocrust sample was placed. To achieve field water-holding capacity, 0.15 mL of $H_2{}^{18}O$ or

138   DNase-free water were added. The glass vials were then sealed with butyl rubber stoppers

139   (Sigma-Aldrich, St. Louis, Missouri, United States) to prevent evaporation. Both labeled and

140   unlabeled controls were incubated in duplicates, for a total of 40 vials. Samples were

141   incubated under a 12 hr photoperiod for 96 hr in an incubator (FOC 225 I, VELP Scientifica,

142   Usmate Velate MB, Italy) to allow the incorporation of ^{18}O into the bacterial DNA. Following

143   incubation, the microcosms were sacrificed, and each biocrust sample was divided into 4

144   bead beating tubes (Qiagen, Hilden, Germany), each containing 0.25 g of soil, and stored at -

145   80 °C until further analysis.

146 Each labeled sample had a non-labeled control, incubated under identical conditions but

147 with DNase-free water instead of ^{18}O water.

148

2.4.2. DNA extraction

150 DNA was extracted from all biocrust samples using DNeasy PowerSoil Pro Kit (Qiagen),

151 according to the manufacturer's instructions. The biomass in hyperarid biocrusts tends to be

152 very low, yielding only minute amounts of DNA. Therefore, each 1 g soil was extracted in

153 batches of 0.25 g, and the extracts were later consolidated to increase DNA yield.

154

2.4.3. SIP gradient preparation and fractionation

156 DNA (ca. 3.5 ng) was subjected to isopycnic gradient centrifugation in a solution of caesium

157 chloride (7.163 M; CsCl, Sigma Aldrich. St Loise, MI, USA) and buffer (0.1 M Tris-HCl at pH 8.0,

158 0.1 M KCl and 1 mM EDTA, all from Sigma Aldrich) to a final density of 1.725 g mL^{-1} as described

159 previously (Jia et al., 2019). The tubes were spun for 44 hr at 177,000 g and then fractionated

160 by water displacement using a syringe pump (NE-300 Just Infusion™ Syringe Pump, NewEra

161 Pump systems, Farmingdale, NY, USA). The refractive index was measured using an AR200

162 digital refractometer (Reichert, Depew, NY, USA) and then the DNA was precipitated using a

163 Polyethylene Glycol 6000 solution (30% PEG 8000 and 1.6 M NaCl), and 30 µg of GlycoBlue

164 Coprecipitant (Thermo Fisher Scientific, Waltham, MS, USA). Copy numbers of the 16S rRNA

165 gene in each fraction were determined by qPCR using a probe-based approach. Primers 338F

166 and 805R (Yu et al., 2005) coupled with a 516P probe (FAM-BHQ1 dual labeled) were used for

10

167   the assay. Per one reaction 10 µL of TaqMan™ Fast Advanced Master Mix (Thermo Fisher

168   Scientific), 0.4 µL of Bovine Saline Albumin (BSA; Thermo Fisher Scientific), 1 µL of each primer

169   (10 µM), 0.4 µL of a probe (10 µM) and 2.2 µL of PCR water was combined and mixed with 5 µL

170   of DNA. After 5 min initial denaturation at 95 °C, cycling program: 40 cycles of 95 °C for 30 sec

171   followed by 62 °C for 1 min was applied. Gene copy numbers were established from a standard

172   curve of *Escherichia coli* 16S rRNA gene.

173

174   2.4.4. PCR and sequencing

175   Following fractionation, all samples (labeled and unlabeled) were amplified using the 16S

176   rRNA primers 515F_mod and 806R_mod (Apprill et al., 2015; Parada et al., 2016). Each reaction

177   consisted of 2.5 µL Green Taq Buffer (Thermo Fisher Scientific), 2.5 µL of dNTP set

178   (Biotechrabbit, Berlin, Germany), 0.1 µL of BSA (Thermo Fisher Scientific), 0.625 µL of each

179   primer (10 µM), 0.125 µL DreamTaq Green DNA Polymerase (Thermo Fisher Scientific) and 17.5

180   µL of PCR water (Sigma). The PCR ran for 38 cycles using the following program: denaturation

181   at 94 °C for 45 sec, annealing at 52 °C for 45 sec, extension at 72 °C for 45 sec, and a final cycle

182   of extension at 72 °C for 10 min. The amplified fragments were sequenced using MiniSeq

183   (Illumina, San Diego, CA, USA) at the UIC sequencing core, University of Illinois, Chicago, Illinois

184   (https://rrc.uic.edu/cores/genome-research/genome-research-core/). DNA extraction and SIP

185   gradient controls, PCR negative controls and mock community (ZymoBIOMICS Microbial

186   Community Standard II Log Distribution; Zymo Research, Irvine, CA, USA) samples (2 of each)

187   were also sequenced to control for contaminants in the sequencing results.

