TESIS DOCTORAL

INOCULACIÓN DE CIANOBACTERIAS FORMADORAS DE BIOCOSTRA PARA RESTAURAR SUELOS DEGRADADOS DE ECOSISTEMA ÁRIDOS

BIOCRUST-FORMING CYANOBACTERIA INOCULATION TO RESTORE DEGRADED SOILS FROM DRYLAND ECOSYSTEMS

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"*You may say I'm dreamer, but I'm not the only one, I hope some day you'll join us, and the world will live as one"*

John Lennon

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Contents

Resumen

La acción combinada del cambio climático y el aumento de la presión antrópica están acelerando la degradación de los ecosistemas (Lal, 2015), la cual afecta negativamente a la biodiversidad, la fertilidad del suelo, la disponibilidad de agua y el bienestar de la población local, siendo uno de los principales problemas ambientales del siglo XXI (UNCCD, 2019). Los procesos de degradación son especialmente relevantes en las zonas áridas, por la fuerte presión antrópica que soportan y las condiciones climáticas adversas que los caracterizan. Por este motivo, las Naciones Unidas, en su Agenda 2030, ha propuesto un objetivo específico para detener y revertir la degradación mediante la rehabilitación ecológica de las tierras secas ya degradadas. Sin embargo, la mayoría de los intentos llevados a cabo para restaurar los ecosistemas en estas regiones secas, mediante las estrategias tradicionales centradas en el establecimiento de la cobertura vegetal, fracasan debido a la escasez de agua (Reynolds et al., 2007), baja fertilidad de los suelos y alta vulnerabilidad a la erosión que caracterizan a estas áreas. Por lo tanto, es necesario investigar estrategias de restauración complementarias, que soporten el fuerte estrés abiótico, mejoren las condiciones del suelo y y que sean viables económicamente.

Debido a las duras condiciones abióticas que se presentan en las tierras secas, las plantas, a menudo, se sitúan en las zonas más favorables dentro del ecosistema, y en las menos favorables, aparecen otras formas de vida con menores requerimientos edáficos y de humedad, como las costras biológicas del suelo o biocostras. Las biocostras son comunidades formadas por organismos poiquilohídricos como bacterias, cianobacterias, arqueas, algas, hongos, musgos o líquenes, los cuales viven en estrecha asociación con las partículas del suelo (Belnap et al., 2016). Esta capa casi continua, que forma "la piel viva del suelo", intercede en numerosos procesos clave de los ecosistemas (Castillo-Monroy and Maestre, 2011; Maestre et al., 2016), afectando positivamente a la estabilidad y fertilidad del suelo (Mazor et al., 1996), regulando la disponibilidad de agua en el suelo y reduciendo la erosión hídrica y eólica (Belnap et al., 2007; Cantón et al., 2014). Aunque, las biocostras son muy resistentes y pueden sobrevivir en condiciones climáticas adversas, numerosos trabajos han demostrado que son muy sensibles a las perturbaciones físicas (ej. al tráfico de vehículos o pastoreo) y a los efectos derivados del cambio climático (Ferrenberg et al., 2015), lo que a su vez reduce su capacidad de proporcionar servicios ecosistémicos clave (Weber et al., 2016). Además, una vez que la actividad que causa la alteración cesa, su recuperación natural tiende a ser muy lenta y no siempre ocurre (Weber et al., 2016). Por este motivo, en las últimas décadas se están desarrollando nuevas estrategias para ayudar en la recuperación de las biocostras ya alteradas o para inducir su nueva formación, y que contribuir así, al restablecimiento de los servicios ecosistémicos perdidos. De las diferentes técnicas desarrolladas hasta el momento, la inoculación del suelo con cianobacterias es una de las más atractivas. Estas bacterias fotoautotróficas son uno de los primeros colonizadores del

suelo (Büdel et al., 2016), y gracias a su capacidad para fijar CO_2 y N_2, mejoran la fertilidad (Mazor et al., 1996), la estabilidad de los agregados (Chamizo et al., 2018) y la capacidad de retención de agua (Colica et al., 2014). Además, las cianobacterias presentan una gran variedad de adaptaciones para hacer frente a condiciones abióticas severas. Por ejemplo, todas las especies pueden permanecer en un estado latente durante los períodos de sequía y la gran mayoría son capaces de sintetizar pigmentos que las protegen frente a los rayos UV (Garcia-Pichel and Castenholz, 1991; Rajeev et al., 2013). Además, se pueden aislar a partir de pequeños fragmentos de biocostra natural y cultivarse ex situ en medio de cultivo líquido para producir grandes cantidades de inóculo en periodos de tiempo relativamente cortos. Todas estas características convierten a las cianobacterias en magníficas candidatas para contribuir a la rehabilitación ecológica de las tierras secas.

Aunque estudios previos, en condiciones de laboratorio, han demostrado la viabilidad del uso de la inoculación de cianobacterias para promover la formación de una nueva biocostra que mejora las propiedades del suelo (Acea et al., 2003; Malam-Issa et al., 2007; Chamizo et al., 2018), esta tecnología aun no está lo suficientemente desarrollada como para aplicarse de forma generalizada en los procesos de restauración en tierras secas. Esto se debe, en cierta medida, a que una gran parte de los estudios se han centrado en el uso de una sola especie, *Microcoleus vaginatus*, mientras que apenas se han probado otras especies (Hu et al., 2002; Chen et al., 2006; Wang et al., 2009). Las especies de cianobacterias pueden presentar grandes diferencias en la facilidad para aislarlas y cultivarlas, y en su capacidad para colonizar el suelo y formar biocostras, así como en su tolerancia al estrés abiótico y en su efecto sobre la calidad del suelo (Rossi et al., 2017). Por lo tanto, un examen preliminar para evaluar su crecimiento y efecto sobre las propiedades del suelo en el laboratorio constituye un paso esencial para elegir entre las diferentes alternativas disponibles. Por ejemplo, la escasez de agua y las propiedades fisicoquímicas del suelo son dos de los principales factores que afectan a la supervivencia y colonización de las cianobacterias en condiciones de campo (Bu et al., 2014; Fernandes et al., 2018), pero rara vez se han considerado estos factores en los experimentos publicados realizados en laboratorio. En este sentido, el uso de especies nativas se vislumbra como la opción más atractiva ya que es más probable que ya estén adaptadas a las condiciones abióticas locales (Giraldo-Silva et al., 2019a). Además, trabajando con especies nativas se reduciría la modificación de la comunidad nativa de microorganismos. Por esa razón, esta tecnología se beneficiaría de la identificación y puesta a punto de nuevas especies nativas que pudieran aportar aptitudes y funciones ecológicas que permitieran, por un lado, resistir las duras condiciones en el campo y, por otro, colonizar suelos con diferentes características fisicoquímicas.

La única experiencia exitosa a gran escala de esta tecnología en el campo se ha llevado a cabo en China, en ecosistemas de dunas de arena que fueron previamente estabilizadas (Chen et al., 2006; Wang et al., 2009), mientras que la mayoría de los intentos de inoculaciones del suelo con cianobacterias en los desiertos del oeste de EEUU no mostraron resultados satisfactorios (Kubeckova

et al., 2003; Faist et al., 2020). Esto demuestra que la aplicación de cianobacterias en el campo sigue siendo un desafío, debido sobre todo a la alta radiación UV y las sequías prolongadas que caracterizan las tierras secas, y por el efecto de la erosión hídrica y eólica que desplazan el inóculo antes de que este se asiente definitivamente sobre la superficie del suelo. Por lo tanto, se han propuesto diferentes estrategias para mejorar la supervivencia y el establecimiento de las cianobacterias en condiciones de campo. Una de estas estrategias consiste en aumentar gradualmente la radiación UV y reducir la disponibilidad de agua durante la fase de cultivo para endurecer el inóculo y adquiera mayor capacidad para soportar el estrés abiótico al que se enfrentará en el campo (Giraldo-Silva et al., 2019b). Las primeras pruebas realizadas que usaron esa estrategia mostraron resultados positivos en 13 de las 20 cepas de cianobacterias probadas (Giraldo-Silva et al., 2019b), sin embargo, la viabilidad del inóculo pre-acondicionado aún no se ha probado bajo condiciones de campo. Otra posibilidad es reducir el estrés tras la inoculación (pe: cubrir con una malla de yute), lo cual ya ha mostrado resultados prometedores para la rehabilitación de biocostras utilizando como inóculo propágulos de biocostra natural previamente cultivados en el invernadero (Bowker et al., 2020). Sin embargo, su aplicación en inoculaciones utilizando exclusivamente especies de cianobacterias aún no se ha ensayado.

