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DEVELOPMENT OF INDUCED BIOLOGICAL SOIL CRUSTS AND THEIR EFFECT ON SOIL PROPERTIES

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person no material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Dichiarazione

Con la presente affermo che questa tesi è frutto del mio lavoro e che, per quanto io ne sia a conoscenza, non contiene materiale precedentemente pubblicato o scritto da un'altra persona né materiale che è stato utilizzato per l'ottenimento di qualunque altro titolo o diploma dell'Università o altro istituto di apprendimento, a eccezione del caso in cui ciò venga riconosciuto nel testo.

Riassunto

Scopo: Lo scopo della presente tesi è stato quello di investigare l'effetto dell'inoculo di cianobatteri sulla struttura e lo sviluppo di croste biologiche indotte durante i primi stadi di colonizzazione di suoli sabbiosi.

Metodi e Risultati: La sperimentazione è stata condotta in condizioni di laboratorio, su diverse croste cianobatteriche ottenute mediante inoculazione di due differenti ceppi cianobatterici filamentosi, *Schizothrix* cf. *delicatissima* AMPL0116 e *Leptolyngbya ohadii*, entrambi isolati da croste biologiche naturali campionate nel Deserto Hopq (Mongolia Interna, Cina) e nel Deserto del Negev (Israele), rispettivamente. L'inoculazione è stata condotta in microcosmo, mediante una metodologia preventivamente progettata e ottimizzata di dispersione della biomassa. Dopo aver determinato la strategia di dispersione più fattibile ed efficace, i microcosmi sono stati incubati in condizioni di stress all'interno di una camera di crescita, in assenza di nutrienti e con limitata disponibilità di acqua. Durante la loro crescita su suolo sabbioso, i cianobatteri secernono grandi quantità di esopolisaccaridi (EPS) che incollano i tricomi alle particelle di suolo, in una matrice polimerica extracellulare tridimensionale, inducendo la formazione di croste relativamente spesse in poche settimane. I risultati hanno evidenziato una pronunciata capacità di *S. delicatissima* AMPL0116 e *L. ohadii* di compattare la sabbia fine, suggerendoli come inoculanti idonei per prevenire l'erosione del vento e dell'acqua su suoli non consolidati.

Conclusioni: Questo studio evidenzia notevolmente il ruolo fondamentale degli EPS prodotti dai i due ceppi cianobatterici nella determinazione dell'aggregazione e stabilità del suolo durante i primi stadi della colonizzazione del suolo nudo sabbioso.

Impatto del lavoro: Con la presente tesi di dottorato è stato compiuto un importante passo avanti a livello di laboratorio nella comprensione del ruolo svolto dalla secrezione di EPS, un processo che ha portato alla colonizzazione e alla stabilizzazione del suolo nudo, aumentando la disponibilità di acqua e nutrienti per la microflora risedente nelle croste biologiche. I risultati ottenuti in questa tesi potrebbero portare a considerare i due ceppi cianobatterici come candidati idonei per una futura applicazione direttamente in campo al fine di contrastare gli effetti della desertificazione e riabilitare ambienti degradati.

Abstract

Aim: The objective of the research in this thesis was to investigate the effect of inoculated cyanobacteria on the structure and development of induced biological soil crusts during the first stages of the colonization of sandy soils.

Methods and Results: The research was conducted, under laboratory conditions, on several induced cyanobacterial crusts obtained by inoculating two different filamentous cyanobacteria, *Schizothrix* cf. *delicatissima* AMPL0116 and *Leptolyngbya ohadii*, both isolated from natural biocrusts collected in Hopq Desert (Inner Mongolia, China) and Negev Desert (Israel), respectively. Inoculation was conducted in microcosms, preventively designing and optimizing biomass preparation and dispersion methodology. After determining the most feasible and effective dispersion strategy, microcosms were incubated under stressing conditions in a growth chamber, in the absence of nutrients and with a limited water availability. During their growth on sandy soils, cyanobacteria excreted large amount exopolysaccharides (EPSs) which glued trichomes to soil particles, in a three-dimensional extracellular polymeric matrix, inducing the formation of a relatively thick crusts in a few weeks. The results pointed out at a pronounced capability of *S. delicatissima* AMPL0116 and *L. ohadii* to compact fine sand, and suggest the strains as suitable inoculants for unconsolidated soils for preventing erosion due to wind and water precipitation.

Conclusion: This study prominently points out the paramount role of EPSs produced by the cyanobacterial strains in determining the soil aggregation and stability during the first stages of the colonization of bare sandy soils.

Impact of the study: In this thesis, an important step forward was made at laboratory scale on the understanding the role of the EPSs secretion, a process that lead to the colonization and the stabilization of bare soils, enhancing the availability of water and nutrients for the microflora residing in the biocrust. The results obtained in this thesis could lead to consider the two cyanobacterial strains as suitable inoculants

candidates for a future application directly in the field in order to counteract the effects of desertification and to rehabilitate degraded environments.

PUBBLICATIONS ON INTERNATIONAL JOURNALS:

- **Mugnai, G.,** Rossi, F., Felde, V.J.M.N.L., Colesie, C., Büdel, B., Peth, S., Kaplan, A., De Philippis, R., 2017. Development of the polysaccharidic matrix in biocrusts induced by a cyanobacterium inoculated in sand microcosms. Biology and Fertility of Soils. doi:10.1007/s00374-017-1234-9.

- Rossi, F., **Mugnai, G.,** De Philippis, R., 2017. Complex role of the polymeric matrix in biological soil crusts. Plant and Soil. doi:10.1007/s11104-017-3441-4.

ABSTRACTS

- De Philippis, R., Rossi, F., Swenson, T., Couradeau, E., **Mugnai, G.,** Northen T. (2017). Extracellular polymeric matrix in biocrusts: its role as a repository for essential exometabolites. In 4th International Conference on Microbial Diversity 2017 - Drivers of microbial Diversity (MD2017). Bari; Italy, October 24th-26th, 2017.

- Chamizo De la Piedra, S., **Mugnai, G.,** Rossi, F., Ciani, M., Pastacaldi, C., De Philippis, R. (2017). Increasing soil stability and fertility through cyanobacteria inoculation to combat land degradation processes in drylands. In Drylands, Deserts and Desertification 2017 (DDD), Sede Boqer, Israel, 6th-9th November 2017.

- Chamizo De la Piedra, S., **Mugnai, G.**, Rossi, F., Roncero-Ramos, B., Román, J.R., Rodríguez-Caballero, E., Cantón, Y., De Philippis, R. (2017). Inducing biocrust development by cyanobacteria inoculation to restore dryland soils. In

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- **Mugnai, G.**, Rossi, F., Chamizo De la Piedra, S., De Philippis, R. (2017). Inducing the formation of cyanobacterial crust by cyanobacterization: elaboration and optimization of a proficient cyanobacteria inoculation methodology. In: 1st World Conference on Soil and Water Conservation Under Global Change (CONSOWA), Lleida, Spain, 12th-16th June 2017 (poster).

- **Mugnai, G.**, Rossi, F., De Philippis, R. (2017). Cyanobacterial crust induction using two non-previously tested cyanobacterial inoculants: crusting capability and role of EPSs. In: European Geosciences Union (EGU2017), Vienna, Austria, 23th-28th April 2017 (**oral presentation**).

- **Mugnai, G**., Rossi, F., Felde, V.J.M.N.L., Viti, C., De Philippis, R. (2016). Effect of inoculated cyanobacteria on the structure and development of induced biological soil crust. In 3rd International Workshop on Biological Soil

Crusts (BIOCRUST3), Moab, Utah, USA, 26th – 30th September 2016 (**oral presentation**).

MANUSCRIPTS IN PREPARATION:

- **Mugnai, G.,** Rossi F., Chamizo De la Piedra, S., De Philippis, R., 2017. Inducing the formation of cyanobacterial crust by cyanobacterization: elaboration and optimization of a proficient cyanobacteria inoculation methodology.

- **Mugnai, G.,** Rossi, F., Felde, V.J.M.N.L., Colesie, C., Büdel, B., Peth, S., Kaplan, A., De Philippis, R., 2017. Effect of different water regimes on the development of cyanobacterial crusts induced in a microcosm experiment by cyanobacteria inoculation.

AWARDS DURING THE PHD:

- Full scholarship, including travel, lodging and conference registration fee, for the participation to the 3rd International Workshop on Biological Soil Crusts (BIOCRUST3), Moab, Utah, USA, 26th-30th September 2016.

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Preface

This PhD thesis was focused on improving the knowledge regarding the cyanobacterization technique and the role of EPS in biocrusts. In my thesis, I tested two so far never used inoculants, *Schizothrix* cf. *delicatissima* strain AMPL0116 and a strain of *Leptolyngbya ohadii*, for carrying out a large number of inoculation experiments in microcosms aimed at fulfilling some key knowledge gaps concerning the inoculation methodology, the formation of cyanobacteria-sand aggregates during the development of cyanobacterial crusts, and the release of the extracellular polymeric matrix (EPM) synthesized by the inoculants.

The introduction section aims to provide a solid background for the experiments. The experimentation history in the field of cyanobacterization was enlightened, pointing out at the potential flexibility of this technology, which can be employed either to improve the productivity of agricultural soils or to counteract land degradation in arid and semiarid environments. In doing so, the cornerstone aspects of the process and the existing knowledge gaps were outlined. A particular focus is put on the characteristics and the role of EPM, that provides a wide array of services to the complex soil communities developed following cyanobacterial colonization.

The central body of the thesis is structured in chapters corresponding to as many manuscripts, either published, submitted or on the way to be submitted. Each chapter deals with different specific aspects, in particular with:

I) The dependence of cyanobacterial growth from sand granulometry, an issue previously only investigated by Rozestein et al. (2014). In this chapter, an exhaustive study on how the dimensions of sand grains affect the success of soil colonization by the inoculated cyanobacterium is reported.

II) The possibility of performing the inoculation of sand collected in the Negev Desert, Israel, with the allochthonous strain *S. delicatissima* AMPL0116. This study explores the possibility of inoculating a soil with strains not necessarily isolated from the same environment, contrary to most of the previously existing studies (Rossi et al., 2017), and extensively describes the process of sand colonization and EPS release into the soil.

III) The capability of a desert-isolated strain of *L. ohadii* to form cyanobacterial crusts and the dependence of the process from the way biomass was distributed on the microcosms and from the amount of water provided. The latter information may have an enormous importance in view of designing and evaluating the economical feasibility of future large-scale treatments based on the inoculation of cyanobacteria in desert soils. Indeed, watering can be a necessary and costly support for the success of this technology, but there are no information as whether it can be reduced, or even avoided, only relying on the non-rainfall water resources to support the colonization of soils by cyanobacterial inoculants. This was extensively investigated and described in this chapter.

Collectively, in the manuscripts are reported the methods specifically developed for measuring the increase in compressive strength, aggregate stability and water repellency at the sand surface due to the formation of the crusts during the incubation time. Moreover, a methodology to extract, quantify, and analyze the EPM components having different gelification grades and condensation was developed, improving and implementing pre-existing methodological approaches (the workflow to the final methodology is stressed out in the experimental method section of the thesis). In this way, the formation of the EPM, which is a key component of cyanobacterial crusts on which the crusts rely on for forming and enduring, was for the first time observed and studied even during the first delicate phases of development. In addition, the

application of the analytical methods allowed to detect the factors affecting the characteristics of the EPM, and its amount in the cyanobacterial crusts.

The experimental work reported in this thesis and the data obtained with the two different inoculants represents a key contribution for refining and implementing this biotechnology aimed at counteracting soil loss and at reverting desertification processes, even in highly abiotic stressing environments.

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1. Introduction

1.1 Cyanobacterial inoculation techniques and development of biotechnological approaches for soil improvement.

Microorganisms are the largest and most diverse biotic group in soil (Wani et al., 2015). They are an integral part of soil ecosystem and their activity is very important for soil structure maintenance and functionality (Winding et al., 2005). Although the biological component accounts only for a tiny fraction (<0.5%) of the total soil volume, microorganisms are key players in the cycling of nitrogen, sulphur, and phosphorus, and in the decomposition of organic residues (Nielsen and Winding, 2002). Microorganisms and their organic products (especially extracellular polysaccharides) contribute to the development of soil structure. They also affect water holding capacity, infiltration rate, crusting, erodibility, and susceptibility to compaction (Elliott et al., 1996).

The ecology, activity and population dynamics of microorganisms in soil can be affected by several factors. Those include: the availability of nutrients, carbon and energy sources, mineral nutrients, growth factors, ionic composition, available water, temperature, pressure, air composition, electromagnetic radiation, pH, oxidation–reduction potential, surfaces, spatial relationships, genetics and interactions between microorganisms (Nannipieri et al., 2003). Microbial communities adapt differently to environmental fluctuations, by physiological adjustments by varying individual activity, and by favouring the perpetuation of those species having more favourable characteristics (Winding et al., 2005). There is a limited number of microbial groups that are able to withstand a wide range of environmental conditions, and affect soil processes despite the contingent dominant stressing forces.

Among them, cyanobacteria are oxygenic phototrophic microorganisms that are able to colonize many nutrient-poor and abiotically stressing substrates and are

remarkably well adapted to a wide range of environmental conditions (Issa et al., 2007). Their eco-physiological resilience and high tolerance to stresses such as high irradiance, salinity and temperature are responsible for their capability of colonizing a huge variety of habitats where they are largely responsible for providing the most important ecosystem services. Due to their high pervasiveness, they can be encompassed between microbial ecosystem engineers (Jones et al., 1994). They modulate the availability of resources to other species and cause physical state changes in biotic or abiotic habitat components (Jones et al., 1997). They are considered the most successful forms of life ever to have emerged, and they have had a major influence on Earth for at least the last 2.95 billion years (Planavsky et al., 2014; Whitton, 2012). Phylogenetic analysis of 16S rRNA genes showed that they are a diverse, monophyletic phylum of organisms within the bacterial radiation (Abed et al., 2009). They exist in different morphologies including unicellular and filamentous forms (Castenholz et al., 2001). The unicellular types exist as single cells, suspended or benthic, or aggregates, while the filamentous types may be thin or thick, single trichome or bundles either with or without a sheath (Abed et al., 2009).

One major ability of cyanobacteria is the excretion of large amounts of extracellular polymeric substances (EPSs). The excretion of EPSs by microorganisms is a key physiological step allowing engineering processes (West, 1990). EPS excretion fulfils a variety of different roles: it mediates the attachment of organisms to sediments to form biofilm on solid surfaces, affects water infiltration, percolation, retention and evaporation of water in and from the soil, reduces soil erosion, and affects seedling emergence (Jones et al., 1994). Also, EPSs serve as a boundary between the bacterial cell and its immediate environment, protecting the cells against unfavourable factors (e.g desiccation, extreme temperature, salinity, UV irradiance), antibacterial agents (e.g. antibiotics, antibodies, bacteriocins, phages, phagocytic cells, surfactants), or predation by protozoans (De Philippis and Vincenzini, 1998).

In the past, several studies were focused on the potential use of cyanobacteria and microalgae as bioconditioners and biofertilizers (Barclay and Lewin, 1985; Falchini et al., 1996; Hamdi, 1982). The practice of using cyanobacteria as soil

improvers and fertilizers (cyanobacterization) has been known and studied since the second half of 1900's. In preliminary experiments conducted in Japan in the 1950's, cyanobacteria were employed as a "green manure", and the decomposition of the inoculated nitrogen-fixing cyanobacteria by the action of various soil bacteria increased the N-source in the field (Watanabe and Kiyohara, 1959).

There is considerable amount of literature indicating that this practice can address multiple purposes that include: increase in crop yields (Prasanna et al., 2012), improvement of soil quality (Maqubela et al., 2009), protection from plant diseases (Prasanna et al., 2013), reclamation of salt-affected soils (Prasanna et al., 2008), bioremediation of polluted soils (Wolicka et al., 2009), protection of soil surface from erosion (Falchini et al., 1996), prevention of weed growth (Saadatnia et al., 2009), seedling emergence (Rogers and Burns, 1994) and restoration of soils damaged by fire (Acea et al., 2003). Since the 1950's (earliest source available), most of the research on the potential use of cyanobacterization has been focused on producing improvement of soil quality, soil health, as well as increasing yields and quality of crops. The application of nitrogen-fixing cyanobacteria as green fertilizers has been widely studied in a large number of field trials. Studies particularly focused on the development of cheap and easily applicable technologies for developing countries (e.g., Hamdi, 1982; Kaushik, 2009), representing also a easily accessible tool even for small farmers (D'Acqui, 2016). During the 1960's and the 1970's, these types of studies were partially supplanted by the increasing use of chemical fertilizers and pesticides, that were more practically and economically viable although not eco-friendly. Recently, from the early 2000's, problems associated with the use of chemicals in agriculture set the scientific attention on elaborating ecologically-friendly methods to support a sustainable agriculture (Singh et al., 2011).

Alongside interest in cyanobacterization-based approaches for the management of agriculture soils, an additional emerging source of interest in cyanobacterization is the possibility of using this methodology to limit and possibly reverse the effects of desertification in arid and semiarid lands (Lan et al., 2014, see section 1.1.2).

1.1.1 Direct and indirect effects of cyanobacterization on soil productivity and fertility.

Soil enrichment with cyanobacteria has two main classes of effects: *i*) it can enhance soil productivity and fertility, and *ii*) improve soil chemo-physical characteristics.

Notwithstanding some uncertainties concerning the relationships between cyanobacteria and plants, much of the positive effects on soil fertility is certainly due to the immission in the soil, by cyanobacteria, of a wide array of substances. Prominently, they release C (derived by CO_2 organication through photosynthesis) and N. N fixation activity, incompatible with the presence of O_2, is carried out by some species by differentiating heterocysts (spatial separation) and by fixing N during the night by others (temporal separation). Next, they release phytormones, vitamins, aminoacids and phosphorus (Abdel-Raouf, 2012; Rodríguez et al., 2006). As an indirect mean of affecting plant growth and metabolism, cyanobacteria have a positive effect on the beneficial members of plant endophytic microbial community (Priya et al. 2015), and the enhancement of soil enzymatic activities (Hu et al., 2012). According to Singh et al. (2016), cyanobacteria also contribute in: *i*) enhancing the solubilization and mobility of nutrients, *ii*) limiting the mobility of heavy metals and xenobiotics, *iii*) mineralizing simple organic molecules, *iv*) protecting plants from pathogens and diseases, *v*) stimulating plant growth and *vi*) improving soil quality. Saadatnia et al., (2009) cite also the decrease in soil salinity and the prevention of weed growth, while others (Mandal et al., 1999; Prasanna et al. 2008) cite curbing of ammonia volatilization, reduction of methane emission, reduction of oxidizable organic matter and pesticide degradation.

According to field trial studies, cyanobacterization might be an effective tool for different cultures, including rice, wheat, cotton, legumes and vegetables (Manjunath et al., 2016). Effects were particularly detected concerning cultivation yields, seed germination, seedling growth, quality and number of grains per panicles, plant size, number of tillers, ears and spikelets (Gupta and Lata, 1964; Vaishampayan et al., 2001). In a major number of studies, the employment of members of the genus

Anabaena, *Aulosira*, *Nostoc*, *Calothrix* and *Tolypothrix* resulted beneficial to soil microbial population, pH, electrical conductivity (EC), redox potential, enzymatic activity, crop growth and nitrogen provision (Hamdi, 1982; Maqubela et al., 2010; Swarnalakshmi et al., 2007). Some authors reported N gains of 80 kg/N per hectare, and increases in crop yields reaching 114.8% (Singh, 1961). In one study (Sankaram, 1971), the use of *Tolypothrix tenuis* provided a rice yield comparable to that utilizing 45 Kg N/ha. More recently, de Mulé et al. (1999) demonstrated that *T. tenuis* addiction to rice seedlings contributed hugely to C increase (241%). On the other hand, some authors registered not significant effects of cyanobacterization (Grant et al., 1985; Irisarri et al., 2006).

The capacity of enriching the soil is coupled with the capability of improving soil quality and structure. Through the production of EPSs, cyanobacteria promotes the formation of stable sediment macro-aggregates (Falchini et al., 1996; Malam-Issa et al., 2007). Both Chesters et al. (1957) and Greenland et al. (1962) identified polysaccharides as the paramount players in soil aggregation, while they were recognized by Chenu (1995) as important in soil formation. The importance of EPS excretion in cyanobacterization was pointed out more recently by Malam-Issa et al. (2007) and Maqubela et al. (2010), while Hu et al. (2003a) observed a positive correlation between EPS productivity and sand stabilization. The synergistic action of cyanobacterial filaments and EPSs concur in increasing soil aggregate stability, that is an index of soil degradation (Cerdà, 2000), counteracting the action of wind and water erosion. Cyanobacteria filaments and EPSs have been recognized to promote the coating, enmeshment, binding and gluing of aggregates and isolate mineral particles (D'Acqui, 2016), in addition to promoting the connectivity between aggregates and associated pore network spaces (Chenu and Cosentino, 2011).

Beside consolidating the structure of cyanobacterial biofilm and soil structure, EPSs can condition soil/water relations and water distribution. They constitute a gelatinous component that regulates water uptake and water loss (Colica et al., 2014). EPSs can influence water distribution affecting soil sorptivity and hydraulic conductivity (Rossi et al., 2012). It is commonly accepted that EPSs are essential in

conferring drought and freeze stress tolerance to cyanobacteria which can preserve photosynthetic activity even in semiarid and arid environments (Tamaru et al., 2005).

1.1.2 Cyanobacterization to counteract desertification.

The growing international awareness of the fast pace of climate change, together with the constant increase in the extension of land degradation, claimed scientific attention toward mitigating low-cost biotechnological approaches. The control of desertification is among the largest environmental challenges and it highlights the complexity and urgency of management plan in drylands (Reynolds et al., 2007). The United Nations (UN) used the term desertification to indicate "land degradation in arid, semi-arid and dry sub-humid areas resulting from various factors, including climatic variations and human activities'' (UNCCD., 1994). Desertification produce changes in soil properties, vegetation or climate, which results in a persistent loss of ecosystem services that are fundamental to sustain life (D'Odorico et al., 2013). This "long-term" phenomenon affects large dryland areas around the world. Understanding how drylands might respond to the alterations in climate, land cover, and land use, is extremely important for global sustainability (Maestre et al., 2016).

Drylands cover about 41% of the Earth's land surface and are home to about 2 billion people live (Table1), of which approximately 72% live in developing countries while only 28% live in industrialized countries (Fig. 1) (Yirdaw et al., 2017).

Table 1. Degraded area and population distribution in dryland zone.

Dryland zones	Size of the area (million km^2)*	Share of global area (%)*	Degraded area (million km^2)**	Degraded area (%)	Population***	Population density (km^2)
Dry sub-humid	12.96	8.7	2.5	19.5	909,972,000	70
Semi-arid	22.67	15.2	4.8	21.2	855,333,000	38
Arid	15.17	10.6	4.5	28.7	242,780,000	16
Total dryland	50.80	34.5	11.8	23.1	2,008,085,000	40

Sources:

*UN 2011. Global drylands: a UN system-wide response. Environment management Group. 132p. http://www.unccd.int/Lists/SiteDocumentLibrary/Publications/Global-Drylands-ENG.pdf.
**Zika and Erb 2009.
***UNCCD 2011.Desertification: a visual synthesis. Bonn. 50 p.

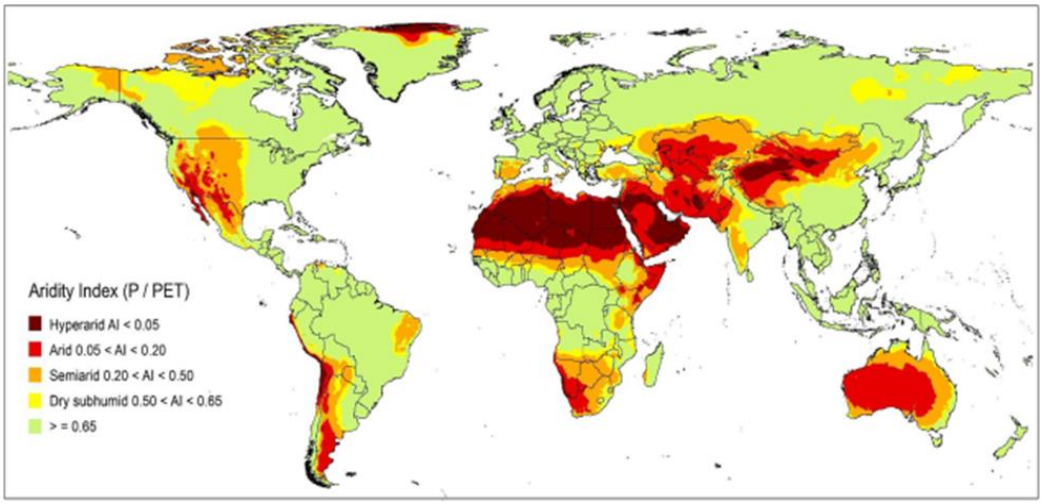

Fig. 1 Global map of estimated aridity index (AI) with a spatial resolution of 10 arc minutes. The AI is defined as the ratio of yearly precipitation to average yearly potential evapotranspiration by the United Nations Environmental Programme. The classification of drylands is: hyperarid AI < 0.05; arid 0.05 < AI < 0.20; semi-arid 0.20 < AI < 0.50 and dry sub-humid 0.50<AI<0.65. Data of the AI were obtained from the global aridity map of the FAO (2014) (Available at: http://www.fao.org/nr/aquastat) (Hu et al., 2017).

It is estimated that 10–20% of drylands are already degraded and about 12 million ha are degrading each year (Reynolds et al., 2007). One of the most recent climatic projections suggests an increase in the extent of global drylands that will expand by 23% and 11% and will respectively cover a total of 56% and 50% of the global land surface by the end of this century (Huang et al., 2015). Approximately 78% of the newly expanded dryland areas will be located in developing countries, where drylands will extend for approximately 61% of these countries area by the end of the twenty-first century (Huang et al., 2015).

In this context, understanding the drivers of land degradation, which includes both natural and social factors, is challenging due to the complex nature of degradation causes and to the complicated interactions between them (Nkonya et al., 2016).

Desertification occur when principal drivers of land degradation such as erosion by water and wind, chemical degradation (including acidification, salinization, fertility depletion, and decrease in cation retention capacity), physical degradation (comprising crusting, compaction, hard-setting, etc.), and biological degradation (reduction in total and biomass carbon, and decline in soil biodiversity) (Sivakumar, 2007) exceed the threshold of ecosystem resistance and resilience (Yirdaw et al., 2017) (Fig. 2).

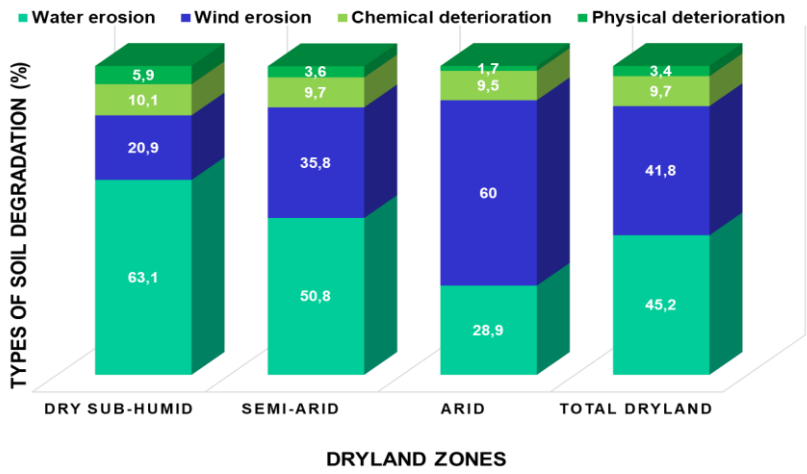

Fig. 2 Drivers to soil degradation in dryland areas. (Data source: UNEP1997).

In addition, other drivers are unsustainable land management such as livestock overgrazing, deforestation, mining, bush encroachment, invasion by non-native plant species, intensive agricultural activity, forest fires and urbanization (Kosmas et al., 2014; Yirdaw et al., 2017). Desertification has a critical impact on rangeland biodiversity, soil erosion, soil nutrient cycling and hydrological processes further diminishing the ability of drylands to provide life-supporting ecosystem services under climate change. Also, income losses to farmers and pastoralists due to loss of land productivity also entail costs for land rehabilitation (Zika and Erb, 2009).

Soil reclamation in drylands and in areas prone to desertification can be achieved using biological, chemical and physical means. Among the possible solutions, cyanobacterization was deemed a promising biotechnological tool to address dryland restoration and rehabilitation (Issa et al., 2007; Pandey et al., 2005).

Several studies reported that successful cyanobacterization in arid and semiarid environment, induced the formation of cyanobacterial-dominated biolayers (defined cyanobacterial crusts), which can further develop to biological soil crusts (BSCs). To distinguish these artificial communities from the naturally developed ones, in some studies they are called man-made, or induced biological soil crusts (IBSCs, Chen et al., 2006; Lan et al., 2014; Wang et al., 2009).

Natural BSCs are important components of arid and semiarid soils, although they are diffused in a wide array of environments and climates. They are constituted by a complex soil-surface communities, constituted by a phototrophic fraction, represented by cyanobacteria and microalgae, and a heterotrophic fraction, represented by different bacterial and fungal taxa. In addition, plants such as mosses and liverworts can be recruited in these communities. Depending on the degree of the BSCs development, they are also constituted by bryophytes and lichens (Belnap and Lange, 2002). Based on the dominant species, BSCs can be classified as algae and cyanobacterial crusts, lichen crusts and moss crusts (Lan et al., 2013).

BSCs are involved in several ecologically keystone processes in drylands. Those include increasing soil stability, reducing wind and water erosion, increasing moisture and nutrient content at the topsoil, and establishing complex relations with

plants, influencing their establishment and growth (Bowker, 2007; Pointing and Belnap, 2012). Most notably, BSCs determine the distribution of sinking and shedding areas in desert ecosystems by affecting water penetration (Rossi et al., 2012b) and play a vital role in biogeochemical cycles and geomorphological process in desert ecosystems (Zhang et al., 2009).

Given their ecological importance, many studies have focused on introducing BSCs, or BSC material for halting and reverting land degradation processes, fostering the re-establishment of vegetation in arid and semi-arid regions (Hu and Liu, 2003). The transplant of crust fragments (e.g., crust slurries) was successfully attempted, proving beneficial as a restoration tool (Belnap, 1993; Bowker, 2007; Maestre et al., 2006). Nonetheless, this approach is hampered by the difficulty of producing inoculum for large scale treatments, and the need to exploit sacrificial areas to obtain it (Rossi et al. 2017). In this sense, the option of inducing BSC formation through cyanobacterization appears more viable for large-scale treatments.

The capability to grow on unconsolidated soils is typical of filamentous cyanobacteria of genera such as *Anabaena*, *Cylindrospermum*, *Microcoleus*, *Nostoc*, *Oscillatoria*, *Phormidium* and *Schizothrix* (Rossi et al., 2017a). Between them, members of the genera *Microcoleus*, *Schizothrix* and *Leptolyngbya* proved valid as inoculants under stressing conditions. Their pioneering and surviving capability in abiotically stressing environments, and their capability to form BSCs, is due to their special features, including relatively rapid growth, migration (gliding), and their extraordinary ability to tolerate desiccation, radiation, extreme temperatures, high pH, and salinity (Lan et al., 2013). After BSCs have formed, their development and succession is affected by microenvironment characteristics such as soil structure and types, radiation intensity, topographic attributes, and availability of water and nutrients (Lan et al., 2013). Owing to the action of filaments and extracellular polymeric substances (EPSs), they conglomerate the soil forming stable biolayers that are subsequently colonized by other species (Hu et al., 2002; Rossi et al., 2015) that find nutrients and a moisture substrate.

The majority of cyanobacterization experiments on desert soils so far have been conducted in indoor trials (e.g., Rozenstein et al., 2014), whilst a minority of them was conducted in open field. Several aspects of cyanobacterial crust formation, the influence of some factors, and the possibilities of improving the process of induction and its outcomes were investigated by different authors (Table 2)

Table 2. Literature overview of inoculation experiment of cyanobacterial strains in desert ecosystem.

Cyanobacterial Strain	**Study type**	**Soil**	**Properties and/or processes studied**	**Reference**
Microcoleus vaginatus *Scytonema javanicum*	Microcosm	Sandy soil	Application of sodium alginate in induced biological soil crusts: enhancing the sand stabilization in the early stage.	(Peng et al., 2017)
Nostoc Vaucher *Phormidium Kützing* *Scytonema arcangeli*	Field	Sandy soil	Effects of the combined application of cyanobacteria and different concentrations of soil fixing chemicals.	(Park et al., 2017)
Nostoc sp. *Phormidium sp.* *Scytonema arcangeli*	Microcosm	Sandy soil	Combined application of cyanobacteria with soil fixing chemicals for rapid induction of biological soil crust formation.	(Park et al., 2017)
Microcoleus vaginatus	Microcosm	Sandy soil	Effect of shifting sand surface on IBSCs.	(Lan et al., 2017)
Nostoc sp.	Microcosm	Sandy soil	Effects of superabsorbent polymer on induced biological soil crust.	(Park et al., 2015)

Microcoleus vaginatus	Microcosm	Sandy soil	Effect of sand grain size on the development of cyanobacterial biocrusts.	(Rozenstein et al., 2014)
Microcoleus vaginatus *Scytonema javanicum*	Field	Dune	Morphological and ecophysiological characteristics of IBSCs in the field.	(Wu et al., 2013)
Microcoleus vaginatus *Scytonema javanicum*	Field	Dune	Effects of sand burial stress on the early development of IBSCs.	(Rao et al., 2012)
Microcoleus vaginatus *Scytonema ocellatum* *Phormidium sp.* *Synechococcus* sp.	Microcosm	Sandy soil	Effect of different proportions of *M. vaginatus* on the structure and function of IBSCs.	(Zheng et al., 2011)
Microcoleus vaginatus	Microcosm	Sandy soil	Effects of drought and salt stresses on man-made cyanobacterial crusts.	(Lan et al., 2010)
Microcoleus vaginatus *Scytonema javanicum*	Field	Dune	Influence of dew on biomass and photosystem II activity of induced cyanobacterial crusts.	(Rao et al., 2009)
Microcoleus vaginatus *Scytonema javanicum*	Field	Dune	Colonization and development of biological soil crusts.	(Wang et al., 2009)
Microcoleus vaginatus	Microcosm	Sandy soil	Effects of sand burial on biomass, chlorophyll fluorescence and extracellular polysaccharides of man-made cyanobacterial crusts.	(Wang et al., 2007)
Microcoleus vaginatus *Phormidium tenue*	Field	Dune	Enzymes activities in shift dune and fixed dune.	(Tang et al., 2007)

Microcoleus vaginatus *Scytonema javanicum* *Phormidium tenue*	Field	Dune	Investigate the relationships between desert algal biomass and compressive strength of the crusts.	(Xie et al., 2007)
Microcoleus vaginatus	Field	Dune	Colonization and development of biological soil crusts.	(Chen et al., 2006)
Microcoleus vaginatus	Microcosm	Sandy soil	Understanding the cementing mechanism, comparing natural crusts (dominated by *M. vaginatus*) and artificial crusts formed by *M. vaginatus*.	(Hu et al., 2002)
Microcoleus vaginatus *Phormidium tenue* *Scytonema javanicum* *Nostoc sp.* *Desmococcus olivaceus*	Microcosm (test trays)	Sandy soil	Ability of some cyanobacterial strains to aggregate sand grains against wind erosion.	(Hu et al., 2002)
Nostoc commune *Lyngbya*	Microcosm (test trays)	Sandy soil	Wind transport of sand surfaces crusted with photoautotrophic microorganisms.	(McKenna Neuman et al., 1996)
Microcoleus sp. *Nostoc sp.*	Microcosm (test trays)	Sandy soil	Run-off experiments with cyanobacterial crusts.	(Mazor et al., 1996)

Some groundbreaking studies were carried out in Inner Mongolia, China, where EPS-producing cyanobacteria were inoculated over large experimental areas ranging from 35 to 1,395 square meters (Chen et al., 2006; Lan et al., 2014; Wang et al., 2009). The application of the technology proved valid to pursuit desertification control. Follow-up studies brought to collect data (summarized in Table 3) that testify that, although effects were affected by site morphology (leeward and windward slopes, shorter or longer exposition to sunlight) there was a significant transition from bare

sand to soil, increase in nutrient contents, changes in soil chemo-physical characteristics, increase in soil stability and increase in microbial and plant diversity (Lan et al. 2014, Wang et al., 2009).