188

2.5. Bioinformatic analysis

190 All the bioinformatic and statistical analyses were done in R V4.1.1 (R development core

191 team, 2013). Labeling of bacteria was detected using differential abundance analysis as

192 described in Angel (2019). Briefly, the sequences were processed using the DADA2 package

193 V8.8 (Callahan et al., 2016) for quality filtering, denoising, read-merging, chimera removal,

194 constructing amplicon sequence variants (ASV) tables, and taxonomic assignment. Detection

195 and removal of potential contaminant sequences were performed using the R package

196 decontam V.1.12.0 (Davis et al., 2017). Prevalence filtering of rare ASVs was done using the

197 Phyloseq package V1.36.0 (McMurdie and Holmes, 2013). ASVs that appeared in less than

198 2.5% of the samples were removed. A maximum-likelihood phylogenetic tree was calculated

199 using IQ-TREE2 V 2.1.1. (Minh et al., 2020). Finally, differential abundance analysis was

200 performed using DESeq2 V1.32.0 (Love et al., 2014) to compare the relative abundance of

201 each ASV in the heavy fractions of labeled DNA to the unlabeled heavy fractions (the negative

202 control samples), which allows identifying the bacterial groups that incorporated the water

203 isotope into their DNA. The results were filtered to include only ASVs with a 2-fold log change

204 and a significance value $p < 0.1$.

205

2.6. Predictions of genomic functions

207 Abundances of functional genes based on 16S rRNA gene abundances were performed using

208 PICRUSt2 (Douglas et al., 2019). Abundances of functional genes were predicted based on a

12

209 filtered ASV table containing only ASVs belonging to proliferated bacteria based on the

210 differential abundance modeling. The resulting output is functional identifications that were

211 annotated using the KEGG database to infer functional gene families. Each gene was then

212 classified into a function category and the abundance of genes within each category was

213 averaged. The function categories were chosen based on Meier et al. (2021). In their study,

214 Meier et al. collected biocrusts from the Negev and analyzed bacterial metagenomes in the

215 biocrusts to evaluate the distribution of metabolic potential among bacterial populations. To

216 compare functional potential between various bacterial phyla, they selected metabolic genes

217 encoded in the metagenomic-assembled genomes and grouped them into 10 function

218 categories.

219

220 ## 2.7. Statistical analyses

221 chlorophyll *a* concentrations were visualized as an estimation plot using the dabestr

222 package V0.3.0 (Ho and Tumkaya, 2018). The effect size was calculated as a bootstrap 95%

223 confidence interval. Relative abundances of phyla, Abundances of functional genes and soil

224 properties were compared between natural and post-mining biocrusts using Mann-Whitney

225 tests. The community composition of natural and post-mining biocrusts was assessed using

226 only sequences belonging to proliferated bacteria based on DESeq2 modeling. The weighted

227 UniFrac (Lozupone et al., 2011) was used to calculate the similarity between the natural and

228 post-mining communities, and an adonis model was used to assess whether communities

229 differ significantly from each other (package Vegan V2.6-2; Dixon, 2003).

3. Results

Sample wetting and greening

Most biocrust samples (both natural and post-mining) showed greening within 36 to 48 hr into the 96 hr incubation. By the end, most samples displayed varying degrees of greening, indicating cyanobacterial activity. Generally, post-mining biocrust showed less greening than the natural biocrusts (Fig. 2).
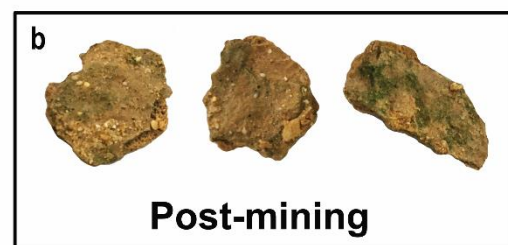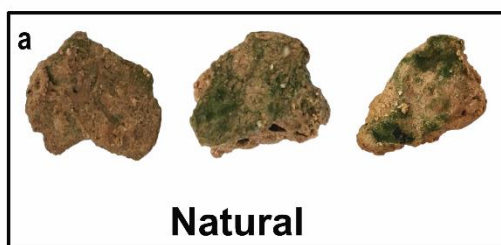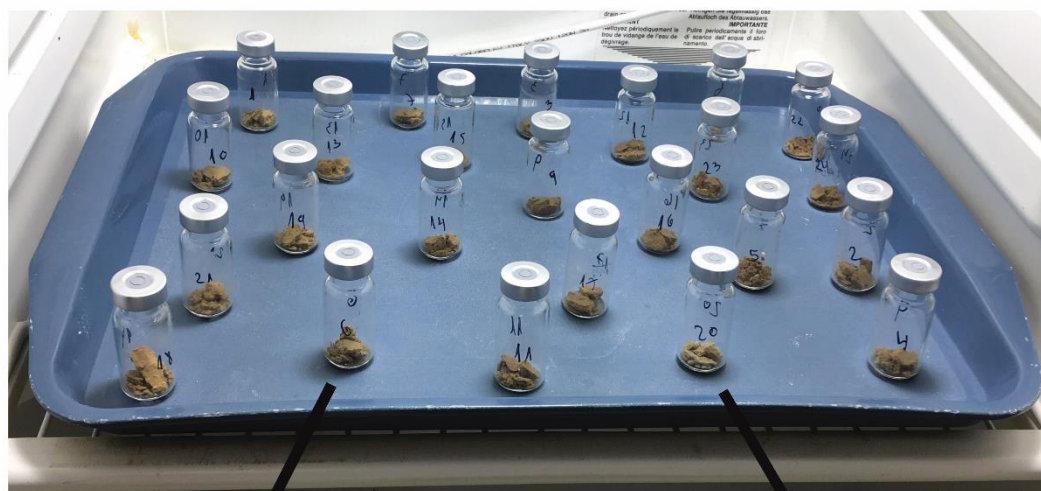

Figure 2: Incubation setup. Top picture – biocrusts in sealed glass vials in the incubator. Bottom picture – natural (a) and post-mining (b) biocrusts following the 96-hour incubation.