Además, una vez que se cosecha la biomasa, esta se debe aplicar en el área objetivo de forma casi inmediata, de lo contrario puede contaminarse o verse afectada por el ataque de patógenos. Esto dificulta su incorporación en las acciones de restauración tradicionales, y por este motivo hay varios grupos de investigación trabajando en el desarrollo de nuevas metodologías y técnicas que mejoren su almacenamiento, transporte y aplicación en las zonas a restaurar. Una de estas alternativas sería la incorporación de los cultivos de cianobacterias en pellets. Por ejemplo, Buttars et al., (1998) demostraron que *M. vaginatus* puede sobrevivir al proceso de peletización en pellets de alginato y que posterior se pueden liberar al suelo con éxito. Por el contrario, otros intentos con la misma especie, pero usando almidón como agente aglutinante, mostraron una alta mortalidad (Howard y Warren, 1998). Es necesario, por tanto, probar su aplicación con otras especies y compuestos de pellets.

Una vez que se ha realizado la inoculación del suelo con cianobacterias es necesario llevar a acabo un seguimiento continuo de la evolución de la nueva biocostra generada, que nos permita evaluar el éxito de la restauración e identificar los posibles factores que puedan limitar la viabilidad del inóculo. Los indicadores más usados para realizar el seguimiento de las biocostras y controlar el éxito de la restauración son la cobertura de biocostra y, sobre todo, su contenido en clorofila. El problema es que los procedimientos que se suelen utilizar para para determinar el contenido de clorofila son lentos y costosos, y producen una alteración de la zona inoculada. Por lo tanto, es necesario desarrollar metodologías alternativas que nos permitan estimar el contenido de clorofila de forma indirecta y no destructiva. Una de las metodologías con mayor potencial para la estima indirecta del contenido de

clorofila de las biocostras es la aplicación de indicadores espectrales calculados a partir de su reflectancia, tal como se viene haciendo desde hace algunos años para las plantas (Haboudane et al., 2002). Sin embargo, hasta ahora, esta metodología no ha sido probada ni adaptada para su uso con comunidades de biocostras, ya sean naturales o artificiales.

El objetivo principal de esta tesis es probar el potencial de la inoculación con especies de cianobacterias formadoras de biocostras para promover el desarrollo de una nueva biocostra que mejore las condiciones de los suelos degradados de tierras secas. Para lograrlo, en primer lugar, se llevó a cabo un experimento para evaluar el potencial de tres cepas nativas de cianobacterias fijadoras de nitrógeno, *Nostoc commune*, *Scytonema hyalinum* y *Tolypothrix distorta*, de forma individual y combinadas formando un consorcio, para restaurar las funciones del suelo. La inoculación se llevó a cabo en tres suelos con diferentes grados de desarrollo provenientes de ecosistemas semiáridos de la provincia de Almería (sureste de España). Los resultados de este experimento demostraron que la inoculación con cianobacterias nativas induce la formación de una nueva biocostra que mejora las propiedades del suelo, relacionadas con su fertilidad, en un período de tiempo corto. De las diferentes cepas probadas, *N. commune* y el Consorcio de las tres cepas fueron los tratamientos que mostraron una mayor capacidad para colonizar y mejorar las propiedades del suelo (CAPÍTULO I). Con el fin de identificar el inoculo que mejor se adapta a condiciones de estrés hídrico, el experimento se repitió simulando dos regímenes de hidratación, uno que correspondía a un año hidrológico húmedo y otro a un año seco en las áreas de origen. En contra de lo que cabría esperar, tanto el desarrollo de la biocostra como las mejoras en las condiciones edáficas fueron similares bajo ambos regímenes de hidratación y para todos los tratamientos de inoculación probados, lo que sugiere que la disponibilidad de agua podría no ser tan importante para la formación de la nueva biocostra mediante la inoculación con cianobacterias, como inicialmente se pensaba. Además, *Noctoc commune*, el cual es conocido por ser un buen tolerante frente a la desecación, mostró una mayor capacidad de crecimiento bajo escenarios de restricción de agua, convirtiéndose de esta forma en un buen candidato para restaurar áreas degradadas en zonas áridas (CAPÍTULO II). Posteriormente, se evaluó la viabilidad de esas mismas cepas bajo condiciones de campo inoculando un consorcio de ellas en suelos de nuestras tres áreas de estudio. Para ello, previamente, se llevó a cabo el cultivo en fotobiorreactores de 100 L utilizando un medio de cultivo hecho a base de fertilizantes, lo que reduce considerablemente el coste de producción biomasa (Roncero-Ramos et al., 2019). Este experimento no generó los resultados esperados, pues se encontraron valores similares de clorofila *a*, absorción espectral de clorofila y albedo en las parcelas inoculadas y en las no inoculadas, dos años después de la inoculación. La baja tasa de éxito en campo motivó la realización de un segundo experimento para evaluar el efecto de técnicas dirigidas a la aclimatación o endurecimiento del inóculo, en el éxito de la inoculación. Para ello, los cultivos se aclimataron aumentando la radiación solar y disminuyendo el suministro de agua progresivamente antes de la inoculación. A pesar de ello, 6 meses después de

inocular el suelo con el inóculo acondicionado se obtuvieron resultados poco exitosos y similares para parcelas en las que se aplicó el inóculo aclimatado y sin aclimatar. Finalmente, se evaluó el efecto de la cobertura de las parcelas con una red de plástico y una malla de fibra vegetal reciclada que aportaba sombra y mejoraba las condiciones ambientales del inoculo una vez instalado en campo. La combinación de la inoculación con cianobacterias no acondicionadas y posteriormente cubiertas por la malla vegetal dio lugar a una mayor colonización, contenido de clorofila, picos de absorción espectral de la clorofila más profundos y albedo más bajo que las parcelas descubiertas (CAPÍTULO III).

Con el objetivo de desarrollar una metodología que permitiese mejorar el almacenamiento y la aplicabilidad del inóculo, se desarrolló un experimento paralelo para evaluar la supervivencia y el establecimiento de las cianobacterias después de ser encapsuladas en pellets. Para ello, se seleccionaron tres cepas de cianobacterias pertenecientes a géneros de fijadoras (*Nostoc* y *Scytonema*) y no fijadoras (*Leptolyngbya*) de nitrógeno. Las diferentes especies y el consorcio se incorporaron en pellets hechos a base de arena y arcilla y se probaron sobre suelos de tres sitios degradados de Australia. Los resultados obtenidos en este experimento mostraron que los pellets pueden disolverse completamente, y el inóculo se extiende hacia zonas subyacentes en todos los tratamientos probados. Los pellets que incorporaron *Scytonema* y el Consorcio mostraron mayor biomasa que los que incluían *Nostoc* y *Leptolyngbia* al final del período de incubación. Además, el almacenamiento de los pellets durante 30 días produjo una reducción de en el contenido de clorofila *a* en todos los tratamientos, aunque al menos el 50% de la biomasa aún estaba presente (CAPÍTULO IV).

Finalmente, se estudió el potencial del uso de la reflectancia de la superficie del suelo para el seguimiento no destructivo de la biocostra inducida. El análisis de la respuesta espectral de diferentes comunidades de biocostras reveló que algunas transformaciones aplicadas a las firmas espectrales, tales como el continuo quitado y la primera derivada de la reflectancia, así como los índices de diferencia normalizada y los índices verdes estándar, tanto hiperespectrales como de banda ancha, se pueden utilizar de forma efectiva para este propósito. Sin embargo, esta metodología debe adaptarse de forma específica a cada tipo de biocostra. Para resolver esta limitación aplicamos un "random forest" que combinaba la información espectral con datos de la cobertura de cada tipo de costra. Este modelo no lineal dio muy buenos resultados cuando se aplicó al conjunto de la base de datos (CAPÍTULO V).

