EFFECT OF BIOCRUSTS ON BACTERIAL COMMUNITY COMPOSITION AT DIFFERENT SOIL DEPTHS IN MEDITERRANEAN SEMI-ARID ECOSYSTEMS.

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ABSTRACT

This study analyzed the influence of biocrusts on the chemical properties and bacterial diversity and community composition in the underlying soils along a depth gradient (the biocrust (C1), middle (S2) and deep (S3) soil layers) in two semi-arid Mediterranean ecosystems. Organic carbon, pH, electric conductivity and calcium carbonate content were estimated by wet oxidation, potentiometrically (pHmeter), with a conductivity-meter and volumetrically with a Bernard calcimeter, respectively. Bacterial diversity and community composition were estimated by 16S rRNA gene high-throughput amplicon sequencing. Chemical properties in C1 were significantly different from the other soil layers, showing higher organic carbon content and lower pH ($p<0.05$). The relative abundance of several bacterial taxa, such as *Bryocella, Methylobacterium, Segitebacter and Actinomycetospora* showed significant positive correlations with organic carbon ($r = 0.53$ to 0.75) and negative with pH ($r = -0.72$ to -0.84), and were also highly correlated with each other ($p<0.01$), suggesting a bacterial co-occurrence pattern associated with the biocrust. On the contrary, other bacterial taxa, such as *Euzebyaceae, Truepera, Alphaproteobacteria* and *Caldinilaceae*, showed positive correlations with electrical conductivity and calcium carbonate, and were also correlated

with each other ($p<0.01$), in a second type of co-occurrence pattern associated with bare soil. The C1 and S2 layers had several taxa in common, while S3 layers had taxa common to bare soil, suggesting that the effect of biocrusts was limited to the first centimeters of soil and progressively decreased in depth. Bacterial diversity was lower in C1 than in the underlying layers and increased progressively from biocrust to deeper soil layers. The results suggest that the diversity and composition of soil microbial communities in biologically crusted sites in Mediterranean semi-arid environments are mainly controlled by chemical properties which in turn are modified by the biocrust along a depth gradient.

Keywords: Soil bacterial co-occurrence patterns, high-throughput sequencing, pH, salinity, soil organic carbon, calcium carbonate content.

1, INTRODUCTION

About 41% of the Earth's land surface is drylands, which are exposed to expanding degradation and desertification as a consequence of global climate change and the exponential growth of the human population (Maestre et al., 2012). According to Huang et al. (2016), drylands will increase 23% globally by the end of the 21st century. Biological soil crusts (hereinafter "biocrusts") are a highly representative dryland community of organisms which live in the upper millimeters of the soil surface, occupying up to 70% of the surface in arid and semi-arid areas around the world (Belnap, 2003; Belnap et al., 2001). Biocrusts are made up of different types of organisms like mosses, soil lichens, green algae, fungi, cyanobacteria and other bacteria (Belnap et al., 2016), closely bound to soil particles (Belnap and Gardner, 1993). These highly specialized organisms can survive and develop under the extreme conditions of arid environments (Coe et al., 2014) where vascular vegetation cannot thrive (Bowker et al., 2016). Their role in arid and semi-arid ecosystems is essential (Belnap and Lange, 2001; Xiao et al., 2014), affecting soil functionality (Bastida et al., 2014).

Biocrusts influence the physical and chemical properties of the underlying soil by reducing erosion (Guo et al., 2007; Maestre et al., 2005) and improving primary productivity, stability and fertility of soil for plants (Ferrenberg et al., 2017). Soil colonized by biocrusts has better nutrient storage (C, N and P) than bare soil (Delgado-Baquerizo et al., 2015), as they contribute to increasing aggregate stability and water content at 33 and 1500 kPa in the soil surface (Chamizo et al., 2012a), which is also less saline (Kakeh et al., 2018) than bare soil. Biocrusts influence soil hydrological cycles (Warren, 2001; Whitney et al., 2017), screen the soil from high solar radiation (Miralles et al., 2012) and improve cation exchange capacity and soil porosity (Belnap, 2006). Moreover, biocrusts , increase soil enzymatic activities involved in carbon (β-glucosidases, invertases, cellulases), nitrogen (protease BAA, protease-cellulase, nitrate reductase, urease) and phosphorus cycles (phosphodiesterase, phosphatase) (Castillo-Monroy et al., 2011; Miralles et al., 2012a), and are therefore essential to biogeochemical cycles (Bastida et al., 2014; Elbert et al., 2012; García-Pichel et al., 2003; Miralles et al., 2012a). Biocrusts also secrete secondary acid metabolites (e.g., lecanoric acid and usnic acid; Jorge-Villar et al., 2011), enriching labile C in the underlying soil (Bastida et al., 2014), carbohydrates and polyphenols (Miralles et al., 2013), and leading, in turn, to an increase in the size of the microbial communities in them (Miralles et al., 2012b; Bastida et al., 2014).