14

242   ## Soil properties

243   EC and NO_3^- were significantly higher in natural biocrusts compared to post-mining biocrusts

244   (EC: t = 2.89, p < 0.05; NO_3^-: t = 4, p < 0.01; Table 1). Soil organic matter was also significantly

245   higher in the natural biocrusts (t = 3.77, p < 0.01; Table 1). pH was slightly higher in natural

246   biocrusts; however, the differences were not statistically significant (pH: t = 1.41, p = 0.19;

247   Table 1).

248

249   Table 1: soil properties for natural and post-mining biocrusts. The numbers represent the
250   means for each property and the standard deviation. Significant differences are marked with
251   an asterisk (* = p < 0.05; ** = p < 0.01).

252

Plot type/Soil property	Natural	Post-mining
pH	7.6 ± 0.12	7.5 ± 0.1
EC	26.22* ± 9.38	9.94 ± 8.39
NO3	84.82** ± 36.69	14.75 ± 13.57
Soil organic matter	1.2** ± 0.19	0.81 ± 0.12

253

254

255   ## Chlorophyll *a*

256   The estimation plot revealed an effect size estimate at 1.42 (95CI -0.432; 3.03; Fig. 3). In the

257   natural samples, there was no clear clustering according to the soil water content i.e., dry or

258   hydrated (following 96 hr incubation with water). In fact, there was a larger variance between

259   samples collected after incubation (Fig. 3). Hydrated post-mining biocrusts had consistently

260   higher chlorophyll *a* concentrations compared to dry biocrusts. It Is also apparent that the

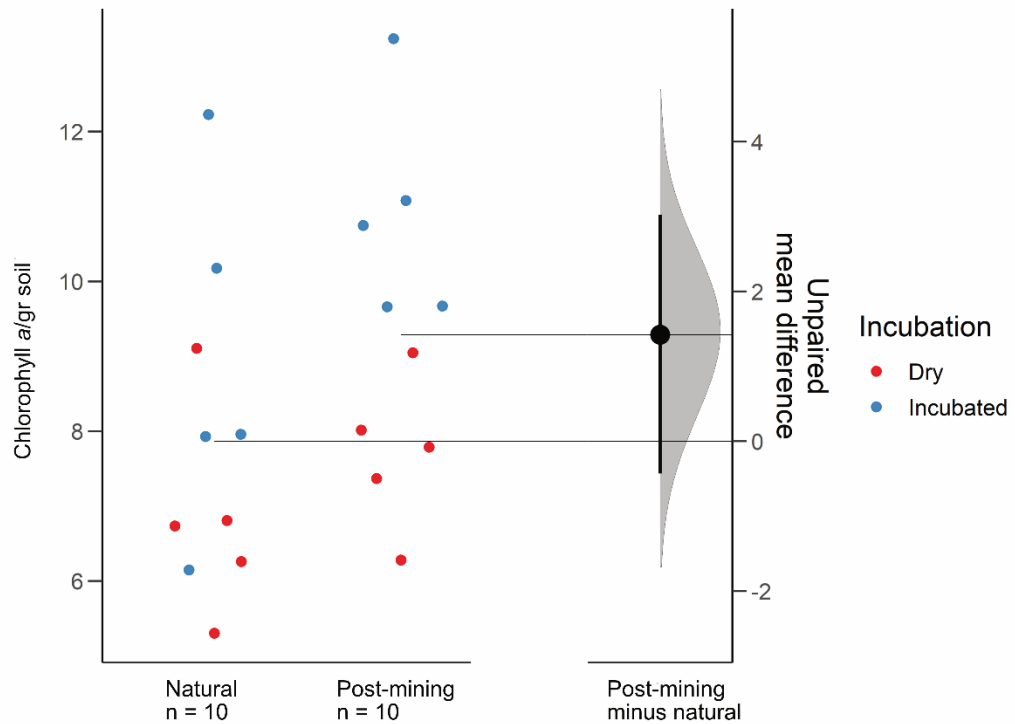261   variance between samples was smaller in the post-mining biocrusts (Fig. 3).

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Figure 3: Estimation plots of chlorophyll *a* concentrations. Dots represent the biocrust samples, and colors represent either dry or incubated soil.

Sequencing and differential abundance modeling

Sequencing resulted in 47,311 reads per sample on average (Table S2) and 10,275 ASVs

(Table S3). Following decontamination and filtering, 86% of the ASVs were removed (Table

S3). However, they accounted for only 16% of the total reads. Out of the remaining 1,404

ASVs, 1,266 in total were labeled and used for the differential abundance modeling (Table

S3). Each sequence in the labeled samples was compared to its corresponding negative

control, and the Log_2-fold change in labeled sequences was evaluated to determine whether

an ASV could be considered truly labeled (i.e., belonging to growing bacteria) based on the

significance threshold. One of the natural biocrust samples (no. 1; Fig. 4) displayed much

higher labeling than the other 4 samples (414 ASVs passed, out of a total of 1,093; Fig. 4).