En resumen, los resultados de esta tesis proporcionan información valiosa para la mejora de las acciones de rehabilitación del suelo basadas en la aplicación de una biotecnología basada en la incoculación de cianobacterias formadoras de biocostras, para su aplicación en zonas áridas. En primer lugar, los experimentos de laboratorio realizados demuestran la viabilidad del uso de un

consorcio de cianobacterias nativas para promover la formación y el desarrollo de una nueva biocostra que mejora notablemente las propiedades clave de diferentes suelos degradados. Además, su aplicación exitosa sobre suelos con diferentes propiedades fisicoquímicas, incluyendo suelos de textura fina y sustratos de cantera, proporciona nuevos avances para desarrollar una tecnología aplicable en diferentes entornos. También hemos comprobado que *N. commune* puede sobrevivir y colonizar el suelo con muy poca disponibilidad de agua, lo que demuestra su gran potencial para restaurar suelos de ecosistemas donde el agua es un recurso limitante. Sin embargo, la inoculación directa del suelo en campo con el consorcio de cianobacterias nativas no mostró los resultados esperados. El bajo éxito se explicaba principalmente porque cuando se secó el suelo el inoculo se desprendió de la superficie del suelo y fue arrastrado por el viento y por la escorrentía. El uso de procedimientos para la mejora del hábitat redujo considerablemente el estrés abiótico y la inestabilidad del suelo, mejorando de esta forma significativamente la supervivencia y el establecimiento del inóculo. Sin embargo, aunque estos resultados son prometedores de cara a su aplicación a escala local, se necesitan más estudios para el desarrollo de técnicas de mejora del hábitat que puedan aplicarse a escalas más grandes. Por otro lado, nuestros resultados muestran que algunas cianobacterias, especialmente *Scytonema* sp. y el Consorcio, se pueden incorporar con éxito en pellets hechos a base de arena y bentonita. Aunque durante el almacenamiento del pellet se pierde parte de la clorofila al menos el 50% de la biomasa total cianobacterias sobrevivió. Por lo tanto, esta tecnología, aunque prometedora, necesita ser revisada y refinada en futuro estudios antes de que pueda aplicarse en situaciones reales de campo. Finalmente, la tesis presenta el primer estudio donde las propiedades espectrales de las biocostras se emplean con éxito para la estimación no destructiva de biomasa en biocostras. Esta información puede incorporarse a un coste razonable en los programas de monitoreo para la evaluación de proyectos de rehabilitación basados en el uso de biocostras.

Summary

In drylands, the largest earth biome, the coupled action of climate change and rising human pressure are causing accelerated land degradation (Lal, 2015). This negatively affects biodiversity, soil fertility, water availability, and local population wellbeing, being one of the major environmental issues of the 21st century (UNCCD, 2019). For all these reasons, United Nations, in the Agenda 2030 has proposed a specific goal to stop and reverse ongoing land degradation by the ecological rehabilitation of already degraded drylands. However, most attempts to restore drylands ecosystems by using traditional strategies focused on plant cover establishment fails due to the water scarcity, low fertility and high vulnerability to erosion that characterize these areas (Reynolds et al., 2007). Thus, it is necessary to investigate complementary restoration strategies adapted to strong abiotic stress that can contribute to the recovery of these ecosystems in a cost-effective manner.

Due to the harsh abiotic conditions imposed in drylands, plants are often restricted to the most favorable position within the landscape. In open and less favourable inter-plant spaces, other life-forms with lower edaphic and moisture requirements appear, such as poikilohydric communities of cyanobacteria, algae, fungi, bryophytes and lichens, living in close association with soils particles, and known as biological soil crusts or biocrusts (Belnap et al., 2016). By covering the soil surface, biocrusts form an almost continuous live-skin that intercede in numerous key ecosystems processes (Castillo-Monroy and Maestre, 2011; Maestre et al., 2016), positive affecting soil stability and fertility (Mazor et al., 1996), regulating water balance and reducing water and wind erosion (Belnap et al., 2007; Cantón et al., 2014). However, they are very sensitive to physical disturbance (e.g., vehicular traffic or grazing) and climate change (Ferrenberg et al., 2015), which in turn results in a reduction of their ability to provide key ecosystems services (Weber et al., 2016). In addition, once the activity that causes the disturbance ceases their natural recovery, when possible, tends to be very slow (Weber et al., 2016). Within the last decades, several innovative techniques are being developed to assist in the recovery of disturbed biocrust or to induce the formation of new ones, thereby reinstating the lost ecosystem services. From the different techniques already developed, soil inoculation with biocrust-forming cyanobacteria propagules is among the most attractive for several reasons. For example, theses photoautotrophic bacteria are among the first colonizers of soils (Büdel et al., 2016), enhancing soil fertility (Mazor et al., 1996), soil stability (Chamizo et al., 2018) and improving water retention (Colica et al., 2014) due to their capability to fix CO_2, and N_2. Moreover, cyanobacteria poses several behavioural and physiological adaptations to cope with harsh abiotic conditions, such as dormancy during desiccation or the releasement of sunscreen pigments to protect from UV (Garcia-Pichel and Castenholz, 1991; Rajeev et al., 2013). In addition, they can be isolated from small amount of natural biocrusts, and cultured ex-situ in nutrient media to produce large

quantities of inoculum. All these features convert cyanobacteria in a good candidate to be employed for the ecological rehabilitation of degraded zones in drylands.

Previous studies under laboratory conditions proved the feasibility of using cyanobacterial inoculation for promoting artificial biocrust formation and improve soil properties (Acea et al., 2003; Malam-Issa et al., 2007; Chamizo et al., 2018). However, this technology, is far for being applied widespread, as the majority of the literature focused on the use of a single species, *Microcoleus vaginatus* (Hu et al., 2002; Wang et al., 2009; Xie et al., 2007). Cyanobacteria can be very different regarding feasibility to be isolated and cultured, biocrust formation capability, tolerance to environmental stress and effect on soil quality (Rossi et al., 2017). Therefore, a preliminary laboratory screening of the cyanobacterial growth and its effect on soil properties is an essential step to choose among potential inoculants. For example, water scarcity and soil physicochemical properties constitute two of the main factors affecting cyanobacterial survival and colonization under field conditions (Bu et al., 2014; Fernandes et al., 2018), but indoor studies addressing the sensitivity of different species to these factors are scarce. In this sense, the use of native species should be a preferable option because they can be already preadapted to local conditions (Giraldo-Silva et al., 2019a). Also, we avoid substantial modification of indigenous community. For that reason, this technology would benefit from the search and exploitation of new and native desert species with broader ecological aptitudes and functions for resisting field desiccation and colonize soils with differing physicochemical features.

Successful large-scale field application of cyanobacteria to combat soil degradation has been only conducted on previously stabilized sand dunes in China (Chen et al., 2006; Wang et al., 2009). In contrast, several soil inoculations with cyanobacteria were attempted at different deserts in western USA, showing poor results (Kubeckova et al., 2003; Faist et al., 2020). This demonstrated that cyanobacteria application in the field is still a challenge because of the high UV radiation and the prolonged droughts that characterize the arid environments and the negative effect of soil and wind erosion, reducing the odds of survival. Thus, different strategies have been proposed to enhance cyanobacterial survival and establishment under field conditions. One possibility consists on improving the capacity of cyanobacteria to face abiotic stress in the field by gradually increasing UV radiation and reducing water availability during culturing (Giraldo-Silva et al., 2019b). The first outdoor tests using that strategy showed positive fitness results in 13 of the 20 cyanobacterial strains tested (Giraldo-Silva et al., 2019b), however the viability of the preconditioned inoculum have not been tested under field conditions yet. Other possibility that has shown promising results for the rehabilitation of mixed biocrusts communities in the field is to implement habitat amelioration procedures (e.g., jute mesh to promote shade; Bowker et al., 2020), but its application to facilitate cyanobacterial biocrust establishment after the inoculation has yet to be tackled.

In addition, once biomass is harvested it should be applied onto the target area in the short-term, otherwise it can get contaminated or be affected by pathogens. This fact difficult the restoration actions based on cyanobacterial inoculation, thereby different groups are currently working on the development of new methodologies to enhance inoculum storage, transferral and applicability to the target areas. One of these alternatives is incorporating cyanobacteria cultures into extruded pellets. Buttars et al. (1998) showed that *M. vaginatus* survived pelletization and successfully escaped from crushed alginate pellets. In contrast, other attempts with the same species using starch as binder agent showed high mortality (Howard and Warren, 1998). Thus, it is necessary to test its application with other species and pellet compounds.

