The role of grain size and inoculum amount on biocrust formation by *Leptolyngbya ohadii*

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Abstract

Cyanobacteria are widespread prokaryotic organisms that represent feasible biotechnological tools to set up valid approaches to counteract desertification. Their peculiar physiological traits, and their resilience to abiotic stresses, allows their application on abiotically constrained soils to trigger their stabilization. A successful cyanobacteria inoculation results in the formation of cyanobacterial biocrusts, complex microbial communities characterized by tangled filament meshes imbued in a matrix of self-secreted extracellular polysaccharides (EPS) that keep loose sediments and aggregates firmly in place. However, the capability to form stable cyanobacterial biocrusts is not common to all the species, and a mix of factors can hamper the success of the treatment, notably inoculum amount, and substrate characteristics.

The aim of this work was to assess the influence of inoculum quantity and substrate granulometry on the physical stability of cyanobacterial biocrusts induced by inoculating the strain *Leptolyngbya ohadii* in a microcosm experiment, under laboratory conditions. After applying three different initial inoculum amounts on two different sand granulometries (medium and coarse sand), we assayed aggregate stability, physical stability and surface hydrophobicity on the resulting biocrusts during a 30-day incubation. Also, the features and the role of the EPS synthesized by *L. ohadii* were studied following their isolation, characterization, and direct application on the sand. The two EPS fractions produced by the strain, one more soluble and easily released in the surrounding medium (released polysaccharides, RPS) and one solidly attached to the filaments (glycocalyx EPS, G-EPS), were separately tested.

Cyanobacterial biocrusts visibly formed in all the microcosms after 15 days. However, we observed a strong effect of sand granulometry in affecting aggregate stability and tensile strength, both of which appeared weaker on coarse sand. A higher amount of initial inoculum was necessary to produce stable biocrusts on coarse sand compared to medium sand. Also, we observed how the inoculation of EPS alone did not sort most of the significant effects that we detected by inoculating the whole culture, pointing at the importance of the action of the cyanobacterial filaments in soil conglomeration. However, a significant increase in physical stability was achieved by inoculating G-EPS on medium sand, suggesting the involvement of this fraction in biocrusts structuration.

This work analyzes for the first time the effects of the variable grain size and inoculum amount in the achievement of physically stable biocrusts by cyanobacteria inoculation. The results that we obtained are useful in improving and optimizing the process of biomass preparation and dispersion for future indoor and outdoor studies.

Keywords: biocrusts, inoculation, aggregate stability, tensile strength, RPS, G-EPS.

1. INTRODUCTION

Cyanobacteria are phototrophic prokaryotes commonly found in a wide array of habitats on Earth, in temperate and extreme climates, in marine and fresh waters and from soil to rocks (Rossi and De Philippis, 2015). Many cyanobacterial species can colonize arid and hyper-arid substrates, even affected by salt stress, at a temperature range of 45-70 °C, pH lower than 4-5 (with optimum between 7.5-10) and under severe drought (Singh et al., 2016). Several authors already described their capability to promote the stability of soil aggregates against breakdown by wetting and physical forces (Malam-Issa et al., 2007; Rogers and Burns, 1994). Also, the presence of cyanobacteria positively affects the level of soil organic carbon and influence water distribution (Chamizo et al., 2018b; Colica et al., 2014; Muñoz-Rojas et al., 2018; Singh et al., 2016). Owing to these features, some selected strains revealed effective inoculants in arid and hyper-arid environments to promote sand dune stabilization and desertification reversal (Lan et al., 2014; Li et al., 2014), in a technology known as cyanobacterization (D'Acqui, 2016). Cyanobacteria aggregate the sand by forming a dense and sticky network constituted by filaments and exopolysaccharides (EPS) that bind together sand particles, forming cyanobacterial biocrusts (or algal biocrusts). These early formations represent the incipient developmental phase of complex and ecologically relevant microbial communities known as biological soil crusts, whose impact on arid and hyper-arid environment has been widely discussed (Belnap and Weber, 2013; Bowker, 2007; Weber et al., 2015). In the crusting process, the release of cyanobacterial EPS in the surrounding environment has several important implications. Their amphiphilic and hygroscopic nature allows to maintain hydration and tolerate desiccation, they shield cells from excessive solar irradiation and from protozoan predation, protect from freezing and salt stress, while chelating and immobilizing nutrients from the surrounding environment (Li et al., 2001; Rossi et al., 2018; Rossi and De Philippis, 2015; Swenson et al., 2018). On the other hand, EPS regulate the attachment to surfaces by changing the hydrophobicity of the cyanobacterial filaments (Mazor et al., 1996), gluing unitary sand particles into cohesive biocrust layers (Malam Issa et al., 2001).

The choice of the most feasible cyanobacterial inoculants is a very important starting point before attempting any cyanobacterization treatment. Inoculants must fit with a given soil type and environmental conditions, so that a treatment is tailor-made for a specific environment to rehabilitate (Malam-Issa et al., 2007; Singh and Kaur, 2009). In this framework, the physiological characteristics of the strain are a very important factor impinging on its capability to survive and form stable biocrusts. For example, the gliding motility, typical of some species such as the model inoculant *Microcoleus vaginatus*, allows avoidance of strong sunlight and propulsion towards

increasing moisture gradient, away from surface-desiccation fronts (Garcia-Pichel and Pringault, 2001). The gliding process also allows a wider release of EPS, enhancing the crusting process (Mugnai et al., 2018b). The chemical quality of EPS is highly related to their capability to glue loose sand together (Attou et al., 1998; Hu et al., 2003; Malam Issa et al., 2001), although the information on the most influential EPS features is still limited. The monosaccharide composition and the molecular size of cyanobacterial EPS so far analyzed are highly variable, and generally complex (Mugnai et al., 2018a; Pereira et al., 2009; Rossi and Philippis, 2016). Furthermore, EPS features are significantly affected by nutrient levels (Mager and Thomas, 2010; Rossi and Philippis, 2016), water availability (Mugnai et al., 2018a), or soil type (Chamizo et al., 2018a), so that they cannot be considered absolute for a given strain, but differing according to abiotic conditions (Mugnai et al., 2018a). By determining variations in EPS chemical quality, environmental parameters affect the crusting capability of cyanobacterial inoculants. Additional factors can hamper or condition cyanobacteria establishment and biocrust formation. One is inoculum quality, that include the amount, preparation and dispersion of the biomass. At the moment, standardized protocols do not exist, and methodologies have been diverse (Hamdi, 1982; Rossi et al., 2017). Other known factors impinging on cyanobacterization success are unfavorable soil pH, low P levels, low water availability, and, importantly, granulometry of the substrate (Rossi et al., 2017; Rozenstein et al., 2014). Rozenstein et al. (2014) found that cyanobacterial biocrusts develop more rapidly on finer substrates, while they resulted patchy and discontinuous on coarse sand. However, we have no indications concerning how substrate granulometry influences tensile strength and aggregate stability of biocrusts. The large number of factors at play during biocrust formation explains the highly variable responses so far registered by employing different inoculants, both in desert environments and in agricultural settings (Rossi et al., 2017). Some inoculants only form thin films at the soil surface, while others form crusts with aggregation and physical stability that are too poor to withstand erosion events typical of arid and semiarid environments (Hu et al., 2002; Rossi et al., 2017). Wind erosion can dismantle the incipient biocrust structure if its threshold friction velocity does not exceed wind forces experienced in a given environment (Belnap and Gillette, 1998). A deeper knowledge on the paramount factors influencing inoculant establishment and stable cyanobacterial biocrust formation is important to select novel and performing candidates and employ them in the frame of optimized procedures. *Leptolyngbya ohadii* is a model strain that we employed in a previous study (Mugnai et al., 2018b). Following an optimized inoculation protocol, it revealed a very good crust former, useful for testing how the variation of different parameters affect the cyanobacterization outcome. We utilized the strain to investigate how water availability impinges on the physical characteristics of biocrusts, and on the chemical quality of EPS (Mugnai et