Chemical soil properties, such as pH, soil organic carbon and salinity have been shown to have an essential role in the diversity and composition of soil microbial communities (Canfora et al., 2014; Fierer et al., 2012, 2007; Fierer and Jackson, 2006; Goldfarb et al., 2011; Lauber et al., 2009; Upchurch et al., 2008). Thus, biocrusts could indirectly influence the microbial communities in the underlying soil through their effect on chemical soil properties. Nevertheless, the potentially important effect of biocrusts on the diversity and composition of the microbial communities in the underlying soil has scarcely been explored. Biocrust influence on chemical and biochemical soil properties is known to be limited to the first millimeters of the colonized substrate, with gradual decrease in total organic carbon content, total nitrogen and hydrolytic enzymes, and increase in soil pH from biocrust to deeper soil layers (Kakeh et al., 2018; Miralles et al., 2012a). Kakeh et al. (2018) also found vertical variation in other chemical soil properties, such as the carbonate content, which decreased with depth under the biocrust. However, despite their

74 influence on chemical soil properties, which could, in turn, modify the microbial communities of the soil,
75 there are still few studies analyzing the effect of biocrusts on the bacterial composition of the underlying
76 soil in a vertical depth gradient. High-throughput sequencing and metagenomic techniques enable detailed
77 study of microbial communities associated with biocrusts and changes in these communities from biocrusts
78 to deeper soil layers. This could greatly improve our understanding of the ecological processes in the
79 biologically crusted arid and semi-arid ecosystems today so seriously threatened.
80 The aim of this study was to analyze the influence of biocrusts on chemical soil properties, soil microbial
81 community composition and diversity, and their relationships in a vertical soil depth gradient underlying
82 the biocrusts, comparing two different areas with similar environmental conditions, but in different stages
83 of degradation in a Mediterranean semi-arid ecosystem. We hypothesized that biocrusts influence the
84 chemical properties of the underlying soil substrate, and thereby, indirectly the bacterial communities
85 associated with the local conditions generated by them with respect to bare soils. In turn, the effect of
86 biocrusts on the chemical soil properties and soil microbial communities is diluted in deeper soil layers
87 under biocrusts.
88
89 **2. MATERIALS AND METHODS**
90 **2.1 Study areas**
91 Two study areas, Amoladeras (AMO) (36º50'5''N, 2º15'1''W) and Balsa Blanca (BB) (36º56'26''N,
92 2º01'58.8''W), both located in the Cabo de Gata-Níjar Natural Park (Almería, SE Spain) were selected as
93 representative of Mediterranean semi-arid environments. Both study areas are coastal-steppe ecosystems
94 widely distributed in a piedmont area with a landscape of alluvial fans (glacis) formed by flat open areas
95 with a gentle slope (2-6%) in the Cabo de Gata Natural Park. In both areas, the climate is semi-arid with
96 random rainfall patterns and mean annual precipitation of about 240 mm falling mainly in winter. The high
97 interannual variation is mainly recorded during the spring and autumn. The mean annual temperature is
98 about 18ºC, with long warm, dry summers and annual evapotranspiration of 1390 mm (Aranda and

99 Oyonarte, 2005; Rey et al., 2011). The predominant soils are shallow Calcaric Lithic Leptosols (WRB,
100 2006) with lithic contact (a very powerful petrocalcic horizon) no more than 25 cm deep, and a silty-to-
101 sandy loam texture, with a predominantly sandy granulometric fraction (58.56% at AMO and 58.56% at
102 BB), followed by silt (31.03% at AMO and 31.73% at BB) and clay (10.51% at AMO and 9.7% at BB)
103 (Miralles et al., 2012d). The vegetation is grassland/shrub with predominance of *Stipa tenacissima* L., but
104 with a large number of other species, such as *Chamaerops humilis* L., *Rhamnus lycioides* L., *Asparagus*
105 *horridus* L., *Olea europaea* L. var. plants sylvestris Brot., *Pistacia lentiscus* L. and *Rubia peregrina* L. In
106 general, the dispersed vegetation cover (between 18 and 65%; Rey et al. 2011) is heterogeneous, forming
107 complex vegetation patterns with open areas between plants. These are often colonized by biocrusts, mainly
108 lichens, such as *Diploschistes diacapsis* (Ach.) Lumbsch, cyanobacteria and moss, or occupied by rock
109 fragments, regolith outcrops or bare soil (Maestre and Cortina, 2002; Rey et al., 2011). At AMO perennial
110 plants cover about 20% of the surface and annual plants about 10%, with biocrusts in the open spaces
111 between plants covering about 27% of the soil surface, and abundant stones (around 13%) and rocky
112 outcrops (around 14%) (Miralles et al. 2012d). At BB, perennials cover about 45% of the soil surface and
113 annuals about 5%. Biocrusts cover about 25% of the surface surrounding plants, and stones about 8%
114 (Miralles et al., 2012d). At both AMO and BB, *Diploschistes diacapsis* is one of the most abundant and
115 representative lichens colonizing the bare space on the soil substrate (Chamizo et al., 2012b; Ladrón de
116 Guevara et al., 2015; Miralles et al., 2012d).
117
118 **2.2 Experimental design and sampling**

119 These two study areas were selected because: (i) They are representative of Mediterranean semi-arid
120 ecosystems, (ii) biocrusts are very well represented, covering a high percentage of the total surface of the
121 soil in the clearings between plants at both sites, (iii) biocrusts dominated by the lichen *D. diacapsis* are
122 very abundant at both AMO and BB, and (iv) both sites have similar geology, topography, vegetation,
123 climate and soil types. However, AMO is more degraded than BB (López-Ballesteros et al., 2018; Rey et

al., 2011), and has less plant cover, rocky outcrops on the calcareous crust surface and more gravel and biocrust cover (Rey et al., 2011). The soil was also shallower at AMO than at BB (Rey et al., 2011). Soil at AMO and BB have basic pH, but the calcium carbonate content is higher at AMO than at BB and, on the contrary, the soil humic acid content is higher at BB than at AMO (Miralles et al., 2012d). Worse degradation at AMO than BB may be due to its proximity to urban populations and human economic activity until the late 50s. Land was used for wood, fiber and extensive agriculture, and later abandoned, resulting in the area's degradation, and leaving only this vestige of its historical uses (López-Ballesteros et al., 2018).