16

276   Excluding sample 1, 38 out of 975 ASVs total passed the significance threshold for Log_2 fold

277   change. In post-mining samples, the number of labeled reads was more consistent among

278   the different samples (Fig. 4). 68 ASVs out of 874 ASVs total passed the threshold for Log_2 fold

279   change. Altogether, the number of labeled ASVs did not differ significantly between natural

280   and post-mining samples (natural sample 1 was excluded, natural community mean = 9.5,

281   post-mining community mean = 13.6, W= 9, p = 0.9).
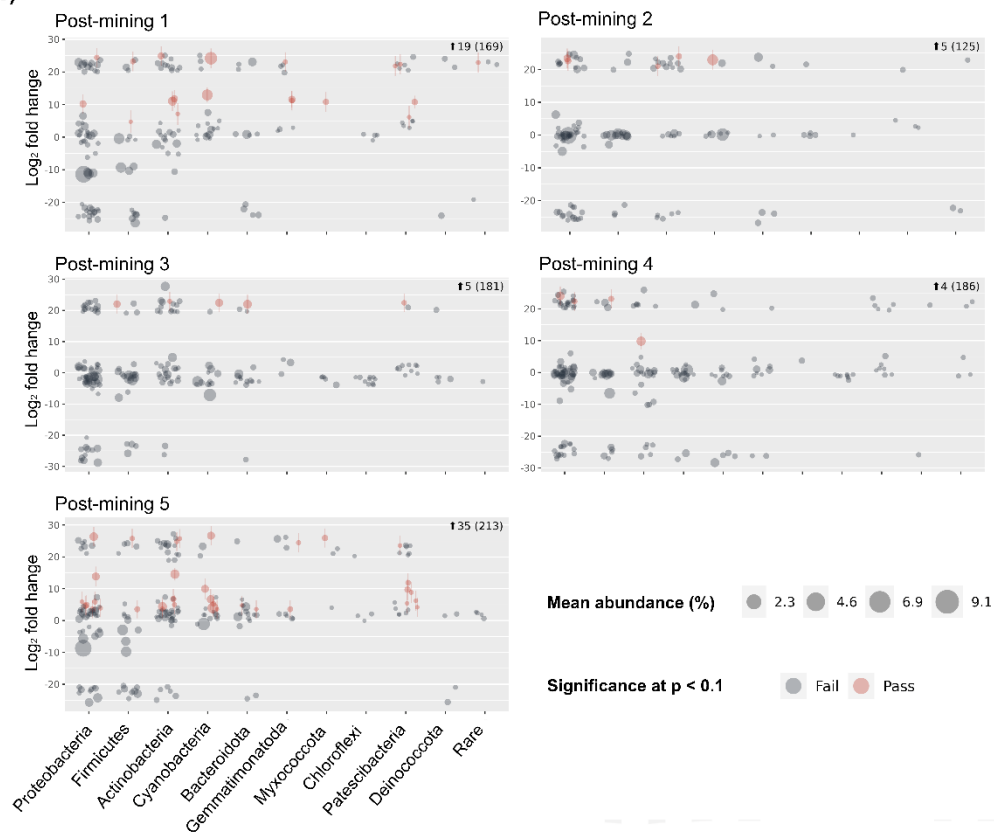
282

(a)

(b)

283
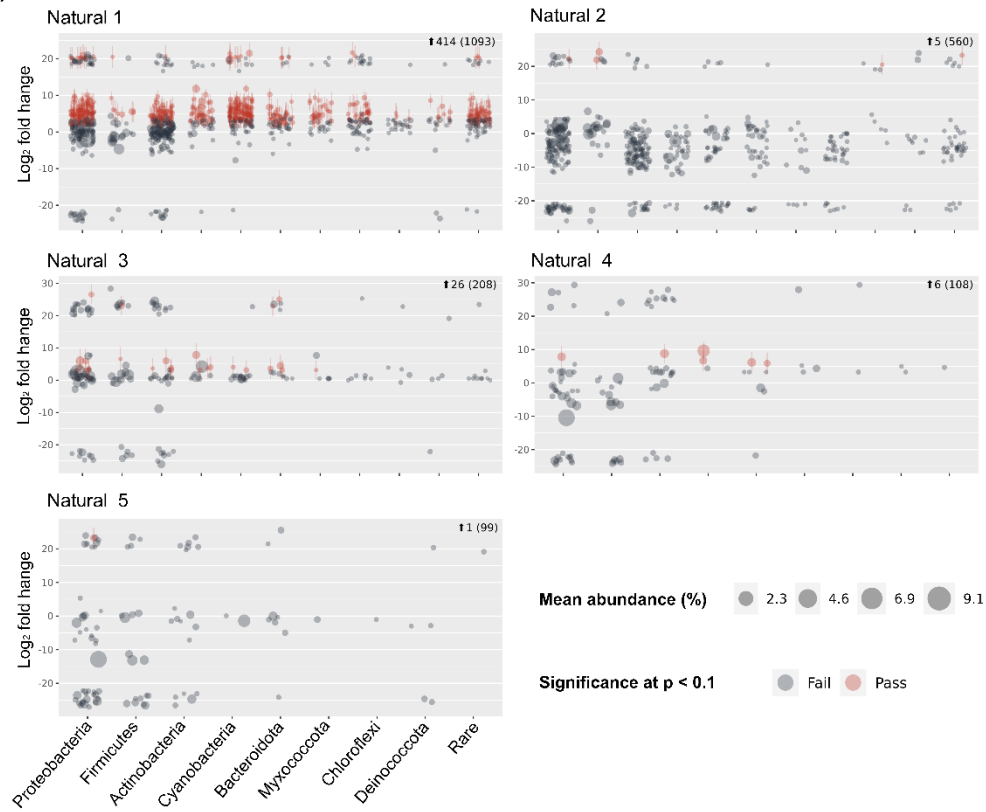
284　Figure 4: community composition of proliferated bacteria in natural (a) and post-mining (b)
285　biocrusts. Each graph represents a different sample. Red dots indicate labeled ASVs, and
286　grey dots indicate unlabeled ASVs, based on Deseq2 modeling.