Table 3. Changes in soil physic-chemical characteristics and nutrient content of desert soil inoculated with a mixed culture of *M. vaginatus* and *S. javanicum* in Hopq Desert, Inner Mongolia, China. This experiment represents the first known large-scale cyanobacterization attempt to counteract desertification in an arid environment (adapted from Rossi et al., 2017).

Time from inoculation														
	control[a]	control[b]		18d[b]		1y[b]		2y[b]		3y[a]		3y[b]		8y[a]
		ww	*lw*	*ww*	*lw*	*ww*	*lw*	*ww*	*lw*	*su*	*sh*	*ww*	*lw*	*su*	*sh*
Water content (%)	0.24									0.29	0.45			0.29	0.44
Thickness (mm)		0	0	0.99	1.02	1.31	1.75	2.18	2.78	4.36	8.33	2.34	3.38	3.90	9.05
Compressive strenght	0	0	0	na	na	na	na	na	na	29.57	66.74	na	na	35.54	106.00
Sand (%)	99.50									92.65	84.50			91.19	83.83
Silt (%)	0.33									6.00	14.15			7.46	14.86
Clay (%)	0.17									1.35	1.35			1.31	1.31
Org. C ($g\ Kg^{-1}$)	0.51	0.33	0.49	2.67	2.71	3.05	3.95	5.65	7.01	5.02	11.47	6.23	8.83	7.28	17.09
Total N ($g\ Kg^{-1}$)	0.08	0.18	0.19	0.43	0.45	0.37	0.45	0.47	0.61	1.10	1.38	0.66	0.79	1.63	1.77
Total P ($g\ Kg^{-1}$)	0.06									0.17	0.19			0.18	0.23
C/N ratio		1.85	2.55	5.97	6.35	8.09	8.39	11.43	11.88	9.49	11.14	9.49	11.14	0.75	3.31

d=days, y=years, lw=leeward slope, ww=windward slope, su=sunny side of the dunes, sh=shady side of the dunes

Artificial rapid cultivation of IBSCs can provide a novel alternative to traditional biological methods for controlling soil and water loss (Bu et al., 2014a) and was proposed for the rehabilitation of soil ecosystem functions of degraded soil (Li et al., 2014).

1.1.3 Towards an open field, large scale cyanobacterization treatment.

Any cyanobacterization approach must be addressed considering the objectives to pursuit. We have to distinguish between the meaning of the terms "rehabilitation" and "restoration". Although both terms are used interchangeably in the literature, the term "rehabilitation" is defined as the recovery of ecosystem processes, productivity and services, without necessarily achieving a return to "pre-disturbance" conditions (Aronson et al., 1993). On the other hand, any "restoration" effort requires knowledge of composition, structure, pattern, and resilience of these degraded ecosystem, in order to achieve a complete or near-complete return to a pre-existing state (Bowker, 2007). However, since it is rarely possible to determine exactly how historic or prehistoric ecosystems look like, or how they functioned, restoration efforts may be plagued by ambiguities in both their goals and criteria of success (Aronson et al., 1993). Therefore, rehabilitation can be seen as a more direct mean of raising ecosystem productivity and increase fertility.

In order to apply successfully cyanobacterization on large scale, it is necessary to elaborate a reproducible methodology that can be modified to adapt to different contexts and to pursuit different goals. Before applying cyanobacterization in a given setting, several test trials must be conducted at several levels: *i*) an indoor/laboratory scale, *ii*) an outdoor and *iii*) "habitat amelioration approaches" (HAP) (Fig. 3).

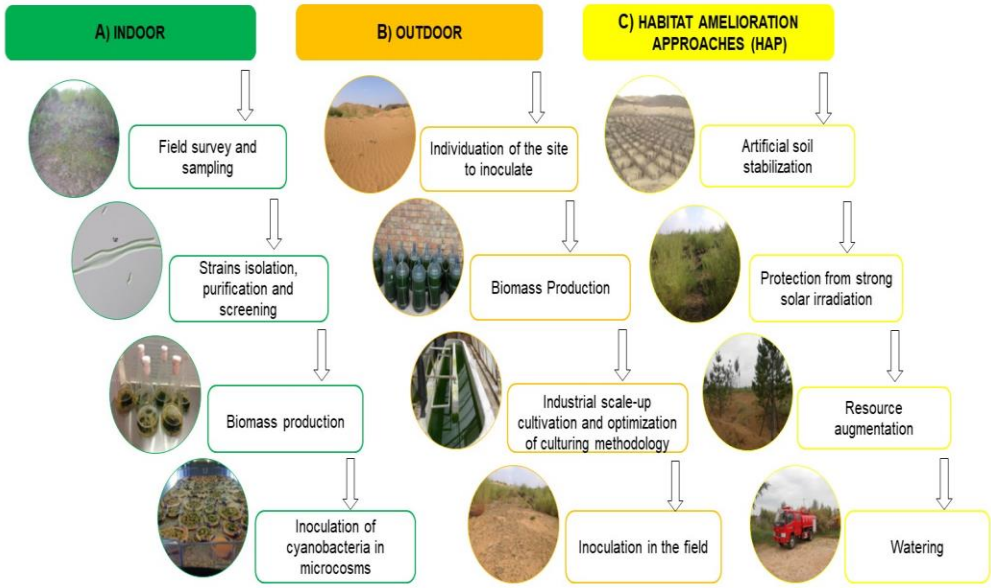

Fig. 3 General workflow for the development of a cyanobacterization approach from lab-scale to large field-scale.

A first guideline for the development of a cyanobacterization approach in arid environment is given by the project "Engineering Application of Integrative Artificial Algal Crust Technology in Shifting Sand Stabilization procedure for a large area", funded by the Chinese government and involving the Chinese Academy of Sciences (CAS) and Chinese Academic Institutions. This was the first cyanobacterization attempt over large desertified areas, proposing an experimental workflow that included indoor/laboratory phase trials and outdoor trials to address soil rehabilitation in desertified and abiotically stressed areas (Rossi et al., 2017).

The indoor phase is the "heart" of the technology development. It is dedicated to isolating, testing and selecting proficient inoculants, and it is the focus of the experimental work of this thesis. It is important to increase the number of new potential cyanobacterial candidates to test as inoculants, as it will concur in increasing the possibilities of the technology.

Several effective treatments benefited from using strains allochthonous to the area to inoculate (e.g., Ghosh and Saha, 1993; Tomaselli and Giovannetti, 1993), while

in other cases autochtonous strains were employed (e.g., Giller, 2001; Maqubela et al., 2010; Mazhar and Hasnain, 2011). This latter option allows to isolate strains that are already adapted to the environmental characteristics of the area, and thus potentially of interest. Most of the strains so far tested as inoculants in arid and semiarid settings were isolated from natural BSCs. Common procedures are the following:

i) Collection of organo-mineral samples where cyanobacterial growth is more evident at a depth of 0–5 cm for virgin soils and 0–10 cm for cultivated soils. The collection usually was performed using metal (circle knife, spoon) or plastic instruments, and specimen enclosed in plastic (bags, falcon tubes or petri dishes) or glass sterile containers. Information regarding the soil characteristics (texture, pH, mineral composition), geographical position (latitude), and environmental characteristics (temperature, relative humidity, irradiance) are usually registered for extrapolating an optimal growth settings for the future isolates.

ii) Samples are dipped in liquid nutrient media ("suspension inoculation method") or dispersed on agarized nutrient media. The most common medium for cyanobacteria cultivation are BG11 and its counterpart, N-free $BG11_0$ (Rippka et al., 1979). In case of samples collected in saline environments, feasible culture media are: enriched sea water AMA (De Philippis et al., 1998) and ASN III (Rippka, 1988).

iii) Once a greenish coloration is visible in the culture media, the obtainment of monocultures can be achieved through repeated striking on agarized growth medium until the formation of single colonies. This method has been applied in order to isolate filamentous cyanobacteria with gliding motility especially non-sheathed Oscillatoriaceae and some of the Nostocacales such as *Anabaena, Cylindrospermum*, and *Nodularia* (Castenholz, 1988). Alternatively, isolation can be achieved by picking up single cells or filaments with a micropipette under a microscope. This method uses transparent glass depression slides where the trichomes are pipetted. Thereafter, under a dissecting scope, one or a few trichomes are "pulled out" of the suspension with a glass capillary with the end pulled out to an appropriate diameter over a small flame. After blowing out the trichomes into the next depression, a new capillary is used to select a single trichome with the procedure repeated until the trichome has been

washed several times until to obtain axenic one. Sheathed trichonaes are particularly difficult to clean. A wash of selected filaments in about 0.3% (v/v) phenol in the dark for 4-6 h may greatly enhance the chances of isolation. A third option applies when isolating planktonic cyanobacteria have a significant portion of their cell volume constituted by gas vesicles. In this case, contaminants (other types of organisms) may be separated from gas-vesiculate cell types by centrifugation (Castenholz, 1988). An option to increase cyanobacterial unit population before plating is the filtration of the cell suspension through membranes with known pore sizes (Temraleeva et al., 2016). Sulphide selection gradients which inhibits or kills the associated contaminants (Bolch and Blackburn, 1996), or isolation using movement in unidirectional light and different light intensities on smooth or scored agar (de Chazal et al., 1992) are other options.

iv) In order to obtain axenic cultures, it is possible diminish the charge of eukaryotic and bacterial contaminants (e.g., Choi et al., 2007; Ferris and Hirsch, 1991; Rippka, 1988; Won et al., 2010), selecting the type of antibiotic according to the degree and type of contamination. Cycloheximide and nystatin are often used to purify the culture from eukaryotic contaminants (Berg et al., 2009; D'Acqui, 2016; Ferris and Hirsch, 1991). Among the different antibiotic (both bacteriostatic and bactericidal agents), imipenem seems to be one of the most useful chemicals to obtain the isolation and the purification of cyanobacterial strains (Bolch and Blackburn, 1996; Choi et al., 2007; Ferris and Hirsch, 1991; Won et al., 2010).

Unialgal cultures can be assigned genus and species by morphology (Komarek et al., 2014), and/or by molecular means (i.e. sequencing of the 16S ribosomal DNA). Today, both are extremely necessary for a complete characterization. Once identified, the isolate must be validated as inoculants, undergoing lab-test screening for its capability to grow and form crusts, and for its tolerance to abiotic stresses (Fig. 3a, see next section).

If attention must be paid to inoculant selection, an equal attention must also be deserved to: *i*) set the amount of biomass to cast per square meter, *ii*) select the modality of biomass preparation (e.g., dried biomass, liquid suspension) and *iii*) select the dispersal methodology to disperse the biomass on the available surface. During the

experimental work of this thesis, time was dedicated to elaborate proficient inoculation strategies that are discussed in section 3.1.

The outdoor phase of the technology development must be aimed to test the selected strains in the area intended for inoculation (small plot experiments), under the biotic and abiotic conditions characterizing the environment (Fig. 3b). The soil type, the morphological conformation of the site (mobile dune, dune slack, distribution of windward and leeward slopes) and environmental characteristics have to be considered.

Also, there is the need to select a feasible and stable mass-cultivation system for growing the selected strains. Biomass production starts in small volumes (50 to 100 mL) to increasingly larger volumes. Several studies carried out in China employed open-mixing raceway ponds or high rate algal pond systems (Fig. 4).

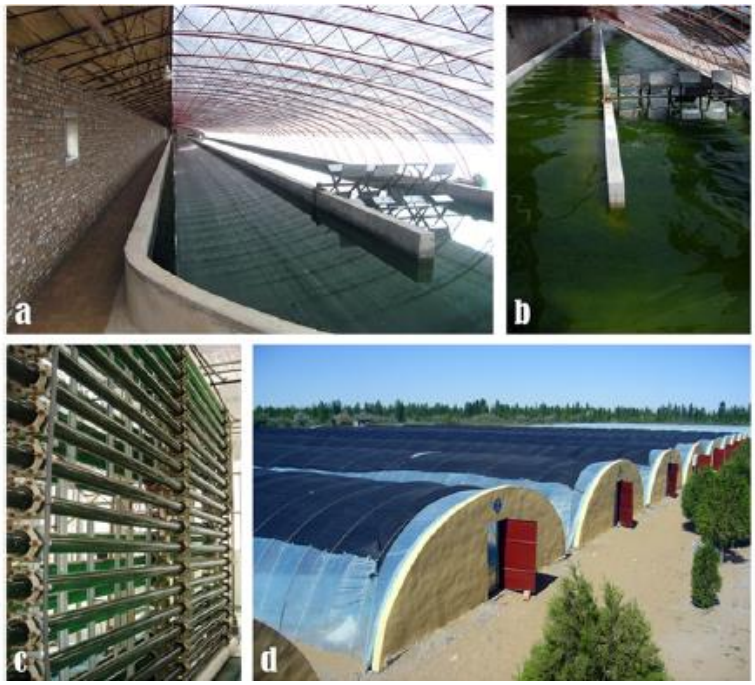

Fig. 4 Facilities for biomass production operated by CAS in Inner Mongolian Desert, China. In order to produce an inoculum for 100 hL raceway ponds (a, b) biomass is grown from small volumes to pipeline-type reactors (c). In order to exploit sunlight, raceway ponds were built inside greenhouses (d).

An important step which is still under optimization is the selection of a culture dispersal methodology. Two culture dispersal methodology are currently under study/evaluation: a field dispersion (e.g., spray-inoculation) and an aerial dispersion. While the latter typology is still in the infancy of the study, the former is reported in some publications (Colica et al., 2014; Rossi et al., 2017).

Cyanobacterization in the field can be accompanied by technological interventions aimed at lowering abiotic stress levels. These interventions, billed as "habitat amelioration approaches" (HAP) (Rossi et al., 2017; Bowker, 2007) may be useful to lower stress barriers and increase the success of cyanobacterization that they might hinder. HAP can include: *i*) soil stabilization through the implantation of straw checkerboards or chemicals or sand-binding vegetation, *ii*) protection from strong solar irradiation by introducing plants *iii*) resource augmentation through the addition of nutrients, introduction of perennial plants that enhance moisture status and *iv*) watering through the use of irrigation systems (e.g. tank truck) (Fig. 3c).

Notwithstanding the promising results, the technology has to be implemented by lowering the costs of actuation. Hu et al. (2012) reported that the main difficulty is the high cost regarding the cultivation and the transport of the cyanobacterial mass culture (Hu et al., 2012), especially when dispersed as liquid suspension. As of 2011 it was estimated that the cost of the application of this technology was roughly 0.15¥ (which equals 0,02$) per m^2, not taking into account the expenses for building infrastructures (e.g., laboratories, biomass growing facilities). The figure includes the salary of occasional workers, electricity, water and transport up to a distance of 300 km (Hu et al., 2012).

1.1.4 Selection of inoculants for abiotically stressing systems.

Understanding the reasons for the success/failure of a cyanobacterization treatment means quite often carrying out a multifactorial study. If the selection of proficient inoculants is an essential prerequisite for the success, Grant et al., (1985) cite unfavourable soil properties (low pH, low available P), climatic factors (heavy rains, too low or too high light intensities), biotic factors (competition, grazing) and the quality of the inoculum as causes of cyanobacterization failure. The influence of each factor must be evaluated at laboratory phase, considering the characteristics of the environment that will be inoculated, after the individuation of feasible candidates.

Notwithstanding the limited morphological differences, cyanobacteria may display a different capability of biofertilizing and bioconditioning, or may possess only one of the two (Maqubela et al., 2010). When aiming at testing isolated strains for highly stressed systems, two capabilities are important: *i*) that of blooming and colonizing notwithstanding harsh conditions, and *ii*) that of promoting soil aggregation and stability. Soils subjected to degradation suffer generally from poor physical structure, the latter playing a key role in water balance and placing of organic matter (Dexter, 1988). The loss of soil structure is associated with increase in salinity, sodicity, compaction, reduction of aeration, water infiltration, and a higher susceptibility to erosive forces (Goldberg et al., 1991; Rengasamy et al., 2003). Hence, the primary desired effect of cyanobacterization is an enhancement of soil cohesion, especially where it is poorly structured and unconsolidated.

One guideline study for determining stabilization capability of (potential) inoculants was conducted by the research group of Prof. Chunxiang Hu at CAS (Hu et al., 2002a; Hu et al., 2002b; Hu et al., 2003a, 2003b, 2003c; Hu and Liu, 2003). Four desert-dweller filamentous cyanobacterial strains (*M. vaginatus, Phormidium tenue, Scytonema javanicum* (Kutz.) and *Nostoc sp.*) were inoculated in microcosms and then tested for their tolerance to artificially-generated laminar wind at the Lanzhou Institute of Desert Research, China. *M. vaginatus* and *P. tenue* were the strongest among the 5

species, while the other species produced a weaker aggregation (Hu et al., 2002a). For the first time, it was demonstrated that even strains autochthonous to the same constrained environment may show different potential as inoculants.

M. vaginatus is the dominant species in most desert soils (Belnap, 1993; Hu et al., 2003c; Hu and Liu, 2003b), and it is considered as a model inoculant due to its pioneering capability. It can represent up to 70% of the living cover in drylands (Belnap et al., 2001). It is constituted by a thick sheath enveloping filament bundles. It is the more abundant cyanobacterial species in BSCs and the first contributor to their structural stability due to the action of filament bundles and EPSs. It was demonstrated that *M. vaginatus*, although not able to synthesize UV-screening pigment alike members of the genus *Nostoc* and the desert-dweller *S. javanicum,* glides up and down within the first millimetres of soil to avoid strong sunlight and reach moistured spots. Members of the genera *Scytonema*, *Nostoc* and *Calothrix* are typically organisms of the uppermost layer of BSCs (Hu et al., 2003d; Rosentreter and Belnap, 2001), as they possess UV-screening pigments scytonemin and/or mycosporine aminoacid-like substances (Garcia-Pichel and Castenholz, 1991). Notwithstanding, they are less efficient than *M. vaginatus* as inoculants. This clearly demonstrates the need to test every candidate notwithstanding its theoretical advantages/disadvantages.

By stabilizing unstable soil surfaces, cyanobacterial counteract wind erosion and thus land degradation (Belnap and Gillette, 1998). Soil aggregation increment after cyanobacterization can be measured before and after the treatment, or during cyanobacterial crust development, by artificially creating the forces leading to aggregate dispersion in nature. Examples are the simulation of raindrop impact, or the sudden immersion in water (Rohošková and Valla, 2004). The method proposed by Le Bisonnais et al. (Le Bissonnais, 1996) is based on a combination of treatments: rapid hydration, slow hydration and a wet stirring treatment before evaluating aggregate stability by a dry and wet sieving approach. A field kit to determine aggregate stability was proposed by Herrick (Herrick et al., 2001). An increase in mechanical stability of the soil after inoculation can be assessed by the use of electronic micropenetrometers, both in laboratory and in the field (Drahorad and Felix-Henningsen, 2013). The

possibility to perform these measurements on indoor microcosms allows to compare the stabilizing effect of more strains in parallel, evaluating the increase in compressive strength during cyanobacterial crust development.

Other "desirable traits" of new inoculants concern the high productivity of EPSs (see chapter 2) and the resilience of the candidate strains to abiotic stresses such as UV irradiation, drought and salinity, which characterize desert environments. EPS productivity can be assessed for strains grown in liquid suspension and for soil-inoculated strains. The “Alcian blue method” allows to have a general evaluation of EPS production and distribution. Commonly used on liquid cultures, the cationic dye Alcian blue in 3% acetic acid binds to the EPS negative charges, and its distribution reflecting that of EPSs can be observed under light microscopy (Rossi et al., 2012a). Methods to quantify EPSs excreted by inoculants in the soil have been optimized during this PhD experimental work, and are reported in section 3.2 of this thesis.

A preliminary lab-screening parameter of possible candidates is the growth velocity in liquid culture that could be a paramount factor in the biomass production stage for field inoculation (see section 5.1). Although this not implies necessarily the capability to grow well and fast in the field, it is a promising trait in the light of the economical balance of the biomass production process.

1.2 The role of extracellular polymeric substances (EPSs) in cyanobacterial crust development: the formation of the extracellular polymeric matrix.

The excretion of EPSs is an important physiological process from the first stage of BSC development, when bare soil is colonized by sheathed filamentous cyanobacteria (Belnap and Eldridge, 2001). Incipient cyanobacterial crusts, representing an early stage of BSCs (Lan et al., 2013), are a nutrient-rich substrate easily colonizable by other phototrophic species and heterothrophic bacteria. At the moment, although several authors recognize the importance of EPSs in BSC - water relations and survival (Mager and Thomas, 2011; Mazor et al., 1996), consider their amount an index of soil stability (Hoppert et al., 2004) and of the metabolic capacity of the community (Bu et al., 2014), information concerning their physiochemical properties and their modifications in space and time is still limited.

While the roles of cyanobacterial EPSs have been pointed out by several researchers (De Philippis and Vincenzini, 1998; Mager and Thomas, 2011; Mazor et al., 1996; Rossi and De Philippis, 2015), the majority of these scientific advances were attained under laboratory conditions, growing the strains under optimal settings (biotic parameters and nutrient provision). Such studies cannot provide significant data concerning the EPS production in natural outdoor conditions (e.g., cyanobacterial field colonies), or simply in simulated conditions reflecting those encountered outdoor. Indeed, one study recently published and reported in this thesis (see section 4, publication II) shows that the chemical and macromolecular characteristics of the EPSs produced in liquid cultures under optimal growth conditions or inoculated in microcosms under stressing conditions may be significantly different (Mugnai et al., 2017). This study supports other previous studies underlining how chemical characteristics and amount of secreted EPSs may be strongly affected whether they face nutrient limitations and constraints, or optimal growth conditions (Brüll et al., 2000; Huang et al., 1998).

1.2.1 Cyanobacterial EPS: physico-chemical characteristics and roles.

It is commonly accepted that cyanobacterial synthesis of EPSs, which is energy-consuming, has important ecological implications for its producers (Li et al., 2001). Several studies supported that it is a physiological mechanism increasing survival when facing adverse environmental conditions. Several known roles of EPSs are reported in Table 4.

Table 4. Known major roles of microbial EPS.

Role	Details	References
Cell adhesion and cohesion	Enhancement of capability of cell to bind to solid substrates, and enhancement of bounds between cells. They support the formation of biomineral layers and influence the physico-chemical properties of cell aggregates (charge, viscosity, flocculation).	(De Philippis and Vincenzini, 1998; Rossi et al., 2012; Xiao and Zheng, 2016)
Tolerance against desiccation and freezing	EPS constitute a hydrated surrounding of the cells that control the uptake and the release of moisture. EPS were demonstrated to prevent the drought-impairment of O_2 evolution. Also, EPS were demonstrated to enhance the resilience to freezing and thawing.	(Pereira et al., 2009; Tamaru et al., 2005; Varin et al., 2012)
Protection from external specific and non-specific threats	Protection against protozoan predation, antibiotics, host defenses, lysis from other bacteria and viruses. Capsulated cells are less efficiently digested than noncapsulated. Less condensed EPS are more easily digested, possibly due to the less abundant proteic portions included.	(De Philippis and Vincenzini, 1998; Li et al., 2001; Pereira et al., 2009)

Protection from UV-radiation	The UV-screening pigments scytonemin and mycosporine aminoacid-like substances (MAAs) are contained in the sheath of several cyanobacterial species. In addition, the thickness of the EPS casing is a barrier hindering the radiation from reaching the cells.	(Garcia-Pichel and Castenholz, 1991, 1993; Rossi and De Philippis, 2015)
Cell gliding	Some cyanobacteria are motile by gliding. The junctional pore complex system (JPC), observed on the cell wall of *Phormidium uncinatum* and *Anabaena variabilis*, is a structure constituted by a proteic scaffolding and fibrils, and operates thanks to EPS extrusion that provides the thrust. JPC was demonstrated to be involved in cell propulsion.	(Belnap et al., 2004, Hoiczyk, 1998; Hoiczyk and Baumeister, 1998; Ohad et al., 2005)
Nutrient and mineral accumulation	EPS secretions, due to their ionic nature, help the accumulation by ionic interactions, of minerals and nutrients.	(Sutherland, 1994; Welch and Vandevivere, 1994)
Support to photosynthetic systems	EPS help the re-establishment of damaged photosynthetic apparatus after the state of dormancy.	(Harel et al., 2004)
Protection of nitrogenase against the harmful effects of oxygen	EPS is capable of creating an effective barrier for oxygen transfer to the cells, protecting nitrogenase from the inhibition due to the presence of this gas.	(Sabra et al., 2000)

Cyanobacteria produce EPSs excreted as sheaths and capsules, or unevenly dispersed as mucilage (Rossi and De Philippis 2015b) depending on their chemical features, and on abiotic factors (e.g., available ions, pH). Many structural aspects of these outermost structures are still to be deeply investigated. Capsules and sheaths are attached to cells probably through non-covalent interactions, or covalently bound to phospholipids and lipid-A molecules, as observed for other bacteria (Roberts, 1996).

Many features of cyanobacterial EPSs can be found described in details in past publications (De Philippis and Vincenzini, 1998; Paniagua-Michel et al., 2014; Pereira et al., 2009; Philippis et al., 2001; Rossi and Philippis, 2016). They can be constituted by up to 15 sugar moieties (Pereira et al., 2009), organized in complex repeating units with molecular weights (MW) up to 1–2 MDa. Non saccharidic components like peptides, lipids and nucleic acids may also present. Glucose, galactose, arabinose, xylose and uronic acids have been frequently detected in major amounts. In addition, methyl, pyruvyl, succinyl and sulphate groups were also detected in some cases. The presence of hydrophilic moieties on one side (sulphated sugars, uronic acids and ketal-linked pyruvyl groups, among others), and hydrophobic on the other (acetyl groups, dehoxysugars and peptides) confers an amphiphilic character to EPS conferring a great plasticity in organisms' response to surrounding environment (Rossi and Philippis, 2016). Hydrophobic EPS fractions are more involved in the adhesion to solid surfaces, while hydrophilic fractions are more involved in binding minerals, nutrients and water molecules (Rossi et al., 2012a). Some cyanobacteria are also reported to excrete cellulose (de Winder et al., 1990), which is often localized in the sheath (Stuart et al., 2016).

1.2.2 The EPS supra-structure: the extracellular polymeric matrix (EPM).

EPSs excreted by cyanobacterial on solid substrates accumulate in a heterogeneous extracellular polymeric matrix (EPM) structured in fractions having varying condensation degrees, ranging from slime to solid gel (Fig. 5).

Fig. 5 Microscopical image showing the EPM of a cyanobacterial crust of *Schizothrix* cf. *delicatissima*. Picture by GM.

It is a highly hydrated semi-solid mesh that glues together organisms and soil particles in a three-dimensional network. It provides a wide array of services to the crust community, from conferring physical integrity and stability, to providing an optimal microenvironment with increased moisture, nutrients, and protection from harmful biological and physical agents. The physical state of EPM is governed, to a large extent, by environmental parameters (e.g., pH, available ions).

Being the result of microbial synthesis and demolition, EPM of natural communities is in continuous modification and rearrangement, with its composition and distribution varying spatially and temporary according to the prevailing activities

of the biofilm (Wingender et al., 1999). The presence of an embedding extracellular matrix allows consortial activities, that are needed by microorganisms to maximize their fitness through cooperative interactions. Synergistic activities enhance the resilience to stress factors and oligotrophic conditions (Wingender et al., 1999). Within the EPM, the spatial arrangement creates gradients of oxygen (determining aerobic and anaerobic habitats), other electron acceptors, as well as organic substrates (e.g., proteins, peptides, aminoacids and colloids), and pH value (Costerton et al., 1987; Kepkay, 1994; Mayer et al., 1995; Wingender et al., 1999). Some processes such as the accumulation of nutrients and other substances from the bulk soil water, gene exchange, and quorum sensing are favoured.

Although the polysaccharidic component is a prevalent EPM component (up to 90%) in most of the cases (Al-Thani, 2015) other components may be significantly present as a result of secretion processes or cellular release after lysis (Wingender et al., 1999). Proteins, nucleic acids, and amphiphilic substances as (phospho)-lipids can be often detected in the extracellular space. Extracellular proteins may establish hydrogen bonds within EPM (Dignac et al., 1998); some can be glycosylated to create glycoproteins, or substituted with fatty acids to form lipoproteins. One main function of extracellular proteins is to act as enzymes for digestion of exogenous macromolecules (Wingender et al., 1999).

Several authors have attempted to define the different EPM fractions observed in complex biofilms. Some used the term “slime” to indicate EPM fractions that are loosely bound to cells and soil sediments, and less condensed although not dissolved. Dissolved EPM fractions are referred to as “colloidal” (Nielsen and Jahn, 1999). A generic distinction used by some authors is between “bound” EPSs (sheaths, capsules, condensed gels, loosely bound polymers, attached organic material) and “soluble” EPSs (soluble polymers, colloids, slimes) (Nielsen et al., 1997). Operationally, the more easily recoverable fraction is that which is less condensed, and weakly attached to cells and sediments (loosely-bound EPSs, LB-EPSs) (Fig. 6). A second fraction consists of those macromolecules with a higher level of gelification and thus thickened, having strong bonds with cells and sediments (tightly-bound EPSs, TB-EPSs). This

fraction may include more sub-levels of gelification and the extent of recovery is strictly determined by the extraction methodology (see section 3.2). In addition to LB-EPS and TB-EPS fractions, we can identify a third "glycocalix" fraction (G-EPS) (Wingender et al., 1999), which includes the outermost cellular structures firmly attached to the cells (capsules or sheaths). G-EPSs may be either containing filaments or hollow following filament migration.

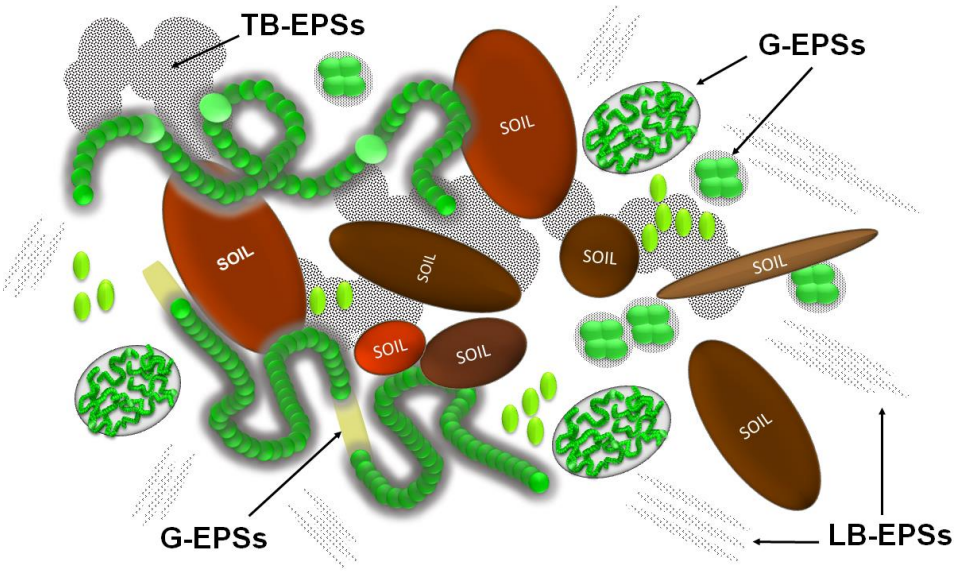

Fig. 6 Extraction and analysis of EPS from complex microbial associations: the case of BSCs (retrieved from Rossi et al., 2017).

Arid soil pioneers like the non-heterocystous cyanobacterium *Microcoleus vaginatus*, members of the genera *Schizothrix* or *Hydrocoleum* forms filaments constituted of rope-like bundles of trichomes encased in tubular exopolysaccharidic sheaths (Garcia-Pichel and Wojciechowski, 2009), resulting from one or more secretion events. This is recognized as the first step in BSC formation. Cyanobacterial sheaths can be considered a central element in the formation of EPM, as they constitute the first accumulation of EPS material on which early stages of BSCs are structured (Rajeev et al., 2013). Organized in "large bodies" (~100 μm), sheaths bind soil particles stabilizing soil against erosion by wind or water (Belnap and Büdel, 2016;

Belnap and Gardner, 1993). In studying early-stage BSCs from Gurbantunggut Desert, China, Zhang (Zhang, 2005) observed that cyanobacterial sheaths either contained filaments, or were leftover material, after filament migration or death. Empty sheaths remain solidly attached to the organo-mineral material as a cement stabilizing the crust structure. Unless crashed by compressional disturbances, a primitive discernible organization of the EPM is visible from the first stages of development of BSCs by microscopical observations. Three month-old artificial cyanobacterial crusts obtained by inoculating the cyanobacterium *Schizothrix delicatissima* AMPL0116 were constituted by an EPM distinctly organized in a LB-EPSs and a TB-EPS fraction (Fig. 7 and section 4; publication II; Mugnai et al., 2017).

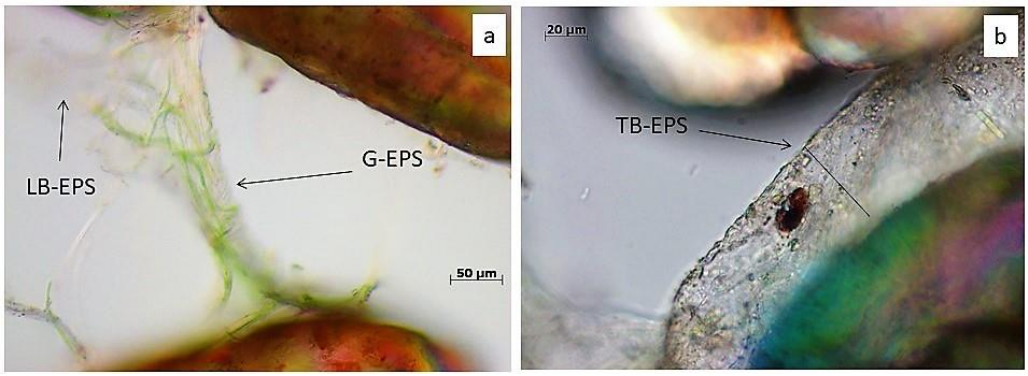

Fig. 7 Microscopical images of the operationally-defined EPM fractions in a 15-day-old induced cyanobacterial crusts obtained by inoculating the cyanobacterial strain *Schizothrix* cf. *delicatissima* AMPL0116 in microcosms. G-EPS, glycocalix EPS (encompassing sheaths and capsules); LB-EPS, loosely bound EPS; TB-EPS, tightly bound EPS. a) Filaments and sheaths of S. delicatissima gluing two adjacent sand particles. The picture underlines the prominent role of EPS in sediment cohesion. b) TB-EPS covering a sand grain surface. Pictures by GM.