al., 2018b). The present work aims at an additional step forward, which is to employ *L. ohadii* to investigate if and how the amount of initial inoculum and the granulometry of the substrate influence several biocrust parameters, namely tensile strength, aggregate stability and surface hydrophobicity. Expecting, by literature, a different inceptive colonization between coarse and fine substrates, we concurrently hypothesized a significant difference in the physical stability of the induced biocrusts. Additionally, the role of the EPS excreted by the strain on sand aggregation process was also investigated.

2. MATERIAL AND METHODS

2.1. Sand inoculation experiments

2.1.1 Organism and culture conditions

L. ohadii was kindly provided by the Department of Plant and Environmental Sciences, The Hebrew University of Jerusalem, Israel. It is a filamentous, non-heterocystous cyanobacterium isolated from biocrusts collected in the Nizzana region of the Negev Desert, Israel (Ohad et al., 2010; Raanan et al., 2016). Its morphological characteristics were first described in Mugnai et al. (2018b). It does not produce an exocellular sheath when grown in liquid culture, although a more condensed EPS fraction remains attached to the filament (hereafter referred to as "glycocalyx EPS", G-EPS) and one more soluble (hereafter referred to as "released polysaccharides", RPS) is released in the culture medium.

L. ohadii was grown in BG11 medium (Rippka, 1988) for 30 days in Pirex flasks in an orbital incubator (INNOVA 44-R, New Brunswick) at a temperature of 30 °C, at low light intensity of 15 µmol photons m^{-2} s^{-1} and a constant stirring of 100 rpm until reaching the stationary phase.

2.2.2. Isolation and characterization of EPS from liquid culture

RPS were isolated by centrifuging the culture at 4,000 × *g* for 30 min at room temperature. The supernatant was enclosed in nitrocellulose tubular dialysis membranes (Medicell International, 12-14,000 MWCO) and dialyzed against 10 volumes of distilled water for 48 h under continuous stirring, with two changes of water, and then concentrated by using an orbital evaporator at 35 °C. G-EPS were extracted from the pellet obtained after RPS removal, washing the resulting pellet with 0.5% NaCl solution and then extracting it with 4 mL hot (80 °C) distilled water for 1 h. After

centrifuging at 4,000 × *g* for 30 min, the G-EPS-containing supernatant was collected. Both RPS and G-EPS were eventually lyophilized and used for macromolecular characterization. Additionally, RPS and G-EPS were used to test their sole effect when applied to the sand, as compared with the whole cyanobacterial biomass.

The monosaccharidic composition of the two fractions was determined by ion-exchange chromatography (IEC) on dried EPS samples after hydrolyzation with trifluoroacetic acid according to Mugnai et al. (2018b). Samples were analyzed by using a Dionex ICS-2500 ion exchange chromatograph (Dionex, Sunnyvale, CA) equipped with an ED-50 detector with a gold-working electrode, and a Carbopac PA1 column of 250 mm length and 4.6 mm internal diameter (Dionex, Sunnyvale, CA). Chromatographic conditions were in accordance with Chen et al. (Chen et al., 2014). Unknown sugars were identified by injecting several concentrations of standard monosaccharides and plotting response area as function of concentration.

The apparent molecular weight (MW) was determined by Size-Exclusion Chromatography (SEC). EPS were dissolved in deionized water and injected in a Varian Pro-Star liquid chromatographer (Varian Inc., USA) equipped with two PolySep – GFC–P 6000 and 4000 columns (Phenomenex, USA) connected in series, and a refractive index detector. The eluent was HPLC-grade water at a working flow of 0.4 mL min^{-1}. Dextran at known MWs (2,000 kDa, 1,100 kDa, 410 kDa, 150 kDa, 50 kDa) purchased from Sigma Aldrich, was used as a reference standard to determine MW classes according to retention time.

2.2.3. Sand inoculation with L. ohadii and with isolated EPS

Biomass and EPS (RPS and G-EPS) obtained from *L. ohadii* were inoculated in microcosms constituted of Petri dishes of 92 mm (diameter) x 16 mm (depth), containing 80 g of sand alternatively with a granulometry of 0.3 – 0.6 mm (henceforth medium sand, MS) or 0.8 – 1.25 mm (henceforth coarse sand, CS). The sand was a commercial dried silica sand (VAGA s.r.l., Pavia, Italy) with known chemical and mineralogical composition (Tab. S1) that was autoclaved twice before use. The experimental design is summarized in Table S2. A total of 90 microcosms (45 microcosms containing MS and 45 microcosms containing CS) were prepared and divided in two subsets (one to receive culture inoculum and one to receive EPS suspension) to serve in two temporally separated experimental phases. In the first experimental phase, 36 microcosms, 18 containing MS and 18 containing CS, were inoculated with cyanobacterial biomass, preventively treated according to Mugnai et al. (2018a, b). Briefly, the culture medium was removed by centrifugation, trichomes fragmented with sterile tweezers, and eventually re-suspended in distilled

water in a volume to provide enough inoculum for all the microcosms. After determining the dry weight (DW), the biomass was applied on the sand, drop by drop with a sterile 10 mL pipette, using a "double spiral dispersion" inoculation methodology, i.e. applying the biomass following a first spiral line and then by applying a second spiral in the empty spaces left on the sand by the first spiral (see the sketch in Figure 2a). The inoculum was applied in volumes corresponding to 0.15, 0.45 and 0.75 mg cell dry weight (CDW)/cm^2, calculated based on cell DW, and considering microcosm surface area. In addition, 12 microcosms (6 with MS and 6 with CS) were not inoculated and served as control. In a second experimental phase, a total of 36 microcosms (half containing MS and half containing CS were inoculated alternatively with RPS and G-EPS suspended in deionized water, in amounts corresponding to 6.8, 13.5 and 27.1 µg EPS/cm^2. Such amounts corresponded to the highest values of EPS contents detected in 30-day-old cyanobacterial biocrusts induced in the first experimental phase by applying respectively the lowest, the intermediate and the highest quantity of initial inoculum (see Tab. 4 and section 2.2.7 for EPS quantification method). Six non-inoculated microcosms containing MS (3) and CS (3) served as control. Inoculated microcosms and controls were incubated for 30 days in a plexiglass growth chamber under controlled temperature (30°C), illumination (45 µmol photons m^{-2} s^{-1}) and relative humidity (RH, 0 %) conditions. RH was monitored with a digital hygrometer (VWR, USA), having an accuracy of 5%. Every two days, 0.4 mm distilled water was sprayed over each microcosm, including the control, according to Mugnai et al. (2018b). Microcosms inoculated with cyanobacterial biomass (first experimental phase) were collected and analyzed 15 and 30 days. Microcosms with EPS suspensions (second experimental phase) were collected only after 30 days.