287

Composition of the proliferated bacterial community

289　Figure 5(a) depicts a PCoA ordination based on weighted UniFrac metric showing that the

290　biocrust samples do not cluster according to plot type (natural sample number 1 was

291　excluded). Furthermore, the adonis test revealed no significant differences in community

292　composition (Weighted UniFrac ~ Plot type; $F = 1.23$, $R^2 = 0.15$, $p = 0.21$). A comparison of

293　phyla relative abundances reveals higher abundances of Cyanobacteria and Actinobacteria

294　in post-mining samples, and higher abundances of Firmicutes and Proteobacteria in natural

295　samples (Fig. 5(b)). However, none of the abundances differ significantly between groups

296　(Table S4). A Venn diagram of unique and overlapping sequences reveals that only 8 out of

297　88 labeled sequences appear both in natural and post-mining samples (Fig. S2). However,

298　phylogenetic trees depicting the different proliferated bacterial groups indicate that, for the

299　most part, sequences that appear in natural and post-mining biocrusts belong to the same

300　orders/classes. In the phylum Cyanobacteria, labeled sequences belonged to two classes,

301　and most sequences in both natural and post-mining samples belonged to the class

302　Cyanobacteria, with a slightly higher prevalence in the post-mining samples (Fig. S1). The

303　class Bacteroidia, belonging to the phylum Bacteroidota, had a similar prevalence for natural

304　and post-mining samples (Fig. S1). The trend was similar in the class Bacilli, belonging to the

305　phylum Firmicutes (Fig. S1). In the Alphaproteobacteria phylum, the orders

306　Rhodobacteriales, Rhizobiales and Sphingomonadales appeared in both natural and post-

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307   mining samples (Fig. S1). The phylum Gammaproteobacteria appeared only once in post-

308   mining samples but was more prevalent in natural samples (Fig. S1). The phylum

309   Actinobacteria was more prevalent in post-mining samples, yet the orders Frankiales,

310   Micrococcales and Propionibacteriales appeared in both natural and post-mining samples

311   (Fig. S1).