Analysis conducted on BSC samples from desert areas showed that, notwithstanding the stressful environmental conditions, EPM resulted of a significant compositional complexity. Up to 13 different types of sugars were identified. They included the hexoses galactose, fructose and glucose, which had the highest relative abundance, the dehoxy-sugars fucose and rhamnose, the amino-sugars galactosamine

and glucosamine, and the pentose ribose. In addition, uronic acids, namely galacturonic and glucuronic acids, were also detected (Chen et al., 2014; Colica et al., 2015). EPS were of a MW comprised between 2 M and 485 kDa (in the range characterizing EPS produced by cyanobacteria) or between 72.6 kDa and 0.34 kDa, (small saccharides, dimers and monomers). In a further study, the composition and MW distribution of LB-EPSs and TB-EPSs, extracted with an optimized methodology (see section 3.2), were determined and compared. While the two fractions had a similar compositional pattern, they appeared to be different in MW distribution. The 90% of TB-EPSs had a MW between 2 M and 0.76 MDa, while the less condensed fractions were polymers in the lower MW ranges (Chen et al., 2014). By studying EPM of three to eight-year-old induced BSCs in a semiarid environment, it was observed that TB-EPSs seem more involved in providing a structural role than LB-EPSs, which are loosely bound to filaments and sediments (Chen et al., 2014).

1.2.2.1 The role of extracellular polymeric matrix in biological soil crust-water relations.

EPM is essential for the BSCs to cope with water stress. The following cited studies clarified how EPSs, due to their amphiphilic nature and chemo-physical characteristics *i*) delay water movement when soil sorptivity is high, and contribute to the creation of viable waterways when sorptivity is low, *ii*) regulates water uptake and water loss from the cells (Pereira et al., 2009), and *iii*) reduce evaporation loss and increase soil water-holding capacity (Mager and Thomas, 2011).

The role of EPM of BSCs in affecting soil infiltrability was investigated in two different studies (Colica et al., 2014; Rossi et al., 2012b), considering soil textures ranging from silt loam to sandy, according to the USDA classification (e.g., Groenendyk et al., 2015). Although soil texture resulted the paramount factor affecting sorptivity, the application of a simple non-destructive EPS-extraction method (detailed in Rossi et al., 2012b) resulted in a more compacted crust structure that lost the ability to absorb water. This strongly supported the idea of a contribution of the EPM in

structuring BSC waterways. Studies on BSCs collected on sandy soils, where water infiltration velocity is high, depicted a different scenario. A significant correlation between EPS content and soil sorptivity was found. In this context, the onset of BSCs diminishes remarkably water infiltrability up to 90% (Colica et al., 2014). The correlation observed supports the notion that EPS swell following the contact with water, reducing the volume of soil pores (Fischer et al. 2010a), promoting the maintenance of the moisture in the very first soil layers from which it would evaporate or seep away easily.

Other investigations underlined how EPM is *i)* fundamental to retain humidity and *ii)* fundamental for water uptake from non-rainfall water sources (i.e., dew, fog and plant guttation) (Colica et al., 2014). In such studies, the capability of BSCs to retain water against evapotranspiration in a hyper-arid setting was correlated to the presence of EPSs, especially those having a high MW. After removing 90% of EPSs from BSC samples, utilizing the previously mentioned non-destructive method, water uptake capability decreased sensibly, to being not statistically different from that of bare sand.

1.2.2.2 Extracellular polymeric matrix in biological soil crusts as a direct and indirect source of nutrients.

EPM promotes the association in consortia that ease the depolymerisation processes of complex compounds to molecules easily assimilable even by community members having a smaller genome, a less specialized lifestyle, and not possessing a full range of degradative enzymes (Medie et al., 2012). EPM itself represent a notable C source. According to (Chenu, 1993), EPSs may represent up to 50% of cellular biomass, and were hypothesized to be the primary substrate respired after heavy rainfall events (Fischer, 2009; Thomas et al., 2008; Thomas and Hoon, 2010). A study on the enzymatic activity in induced BSCs of different years, was coupled with a study on the characteristics of the EPM fractions (Chen et al., 2014). This study suggested that the enzymatic activity was mostly directed towards LB-EPSs, while TB-EPSs appeared more “preserved” from biological activity. In this light, a study conducted by (Decho

and Lopez, 1993) on the digestibility of EPS-producing bacterial cells, demonstrated an higher resilience to digestion of G-EPSs, which has more stable, ordered and definite secondary structures, in comparison with less condensed EPSs. This supports the idea that slime diffusing in the soil is the principal more easily degradable EPS fraction, while TB-EPSs and G-EPSs, due to their lower degradability, constitute more a "structural skeleton" of BSCs.

EPM may also act as a nutrient accumulator, concentrating dissolved organic matter (DOM) with compounds containing C, N, P and trace metals which are essential for cell metabolism (Flemming and Wingender, 2010; Wolfaardt et al., 1999). In this respect, such a nutrient source may be of importance for vegetation establishment, determining an increase of essential nutrients in plant tissues (Zhang et al., 2006).

1.2.2.3 Extracellular polymeric matrix enhances the tolerance of biological soil crusts to light stress and salt stress.

The excretion of an EPM helps the crustal community dealing with light stress and salt stress.

Since the early stage of cyanobacterial crust formation, EPM envelope shield cells from the excess of light and UV radiation in two ways: *i*) physically, by increasing the path length for the light to reach the cells, and *ii*) by the presence in the polysaccharidic envelopes of UV-screening pigments that some strains are able to synthesize, namely scytonemin and mycosporine-aminoacid like substances (MAAs). Both pigments are synthesized in response to light stress, and their presence can be checked by proper extraction methods and subsequent spectrophotometric revelation (Rossi et al., 2012a).

In hypertonic environments, EPS synthesis preserve cells from water loss. Ozturk and Aslim (2010) demonstrated that EPS synthesis can be affected, in composition and abundance, by NaCl concentrations, and that EPS production

conditions strain survival. Under salt stress, the oscillatorean *Microcoleus vaginatus* produces low molecular weight EPSs that help preserve the photosynthetic activity (Chen et al., 2003), and act as a buffer compound for the accumulation of water (L.-Z. Chen et al., 2006).

EPSs excreted by cyanobacteria can ease salinity stress during plant growth reducing the content of Na^+ available for plant uptake (Ghalab et al., 2016).

These data suggest that the development of EPM is essential for BSC development notwithstanding salt stress, which is commonly encountered in desert environments.

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2. Aim of the thesis

The aim of the present thesis was to expand the knowledge on some key aspects of the cyanobacterization process which have not been clarified by other studies so far.

In particular, the main experimental work was focused on:

i) Designing and testing an optimal inoculation procedure, with particular attention to the initial inoculum concentration and the biomass dispersion procedure.

ii) Demonstrating that proper selected cyanobacterial inoculants can be applied on oligotrophic unconsolidated substrates, without nutrient sustainment, and with a minimal water supply. In this frame, the effect of different water regimes was investigated.

iii) Assessing the effects of the development of the cyanobacterial crust on soil stability and hydrological properties.

iv) Investigating the development of the cyanobacterial crusts following the successful cyanobacterization of soil microcosms.

v) Characterizing the exopolysaccharidic matrix of incipient cyanobacterial crusts, constituted by exopolysaccharides excreted by the colonizing strains.

vi) Investigating the possible roles of the different fractions of the extracellular matrix in cyanobacterial crust development.

A deeper knowledge in these aspects will contribute in paving the way for future small and large scale studies aimed at the application of this technology in order to counteract soil degradation and desertification spread. In addition, this thesis will shed

light on the mechanisms of soil colonization and aggregation by cyanobacterial inoculants, stressing the contribution of the extracellular polymeric matrix.

3. Materials and Methods

3.1 Cyanobacterial inoculation in microcosm: elaboration and optimization of a biomass dispersion methodology.

The experimental activity of this thesis was spent on studying, at laboratory level, some aspects that will turn out very important in the perspective of future large-scale, outdoor approaches.

Biomass preparation and dispersion, and biomass maintenance thereafter are crucial steps in the cyanobacterization technology. According to literature, a recognized standardized cyanobacterization methodology does not exist so far, with existing examples varying even largely. In different studies, cyanobacterial biomass was dispersed dried, in suspension, with/without the additions of nutrient, and/or mixed with sand, lime or gravel (Rossi et al., 2017).

In general terms, it is important to reduce as much as possible the costs related to biomass preparation, and secondly, it is important to abate those associated with the sustainment of incipient cyanobacterial crusts after the treatment.

The more direct cyanobacterization approach consists in inoculating biomass in suspension, directly withdrawn from the reactor or raceway pond in which it was grown. Outdoor, large-scale experiments conducted in the Hopq Desert, previously described, utilized cyanobacterial suspension after removing the culture medium by let it sedimenting in open-raceway ponds. The documented success of these field trials demonstrated that properly selected strains can thrive by drawing on sand minerals, not necessarily needing artificial nutrient sustainment. The employment of nutrient amendments accompanying the inoculation might result economically-prohibitive in view of large-scale field approaches.

Nonetheless, in all these cases incipient cyanobacterial crusts were largely watered, intermittently, for several days after biomass dispersion (Wang et al., 2009). The importance of water provision, and hydration after biomass dispersion has been

evidenced also in other studies (e.g., Shatta et al., 2014). Unfortunately, this would not always be possible, or would result too onerous, especially in desert environments far from any type of facility.

Another important question is whether sand granulometry influences cyanobacterial crust development. In other words, will it suffice to select inoculants based on environmental conditions, or a selection must be made also considering grain size on a same texture basis? Considering the study employing the model inoculant *Microcoleus vaginatus* (Rozenstein et al., 2014), cyanobacterial crusts seem to develop faster on small-grained substrates.

An additional point of interest in this experimental work was the standardization of an effective biomass dispersion methodology, allowing to disperse the biomass as uniformly as possible on the microcosms.

Considering all these premises, several preliminary experimentation trials were conducted to verify:

i) The possibility of inoculating cyanobacterial strains without nutrient addition;

ii) The minimum inoculum concentration (MINoC) to achieve cyanobacterial crust development;

iii) The influence of the biomass dispersion methodology; several dispersion methodologies were tested to determine the optimal one to achieve an even distribution of cyanobacterial cells on the microcosm;

iv) The influence of different substrate grain sizes on cyanobacterial crust development.

(i) The strains *Schizothrix* cf. *delicatissima* AMPL0116 and *Leptolyngbya ohadii* were inoculated in microcosms after having been washed 3 times with distilled water to remove growth medium. Beside excluding the sustainment of the nutrients, this procedure also excludes the contribution in the aggregation process by the released polysaccharides excreted during growth in the liquid media. *S. delicatissima* and *L. ohadii* demonstrated to be able to form stable cyanobacterial crusts with a minimal water provision (see sections 4; publication III)

(ii) The strain *L. ohadii* was inoculated in microcosms in three different amounts (0.15, 0.45 and 0.75 mg dry weight/cm^2). While the minimum amount produced inconsistent cyanobacterial crusts, stable aggregates were induced with the other two concentrations. However, the highest concentration tested produced an excessive stratification of filaments, which could result in an obscuration of the nethermost layer deposited. By these means, the MINoC was set as 0.45 mg/cm^2 and was employed in the following experiments.

(iii) The strain *L. ohadii* was dispersed utilizing four methodologies: random, radial, spiral and double spiral (Fig. 1 a-d). After 15 days of incubation, the random dispersion produced unconsistent cyanobacterial crusts that were barely visible but in a few little spots (Fig. 1a´). In addition, the small amount of crust that formed tended to peel off. A similar effect was observed after radial dispersion (Fig. 1b´). The spiral dispersion induced a limited and non-homogenous crust coverage (Fig. 1c´). Conversely, applying the double spiral dispersion, cyanobacterial crusts appeared more consistent. More extensive green spots were visible. In addition, cracks and curls were visible on the surface of the microcosms, indicating a profusive microcosm colonization (Fig. 1d´).

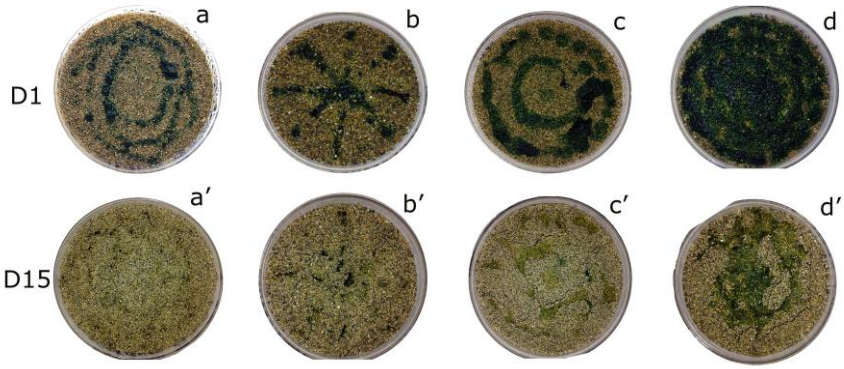

Fig. 1 Inoculation of *Leptolyngbya ohadii* in microcosms using a random (a), a radial (b), a spiral (c) and double spiral (d) dispersal methodology, dispersing a biomass corresponding to 0.45 mg dry weight/cm^2. The same microcosms after 15 days of incubation was reported (a´, b´, c´, d´).

All further experiments were conducted distributing the biomass by double-spiral methodology (schematized in Fig. 2).

Fig. 2 Double spiral dispersion (schematized in A) dispersed on the microcosm (B). It allows a homogeneous coverage of the microcosm surface (C).

(iv) The strain *L. ohadii* was inoculated in microcosms containing sand having two different grain sizes: 0.3 – 0.6 mm (medium sand, MS) and 0.8 – 1.25 mm (coarse sand, CS). The inoculation in an amount corresponding to 0.45 mg/cm^2 produced cyanobacterial crusts on both types of substrates, demonstrating its plasticity. A further experiment in which the effect of

grain size on cyanobacterial crust development is detailed in section 4; publication I.

All inoculation experiments were conducted using culture suspension deprived of the growth medium and subjected to fragmentation in a sterile plastic tube. Fragmentation was aimed at obtaining even suspensions without the presence of bundles or flakes, which would hinder dry weight determination and uniform dispersion in the microcosms. Notwithstanding the initial fragmented form (Fig. 3), the biomass was able to colonize the available space, forming resistant bundles across the microcosms (section 4; publication III).

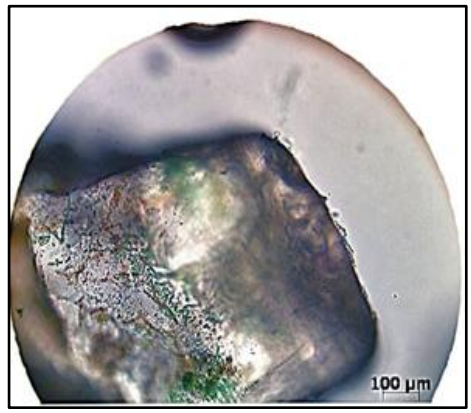

Fig. 3 Microphotograph showing fragmented biomass of *L. ohadii* adhering to a sand grain. Picture by GM.

3.2 Extracellular matrix analysis: advances in improving extraction and analytical methodology.

An important part of the experimental activity was dedicated to optimizing the procedures for extracting the EPM of induced cyanobacterial crusts, and study their role in soil aggregation.

Existing extraction procedures rely on the fact that EPS fractions (which are the "building blocks" of EPM) have varying levels of solubility. The immediately soluble fractions can generally be removed washing with H_2O, whereas more hydrophobic fractions are not expected to be recovered in this way. Less soluble fractions can be recovered by applying proper extraction methods. For the majority of studies on complex microbial biofilms, extraction methodologies can be encompassed in the following procedural scheme (Table 1).

Table 1 Theoretical procedural workflow for extracting EPS from organo-mineral microbial aggregates (Rossi et al. 2017).

Operation		**Details**
1)	Sampling	Collection of BSCs from natural setting or from laboratory setting (e.g., microcosms).
2)	Preparation of samples for extraction procedure	Preparation procedures may include washing or homogenization without disruption of the cells. Homogenization is aimed at dispersing soil aggregates and allows to eventually perform normalization on a dry soil basis.
3)	Extraction procedure	Selection of the most suitable procedure to recover EPS from the organo-mineral layer.
4)	Purification of EPS	After extraction, EPS can be purified to remove non-carbohydrate components or salts. Treatment generally include dialysis to remove salts, and use of proteases to remove peptides.
5)	Analysis of EPS	This phase includes the preparation of the samples for the different possible analytical and instrumental procedures (e.g., hydrolyisis, removal of coarse particulate).

There is not a universally followed extraction method to recover EPS from biocrusts. The existing extraction procedures that appeared recently in literature were

adapted from methods previously applied to other types of biofilms, or to cyanobacterial strains grown in liquid cultures (Rossi et al. 2017).

It has to be stressed that if the analysis is aimed only at the carbohydrates present in the EPS, the maintenance of cell integrity is a prerequisite. In this case, the extraction procedure must be selected in a way to not cause cell leakage, or the EPS will be contaminated with intracellular material. When the extraction causes cell lysis, the subsequent analysis, whether simple quantification or macromolecular characterization, is performed on "total carbohydrates" and not on EPS. The determination of cell lysis after extraction is not straightforward, since some intracellular substances, e.g. proteins and nucleic acids, may be naturally present in the extracellular environment. The use of truly intracellular compounds as markers for cell lysis was suggested. One compound is ATP, although in past studies some concerns regarded the level of accuracy in measuring this molecule (Grotenhuis et al., 1991). Another suggested marker is the enzyme glucose-6-phosphate dehydrogenase (G6PDH) (Platt et al., 1985). Stuart et al. (Stuart et al., 2016) used this enzyme to evaluate the extent of cell lysis after EPS extraction from cyanobacterial mats. Another parameter supporting the loss/maintenance of cell integrity after EPS extraction from cyanobacterial mats is the content of chlorophyll in the extracts, although the reliability is related to the abundance of the phototrophic fraction of the community.

Extraction procedure is generally a combination of chemical and physical approaches, based on considering the major type of interactions that keep EPS together in EPM, namely van der Waals forces, electrostatic interactions, hydrogen bonds, hydrophobic interactions and covalent bonds (Christensen, 1999). The application of only physical methods (addition of water followed by centrifugation, mixing, shaking or sonication) gives lower EPS yields than combining with chemical methods (Nielsen and Jahn, 1999). Physical methods alone result in a minimal, if not null, release of bound EPS. Chemical methods include the use of a wide array of substances that are meant to facilitate the release of TB-EPS. Extractants include pyridine acetate, used for *Escherichia coli* (Pelkonen et al., 1988), NaOH (Sato and Ose, 1980) and NaCl used for *Pseudomonas aeruginosa* (May and Chakrabarty, 1994). The use of alkali (e.g.,

NaOH) leads to the ionization of charged groups in EPS, due to their isoelectric point that is generally below pH 4–6. The result is a strong repulsion within the EPM and the increase of water solubility of more condensed fractions, although this process seldom leads to the removal of G-EPS. The structural order of EPM can be shifted to disorder on heating or removal of ions (Sutherland, 1999). For example, the removal of cations such as Ca^{2+} and Mg^{2+} using complexant agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol - bis(ß-aminoethyl-ether) - N, N, N′, N′ – tetraacetic acid (EGTA) strongly compromises the stability of the EPS strands, and EPM tends to fall apart. In a study dedicated to the comparison of several methods for the extraction of EPS from soil biofilms, Redmile-Gordon et al. (Redmile-Gordon et al., 2014) suggested that the best method is based on the use of cation exchange resins as they are capable of maintaining the integrity of the cells thus preventing the contamination of the extracted EPS with humified soil organic matter.

The extraction procedure can be aimed at a general quantification of EPS in a BSC sample, or at recovering specific fractions for separate quantifications. A method to extract and quantify EPS from intertidal sediments proposed by Underwood et al. (Underwood et al., 1995) was recently successfully applied to quantify EPS in BSCs (Colica et al., 2014; Rossi et al., 2012). It consists of extractions in 0.1M Na_2EDTA of small amounts (~100 mg) of homogenized BSC for 15 min at room temperature. The extracts can be assayed for total extracellular carbohydrate amount by applying phenol-sulfuric acid assay (Dubois et al., 1956). To quantify actual EPS (the fraction with a MW $\geq$ 100 kDa), the quantification must be performed after treating the extract with ethanol (70% final concentration) (Decho and Lopez, 1993). According to some authors, treatment with EDTA may cause cell wall destabilization due to divalent cations removal causing cell leakage (Nielsen and Jahn 1999). Nonetheless, Underwood et al. (1995) observed low, if not null, intracellular contamination extracting intertidal sediments. In using EDTA extraction on cyanobacterial biofilms, Stuart et al. (Stuart et al., 2016) ruled out cell lysis.

The extraction efficiency of the method in analysis, preliminary to those published in Rossi et al. (2012), was evaluated by extracting EPS from natural BSCs

collected in North American deserts (F. Rossi, personal communication). The analysis was conducted employing two BSC typologies (described in Rossi et al., 2012), one collected in the Chihuauan Desert, and one in the Mohave Desert, that were different for relative abundances of species (significant differences in cyanobacterial and proteobacterial relative abundances), and in the percents of sand and silt contents.

These results pointed out that repeated extractions may increase extraction yields. At least five extractions were needed to remove all the extracellular carbohydrates, although the carbohydrates removed with the first two extractions represented over 60% of the total amount recovered with the sum of all the extractions (Fig. 4) (F. Rossi, *personal communication*).

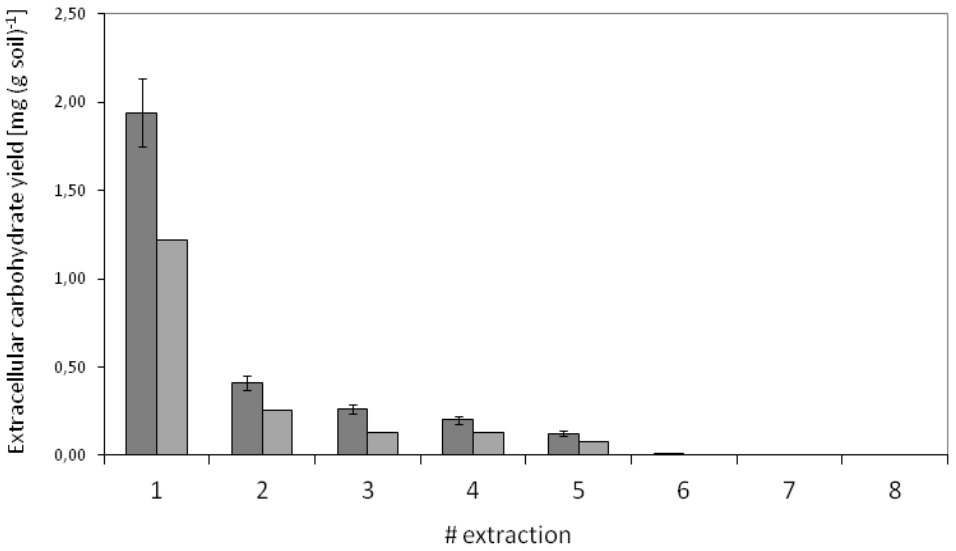

Fig. 4 Repeated extracellular carbohydrate extractions from two different typologies of BSCs from North American deserts, utilizing the method of Underwood et al. (1995) for the first time on BSCs. Dark brown bars, Chihuahuan Desert crust; light brown bars, Mohave Desert crust. Yields were expressed as mg of extracellular carbohydrates (g soil) $^{-1}$ for each extraction on each sample. Each sample type was extracted in triplicate and results were expressed as mean value ±SD (Rossi et al. 2017).

Although the needed number of extractions may depend on the typology of crust and soil texture, the method is relatively rapid, and allows to process multiple samples simultaneously.

In order to confirm the reliability, the above methodology also for the cyanobacterial crusts induced in microcosms, several trials were conducted before formal experimentations. Several repeated extractions were conducted for single samples to determine the amount of LB-EPS and TB-EPS per gram of crust that is possible to recover with every single treatment (Fig. 5 and 6).

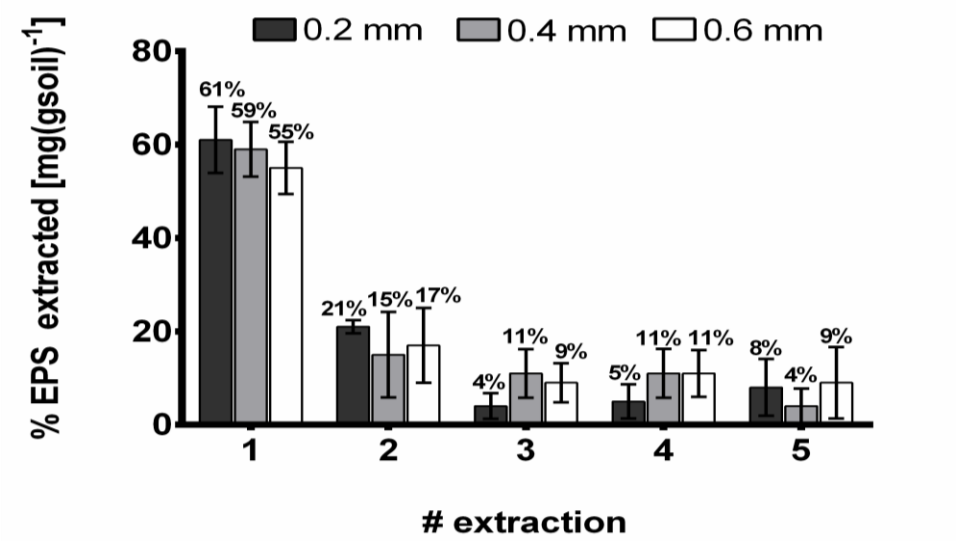

Fig. 5 Repeated water extractions to recover LB-EPS on 90-day old cyanobacterial crusts induced by *L. ohadii* inoculation in microcosms. Cyanobacterial crusts developed under three different water regimes (0.2mm 0.4mm 0.6mm water received every two days; see section 4; publication III). Extraction yields were expressed as mg of extracellular carbohydrates per gram of dry crust. Extractions were conducted on experimental triplicates (n = 3), and SD is reported as error bars.

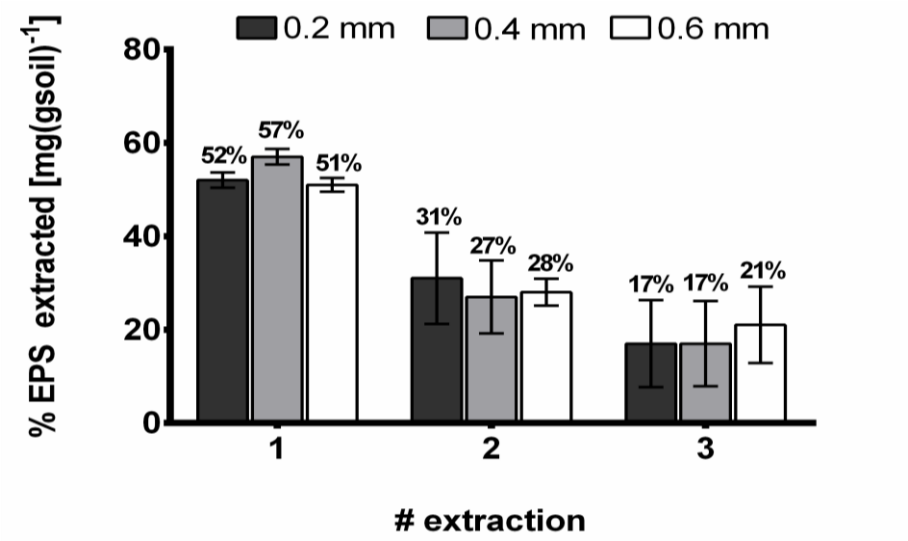

Fig. 6 Repeated Na_2EDTA extractions, to recover TB-EPS, performed on 90 day-old cyanobacterial crusts induced by inoculating *L. ohadii* in microcosms. Cyanobacterial crusts developed under three different water regimes (0.2mm 0.4mm 0.6mm water received every two days; see section 4; publication III). Extraction yields were expressed as mg of extracellular carbohydrates per gram of dry crust. Extractions were conducted on experimental triplicates (n = 3), and SD is reported as error bars.

These results prove that at least three extractions with water and Na_2EDTA solutions are needed for a representative fraction recovery, although from 50 to 60 % of EPS are already recovered with the first extraction.

The procedure described in Figure 7 synthesizes the optimization of the pre-existing methodology to recover LB-EPS, TB-EPS and G-EPS from induced cyanobacterial crusts. The procedure includes recovering LB-EPS by water extraction and then recovering TB-EPS by extracting with 0.1 M Na_2EDTA. These two treatments in sequence will leave a pellet of sediments, cells and G-EPS. Considering the results obtained in the preliminary trials, every step of the extraction process was repeated several times.

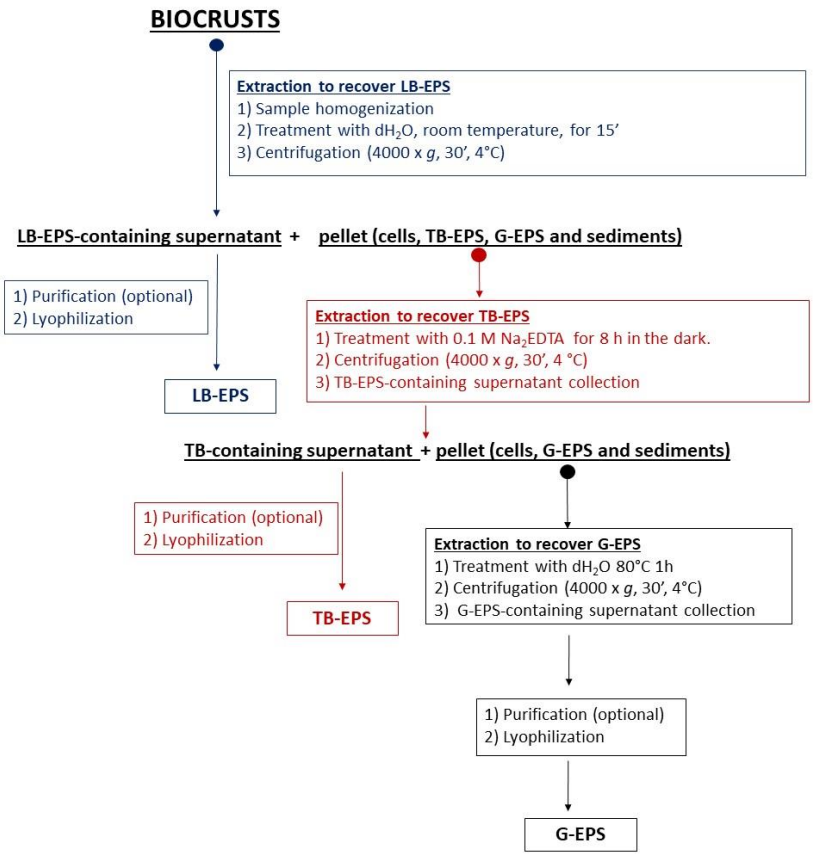

Fig. 7 Schematization of the procedure to extract the three main EPS fractions (LB-EPS, TB-EPS and G-EPS) that was optimized to be employed for the experimental work of this thesis.

Considering the results obtained in preliminary trials, this methodology has been applied successfully to natural BSCs in some recent studies (Chen et al., 2014; Colica et al., 2014, 2015; Rossi et al., 2012).

An important step forward was to elaborate a method to extract the fraction most intimately associated to cells (G-EPS). After testing several known methodologies (Rossi and Philippis, 2016), the most effective method resulted a combination of 1.5 % NaCl treatment, followed by hot water (80 °C) extraction for 60 min. The effectiveness of the method in removing the sheath was tested on *S.* cf. *delicatissima* (Fig. 8).

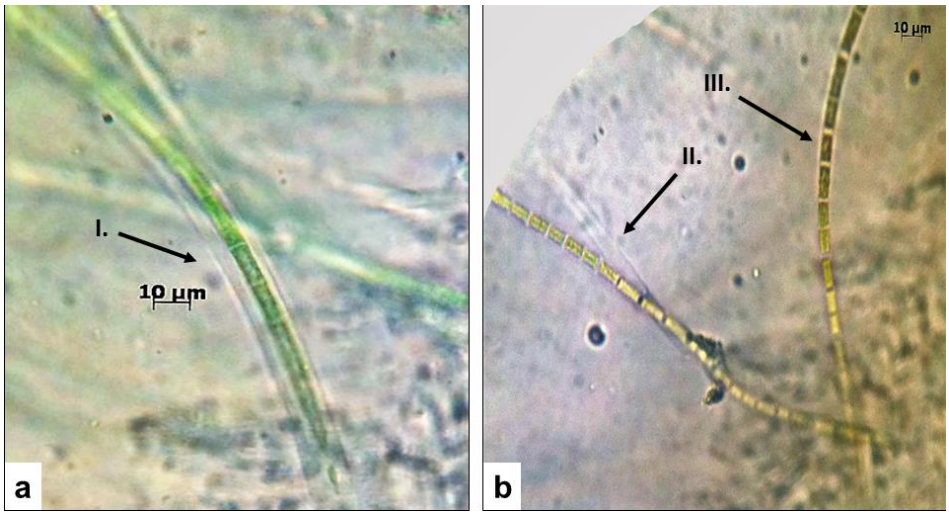

Fig. 8 Microscopical observation of the filaments of *S.* cf. *delicatissima* AMPL0116 with intact sheaths (indicated with I.) (a), and of the filaments after washing with 1.5 % NaCl and then extracting with hot water at 80 °C (b). Note the detached sheath from a filament (indicated with II.) and a sheath-deprived filament (indicated with III.)

Following the recovery of the three fractions, the phenol-sulfuric acid assay can be used to quantify them and/or further purify the fractions, or treat them for further analytical analysis. The purification processes often require precipitation in alcohol/acetone (we recommend isopropyl alcohol or ethanol) of the extract. Although the process removes impurities, it also removes low MW polymers that will not be detected in further macromolecular analysis (e.g., gel permeation chromatography). The level of purification strictly depends on the aim(s) of the investigation and may involve further purification steps depending on the hydrophobicity/hydrophilicity of the macromolecules to be removed. When not of interest, proteins may be removed by using proteases, phenol extraction or gel filtration chromatography. DNA or RNA may be removed by using nucleases.

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4. Results

Publication I

Marginal note

The following manuscript investigate the capability of a strain of *Leptolyngbya ohadii* in forming stable cyanobacterial crusts in a microcosm experiment. These inoculation experiment was aimed at evaluating the influence of sand granulometry on cyanobacterial crust growth, using three different concentrations of inoculum.

In addition, a particular focus was put on the role of the two EPM fractions with different water solubility in the aggregation process and their influence in the hydrological properties of induced cyanobacterial crusts.

The candidate directed, and/or contributed preeminently to, all the activities described in the manuscript, in particular to:

i) Designing the experimental procedure, including choosing the sand granulometry and the growth of the microcosms;

ii) Producing and preparing the cyanobacterial biomass that was employed as inoculum;

iii) Managing of all the microcosm incubation equipment and setting up of the incubation conditions;

iv) Performing the inoculation in microcosms employing a dispersion methodology that I optimized during this PhD thesis;

v) Performing, the analysis to assess cyanobacterial crust growth, and the production and the characteristics of the EPM.

vi) Performing analysis to determine the hydrological and physical properties of the cyanobacterial crusts (except for the reflectance measurements, which was conducted in a properly equipped laboratory by specifically trained operators);

vii) Performing the instrumental analytical analysis to determine the characteristics of the EPM;

viii) Conducting the analysis and interpretation of the data, including the statistical analysis;

ix) Producing the final draft of the manuscript for publication;

Inducing the formation of cyanobacterial crust by cyanobacterization: elaboration and optimization of a proficient cyanobacteria inoculation methodology.