Sampling was done in June 2016 at both the AMO and BB sites, following a factorial design in which three composite samples (a mixture of seven subsamples) were taken from the following layers: i) Biocrust (C1) dominated by the lichen *D. diacapsis* was extracted with a small spatula from the upper few centimeters of the surface (over 0.5 cm) in large clearings (over 2×2 m with no significant vegetation); ii) Middle soil layers (S2): samples were taken from right below where the biocrusts had been removed down to a depth of three centimeters; iii) Deep soil layers (S3): samples were collected from a depth of 3 to 10-15 cm along the same profile; iv) Bare soil (BS) without biocrusts was collected from the surface to a depth of 10-15 cm in areas close to the crusted clearings previously sampled. Then, a total of 24 samples (12 samples at AMO and 12 at BB) were taken to the laboratory in isothermal bags. Samples, including biocrusts, were homogenized and sieved through a 2-mm screen (C1). A part of these samples was stored in polythene containers at -80ºC for later DNA extraction and next-generation sequencing (NGS) analysis.

2.3 Chemical soil properties

Soil organic carbon (OC) was determined by the Walkey and Black (1934) method (modified by Mingorance et al., 2007). Soil pH and electrical conductivity (EC) were measured in a 1:1 aqueous suspension using a pHmeter (Crison BASIC 20 Crison, Barcelona, Spain) for soil pH measurements and a digital conductivity meter (Crison 522, Barcelona, Spain) for EC measurements. Soil calcium carbonate content was measured with a Bernard calcimeter (Loeppert and Suarez, 1996).

151

152 **2.4 DNA extraction, high-throughput sequencing and bioinformatics analysis**

153 DNA was extracted from 0.5 g of soil using the DNeasy PowerSoil kit (QIAGEN-MoBio Laboratories,
154 Carlsbad CA) following the manufacturer's protocol (Mo-Bio, 2016). Blank controls were included to
155 check for any contamination during DNA extraction. DNA concentration and purity were estimated
156 spectroscopically using Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA).

157 A Miseq sequencing platform (Illumina) with paired reads and V3 kit chemistry (300 + 300 cycles) was
158 used for amplicon sequencing of 16S rRNA gene libraries (V4-V5 domains). PCR negative controls were
159 used (1 for every 96-well plate = 4 per MiSeq run). PCR were checked for contamination (no bands present)
160 and then sequenced on the MiSeq to be sure that no substantial reads were coming through on this barcode
161 combination. Multiplexing used a dual indexing strategy with eight Nextera XTv2 kit base pair indexes.
162 Primers and library construction were as previously described (Sánchez-Marañón et al., 2017).
163 Bioinformatic analyses were carried out using Mothur v.1.39.5 (Schloss et al., 2009). Identification and
164 elimination of chimeras was performed with CHIMERA-UCHIME.

165 Bacterial community diversity was assessed by number of observed Operational Taxonomic Units
166 (OTUs) (Sobs) and the Chao1, Shannon and Pielou (J') indices. All indices were calculated with the number
167 of OTUs defined at an evolutionary distance of 0.03 (97% similarity of 16S rRNA sequences) with the
168 equations described and implemented in Mothur.

169 Sequence taxonomic classification was done using a Bayesian classifier (Wang et al., 2007) with Silva
170 v.123 (https://www.arb-silva.de/no_cache/download/archive/release_123/) as the reference database.
171 Bacterial abundance was expressed as a percentage of the total number of sequences in each sample. Taxa
172 with total abundance over 0.1% were selected for statistical analysis. Sequences classified as Archaea,
173 Mitochondria or Chloroplast were removed from the respective datasets.

174

175 **2.5 Statistical analysis**

176 Samples were grouped by a two-factor (fixed-effect) design: (i) Study sites (Amoladeras, AMO, and
177 Balsa Blanca, BB) and (ii) sampling layer (biocrusts dominated by *Diploschistes Diacapsis* lichen (C1),
178 soil under the biocrust at two different depths (middle layer, S2, and deep layer, S3), and bare soil (BS).

179 Differences in chemical parameters, relative abundance of soil bacterial taxa and diversity indices
180 between groups of samples were assessed using two-way Permutational Multivariate Analysis of Variance
181 (PERMANOVA) and the above mentioned two-factors and their interactions (Anderson et al. 2008). Then
182 pairwise comparisons were made using a multivariate analogue of the *t* test, and finding the probability
183 levels by permutation (Eldridge et al., 2016). Partial pairwise tests were performed on subgroups of samples
184 to compare each layer to the same one at the other site or different layers at the same site. PERMANOVA
185 uses geometric partitioning of multivariate variation in the space of a chosen dissimilarity measure by
186 means of permutation tests to find the P values. The similarity matrix of the samples was constructed using
187 Bray Curtis distances for multivariate PERMANOVA. Moreover, two-way Permutational Univariate
188 Analysis of Variance (PERANOVA) was also performed using Euclidean distances to check the effects of
189 the two factors on each individual variable. Both statistical analyses are nonparametric tests that retain the
190 advantages of the classical partitioning methods, such as ANOVA, without the parametric assumptions
191 (Anderson, 2001). Canonical Analysis of Principal Coordinates (CAP) was performed to group by biocrusts
192 and soil layers and also by site according to the bacterial communities. The Monte Carlo test was used in
193 reduced models assigning unrestricted permutations to assess the statistical significance of the first and
194 second canonical axes (Zhang et al., 2011).