312

(a)
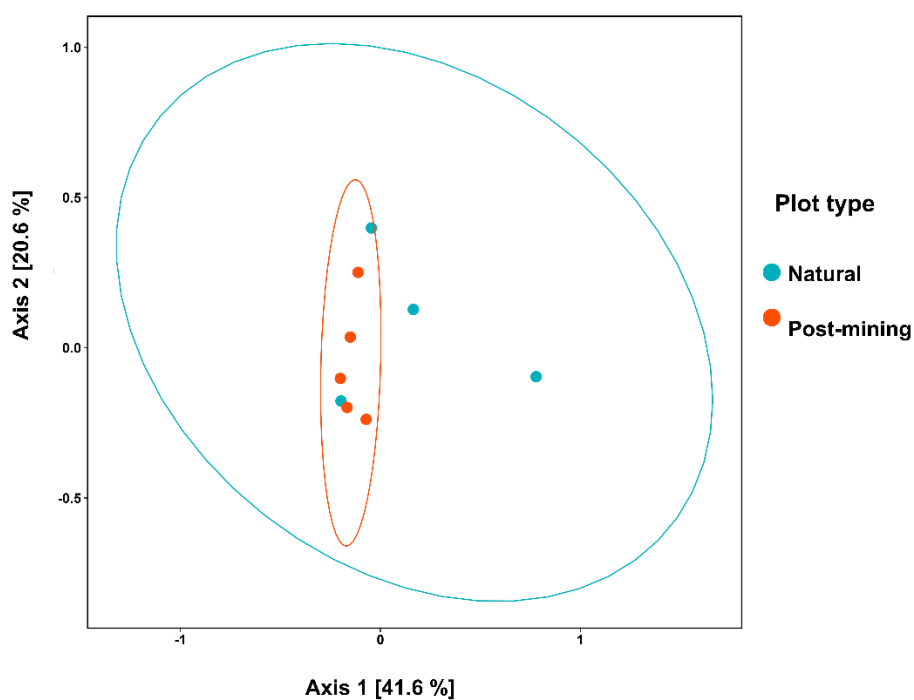
(b)
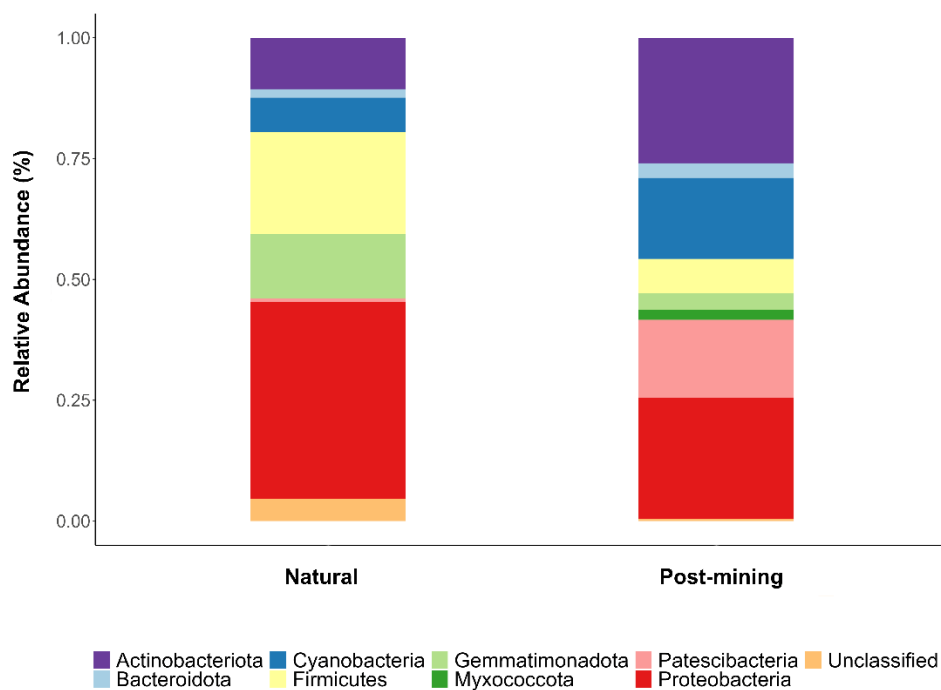
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314 Figure 5: Composition of proliferated community. Top figure (a) depicts a PCoA ordination of
315 community composition based on weighted UniFrac similarity metric. Blue dots are natural
316 samples and pink dots are post-mining samples. The ellipses represent 95% confidence
317 intervals; the bottom figure (b) depicts a bar plot of phyla relative abundance (%) in natural
318 and post-mining biocrusts.

Predictions of genomic functions

320 Abundances of 10 function categories (listed in Table S5) were compared between natural

321 and post-mining biocrust samples. Abundances were generally higher in post-mining

322 compared to natural biocrusts (Fig. 6; Table S5). Also, the variance between samples was

323 larger in post-mining biocrust (Fig. 6). However, the differences between plot types were not

324 statistically significant in any of the function categories (Table S5).
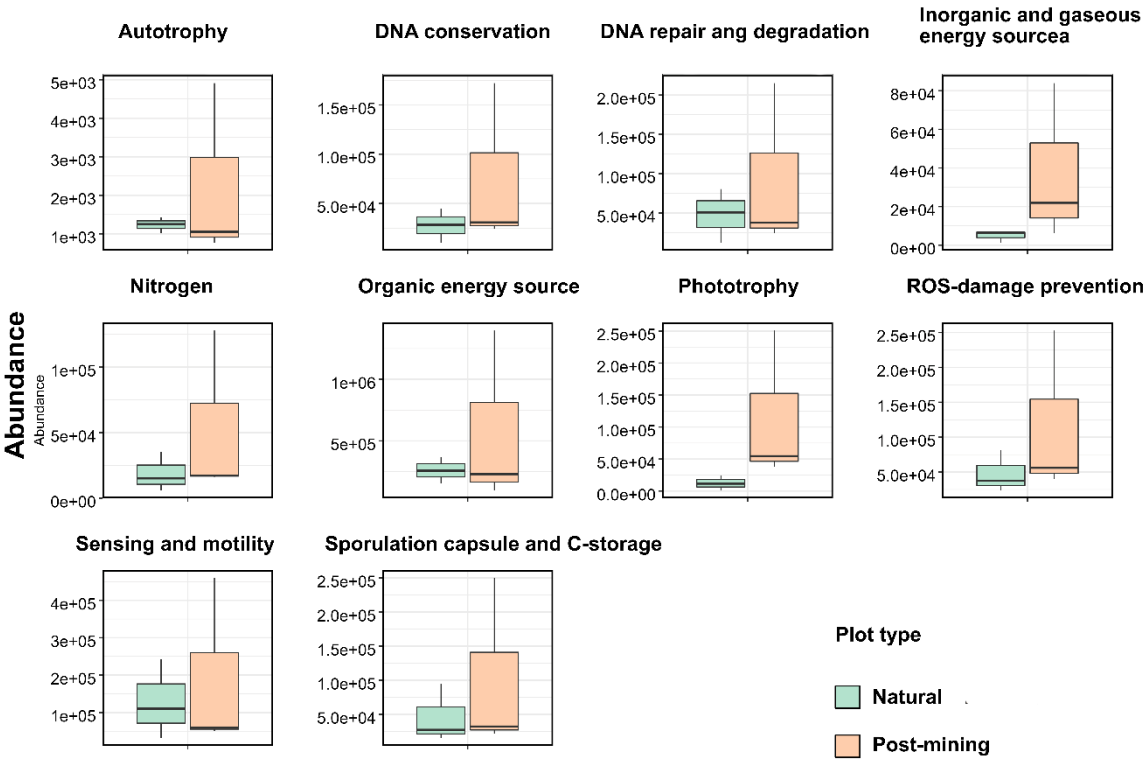
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327 Figure 6: boxplot of functional predictions. The Y axis represents functional gene
328 abundances. The line represents the median, and the whiskers represent the range.