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ABSTRACT

Inoculation of cyanobacteria (cyanobacterisation) is a potential tool to address soil rehabilitation. A successful inoculation process leads to the formation of cyanobacterial crusts, which are an early stage of biological soil crusts (BSCs). The onset of these complex microbial communities brings to several benefits for the environment, ameliorating nutrient status and soil chemo-physical characteristics.

In order to optimize this promising technology, several aspects related to its application need to be implemented, and several mechanisms at the basis of cyanobacterial crust formation completely understood. For example, the influence of substrate characteristics and of the synthesis of the extracellular polymeric matrix (EPM) in BSC formation have been minimally investigated.

In this study, we evaluated the influence of sand granulometry and initial inoculum amount on the capability of a strain of *L. ohadii* to form stable cyanobacterial crusts in a microcosm experiment.

Three different concentrations of biomass, and two sand types having different grain size were tested. A particular attention was paid to analyzing the characteristics of EPM in the process of crust formation.

The results demonstrated the importance of the investigated parameters in the success of cyanobacterization, proving that substrate granulometry influences is an important factor to consider, as it conditions both the capability of the inoculant to produce stable sand aggregates, and affects the features of the EPM.

INTRODUCTION

The use of cyanobacteria as inoculants (cyanobacterisation) has a widely recognized potential in environmental management to address soil conditioning and biofertilizing. Due to their ability to absorb large amounts of water, bind soil particles by cyanobacteria filaments and their sticky exopolysaccharides and the ability to fix the atmospheric carbon and nitrogen, the dispersion of cyanobacterial biomass in the soil can positively affect soil physico-chemical characteristics such as water holding capacity, nutrient status, and soil stability (Rogers and Burns 1994; Hu et al. 2002; Singh et al. 2016). A high number of filamentous cyanobacteria belonging to the orders Oscillatoriales and Nostocales have been tested since the 1950's as green fertilizers and to improve soil physicochemical properties (Rossi et al. 2017). Investigations have shown that the introduction of an efficient microbial community in the soil ecosystem can improve soil quality, soil health, growth, yield and quality of crops (Singh et al. 2011).

The resilience to environmental constraints owned by cyanobacteria allows employing the technology also in semiarid, arid and hyper arid environments for ecosystem rehabilitation and restoration, where drought, salinity, and strong solar irradiation are common stressors. In this frame, some large scale trials conducted by Chinese scientists employing *Microcoleus vaginatus* and *Scytonema javanicum*

brought very promising results (Zheng et al. 2010; Lan et al. 2014). This pivotal large-scale experiment in hyper-arid environment led to the development of induced cyanobacterial crusts, which represent an incipient stage of biological soil crusts (BSCs) or biocrusts. BSCs are complex communities commonly naturally found in arid and semiarid landscapes, where they exceed the total coverage of grasses and shrubs (Zheng et al. 2010), and where they play a central ecological role.

Since the success of cyanobacterization can be hampered by biotic and abiotic factors (Grant et al. 1985), it is important to identify, and improve, the cornerstone steps to apply this technology.

A special attention must be paid to the selection of the inoculants to employ according to abiotic stressors and soil chemo-physical characteristics of the site to rehabilitate. The capability to work as biofertilizers and/or bioconditioners, and the resilience once cast on the soil, are strongly dependent on the physiology of the strain and its capability to fit the characteristics of the site.

Notwithstanding the limited morphological differences, even inoculants isolated from the same highly stressing environment can display different potential roles once they are put to the test.

Before moving to a successful outdoor experimentation, it is necessary to perform targeted laboratory screenings in order to select the most suitable species (in terms of mass culture, EPS production, capacity for soil stabilization) and inoculation methodologies. In addition, in order to expand the possibilities of the technology, the research must move towards testing always new strains with more and better qualities. Recently, the inoculation of *Schizothrix* AMPL0116, isolated from a Chinese Desert, proved to trigger the development of stable cyanobacterial crusts in microcosms, on a poor sandy substrate with very limited water supply (Mugnai et al. 2017). The study supported the notion that the capability to produce an extracellular polymeric matrix (EPM) is a good criteria for selecting candidates for environments subjected to erosive forces. In addition, it underlined that it is possible to identify candidates able to produce a significant sand aggregation notwithstanding nutrient and water constraints, paving the way to economically-sustainable large-scale field approaches.

In this study, we tested the capability of the strain *Leptolyngbya ohadii* to form stable cyanobacterial crusts in a microcosm experiment. *L. ohadii* has never been tested as inoculant so far, whilst being a strain strongly adapted to harsh environmental conditions. It was isolated from biocrusts collected in the Negev Desert, Israel, where the abundance of the genus *Leptolyngbya* equals that of *M. vaginatus* (Raanan et al. 2016a). The focus of this study is: *i*) to determine the capability of *L. ohadii* to form stable organo-mineral soil aggregation and promote cyanobacteria biocrust development, and *ii*) to study the role of EPM in the process. In addition, we evaluated the influence of *iii*) sand granulometry and *iv*) initial inoculum density on the aggregation process.

MATERIAL AND METHODS

Culturing of *L. ohadii* in liquid suspension and optical microscope observations.

L. ohadii is a filamentous non-heterocystous cyanobacteria isolated from biocrusts collected in Nizzana field station, Israel, and formerly identified as *M. vaginatus* (Ohad et al. 2010; Raanan et al. 2016b). The strain is interesting in that is able to survive with the morning dew as the sole source of water and tolerate severe and relatively fast desiccation processes (Murik et al. 2017; Oren et al. 2017). The strain was kindly provided by the Department of Plant and Environmental Sciences, The Hebrew University of Jerusalem, Israel.

L. ohadii was grown in BG11 medium (Rippka 1988) for 90 days in Pirex flasks in an orbital incubator (INNOVA 44-R, New Brunswick) at a temperature of 30 °C, light intensity of 15 µmol photons m^{-2} s^{-1}, and a constant stirring of 100 RPM until reaching the stationary phase.

Inoculation of *L. ohadii* in microcosms.

L. ohadii was inoculated in microcosms that constituted of plastic Petri dishes having dimensions of 92 mm (diameter) x 16 mm (depth), filled with 60 g sand. We employed a commercial dried silica sand (VAGA s.r.l., Pavia, Italy) with two different grain sizes: 0.3 – 0.6 mm and 0.8 – 1.25 mm, determining microcosms with medium sand (MS) and coarse sand (CS), respectively. The chemical and mineralogical features of the sand are reported in Table 1. Before the use, sand was autoclaved twice to kill the pre-existing microflora.

Table 1. 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

Before being dispersed, the biomass was separated from the culture medium by centrifugation at 4000 x *g* and the pelleted filaments fragmented in a sterile plastic tubes using a sterilized spatula. Finally, the biomass was suspended in distilled water to constitute the inoculum, of which the dry weight was determined. The inoculum was dispersed spirally on the microcosms, drop by drop, using a sterile 10 mL pipette, with

a “spiral-dispersion” methodology that allows to distribute the inoculum evenly on the microcosm surface (Mugnai et al. 2017). The biomass suspension was applied to the microcosms in volumes corresponding to three different amounts: 0.15, 0.45 and 0.75 mg dry weight (DW) per square centimeter. The three different amounts were distributed in a number of microcosms always corresponding to experimental triplicates (n = 3). In addition, three microcosms containing only MS and CS (n = 3) (without the inoculum) were used as controls.

Microcosms were incubated for 30 days in a plexiglass growth chamber with controlled temperature (30 °C), illumination (45 µmol photons m^{-2} s^{-1}) and relative humidity (RH, 0 %). Every two days, 0.4 mm distilled water were sprayed over each microcosm. Such amount corresponds to the non-rainfall water (dew) registered on the playa in the Negev desert (Heusinkveld et al. 2006). Given the constant 0% RH registered in the growth chamber, this was the only water source available to the microcosms.

Soil water repellency.

Soil water repellency (WR) of the cyanobacterial crusts was assessed by applying the water drop penetration time (WDPT) test and the repellency index (RI) method.

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

RI was determined according to Mugnai et al. (2017) after 15 and 30 days of incubation 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 repellent soil.

Reflectance.

Surface reflectance (SR) was measured after 30 days of incubation with an ASD hand held portable spectroradiometer (ASD Inc., Boulder, Colorado, USA) in the laboratory, 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. Previous to measurements, the spectroradiometer was calibrated using a 99% Spectralon(r) panel. Two spectra per sample were taken, each one consisting on the internal average of three individual spectra. 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).

Soil stability.

Aggregate stability (AS) of cyanobacterial crusts was measured after 15 and 30 days of incubation using the single water-drop test (Imeson and Vis 1984). Dried cyanobacterial crusts were first sieved using 4.0 and 4.8-mm mesh sieves. Next, 0.1 g water drops were allowed to fall for 1 m onto crust 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).

Penetration resistance (PR) of the crusts was measured using a digital force gauge (Mark-10 Model M7-5, 25N, Mark-10 Corp, USA) equipped with a cone tip (0.5 mm length, 0.6 mm diameter). Cyanobacterial 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. The surface peak value was read out as the mean maximum PR (PRmax) of the upper surface of the crust. This value (reported in N units) is equivalent to the breaking point of the crust. PR max on each sample was calculated as a mean of 5 penetration-resistance readings (instrumental replicates, n = 5).

Quantification of chlorophyll and total carbohydrates in cyanobacterial crusts.

The contents in chlorophyll and carbohydrates were determined after 15 and 30 days of incubation.

After cyanobacterial crusts were homogenized with mortar and pestle, chlorophyll was extracted by treating samples with ethanol at 80 °C for 5 min, cooling at 4 °C for 30 min, and then centrifuging at 4000 x *g* to separate the pigment-containing supernatant from the pellet. Chlorophyll content was finally quantified according to Castle et al. (2011), and expressed on a g dry crust basis.

Total carbohydrate content was determined by applying the phenol-sulfuric acid assay (Dubois et al. 1956) on 10-30 mg of homogenized cyanobacterial crusts. 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. Results were expressed on a mg per dry crust basis.

Extracellular polymeric matrix extraction and analysis.

EPM was extracted from cyanobacterial crusts as two different operationally-defined fractions: tightly-bound EPS (TB-EPS), which are more condensed and stably attached to cells and sediments, and loosely-bound EPS (LB-EPS), which encompass those fractions with a higher solubility that, although not dissolved, are less compacted and are easily dispersed in the extracellular environment. The extraction methodology is in accordance with Mugnai et al. (2017), and with the protocol found in Rossi et al. (2017). For each sample, 20 g of homogenized cyanobacterial crust was extracted for 15 min with distilled water to recover LB-EPS. After centrifuging at 3,500 x *g* for 20 min, a 0.1 M Na_2EDTA solution was added to the pellet for 16 h to detach, and then recover by centrifugation (same parameters as above) TB-EPS. Both fractions were quantified applying the phenol-sulfuric acid assay, previously described, to 1 mL extract.

The apparent molecular weight (MW) distribution of LB-EPS and TB-EPS was determined by size-exclusion chromatography as described in Mugnai et al. (2017). LB-EPS and TB-EPS dissolved in deionized water were 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 (2000 kDa, 1100 kDa, 410 kDa, 150 kDa, 50 kDa) purchased from Sigma Aldrich, was used as standard.

Statistical analysis.

The significance of the data was validate 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 $P \geq 0.05$. Letters indicate a significant difference ($P < 0.05$) between the three different biomass

applications; symbols indicate a significant difference ($P < 0.05$) during the incubation time under each of the three biomass dispersions. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA.

RESULTS

Development of cyanobacterial crusts.

L. ohadii inoculation produced the formation of stable cyanobacterial crusts in a time span of three days after the spiral dispersion of the biomass (Fig. 1). The spiral dispersion methodology (Fig 1a) allowed a homogeneous coverage of the microcosm surface (Fig. 1b) producing a consistent and stable cyanobacterial crust (Fig. 1c).

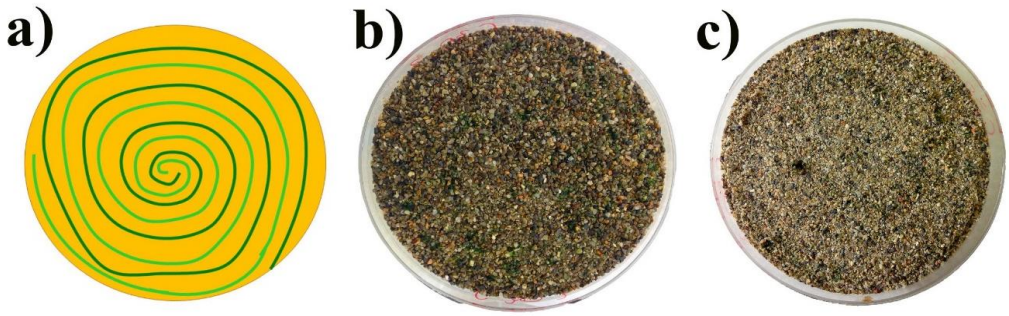

Fig. 1 Exemplification of cyanobacterial crust induction. (a) Schematization of spiral dispersion applied on a microcosm. (b) Microcosm after the inoculation of *L. ohadii* applying 0.75 mg/cm^2 of biomass suspension on CS. (c) The same microcosm after 30 days of incubation.

The thickness of the induced cyanobacterial crusts resulted depending both from the initial inoculum amount and the granulometry of the substrate (Table 2).

Table 2. Thickness of induced cyanobacterial crusts measured after 30 days of incubation with a caliper. Values were calculated as means of three independent measurements and expressed as mean (±SD).

Granulometry	**MS**				**CS**			
Inoculum concentration (mg/cm^2)	**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
Thickness [mm]	nd	4.9 (0.05)	7.8 (0.05)	7.4 (0.05)	nd	nd	7.8 (0.05)	9.7 (0.05)

Soil water repellency.

The results of WDPT indicate that inoculating 0.15 mg/cm^2 of biomass produced cyanobacterial crusts which resulted hydrophilic at the surface (penetration time < 5 sec) after 15 and 30 days of incubation both on MS and CS. Conversely, inoculating 0.45 mg/cm^2 biomass produced more hydrophobic cyanobacterial crusts on which droplet infiltration showed a significant delay after 15 days on MS and CS, and also on 30 days except on MS. The higher biomass concentration applied (0.75 mg/cm^2) produced always cyanobacterial crusts on which water penetration time is significantly longer, suggesting a higher hydrophobicity (Table 3).

Table 3. WDPT and RI measured at the surface of induced cyanobacterial crusts. Values are expresses as mean of three independent replicates (n = 3), and SD is reported between parenthesis. Different small letters indicate a significant difference between the values measured on cyanobacterial crusts induced with different amounts of initial inoculum. Symbols indicate a significant difference ($P < 0.05$) between the three inoculation different amounts of initial inoculum.

Granulometry		**MS**				**CS**			
Inoculum concentration (mg/cm^2)		**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
WDPT [sec]	**After 15 days**	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$>10^{b*}$ (1.16)	$>20^{c*}$ (6.26)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	6.06^{b} (1.16)	$>30^{b*}$ (6.26)
	After 30 days	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{ab}$ (1.09)	9.71^{b} (3.51)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	7.55^{a} (6.54)	10.51^{a} (9.11)
RI	**After 15 days**	$<1^{a}$ (0.11)	1.91^{a} (0.5)	$>10^{b}$ (3.3)	$>10^{b*}$ (2.65)	1.04^{a} (0.43)	3.53^{a} (0.05)	$>20^{b*}$ (0.67)	$>20^{b*}$ (2.69)
	After 30 days	$<1^{a}$ (0.16)	1.24^{a} (0.1)	5.96^{a} (4.84)	6.89^{a} (0.60)	1.14^{a} (0.62)	2.11^{a} (0.53)	1.82^{a} (0.52)	1.21^{a} (0.49)

RI resulted significant on cyanobacterial crusts induced by inoculating 0.45 and 0.75 mg/cm^2 biomass. Generally, RI showed a decrease from 15 to 30 days of incubation, especially on cyanobacterial crusts grown on CS. Soils inoculated with the lowest cyanobacteria biomass showed average values of 1.24 and 1.90, medium and coarse sand respectively, indicating subcritical repellent soils. After 30 days of incubation, RI drastically decreased to values lower than 1.95 on CS, while on MS average RI was 5.96 and 6.89 on cyanobacterial crusts induced by inoculating 0.45 mg/cm^2 and 0.75 mg/cm^2 biomass, still indicating a high water repellence (Table 3).

Surface reflectance.

The spectral curve of the soils 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 in the infrared region (Fig. 2a and 2b). Two main absorption features were observed in the VIS region, around 500 and 670 nm, corresponding to the absorptions by carotenoids and chlorophyll *a* pigments, respectively (Fig. 2c and 2d).

Cyanobacteria inoculation and induced biocrust development darkened the surface and led to lower reflectance in inoculated than in control soils. On MS, inoculation with 0.45 and 0.75 mg/cm^2 biomass produced crusts showing lower reflectance than inoculating with 0.15 mg/cm^2 and control soils. On CS, cyanobacterial crusts induced with the highest cyanobacteria concentration showed lower reflectance than the other inoculation treatments and control soils. Regarding absorption peaks by photosynthetic pigments, no differences were found in the absorption peak at 500 nm among the different soils. However, there were differences in the absorption peak at 680 nm due to chlorophyll *a*, which showed an increasing depth as the amount of inoculum increased (Fig. 2c and 2d).

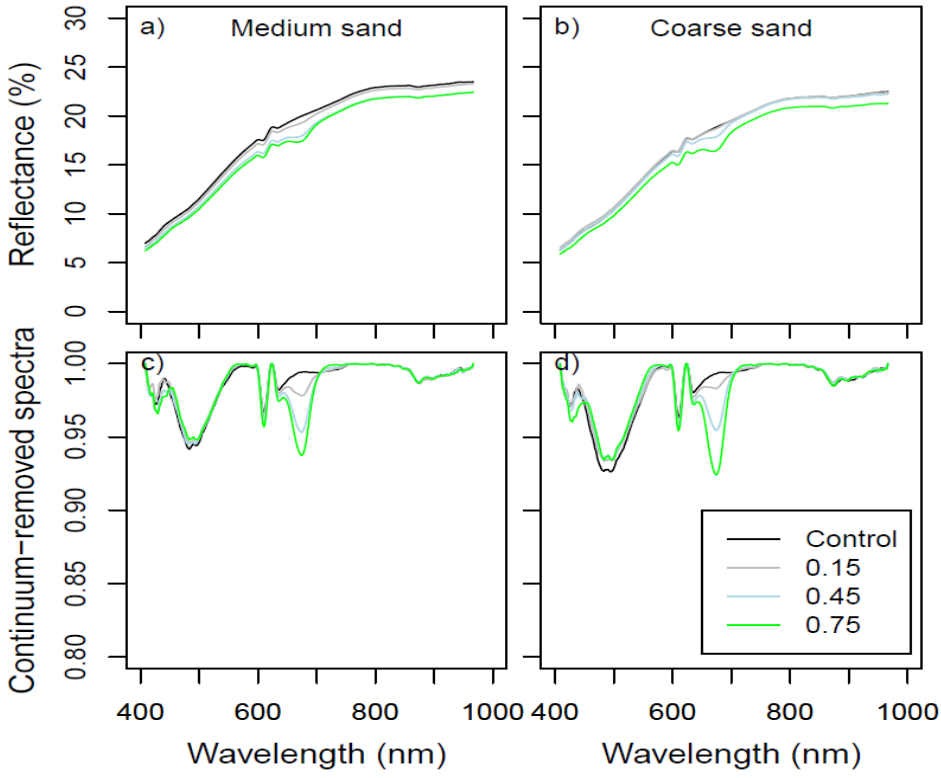

Fig. 2 Spectral absorptions in cyanobacterial crusts formed on medium sand (a) and coarse sand (b) after 30 days of incubation. Chlorophyll *a* content of control soils (sand) and cyanobacterial crusts produced by inoculation of *Leptolyngbya ohadii* on MS (c) and CS (d) sand after application of three different amounts of inoculum (0.15, 0.45 and 0.75 mg/cm^2).

Aggregate stability and penetration resistance.

AS measurement pointed out at significant differences between cyanobacterial crusts grown on MS and on CS (Table 4). On MS, after 30 days the presence of stable aggregates was present for all the three initial inoculum amounts, with the average number of drops needed to disperse the aggregates in accordance with the amount of dispersed biomass. On the other hand, no stable aggregate was detected on CS. Although aggregates were visible, they resulted so fragile that the number of drop impacts necessary to disrupt the aggregates was generally very low.

Table 4. AS and PR of cyanobacterial crusts grown in microcosms with MS and CS. AS, measured after 15 and 30 days of incubation, was expressed as the average number of water drops needed to disperse aggregates (n = 15). PR was measured after 30 days of incubation. Different small letters indicate significant differences ($P < 0.05$) between the values obtained employing different inoculum concentration.

Granulometry		**MS**				**CS**			
Inoculum concentration (mg/cm^2)		**Control**	**0.15**	**0.45**	**0.75**	**Control**	**0.15**	**0.45**	**0.75**
Aggregate stability [Average number of drops]	**after 15 days**	nd	nd	3.40^a (0.39)	3.78^a (0.50)	nd	nd	nd	nd
	after 30 days	nd	2.45^a (0.12)	3.36^b (0.22)	4.11^c (0.39)	nd	nd	nd	nd
Maximum penetration resistance [N]	**after 30 days**	nd	0.11^a (0.07)	0.30^b (0.08)	0.65^c (0.12)	bld	bld	0.06^a (0.04)	0.29^b (0.09)

nd: no aggregated detected, bld: below the limit of detection of the force gauge instrument

PR measurement performed on MS supported the data obtained with AS measurements. The compressive strength of induced cyanobacterial crusts resulted in accordance with initial inoculum amount, after 30 days. On CS, only the application of 0.75 mg/cm^2 of inoculum produced cyanobacterial crusts showing a significant compressive strength, that resulted similar to the one showed by cyanobacterial crusts induced with an initial inoculum of 0.45 mg/cm^2 on MS.

Phototrophic abundance and total carbohydrate content.

The amount of initial inoculum influenced chlorophyll content of cyanobacterial crusts grown on both types of substrate after 15 days of incubation. On both MS and CS, the application of the lowest inoculum amount (0.15 mg/cm^2) did not produce a significant increase of the pigment content from 15 to 30 days of incubation. On MS, the application of 0.75 mg/cm^2 produced a roughly proportional increase of the pigment concentration from 15 to 30 days of incubation (Fig. 3a); on CS such increase is produced by applying either 0.45 and 0.75 mg/cm^2 (Fig. 3b).

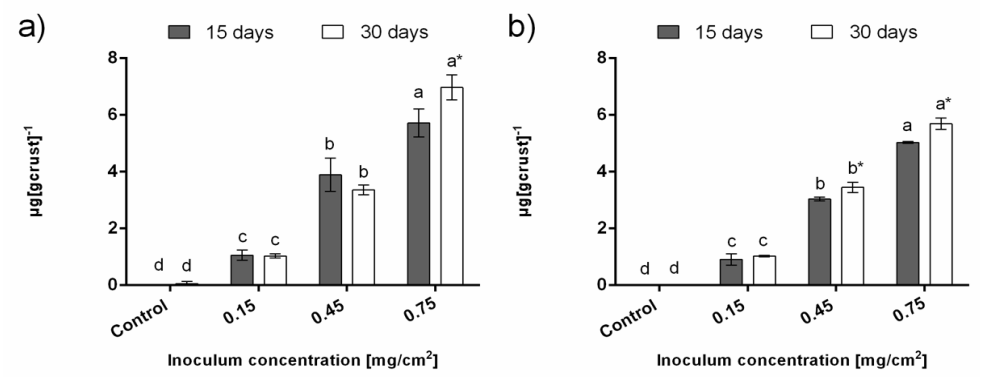

Fig. 3 Chlorophyll content (expressed as µg per g dry crust) of cyanobacterial crusts grown on MS (a) and CS (b) after 15 and 30 days from biomass dispersion. Values are means of three experimental replicates (n = 3) and error bars represent SD. Small letters indicate significant differences between values measured on cyanobacterial crusts induced with different amounts of initial inoculum. Asterisks indicate a significant increase in the value from 15 to 30 days of incubation for a same initial inoculum amount.

Total carbohydrate content in cyanobacterial crusts was influenced by the amount of initial inoculum and by substrate granulometry (Fig. 4). The higher the initial inoculum amount, the higher the carbohydrate content at the end of incubation time.

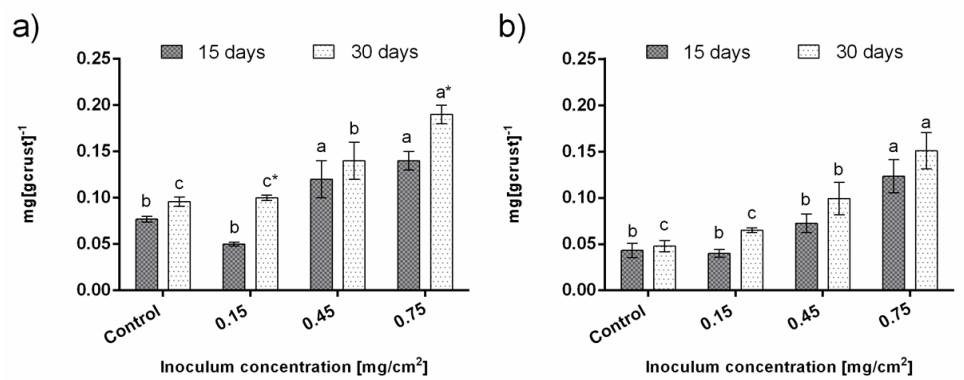

Fig. 4 Total carbohydrate content in cyanobacterial crusts grown on CS (A) and MS (B) after 15 and 30 days from biomass dispersion. Values are means of three experimental replicates (n = 3) and error bars represent SD. Bars with the same small letters are not significantly different. Asterisks indicate indicates a significant increase in the value from 15 to 30 days of incubation for a same initial inoculum amount.

Extracellular polymeric matrix analysis.

EPM was analyzed as LB-EPS and TB-EPS. The amount of LB-EPS in cyanobacterial crusts developed on MS and CS was in agreement with the initial inoculum amount after 15 days (Fig. 5). In most of the cases, LB-EPS content decreased from 15 to 30 days of incubation.

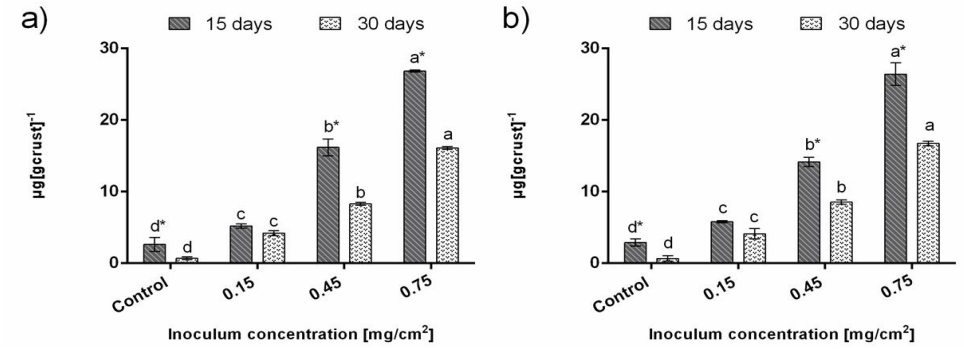

Fig. 5 LB-EPS content of cyanobacterial crusts induced by inoculating 0.15, 0.45 and 0.75 mg/cm^2 of biomass dry weight on MS (a) and CS (b). Values are means of three experimental replicates (n = 3) and SD is reported as error bars. The same small letter indicates a non significant difference between values calculated on cyanobacterial crust induced by inoculation different initial inoculum amounts. Asterisks indicate a significant increase in the values from 15 to 30 days of incubation for a same initial inoculum amount.

In the case of TB-EPS, initial inoculum amount did not affect the amount, as the content of the fraction did not result significantly different from that of the control, except for the 0.15mg/cm^2 on MS (Fig. 6).

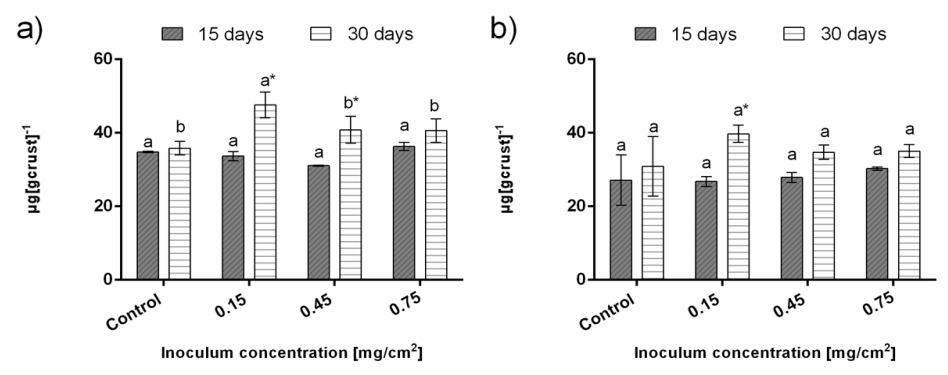

Fig. 6 TB-EPS content of cyanobacterial crusts induced by inoculating 0.15, 0.45 and 0.75 mg/cm^2 of biomass dry weight on MS (a) and CS (b). Values are means of three experimental replicates (n = 3) and SD are reported as error bars. A same small letter indicates a non significant difference between values calculated on cyanobacterial crusts induced by inoculating different initial inoculum amounts. Asterisks indicate indicates a significant increase in the value from 15 to 30 days of incubation for a same initial inoculum amount.

In terms of MW distribution profiles, LB-EPS showed a similar pattern, both on MS and on CS after 30 days (Fig. 7a, b). The fraction was mainly constituted by molecules belonging to three main size ranges, corresponding to apparent MWs of 2000-1100 kDa (representing from 30% to 37%), 450-150 kDa (representing from 27% to 32%) and 150-50 kDa (representing from 30% to 20%). A smaller percentage of molecules (from 6-7%) had an apparent MW in the range 1100-410 kDa. Results showed that MW distribution of LB-EPS was not influenced neither by inoculum amount or by sand granulometry.

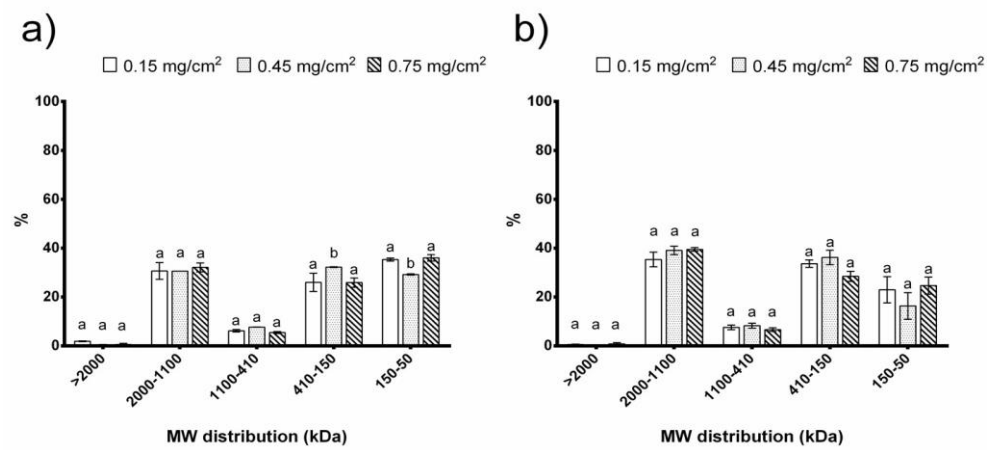

Fig. 7 MW distribution of LB-EPS extracted from cyanobacterial crusts grown on CS (a) and MS (b), induced by inoculating 0.15, 0.45 or 0.75 mg/cm^2 of biomass dry weight. Values are means of three experimental replicates (n = 3) and SD are reported as error bars. The same small letters indicate a non-significant difference within the same range of MW.

On the other hand, TB-EPS MW distribution profile analysis underlined significant differences according to the granulometry of the substrate. On CS (Fig. 8a), TB-EPS resulted of polymers almost totally distributed in the range 2000-1100 kDa; (representing from 90% in the intermediate and higher cyanobacteria biomass and 50% in the lowest), conversely, on MS (Fig. 8b) TB-EPS resulted having a more variable apparent MW, constituted for 34% by molecules with an apparent MW between 2000-1100 kDa, 42% of molecules between 410-150 kDa, 19% of molecules between 1100-410 kDa.

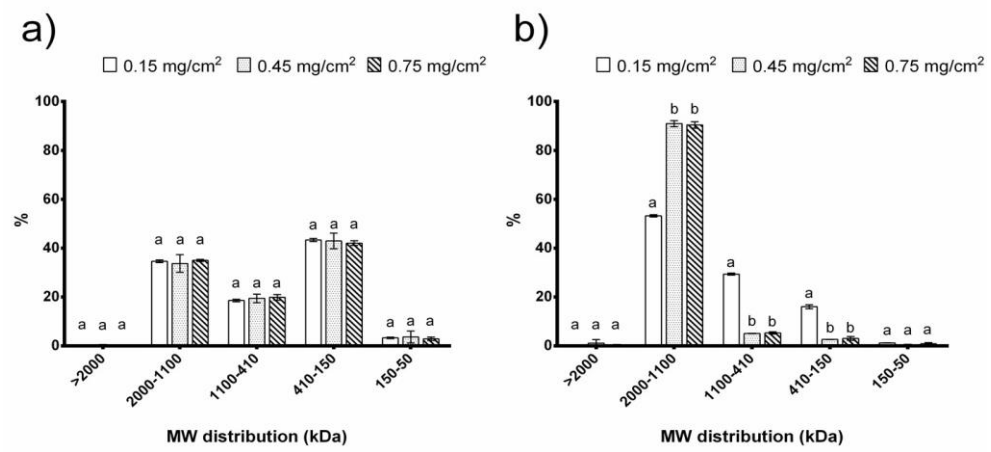

Fig. 8 MW distribution of TB-EPS extracted from cyanobacterial crusts grown on MS (a) and CS (b), induced by inoculating 0.15, 0.45 or 0.75 mg/cm^2 of biomass dry weight. Values are means of three experimental replicates (n = 3) and SD are reported as error bars. The same small letters indicate a non-significant difference between the same category of MW.

DISCUSSION

The inoculation of *L. ohadii* on sandy soil produced visible cyanobacterial crusts, both on MS and on CS. All the investigated variables resulted affected, to varying degrees, both by initial inoculum amount, and substrate granulometry. Interestingly, most of the detected characteristics resulted already present after 15 days of incubation, demonstrating the reliability of the strain as crust former. The effects of biomass dispersion, and initial inoculum amount, reflect in the lower values of SR. The strain produced more consistent crusts on MS than on CS, as supported by AS and PR tests. While on MS inoculation led to an increased aggregate stability and compressional strength compared to controls, on larger grain size sand it did not produce consistent aggregates, apart from when induced with the highest initial inoculum amount. This means that on CS cyanobacterial crusts are not enough consistent, and possibly need more initial inoculum or longer incubation times than the one tested in this work.