2.2.4. Spectral measurements

Surface reflectance (SR) was used as a non-destructive measure of cyanobacterial biocrust development (Rozenstein et al., 2014). SR was measured at the end of incubation (30 days) with an Analytical Spectral Device (ASD) hand held portable spectroradiometer (ASD Inc., Boulder, Colorado, USA), under constant light conditions. The instrument had a sampling interval of 3.5 nm from 325 nm to 1075 nm and was equipped with an optic fiber which was placed 16 cm above the soil sample, to measure the total surface of the microcosm. Before measurements, the spectroradiometer was calibrated using a 99% Spectralon(r) panel. Two spectra were taken per sample, each one consisting on the internal average of three individual spectra, resulting in six pseudo-replicates that were averaged to obtain the mean spectrum for each sample. Then, the three independent replicates per treatment were averaged to obtain the mean spectral curve per inoculum

amount and granulometry. All reflectance values were expressed proportional to the 99% Spectralon standard. Data were acquired with the RS^3 Spectral Acquisition Software on a laptop connected to the spectroradiometer. After data collection, data pre-processing was performed by removing noisy bands in the range between 325 and 400 nm and between 950 and 1075 nm and later application of a cubic polynomial smoothing filter (Savitzky and Golay, 1964).

Spectral absorptions at specific wavelengths were extracted using the continuum removal (CR) technique (Clark and Roush, 1984). This technique normalizes comparison between samples by rationing the measured spectrum to the estimated reflectance of a common baseline which has a value of 1.0. Values equal to 1.0 indicate no absorption, while values lower than 1.0 indicate the presence of absorption features. The continuum-removal was computed using ENVI 4.3 (ITT VIS, Boulder, CO, USA).

2.2.5. Crust thickness and stability measurement

The thickness of the cyanobacterial biocrusts was measured with a caliper after 30 days of incubation. Aggregate stability (AS) of the cyanobacterial biocrusts was measured applying the single water-drop test (Imeson and Vis, 1984). Biocrust aggregates were first sieved to 4.0 and 4.8-mm size. Next, 0.1 g water drops were allowed to fall from 1 m height onto biocrust aggregates placed on a 2.8-mm metal mesh sieve. The number of drops necessary to disrupt the aggregates was counted and used as a stability index. For each sample, the final number of drops needed was calculated as a mean of at least 15 instrumental replicates (n = 15).

Tensile strength was measured using a digital force gauge (Mark-10 Model M7-5, 25N, Mark-10 Corp, USA) equipped with a cone tip (0.5mm length, 0.6mm diameter). Crusts were oven-dried (40°C) for 4 h before each measurement. Samples were placed onto a lifting table that was raised up until probe–soil contact produced biocrust break-down. The registered peak value corresponded to the breaking point of the crust aggregates, and to their tensile strength. Five instrumental replicates were conducted for each sample (n = 5).

2.2.6. Soil water repellency

Surface water repellency (SWR) of induced cyanobacterial biocrusts was assessed by the water drop penetration time (WDPT) test and repellency index (RI) measurement.

WDPT test was performed on the samples after 15 and 30 days of incubation, according to Bisdom et al. (1993). A drop of distilled water of 50 (± 5) μL was released from a height of 10 mm

on the biocrust surface, and the time for complete drop infiltration was recorded. For each sample, the final infiltration time was calculated as a mean of 10 instrumental repetitions (n = 10). Samples were considered as wettable when WDPT < 5 s, slightly water repellent when WDPT ranged between 5 and 60 s, and strong water repellent when > 60 s (Bisdom et al., 1993).

RI was determined on the samples after 15 and 30 days of incubation according to Mugnai et al. (2018a), utilizing a miniaturized tension infiltrometer (Lichner et al., 2013). After calculating the sorptivity of distilled water and 95 % ethanol at – 2 cm head pressure, RI was calculated applying the following formula:

$$RI = 1.95 \sqrt{\frac{S_E}{S_W}}$$

where S_E is the sorptivity of 95 % ethanol and S_W is the sorptivity of distilled water, while the factor 1.95 accounts for differences in viscosity and surface tension between the two liquids (Tillman et al., 1989). A soil with a RI = 1 (i.e. where $S_E = S_W$) is considered non-repellent, whereas a subcritical repellency is characterized by RI values greater than 1 and lower than 1.95. Values greater than 1.95 indicate a critically water repellent soil.

2.2.7. Extraction and quantification of EPS in cyanobacterial biocrusts

EPS were extracted and quantified in the biocrusts after 15 and 30 days since inoculation according to previously set protocols (Rossi et al., 2018). 20 g of dry homogenized crust were suspended for 15 min in distilled water and then centrifuged at 4000 x *g* for 30 min at 4 °C to recover the soluble EPS fraction. Then, the EPS fraction more condensed around sediments and filaments was extracted by treating the pellet with 0.1 M Na_2EDTA for 16 h at room temperature, then recovering it by centrifuging at 4000 x *g* for 30 min at 4°C. Finally, the two fractions were blended and quantified as total EPS. The quantification was done by the phenol-sulfuric acid assay (Dubois et al., 1956). Briefly, 1 ml 5% phenol was added to the sample, followed by the addition of 5 ml H_2SO_4. The mix was incubated at room temperature for 10 min, water-cooled for 15 min and then analyzed spectrophotometrically at 488 nm. D-glucose at different concentrations was used as a reference standard.

3. STATISTICAL ANALYSIS

All the analysis was conducted in experimental triplicates (N = 3); a minimum number of three instrumental replicates was always conducted for each measurement (n = 3). To analyze whether the results were significantly different, data were treated using one-way analysis of the variance (ANOVA) at 95% of the significance, followed by Tukey's honest significance difference (HSD) post-hoc test. Results were considered significant at $P \leq 0.05$. Prior to ANOVA, data Independence (Pearson's Chi-square test), normal distribution (D'Agostino-Pearson normality test), and homogeneity of variance (Bartlett's test) were assessed. To correlate parameters, linear regression analyses were performed, and r^2 and P values are reported for each case. Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad software, USA).

4. RESULTS

4.1.Monosaccharide composition and apparent molecular weight of EPS from *L. ohadii*

Overall, 13 different monosaccharide moieties were detected by analyzing the EPS produced by *L. ohadii* in liquid culture (Fig. 1a). IEC unveiled a marked difference between G-EPS and RPS. The former fraction was dominated by glucose (accounting for 48% of the components), characterized by higher content of ribose (21%) than RPS, and slightly higher amounts of galacturonic acid (5%). On the other hand, RPS contained glucuronic acid as the dominant moiety (accounting for nearly 40% of the detected constituents), had higher contents of rhamnose, galactosamine, galactose, mannose and xylose, than G-EPS.