Finally, once the cyanobacteria inoculation has been carried out, the monitoring of the induced biocrust is crucial in order to assess the restoration success and to identify potential factors that constrains inoculum viability. Generally, this is done by measuring induced biocrust coverage or chlorophyll *a* content. However, laboratory procedures to determine chlorophyll *a* content are costly and time consuming, and the disturbance of the inoculated crust during samples collection is unavoidable. Thus, the development of an indirect and non-destructive methodology for chlorophyll *a* quantification in biocrusts is of high interest. In this sense, soil surface reflectance shows high potential to be used for chlorophyll *a* estimation in biocrust as it is already done for plants (Haboudane et al., 2002), however this methodology has not been previously tested and refined for natural or artificial biocrust communities so far.

Under this framework, the main objective of this thesis is to test the potential of native biocrust-forming cyanobacteria inoculation to induce the formation of a new biocrust able to enhance soil physicochemical properties in drylands' degraded soils. To achieve it, firstly, a microcosm experiment was set to test the potential of three native nitrogen-fixing cyanobacteria strains, *Nostoc commune*, and the non-previously tested *Scytonema hyalinum* and *Tolypothrix distorta*, each strain alone and in a consortium (equal mix), as inoculants to restore soil functions. Cyanobacterial inoculation was conducted over three soils with different degrees of soil development from semiarid ecosystems of the province of Almería (SE Spain). Our results revealed that native cyanobacteria inoculation can bring to the formation of an artificial biocrust that enhance soil properties related to soil fertility in a short period. From the strains tested, *N. commune* and the Consortium showed the best results (CHAPTER I). This experiment was repeated simulating hydration regimes that corresponded to wet and dry hydrological years in the origin areas, in order to identify the cyanobacterial inoculant that best performed under water stress conditions. Surprisingly, similar biocrust development and improvement in soil edaphic conditions were observed under both hydration regimes for all inoculation treatments, suggesting that water availability might not be as important for cyanobacterial biocrust formation as previously reported. During this experiment, the performance of the well-known desiccation-tolerant *Nostoc commune* was remarkable, showing a

greater capacity to growth under water-restrictive scenarios than all other species and being a good candidate for restoring arid degraded areas (CHAPTER II). Afterwards, the viability of the strains was evaluated under field conditions by inoculating a consortium of them on soils from our three study areas. To achieve it, previously, th culturing of each species was carried out in photobiorreactors of 100L using media made with fertilizers, reducing the overall cost of the biomass production (Roncero-Ramos et al., 2019). The direct field application of this indigenous cyanobacterial consortium (*N. commune*, *S. hyalinum* and *T. distorta*) did not significantly facilitated the formation of a new biocrust, as similar values of chlorophyll *a*, chlorophyll spectral absorption and albedo were found in inoculated and control plots 2 years after inoculation, although soil organic carbon content increased. Thus, a second experiment was carried out in the field to test the effect of inoculum preconditioning and the use of habitat amelioration techniques on the inoculation success. Moreover, previously, the cultures were progressively pre-conditioned to decreasing water supplies before inoculation. However, 6 months after inoculating the hardened inoculum, the similar results were shown by non-conditioned and conditioned plots. Afterwards, the cover of the plots with a plastic fiber grid and a recycled vegetal fiber mesh was evaluated. The combination of non-conditioned cyanobacteria covered with the vegetal mesh resulted in higher colonization, chlorophyll *a* content, deeper chlorophyll *a* spectral absorption peaks and lower albedo than uncovered plots 6 months after inoculating (CHAPTER III).

In parallel, with the aim of developing a proficient technology to enhance inoculum storage and applicability, we evaluated the survival and establishment of cyanobacteria encapsulation in pellets. Thus, two representative N-fixing genera (*Nostoc* and *Scytonema*), a non-heterocystous filamentous genus (*Leptolyngbya*), and a consortium (equal mix) of all strains were encapsulated in pellets composed of sand and bentonite and incubated on soils from three degraded sites in Australia. This pelletization tests showed that pellets can dissolve completely and spread out in all treatments tested. From the different species, *Scytonema* and the Consortium showed the best results, with higher biomass than *Nostoc* and *Leptolyngbia* at the end of the incubation period. Also, the storage of the pellets for 30 days produced a reduction in chlorophyll *a* content in all treatments, although at least approximately 50% remained (CHAPTER IV).

Finally, the potential of soil surface reflectance measurements for non-destructive monitoring of induced biocrusts was studied. To do this I explored the relationship between different spectral traits of a wide range of biocrusts samples and their chlorophyll *a* content. This spectral analysis revealed that spectral transformation such as continuum removal and first derivate of reflectance, as well as, normalised band ratios and standard hyperspectral and broad band indices can be used for general indirect chlorophyll quantification in biocrusts. However, such approaches need to be adapted to each specific biocrust type. Interestingly, we found that the need for a specific calibration for each

crust type can be sorted out by the combination of spectral measurements with non-linear random forest models (CHAPTER V).

In summary, the findings of this thesis provide valuable insights to improve soil rehabilitation actions based on the application of this biotechnology on drylands' soils. First, our laboratory experiments demonstrate the feasibility of using native cyanobacterial consortium to promote the formation and development of new biocrust that improve key properties of different degraded soils from three Mediterranean ecosystems. Moreover, their application over soils with differing physicochemical properties, including fine-textured soils and mine tailings substrates, provides new advances for developing a proficient technology. Also, we demonstrate that *N. commune* can survive and colonize the soil with very low water availability, showing a good potential to be used as inoculant in water limited ecosystems. However, the attempt to reinstate soils functions under natural field conditions by means of direct soil inoculation with the indigenous consortium showed poor outcomes. The poor results obtained was due to the early detachment of the inoculum from the surface after desiccation, thereby the propagules were probably washed away due to the overland flow and wind. The use of habitat amelioration procedures that reduce abiotic stress and soil instability significantly improved inoculum survival and establishment. However, although promising at local scale, more studies are necessary for the development of habitat amelioration techniques that can be applied at larger scales. On the other hand, our results showed that some cyanobacteria inoculants, specially *Scytonema* sp. and the Consortium, can be successfully incorporated in extruded pellets composed of commercial bentonite powder and sand. Nevertheless, the pellet storage for 30 days significantly reduced chlorophyll *a* content in all the inoculation treatments, though at least the 50% of total cyanobacterial biomass survived. Thus, this technology, although promising, need to be further revised and refined in future studies. Finally, this is the first study where biocrust spectral traits are successfully employed for non-destructive estimation of biocrust biomass. This information can be incorporated at a reasonable cost into monitoring programs for the evaluation of biocrusts rehabilitation projects.

Introduction

Drylands: features and threats

Land degradation is considered as one of the major global threat for human well-being of this century (Lal, 2015), raising the risks of food insecurity, migration or conflict, and leading to unpredictable biodiversity loss and reduction of key ecosystem services (UNCCD, 2019). Land degradation issues are especially critical in drylands, which represent approximately the 45% of the Earth surface (Prăvălie, 2016), and constitute the home of nearly the 40% of the total human population, including some of the poorest and most vulnerable on the planet (MEA, 2005). In these areas, the coupled action of aridity, climate change and growing human pressure has turn out in accelerated ecosystems degradation (D'Odorico et al., 2013). Consequently, from 10% to 20% of the world drylands are already degraded (FAO, 2016), and additional 12 million hectares are degraded each year (Brauch and Spring 2009). Besides, ongoing population growth and climate change are expected to exacerbate desertification risk (IPCC, 2014), resulting in a reduction of their capacity to provide crucial services to society that seriously threat the livelihoods of 2 billion people (D'Odorico et al., 2013). In addition to local effects of degradation process on soil fertility, erosion, biodiversity and population, dryland degradation has adverse off-site impacts that rise up this regional problem to a global concern. For instance, drylands comprise 27% and 97%, respectively of the global soil organic and inorganic carbon reserves (Yirdaw et al., 2017), and they control the trend and inter-annual variability of global terrestrial ecosystem capacity to fix C (Ahlström et al., 2015). Moreover, dust storms resulting from reduced vegetation cover and soil degradation may lead to air quality problems both locally and far away (Pointing and Belnap, 2012). Thus, drylands degradation is a top concern for many local, regional and international administrations (Menz et al., 2013), and one of the major goals for the United Nations in the 2030 Agenda for Sustainable Development is to reverse land degradation and to ecological restore the already degraded areas by 2030. However, most of the rehabilitation projects often show unsatisfactory results in drylands. Some of the main constrains for restoration succeed in these regions are poorly developed soils, scarce and unpredictable precipitation and high potential evapotranspiration rates (Reynolds et al., 2007), which limited vegetation growth and survival and imped the success of traditional restoration approaches that are mainly focused on the recovery of plant cover (James et al., 2013). This motivated the development of innovative tools that combined traditional practices and the imitation of natural processes (Cortina et al., 2011). Although, some of these techniques, such as the use of native species (Ramón Vallejo et al., 2012), applications of amendments that enhance soil fertility (Luna et al., 2018), seed-coating (Brown et al., 2019), the pre-conditioning of seedling (Vilagrosa et al., 2003) or the design of water harvesting techniques (García-Ávalos et al., 2018; Valdecantos et al., 2014), raised vegetation survival rates, the success of restoration actions is still not guaranteed. Indeed, unsatisfactory results are more likely to occur at

ecosystems with aridity values over 0.7, where abrupt decays in multiple ecosystem attributes such as soil fertility and microbiota functionality can hinder restoration efforts (Berdugo et al., 2020). Therefore, it is necessary to adopt a different framework that integrates the inherent abiotic constrains and eco-hydrological complexity that characterize these regions in order to return ecosystems to its historic trajectory (Merino-Martín et al., 2012; Yu et al., 2008).