195 Pearson's correlation coefficients (r) were used to assess the relationship between chemical soil
196 properties and soil bacteria abundance and diversity at the lowest classification level (subgroup-to-genus).
197 The soil bacteria with highest significant correlations at $p < 0.01$ and positive correlation coefficients ($r > 0.8$)
198 were grouped together in co-occurrence patterns to simplify the correlations found for the different
199 variables.

Network analysis was used to show graphically the relationships between the variables found from the Pearson's correlations. A network study based on modularity methods (Blondel et al., 2008) was carried out for this purpose using the Force Atlas 2 algorithm (Jacomy et al., 2014).

The statistical package PRIMER-E + PERMANOVA software (PRIMER-E Ltd., Plymouth Marine Laboratory, UK) for Windows was used for PERMANOVA, PERANOVA and CAP analysis. Correlations were performed with the R software (http://www.R-project.org) and "ggplot2" package (Viechtbauer, 2010; Wickham, 2009). Gephi 0.9.2 software was used to obtain the network analysis (Bastian et al., 2009).

3. RESULTS

3.1 Chemical properties in biocrusts, underlying soil and bare soil.

The OC content was comparatively higher in biocrusts (7.29% at AMO and 5.95% at BB) than in the rest of the soil layers and progressively decreased downward to deeper soil layers at both AMO and BB (Table 1). pH was acidic in C1 (5.94 at AMO and 5.54 at BB), but basic in the soil layers at both study sites. $CaCO_3$ content was also lower in C1 than in the soil layers at both AMO and BB. EC was generally higher in all layers at AMO than at BB, and highest in AMO BS (Table 1).

PERMANOVA analysis comparing the chemical properties showed significant differences in both factors (site and layers), but not in their interaction. Nevertheless, significant differences were observed in the partial Pairwise Test in S2 layer chemical properties at AMO and BB (Supplementary Table 1). In particular, the two-way univariate PERANOVA showed that soil carbonate content was significantly higher in S2 at AMO than at BB, however, there were no significant differences in chemical properties in the rest of the layers between AMO and BB (Supplementary Table 2).

The partial Pairwise Test comparing chemical properties between layers at each study site separately showed that the chemical properties in the biocrust (C1) were significantly different from the soil layers (S2, S3 and BS) at AMO and BB (Supplementary Table 1). The univariate PERANOVA analysis showed that C1 layers had a significantly higher OC and significantly lower pH than S2, S3 and BS layers both at

AMO and BB. The soil carbonate content was significantly lower in C1 than in S2 and BS soil layers at AMO, but it was only significantly lower in C1 than in S3 at BB (Supplementary Table 2).

3.2 Diversity of the bacterial communities in biocrusts, underlying soils and bare soils.

Sequencing the 16S rRNA gene (V4-V5) amplicons with the Illumina MiSeq system resulted in a total of 768882 sequences after eliminating those nonaligned and chimeras with an average length of 370 base pairs (bp). High-quality sequences ranged from 17721 to 50711, using the lowest value to calculate alpha-diversity parameters. The diversity indices (Sobs, Chao1, Shannon and Pielou) were comparatively lower in biocrusts (C1) than in the soil layers (S2, S3 and BS) and progressively increased with depth from upper to deeper soil layers at both AMO and BB (Table 2). These indices were also comparatively higher in soils underlying biocrusts (S2 and S3 layers) than in bare soil (BS) at both AMO and BB (Table 2). Nevertheless, PERMANOVA analysis diversity indices did not show significant differences between AMO and BB (Supplementary Table 1). The univariate PERANOVA analysis did not show any significant differences between AMO and BB in the Chao1, Shannon and Pielou indices in the partial Pairwise Test layer-by-layer comparison (C1, S2, S3 and BS), although the Sobs was significantly higher in the S2 layer at AMO than at BB (Supplementary Table 2).

The partial Pairwise Test comparing the diversity indices between the layers at each study site separately, showed significant differences only in the Sobs and the Chao1 index between C1 and soil layers at both AMO and BB, even though the Chao1 index did not show significant differences between C1 and BS at AMO (Supplementary Table 2). The Shannon index showed significant differences between C1 and the S3 and BS soil layers, but C1 was not significantly different from S2 at AMO. At BB, C1 was not significantly different from the soil layers, while S3 was significantly different from S2 and BS. The Pielou index did not show any significant differences between the biocrust and soil layers at AMO, but did show significant differences between S3 and BS at BB (Supplementary Table 2).

3.3 Bacterial community composition in biocrusts, underlying soil and bare soil.

The taxonomic analysis detected a total of 16 dominant phyla, of which *Acidobacteria, Proteobacteria, Bacteroidetes, Planctomycetes* and *Actinobacteria* dominated in both study areas, although in different proportions (Supplementary Figure 1). The most abundant phylum in C1 was *Bacteroidetes*, while in the S2, S3 and BS soil layers it was *Acidobacteria* (Supplementary Figure 1) at both AMO and BB. The Cyanobacteria phylum decreased progressively with depth, and was much less abundant in S2 than in C1 and practically non-existent in S3. The relative abundance of this phylum was very low in BS layers at AMO and BB (Supplementary Figure 1).

The taxonomic analysis showed a total of 150 taxa at the lowest classification level (subgroup to genus) with a relative abundance over 0.1%. The PERMANOVA statistical analysis showed significant differences between bacterial taxa at the lowest classification level (subgroup-to-genus) in the two factors: site (AMO and BB) and layer (C1, S2, S3 and BS) (Supplementary Table 1).