329

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Discussion

In this study, we examined which groups of the biocrust bacterial communities grow after hydration using a SIP assay and differential abundance and diversity modelling. We hydrated and incubated the biocrusts for 96 hr expecting bacterial growth, yet very little growth was detected. Only 3.9% of the natural and 7.7% of the post-mining biocrusts' ASVs were identified as truly labeled by the stable isotope. Post-mining biocrusts had a slightly higher number of labeled ASVs compared to natural biocrusts but, the differences were not significant. Also, the composition and taxonomic identity of the growing communities did not significantly differ between natural and post-mining biocrusts.

Biocrust organisms are known to resume activity quickly following hydration, resuming functions such as damage repair, germination, nutrient cycling, and growth (Harel et al., 2004, Rajeev et al., 2013, Green and Proctor, 2016, Thomas et al., 2022). Hydration was also demonstrated to change the biocrust bacterial communities (Angel and Conrad, 2013; Štovícek and Gillor, 2022). In a $H_2^{18}O$ SIP assay using the Negev Desert biocrusts from arid and hyperarid regions, samples were hydrated and incubated for three weeks at maximum water holding capacity. Within days, changes in the labeled bacterial community composition and abundance were observed, indicating growth (Angel and Conrad, 2013). Similarly, biocrusts collected in the Negev Desert Highlands during a rain event and subsequent desiccation, demonstrated an increase in Cyanobacteria and decrease in Actinobacteria relative

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351   abundance (Baubin et al., 2021), implying selective activation of bacterial taxa in the

352   hydrated biocrust.

353

354   In other $H_2^{18}O$ SIP assays on soil bacterial communities, a quick response to re-wetting was

355   observed, and bacterial growth was evident within 24 to 72 hours of incubation (Blazewicz et

356   al., 2014, Aanderud et al., 2015). Thus, we assumed that hydration and incubation of

357   hyperarid biocrusts under favorable conditions would result in growth. Previous studies

358   examining the effect of a physical disturbance (repeated trampling) on biocrust

359   communities, revealed a decrease in the amount of extractable DNA, lower chlorophyll *a*,

360   and a decrease in biomass and cyanobacteria abundance (Kuske et al., 2012; Steven et al.,

361   2015; Chung et al., 2019). However, these studies investigated a localized disturbance

362   compared to mining disturbance, where the biocrust is completely removed over large

363   spatial scales. Moreover, the previous studies were conducted in environments that were

364   less extreme than the hyper-arid Zin Valley. Therefore, we expected the damage to the

365   biocrust in Zin post-mining sites to follow similar patterns but to be more conspicuous than

366   the previously reported disturbed biocrusts (Kuske et al., 2012; Steven et al., 2015; Chung et

367   al., 2019). Our previous report (Gabay et al., 2022) supported this notion; we demonstrated

368   differences in bacterial communities in natural and post-mining biocrusts, expecting these

369   differences to be reflected in the proliferating communities of these biocrusts.

370

371 Our previous survey (2017) also revealed significantly lower abundances of cyanobacteria

372 and chlorophyll *a* concentrations in post-mining biocrusts (Gabay et al., 2022). Out of the

373 four mining sites surveyed, Gov (which was restored in 2007) showed the most considerable

374 shift in biocrust community following mining. However, in the current study, we sampled

375 post-mining biocrusts at a different location in the Gov mining site (~500 m away from the

376 original plot) due to technical constraints. In the new location we found that the

377 photosynthetic potential of the biocrust in the post-mining plots did not differ from the

378 natural biocrust. These results highlight the importance of microenvironments in shaping

379 the functionality of biocrusts (Garcia-Pichel and Belnap, 1996). The similarities in active

380 communities and photosynthetic potential could be due to more developed biocrusts in the

381 new sampling locations compared to the previous ones.

382

383 Photosynthetic activity is usually observed in biocrusts within minutes to hours after

384 hydration by either dew or rain (Harel et al., 2004; Lange, 2003). In our experiment, we

385 hydrated the biocrusts to capacity and then incubated the samples for 96 hr. During the

386 incubation, most biocrust samples displayed some degree of greening, with more greening

387 in the natural biocrusts (Fig. 2). This indicates that the photosynthetic bacteria in the

388 biocrust were activated upon wetting. Yet, no significant differences were detected between

389 natural and post-mining biocrusts chlorophyll *a* concentrations (Fig. 3) or abundances of

390 photosynthesis related genes (Fig. 6). This implies that similar abundances of photosynthetic

391 bacteria were activated upon wetting in both biocrusts, yet they barely proliferated (Fig. 4).