The dispersion on the microcosms is followed by EPS synthesis which appears to be a main driver to crust development. Much of the EPS excreted are LB-EPS, characterized by higher water solubility than TB-EPS and easily dispersed in the soil

medium. The amount of LB-EPS after 15 days is in accordance with initial inoculum concentration, whereas the values decrease in 30-day-old cyanobacterial crusts. The amounts of TB-EPS are not in agreement with initial inoculum amount, characteristic that was observed for LB-EPS, and are on the whole constant throughout incubation time. The hypothesis of LB-EPS excretion in response to abiotically stressing conditions was claimed by Mugnai et al. (2017) studying the development of cyanobacterial crusts in microcosm by inoculating *Schizothrix* cf. *delicatissima*.

The analysis of WR after 15 days suggests that the detected hydrophobicity is related to initial inoculum amount. WR values appear also to have a similar pattern to those of LB-EPS amount. In this light, the decrease of WR after 30 days may be reflecting the decrease of LB-EPS amount.

SEC chromatography pointed out at significant differences between the distribution patterns of LB-EPS and TB-EPS. MW distribution of LB-EPS is not influenced by initial inoculum amount, nor by substrate granulometry, and constituted by polymers representing similarly different size ranges; conversely, TB-EPS extracted from cyanobacterial crusts grown on CS were constituted for the most (up to 80-90%) by polymers in the range 2000-1100 kDa. This is a proof of the fact, already reported from studies conducted in liquid culture, that polymer assembly process in cyanobacteria may be affected by external factors (Rossi and Philippis 2016). In our case, this difference could be driven by the difference in sand granulometry. TB-EPS is the fraction of EPM that is more condensed and more conserved from degradation, and it is the fraction involved in conferring structural stability to biocrusts (Chen et al., 2014). These results point out at a possible physiological response to the need of conglomerating sediments of a larger grain size, contrary to smaller grain size, on which TB-EPS are represented by polymers more distributed in the different MW ranges analyzed.

L. ohadii proved to be a good candidate for inoculation under abiotically stressing conditions, showing a behavior that differs from that of previously tested strains (Mugnai et al., 2017; Rozestein et al., 2014). It is a strain able to form stable cyanobacterial crusts in a very short time with a limited water provision and without

the need of nutrient supplies. Nonetheless, it shows a better performance as inoculants when dispersed on fine-grained substrates, than on more coarse-grained ones, where possibly higher inoculum concentration or longer development times are needed to produce more stable aggregates and higher penetration resistance to cyanobacterial crusts.

This work demonstrated that a minimal initial inoculum concentration has to be respected to obtain stable cyanobacterial crusts, as lower concentrations, at least in the short run, could affect surface stability, the formation of aggregates and the proper synthesis of EPM.

After 15 days, the strain produces an increase in surface water repellency, conferred most probably by the release of LB-EPS in the substrate.

At the same time, the presence of TB-EPS, which are more conserved in quantities than LB-EPS during incubation time, seem to be involved in sand conglomeration, with the MW distribution of this EPM fraction affected by substrate granulometry.

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Publication II

Marginal note

The following manuscript describes a cyanobacterization study in which the strain *Schizothrix* cf. *delicatissima* AMPL0116, newly isolated from a desert environment, was inoculated in a microcosm experiment to determine the formation of cyanobacterial crusts. The effects of inoculation were followed and described.
In addition, a biochemical analysis was conducted on the EPM keeping together the bio-aggregates.

The candidate designed, and contributed preeminently to, all the activities described in the manuscript, in particular to:

i) Isolating and purifying the strain;
ii) Extracting the 16S ribosomal DNA for strain identification to a molecular level;
iii) Deposit of the rDNA sequence in the databank;
iv) Collecting the sand in the Negev Desert;
v) Biomass production to perform the inoculation;
vi) Designing and performing the inoculation in microcosms;
vii) Analyzing the content of chlorophyll *a* and total carbohydrates;
viii) Producing the microphotographs included in the manuscript, reporting the development of cyanobacterial crusts;
ix) Extracting and quantifying the three EPM fractions, namely LB-EPS, TB-EPS and G-EPS;
x) Performing the acquisition, analysis and interpretation of data, including the statistical analysis;
xi) Contributing to the optical microscope imaging;
xii) Contributing to the instrumental analytical procedures to determine monosaccharidic composition and molecular weight distribution of the EPM;

xiii) Producing a final draft of the manuscript for submission and participate in the journal revision process;

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ORIGINAL PAPER

Development of the polysaccharidic matrix in biocrusts induced by a cyanobacterium inoculated in sand microcosms

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Abstract Soil inoculation with cyanobacteria (cyanobacterization) is a biotechnological method widely studied to improve soil quality and productivity. During their growth on soil, cyanobacteria excrete exopolysaccharides (EPSs) which glue trichomes to soil particles, in a three-dimensional extracellular polymeric matrix. EPS productivity is an important screening parameter to select proficient inoculants and is affected by growth conditions and abiotic stresses. In this study, we evaluated the capability of the cyanobacterium *Schizothrix* cf. *delicatissima* AMPL0116 to form biocrusts when inoculated in sand microcosms under stressing conditions, and the characteristics of the synthesized polymeric matrix. In parallel, we evaluated the characteristics of exopolysaccharidic exudates of the strain when grown in liquid culture, under optimal growth setting. Our results pointed out at significant differences of the exopolymers produced in the two conditions in terms of monosaccharidic composition and molecular weight distribution, and proved the capability of *S.* cf. *delicatissima* AMPL0116 to form stable bioaggregates on sandy soils.

Keywords Cyanobacteria · Biocrusts · Cyanobacterization · Microcosm inoculation · EPSs · Extracellular polymeric matrix

Introduction

The practice of cyanobacterization (use of cyanobacteria as soil fertilizers and conditioners) has been studied along the last 60 years, especially in order to increase crop yields from agricultural soils (Hamdi 1982; Priya et al. 2015; D'Acqui 2016). A successful inoculation procedure leads to the formation of organo-sedimentary layers at the topsoil (biocrusts), in which the action of cyanobacterial filaments and cyanobacterial-secreted exopolysaccharides (EPSs)

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determines a conglomeration of soil particles (Rossi and De Philippis 2015). Cyanobacterization can improve both soil structure (Maqubela et al. 2012) and soil fertility due to the release, by cyanobacteria, of bioactive substances, N, vitamins, polypeptides, and amino acids (Rodríguez et al. 2006). These positive effects are not only limited to the biocrust thickness but also extends to the subcrust (Guo et al. 2008).

Many studies concur in indicating cyanobacteria as valid eco-friendly biotechnological tools for improving the quality and harvest yields in arable lands and for the treatment of degraded and desertified environments (Wu et al. 2014; Lan et al. 2014; D'Acqui 2016). Their proven capability to withstand adverse climatic conditions make them feasible candidates even for the treatment of environments characterized by extreme drought, strong solar irradiation, and nutrient scarcity (Lan et al. 2014). Notwithstanding their limited differences in form, their stress tolerance and the response to environmental characteristics can vary when soil-inoculated. Indeed, Grant et al. (1985) observed that the success of cyanobacterization depends upon several variables, including soil properties and climatic and biotic factors. In this light, the selection of proficient inoculants according to the environmental characteristics and constraints is a key step for elaborating effective approaches. Notwithstanding the rather large number of cyanobacterial strains that have been tested as inoculants in laboratory and open field trials, there are still knowledge gaps regarding the physiological characteristics that make them good or bad candidates (Rossi et al. 2017).

Several studies indicated the excretion of EPSs as a key physiological process affecting the outcome of the inoculation process (Hu et al. 2003; Malam-Issa et al. 2007; Maqubela et al. 2010). One reason likely lies in that these mucilaginous exudates increase the inoculant resistance to adverse conditions (Pereira et al. 2009) and the extracellular polymeric matrix (EPM) in which they spatially organize in biocrusts constitutes an optimal microenvironment in terms of nutrient concentration and moisture balance (Or et al. 2007).

Additionally, and most importantly, EPS excretion improves soil stability (Hu et al. 2003; Malam-Issa et al. 2007; Maqubela et al. 2010) and moisture content at the topsoil (Colica et al. 2014), determining ameliorated conditions allowing a higher biological activity.

The importance of EPS productivity and characteristics as a screening criteria for inoculant selection is suggested by some studies (Brüll et al. 2000; Hu et al. 2003). However, these two parameters can be profoundly influenced by environmental conditions and abiotic stresses (Huang et al. 1998; Mager and Thomas 2010; Rossi and De Philippis 2015). It was suggested that cyanobacteria produce more chemically simple EPSs when growing under natural challenging conditions than when they are cultured in a laboratory with full availability of nutrients and optimal growth settings (Mager and Thomas 2011).

In order to evaluate the effect(s) of abiotic and nutritional stresses on EPS excretion in the cyanobacterization process, we compared the characteristics of EPSs produced by a desert-dweller strain, *Schizothrix* cf. *delicatissima* AMPL0116, when grown in liquid suspension and when dispersed on sandy soil in a microcosm experiment under water and nutrient stress, to simulate inoculation in an arid setting. The objectives of the study are to evaluate the differences in two key characteristics of EPSs: monosaccharidic composition and molecular weight (MW) distribution of EPSs produced in these two conditions, and to study, for the first time, the formation of EPM during the initial stages of biocrust development.

Material and methods

Isolation and identification of the cyanobacterium used as inoculant

The genus *Schizothrix* belongs to the order Oscillatoriales (Komárek et al. 2006) and has been commonly found in biocrust communities in arid and semiarid environments (Alwathnani and Johansen 2011; Dojani et al. 2014). Additionally, it was observed as a colonizer, in a network with cyanobacteria belonging to the genus *Microcoleus*, on unstable, coarse-grained substrates (Danin et al. 1998).

S. cf. *delicatissima* AMPL0116 was isolated from biocrusts collected at the edge of Hobq Desert, Inner Mongolia, China (40° 21′ 58″ N–109° 50′ 42″ E). The environment is characterized by moving sand dunes (average height 5 m) for 61% of the landscape. The climate is semiarid, temperate, and continental monsoon, with an average temperature of 6.1 °C and annual precipitation and evapotranspiration of 293 and 2448 mm, respectively (Rao et al. 2009).

Biocrusts were collected using a circle knife with a diameter of 7.5 cm. Crusts were transported to the lab and oven dried at 35 °C for 24 h. Crust fragments were immersed in sterile liquid BG11 medium (Rippka et al. 1979) under illumination of 20 μmol photons m^{-2} s^{-1} and at 25 °C, until green coloration was visible. Afterwards, liquid culture was repeatedly stricken on BG11-containing agar plates, maintained in the conditions described above. Single colonies were picked up with a sterile spatula from the agar and suspended again in liquid BG11 medium, in Pyrex flasks. The latter were incubated in an orbital incubator (Innova 44B, New Brunswick, USA) under continuous illumination (light intensity = 10–14 μmol photons m^{-2} s^{-1}). In order to purify the culture, it was transferred in BG11 medium supplemented with cycloheximide (purchased from Sigma Chemical Co.) at a concentration of 100 μg mL^{-1}, to eliminate eukaryotic contamination. The culture was then incubated in the dark for 22 h with three other antibiotics (ampicillin, rifampicin, and ciprofloxacin), which selectively killed growing heterotrophic bacteria

(Ferris and Hirsch 1991). The presence of only one cyanobacterial species was assessed by optical microscopic observations, while the possible presence of heterotrophic contaminants was excluded by plating the culture on agarized Luria-Bertani medium and then incubating it at 30 °C to observe possible colony formation. The identification of the strain was carried out by traditional (light microscopy). Samples of the culture were observed using several different light microscopy techniques (Axioskop and Stemi 200-C, Carl Zeiss, Jena, Germany), and genus and species were assigned by appropriate taxonomic keys (Komárek and Anagnostidis 1999, Komárek and Anagnostidis 2005).

The isolate was also identified by sequencing the 16S ribosomal DNA (rDNA) gene. Genomic DNA extraction was carried out using the protocol reported by Sinha et al. (2001). The amount of extracted DNA was quantified using NanoDrop (ND-1000, NanoDrop Inc., Wilmington, DE, USA). Bacterial 16S rDNA gene fragments were PCR-amplified using the cyanobacterial specific primers, forward primer CYA359F (5′-GGGG AATYTTCCGCAATGGG-3′), and an equimolar mixture of CYA781Ra (5′-GACTACT GGGGTATCTAATCC CATT-3′) and CYA781Rb (5′-GACTACA GGGGTATC TAATCCCTTT-3′) (Nübel et al. 1997).

PCR mixtures were set up in volumes of 50 μL and contained 10 μL Red Load Taq Master 5X (Larova, GmbH, Germany), 2.5 μL of each primer (CYA359F and CYA781R) (Integrated DNA Technologies, USA), 33 μL PCR grade H_2O (Larova, GmbH, Germany), and 2 μL of template DNA. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturing at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 3 min, and a final extension for 2 min at 72 °C. Agarose gel electrophoresis (with addition of 10 μg mL^{-1} ethidium bromide) was used to check the size of the amplicons. The purification was made using the MinElute PCR purification Kit (Qiagen, Germany). Sequencing of the 16S rRNA was performed using the BigDye Terminator cycle sequencing chemistry from Applied Biosystems (ABI), ABI PRISM 3730xl DNA Analyzer and ABI's data collection and Sequence analysis softwares.

The cyanobacterium, identified as *S.* cf. *delicatissima* AMPL0116, was deposited at the Freshwater Algae Culture Collection (FACHB-collection) at the Institute of Hydrobiology of the Chinese Academy of Sciences, Wuhan, China, with deposit number FACHB2129.

Separation and characterization of EPSs obtained in liquid cultures

In order to produce the biomass for the inoculation in microcosms, the cyanobacterium was grown for 90 days in liquid cultures under the following conditions: a temperature of 30 °C, light intensity of 15 μmol photons m^{-2} s^{-1}, and a constant agitation of 100 rpm in an INNOVA 43R orbital incubator (INNOVA, New Brunswick). The released polysaccharides (RPSs) and the sheath were recovered from the liquid culture as follows: RPSs were extracted by centrifuging the culture at 4000×*g* for 30 min and concentrating the supernatant by evaporation. Afterwards, the supernatant was mixed with two volumes of cold (4 °C) isopropyl alcohol. After 8 h at 4 °C to allow the precipitation of RPSs, samples were centrifuged at 4000×*g* for 15 min and the pelletized RPSs resuspended in distilled water and lyophilized. Pelletized cyanobacterial cells after RPS recovery were washed with 1.5% NaCl solution and then extracted with 4 mL of distilled water at 80 °C for 1 h. After centrifuging at 4000×*g* for 30 min, the sheath-containing pellet was resuspended in distilled water and lyophilized. The effectiveness of the method in achieving a significant sheath extraction was checked and validated by optical microscope observations of the treated biomass in order to evaluate cell integrity and the significant presence of sheath-deprived filaments (Fig. S1).

The monosaccharidic composition of RPSs and of the sheath was determined by ion-exchange chromatography (IEC) (see the dedicated paragraph below). Before the analysis, 1 to 5 mg of lyophilized EPSs was hydrolyzed with 1 mL 2 N trifluoroacetic acid (TFA) at 120 °C for 120 min in screw-cap vials and then cooled on ice. Hydrolysates were filtered using centrifugal filters (AMICON Ultra-4, Billerica, MA) and evaporated 3 times in an orbital evaporator always resuspending in HPLC-pure water to remove TFA.

The MW distribution was determined by size exclusion chromatography (SEC), as described below in the dedicated paragraph, on the lyophilized EPSs previously solubilized in HPLC-grade water.

Inoculation of the strain in microcosms

Cyanobacterial biomass was inoculated in microcosms that constituted of two different types of plastic Petri dishes, having dimensions of 92 mm (diameter) × 16 mm (depth) and 60 mm (diameter) × 15 mm (depth) filled with 60 and 30 g sand, respectively.

In order to use an oligotrophic substrate typical of arid environments, sand was collected near Tlalim, Negev Desert, Israel (30° 58′ 05″ N–34° 38′ 10″ E). The environment where the sand was collected is hyperarid and characterized by sandy dunes with the presence of sparse subshrub vegetation. The sand, which has an alkaline pH (= 8.1), was collected far from any type of plants, on sandy dunes. Mobile sand is usually devoid of any coatings due to frequent abrasion by wind and has generally very limited C and N contents

(Felix-Henningsen et al. 2008; Roskin et al. 2011; Zaady et al. 2016). The sand was preventively sieved in order to work with a grain size ranging between 200 and 500 μm (medium sand, according to US Soil Taxonomy). It is well documented that biocrusts grow better on substrates with small granulometry (Rozenstein et al. 2014; Zaady et al. 2016), due to the higher field capacity/water availability.

Before inoculation, the cyanobacterial biomass produced in liquid cultures was separated from the culture medium by centrifugation at 4000×*g*, and the pelleted filaments were fragmented in sterile plastic tubes using a sterilized spatula. Eventually, the biomass was resuspended in distilled water in a volume calculated to provide sufficient inoculum for all the microcosms. Biomass was dispersed spirally on the microcosms, drop by drop, using a sterile 10-mL pipette, in volumes corresponding to 30 mg cell dry weight (CDW) (roughly corresponding to 2 g L^{-1} chlorophyll *a*) for the larger dishes and 15 mg CDW for the smaller ones. The spiral dispersion approach was conceived after several test trials with the aim to distribute the cells as uniformly as possible on the available surface of the microcosms. The quantity of the inoculum to apply was determined after preliminary test trials, applying increasing concentrations of biomass (from 10 to 30 mg CDW). While the lower concentrations produced inconsistent cyanobacterial crusts, concentrations in the higher range overflowed the microcosms, making it difficult to determine the actual crust development.

Inoculated microcosms were maintained inside a Plexiglas incubator with controlled temperature (30 °C) and light (45 μmol photons m^{-2} s^{-1}) for 90 days. Light was measured using a quantum meter (Spectrum Technologies, Plainfield, IL, USA). Relative humidity (RH) and temperature were monitored with a portable digital hygrothermometer (VWR, USA) having an accuracy of 5%, placed inside the incubation chamber. During the incubation time, each microcosm was provided with a total of 0.4 mm distilled water every 2 days, an amount roughly corresponding to the nonrainfall water abundance (dew) registered in the area where the soil was collected (Heusinkveld et al. 2006). Dew is present 200 nights per year, in amounts of 0.2–0.4 mL per square meters (mm) per day. In order to calculate the quantity of water to provide to each microcosm, we calculated the amount equivalent to a 3-month period and normalized it for the surface of the microcosms. Since a 0% RH was maintained in the growth chamber, the provided water amount represented the lone source for the microcosms. Noninoculated microcosms ($N = 3$) served as controls and were incubated under the same conditions and water provision as the samples.

Every 15 days, three large microcosms ($N = 3$) and three small microcosms ($N = 3$) were randomly collected to perform analyses regarding EPSs and growth parameters (chlorophyll *a*, total carbohydrates), respectively. All the analyses on biocrusts were performed after collecting the crusts with a clean spatula to a depth corresponding to their thickness (see Fig. 2c), homogenized in a mortar, and weighed.

Measurement of total carbohydrate and chlorophyll content of the biocrusts

Total carbohydrates were determined by suspending 30–50 mg homogenized crust in 1 mL distilled water and then applying the phenol-sulfuric acid method (Dubois et al. 1956) as follows: 1 mL of sample suspension was mixed with 1 mL 5% phenol, followed by 5 mL H_2SO_4 in screw-cap glass vials for 10 min. Afterwards, vials were dipped in cool water for 15 min and then the reaction mix was analyzed by determining the absorbance at 488 nm with a UV-VIS spectrophotometer (Varian, Cary 50). Calibration was performed using D-glucose at different concentrations as a reference standard.

Chlorophyll *a* in biocrusts was determined as reported by Castle et al. (2011): 1 g of homogenized crust was treated with 5 mL ethanol at 80 °C for 5 min and then incubated at 4 °C for 8 h. Subsequently, samples were centrifuged at 3500×*g* for 15 min and the pigment-containing supernatant was recovered. The extraction method was applied twice to each sample in order to achieve a full pigment recovery. Chlorophyll *a* content was determined by measuring the absorbance (*A*) at 665 nm. Each value was corrected ($A_{(665c)}$) by subtracting the absorbance at 750 nm to account for residual scattering of the solution. Chlorophyll *a* concentration was calculated by applying the formula reported by Ritchie (2006), taking into account the amount of crust (g crust) that was treated:

$$\mathrm{Chl}a\left(\mathrm{mg\ L^{-1}}\right) = \frac{[11.9035 \times A(665c)] \times V}{(\mathrm{g}\,crust) \times L}$$

where *V* is the volume of the extract and *L* is the optical path length.

Measurement of soil water repellency

For the determination of soil water repellency (SWR), we used two different methods. For the first, we employed a miniaturized tension infiltrometer as described by Lichner et al. (2013) to determine the repellency index (RI). Here, we measured the sorptivity of water and ethanol for each sample and then calculated the RI using the following formula:

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

where S_E is the sorptivity of ethanol and S_W is the sorptivity of water, and the factor of 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 nonrepellent, whereas a subcritical repellency is characterized by RI values greater than 1, but finite

(according to Tillman et al. 1989, S_W in a critically repellent soil will be 0, so the RI will be infinite). Further, we determined the contact angle (CA) of the soil using the Wilhelmy plate method (Bachmann et al. 2003) on disturbed samples using a contact angle tensiometer (DCAT 11, DataPhysics, Filderstadt, Germany), measuring the advancing contact angle. Both measurements were performed at least in triplicates.

Extraction, quantification, and characterization of loosely bound EPSs and tightly bound EPSs

The EPSs were extracted from the biocrusts grown in microcosms as two operationally defined fractions: one less condensed and more water soluble (loosely bound EPSs, LB-EPSs) and one having a higher level of gelification and being more tightly attached to cells and sediments (tightly bound EPSs, TB-EPSs). The partitioned extraction is meant to evidence eventual differences between the two fractions which, according to a previous study (Chen et al. 2014), likely play different roles within the crust system. The two fractions were extracted slightly modifying the methods reported by De Brower and Stal (2001), Rossi et al. (2012), and Chen et al. (2014).

Modifications from the published methods consisted of including repetitions of the extraction procedures to achieve consistent fraction recovery. In order to recover LB-EPSs, homogenized crusts were extracted with distilled water at room temperature for 15 min. Next, samples were centrifuged at 3500×*g* for 30 min to recover the LB-EPS-containing supernatants. For each experimental replicate, the water extraction procedure was repeated five times, and the supernatants from each replicate (*N*) were collected together. In order to recover TB-EPSs, the resulting pellet was treated with 5 mL 0.1 M Na_2EDTA for 16 h at room temperature before centrifuging at 3500×*g* for 15 min. The extraction was repeated three times in total on each replicate (*N*), the latter two extractions protracted for 120 min each. Eventually, the three supernatants from the three sequential extractions were collected together.

LB-EPSs and TB-EPSs in the extracts were quantified by applying phenol-sulfuric acid assay, previously described, on 1 ml aliquots of the extracts. No chlorophyll *a* was found in the extracts, suggesting that the extraction procedures did not damage cells causing the leakage of intracellular carbohydrates.

The monosaccharidic composition of LB-EPSs and TB-EPSs was determined by IEC as described in the following paragraph. Before IEC analysis, the extracts were hydrolyzed adding 1 part of extract to 1 part of 4 N TFA in screw-cap vials, for 120 min, at 120 °C. In the case of TB-EPSs, prior to the hydrolysis, extracts were put in 12–14 k MW cutoff nitrocellulose dialysis tubes (Medicell International Ltd., London) and dialyzed for 24 h against distilled water to remove Na_2EDTA that could interfere with the chromatographic analysis.

Apparent MW distribution of the two fractions was determined centrifuging EPS extracts at 13,000×*g* in Eppendorf tubes to remove the coarse particulate and then analyzed by SEC as described in the following paragraph.

Chemical and macromolecular characterization of the EPSs

After the hydrolytic procedures reported above, the EPSs obtained in liquid cultures or in microcosms were analyzed for their monosaccharidic composition by a Dionex ICS-500 chromatographer (Dionex, Sunnyvale, CA), equipped with an ion-exchange column (CarboPac PA1) and an ED 50 electrochemical detector with a gold-working electrode. Chromatographic conditions were those reported by Chen et al. (2014). The eluents used were Milli-Q-grade water (A), 0.185 M sodium hydroxide (B), and 0.488 M sodium acetate (C). In the first stage of the analysis (from injection time to 7 min), the eluent was constituted by 84% A, 15% B, and 1% C; in the second stage (from 7 to 15 min), the eluent was constituted by 0% A, 50% B, and 50% C; in the final stage (from 15 to 30 min), the eluent was that of the first stage. The working flow was 1.00 mL min^{-1} with running times of 30 min. Response factors of each sugar were determined by injecting known concentrations of pure monosaccharide standards (purchased from Sigma-Aldrich).

The MW distribution was determined by injecting the samples 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, 1.1, 0.41, 0.15, 0.05 M), purchased from Sigma-Aldrich, was used as standards.

Statistical analysis

Data sets were compared by applying Student's *t* test or one-way analysis of variance (ANOVA) at the 95% significance level. Every measurement was performed in instrumental triplicates ($N = 3$) for which a mean value was calculated. Mean values and SDs were calculated at least on three different independent replicates (statistical replicates, $N = 3$). Where needed, regression analysis to correlate parameters was done, reporting r^2 and *P* values in each case. For all statistical analysis apart from RI and CA measurements, GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used. For the statistical analysis of IR and CA, Student's *t* test was used with Microsoft Excel, version 2013.

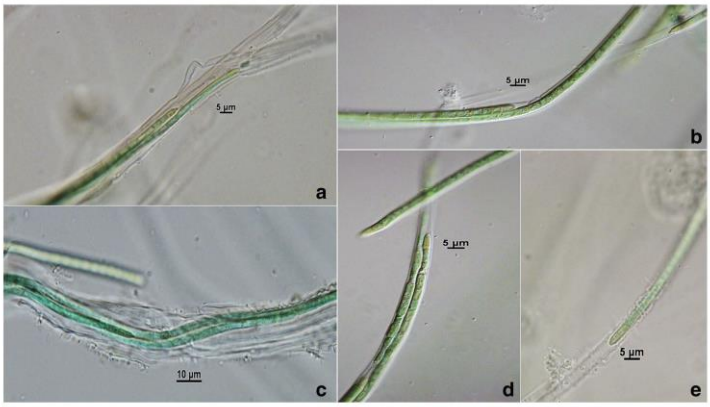

Fig. 1 **a**–**e** Optical microscope images of *S.* cf. *delicatissima* AMPL0116 evidencing the sheath

Results

Identification of the strain

The morphology of the strain allowed to identify it as *Schizothrix* cf. *delicatissima* (Fig. 1). It is characterized by solitary, sometimes pseudobranched trichomes (100–600 µm long, 5–6.5 µm thin) (Fig. 1a, b) surrounded by a thick smooth sheath (Fig. 1c) containing 1–2 trichomes (Fig. 1d). The sheath is wavy and sometimes lamelled (Fig. 1e). Cells are (4)6–8(10)× longer than wide.

The DNA sequence of the 16S rRNA gene obtained from this study was deposited in GenBank with accession number KY120205. 16S rDNA analysis showed 98% identity to the 16S sequence of *Schizothrix* sp. UAM 402 (KF544967) and *Schizothrix* sp. UAM 404 (KF544969) from the NCBI 16S

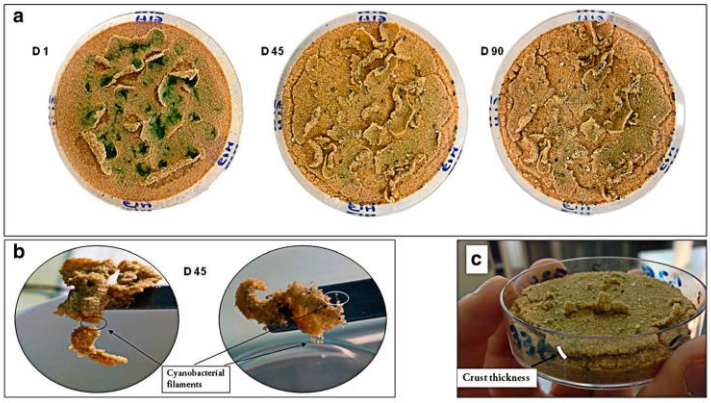

Fig. 2 **a** Development of cyanobacterial crusts from the day after inoculation (day 1, D1), halfway in the incubation time (day 45, D45), and at the end of incubation time (day 90, D90). **b** Detached 45-day-old crust shred, disclosing the presence of cyanobacterial bundles. **c** Semi-side view picture of a 90-day-crust showing a thickness of approximately 2 mm

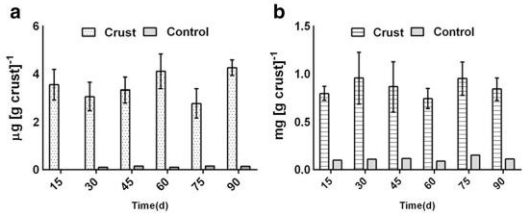

Fig. 3 **a** Chlorophyll *a* and **b** total carbohydrate content in cyanobacterial crusts induced by inoculation of *S.* cf. *delicatissima* AMPL0116 (values represent the mean of $N = 3$, error bars represent SD). No significant difference was found between values along the incubation period, in the case of chlorophyll *a* and in the case of total carbohydrate content

Ribosomal RNA Sequences Database using BLAST (Altschul et al. 1990).

Development of biocrusts

The inoculation of *S. delicatissima* AMPL0116 produced visually evident biocrusts (Fig. 2a), with obvious modification from the day after the inoculation to the end of the incubation period. At day 1, the spiral inoculation path on the surface of the microcosm was still visible. The first clear signs of sand aggregation started to appear, with cyanobacterial biomass forming flakes and determining upturned/curled features of the microbial-sediment structures. At day 45, crusts presented diffused cracks on the surface, and crusting borders appeared extended to reach the microcosm limits. All these characteristics accentuated in 90-day-old crusts. A close examination of pieces of crusts unveiled the massive presence of filament bundles collating aggregates (Fig. 2b).

A side view observation of the crusts allowed observing horizontal structuring, with demarked thickness limits of roughly 2 mm (Fig. 2c).

Chlorophyll *a* and total carbohydrate content of biocrusts

Chlorophyll *a* content in the crusts did not show significant variations during incubation time (Fig. 3a). The analysis of variance ruled out the significance of the observed fluctuations, averagely between 3.5 and 4.3 µg (g soil)$^{-1}$ ($P = 0.052$).

Similarly, total carbohydrate content did not vary significantly ($P = 0.683$), ranging averagely from 0.74 to 0.96 mg per gram of crust (Fig. 3b). As expected, the values of the two parameters were significantly higher in the biocrusts, in comparison with the controls.

Soil water repellency

The CAs did not increase during the entire experiment and no significant differences were observed between the crust and control at any time (Fig. 4). A soil is considered water repellent if the CA exceeds 90°, but the fact that CAs in our experiment were well below 20° at any time shows that no SWR was induced due to cyanobacterial growth.

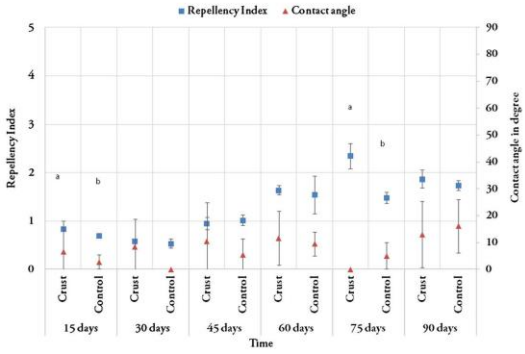

Fig. 4 Soil water repellency of the crusts. Both repellency indices (squares) and contact angles (triangles) show no strong effect of the inoculation on Error hydrophobicity over time. bars symbolize standard hydrophobicity over time. deviations of at least $N = 3$. Different letters indicate hydrophobicity over time. significant differences (only the case of RI)

Table 1 Monosaccharidic composition (expressed as moles of the single monosaccharide divided by the total amount of moles of monosaccharides in the EPS × 100)

Monosaccharidic composition (moles %)		
	RPSs	Sheath
Fuc	tr	0.64 (0.24)
Rha	tr	0.84 (0.61)
GalN	0.96 (0.53)	0.20 (0.05)
Ara	4.47 (0.54)	11.69 (1.01)*
GlcN	8.15 (1.94)	nd
Gal	11.30 (0.84)	11.22 (2.38)
Glc	53.54 (0.51)*	40.86 (0.57)
Xyl	8.36 (1.44)	20.05 (1.03)*
Fru	0.32 (0.19)	0.78 (0.46)
Rib	5.80 (1.21)*	1.57 (1.21)
GalA	nd	1.26 (0.28)
GlcA	5.44 (1.30)	10.87 (1.39)*

Contents labeled as *tr* were found not in all the biological replicates and always in contents smaller than 1%. Values are means of three determinations ($N = 3$) and SDs are reported in brackets. Values marked thus * are significantly higher than their counterpart in the other fraction

Fuc fucose, *Rha* rhamnose, *GalN* galactosamine, *Ara* arabinose, *GlcN* glucosamine, *Gal* galactose, *Glc* glucose, *Man* mannose, *Xyl* xylose, *Fru* fructose, *Rib* ribose, *GalA* galacturonic acid, *GlcA* glucuronic acid, *nd* not detected, *tr* detected only in traces

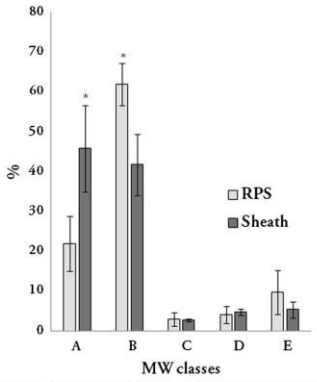

Fig. 5 Molecular weight distribution of RPS and sheath of *S.* cf. *delicatissima* AMPL0116 within 5 MW classes (A–E). A, higher than 2 MDa; B, between 2 and 1.1 MDa; C, between 1.1 MDa and 410 kDa; D, between 410 and 150 kDa; E, between 150 and 50 kDa. Values are means of three determinations. Values marked with * indicate a significant difference between the RPS and the sheath

The same is true for the RI, with the slight difference that a small increase was observed over time and that mean comparisons between control and crust yielded significant differences in two cases, namely at 15 and 75 days (Fig. 4). However, in most cases, RI was (close to 1) below 1.95 indicating nonrepellent soil crusts, and only for the crust at 75 days of incubation time, RI is slightly higher than 1 corresponding to subcritical water repellency. The results of RI are, therefore, generally consistent with the very low values for the CA.

Characterization of released and sheath polysaccharides in liquid culture

EPSs excreted by *S. delicatissima* AMPL0116 in liquid cultures were composed of 12 different types of monosaccharides: dehoxy-sugars, fucose and rhamnose; pentoses, arabinose, xylose, and ribose; aldo-hexoses, glucose and galactose; cheto-hexose, fructose; amino-sugars, galactosamine and glucosamine; and acidic sugars, galacturonic and glucuronic acids. Overall, glucose, galactose, xylose, arabinose, glucosamine, ribose, and glucuronic acid were the sugars detected at higher relative percentages (Table 1). Globally, galactose, glucose, and xylose represented 73.20 and 72.13% of the relative abundance in the RPSs and in the sheath, respectively. However, the two fractions showed some pronounced differences. While the former does not contain galacturonic acid, the latter does not contain glucosamine. In addition, some constituents showed different internal molar percentages (Table 1).

SEC analysis showed that both the RPSs and the sheath were mostly composed of high MW fractions (Fig. 5). The total percentages of the two fractions with a MW between 1 and 2 MDa and with a MW higher than 2 MDa accounted for more than 80% and for nearly 90% in RPSs and in sheath, respectively. However, the RPSs and the sheath differed significantly for what concerns the ratio between the two molecular fractions having the highest MW. Unpaired *t* test comparison ruled that while RPSs were constituted by a higher number of molecules with MW in the range between 2 and 1.1 MDa ($P = 0.010$), the sheath was constituted by a higher number of molecules with a MW higher than 2 MDa ($P = 0.015$).