The main feature that characterizes drylands regions is water scarcity. This, in conjunction with the frequent edaphic limitations, leads to patchy landscapes in which vegetation occupy most favourable positions (Maestre and Cortina, 2002; Puigdefábregas et al., 2005), and act as fertility islands (Armas et al., 2008). During rainfalls, runoff is generated in open areas and redistributed to vegetated patches, where water and dissolved nutrients are trapped and stored (Ludwig et al., 2005; Mayor et al., 2008). Water redistribution from open to vegetated patches maximize resource availability for plants and the overall ecosystem functioning. On less favorable areas, other life forms with lower edaphic and moisture requirements, such as biological soil crusts (biocrusts), appear (Rodríguez-Caballero et al., 2019). These are poikilohydric cryptogamic communities dominated by autotrophic organisms such as bryophytes, lichens, cyanobacteria or microalgae, but also including heterotrophs and decomposers (bacteria, archaea, fungi and microarthropods) (Belnap et al., 2016), that live in intimate association with soil particles on the first centimeters of the topsoil (Figure 1). Biocrusts are often among the first colonizers in primary succession and whereas they are eventually displaced by vascular plants in low abiotic stress systems, they tend to persist as a permanent life-form in undisturbed high abiotic stress systems (Bowker, 2007). Therefore, drylands restoration projects would benefit from the consideration of biocrusts recovery as precursor of natural succession that improves edaphic conditions, help to recover ecosystem structure and function and facilitates plant colonization and survival in latter stages.

Biocrusts: ecosystems engineers in drylands

Natural succession starts with the colonization of bare soils by filamentous cyanobacteria and eukaryotic algae, creating an incipient matrix that facilitates the later colonization of lichens, mosses and microfauna (Weber et al., 2016). As such, they create an almost continuous living skin that mediate in most of the soil structural and functional processes (Castillo-Monroy and Maestre, 2011; Maestre et al., 2016), particularly in those involving early stage soil succession on degraded soils (Weber et al., 2016). They are able to fix atmospheric carbon and nitrogen, influencing global biogeochemical cycles (Rodriguez-Caballero et al., 2018a), and contributing to increase soil fertility (Chamizo et al., 2012). As a result of their biological activity they produce and exudate exopolysaccharides (EPS) that bind soil particles together (Rossi and De Philippis, 2015a), contributing to the formation of larger and more stable aggregates, thus enhancing soil structure and stability (Mazor et al., 1996). Consequently, they positively affect water balance by influencing the

water run-off infiltration thorough soil pores, evaporation and soil moisture (Chamizo et al., 2017), and they contribute to reduce wind and water erosion (Belnap et al., 2007; Cantón et al., 2014). They increase soil surface roughness, enhancing the water storage capacity, which also contributes to an increase in water infiltration and a reduction of soil erosion (Rodríguez-Caballero et al., 2012), and facilitates dust deposition (Reylds et al., 2001). Furthermore, due to the magnitude of all the ecosystems services they provide (Rodríguez-Caballero et al., 2018b), biocrusts have been described as ecosystem engineers in drylands (Bowker et al., 2006), and have been proposed as indicators of ecosystem ecological health (Zhang, 2005).

Figure 1. A close view of a cyanobacteria-dominated biocrust at El Cautivo, Almería (Spain).

Biocrusts are highly sensitive to disturbances derived from human activities such as living stock grazing, vehicular traffic, mining, agriculture or construction (Weber et al., 2016), and from natural processes (e.g. fire, soil deposition, etc.). Theses disturbances can easily break biocrust structures, converting them to a non-functional state. In addition, different experiments around the world also demonstrated that global warming and the changes in precipitation patterns also affect biocrust diversity, composition and functioning (Fernandes et al., 2018; Maphangwa et al., 2012; Reed et al., 2012). Consequently, the actual global biocrust's cover (12.2%) is expected to decrease in 27-39% by 2070 (Rodriguez-Caballero et al., 2018a), and this situation could be exacerbated by a general community shift from mature to incipient stages of succession (Ferrenberg et al., 2017). All these disturbances will lead to a loss in the ability of the biocrust to provide key ecosystem functions, such as an alteration of C balance (Maestre et al., 2013), a reduction of N_2 fixation rates (Rodriguez-Caballero et al., 2018a), or a decrease in their capacity to protect against soil erosion (Chamizo et al., 2017).

Once the activity that originates these negative impacts ceases, passive rehabilitation can eventually occur. However, biocrust recovery after a disturbance, when possible, tends to be slow, ranging from years to millennia depending on disturbance type, duration and severity and the climate and soil

conditions of the disturbed area (Weber et al., 2016). Among these factors, water availability is the more determinant, and it has been corroborated for several studies that drier climates appear more likely to have longer recovery times than wetter ones (Weber et al., 2016). For all these reasons, the assisted recovery by inoculation of biocrusts communities has arisen as a way to: i) speed biocrust recovery rates, ii) restore lost ecosystems functions of importance, and iii) reinstate lost biodiversity and ecosystem resilience (Bowker, 2007).

Biocrust-based restoration in drylands

During the last decades, several strategies to recover biocrusts have been explored. These can be classified in two main groups attending at the origin of the biocrust inoculants: 1) the translocation of field-collected biocrust propagules to the target area or, 2) the use of small amounts of native biocrust for ex-situ cultivation under optimal conditions and later inoculation.

The first approach consists of collecting biocrust propagules from a neighboring undisturbed area and spreading of the collected material on the area to be restored (Antoninka et al., 2018). Translocation of field-collected inoculum methodology has the advantages that the inoculant is pre-adapted to local field conditions, the native genetic pedigree is ensured, and no costs from cultivation and transport of biocrusts propagules are assumed. Thus, it has been the most widely tested practice so far, reporting promising results in some small scale field settings (Ballesteros et al., 2017; Chiquoine et al., 2016; Condon and Pyke, 2016). However, it is potentially unsustainable for large-scale restorations because it requires the disturbance of intact natural biocrusts in donor areas to improve their presence in a degraded area (Antoninka et al., 2019). For this reason, this approach is mostly recommended only in sites where a disturbance is previously planned, thereby the pre-existing biocrust material can be salvaged in advance and serve to rehabilitate the same area or a nearby degraded zone once the disturbance ends (Chiquoine et al., 2016).

The second approach consist of using small amounts of native remnant biocrusts that can be cultivated under optimal conditions to produce large quantities of inoculum to face large-scale restorations. Two main inoculum-culturing procedures have been tested: nursery-based production and laboratory cultivation-based. i) The nursery-based production process have been developed for obtaining inoculants based on cyanobacterial communities, lichens, mosses or a mix of them (Antoninka et al., 2018, 2015; Ayuso et al., 2017; Bowker and Antoninka, 2016). To do this, field harvested biocrusts propagules are normally incubated in the greenhouse under optimized conditions of temperature, light and moisture to promote fast growth (Doherty et al., 2015). Once enough biomass has been produced, it can be harvested and used to inoculate degraded zones in the original area (Antoninka et al., 2019). Following this procedure a significant amount of inoculum can be grown at a reasonable time (Ayuso et al., 2017; Bethany et al., 2019), and there were some tentative

to applied the nursery-grown inoculum (e.g., mosses or mixed communities) to field plots, although showed limited results (Xiao et al., 2011; Antoninka et al., 2018). Nevertheless, this approach also presents some important drawbacks. First, the microbial composition of the inoculum cannot be fully guaranteed as some components of the original biocrusts material can grow faster than others, thereby important community shifts may occur if inoculum is reuse for more than one incubation (Bethany et al., 2019). Second, the unavoidable presence of infectious pathogens (e.g., bacteriophages) in the original biocrusts remnants can negatively affect biocrust growth (Bethany et al., 2019; Sorochkina et al., 2018). ii) Alternatively, biocrust-forming bacteria, cyanobacteria and algae can be isolated and cultivated in the laboratory to restore soils functions. While a few studies explored the use of algae and bacteria (Sadeghi et al., 2017) for biocrust rehabilitation, the use of cyanobacteria is gaining interest in recent years because of its key role in initial soil formation and the feasibility to be isolated and mass cultured (Maestre et al., 2017; Rossi et al., 2017).