[Near Figure 1]

SEC revealed that the two EPS fractions were polydisperse polymers, constituted by macromolecules having a large MW distribution. However, while G-EPS showed fractions distributed in four MW classes ranging between 2,000 kDa and 50 kDa, RPS showed a predominant (71%) fraction having an apparent MW between 2,000 kDa and 1,100 kDa (Fig.1 b).

4.2. Effects of the inoculation of *L. ohadii* biomass

4.2.1 Effects on cyanobacterial biocrust development and stability

The "double spiral dispersion" methodology (sketched in Fig. 2a) allowed an even distribution of the biomass on the microcosm surface (Fig. 2b), determining the formation of cyanobacterial

biocrusts on MS (Fig. 2c) and CS (Fig. 2d) with a significant thickness on both substrates (Fig. 2e, f). The thickness was in accordance with initial inoculum amount and influenced by substrate granulometry (Tab. 1).

[Near Fig. 2]

On CS, a consistent thickness was observed only in the crusts obtained by applying the intermediate and the highest inoculum amounts, while in those obtained by applying the lowest amount, crusts were very thin and fragile, not allowing to obtain solid measurements. On MS, a coherent crust (7.03 ± 0.41 mm thick) was observed even applying the lowest amount of inoculum (Tab. 1). The thickness increased with the increase of the inoculum amount, showing comparable values in the biocrusts obtained by applying 0.45 and 0.75 mg CDW/cm^2.

Table 1. Thickness of cyanobacterial biocrusts measured at t = 30 with a caliper. MS: medium sand (granulometry 0.3 – 0.6 mm); CS: coarse sand (granulometry 0.8 – 1.25 mm). Different letters indicate significant differences ($P < 0.05$) between the values employing different inoculum concentration. – absence of a measurable thickness. Values are expressed as mean ± (SD).

Sand Granulometry	**MS**				**CS**			
Inoculum (mg CDW/cm^2)	**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
Thickness (mm)	-	7.03^b (0.41)	7.85^a (0.75)	8.48^a (0.50)	-	-	7.33^b (0.73)	8.53^a (0.75)

Surface reflectance analysis at t = 30 d showed that cyanobacterial biocrust development darkened the surface and led to a decrease in reflectance in the visible (VIS) region (400-700 nm) compared to controls. The spectral curve was characterized by low reflectance in the blue region and increasing values towards the green and red regions, followed by a steeper increase in reflectance near the infrared region (700-950 nm; Fig. 3a, b). On MS, reflectance was lower in the biocrusts induced by inoculation with higher inoculum amounts (0.45 and 0.75 mg CDW/cm^2) than in those with low amounts (0.15 mg CDW/cm^2), and controls. Differences between control and inoculated soils were also reflected in the absorption at 680 nm due to chlorophyll *a* content. This absorption peak was absent in the controls and present in the inoculated sand, showing an increasing depth as the increasing initial inoculum amount increased (Fig. 3c, d). Another absorption peak was observed at 500 nm in both control and inoculated soils associated to the presence of iron oxides in the studied sandy substrates (Tab. S1), which can interfere with absorption by carotenoids, which also absorb at 500 nm (Weber et al., 2008).

[Near Figure 3]

Stability of biocrust aggregates was very low, with only less than five drops needed to break down the aggregates. However, we found some differences in aggregate stability among the biocrusts induced by the different inoculum concentrations on the MS. With this granulometry, after 30 days of incubation, the number of water droplets needed to disperse aggregates increased significantly according to the amount of initial inoculum (Tab. 2). On the other hand, cyanobacterial biocrusts on CS presented aggregates that were too weak to give consistent values when applying this method of analysis.

Table 2. Aggregate Stability (AS, expressed as the number of drops needed to disperse sand aggregates) and tensile strength, expressed as Maximum Penetration Resistance (MPR), expressed in kPa, of cyanobacterial biocrusts developed on MS and CS. AS was measured at t = 15 d and t = 30 d. Tensile strength was measured at t = 30 d. Different small letters indicate significant differences ($P < 0.05$) between the AS or the tensile strength values obtained employing different inoculum concentration. Dash (-) indicates an AS under the detection limit of the method. Values are expressed as mean ± (SD)

Sand type		**MS**				**CS**			
Inoculum (mg DW/cm^2)		**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
AS (average number of drops)	**15d**	-	-	3.40^{c} (0.39)	3.78^{b} (0.50)	-	-	-	-
	30d	-	2.45^{d} (0.12)	3.36^{bc} (0.22)	4.11^{ab} (0.39)	-	-	-	-
MPR [kPa]	**30 d**	1.70^{c} (0.09)	1.49^{c} (0.53)	7.59^{b} (1.74)	13.74^{a} (2.45)	2.37^{b} (0.99)	1.66^{b} (0.22)	1.22^{b} (0.91)	6.19^{a} (1.93)

Tensile strength of the biocrusts, measured at t = 30 depended upon sand type and inoculum amount (Tab. 2). On MS, cyanobacterial biocrusts showed a tensile strength that increased with the increase on inoculum amount. On CS, while no significant increase was observed between applying the lowest and the intermediate inoculum amount, a significantly increased tensile strength (from 1.22 ± 0.91 to 6.19 ± 1.93 kPa) was detected in the biocrusts obtained by applying the highest inoculum amount.

4.2.2 Effects on surface water repellency (SWR)

At t = 15 d, WDPT test revealed a strong SWR of cyanobacterial biocrusts obtained by applying 0.45 and 0.75 mg CDW/cm^2 of inoculum on both MS and CS (Tab. 3). No SWR was revealed by WDPT test with the application of only 0.15 mg CDW/cm^2. From t = 15 d to t = 30 d, values decreased significantly in all the cases.

Table 3. Water drop penetration time test (WDPT) and repellency index (RI) of induced cyanobacterial biocrusts. Different letters indicate a significant difference among the treatments, and between t = 15 d and t = 30 d of incubation, on each sand type. Values are expressed as mean ± (SD).