Quantification and characterization of EPS in biocrusts

The amount of EPSs extracted from biocrusts (i.e., LB-EPSs plus TB-EPSs) increased during the whole incubation time, from 93.65 ± 8.37 to 205 ± 19.97 μg, per gram of crust. The increment of EPSs is due to the increase of the LB fraction. This fraction increased significantly, from roughly 43.6 (day 15) to around 108 μg per gram of crust (day 90) (Fig. 6a) with a positive correlation with the age of biocrusts ($r^2 = 0.69$,

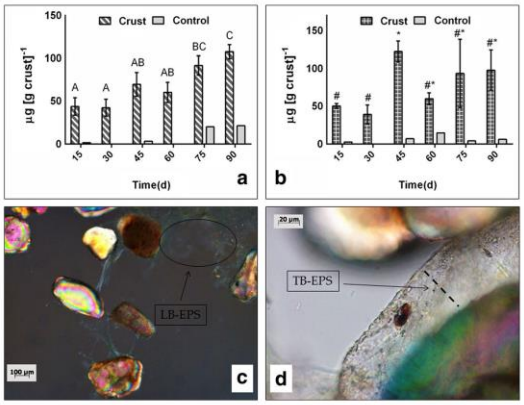

Fig. 6 LB-EPS (**a**) and TB-EPS (**b**) contents of cyanobacterial crusts during their development. Values are the means of three replicates. Values marked with different symbols are significantly different ($P < 0.05$). **c** Optical microscope image showing sand particles embedded in EPS and cyanobacterial filaments; hydrophobicity over time. LB-EPSs are pointed out. **d** Close-up of a sand grain covered by TB-EPSs. The pictures were taken on cyanobacterial crusts of 90 days

$P < 0.01$). During the whole incubation time, the analysis of variance pointed out that the content of TB-EPSs did not vary significantly ($P = 0.001$), although t test analysis between values at 15 and 90 days showed a significant increase ($P = 0.019$), from roughly 50 to 95 µg per gram of crust (Fig. 6b).

Optical microscope observations conducted on a 90-day biocrust showed distinctively the presence of LB-EPSs (Fig. 6c) and TB-EPSs (Fig. 6d) constituting the EPM. After 15 days of incubation, EPSs extracted from biocrusts were prominently composed of glucose, galactose, and xylose (Table 2), the three accounting overall for roughly 85% of LB-EPSs and 87% of TB-EPSs. Compared to liquid suspension, arabinose, ribose, and uronic acids were detected in lower relative amounts, with uronic acids detected only in traces. On the contrary, rhamnose resulted in higher relative abundances throughout the incubation time.

LB-EPSs and TB-EPSs showed significant compositional differences. Besides the three dominant neutral sugars, the former contained arabinose and glucosamine, with other constituents detected only in traces. TB-EPSs showed conversely substantial amounts of fucose, rhamnose, and galactosamine and higher relative abundance of xylose. At the end of the incubation time (day 90), TB-EPSs showed essentially galactose, glucose, and xylose (representing overall roughly 90% of the relative abundance) and rhamnose (6.51%), while the other sugars were detected only in traces. In addition to the three dominant neutral sugars, LB-EPSs contained arabinose (4.08%) and glucosamine (8.36%).

SEC analysis showed that EPSs extracted from biocrusts had varying MWs (Fig. 7) with LB-EPSs and TB-EPSs having markedly different MW distribution profiles. LB-EPSs resulted composed of molecules with an apparent MW in the range 2–1.1 MDa, roughly from 54 to 90%. TB-EPSs resulted averagely from 38 to 45% molecules with an apparent MW between 150 and 50 kDa, for roughly 26 to 30% molecules between 410 and 150 kDa, for 10% between 1.1 MDa and 410 kDa, and for roughly 16 to 20% between 2 and 1.1 MDa. Another difference between the fractions was represented by molecules with an apparent MW higher than 2 MDa. While in LB-EPSs they represented from 7 to 15%, in TB-EPSs, they were detected, for the most, in traces.

Discussion

This study contributes to implement our understanding of the interactions existing between microorganisms and minerals at biogeochemical interfaces in soil, choosing a methodology that can be encompassed between the so-called artificial soil maturation experiments (Pronk et al. 2017). In particular, we worked toward examining biotic and abiotic modifications of a sandy substrate after the introduction of a cyanobacterial inoculant in a designed laboratory study.

The inoculation of *S. delicatissima* AMPL0116, carried out following a dispersal procedure that was optimized for the purpose, induced stable cyanobacterial crusts. Inoculated microcosms were maintained throughout the experiment under continuous illumination, only providing a minimal amount of water. Notwithstanding these highly stressing conditions, the inoculated biomass organized in sheets of filaments and aggregated sand. During incubation time, the biosedimentary structures formed upward curls and an increasing number of cracks. In natural conditions, the

Table 2 Monosaccharidic composition (expressed as moles of the single monosaccharide divided by the total amount of moles of monosaccharides in the EPS × 100) of LB-EPSs and TB-EPSs extracted from biocrusts of different ages

Monosaccharidic composition (moles %)																
Time (days)	15		30		45		60		75		90					
	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs	LB-EPSs	TB-EPSs				
Fuc	tr	1.74 (1.44)	tr	tr	1.18 (0.26)	1.23 (0.17)	tr	tr	tr	tr	tr	tr				
Rha	tr	4.76 (2.43)	tr	4.66 (3.00)	1.13 (0.27)	7.09 (2.66)	3.52 (2.40)	4.08 (3.11)	3.86 (1.54)	6.64 (3.03)	tr	6.51 (5.64)				
GalN	tr	1.60 (1.08)	tr	2.95 (0.86)	4.22 (4.60)	1.39 (1.53)	1.41 (1.30)	tr	1.45 (0.67)	1.65 (0.37)	tr	tr				
Ara	3.90 (0.58)	tr	2.76 (1.88)	1.08 (0.21)	3.06 (0.41)	1.51 (1.35)	4.97 (2.83)	tr	4.64 (1.04)	tr	4.08 (1.96)	tr				
GlcN	6.72 (3.12)	1.33 (0.93)	9.31 (5.04)	tr	11.67 (3.25)	1.19 (0.91)	8.04 (2.80)	tr	14.25 (2.00)	tr	8.36 (3.48)	tr				
Gal	25.87 (0.16)	19.09 (5.67)	22.25 (0.29)	20.50 (2.00)	17.49 (11.68)	21.75 (4.36)	14.81 (4.40)	22.92 (5.12)	22.60 (8.76)	24.43 (2.23)	24.92 (5.72)	22.85 (6.43)				
Glc	57.64 (10.18)	59.75 (2.40)	55.80 (9.00)	61.56 (6.81)	50.38 (6.67)	56.15 (2.38)	53.73 (3.41)	58.42 (5.07)	44.15 (9.78)	55.87 (0.79)	51.56 (9.06)	57.89 (0.00)				
Xyl	2.10 (0.98)	8.10 (1.75)	4.11 (2.42)	6.20 (0.11)	4.21 (0.85)	8.03 (2.50)	5.11 (1.43)	8.04 (0.24)	7.65 (1.20)	8.28 (0.47)	7.47 (3.26)	8.99 (2.64)				
Fru	tr	1.18 (0.92)	2.99 (3.12)	tr	4.82 (3.09)	tr	1.76 (1.54)	tr	tr	tr	tr	tr				
Rib	tr	1.34 (1.20)	tr	1.40 (0.62)	1.84 (1.81)	1.09 (1.37)	0.18 (0.31)	tr	tr	tr	tr	nd				
GalA	tr	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd				
GlcA	nd	tr	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd				

Values represent the mean values from $N = 3$ and are expressed as mean (±SD). Contents labeled as *tr* (traces) were found not in all the biological replicates and always in contents lower than 1%, averagely

nd not detected

morphology of the biocrusts strictly depends on the characteristics of the soil and on climate (Belnap et al. 2001). Morphological characteristics similar to those observed in our experiment were found in biocrusts in Mohave Desert (Williams et al. 2012). The formation of cracks was explained as due to shrinking and swelling of clay minerals (Williams et al. 2012) and to the viscoelastic properties of EPSs (Navarini et al. 1992), while similar curls were observed upon drying in cyanobacteria-dominated biocrusts (Skujins 1991). In natural dry hot environments, the formation of cracks provides open pathways to increase soil permeability and aeration under the sheets (Williams et al. 2012), leading to new colonizable niches with ameliorated moisture regimes (Danin et al. 1998). In our study, filament bundles became visible when biocrusts were fragmented, testifying the colonization capability of the strain, which was originally dispersed only in fragmented form.

At the same time, our data suggest a restrained growth, testified by the statistically nonsignificant increase in chlorophyll *a* and total carbohydrate content during the incubation time. We assume that the absence of growth found in our study was due to the very limited water supply and very low humidity. Nonetheless, in a similar microcosm experiment employing the Oscillatorean strain *Microcoleus vaginatus* in less stressing conditions, the authors also observed a nonsignificant increase of proteins, chlorophyll, and carbohydrates (Rozenstein et al. 2014). The same authors suggested the nonreliability of these parameters to measure the inceptive colonization of biocrusts, showing the greater consistency of techniques such as electronic microscopy or reflectance spectroscopy.

In this study, microscopical observations of the dismantled crust structure clearly showed the presence of a mucous-dispersed fraction (which we referred to as LB-EPSs) and a fraction enveloping, or strictly connected to, filaments and sand grains (which we referred to as TB-EPSs). Our data showed a constant increase of the former less condensed and more soluble fraction, in correlation with incubation time. Apparently, stressing conditions elicited EPS synthesis at the expense of cell growth. The stimulating effect of a reduced moisture level on extracellular carbohydrate synthesis has already been reported by Mazor et al. (1996). This is not surprising considering the role of EPSs in increasing water-stress tolerance and their contribution in crust-water relations (Hill et al. 1994; Colica et al. 2014).

Crust development did not further increase in water repellency. A soil with a CA between 0° and 90° was defined by Lamparter et al. (2006) as "subcritically water repellent." Concerning biocrusts in the Negev Desert, Keck et al. (2016) reported subcritical SWR with similar CAs. Since the values for RI and CA of our study were even smaller, we can conclude that crust inoculation caused SWR only to a very small, subcritical degree in our samples. Most probably, very

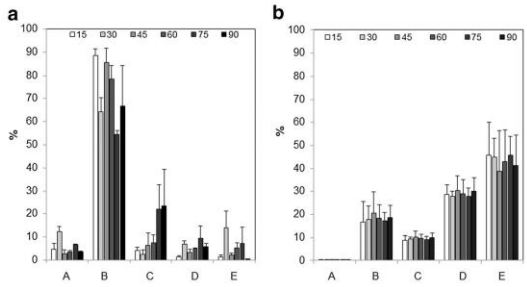

Fig. 7 **a** MW distribution of LB-EPSs and **b** TB-EPSs within the five weight classes (A–E) during cyanobacterial crust development (evaluated after 15, 30, 45, 60, 75, and 90 days). A, higher than 2 MDa; B, between 2 and 1.1 MDa; C, between 1.1 MDa and 410 kDa; D, between 410 and 150 kDa; E, between 150 and 50 kDa ranges. Values are means $N = 3$ and SDs are reported in the figure

young crusts, having only 3 months of age, are not yet capable of causing significant changes in a macroscopic feature like SWR.

When cultured in liquid suspension, *S.* cf. *delicatissima* AMPL0116 excreted compositionally rich EPSs, which are partly released in the medium (RPSs), and partly constitute the thick sheath surrounding the trichomes. RPSs and sheath resulted to be compositionally similar, with minimal differences. Both EPS fractions also share the characteristic of being mostly composed of macromolecules with a MW higher than 1 MDa, thus confirming that most cyanobacterial EPSs are characterized by high MW (Pereira et al. 2009).

EPSs extracted from the biocrusts showed a compositional pattern similar to that produced by the strain in liquid culture. Notably, galactose, glucose, and xylose resulted to be the most abundant components, indicating that they represent the polysaccharidic "core" of the EPSs produced by this strain. At the same time, some components were detected in different relative abundances.

Compared to EPSs produced in liquid culture, EPSs from biocrusts showed a higher content in rhamnose, detected mainly in TB-EPSs, while it showed lower abundances of uronic acids and arabinose. The higher content in rhamnose, which is a deoxysugar having a hydrophobic character that favors the attachment to solid surfaces (Pereira et al. 2009), may explain the tendency of the TB-EPSs to be tightly bound to sand particles. Interestingly, while sugars composing LB-EPSs were in similar internal molar ratio throughout the incubation period, TB-EPSs bore significant differences. While the "core" components and rhamnose had similar abundances from 15 to 90 days, other components were only detected in traces at the end of incubation time. Most probably, being in oligotrophic and abiotic stressing conditions, the strain produced compositionally simpler exopolymers compared to the liquid culture, where it experiences optimal abiotic conditions and excess of nutrients, as suggested by Brüll et al. (2000).

Studying the early development stages of biocrusts, Zhang (2005) observed that cyanobacterial sheaths deprived of the inner producing filament (that either migrated or died) provided the major contribution to sediment cohesion, appearing in condensed form attached to sand grains. In this view, TB-EPSs might derive from the sheath material produced by *S. delicatissima*. Stressing conditions seem to restrain the synthesis of this fraction while they do not seem to affect the synthesis of LB-EPSs. If this is the correct interpretation, this study confirms the previously hypothesized influence that nutrient status (notably the availability of N and P) and abiotic stress have on EPS excretion (Huang et al. 1998; Brüll et al. 2000). At the same time, these results point at a possible different excretion mechanism of LB-EPSs, and TB-EPSs, evidenced by the analysis performed during incubation time.

The same factors impinging on the differences in composition might be the cause of the differences in the MW distribution profiles of EPSs between liquid culture and microcosms. The more variegate distribution profile in microcosm, i.e., the conspicuous higher presence of polymers with apparent MWs lower than 1 MDa, is compatible with a downregulation of those enzymes involved in polymer assembly and elongation (Pereira et al. 2009). Again, this is true for TB-EPSs, while LB-EPSs do not seem to be affected by different growth conditions, maintaining a MW distribution profile similar to that observed in liquid culture.

This study provides, for the first time, an insight on the chemical and macromolecular characteristics of EPSs produced by cyanobacterial inoculants in the first stages of their colonization of sandy soil. The EPS synthesis appeared significantly altered under stressing conditions in soil compared to liquid culture, both in composition and MW distribution, with special regard to the more condensed EPS fraction (TB-EPSs). No alterations were observed for the more dispersed and soluble fraction (LB-EPSs). This suggests an active role of the latter fraction in fostering the formation of cyanobacterial crusts under stressing conditions, with its

synthesis representing the larger investment for the strain in the conditions in which this experiment was carried out.

Notwithstanding its restrained growth, the strain *S.* cf. *delicatissima* AMPL0116 proved to be a good candidate as inoculant and deserved further studies.

Conclusions

In conclusion, this study clearly showed that the process of colonization of sandy soil started immediately after the inoculation. *S. delicatissima* was capable of establishing a relatively thick crust in a very short period of time, releasing a rather large amount of EPSs in a few weeks. On the other hand, the period of time was probably too short for inducing also a change in the hydrological properties tested, possibly because the distribution of the trichomes and of the EPSs on the surface and on the bulk of the crust was not yet homogeneous enough.

The result of this study is that the chemical and macromolecular characteristics of the EPSs produced in liquid cultures under optimal growth conditions or in the sandy soil under stress conditions are significantly different. Moreover, the two EPS fractions, TB-EPSs and LB-EPSs, interestingly showed significant differences, particularly in their MW distribution. These previously unreported observations clearly point out the need of investigating on how the expression of the genes involved in EPS biosynthesis is regulated by the environmental conditions in cells growing in sand.

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SUPPLEMENTARY MATERIAL

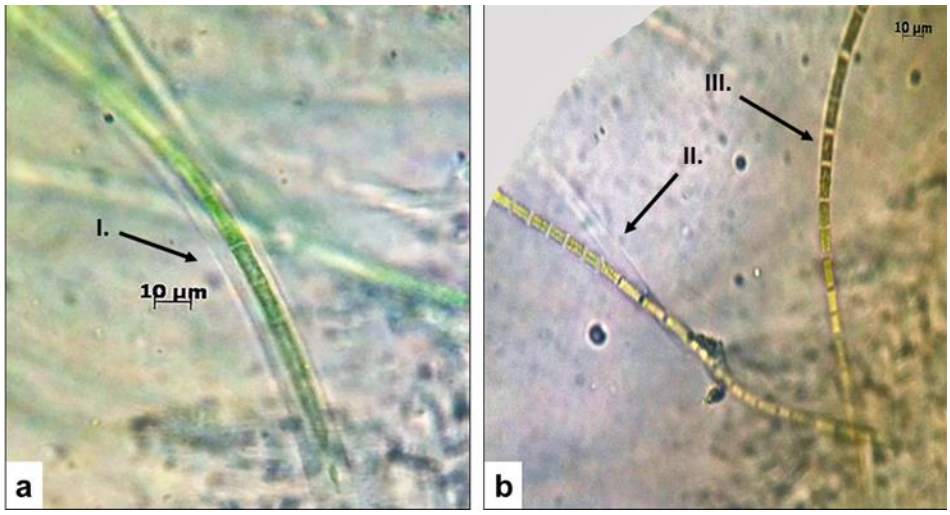

Fig. S1 Microscopical observation of the filaments of *S.* cf. Delicatissima AMPL0116 with intact sheaths (indicated with I.) (a), and of the filaments after washing with 1.5% NaCl and extraction with hot water at 80 °C (b). Note the detached sheath from a filament (indicated with II.) and a sheath-deprived filament (indicated with III.).

Publication III

Marginal note

The following manuscript deals with evaluating how water regime affect the development of cyanobacterial crusts induced by inoculating the cyanobacterium *Leptolyngbya ohadii*, in a microcosm study.

The candidate directed, and contributed preeminently to, all the activities described in the manuscript, in particular to:

i) Designing the experimental procedure, including deciding the water regimes to apply;

ii) Managing of all the microcosm incubation equipment and setting up the incubation conditions;

iii) Performing the inoculation in microcosms;

iv) Contributing in the production of the microscopical images;

v) Performing all the analysis to assess the growth of cyanobacterial crusts (content of chlorophyll *a* and total carbohydrates) and to evaluate the abundance of two EPM fractions characterized by a different grade of water solubility, in the organo-mineral aggregates;

vi) Performing the analysis of the hydrological and physical properties of the cyanobacterial crust (except the water retention analysis, which was performed by skilled operators in a properly equipped laboratory);

vii) Performing the analytical instrumental analysis to determine the monosaccharidic composition and the molecular weight distribution of the abovementioned EPM fractions;

viii) Performing analysis and interpretation of data, including the statistical analysis;

ix) Producing a final manuscript draft for publication.

Effect of different water regimes on the development of cyanobacterial crusts induced in a microcosm experiment by cyanobacteria inoculation.

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ABSTRACT

The rehabilitation of degraded soils is one of the possible approaches to counteract desertification. The fixation of shifting sand dunes is a major task in this frame.

Cyanobacterization (the employment of cyanobacteria as soil inoculants) is one important tool that can be used to achieve this goal. Cyanobacteria excrete exopolysaccharides (EPSs) which enforce the action of the filaments in conglomerating sand grains and conferring resistance to wind and water erosion to the soil. A key step is the selection of feasible inoculants, especially when aiming to apply the technology in abiotically constrained sites. To implement the technology, it is important to select always new potential inoculants basing on the capability to withstand abiotically stressing conditions, and form stable cyanobacterial/soil aggregates (cyanobacterial crusts) in a short time, with a limited request of water and without the need of adding nutrients.

The main goal of this study was to test the feasibility of a *Leptolyngbya ohadii* strain as an inoculant in microcosms containing sand which was collected in the Negev Desert, where also the strain was isolated.

The development of cyanobacterial crusts was induced under three different water regimes, corresponding to naturally occurring dewfall regimes in the Negev Desert. The development of the crusts was followed at regular intervals and the formation of the extracellular polymeric matrix, composed by the exopolysaccharides (EPSs) excreted by *L. ohadii*, was studied.

L. ohadii was able to form resistant cyanobacterial crusts having a remarkable thickness in the time span of two weeks. This process was possibly enhanced by the capability of the strain to glide down in the sand, producing a vertical structuring. At the same time, the different water regimes applied, appeared to impinge on the characteristics of the extracellular polysaccharidic matrix, notably on its molecular weight distribution.

This study enlightens the promising characteristics of *L. ohadii* as a sand stabilizer on oligotrophic substrates, producing cyanobacterial crusts that can enhance soil resilience to erosion forces. This capability is likely owing to the movement capability of the strain resulting in a microcosm vertically enriched in extracellular products. This study also enlightens, for the first time, the effects of different water regimes on the development of the extracellular polymeric matrix.

INTRODUCTION

Land degradation in drylands is a current major problem which is estimated to pose a threat for some 250 million people in the developing world, and urges to promote research and the elaboration of a common framework for managing dryland systems (Reynolds et al., 2007). The control of desertification is among the most urging environmental challenges and poses the need of elaborating new and eco-friendly biotechnological strategies. In this frame, rehabilitation or reallocation can be used to repair ecosystem functions damaged by land degradation and raise ecosystem productivity for the benefit of local people (Aronson et al., 1993).

Soil inoculation with cyanobacteria (cyanobacterization) is a promising biotechnological tool to ameliorate the topsoil environment, improving the tolerance to erosion and the physical stability, as well as promoting fertilization (Rossi et al., 2017a). Several studies demonstrated the rehabilitating effects of large-scale cyanobacterization of sand dunes in the Hopq Desert (China), producing desertification reversal (Chen et al., 2006; Lan et al., 2014; Wang et al., 2009). A successful cyanobacterization induces the formation of cyanobacterial-dominated biofilms at the soil surface (cyanobacterial crusts), recognized as a first level of development of biological soil crusts (BSCs) (Lan et al., 2013). BSCs are highly specialized communities covering the soil in the open spaces in areas with low water availability worldwide. Since BSCs are known to positively influence soil stabilization, water retention, and soil fertility, their onset can significantly lower stress barriers, and possibly turn a degraded steady state system to a more desired alternative one (Bowker, 2007). Cyanobacterization on degraded soils can kickstart the establishment of a self-sustaining ecosystem at the topsoil, by firstly stabilizing the terrain, and then promoting the cross-linking of mineral particles and organic matter accumulation (Gypser et al., 2016).

Being prominent producers of EPSs, cyanobacteria produce the first crucial soil stabilization, mediating the conglomeration between organisms and sediments. The synthesis of EPSs modify soil/water relations and water distribution, and confers

tolerance to drought stress (Tamaru et al., 2005). EPSs accumulate in a gelatinous extracellular polymeric matrix (EPM) that regulates water infiltration, percolation, retention and evaporation in and from the soil (Colica et al., 2014; Or et al., 2007; Rossi et al., 2012, 2017b; Volk et al., 2016).

The selection of always new proficient cyanobacterial inoculants is a crucial step to improve the potentiality of the cyanobacterization technology, as recent papers underline (Mugnai et al., 2017; Rossi et al., 2017a). The capability to colonize and survive with low water and nutrient requirements is a paramount screening criterion to select feasible cyanobacterial inoculants to employ in extreme environments, and is desirable for the economy of successive large-scale applications.

The Negev Desert was defined by Krumbein and Jens (1981) as one of the most extreme environments on this planet, being characterized, among others, by very low water availability. In such harsh arid environments, water is the most important limiting factor for plant colonization (Jacobs et al., 1999; Ouyang et al., 2017). Here, dew is an important source of moisture, as it contributes substantially to the annual water balance (Jacobs et al., 1999; Ohad et al., 2010; Uclés et al., 2014), being still present during summer months, where drought stress is higher (Malek et al., 1999). One study (Jacobs et al., 1999) showed the importance of dew deposition in the Negev Desert, and how it affects the daily water balance at the soil surface. During lengthy rainless seasons, dew may represent the only source of water for microorganisms (Heusinkveld et al., 2006). BSCs are naturally hydrated by early morning dew and dehydrate thereafter with rising sunlight and temperature (Kappen et al., 1979; Raanan et al., 2016b). BSC organisms have cyclic active/dormant states based on the availability of water (Garcia-Pichel and Pringault, 2001), and highly specialized physiological processes enabling the switching between these two conditions (Rajeev et al., 2013). In the Negev Desert, *Phormidium*, *Trichocoleus*, *Leptolyngbya* and *Microcoleus* are abundant genera (Hagemann et al., 2014). The isolation and screening of strains indigenous to such an environment could lead to the selection of proficient inoculants. Although several studies describe *Leptolyngbya ohadii* as BSC-dwelling species (Murik et al., 2017; Oren et al., 2017; Raanan et al., 2016b, 2016c), there is still

a lack of information concerning its contribution in increasing the stability and resilience of BSCs and, having never been tested as inoculant, there is no information on its colonizing capability on bare sand.

The objective of this paper is to investigate the capability of *L. ohadii* to form cyanobacterial crusts in a microcosm experiment, on sand collected directly in the Negev Desert, applying three different water regimes in amounts similar to those of morning dew it may receive in that area. We hypothesized that the amount of available water would lead to eventual differences in the characteristics of the cyanobacterial crusts, in terms of morphology, hydrological characteristics, and amount and characteristics of the EPM produced by the strain during sand colonization.

MATERIAL AND METHODS

Growth conditions and morphological characterization of the strain.

L. ohadii was kindly provided by the Department of Plant and Environmental Sciences of the Hebrew University of Jerusalem, Israel. Information concerning the isolation procedure and the molecular identification of this strain can be found in (Murik et al., 2017; Oren et al., 2017; Raanan et al., 2016b, 2016c). The strain was grown in liquid BG-11 medium (Rippka et al., 1979) at 30 °C in an orbital incubator (Innova 44R, New Brunswick) under a continuous illumination of 15 µmol photons m^{-2} s^{-1}.

Morphological characterization of the strain was carried out using several different light microscopy techniques (Axioskop and Stemi 200-C, Carl Zeiss, Jena, Germany), utilizing appropriate taxonomic keys (Komàrek and Anagnostidis, 1999, 2005).

Inoculation of the strain in microcosms.

Biomass preparation and dispersion in the microcosms was carried out according to Mugnai et al., (2017). The biomass was separated from the culture medium by centrifugation at 4000 × g, and the pelleted filaments were fragmented in sterile plastic

tubes using a sterilized spatula and re-suspended in distilled water in a volume calculated to provide sufficient inoculum for all the microcosms.

Microcosms constituted of two different plastic Petri dishes: large microcosms, having dimensions of 92 mm (diameter) x 16 mm (depth), and small microcosms having dimensions of 60 mm (diameter) x 15 mm (depth), filled respectively with 60 and 30 g sand, resulting in a bulk density of 1.55 g cm^{-3} (corresponding to a porosity of 42%). The sand used as a substrate was collected near Tlalim, in the Negev Desert, Israel (30°58'05" N - 34°38'10" E). The sand had very low contents of C and N (see Table S1). Before inoculation, the sand was preventively sieved to a grain size between 200 and 500 μm (medium sand), to a texture on which cyanobacterial development occurs more rapidly (Rozenstein et al., 2014). After determining the dry weight of cellular suspension, the biomass was dispersed spirally on the microcosms using a 10 mL sterile plastic pipette in volumes corresponding to 30 mg cell dry weight (CDW; corresponding to 4.6 g L^{-1} chlorophyll *a*) for the large microcosms and 15 mg CDW for small microcosms. Microcosms were subsequently incubated in a plexiglass growth chamber under controlled stable temperature (30 °C), continuous light (45 μmol photons m^{-2} s^{-1}) and a 0% relative humidity (RH) for 90 days.

The total number of microcosms was divided into three subsets, each maintained with a different water supply. In the Negev Desert, although rainfall amount and frequency increase from South to North, dew frequency and input has a more common pattern and occurs in about 200 nights each year (Hagemann et al., 2014; Heusinkveld et al., 2006; Ouyang et al., 2017). Two water regimes were applied to simulate dew received on dune slopes (0.2 mm every two days) and on dune interspaces (0.4 mm every two days), calculated from the data reported by Heusinkveld et al., (2006). A third water regime (0.6 mm every two days) was applied to provide water in "excess" compared to the former two. Since a 0% RH was maintained in the growth chamber, the provided water amount represented the only moisture source for the microcosms.

Every 15 days, large microcosms (n = 3) and small microcosms (n = 3) incubated under each of the three water regimes were randomly collected. The large

microcosms were used to measure growth parameters (chlorophyll-*a*, total carbohydrates), and EPS production and characteristics, while the small microcosms were used to measure surface repellency (water drop penetration time test, repellency index, and water retention), and the physical stability of the cyanobacterial crusts (aggregate stability and penetration resistance measurements). Non-inoculated microcosms (n = 3) incubated under each of the three water regimes served as controls.

Measurement of soil water repellency and water retention.

Soil water repellency (SWR) was assessed by measuring the water drop penetration time (WDPT) and the repellency index (RI).

WDPT was determined by measuring the time required for a drop of distilled water of 50 (± 5) μL to penetrate the crust surface. Measurements were conducted in experimental triplicates (n = 3), with 10 instrumental replicates conducted on each sample. Repellency categories to classify the level of SWR were according to (Bisdom et al., 1993; Chenu et al., 2000; King, 1981).

RI was measured by firstly measuring the sorptivity of water and ethanol at the crust surface using a miniaturized tension infiltrometer (Lichner et al., 2013), and then plotting the values in the following equation (Hallett and Young, 1999):

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

where S_E and S_W are the sorptivity of ethanol and water, respectively, while the numeric factor accounts for differences in viscosity and surface tension between the two liquids (Tillman et al., 1989). According to (Tillman et al., 1989), non-repellent soils have RI = 1, whereas a subcritical repellency is characterized by RI values greater than 1. In the case of RI, measurements were conducted both on the uppermost layer of the cyanobacterial crusts, and on the nethermost layer, after flipping the crust over.

The water potential (WP) indicates how tightly water is bound in the soil and was measured with a dew point potentiometer WP4C (Decagon devices, Pullman, USA). This device uses the chilled mirror dew point technique and is best suited for the

dry end of the water retention curve, namely a matrix potential smaller than -2 MPa. The measurements were conducted on cyanobacterial crust aggregates of roughly 2 g, which were moistened with a spray bottle and allowed to equilibrate in a sealed sample cup overnight. Measurements were conducted on the next day with the WP4C set to precision mode. The samples were allowed to air dry for approx. 30 min between each measurement.

Measurement of total carbohydrate and chlorophyll content of the biocrusts.

Total carbohydrate content was calculated by applying the phenol-sulphuric acid assay (Dubois et al., 1956) on 30-50 mg of homogenized crust suspended in 1 mL distilled water. Briefly, sample suspensions were mixed with 1 mL 5% phenol, followed by 5 mL H_2SO_4 in screw-cap glass vials for 10 min. The vials were water-cooled for 15 min and then the reaction mix eventually analyzed with a UV-VIS spectrophotometer (Varian, Cary 50. Varian Inc.) at 488 nm. Titration was carried out by using D-glucose at different concentrations.

Chlorophyll-a was extracted and quantified according to the method optimized for BSCs reported in Castle et al. (2011). Homogenized crust samples were treated with 5 mL ethanol at 80 °C for 5 min, and then incubated at 4 °C for 8 h. Subsequently, samples were centrifuged at 3500 × *g* for 15 min and the supernatants were measured at 665 nm and 750 nm with a UV-Visible Spectrophotometer (Cary, Varian Inc.). In order to exclude the contribution of the residual scattering of the ethanol solution, the corrected absorbance (A_{665c}) for each sample was obtained by subtracting the absorbance at 750 nm from the value of absorbance measured at 665 nm. Chlorophyll-*a* concentrations were finally calculated using the following equation:

$$\text{Chl-a (mg L}^{-1}) = ([11.9035 \times A(_{665c})] \times V)/((\text{g crust}) \times L)$$

where V is the volume of the extract and L is the optical path length.

EPS matrix quantification and analysis.

EPSs were extracted from cyanobacterial crusts in two operationally-defined fractions, one water soluble (loosely-bound EPSs, LB-EPSs) and one more tightly-bound to cells and sediments (tightly-bound EPSs, TB-EPSs), applying the extraction procedure reported in Rossi et al. (2017).

First, cyanobacterial crusts in the microcosm were dried, homogenized, weighed and treated with distilled water for 15 min. Next, samples were centrifuged at 3500 x *g* at 8 °C for 30 min and the LB-EPS-containing supernatants were collected. The above water extraction was repeated five times for each sample. To recover TB-EPSs, resulting pellets from water extraction were treated with 0.1 M Na_2EDTA for 16 h at room temperature. Next, samples were centrifuged at 3500 × *g* for 30 min to recover TB-EPS-containing supernatants. Na_2EDTA extraction was repeated three times on each microcosm, the last two extractions performed for 120 min each. LB-EPSs and TB-EPSs in the extracts were quantified by the previously mentioned phenol-sulphuric acid assay. Cellular integrity after EDTA extraction was checked by verifying the absence of chlorophyll *a* in the extracts.

Monosaccharidic composition of the two EPS fractions was analyzed according to Chen et al. (2014). Briefly, extracts were concentrated using a rotary evaporator and clarified by ultracentrifugation at 13,000 x g for 10 min in order to remove any remaining coarse particulate. In the case of TB-EPSs, extracts were confined in dyalisis tubes (14,000 Da MW cut-off, Medicell International, London) and dialyzed against distilled water for 24 h, with two changes of water, in order to remove the Na_2EDTA. Extracts were then hydrolyzed by adding 1 part sample to 1 part 4 N Trifluoroacetic acid (TFA) for 120 min at 120 °C, cooled on ice, and then evaporated three times in an orbital evaporator always suspending the samples in HPLC-grade water. Finally, samples were analyzed by a Dionex ICS-2500 ion exchange chromatograph. The chromatograph was 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). Eluents used were deionized water (A), 0.185 M NaOH (B) and 0.488 M sodium acetate (C). Chromatographic conditions were in

accordance with Chen et al. (Chen et al., 2014) and Mugnai et al. (2017). Flow rate was kept constant at 1 mL min^{-1}. Sugars in the extracts were identified and quantified according to reference standards.

EPS fractions were analyzed for size distribution by a Varian ProStar HPLC chromatograph (Varian, USA) equipped with a refractive index detector and two columns for Size Exclusion Chromatography (SEC), Polysep-GFC-P6000 and 4000 (Phenomenex, USA) connected in series.

Samples were analyzed with runs of 70 min and with MilliQ-grade water as eluent at a flowrate of 0.4 mL min^{-1}, using Dextran (Sigma-Aldrich, USA) at different MWs (2, 1.1, 0.41, 0.15, 0.05 M) as standards.

Crust thickness and compressive strength measurement.

The thickness of the cyanobacterial crusts was measured using a caliper.

A crushing test of the cyanobacterial crusts was performed using a digital force gauge (Mark-10 Model M7-5, 25N, Mark-10 Corp, USA) equipped with a cone tip (0.5 mm length, 0.6 mm diameter) in order to assess the mechanical stability of the cyanobacterial crust aggregate. For this, crusts were oven-dried (40 °C) for 4 h before any measurement and then put on a lifting table under the cone probe with the force gauge fixed to the lab shelf. The measurements were conducted on representative aggregate specimen with similar shape and size (roughly 3 square centimetres, roughly 5 g). The measurements were conducted raising up the lifting table until the probe–soil contact produced crust break-down. The registered peak value corresponded to the breaking point of the crust aggregates. Three instrumental replicates (n = 3) were conducted for each sample.