Cyanobacterial inoculation process: a potential tool for restoring biocrust and soil functions

Cyanobacteria are a very diverse prokaryotic group belonging to the domain *Bacteria*, which probably appeared in the earth from 3 billion years ago onwards, constituting the oldest photoautotrophic component of biocrusts (Büdel et al., 2016). Cyanobacteria have developed a variety of adaptations and mechanism that allow them to survive under very stressful conditions, being present in all the continents and constituting one of the main primary producers in dryland environments. For instance, their vegetative cells enter on a dormant state during desiccation, and are able to rapidly get active again upon wetting (Rajeev et al., 2013). Indeed, they can survive with only small amounts of non-rainfall water inputs, e.g. during dewfall events (Büdel et al., 2008; Jung et al., 2019). They produce pigments, such as carotenoids or scytonemin, that protect against photo-oxidative damage (Colyer et al., 2005) and UV radiation (Garcia-Pichel and Castenholz, 1991), and there are some species, such as *Microcoleus vaginatus* or *Oscillatoria* sp., that reside below the soil surface during unfavourable periods and migrate vertically only when soil pore water is available (Pringault and Garcia-Pichel, 2004; Rajeev et al., 2013). Moreover, most cyanobacteria secrete large amounts of exopolysaccharides (EPS) that serve as a repository of hydrophilic substances, e.g. uronic acids, contributing to increase water uptake and reduce moisture loss (Colica et al., 2014). The EPS matrix also plays a fundamental protective and structural role, reducing wind and water erosion (Rossi et al., 2018; Rossi and De Philippis, 2015b) and contributing to initial biocrust formation. All these characteristics allow them, on the one hand, to colonize highly degraded and poor substrates subjected to very harsh climatic conditions and, on the other hand, to improve soil properties such as water availability or stability. In addition, cyanobacteria can be isolated from soils and cultured ex-situ in bioreactors under optimal grow conditions to produce large quantities of inoculum (Guedes et

al., 2013). Thus, the production of enough inoculum to face large scale restoration can be accomplished in a period of weeks (Liu et al., 2013), rather than several months as shown by the

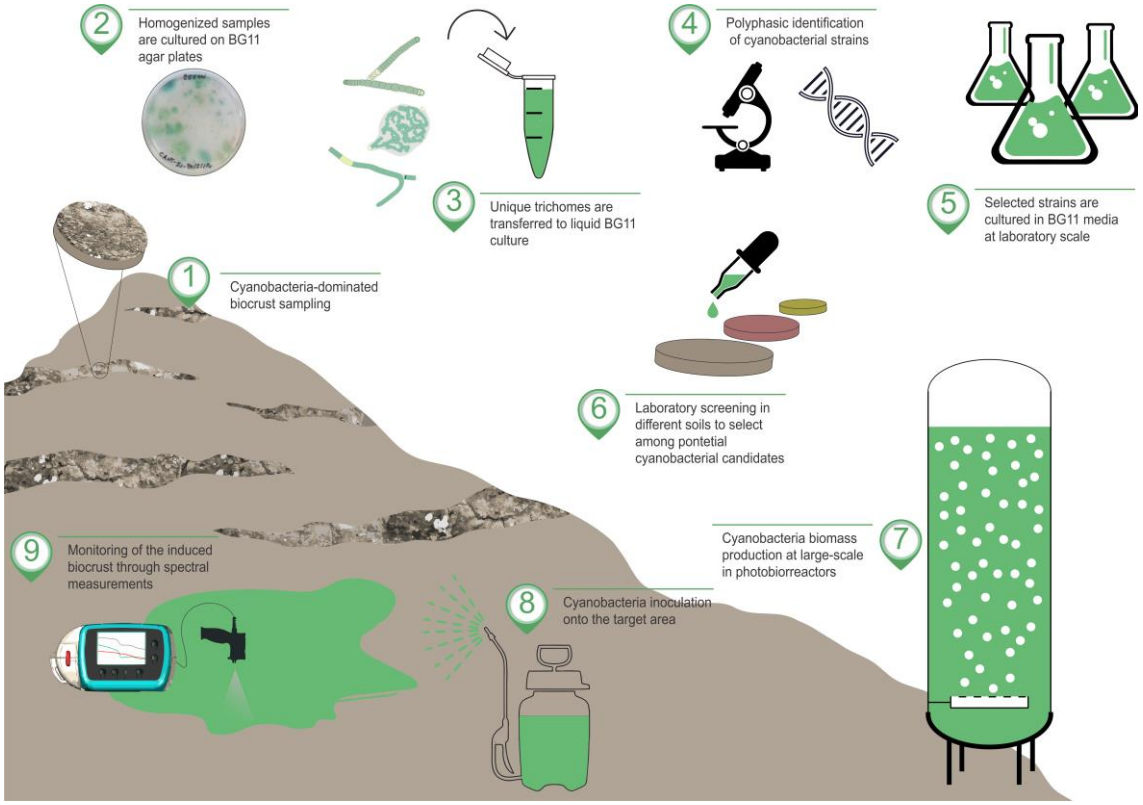

Figure 2. A general overview of the different stages of soil inoculation with cyanobacteria: isolation, identification, culturing, inoculation and monitoring.

nursery-based method production rates (Ayuso et al., 2017).

Isolation, identification and species selection

The process for obtaining cyanobacteria inoculants starts with the collection of natural biocrust samples in the field (Figure 2). Then, a small portion of the biocrust is inoculated in a specific liquid nutrient media (BG11) for growing cyanobacteria (Rippka et al., 1979). Afterwards, there are two main procedures for obtaining single cultures: 1) streak inoculation of the cultures on agar medium for obtaining colony-forming units and, 2) the collection with a pipette of single cells from the original culture under a microscope. Afterwards, the reliable characterization of cyanobacteria genus and species can be assigned according to the morphology by microscopic observation of isolated cultures. In some cases, identifying morphological specific features can be difficult, thereby the taxonomic assignation can be complemented with molecular markers (i.e. sequencing of the 16s rRNA gene) or ecological features (Komárek et al., 2014). Once the cyanobacteria have been identified, several principles should be considered when selecting species to be used in soil rehabilitation. One crucial characteristic of a determined strain is the feasibility to be mass cultured using scaling-up techniques in order to produce enough inoculum to face large-scale restoration.

Moreover, cyanobacteria species can be vastly different regarding colonizing capability of the soil, tolerance to abiotic stress and their effects on soil functions (Rossi et al., 2017). Therefore, preliminary laboratory tests are essential in order to choose among a pool of potential inoculants.

One selection criterion is the inoculant capability to colonize a given soil type and the persistence and abundance thereafter. In this sense, a great portion of the literature focused on the use of a single species, *Microcoleus vaginatus* (Hu et al., 2002; Kubeckova et al., 2003; Chen et al., 2006; Wang et al., 2009), which is recognized as a pioneer colonizer of arid soils worldwide (Zaady et al., 2010; Zhao et al., 2016). This strain, alone, or in combination with other strains such as *Scytonema javanicum* and *Phormidium tenue*, have been used to successfully promote the formation of an artificial biocrust in sand dunes (Hu et al., 2002; Wang et al., 2009; Xie et al., 2007). Still, the number of desert cyanobacteria species that has been tested is reduced, and more effort is needed in order to evaluate the best candidates to face future restoration projects. Moreover, most of the studies addressing the application of cyanobacteria have been carried out over sand dunes (Hu et al., 2002; Mugnai et al., 2018b; Wang et al., 2009; Xie et al., 2007), whereas its application in fine-textured soils are scarce (Malam Issa et al., 2007). Considering that cyanobacteria soil colonization and development is deeply influenced by the physicochemical characteristics (e.g., soil texture, porosity or fertility) of the soil intended for inoculation (Mugnai et al., 2020; Rozenstein et al., 2014), the transferability of this technology to other environments would benefit from the search for new desert species and their evaluation over a wide range of soil with contrasting properties.