Sand Granulometry		**MS**				**CS**			
Inoculum (mg CDW/cm^2)		**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
WDPT [sec]	**15d**	0.0^{d} (0.01)	0.0^{d} (0.01)	10.98^{bc} (3.67)	$>20^{a}$ (7.64)	0.0^{d} (0.01)	0.0^{d} (0.01)	6.06^{c} (1.16)	$>20^{a}$ (6.26)
	30d	0.0^{d} (0.01)	0.0^{d} (0.01)	1.45^{cd} (1.09)	9.71^{b} (3.51)	0.0^{d} (0.01)	0.0^{d} (0.01)	7.55^{bc} (6.54)	10.51^{b} (9.11)
RI	**15d**	1.04^{c} (0.11)	1.91^{c} (0.5)	12.09^{ab} (3.3)	14.23^{a} (1.94)	0.88^{b} (0.43)	3.54^{b} (1.50)	$>20^{a}$ (0.67)	$>20^{a}$ (2.69)
	30d	1.14^{c} (0.16)	1.24^{c} (0.1)	7.22^{b} (4.06)	7.26^{b} (0.77)	0.86^{b} (0.62)	2.11^{b} (0.53)	1.82^{b} (0.52)	1.89^{b} (0.47)

At t = 15 d, RI analysis showed that cyanobacterial biocrusts on MS induced by applying 0.45 and 0.75 mg CDW/cm^2 biomass showed a critical SWR (RI > 1.95), whereas a sub-critical one was observed applying 0.15 mg CDW/cm^2. At t = 30 d, cyanobacterial biocrusts still maintained a critical or subcritical repellency value, despite a general decline in the values. On CS, although at t = 15 d biocrusts showed a critical SWR according to RI values, only sub-critical values were found for all the treatments at t = 30 d. After 30 d, a significant increase of RI was observed from applying the lowest, the intermediate and the higher inoculum amounts.

4.2.3 Effects on EPS contents in cyanobacterial biocrusts

Total EPS contents of cyanobacterial biocrusts, determined at t = 15 d and t = 30 d, increased showing a positive correlation with the amount of initial inoculum in both MS (r^2=0.98, P =0.07 and r^2 = 0.98, P = 0.06 for t = 15 and t = 30 d, respectively) and on CS (r^2 = 0.98, P =0.09 and r^2 = 0.97, P = 0.1 for t =15 d and t = 30 d, respectively).The highest EPS content in biocrusts both in MS and in CS was detected at t = 15 d (Tab. 4).

Table 4. EPS content in cyanobacterial biocrusts (expressed as μg EPS per g biocrust) that were induced by inoculating *L. ohadii* at three different inoculum amounts, expressed as mg cell dry weight (CDW) per square centimeter. Presented data were treated by subtracting the values detected in non-inoculated microcosms (controls), to correct for background noise. Different letters indicate a significant difference (P <

0.05) in EPS contents by applying different inoculum amounts. MS, medium sand; CS, coarse sand. Values are expressed as mean ± (SD).

Sand Granulometry		MS			CS		
inoculum (mg CDW/cm^2)		0.15	0.45	0.75	0.15	0.45	0.75
EPS µg [g biocrust]$^{-1}$	15d	3.67^{d} (0.77)	13.27^{bc} (1.97)	27.86^{a} (1.39)	2.43^{dc} (1.47)	11.80^{bc} (1.26)	25.95^{a} (3.57)
	30d	3.45^{d} (0.81)	7.84^{d} (1.46)	15.28^{b} (2.58)	3.48^{dc} (0.41)	7.94^{c} (2.17)	15.65^{b} (1.79)

4.3 Effects of the sole EPS application

The application of pure EPS obtained from liquid culture did not produce any significant effect on AS, nor statistically significant variation of SWR. Values of RI lower than 1 (Table 3S) and values of WDPT < 5 sec (data not shown) were always found at t = 30 d. However, when the G-EPS solution was applied on MS, an increase in tensile strength was detected as the applied G-EPS concentration increased, while no significant effects were observed by applying RPS suspension (Table 5). On the contrary, G-EPS and RPS application on CS never affected the surface tensile strength, which showed values not statistically different from the control.

Table 5. Tensile strength (expressed in kPa) measured after 30 days on MS and CS microcosms following G-EPS and RPS application isolated from liquid culture of *L. ohadii*. Different letters indicate significant differences (P < 0.05) between the values obtained employing different EPS concentrations. MS, medium sand; CS, coarse sand. Values are expressed as mean ± (SD).

Sand Granulometry		MS				CS			
EPS (µg/cm^2)		Control	6.80	13.54	27.09	Control	6.80	13.54	27.09
MPR [kPa]	RPS	1.70^{a} (0.11)	2.44^{a} (0.14)	1.69^{a} (0.56)	1.93^{a} (0.24)	2.12^{a} (0.55)	2.57^{a} (0.57)	2.32^{a} (0.67)	3.53^{a} (0.72)
	G-EPS	1.70^{c} (0.11)	2.63^{bc} (0.50)	3.07^{b} (0.56)	5.27^{a} (0.35)	2.12^{a} (0.55)	2.46^{a} (0.92)	2.90^{a} (0.19)	3.15^{a} (0.01)

5. DISCUSSION

In this work, we confirmed the marked capability of *L. ohadii* to form stable cyanobacterial biocrusts on sandy substrates. The inoculation was not supported by any type of nutrient addition,

nor by any pretreatment with stabilizing agents or tackifiers. Although we observed crust formation on both sand types, the grain size of the substrate and initial inoculum concentration significantly affected thickness, hydrophobic properties and physical stability of cyanobacterial biocrusts.

Overall, the strain more easily formed cyanobacterial biocrusts on MS than on CS. The lowest inoculum amount (0.15 mg CDW/cm^2) did not induce cyanobacterial biocrusts with a measurable thickness and tensile strength on CS; however, such amount was enough on MS. Cyanobacterial biocrusts on CS also showed a very poor particle aggregation. Although aggregates were visible, they resulted so fragile that the number of drops necessary to disrupt the aggregates was very low, not allowing obtaining significant and reliable values for AS. On the other hand, the same crusts presented a significant tensile strength when the highest inoculum amount was applied (0.75 mg CDW/cm^2), indicating this as the sole inoculum amount determining a significant effect on CS (Table 2). The impact of different initial inoculum amount and sand grain size on biocrust formation was also supported by SR results. A significant reduction in albedo due to surface darkening owing to biocrust development was observed, although only by applying 0.45 or 0.75 mg CDW/cm^2 on MS, and 0.75mg CDW/cm^2 on CS (Figure 3). It was already evidenced how finer substrates are more supportive to cyanobacterial biocrust colonization (Rozenstein et al., 2014). The larger pore spaces between the grains on CS arguably hindered the spatial spreading of cyanobacterial filaments by imposing the organism a more elaborated development process to fill the voids, surround sand particles and then connect them together steadily. This was recently evidenced by scanning electron microscopy for *Phormidium ambiguum* and *Scytonema javanicum* growing on sandy soils (Chamizo et al., 2018a). While on sandy loam soils the filaments formed a very packed filament network embedding sediments, on sandy soil the biocrusts structure showed frequent air spaces between sand grains which become entangled in a network. In addition to the decrease of albedo by surface darkening, biocrust development was supported by the common absorption feature around 670 nm due to chlorophyll *a*, which was absent in the control sand (Weber et al., 2008). Moreover, this absorption increased as the inoculum amount increased (Fig. 3c and Fig. 3d) demonstrating that crust spectral features can be used to assess crust development. To this regard, a recent paper has shown a strong correlation between chlorophyll content and different spectral indices in natural and induced biocrusts, supporting the possibility of using non-destructive soil surface reflectance measurements for chlorophyll quantification in biocrusts (Román et al., 2019).