The CAP analysis confirmed clear differentiation of the two study sites (AMO and BB) by soil bacterial genus composition. Axes 1 and 2 (CAP1, CAP2) distinguished AMO (lower left cluster) and BB (upper right cluster) (Figure 1a). Although boxplots also showed that most of the bacterial taxa were found at both sites, some bacterial communities were relatively more abundant at one site than the other. Thus, *Pirellulaceae unclassified, Actinobacteria uncultured, Planctomycetales uncultured* and *Rizhobiales uncultured* were more abundant at AMO, and on the contrary, *Isosphaeraceae unclassified, Solibrubacteraceae unclassified, Solibrubacter* and *Tychonema CCAP 1459.11B* were more abundant at BB (Figure 2). Moreover, the CAP analysis showed that all samples were clearly clustered by biocrust and soil layer (Figure 1b). Soil bacterial composition was a highly significant explanatory feature, separating samples along the first and second axes (Figure 1b). Cross-validation of the CAP method was up to 95.83% accurate for the layer factor (Supplementary Table 3). The results also showed that biocrusts (C1 samples, upper right cluster) and soil immediately underlying biocrusts (S2 samples; lower right cluster) were

different from the deeper soil layers and bare soil (S3 and BS samples respectively, upper left cluster). On the contrary, S3 and BS samples were similar to each other.

The boxplots showed also that some soil microbial communities were more abundant or exclusive according to the different biocrusts and soils layers. Thus, the bacterial taxa *Methylobacterium, Bryocella, Proteobacteria-1174-901-12, Oxyphotobacteria, Acidobacteriaceae subgroup 1 unclasiffied, Geodermatophilaceae unclassified, Actinomycetospora, Acetobacteraceae uncultured* and *Acidipila* were exclusive in C1 (Figure 3). However, *Pontibacter, Euzebyaceae uncultured, Truepera, Planctomycetales unclassified* and *Rhodothermaceae uncultured* were exclusive in BS and practically absent in the rest of layers (C1, S2 and S3) (Figure 3), while other bacteria, such as *Alphaproteobacteria unclassified*, *Caldilineaceae uncultured, Longimicrobiaceae* and *Gammaproteobacteria unclassified* were more abundant in BS (Figure 3). The S2 layers had a larger number of bacterial taxa, such as *Beijerinckiaceae, Bryobacter, Rhodocytophaga, FBP ge, Ferruginibacter, Microvirga* and *Nostocales* in common with C1 (Supplementary Figure 2). But more bacterial taxa in S3, such as *0319.7L14, Gaiellales, Rhodoplanes, Gaiella, S085, Rokubacteria sp., MB.A2.108, Xanthobacteraceae and Actinobacteria sp.* were in common with BS (Supplementary Figure 3). Some bacterial taxa gradually decreased from C1 to the deepest soil layers (C1> S2> S3 ≥ BS) (Supplementary Figure 2), while others showed the opposite pattern, gradually increasing from C1 to the deepest soil layers (C1 < S2 <S3 ≤ BS) (Supplementary Figure 3).

3.4 Relationship between bacterial taxa and chemical soil properties in biocrusts and soil layers.

A large number of bacterial taxa were significantly correlated ($p < 0.05$) with chemical soil parameters in the samples from both AMO and BB (Supplementary Tables 4 and 5). In general, exclusive soil bacteria in C1 (Figure 3) and soil bacteria more abundant in C1 and S2 (Supplementary Figure 2) had significant positive correlations with OC and negative with soil pH and calcium carbonate content. On the contrary, the soil bacteria more abundant in BS (Figure 3) showed significant positive correlations with EC and calcium carbonate content, while the soil bacteria more abundant in S3 and BS (Supplementary Figure 3)

were negatively correlated with OC and positively with pH and calcium carbonate content. Table 3 summarizes the correlations between chemical soil properties and the most dominant bacterial taxa.

The soil bacterial genera had a large number of significant correlations with each other. In this group of correlations between soil bacterial genera, seven co-occurrence patterns were found by selecting those bacteria with correlations over 0.8, and selecting the bacterial taxa with the highest correlation ($r > 0.98$) in each group of bacteria as representative of each co-occurrence pattern (Supplementary Table 6). Two groups of co-occurrence patterns were clearly differentiated in this way: i) the first one (Co-occurrence Group 1), led by the bacteria *Acidobacteraceae Subgroup 1 unclassified, Pseudonocardia, Caldilineacea uncultured, Actynomycespora* and *Acidipila* (Figure 4), grouped exclusive or majority soil bacteria in C1, and ii) the second group of co-occurrence patterns (Co-occurrence Group 2), led by the *Rhodotermaceae uncultured, Euzebyaceae uncultured* and *Caldilineacea uncultured* bacteria (Figure 4), grouped exclusive or majority soil bacteria in BS. The network graphic in the network modularity study confirmed the presence of both co-occurrence sets found in the correlation analysis described above (Figure 4).

4. DISCUSSION

4.1 Factors controlling differences in microbial diversity and soil bacterial composition between two different biologically crusted areas in a semi-arid ecosystem

The results did not show any significant differences in the diversity indices between the two study sites, except for the number of observed OTUs (Sobs) index which was significantly higher in S2 at AMO than at BB (Supplementary Tables 1 and 2), possibly because both study sites are in semi-arid ecosystems conditioned by similar environmental factors (climate, geomorphology, vegetation, geological material) and, therefore, subject to similar environmental conditions (Rey et al., 2011).