Aggregate stability.

Aggregate stability was measured using the single water-drop test according to (Imeson and Vis, 1984). After sieving crust samples using 4.0 and 4.8-mm meshes, the remaining aggregates were subjected to water impact from water droplets of 0.1 g (5.8 mm diameter) falling from a height of 1 m, onto aggregates placed on a 2.8 mm metal

sieve. The number of drops necessary to disrupt the aggregates was counted and used as a stability index. In order to reduce the variability, 20 instrumental replicates (n = 20) were conducted on each sample.

Statistical analysis.

The significance of the data was evaluated 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 $P \leq 0.05$. For every set of results, symbols indicate a significant difference in the values between the three water treatments; letters between the value indicate a significant difference revealed between the different sampling steps during the incubation time. 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 5.00 (GraphPad software, USA).

RESULTS

Morphological characterization of the strain.

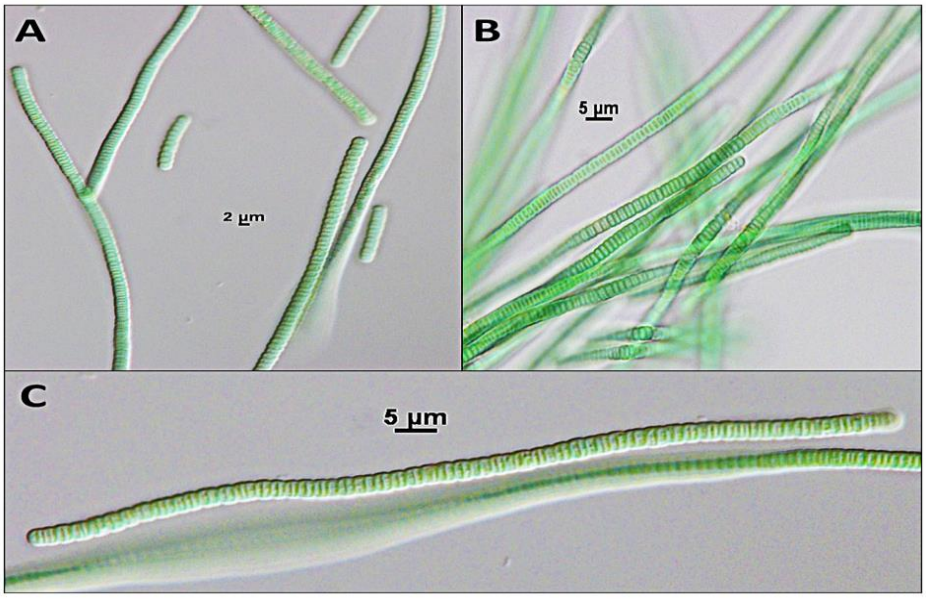

Fig. 1 A-C optical microscope images of *Leptolyngbya ohadii*.

The name *L. ohadii* was suggested by Aarion Kaplan in Raanan et al. (2016) and the species is included in the genus *Leptolyngbya*. So far the strain has not been described validly. The strain exists as an axenic culture and belongs to the deep-branching cyanobacterial cluster D (Shih et al. 2013). The strain has a high degree of similarity with *L. foveolarum* (Rabenhorst ex Gomont) (Anagnostidis and Komárek, 1988). *L. ohadii* is characterized by a thin, fine, and blue-green thallus (Figure 1 A). Filaments appear straight and parallel, sometimes variously curved or tangled together (Fig 1 B). Trichomes appear pale to bright blue-green (0.5) 1-2 µm wide, mostly distinctly constricted at the cross-wall, translucent and not granulated. Cells are monofiliform, slightly barrel-shaped, isodiametric and wider than long (2 µm wide) (Figure 1 C). Cell content is homogeneous or with sparcely distributed granules, pale blue-green; apical cells rounded, almost hemispherical, rarely a little elongated, without calyptra or

thickened outer cell wall. In addition, no sheath material was present around the cells in liquid culture.

Development of cyanobacterial crusts.

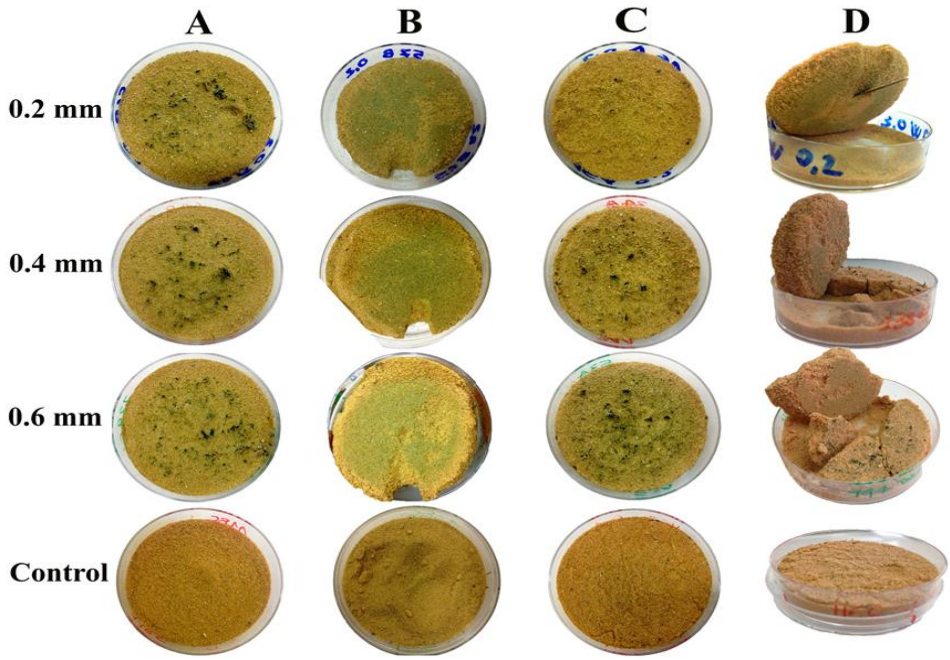

Fig. 2 Development of cyanobacterial crusts under the three water regimes (0.2, 0.4 and 0.6 mm). Upper side (column A) and nethermost layer (column B) of 15 day-old cyanobacterial crusts. (column C) Upper layer of cyanobacterial crusts after 90 days of incubation (column C) and photos showing the cyanobacterial crust thickness (column D) after 90 days of incubation.

Cyanobacterial were visibly formed after 15 days of incubation (Figure 2 A), regardless of the water regime applied. They appeared smooth and flat, with green patches visible on the surface. The aggregation of the sand grains was visually evident from the beginning of the experiment. The observation of the bottom layer of cyanobacterial crusts, after flipping them over, unveiled a diffuse green coloration (Figure 2 column B), which at a microscopical observation resulted determined by the

presence of cyanobacterial filaments that glided through the crust profile. 90-day old cyanobacterial crusts displayed a topcrust green coloration whose intensity appeared in accordance with the amount of water received (Figure 2 column C). Despite the water regime, inoculation produced cyanobacterial crusts having a similar thickness (5.6 mm ± 0.05) (Figure 2 column D). In the control, no growth was evident but only a thin physical sand layer owing to the rearrangement of the surface due to watering, only present where the highest water regime was applied. Microscopical observations showed that cyanobacterial filaments formed a close association with sand particles at the topsoil (Figures 3 A, B). At the same time, microscopical observation of the nethermost crust layers showed also strong presence of cyanobacterial colonization producing a massive sand aggregation (Figures 3 C, D).

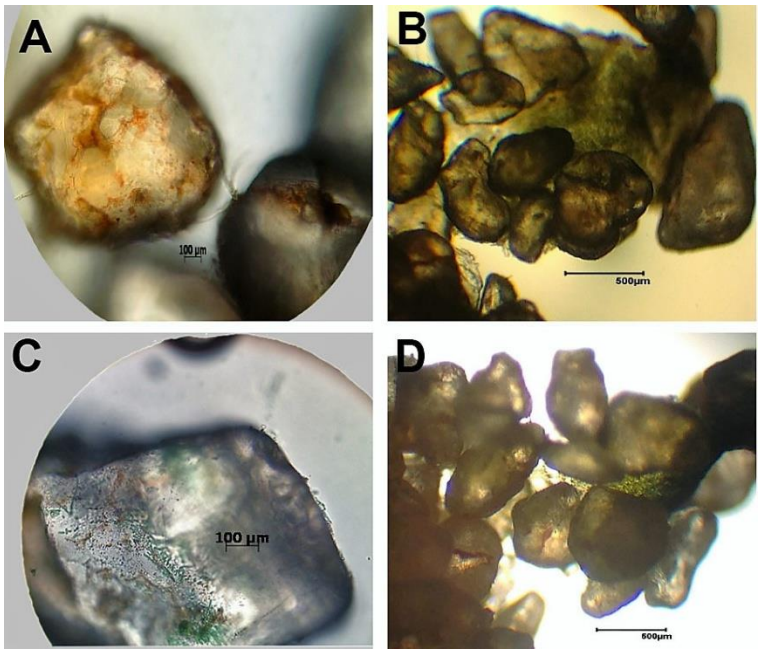

Fig. 3 Optical microscope images showing (A) cyanobacterial filaments connecting sand particles in the uppermost layer, (B) filament meshes conglomerating sand in the uppermost crust layer, (C) cyanobacterial biomass adhering to a sand grain in the nethermost crust layer and (D) filament meshes conglomerating sand in the nethermost crust layer.

Soil water repellency.

WDPT and RI measurements on 15-day old cyanobacterial crusts, showed that crust surface was slightly repellent where the lowest water regime (0.2 mm every two days) was applied. Nonetheless, after 30 days of incubation, SWR results showed only very small differences, few of which were significant (Table 1). The time required for the infiltration of droplets is 21 sec for 15-day old cyanobacterial crusts and 5 sec for the 30-day old cyanobacterial crusts (Table 1). According to Bisdom et al. (1993), Chenu et al. (2000) and King (1981), these samples can be classified as slightly repellent. No SWR was observed for cyanobacterial crusts grown under the other water regimes.

Table 1. Water drop penetration time (WDPT) and repellency index (RI) measured on the uppermost layer of the induced cyanobacterial crusts; values are means of three experimental replicates (n = 3) and values in parentheses represent SD; small letters indicate a significant difference ($P < 0.05$) during the incubation time owing to the different water regimes; asterisks or circles indicate a significant difference ($P < 0.05$) during incubation for a same water regime.

Method	**WDPT [sec]**				**RI [-]**			
Water treatments (mm)	**0.2**	**0.4**	**0.6**	**Control**	**0.2**	**0.4**	**0.6**	**Control**
After 15 days	21^{a*} (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	5.47^{a*} (0.22)	1.29^{a} (0.08)	1.24^{a} (0.09)	$<1^{a\#}$ (0.09)
After 30 days	$5^{b\circ}$ (0.58)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$2.67^{b\circ}$ (0.24)	1.11^{a} (0.03)	1.23^{a} (0.07)	$<1^{a\#}$ (0.19)
After 45 days	$<5^{c}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	1.35^{c} (0.09)	1.11^{a} (0.04)	$<1^{a}$ (0.03)	1.03^{a} (0.08)
After 60 days	$<5^{c}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<1^{d}$ (0.15)	1.13^{a} (0.06)	1.09^{a} (0.03)	$<1^{a}$ (0.07)
After 75 days	$<5^{c}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	1.25^{cd} (0.15)	1.06^{a} (0.03)	1.05^{a} (0.06)	1.00^{a} (0.13)
After 90 days	$<5^{c}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	$<5^{a}$ (0.01)	1.28^{cd} (0.2)	1.01^{a} (0.07)	1.09^{a} (0.06)	$<1^{a}$ (0.17)

Considering the observed filament gliding, RI was measured also on the nethermost layers of 90-day-old cyanobacterial crusts. While cyanobacterial crusts grown under the lowest water regime (0.2 mm) showed a non-significant ($P = 0.072$)

difference in RI between uppermost and nethermost layers, under the other two water regimes the nethermost layer resulted significantly more hydrophobic (Figure 4).

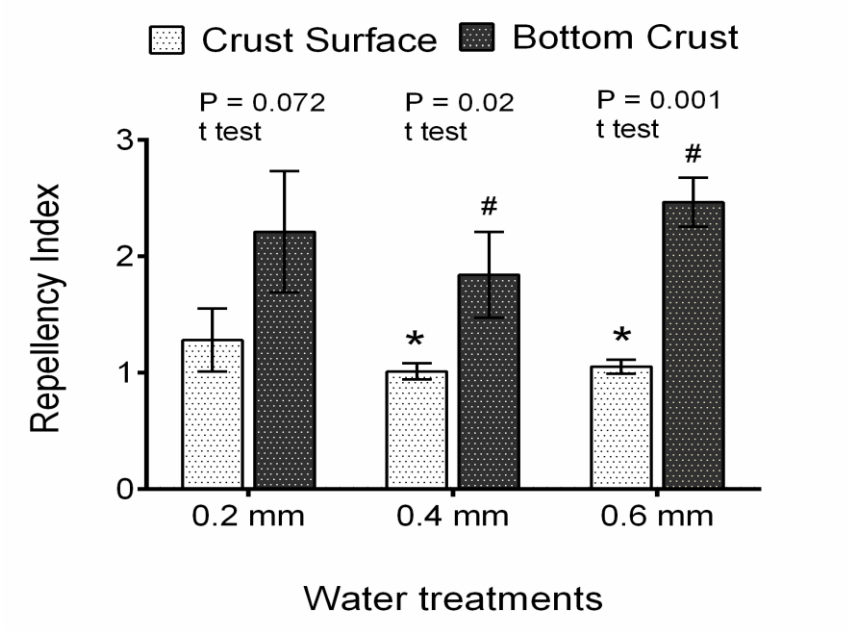

Fig. 4 Soil water repellency measured on the uppermost and the nethermost layers of cyanobacterial crusts grown under the three water regimes. Values are means of experimental replicates (n = 3), error bars represent SD. Symbols indicate a significant difference between the two crust layers.

Water retention.

Water retention of the samples was apparently not strongly affected by the water regime, nor by incubation time. Under all the three water regimes, water content in the cyanobacterial crusts showed a high variability up to pF-value 4.2, but was very low for all samples after that point, indicating that the presence or absence of *L. ohadii* or the EPSs within the samples did not increase the volume of water above the permanent wilting point (Figure 5). However, a small trend can be observed for values below pF 4.2 for all treatments, where the water retention curve behaved more silt-like with time (i.e. a slightly higher amount of water was retained in the soil at the same matric potential in the more mature crusts), but no difference was obvious between the water regimes (Figure 5).

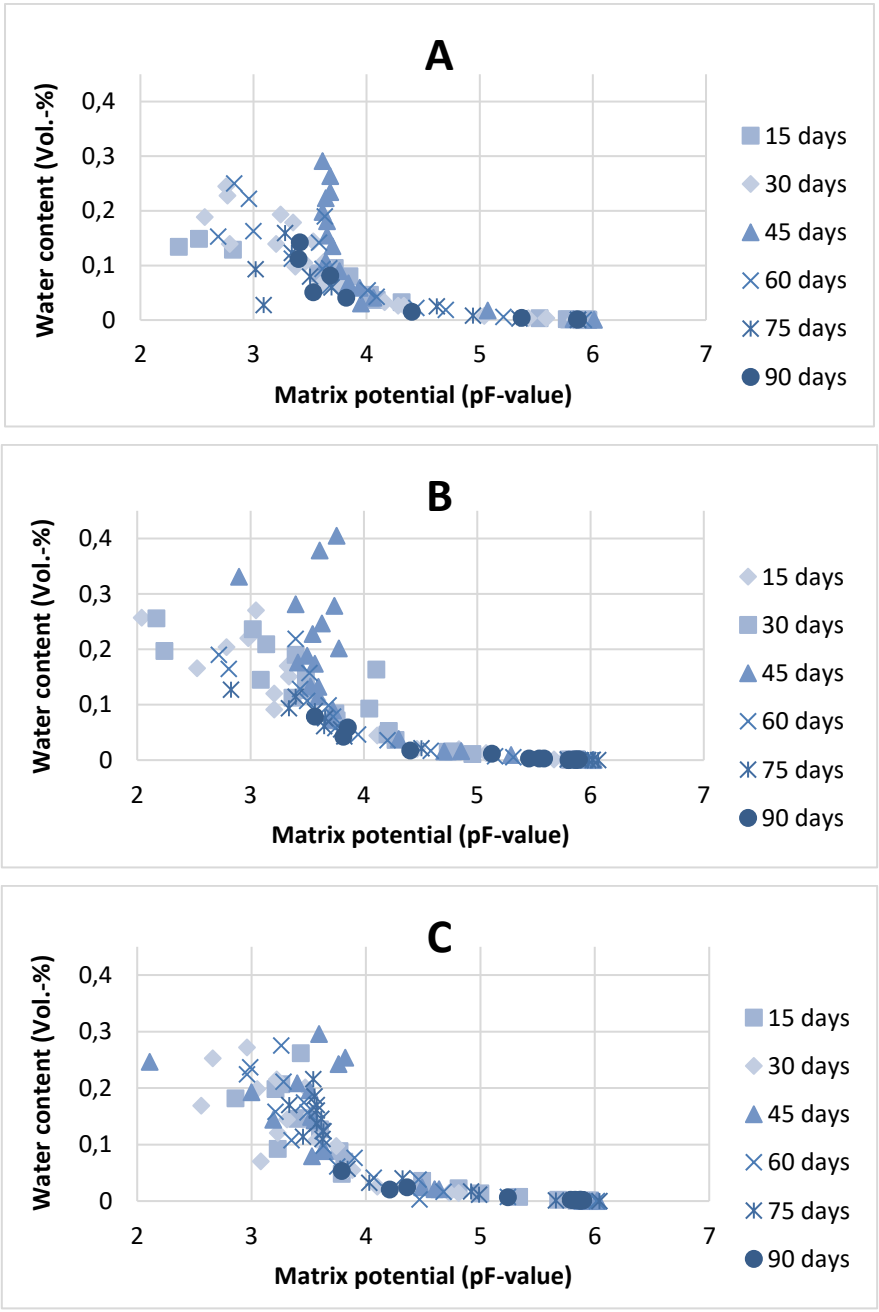

Fig. 5 Soil water retention induced cyanobacterial crusts grown under three different water regimes (0.2 mm, A; 0.4 mm, B; and 0.6 mm, C) measured at different incubation time. Values shown are mean values from at least three determinations.

Aggregate stability and compressive strength of the cyanobacterial crust.

AS of induced cyanobacterial crusts showed a minimal dependence from the water regimes (Table 2 A). A slight increase in AS was detected from 30 to 60 days of incubation for cyanobacterial crusts that received 0.4 and 0.6 mm water, although for 90-day old crusts AS was significantly lower, and not statistically different from that of 30-day-old cyanobacterial crusts.

Compressive strength did not show significant differences correlated to water regime (Table 2 B).

Table 2. (A) AS (expressed as the average number of drops needed to disperse crust aggregates) and (B) PR (expressed as the maximum compression (N) needed to break cyanobacterial crust surface). Values marked with different small letters are significantly different ($P < 0.05$) during the incubation time. Symbols indicate a significant difference ($P < 0.05$) between the three different water treatments for cyanobacterial crusts undergone the same water regime.

Method	**Aggregate stability [Average number of drops]**				**Maximum penetration resistance [N]**			
Water treatments (mm)	**0.2**	**0.4**	**0.6**	**Control**	**0.2**	**0.4**	**0.6**	**Control**
After 30 days	2.95^{a} (0.19)	2.78^{a} (0.26)	2.67^{a} (0.22)	*bld*	0.56^{a} (0.07)	0.60^{a} (0.03)	0.76^{a} (0.07)	*bld*
After 60 days	3.11^{a} (0.17)	3.56^{b} (0.16)	3.06^{b} (0.42)	*bld*	0.59^{a} (0.08)	0.62^{a} (0.09)	0.59^{ab} (0.08)	*bld*
After 90 days	2.83^{a} (0.14)	2.86^{ab} (0.25)	2.83^{a} (0.14)	*bld*	0.46^{a} (0.13)	0.60^{a} (0.12)	0.49^{b} (0.06)	*bld*

bld: below the limit of detection

Total carbohydrate and chlorophyll *a* contents.

Total carbohydrates content showed no significant variation during incubation time, for cyanobacterial crusts grown under the lowest (0.2 mm every two days; $P = 0.01$) and the middle (0.4 mm every two days; $P = 0.01$) water regimes (Figure 6 A). Conversely, an increase in total carbohydrate content during incubation time was observed for cyanobacterial crusts grown under the highest water regime (0.6 mm every two days; r^2

= 0.73; P = 0.03, assuming a linear regression). No significant difference related to the different water regimes was observed, for a same incubation time (P = 0.18).

Chlorophyll content of the cyanobacterial crusts is reported in Figure 6 B. The highest amount was detected in 15 day-old cyanobacterial crusts. Average amounts ranged from 5.40 (in microcosms that received 0.6 mm every two days) to 6.50 µg g^{-1} soil (in microcosms that received 0.2 mm every two days). During incubation time, the phototrophic abundance decreased significantly in a negative correlation with the age of cyanobacterial crusts (r^2 = 0.85; r^2 = 0.95; r^2 = 0.88; P < 0.01 for the 0.2 and the 0.4 mm water regimes, respectively).

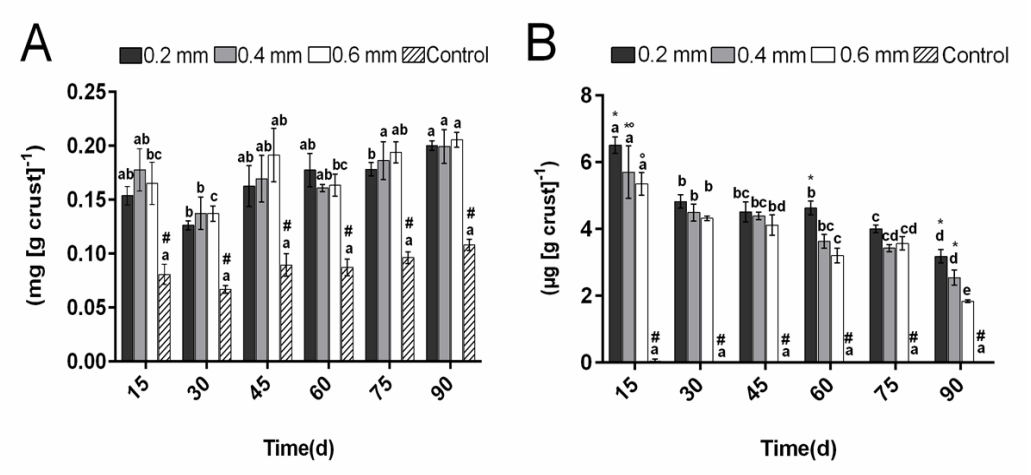

Fig. 6 (A) Total carbohydrates and (B) Chlorophyll a content in cyanobacterial crust induced by inoculation of *L. ohadii*. Values represent the mean of n = 3, error bars representing SD. Letters indicate a significant difference (P < 0.05) in the values during the incubation time under a same water regime. Symbols indicate a significant difference (P < 0.05) between the different water regimes within each sampling step.

Quantification and characterization of EPS in biocrusts

The lowest water regime applied (0.2 mm every two days) determined the development of cyanobacterial crusts containing the relatively highest LB-EPS content, specifically after 15, 30 and 75 days of incubation. The relatively highest content of LB-EPSs was detected after 75 days of incubation (63.6 µg g^{-1}) (Figure 7 A). The middle water regime (0.4 mm every two days) produced cyanobacterial crusts with no statistical

differences in LB-EPSs content during incubation. For cyanobacterial crusts grown under the highest water regime (0.6 mm every two days), a positive correlation between LB-EPS content and incubation time ($r^2 = 0.67$; P = 0.01) was detected.

Regarding TB-EPSs, the contents did not vary significantly (P = 0.01) in relation to the water regime applied, but increased linearly during incubation time under every water regime: from 10.97 to 28.92 μg g^{-1} crust (0.2 mm every two days; $r^2 = 0.7$; P < 0.05), from 13.64 to 25.67 μg g^{-1} crust (0.4 mm every two days; $r^2 = 0.65$; P < 0.05), and from 11.22 to 26.57 μg g^{-1} crust (0.6 mm every two days; $r^2 = 0.73$; P < 0.05).

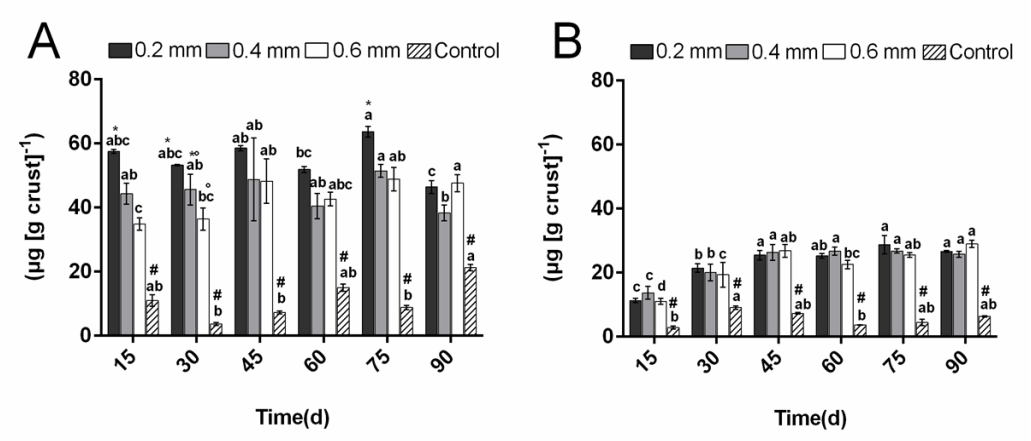

Fig. 7 LB-EPS (A) and TB-EPS (B) contents of cyanobacterial crusts during their development. Values represent the mean of n = 3, error bars represent SD. Letters indicate a significant difference (P < 0.05) in the values between the same water treatment during the incubation time. Other symbols indicate a significant difference (P < 0.05) between the three different water treatments for a same incubation time.

Molecular weight distribution and monosaccharidic composition of the EPM fractions.

Molecular weight distribution analysis of LB-EPSs and TB-EPSs was evaluated after 15 and 90 days of incubation. Results showed that these two fractions are constituted by molecules belonging to several size classes, ranging from (2-1.1 MDa) to the lowest (150-50 kDa) (Figure 8). A size class of polymers with an apparent MW higher than 2 MDa was always detected in very low percentages.

After 15 days, LB-EPSs extracted from induced cyanobacterial crusts resulted constituted for more than 50 % by molecules having an apparent MW higher than 1.1 MDa, despite the water regime that microcosms were subjected to. At the end of the incubation, this fraction significantly decreased in percentage, while we detected a significant increase of the fraction having an apparent MW between 150 and 50 kDa. Results showed that, after 90 days of incubation, the percentage of the LB-EPSs belonging to the MW class between 2 and 1.1 MDa was in accordance with the water regime applied: the higher the water regime, the lower the percentage of the molecules belonging to this MW class. Conversely, the percentage of polymers belonging to the MW range between 150 and 50 kDa increased in accordance to the water regime applied.

Concerning TB-EPSs, the different water regimes influenced strongly the MW distribution profiles. In 90-day old cyanobacterial crusts, the higher the water regime, the significantly higher resulted the percentage of polymers belonging the following MW classes: 2-1.1 MDa, 1.1 MDa - 410 kDa and 410-150 kDa. Conversely, the percentage of polymers in the range 150-50kDa decreased with increasing water regime.

The sand collected in the controls contained amounts of LB-EPSs and TB-EPSs that were too low to be analyzed by HPLC-SEC.

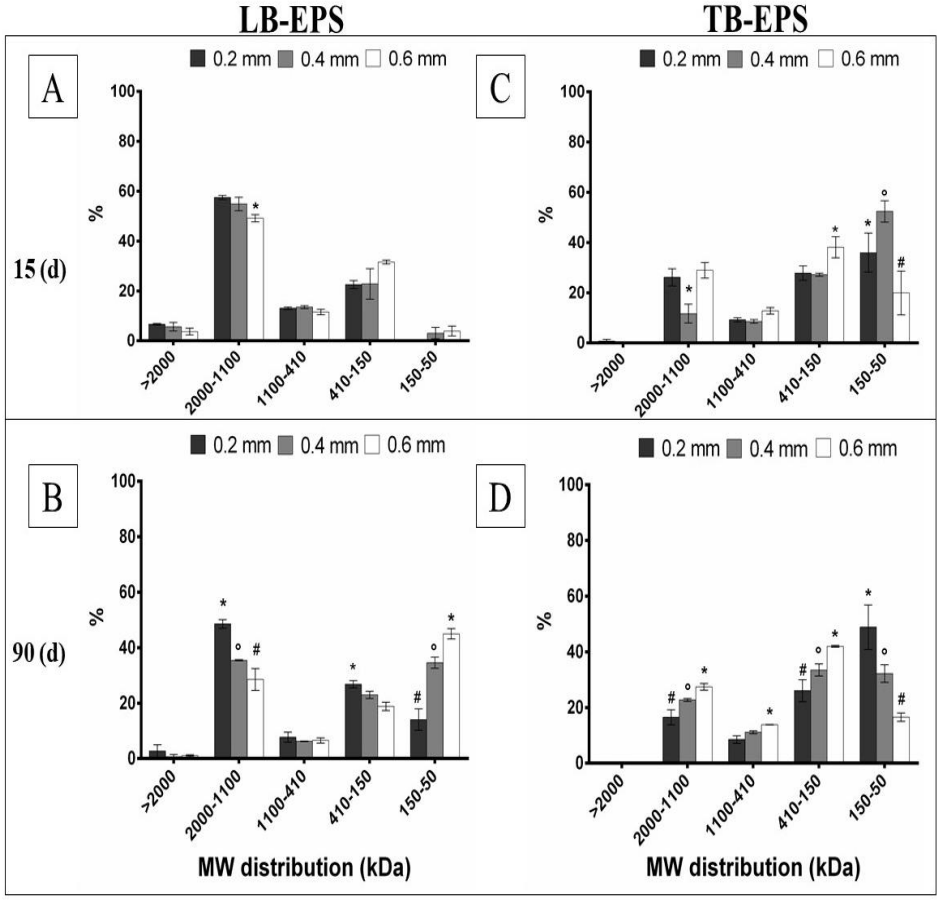

Fig. 8 MW distribution of LB-EPS after 15 (A) and 90 (B) days of incubation, and MW distribution of TB-EPS after 15 (C) and 90 (D) days of incubation, extracted from cyanobacterial crusts grown under the three water regimes. Symbols indicate a significant difference ($P < 0.05$) in the values between the three different water regimes.

Monosaccharidic composition.

The monosaccharidic composition of LB-EPSs and TB-EPSs was determined by IEC for cyanobacterial crusts after 15 days, and at the end of incubation. Results revealed the compositional complexity of the EPM. Up to 13 different types of monosaccharides were identified.

Regarding the LB-EPSs, after 15 days (Figure 9 A), galactose and glucose resulted in relative higher internal molar percentages (32 % and 25 %, respectively) in cyanobacterial crusts that received 0.2 and 0.4 mm water every two days. Conversely,

the percentage of the amino-sugar galactosamine showed the relatively highest amounts in cyanobacterial crusts subjected to the highest water regime (0.6 mm every two days). In 15-day-old cyanobacterial crusts subjected to the 0.4 and 0.6 mm water regimes, fructose and ribose were not detected; ribose was detected again in 90-day-old cyanobacterial crusts (Figure 9 B).

TB-EPSs resulted composed by twelve (when microcosms received 0.2 mm and 0.4 mm water every two days) or thirteen (when microcosms received 0.6 mm every two days) different types of monosaccharides in 15-day-old cyanobacterial crusts (Figure 9 C). Conversely, at the end of the incubation, up to thirteen different monomers were detected, despite the water regime applied. As observed for LB-EPSs, after 15 days of incubation TB-EPSs were constituted by higher percentages of galactose in cyanobacterial crusts that were subjected to the lowest water regime. At the end of incubation, TB-EPSs did not show any significant compositional difference correlating to the water regime applied (Figure 9 D).

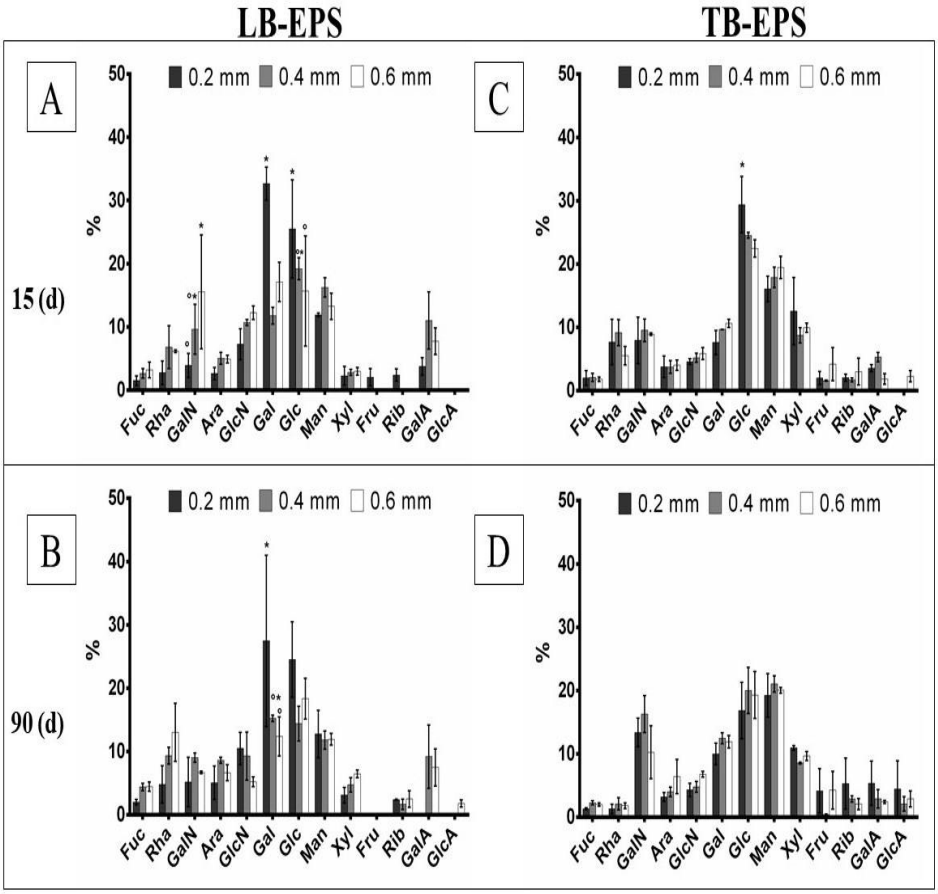

Fig. 9 Monosaccharidic composition of LB-EPSs (A, B) and TB-EPSs (C, D) extracted from biocrusts at 15 and 90 days of incubation (15 days and 90 days), watered using three different water treatments (0.2 mm; 0.4 mm and 0.6 mm). Values represent the mean of n = 3, error bars represent SD. Values marked with different symbols are significantly different ($P < 0.05$) between the three different water treatments.

DISCUSSION

The capability of inoculants to successfully produce cyanobacterial crusts on arid soils is related to the capability of withstanding hydration/dehydration cycles, and to survive with limited water and nutrient input. The synthesis of exopolysaccharidic envelopes is a key process to manage water uptake and water loss, while protecting from strong sunlight and salt stress (Pereira et al., 2009).