Also, the consideration of the functional role previously described of the different species is of special interest for the selection of the most adequate species in each situation. For example, non-heterocystous filamentous strains (e.g. *M. vaginatus*) are naturally present during the primary soil succession and play a key role in initial soil formation and stabilization. Non-motile and N_2-fixing species appear later, enhancing the ecological function of biocrusts, e.g. through their contribution to C and N-cycling or by synthesising sunscreen pigments to protect against UV radiation (Garcia-Pichel and Castenholz, 1991). *Nostocales* belongs to last group, and species of the genus *Scytonema* spp. and *Nostoc* spp. have been employed in the lab showing a strong ability to produce exocellular polysaccharides and to improve soil fertility by fixing atmospheric carbon and nitrogen (Chamizo et al., 2018; D'acqui, 2016; B. Roncero-Ramos et al., 2019). However, their use under real field conditions is still reduced to two studies using Nostoc sp. and *Scytonema javanicum* as inoculants (Kubeckova et al., 2003; Wang et al., 2009). *Tolypothrix* spp., another heterocystous filamentous cyanobacteria genus belonging to *Nostocales*, had not been tested until very recently (Giraldo-Silva et al., 2019a), despite it is an important component of many degraded soil worldwide (Flechtner et al., 2008; Garcia-Pichel et al., 2001; Yeager et al., 2007), and specifically in desert environments such as in south-eastern Spain (Roncero-Ramos et al., 2019). Thus, although it was demonstrated that *Nostocales* play an essential role in enhancing soil functions, they are underexploited yet. In this

view, further studies are needed, not only to prove the feasibility to isolate, culture and inoculate these cyanobacteria genus but also to evaluate the effectiveness of employing them as a part of cyanobacterial consortia that may contribute to the formation of a more resistant and efficient inoculum due to a synergistic effect among components (Hu et al., 2002; Xie et al., 2007). For instance, Xie et al. (2007) demonstrated that the co-inoculation of *Microcoleus vaginatus* and *Phormidium tenue* enhanced soil structure, whereas the sole use of *M. vaginatus* produced weak soil compressive strength. However, our knowledge about the suitability of new candidates, such as *Nostocales*, to be employed in cyanobacterial consortia is still limited, as most of the previous studies incorporated *M. vaginatus* as a majoritarian component. For this reason, more studies to evaluate the effectiveness of employing cyanobacterial consortia for soil rehabilitation are needed.

It is also important to emphasize that water availability has been identified as a key factor affecting cyanobacteria survival under natural conditions (Rozenstein et al., 2014) and determining inoculum viability in restoration settings (Bu et al., 2014). However, most of the previous studies that evaluate the potential of the different cyanobacterial species rarely consider this factor and employ optimal water availability under controlled laboratory conditions. As laboratory conditions differ from the harsh conditions found in the field, before this technology can be applied in real restoration settings, a major challenge for researchers is the search of appropriate native taxa able to cope with water limitation. In this sense, the use of native cyanobacteria from the soils to be restored is a preferable option because they might be already preadapted to specific local conditions (Giraldo-Silva et al., 2019b). Moreover, inoculation of native species may avoid substantial modification of the soil biota by preventing the introduction of foreign species

Figure 3. Microphotographs of heterocystous cyanobacteria. A) *Nostoc commune*, B) *Tolypothrix distorta and* C) *Scytonema hyalinum*. Scale Bar =10 µM

In summary, biocrust restoration by means of cyanobacterial inoculation would benefit from the search of new species that: i) can be isolated and mass cultured at large scale using traditional scaling-up techniques, ii) can be incorporated into the soil without substantial modification of the original indigenous soil biota, iii) contribute with broader ecological aptitudes and functions, and iv) can grow and colonize soils under water stress conditions.

Mass culturing, inoculum preparation and soil inoculation

Initial inoculum production for large-scale cultivation is usually obtained by batch cultures, starting from small volumes (50-500 mL) to increasingly larger containers (Wang et al., 2009). The most critical factors affecting the achievement of optimal yield rates are the maintenance of axenic cultures and the control of light intensity, temperature, aeration and refresh periodicity of the culture medium (Lan et al., 2015; Liu et al., 2013). Once enough biomass to undertake soil inoculation has been obtained, inoculum can be harvested by sedimentation (by turning-off agitation), filtration or centrifugation and inoculated in the field.

There are different procedures in order to apply the inoculants to the target area (Zhao et al., 2016). The most employed is harvesting of cyanobacteria biomass and resuspension in distilled water to an optimal concentration for a direct application onto the soil surface (Muñoz-Rojas et al., 2018; Wang et al., 2009). Some authors added soil powder (sand or lime) to the solution in order to promote early soil formation and inoculum dispersion (Giraldo-Silva et al., 2019b). The ground dispersal can be directly performed for an operator equipped with a back-pack sprayer or, in large-scale inoculation settings, using an off-road tank truck (Wang et al., 2009). For remote or very large areas, it has also been suggested the use of aircrafts that ensure a homogenous inoculum dispersal (Sears and Prithiviraj, 2012). However, the main drawback of this approach is that biomass need to be immediately applied to the soil, otherwise inoculum is exposed to potential contamination by pathogens that would compromise inoculum viability. While this is possible in local field setting, some problems arise when facing large-scale restorations. Thus, recently the efforts focussed on the development of strategies and techniques that facilitate effective inoculum transferral and conservation. For example, some studies proposed the desiccation of the propagules until obtain a fine powder (Dodi and Zaadi, 2006), or the immobilization of cyanobacteria in small hemp cloth pieces (Kubeckova et al. 2003), but there was a general lack of success with almost all the strains tested. Another alternative is the incorporation of cyanobacteria into alginate pellet. This was tested by Buttars et al. (1998) showing promising results, whereas its incorporation in starch pellets resulted in high mortality (Howard and Warren, 1998). Therefore, further studies are needed in order to identify the suitability of different pellet compositions and the tolerance of inoculant candidates to pelletization process and later storage.

Addressing barriers to enhance induced biocrust survival and colonization in the field

Indoor experiments proved that inoculated cyanobacteria can bring to the formation of an induced biocrust over very poor soils, such as post-fire soils (Acea, 2003; Chamizo et al., 2020), mine substrates (Muñoz-Rojas et al., 2018), and very degraded soils (Malam Issa et al., 2007), in a short period of time. Soil inoculation, successfully increases organic carbon, nitrogen and phosphorus

content and improve EPSs content, which is related to changes in soil aggregation (Chamizo et al., 2018; Mugnai et al., 2018a) and increased stability. Consequently, it has been proved that artificial biocrust promoted by cyanobacteria inoculation improve soil fertility, and decrease wind (Hu et al., 2002; McKenna Neuman et al., 1996) and water erosion (Sadeghi et al., 2017). Moreover, cyanobacteria inoculation has been found to favour an accelerated natural colonization of other biocrust components as bacteria or mosses, and different plant species (Acea et al., 2001; Lan et al., 2014). However, although some applications in the field showed positive results (Chen et al., 2006; Wang et al., 2009), other outdoor studies showed a general failure of the amendment to stimulate crust recovery (Kubeckova et al., 2003).

There are a group of factors that can constrain the successful establishment of cyanobacteria after inoculation, including water scarcity, high UV radiation and soil erosion. Thus, two main approaches have recently arisen as a way to reduce the physiological stress and increase resource availability: cyanobacterial hardening and habitat softening. The first approach consists of the pre-acclimation of the inoculants to the climatic conditions found in the natural original areas (Bowker et al., 2019; Giraldo-Silva et al., 2019b). The cultures are subjected to full drying, following by several dry-wet cycles and progressively increasing light and UV radiation exposures in order to match with the conditions found in the target area. The first attempts with cyanobacteria cultures showed the positive fitness results in 13 of the 20 species tested (Giraldo-Silva et al., 2019c), although more studies are necessary in order to test the viability of the hardened inoculum under field conditions. The second strategy implies the use of habitats ameliorations procedures to improve field establishment of cyanobacteria (Bowker, 2007). For example, (Wang et al., 2009) successfully employed straw checkerboards to stabilize sand dunes prior to soil inoculation. Another possibility to improve soil stabilization is the mixture of the inoculum with soil tackifiers (e.g. sodium alginate) to promote rapid cyanobacterial-biocrust formation and increase soil compressive strength (Park et al., 2017; Peng and Bruns, 2018). The use of shade or other cover that reduced UV stress and increase water availability have been also successfully tested in biocrust nursery-based settings in the greenhouse (Ayuso et al., 2017; Bu et al., 2014) and in the field (Antoninka et al., 2019). Nevertheless, its application on direct cyanobacteria inoculations experiments has not been carried out yet. In summary, further studies are necessary in order to fully understand the factors affecting cyanobacterial survival and colonization in the field, and the focus should be paid on the development of facilitation strategies to optimize restoration with cyanobacteria.