We provided supporting evidence that increasing the initial inoculum can promote an effective biocrusts formation on CS. The application of a higher concentration of initial biomass reflected also in increased EPS amounts in cyanobacterial biocrusts (Table 4). Beside contributing

to sand gluing and stabilization, EPS most likely contributed to the increase in SWR, according to RI and WDPT test values (Table 3). This aspect was already observed in cyanobacterial biocrusts induced employing this strain (Mugnai et al., 2018b). This is important to a physiological level in hot drylands, as it extends water permanence in the first mm of soil to the availability of the microbial community, counteracting water loss by evaporation. In contrast, inoculation of other cyanobacterial strains such as *P. ambiguum* and *S. javanicum* on sandy soils was found to not significantly change surface hydrophobicity (Chamizo et al., 2018a). The presence of charged groups on the soluble EPS fractions determines the formation of viscous aqueous solutions (De Philippis and Vincenzini, 1998) that have more difficulty in seeping through the sand. It was previously reported that the EPS extracted from the biocrusts obtained by inoculating *L. ohadii* is characterized by a significant amount of galacturonic acid, especially in the fractions loosely bound to sand grains and filaments (Mugnai et al., 2018b). The complex nature of EPS synthesized by the strain is most likely a significant driver in the stabilization of the sand. In liquid culture, the polysaccharide is partly released in the culture medium (named released polysaccharide, RPS), and partly remain attached to the filaments (named glycocalyx polysaccharide, G-EPS), although not structured like a sheath or a capsule. By characterizing the two fractions for the first time, we observed differences in composition and MW distribution profiles. IEC revealed that although both fractions owned a similar richness in the number of different types of monosaccharides, they showed significant differences in their molar percentages. Notably, G-EPS resulted richer in glucose and ribose, but significantly poorer in rhamnose, galactose, mannose and xylose, than RPS. On the other hand, in RPS we detected marked levels (roughly 40%) of glucuronic acid, a carboxylic sugar that gives a strong negative charge to the polymer. The two fractions were also different in MW distribution profiles. RPS were prominently constituted by high MW polymers, of a molecular size between 2,000 and 1,100 kDa, while the G-EPS showed a MW distribution with three prevalent MW ranges: one between 2,000 and 1,000 kDa, a second between 410 and 150 kDa, and a third between 150 and 50 kDa. Such a diverse monosaccharide composition and MW distribution supports the existence of different supra-structures, and different capabilities to interact with the surrounding environment (Delattre et al., 2016). In the attempt to isolate only the contribute of EPS to biocrust formation, we applied alternatively G-EPS and RPS suspensions to microcosms containing MS and CS, in concentrations corresponding to those detected in cyanobacterial biocrusts induced by applying the three biomass concentrations. EPS application never resulted in the formation of stable aggregates, nor in detectable variations in SWR. This result enlightens how the sole presence of EPS would not suffice to explain most of what we observed concerning SWR, AS and tensile strength of the cyanobacterial biocrusts without considering the

major contribution given by the cyanobacterial filaments. The creation of a filament network is thus confirmed as a prerequisite to surface biological crusting (Chamizo et al., 2018b). However, we found a significant increase in the surface tensile strength in MS microcosms when applying the highest content of G-EPS, while no similar effect was observed employing RPS. We observed that the same amount of G-EPS was not enough to trigger the same effect on CS. The larger pore spaces in CS compared to MS, as previously discussed, probably limited the effect of EPS addition. However, the significant effect that we detected on MS after G-EPS addition suggests this EPS fractions as the most clearly contributing to sand structuration, although it was effective only on one sand type. It is hazardous to try to link EPS chemical composition to their behavior without information on the macromolecular secondary and tertiary structures. However, the detection of a dominant presence of glucose in G-EPS, combined with a large variety of sugar types, support the findings of Hu et al. (2003) that these characteristics are positively related with a stronger sand stabilizing capability. It was previously discussed how the presence of hydrophobic fractions enhances the capability of adhering to solid surfaces (Rossi et al., 2018). Also, G-EPS showed to be a more polydisperse polymer than RPS, being constituted of fractions belonging to different MW ranges. The majority (80%) of the characterized cyanobacterial EPS has an apparent MW of at least 1,000 kDa (Rossi and De Philippis, 2015). It is possible that the synthesis of EPS constituted by different fractions with different MWs is a factor enhancing the sand aggregating capability, better adapting to the uneven porosity of a coarser substrate. Indeed, Hu et al. (2003) demonstrated that the presence of high MW polysaccharides is not necessarily related to a better sand stabilization. The authors showed how *Microcoleus vaginatus*, a known natural cyanobacterial biocrust former often employed in inoculation studies, secretes an EPS with a MW of 380 kDa, and displays a higher sand stabilizing capability than *Nostoc* sp., employed in the same experiment, which produced an EPS with a MW of 460 kDa. On the other hand, *L. ohadii* synthesize RPS prominently characterized by macromolecules with an apparent MW of 1,000-2,000 kDa, and a marked content of glucuronic acid. Due to their higher hydrophilic characteristics, RPS contribute to bind and retain water molecules and nutrients (Rossi et al., 2012), likely playing the role of a selective filter and sustaining the strain in adverse stressing conditions (Swenson et al., 2018). The MW distribution of the two EPS fractions from *L. ohadii* is consistent with what we observed in a previous study in which we analyzed the soluble and the more condensed EPM fractions directly extracted from *L. ohadii* biocrusts. While the soluble EPM fraction was highly characterized by polymers with a MW > 1,100 kDa, the more condensed, less soluble and structural EPM fraction was polydisperse (Mugnai et al., 2018b). Contrastingly, a recent study analyzing the same aspects in cyanobacterial crusts induced by inoculating *P. ambiguum* and *S. javanicum* described a different scenario. The

more hydrophobic EPM fraction, more firmly attached to soil particles, was mainly composed of high MW molecules (1,100-2,000 kDa) while the more hydrophilic, which is released in the surrounding environment, was composed of low MW sugars (< 50 kDa) (Chamizo et al., 2018a). However, *L. ohadii* demonstrated a higher sand aggregation capability than *P. ambiguum* and *S. javanicum* which produced biocrusts with a lower tensile strength on a sandy substrate (Chamizo et al., 2018b). It is also possible that the employment of a strain capable of gliding allowed a wider distribution of EPS in the sand, possibly enhancing sand fixation. Gliding strains migrate hydrotactically (Garcia-Pichel and Pringault, 2001), or phototactically according to other sources (Wilde and Mullineaux, 2017), horizontally and/or vertically (Nadeau et al., 1999), leaving behind a trail of sticky polysaccharidic material (Hoiczyk, 2000) constituted both by G-EPS and RPS. We previously deducted the capability of *L. ohadii* to move downwards in the microcosms, allowing a vertical distribution of the cementing biomaterial, contributing to determine cyanobacterial biocrusts with a notable thickness compared to other inoculants (Mugnai et al., 2018b).