Leptolyngbya ohadii is one of the main filamentous cyanobacteria inhabiting the BSCs in the Negev Desert (Hagemann et al., 2014). It is able to revive after severe and relatively fast desiccation processes (Murik et al., 2017; Oren et al., 2017). According to Hu et al. (2003c) the strain is typically distributed in the deep layers of BSCs. Employed as inoculant in this study, the strain showed the capability to form stable cyanobacterial crusts in a time span of 15 days. The colonization of the microcosms was characterized by a downward gliding of the filaments producing vertical structuring. The downward migration was demonstrated by optical microscope imaging, and by assessing SWR on the nethermost crust layers. This pattern is interesting because *L. ohadii* actively increasing SWR in the deeper regions of the crust could constitute an evolutionary mechanism to cope with dry environments. A higher SWR at a depth of a few mm would impede infiltrating water, preventing it from percolating too fast through the soil, which is likely to increase water availability for the organisms in the top mm of the crust. On the other hand, it is also possible that the substances that caused SWR on the nethermost layer of the crust would have been leeched to deeper layers in a natural environment and hence this finding would have to be attributed to the experimental design which allowed hydrophobic components to accumulate in the bottom of the microcosm. This question could be answered in future studies by removing the soil solution from the microcosms via a ceramic plate on which a suction is applied. The capability of *L. ohadii* to glide from the top to the bottom of BSCs according to light and water availability was already observed by Hagemann et al., (2014) and Raanan et al. (2016a). The capability of sliding in and out from the sheaths contributes strongly to the stability of the topsoil crusts (Raanan et al., 2016a).

We observed that the different water regimes applied did not influence water retention of the cyanobacterial crusts. The recent study by Adessi et al. (2018) showed that the presence or absence of EPSs does indeed alter the water retention characteristics of BSCs quite considerably. Nonetheless, their study was done on natural BSCs, which might be one reason why they found more pronounced effects of EPSs on matric potential. Hence, despite the fact that we could not find strong effects

of any treatment on water retention, this is likely the result of the limitation of our device to measure only accurately in the dry end of the spectrum and the fact that we measured very immature crusts with comparatively low EPSs contents compared to mature natural BSCs.

Despite the visible differences in cyanobacterial crust development during incubation, total carbohydrate content, used as an index for cyanobacterial colonization, did not vary significantly, while chlorophyll content showed a decrease. These data suggest an absence of growth owing to the lack of nutrient supply and limited water availability. We observed similar results also in a previous experiment in which *Schizothrix* cf. *delicatissima* AMPL0116 was inoculated in a similar experimental setting (Mugnai et al., 2017). In a study employing *Microcoleus vaginatus* as inoculant, Rozenstein et al. (2014) sustained the non-reliability of these parameters to assess cyanobacterial incipient colonization, while they suggested the use of other revelation approaches (e.g., reflectance spectroscopy) as possibly more indicative. The reason for the decrease in chlorophyll *a* content is not immediately obvious. The most plausible explanation is that self shading (see the abundance of the cyanobacteria already 15 days after inoculation, Fig. 2) after 15 days time point led to a decline in chlorophyll a (located in the photosynthetic reaction centers) counterbalanced by a possible increase in the light harvesting complexes, the phycobilisomes.

We supplied the microcosms with three different water regimes during incubation, and studied eventual effects on cyanobacterial crust development, and on EPM synthesis and characteristics. Interestingly, the different water regimes did not affect the final thickness of cyanobacterial crusts, nor the compressive strength and aggregate stability. This demonstrates the capability of the strain to form stable crusts despite water scarcity and demonstrate its fast terrain-stabilizing property. Overall, our observation supports the idea that the time to reach a high stability may be even shorter than two weeks, as crust compressive strength and aggregate stability did not vary significantly after such period.

Conversely, our study showed that the productivity, the monosaccharidic composition, and MW distribution of LB-EPSs and TB-EPSs released by the inoculant were influenced by water regime at different degrees. LB-EPSs were found in the highest content where the lowest water regime (0.2 mm every two days) was applied. This could represent a physiological response of the strain to drought stress, as more water-retaining polymer is released in the surrounding medium. Under this water regime, LB-EPS constituted of significantly higher amounts of glucose and galactose compared to the other water regimes, supporting the theory that more stressing conditions elicit the synthesis of more compositionally simple EPS (Mager and Thomas, 2011). At the same time, LB-EPSs were characterized prominently (roughly 50 %) by polymers having an apparent MW in the range 2-1.1 MDa. The presence of soluble HMW EPSs can be the cause of the observed surface SWR and exert a retarding effect on water movement (Mazor et al. 1996). Indeed, according to RI measurement and WDPT test, cyanobacterial crusts after 15 and 30 days of incubation were slightly more repellent when grown under the lowest water regime. In 90-day old cyanobacterial crusts, the percentage of this high MW fraction was found to be lower in cyanobacterial crusts provided with more water availability, counterbalanced by an increase in the percentage of polymers in the range 150-50 kDa. The increase of the percentage of the lower MW fractions of LB-EPSs with increasing water regime suggests an increasing microbial activity leading to the decomposition of the higher polymers under less stressing conditions (Chen et al., 2014). Conversely to LB-EPSs, the content of TB-EPSs was not influenced by water regime. It increased from 15 to 45 days of incubation (before remaining stable thereafter), despite the amount of water received. Water regime did not affect the monosaccharidic composition of this fraction (when evaluated at the end of incubation), although it affected the MW distribution. Under the lowest water regime, TB-EPSs resulted with a MW more uniformly distributed in the different size classes than LB-EPSs, particularly in the range 150-50 kDa. In antithesis to what was observed for LB-EPSs, the increase in water provision resulted in an increase in the percentage of TB-EPSs with a MW in the range 2-1.1 MDa. Overall, these differences suggest different synthetic pathways for the synthesis

of the two EPM fractions (suggested also by our previous study; i.e., Mugnai et al., 2017), and that these can be influenced, alternatively or at the same time, by environmental conditions. This is particularly important considering the supposed different roles of the two factions within the cyanobacterial crust system. While LB-EPSs, more dispersed in the extracellular space, are a notable C source on which BSC microflora feeds, TB-EPSs, less soluble and condensed and with a lower degradability, represent more a "structural skeleton" to cyanobacterial crusts (Chen et al., 2014; Rossi et al. 2017). Given the seemingly restrained growth of the strain, the stability of the induced cyanobacterial crusts was most likely provided by the EPSs excreted by the strain during gliding. In particular, and conversely to LB-EPSs, the content of TB-EPSs was found always increasing despite the water regime at every survey during incubation, suggesting the key support of this fraction in cyanobacterial crust structuring.

In conclusion, *L. ohadii* appears as an excellent inoculant candidate for arid terrains with scarce water and nutrient availability. Its ability to form thick and stable crusts is caused by its capability to glide in the soil producing TB-EPSs, the productivity of which seems to be independent of water regime. At the same time, water regime was shown to influence EPS features, prominently those of LB-EPSs, which resulted prominently synthesized under the lower water availability tested, and that were possibly involved in conferring surface hydrophobicity to cyanobacterial crusts. Future studies should try to assess the performance of *L. ohadii* under field conditions and try to quantify its effect on soil stability and fertility.

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SUPPLEMENTARY MATERIAL

Table S1 C and N contents of control samples for all treatments and time points.

Sample	treatment	description	N (weight-%)	C (weight-%)
109C	0.2 mm	15 day Control	0.004	0.417
110C	0.2 mm	30 day Control	0.005	0.547
111C	0.2 mm	45 day Control	0.002	0.486
112C	0.2 mm	60 day Control	0.003	0.400
113C	0.2 mm	75 day Control	0.002	0.539
114C	0.2 mm	90 day Control	0.003	0.469
115C	0.4 mm	15 day Control	0.003	0.441
116C	0.4 mm	30 day Control	0.003	0.371
117C	0.4 mm	45 day Control	0.004	0.486
118C	0.4 mm	60 day Control	0.003	0.467
119C	0.4 mm	75 day Control	0.003	0.447
120C	0.4 mm	90 day Control	0.006	0.426
121C	0.6 mm	15 day Control	0.002	0.486
122C	0.6 mm	30 day Control	0.001	0.507
123C	0.6 mm	45 day Control	0.001	0.418
124C	0.6 mm	60 day Control	0.001	0.509
125C	0.6 mm	75 day Control	0.001	0.586
126C	0.6 mm	90 day Control	0.001	0.394

5. Results not included in publications

All experiments conducted in the three years of activity were preceded by several pre-trials. The trials produced accessory results that are reported in this chapter.

5.1 Liquid culture *Schizothrix* cf. *delicatissima* AMPL0116 vs *Leptolyngbya ohadii.*

As it is underlined in section 1.1.4, EPS productivity represents a screening *criterium* for the selection of feasible inoculants. EPS productivity can be measured in liquid culture by measuring the increment of released polysaccharides (RPS), and can be measured in the soil by extracting and quantifying EPSs from induced cyanobacterial crusts.

Growing the strains in liquid medium allows to evaluate the time needed by the strain to reach the exponential phase, evaluating the optimal combination of parameters. This is also important in view of producing subsequent large-scale approaches. Liquid suspensions allow to observe whether the strains tend to aggregate in bundles and flakes. In this view, different fragmentation approaches are required in order to perform a uniform inoculation.

The cyanobacteria employed in the experiments, the strain *Schizothrix* cf. *delicatissima* AMPL0116 and the strain of *Leptolyngbya ohadii* were preliminary grown in liquid cultures. The strains were cultivated in an orbital incubator (Innova 44B, New Brunswick, USA) under the following conditions, that resulted optimal to produce the fastest growth: a continuous low light intensity of 15 μmol photons m^{-2} s^{-1}, a temperature of 30 °C, and a constant agitation of 100 rpm. *L. ohadii* showed a higher increment in chlorophyll *a* and OD than *S. delicatissima*, reaching the highest content in chlorophyll after 10 days, and the highest OD after 20 days (Fig. 1A, 1B).

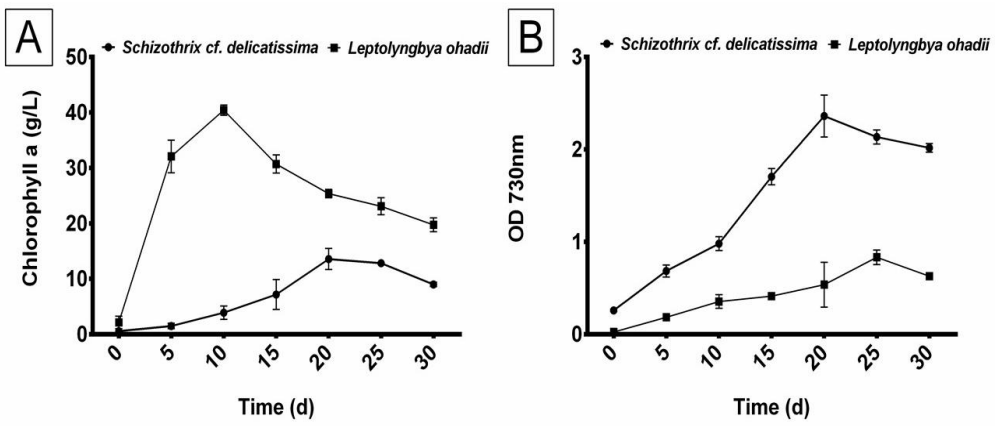

Fig. 1 Chlorophyll content (A), expressed as g per L of culture, and optical density (B) of liquid culture of *Schizothrix* cf. *delicatissima* AMPL0116 and *Leptolyngbya ohadii*. Cultures were grown in BG11 medium, with a 24/24 h light (15 µmol m^{-2} s^{-1}) at 30 °C. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars.

Notwithstanding, *S. delicatissima* showed a higher RPS productivity (roughly 75 mg L^{-1}) compared to *L. ohadii* (roughly 30 mg L^{-1}) during incubation time (Fig. 2).

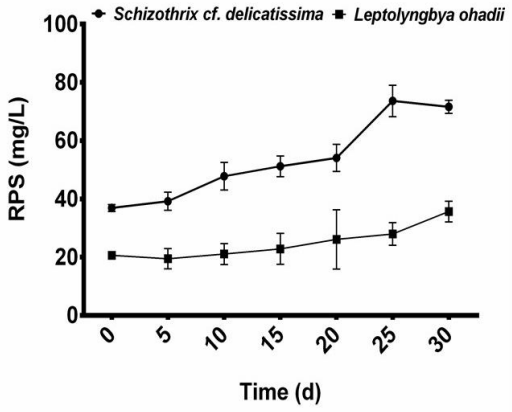

Fig. 2 Amount of released polysaccharides (RPS) expressed as mg per L of culture of *Schizothrix* cf. *delicatissima* AMPL0116 and *Leptolyngbya ohadii*. Cultures were grown in BG11 medium, with a 24/24 h light (15 µmol m^{-2} s^{-1}) at 30 °C. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars.

5.2 Inoculation of *Leptolyngbya ohadii* on sterilized sand.

Two experiments reported in this thesis dealt with the inoculation of the strain of *L. ohadii* in microcosms. One experiment was conducted without sterilizing the sand in the microcosms, in order to observe the growth of cyanobacterial crusts in the presence of an already established sand community (see section 4; publication III). At the same time, preliminary trials were conducted inoculating *L. ohadii* in microcosms with sand collected in the Negev Desert, preventively autoclaved twice, to kill the pre-existing microflora.

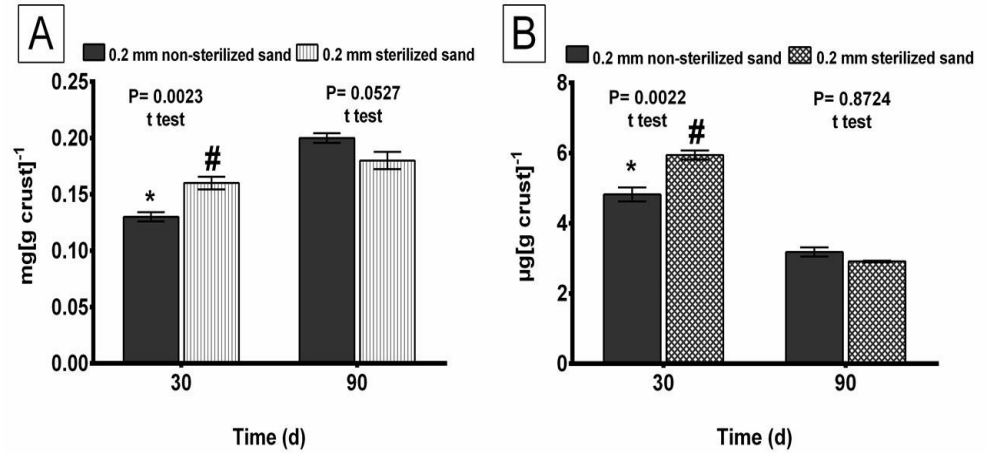

Fig. 3 Total carbohydrates (expressed as mg g^{-1} crust, A) and chlorophyll content (expressed as µg g^{-1} crust, B) of induced cyanobacterial crusts grown on sterilized and non-sterilized sand, with a water provision corresponding to 2 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

Induced cyanobacterial crusts grown on sterilized and non-sterilized sand were compared. Cyanobacterial crusts hydrated with a water amounts of 0.2 mm showed a significantly lower amount of both carbohydrate and chlorophyll contents on non-sterilized sand after 30 days of incubation. Conversely, after 90 days of incubation, no significant difference was detected (Fig. 3). When crusts were hydrated with water amounts corresponding to 0.4 mm, total carbohydrate content did not show significant differences between sterilized and non-sterilized sand after 30 and 90 days (Fig. 4A).

Conversely, chlorophyll content was affected, showing a significantly lower amount after 30 days on non-sterilized sand, while no difference was appreciated after 90 days (Fig. 4B).

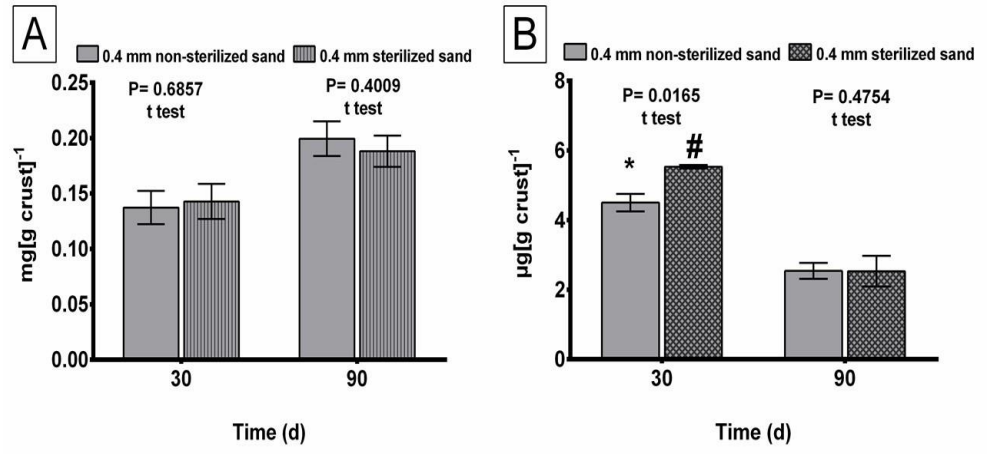

Fig. 4 Total carbohydrates (expressed as mg g^{-1} crust, A) and chlorophyll content (expressed as µg g^{-1} crust, B) of induced cyanobacterial crusts grown on sterilized and non-sterilized sand, with a water provision corresponding to 0.4 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

In cyanobacterial crusts hydrated with a water amount corresponding to 0.6 mm no significant differences were detected between total carbohydrate and chlorophyll contents neither on sterilized or on non-sterilized sand (Fig. 5).

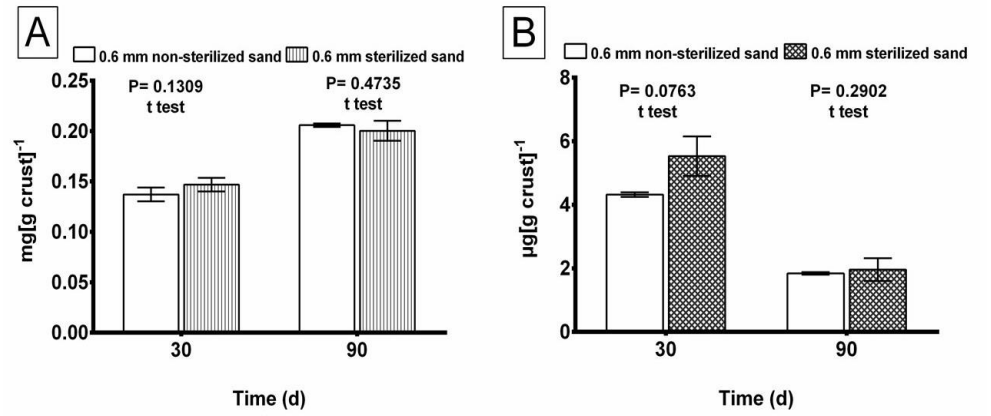

Fig. 5 Total carbohydrates (expressed as mg g^{-1} crust, A) and chlorophyll content (expressed as µg g^{-1} crust, B) of induced cyanobacterial crusts grown on sterilized and non-sterilized sand, with a water provision corresponding to 0.6 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

The content of LB-EPS and TB-EPS was evaluated. With an amount corresponding to 0.2 mm water, LB-EPS resulted significantly higher on sterilized than non-sterilized sand after 30 days, while after 90 days there was not a significant difference (Fig. 6A). Conversely, the content of TB-EPS resulted always significantly lower, after 30 and 90 days (Fig. 6B).

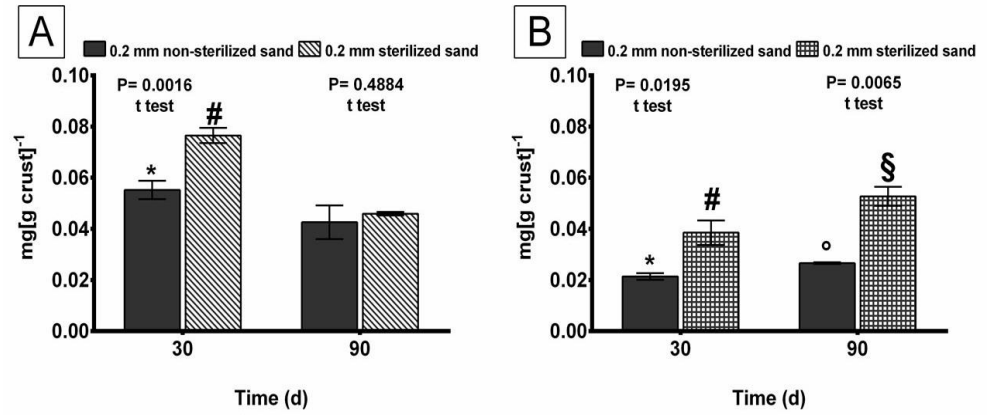

Fig. 6 LB-EPS (A) and TB-EPS (B) (expressed as mg g^{-1} crust) amounts in cyanobacterial crusts induced by inoculating *L. ohadii* in microcosms containing sterile and non-sterilized sand, with a water provision corresponding to 0.6 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

With an amount of water corresponding to 0.4 mm, LB-EPS amount was lower on non-sterilized sand after 15 days, but no significant difference was detected at the end of incubation time (Fig. 7A). The amount of TB-EPS was not significant different between sterilized and non-sterilized sand after 15 days, while it resulted higher on sterilized sand after 90 days (Fig. 7B).

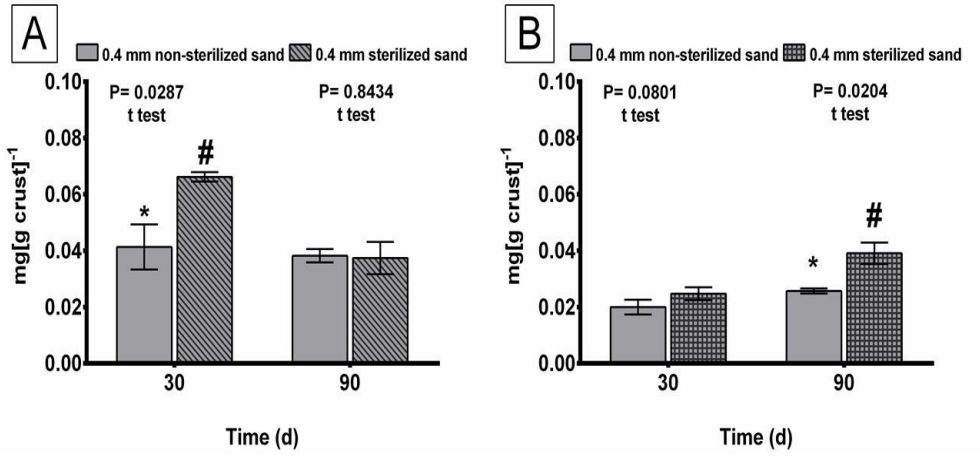

Fig. 7 LB-EPS (A) and TB-EPS (B) (expressed as mg g^{-1} crust) amounts in cyanobacterial crusts induced by inoculating *L. ohadii* in microcosms containing sterile and non-sterilized sand, with a water provision corresponding to 0.6 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

The increase of hydration to an amount of 0.6 mm determined a higher amount of LB-EPS on sterilized sand after 15 days, and higher on non-sterilized sand at the end of incubation time (Fig. 8A). Concerning the content in TB-EPS, no significant difference was detected (Fig. 8B).

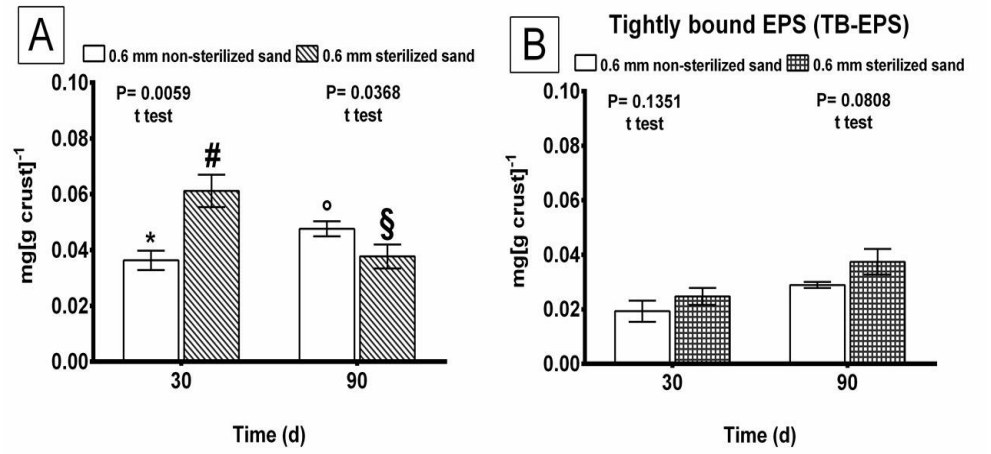

Fig.8 LB-EPS (A) and TB-EPS (B) (expressed as mg g^{-1} crust) amounts in cyanobacterial crusts induced by inoculating *L. ohadii* in microcosms containing sterilized and non-sterilized sand, with a water provision corresponding to 0.6 mm water. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

Water repellency was measured after 90 days of incubation, both on the uppermost, and on the nethermost crust layers after flipping it over. On the uppermost layer, an increase in the RI was detected only applying the lower water regime, on non-sterilized sand (Fig. 9 A). Applying the other two regimes no significant difference was detected. On the bottom layer, the difference between sterile and non-sterilized sand was always significant, under the three water regimes. The most striking difference was detected applying the higher water regime (Fig. 9B).

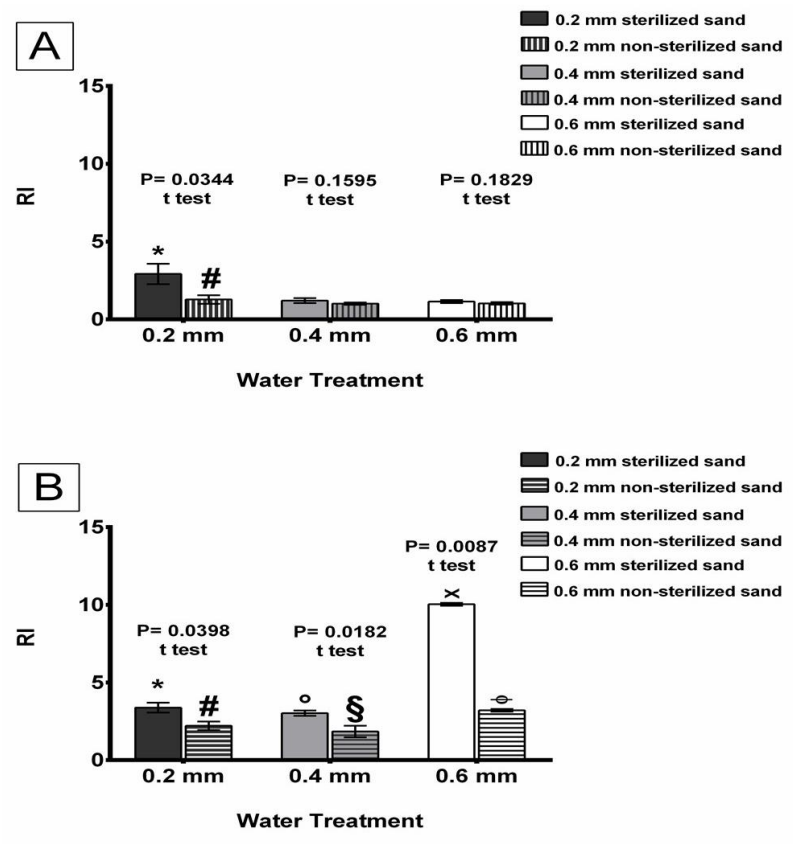

Fig. 9 RI measured on the top of the cyanobacterial crusts grown under the three water regimes (A) and measured on the bottom layers after flipping the crusts upside down (B). Values are means of three independent replicates (n = 3) and SDs are reported as error bars. Different symbols indicate significant differences.

MW distribution of LB-EPS, gauged through SEC analysis, showed that inoculating on sterilized sand, and different water regimes did not produce striking differences in the molecular size patterns of LB-EPS, even though in some cases values showed significant differences (Fig. 10).

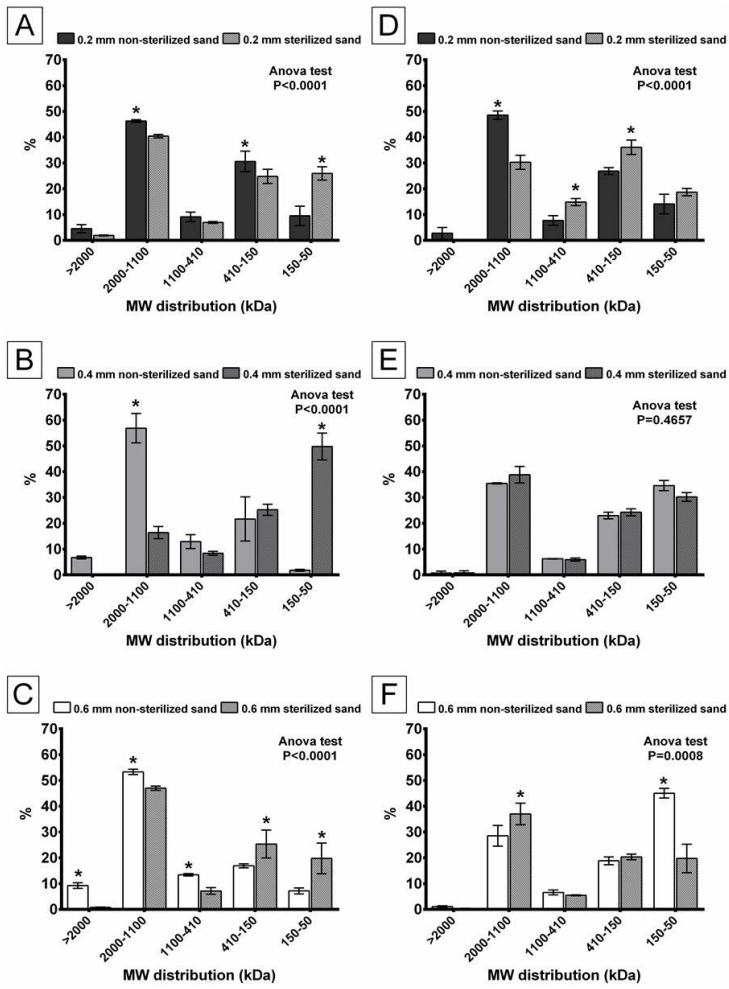

Fig. 10 LB-EPS MW distribution of cyanobacterial crusts grown for 30 days (A, B, C) and 90 days (D, E, F) on sterile and non-sterilized sand under 0.2 mm (A, D), 0.4 mm (B, E) and 0.6 mm (C, F) water regimes. Values are means of three experimental replicates (n = 3) and SDs are reported as error bars. Different symbols stand for significant differences.

5.3 Discussion and Conclusions

In general, while substrate sterilization did not seem to affect total carbohydrate and chlorophyll contents in induced cyanobacterial crusts, it indeed seemed to affected EPS amount. In particular, LB-EPS after 15 days resulted higher in cyanobacterial crusts

grown on sterilized sand, although under water regimes of 0.2 and 0.4 mm the contents did not differ significantly in 90-day-old cyanobacterial crusts. Concerning TB-EPS content, it seems to strongly depend on water regimes. Under 0.2 mm, the content is always higher in cyanobacterial crusts grown on sterile sand; under 0.4 mm the content is higher in cyanobacterial crusts grown on autoclaved sand, but only after 90 days; under 0.6 mm, no significant difference was detected.

Concerning water repellency, the results supported the visually assessed filament migration after inoculation, as RI resulted significantly higher on the bottom on the crust, after 90 days, than that measured on the crust top layers.

Finally, MW distribution pattern showed significant variations, but that all in all does cannot be obviously reconducted to a direct involvement of the substrate sterilization. The most striking data obtained by SEC analysis concern the percentage of the MW size comprised between 2000-1100 kDa in cyanobacterial crusts grown under 0.4 mm water regime, which results notably higher when the substrate was not initially autoclaved. This is only visible after 30 days incubation, while after 90 days the difference was not statistically significant. A higher amount of polymers in this MW range is also present after 90 days under 0.2 mm water regime.

Overall, the data suggest that the pre-existing microflora in non-sterilized sand might feed on the two EPS, or have a boosting effect on LB-EPS in the higher size ranges typical of cyanobacteria (Pereira et al., 2009), due to resource competition stress, already described elsewhere (Rossi and Philippis, 2016).

5.4 References

Pereira, S., Zille, A., Micheletti, E., Moradas-Ferreira, P., De Philippis, R., Tamagnini, P., 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS Microbiology Reviews 33, 917–941. doi:10.1111/j.1574-6976.2009.00183.x.

Rossi, F., Philippis, R.D., 2016. Exocellular Polysaccharides in Microalgae and Cyanobacteria: Chemical Features, Role and Enzymes and Genes Involved in Their Biosynthesis. SpringerLink 565–590. doi:10.1007/978-3-319-24945-2_21.

6. General Conclusions

The work of this PhD thesis aimed to contribute in advancing the knowledge on some key aspects of the cyanobacterization process that are crucial for the future development of an outdoor reproducible biotechnology on a large scale.

The experiments proved that properly selected strains can be used as inoculants on oligotrophic and unconsolidated sandy substrates without any nutrient provision, and with a limited water supply, corresponding only to the non-rainfall source registered in one of the harshest desert environments of the Earth, the Negev Desert. Two filamentous cyanobacteria, *Schizothrix* cf. *delicatissima* AMPL0116 and a strain of *Leptolyngbya ohadii* isolated from desert settings were tested in separate experiments for the first time as inoculants. Results demonstrated that, notwithstanding the common climate of origin, the two strains produced cyanobacterial crusts with different characteristics, underlining the importance of a preliminary microcosm screening in the lab before the application on a large scale of new inoculants.

The results underlined that a successful cyanobacterization procedure depends on the factors that were investigated. Principally, it is important to perform an uniform biomass dispersion. The elaboration of a biomass evenly-dispersing approach led to the development of stable cyanobacterial crusts in microcosms. The characteristics of the cyanobacterial crusts (resistance to compression and aggregate stability) were also dependent on the amount of initial inoculum of biomass.

The thesis shed also light for the first time on the process of formation of the EPM during cyanobacterial crust development. The experimental work included a deep investigation concerning the production and the characteristics of the different EPS fractions of EPM, extracted applying an optimized methodology developed *ad hoc* during the first year of activity. While the formation of cyanobacterial crusts appeared dependent from EPM synthesis, the synthesis itself and the characteristics of the polymer appeared significantly affected by water availability and substrate granulometry. More in general, some of the results supported that the characteristics of

the EPS excreted by cyanobacteria are influenced by abiotic parameters and by the presence of nutrients. Nutrient-rich regimes like the cultivation in liquid medium suspensions promote the synthesis of compositionally more complex polymers, while abiotically and nutrient-constrained conditions lead to the synthesis of compositionally simpler polysaccharides.

A deeper knowledge in these aspects will contribute in paving the way for future small and large-scale studies to apply this technology to counteract soil degradation and desertification spread. In addition, this thesis shed light on the mechanisms of soil colonization and aggregation by cyanobacterial inoculants. In this frame, a new optimized methodology to study EPM formation was put together, representing a valid approach to increase the knowledge about the mechanisms of EPS excretion by cyanobacteria and their role in soil aggregation.

7. Acknowledgments

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Last but not the least, I owe more than thanks to my family members which includes my parents, my sister and my uncle for their support and encouragement throughout my life. To them I dedicate this thesis.