Both study sites showed the same majority phyla, although in different proportions. Proteobacteria and Bacteroidetes were in the majority at AMO and Acidobacteria and Actinobacteria at BB. Cyanobacteria were also well represented at both sites (Supplementary Figure 1). In general, the phyla found at both study

sites are also predominant in biocrusts and their underlying soils in other arid and semi-arid areas worldwide, such as Nyngan, New South Wales, Australia, the Colorado Plateau and Tabernas Desert (SE Spain) (Liu et al., 2017; Steven et al., 2013; Miralles et al., 2020). Nevertheless, the CAP analysis confirmed the clear separation between the samples at AMO and BB by their soil bacterial composition at the lowest classification level (subgroup to genus) (Figure 1a). So some bacterial taxa were more abundant at AMO, whereas others were dominant at BB (Figure 2). The differences between the two study sites in relative abundances and the Sobs index in some microbial communities could be due to differences in some chemical soil properties, such as CE and soil carbonate content, which were significantly higher at AMO than at BB (Table 1 and Supplementary Table 2). Canfora et al., (2014) found that the relative abundance of a number of taxonomic groups changed significantly at different soil sites depending on the soil salt content, although other taxa were almost unaffected by salinity level. The presence of some bacterial genera capable of precipitating calcium carbonate (García et al., 2016) could also be related to the significantly higher concentration of calcium carbonate in the soils at AMO. Some authors have also found different species and microbial genera in secondary calcium carbonate deposits (Achal et al., 2010; Chekroun et al., 2004; García et al., 2016; Rivadeneyra et al., 2006; Rusznyák et al., 2012). On the other hand, some differences in organic carbon pools between study areas might also be influencing the proliferation of different bacterial communities at AMO and BB. Miralles et al., (2012d) found that biocrusts and underlying soils at AMO accumulated a comparatively lower proportion of humic acids and had a lower Humic Acid/Fulvic Acid ratio than at BB. Moreover, the organic matter at BB was associated with higher aromaticity and lower oxidation, whereas at AMO aliphatic and carboxyl content were higher. These differences in soil organic matter at the two study areas could condition a certain specialization in the soil microbial communities, some of which could be more efficient in degrading labile and/or chemically recalcitrant C (Goldfarb et al., 2011).

4.2 Influence of biocrusts on the microbial diversity at different soil depths in two semi-arid ecosystems.

The bacterial richness and alpha-diversity at each study site increased from the biocrust to the deeper soil layers and bare soil (C1<BS<S2<S3) in all indices analyzed (Table 2). Maier et al. (2014) found similar results in the Tabernas Desert (SE Spain), where species diversity and richness (number of recovered OTUs) were significantly higher in below-biocrust soil than in the biocrusts colonized by lichens. One of the possible explanations justifying the lower microbial diversity and biocrust richness (C1) both at AMO and BB could be the production of secondary lichen compounds with antibiotic effects (Boustie and Grube, 2005; Burkholder et al., 1944; Francolini et al., 2004). In fact, several authors have suggested that bacterial growth is controlled in lichens (Boustie and Grube, 2005; Francolini et al., 2004; Ingólfsdóttir et al., 1998), contributing to the production of secondary antibiotic metabolites to prevent the degradation of lichen thalli by fungal, bacterial or other organisms (Grube and Berg, 2009). The progressive increase in the Sobs and Chao1 from the biocrusts to the highest values in deeper soil layers under the biocrusts (C1<S2<S3) at AMO and BB (Table 2), could be because the vertical movement of these antibiotic metabolites by washing is attenuated with depth, still partly affecting the middle soil layer below biocrusts (S2) and only barely in the deepest soil layer (S3). Another possible complementary explanation of the changes in microbial richness and diversity in the vertical gradient from the biocrusts (C1) to the deeper underlying soil layers (S2 and S3) and bare soil (BS) at both AMO and BB, could be the significant differences found in the chemical soil properties between the biocrusts and the soil layers (C1 had a significantly more acidic pH than S2, S3 and BS in both study areas; Table 1 and Supplementary Table 2). Chemical soil properties can have a profound effect on microbial diversity and soil bacterial community composition in semi-arid Mediterranean soils (Sánchez-Marañón et al., 2017). Moreover, the biocrusts dominated by lichens promote surface wetness duration and soil moisture retention in the underlying soil (Chamizo et al., 2012a; Kidron et al., 2009; Kidron and Benenson, 2014). Soil moisture has a strong effect on microbial communities (Moyano et al., 2013) because microorganisms are strongly dependent on water and cannot sustain their normal cell activity below a certain water potential, depending on the species (Angel and Conrad, 2013). Therefore, the favorable moisture conditions in soils underlying biocrusts dominated by lichens could contribute to increasing the microbial richness and diversity in S2 and S3.

4.3 Factors explaining changes in the composition of bacterial communities in biocrusts, soil underlying the biocrusts at different depths and bare soil in two semi-arid ecosystems.

The composition of microbial communities at phylum level was different in all the layers at both AMO and BB sites. The Proteobacteria, Bacteroidetes, Actinobacteria and Cyanobacteria phyla gradually decreased with depth while Acidobacteria progressively increased from C1 to the deeper soil layers (Supplementary Figure 1). At the lowest classification level (subgroup-to-genus), differences in microbial community composition were also observed in biocrusts and soil layers (Supplementary Figure 1). Thus, some bacterial taxa were more abundant or exclusive in C1, at both AMO and BB (Figure 3). Changes in chemical soil properties partly caused by biocrusts, could favor these changes in soil microbial composition. Our results showed that chemical soil properties in biocrusts (C1) were significantly different from the soil layers (Supplementary Table 1), with a significantly higher OC content ($p<0.05$) than the underlying soils (S2 and S3) or adjacent bare soil (BS) (Supplementary Table 2), which decreased progressively from C1 to the deeper soil layers underlying the biocrusts (C1> S2> S3) at both AMO and BB (Table 1). Biocrusts dominated by *D. diacapsis* lichen were characterized by their high production of labile organic carbon, especially carbohydrates and polyphenols (Miralles et al., 2014, 2013), which could explain the high OC content in the C1 layers at AMO and BB. Some authors have shown that the quantity and quality of C substrates are primary drivers influencing soil microbial community composition (Fierer et al., 2007; Goldfarb et al., 2011). Therefore, the differences in OC between C1 and the soil layers could have a crucial role in the soil bacteria composition at AMO and BB. *D. diacapsis* lichen produces certain acidic organic compounds (Jorge-Villar et al., 2011), which could be responsible for the acidic pH in biocrusts and subjacent soil layers being attenuated with depth. The CO_2 generated by the biocrust metabolism (Miralles et al., 2012b) could also contribute to lowering the pH in C1 layers. Several authors have considered soil pH a strong predictor of bacterial community composition (Hartman et al., 2008; Lauber et al., 2009; Sait et al., 2006; Upchurch et al., 2008). Lauber et al., (2009) suggested that the critical influence of pH on soil microbial composition could be due to: i) its influence on numerous soil parameters (nutrient availability, cationic metal solubility, organic carbon characteristics, soil moisture regimen, salinity), which may drive