392

393   The PICRUSt analysis revealed no significant differences in the abundances of genes within

394   any of the function categories examined (Fig. 6). In contrast, a previous study conducted in

395   the Negev Desert Highlands, examined active bacterial communities during a rain and

396   subsequent drying (Baubin et al., 2021). The results indicated an increase in genes related to

397   photosynthesis, light, and sensing following the rain, while the other function categories did

398   not vary significantly. We note that the identity and abundances of the functional genes in

399   the dry biocrusts detected by Baubin et al., (2021) and here (Fig. 6) are similar. Therefore, we

400   propose that the similarity between the post-mining and natural biocrust communities (Fig.

401   5) reflect similar functional potential (Fig. 6). However, low abundance of active ASVs were

402   used to infer the abundances of functional genes, and large variance between samples in

403   post-mining biocrusts could mask significant differences (Table S5).

404

405   The growth patterns of biocrust organisms are affected by local environmental conditions

406   (Kim and Or, 2017). Zin mining fields are in a hyperarid region, where extreme heat events are

407   frequent in the summer, and rains are scarce and unpredicted. Moreover, in recent years

408   there were only two or three rain events during each rainy season (Zin factory meteorological

409   data). Hydration is the most important factor affecting biocrust organisms' growth rate while

410   long desiccation periods negatively affect growth (Zaady et al., 2016). Also, salinity levels in

411   Zin valley soils are high (Table 1; Levi et al., 2021) imposing further stress on the biocrust

412   community. It is known that in high stress environments, biocrust microorganisms increase

413   nutrient availability and accumulation by resuming carbon and nitrogen fixation upon

414   hydration (Aranibar, 2022). The resulting organic carbon and nitrogen compounds can be

415   consumed during the long desiccation periods (Belnap, 2003; Colesie et al., 2014). One study

416   examining microbial nitrogen transformations in biocrusts collected from Succulent Karoo

417   biome in Namibia and South Africa showed that following wetting, nitrogen cycling genes

418   are expressed in biocrust organisms (Maier et al., 2022). Another study examining biocrust

419   samples taken from the Moab Desert in Utah demonstrated a pulse of metabolite release

420   following controlled wetting (Swenson et al., 2018). Based on these reports, and due to the

421   extreme conditions in Zin Valley (Levi et al., 2021), we suggest that hyperarid biocrust

422   communities prioritize functions such as metabolite production, nutrient cycling and

423   preparation for desiccation over growth.

424

425   Natural recovery of biocrusts has been long debated, and is generally estimated to be a slow

426   process, especially in arid sites that experience very short activity periods for biocrust

427   development, such as the hyperarid Zin mining site (Kidron et al., 2020; Weber et al., 2016).

428   The time and trajectory of recovery depend on many factors relating to local climatic

429   conditions and site properties (Belnap and Lange, 2003). One such factor that greatly affects

430   establishment and restoration of biocrusts is the proximity, availability, and dispersal timing

431   of biocrust propagules (Bowker, 2007; Walker et al., 2007). Thus, the low proliferation rates

432   we observed, particularly in post-mining biocrusts, suggest that restoration processes might

433   be much slower than previously estimated. The topsoil from a stockpile is used to cover the

434   mining pits. This soil may not contain a biocrust seed bank because it was probably

27

435 destroyed and buried during the mining processes. Further increase in bacterial biomass

436 might highly depend on the dispersal of biocrust propagules to the site from adjacent

437 natural areas by wind or water. Our results further emphasize the need for active restoration

438 measures in the Zin mines. Such measures include soil inoculation with local cyanobacterial

439 propagules (Acea, 2003; Wang et al., 2009; Zhao et al., 2016; Velasco Ayuso et al., 2017) and

440 increased hydration (Morillas and Gallardo, 2015; Zhang et al., 2018) which were effective in

441 enhancing biocrust establishment and recovery following disturbances (Antoninka et al.,

442 2020).

443

Conclusions

444

445 Low proliferation of biocrust bacteria was detected after wetting suggesting prolonged

446 recovery times of biocrusts following major mechanical disturbances, such as mining.

447 Furthermore, recovery largely depends on site conditions and the ability of biocrust

448 propagules to disperse to post-mining sites. Further research is needed to confirm our

449 hypothesis of low proliferation and thus restoration rates in hyper-arid biocrust bacterial

450 communities.

451

Code and data availability

All data produced in this study and scripts used for community analysis, functional predictions and chlorophyll *a* estimation plot are available at

https://github.com/TaliaJoanne/SIP-experiment-Zin-mines.

The raw 16S sequences are available in the NCBI database under Bioproject ID PRJNA906925, accession numbers SAMN31937891 – SAMN31937900.

Author's contributions

Talia Gabay: Conception, Sample Collection, Incubation, Chlorophyll *a* and DNA extraction, Statistical Analysis, Visualization, Writing-Original Draft Preparation, Writing-Reviewing and Editing. Eva Petrova: DNA-SIP assay, DNA quantification and PCR amplification. Osnat Gillor and Yaron Ziv: Conception, Writing-Reviewing and Editing, Investigation, Supervision. Roey Angel: Conception, Statistical Analysis, Visualization, Supervision, Writing-Reviewing and Editing.

All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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