In conclusion, we demonstrated how the evaluation of the optimal amount of biomass to apply is key in the success of inoculum establishment on the sand, and the development of cyanobacterial biocrusts. This is particularly important when treating substrates with varying granulometry in which coarse particles abound, as compared to finer substrates. We advise to apply an inoculum of at least 0.75mg CDW/cm^2 of biomass when inoculating coarse substrates. The increase of inoculum amount coincided with the presence of a higher EPS content in the sand, which contributes to sand fixation. We also underlined the more prominent role of G-EPS fraction, in other strains organized as sheaths or capsules around the trichomes, in sand fixation. Further studies are needed to provide further confirmation of our observation that the large content of glucose, the low relative abundance of uronic acids, and the polydisperse MW of G-EPS can be considered markers of a propensity of the strain to develop consistent cyanobacterial biocrusts. On the other hand, RPS, more hydrophilic, are probably more involved in determining SWR. To obtain consistent cyanobacterial biocrusts, EPS excretion must be coupled with the creation of a filament network. Finally, the capability of the strain to glide seems to positively affect the strain performance.

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791 **Figure 1. Monosaccharidic composition and MW distribution of EPS produced by *L. ohadii*. a)**
792 Monosaccharide composition of RPS (dark bars) and G-EPS (white bars), expressed as internal molar %.
793 *Fuc*, fucose; *Rha*, rhamnose, *GalN*, galactosamine; *Ara*, arabinose, *GlcN*, glucosamine; *Gal*, galactose; *Glc*,
794 glucose; *Man*, mannose; *Xyl*, xylose; *Fru*, fructose; *Rib*, ribose; *GalA*, galacturonic acid; *GlcA*, glucuronic
795 acid. **b)** MW distribution of RPS (dark bars) and G-EPS (white bars). Different letters indicate a significant
796 difference between G-EPS and RPS ($P < 0.05$).
797

798 **Figure 2. Biomass dispersion approach and biocrusts development. a)** exemplification of the "double
799 spiral dispersion" inoculation methodology employed in this study. **b)** cyanobacterial biomass evenly
800 dispersed on the microcosm surface at t = 0; **c, d)** upper view of cyanobacterial biocrusts at t = 30 d
801 developed on MS (c) and CS (d); **e, f)** thickness of the cyanobacterial biocrusts on MS (e) and CS (f) at t =
802 30 d. MS: medium sand (granulometry 0.3 – 0.6 mm); CS: coarse sand (granulometry 0.8 – 1.25 mm).
803

Figure 3. Reflectance analysis on biocrusts at t = 30 d. Reflectance curves (average of three independent replicates) of biocrusts (three inoculum amounts, 0.15, 0.45 and 0.75 mg CDW/cm^2) and controls on MS **(a)** and CS **(b)**; spectral absorptions measured on the biocrusts and on the controls on MS **(c)** and CS **(d)**.

Table S1. Chemical and mineralogical composition of the sand used as substrate in the microcosms.

Chemical composition (%)	
SiO_2	83.3
Fe_2O_3	2.1
Al_2O_3	6.6
CaO	1.2
MgO	1.5
Na_2O	2.0
K_2O	2.1
Mineralogical composition (%)	
Quartz	61.8

Granitic rocks	16.5
Feldspars	12.7
Others (traces)	9.0

Table S2. Experimental design summarization. Number of microcosms employed in the two experimental phases. Phase 1: inoculation of the biomass on MS and CS microcosms; incubation for 15 and 30 d. Phase 2: Application of the sole EPS (RPS or G-EPS) in liquid suspensions to MS and CS microcosms; incubation for 30 d. Control: non-inoculated MS and CS microcosms.

Phase 1 (biomass application)					Phase 2 (EPS application)			
Sand type	0.15 mg CDW/cm^2	0.45 mg CDW/cm^2	0.75 mg CDW/cm^2	Control	6.8 µg EPS/cm^2	13.5 µg EPS/cm^2	27.1 µg EPS/cm^2	Control
MS	6*	6*	6*	6*	6$^{\#}$	6$^{\#}$	6$^{\#}$	3
CS	6*	6*	6*	6*	6$^{\#}$	6$^{\#}$	6$^{\#}$	3

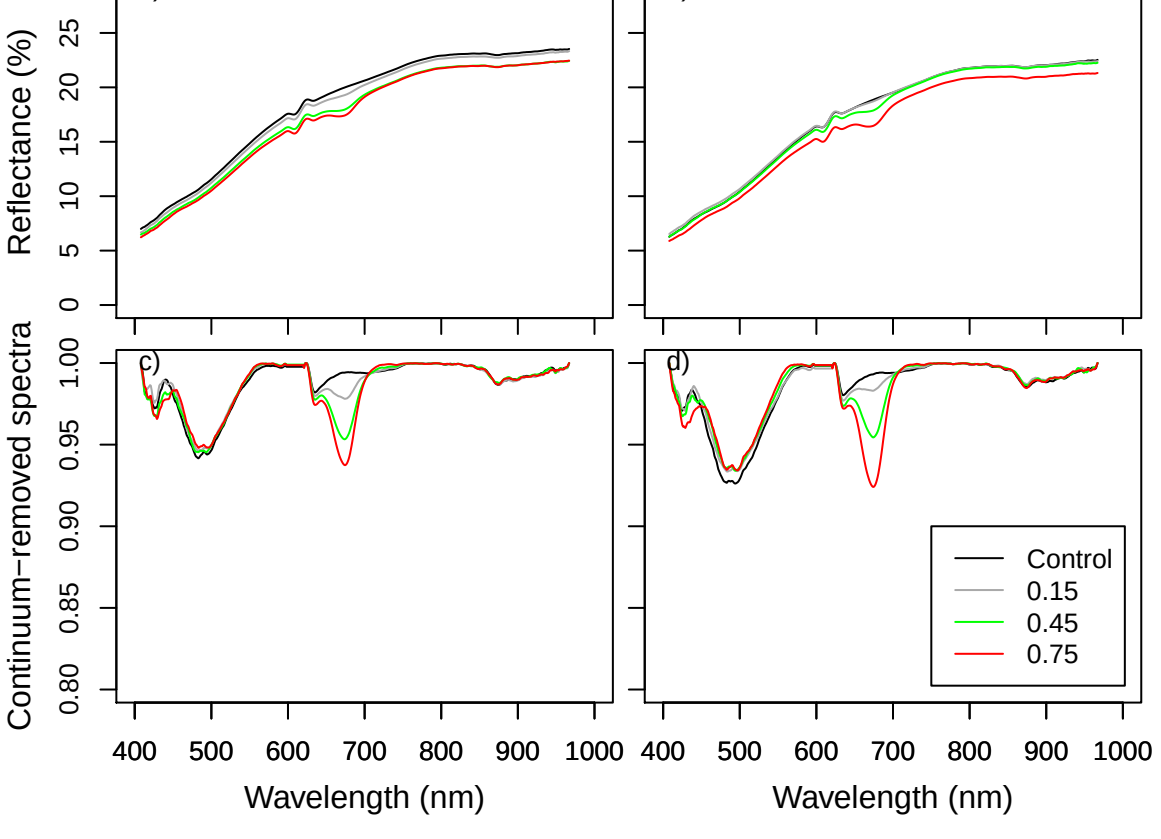
**3 microcosms collected and analyzed at t = 15 d, 3 microcosms collected and analyzed at t =30 d.*

$^{\#}$3 microcosms inoculated with RPS and 3 microcosms inoculated with G-EPS, all incubated for 30 days.