changes in the soil bacteria, and because ii) it conditions a physiological restriction on the soil bacteria, reducing the growth of taxa which cannot survive if the soil pH is outside its optimal growth range. Changes in pH could impose a significant stress that certain soil bacteria might tolerate better than others.

Our results showed highly significant subgroup-to-genus correlations of several bacteria with the soil pH and OC (Supplementary Tables 4 and 5). Specifically, the soil bacteria with the highest relative abundance in C1 (Figure 3) were highly correlated with each other (suggesting the formation of a bacterial co-occurrence group, Figure 4, Supplementary Table 6). In general the soil bacteria in this co-occurrence group showed positive correlations with OC content (r 0.45 to 0.75) and negative correlations with soil pH (r -0.50 to -0.88; Table 3). Such strong correlations with OC and pH suggest that these chemical parameters have an important role in the composition of the bacterial community in C1 at both AMO and BB. Other chemical parameters could also influence the bacterial composition in C1, such as the soil carbonate content which was significantly lower in C1 than the rest of the soil layers at AMO and BB (Table 1 and Supplementary Table 2). The production of acidic organic compounds from biocrusts dominated by lichens (Jorge-Villar et al., 2011) could contribute to reducing the calcium carbonate content in C1. Soil carbonate content was negatively correlated with several exclusive or more abundant soil bacteria in C1 (r -0.41 to -0.59), although those correlations were lower than with the OC or soil pH (Table 3). Other characteristics of the biocrusts could also influence the variability in the microbial community composition, such as the secretion of biomolecules or pigments produced by the lichens *D. diacapsis* (Miralles et al., 2017). These biomolecules are characterized by several physiological functions in biocrusts, such as protection from incident solar radiation and desiccation (Jorge-Villar et al., 2011), antibacterial or antifungal properties as mentioned above (Boustie and Grube, 2005; Francolini et al., 2004; Kosanić and Ranković, 2019). They could also foster a selective environment in which certain bacterial communities proliferate more than others (Cardinale et al., 2008; Grube et al., 2015, 2009).

The CAP analysis showed that C1 and S2 were different from S3 and BS, while S3 and BS layers were similar to each other (Figure1b). This could suggest that biocrusts could have more influence on the bacterial communities of the soil layers immediately underlying them (S2), but a diluted effect in the

deepest soil layer (S3). Thus, some bacterial taxa were more abundant in C1 and S2 (Supplementary Figure 2). Predictably, part of the carbohydrates and polyphenols produced by the dominant *D. diacapsis* in C1 (Miralles et al., 2014, 2013), as well as the above mentioned secondary metabolites produced by it, could also leach to S2. These conditions could create a selective environment for the microbial communities in soil just below the biocrust, in which the soil bacteria in S2 could be better adapted to conditions similar to the biocrusts, explaining why both layers have numerous microbial communities in common (Supplementary Figure 2). However, very few soil bacteria (*Pirellulaceae* uncultured*, Blastocatellia Subgroup 4* unclassified and *Pirellula*) were simultaneously more abundant in S2 and S3 than in C1 and BS, and no bacteria were dominant simultaneously in C1 and S3 layers.

Other microbial taxa were much more abundant or exclusive in BS at AMO and BB or showed a clear gradient increasing their relative abundance from C1 to S3 and BS layers (C1<S2<S3≤BS) (Supplementary Figure 3), suggesting that soil conditions found in both soil layers could favor the growth of those soil bacteria. Curiously, the most abundant or exclusive bacteria in BS (Figure 3) showed highly significant positive correlations with each other (Table 3), constituting a second co-occurrence group (Figure 4) in clear contrast to the first one mentioned above, which was led by the most abundant soil bacteria in C1. The significant positive correlations between calcium carbonate content, pH and EC and soil bacteria more abundant in BS and S3 as well as the significant negative correlations between OC and soil bacteria more abundant in BS and S3 (Table 3), suggest that the salinity, calcium carbonate and low organic matter content could exert a strong influence on the structure of these soil bacterial communities.

The above results have shown that biocrusts have an important direct effect on the underlying soil microbial communities through bacterial taxa selection and their effects on chemical soil properties. Although we did not consider fungal communities in this study, biocrust may also have an impact on them and will be further explored in future studies.

However, biocrust communities are very sensitive to both human and natural disturbance and soil degradation, which decrease the percentage of biocrust cover (Belnap and Eldridge, 2001) and drive changes and reductions in the structure, function and biomass of the biocrusts (Weber et al., 2016). This, in

turn, lowers their functional capacity for capturing and retaining nutrients (Barger et al., 2006) and producing secondary metabolites that could affect the proliferation of soil microbial communities.