Table S3. Repellency index measured after the application of the sole EPS assessed at t = 30 d. The applied quantities are expressed as microgram of EPS (RPS or G-EPS) over square centimeter of medium sand (MS) or coarse sand (CS). Data are expressed as mean ± (SD).

EPS amount	**RPS (CS)**		**RPS (MS)**		**G-EPS (CS)**		**G-EPS (MS)**	
6.8 µg/cm^2	0.74	(0.13)	0.78	(0.04)	0.74	(0.13)	0.78	(0.04)
13.5 µg /cm^2	0.49	(0.08)	1.04	(0.26)	0.67	(0.08)	0.84	(0.04)
27.1 µg/cm^2	0.62	(0.16)	0.69	(0.09)	0.78	(0.08)	0.69	(0.06)
Control	0.71	(0.11)	0.73	(0.07)	0.71	(0.11)	0.73	(0.07)

- Cyanobacterization success requires an optimal initial inoculum amount
- Medium sand is more conductive to biocrust stability than coarse sand
- *L. ohadii* EPS alone cannot elicit sand aggregate stability and water repellency
- G-EPS appear as the fraction more involved in biocrust physical stability


Reflectance (%)
0
5
10
15
20
25
c)
d)
Continuum−removed spectra
0.80
0.85
0.90
0.95
1.00
Control
0.15
0.45
0.75
400
500
600
700
800
900
1000
Wavelength (nm)
Wavelength (nm)

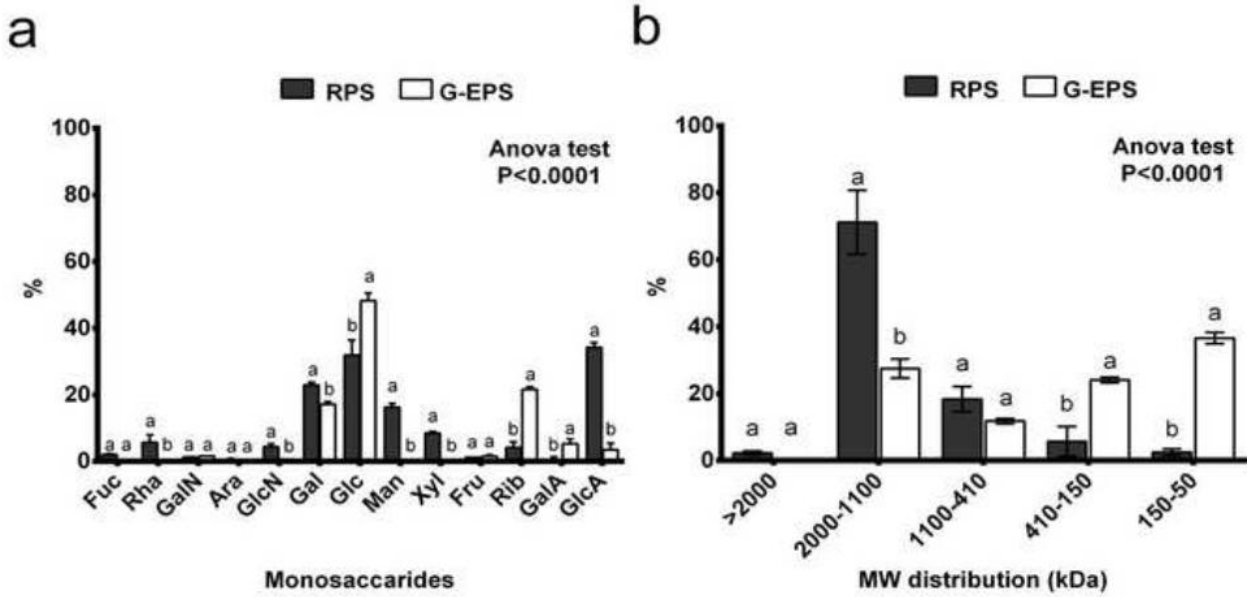

a
RPS
G-EPS
Anova test
P<0.0001
%
Fuc
Rha
GalN
Ara
GlcN
Gal
Glc
Man
Xyl
Fru
Rib
GalA
GlcA
Monosaccarides
b
RPS
G-EPS
Anova test
P<0.0001
%
>2000
2000-1100
1100-410
410-150
150-50
MW distribution (kDa)

Fig. 1

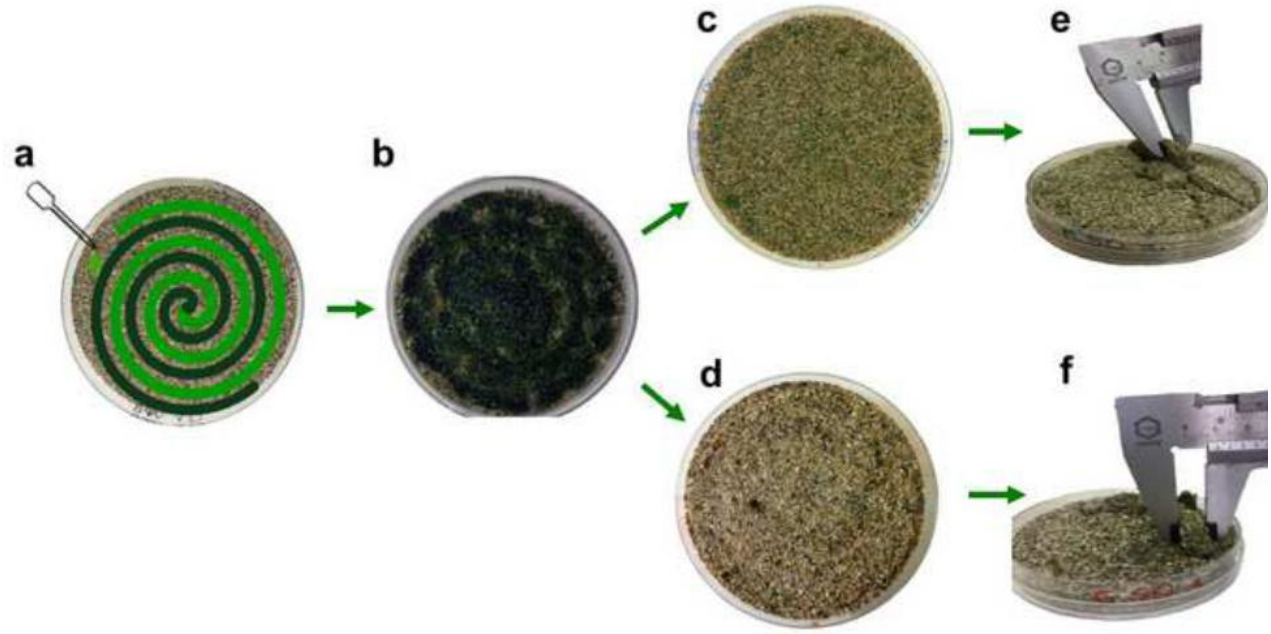


Fig. 2