5. CONCLUSIONS

Our results have shown that the diversity and composition of soil microbial communities in two biologically crusted sites located in a semi-arid Mediterranean ecosystem are mainly controlled by the influence of lichen-dominated biocrusts and chemical soil properties, such as OC, pH, EC and $CaCO_3$.

Biocrusts could influence the diversity and composition of soil bacterial communities directly and indirectly through their effects on key soil parameters, increasing the OC content, acidifying the soil, reducing the soil calcium carbonate content, and by secreting acid organic compounds, as well as through their physiological activity by secreting other secondary metabolites with antimicrobial properties. However, the effects of biocrusts on both chemical properties and microbial communities are limited to the first centimeters of soil, and progressively decrease in depth. Therefore, microbial diversity, as well as soil bacterial community composition were more alike in biocrusts and the soil layers immediately below them, but deeper soil layers under biocrusts were probably hardly affected by the influence of the biocrust, and the bacterial communities were more similar to bare soil.

The microbial co-occurrence patterns are mainly controlled by chemical properties directly affected by the biocrusts, such as higher OC content and acid soil pH. In bare soil, different microbial co-occurrence patterns were formed depending on other chemical soil properties, such as soil salinity and $CaCO_3$ content.

This work corroborates the importance of further study of the relationships between biocrusts and soil bacterial communities, which are essential for the proper functioning of the world's biochemical soil cycles. New global studies are needed to analyze both the physiological role of biocrusts and their effects on chemical soil properties in creating a microhabitat with specific environmental conditions favoring the development of specific microbial soil communities with greater advantages for proliferation than others.

478 This is especially true, because in the current global warming scenario, the effect of biocrusts on the
479 chemical properties and bacterial communities could be compromised.
480
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488
489 **Figure Captions**
490
491 **Figure 1.** Ordination plot produced by Canonical Analysis of Principal Coordinates (CAP).
492 **a)** Distribution of soil samples based on their bacterial communities at the two study sites (AMO:
493 Amoladeras, BB: Balsa Blanca). **b**) Distribution of soil samples based on their bacterial communities in
494 biocrusts and soil layers (C1: biocrust; S2: middle soil layer; S3: deep soil layer; BS: bare soil).
495
496 **Figure 2.** Relative abundance of soil bacteria at the lowest classification level (subgroup-to-genus) at
497 Amoladeras and Balsa Blanca.
498 Footnotes: AMO: Amoladeras, BB: Balsa Blanca. The box represents the relative abundance of soil bacteria
499 (%). The upper and lower lines correspond to the first and third quartiles (interquartile range) and the center
500 line is the median. Whiskers include the deviations of up to a maximum distance of 1.5 times the
501 interquartile range. Values with a deviation over 1.5 are represented as points.
502

Figure 3. Relative abundance of soil bacteria at the lowest classification level (subgroup-to-genus) in each biocrust and soil layer.

Footnotes: C1: biocrust layers; S2: middle soil layers; S3: deep soil layers; BS: bare soils. The box represents the relative abundance of soil bacteria (%), with the upper and lower lines corresponding to the first and third quartiles (interquartile range) and the central line the median. Whiskers include values deviating up to a maximum distance of 1.5 times the interquartile range. Values with a deviation over 1.5 times are represented as points.

Figure 4. Co-occurrence network based on correlation analysis (Pearson > 0.800) for selected bacteria in co-occurrence patterns with the 10 highest correlations detected.

Footnotes: The size of each node is proportional to the number of connections. Density of the edges indicates intensity of the correlation. The resulting soil microbial network is made up of 26 nodes and 52 edges (average degree or node connectivity = 4). The clustering coefficient (how the nodes are integrated into their neighborhood, and therefore, the degree to which they tend to cluster) was 0.33 and modularity was 0.494. Two co-occurrence groups are shown: Co-occurrence group 1 (pink) and Co-occurrence group 2 (green).

Supplementary Figure 1. Bacterial community composition at phylum level in biocrusts and soil layers. A) Amoladeras (AMO); B) Balsa Blanca (BB); C) Biocrust layers (C1) from both sites; D) Middle soil layers (S2) from both sites; E) Deep soil layers (S3) from both sites; F) Bare soil (BS) from both sites.

Footnotes: Fractions of the circular graphs show the average relative abundance of bacterial phyla in the different layers.

Supplementary Figure 2. Relative abundance of soil bacterial taxa at the lowest classification level (subgroup-to-genus) in a decreasing gradient from C1 to S3 or BS (C1>S2>S3≥BS).

Footnotes: C1: biocrust layers; S2: middle soil layers; S3: deep soil layers; BS: bare soils. The box represents the relative abundance of soil bacteria (%), with the upper and lower lines corresponding to the first and third quartiles (interquartile range) and the central line the median. Whiskers include values that deviate up to a maximum distance of 1.5 times the interquartile range. Values with a deviation over 1.5 times are represented as points.

Supplementary Figure 3. Relative abundance of soil bacteria taxa at the lowest classification level (subgroup-to-genus) in an increasing gradient from C1 to S3 or BS (C1<S2<S3≤BS).

Footnotes: C1: biocrust layers; S2: middle soil layers; S3: deep soil layers; BS: bare soils. The box represents the relative abundance of soil bacteria (%), with the upper and lower lines corresponding to the first and third quartiles (interquartile range) and the central line the median. Whiskers include values that deviate up to a maximum distance of 1.5 times the interquartile range. Values with a deviation over 1.5 times are represented as points.

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