Monitoring field establishment of the cyanobacterial inoculum

Since the factors allowing or impeding cyanobacteria to survive and establish are often unknown (Irisarri et al., 2006), continuous monitoring of the inoculated biocrust development and its effect on soil conditions during preliminary stages after inoculation is crucial (Figure 2). In this sense, we will

be able to identify plausible causes that eventually induce inoculum mortality. This has been traditionally done by measuring the chlorophyll a content (Maestre et al., 2006; Dojani et al., 2011), which is a common indicator of the biomass of photosynthetically active organisms within the biocrust community and has been widely used to assess biocrust development and its response to seasonal dynamics and water stress (Bowker et al., 2002; Belnap et al., 2007), anthropogenic disturbances (Kidron et al., 2008) or ongoing climate changes (Ferrenberg et al., 2015). However, laboratory methods for the analysis of chlorophyll are expensive and time consuming, and require destructive sampling, which in turn could affect the recolonization process. Chlorophyll a absorbs incoming solar radiation at specific wavelengths that can be detected trough spectroscopy techniques (Le Maire et al., 2004). Thus, in a similar way as traditionally done in the monitoring of vascular plants (Haboudane et al., 2002) chlorophyll absorption could be used as an accurate, cheap and quick alternative for chlorophyll retrieval in natural and induced biocrust communities. Therefore, this methodology presents a high potential for chlorophyll retrieval and development assessment in biocrust, as well as for the evaluation of biocrusts rehabilitation projects. In addition, this methodology opens a wide range of opportunities for biocrust monitoring at regional scales that would allow for their use in monitoring programs aimed at understanding biocrust responses to ongoing global change and degradation processes. Nevertheless, its application has yet to be harnessed.

Hypothesis

Survival, establishment and growth rate of newly induced cyanobacterial are strongly influenced by soil properties, such as soil texture and fertility. Thus, higher colonization rates and greater improvements of soil conditions after inoculation are expected in less degraded and more fertile soils than in very degraded ones, and also in fine-textured respect coarse-textured soils. Moreover, it has been suggested that the employment of native cyanobacterial blends could contribute to obtain a more resilient inoculum due to a synergistic action among components. Therefore, we hypothesized that the soil inoculation with a native consortium of species would promote better results in comparison to the effect of individual species.

On the other hand, although the ability of some heterocystous cyanobacteria (e.g. *Nostoc*, *Scytonema* or *Tolypothrix* genera) to resist desiccation have been described, water scarcity has been identified as a major abiotic constrain for cyanobacteria survival and performance under natural conditions. Thus, we expected that overall inoculum performance would be better when subjected to a wet regime rather than to a dry one, and the response to differing water regimes would be different among inoculants. In addition to water availability, UV radiation and photooxidation and soil erosion have been also suggested as major cause for inoculum mortality in the field. Thus, given that these factors may hinder biocrust rehabilitation in the field, we thought that cyanobacterial hardening and habitat softening would improve cyanobacterial survival and establishment in the first stages.

Once cyanobacteria inoculum survival is guaranteed, it is necessary to develop new strategies for improving inoculum storage, transferal and applicability. This can be done by incorporating cyanobacteria cultures into extruded pellets. In this sense, we hypothesized that some cyanobacteria would resist pelletization process better than others and, although short-term inoculum storage may theoretically reduce pellet viability to a certain extent, it would be still possible to use it for biocrust restoration.

Finally, the chlorophyll *a* content is the most employed indicator for assess biocrust biomass and development. Thus, we expected that the analysis of biocrust surface reflectance would allow us to infer biocrust chlorophyll *a* content in a similar way as it has been traditionally done for vascular plants. This would allow us to monitor natural biocrust dynamics and to assess the success of large-scale biocrust restoration at a reasonable cost.

Goals

The general purpose of this thesis is to evaluate the feasibility of using biocrust-forming cyanobacteria inoculation to accelerate the formation of a new biocrust able to improve soil conditions in semiarid degraded areas. To achieve this goal, the following specific objectives have been addressed:

1) To test the potential of three selected native nitrogen-fixing cyanobacteria strains, *Nostoc commune*, and the non-previously tested *Scytonema hyalinum* and *Tolypothrix distorta*, as inoculants, alone and in a consortium, to enhance soil properties from three semiarid ecosystems of the province of Almería (SE Spain).

2) To identify inoculant candidates that best perform under water scarcity conditions by simulating contrasted hydration regimes corresponding to dry and wet hydrological years.

3) To test the feasibility of inoculating an indigenous cyanobacterial consortium to induce biocrust formation and improve soil properties under field conditions, and to evaluate the effectiveness of cyanobacterial pre-conditioning and habitat amelioration strategies to improve survival and establishment of the new biocrust.

4) To evaluate the viability of encapsulating three representative cyanobacteria (*Leptolyngbya, Nostoc* and *Scytonema*), individually or as a consortium, into extruded pellets, to facilitate inoculum transferral, application and storage to face scale restoration projects.

5) To explore the potential of surface reflectance measurements, including spectral transformations and the most widely used spectral indexes proposed in the literature, for non-destructive monitoring of biocrust dynamics in natural and restored soils.

Chapter I:

Restoring soil functions by means of cyanobacteria inoculation: importance of soil conditions and species selection

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Abstract

In recent years, soil inoculation with cyanobacteria has become one of the most promising biotechnological strategies for restoring soil functionality in degraded drylands because of their critical role in increasing soil fertility and preventing erosion. Nevertheless, in order to fully exploit this biotechnology on a large scale, it must still be shown whether inoculated cyanobacteria are capable of developing in soils with different physicochemical properties, and new candidates adapted to desert conditions must be explored. To evaluate the potential of cyanobacteria for restoring soil functions of degraded dryland soils, in this laboratory study, we analyzed the effect of inoculating three native N-fixing species (*Nostoc commune*, *Scytonema hyalinum* and *Tolypothrix distorta*), individually and as a consortium, on soil properties from three different semiarid ecosystems in southeast Spain. The biocrust colonization was monitored by determining chlorophyll *a* content (the typical surrogate used for biocrust biomass). Other methodologies, such as the analysis of soil spectral response and image classification were also applied for cover estimation of the biocrust. After three months, all inoculated soils showed cyanobacteria cover of up to 50%, lower albedo and higher chlorophyll *a* content. Cyanobacterial inoculation also improved soil functions, as they promoted a significant gain in total organic carbon and total nitrogen in all soils. Among inoculation treatments, *Nostoc commune* and the mixture of all three species promoted the most cyanobacteria coverage, chlorophyll content and surface darkening, as well as organic carbon and total nitrogen gains in the soil, highlighting their excellent performance in biocrust development.

Keywords cyanobacteria inoculation; soil restoration; soil organic carbon; nitrogen content; semiarid; soil rehabilitation

Introduction

Cyanobacteria are pioneer inhabitants in dryland soils, where they form microbiotic assemblages on the soil surface with other organisms such as lichens, mosses, bacteria, algae and fungi, known together as biological soil crusts (BSCs) or biocrusts (Weber et *al*., 2016). Cyanobacteria are common inhabitants of poorly structured, low fertility soils, where they improve soil conditions, enhancing water availability (Kidron & Yair, 1997; Chamizo et *al.*, 2013), soil fertility and soil stability (Mazor et *al.*, 1996; Chamizo et *al.*, 2012a; Chamizo et *al.*, 2012b). As key promoters in the formation of biocrusts and due to the increasing awareness of their vital ecological roles in drylands, the inoculation of soil with cyanobacteria is attracting growing interest for counteracting soil degradation from intensified desertification and global change (Maestre et *al.*, 2017; Rossi et *al.*, 2017). Cyanobacteria are able to survive long droughts (Potts, 1999; Rajeev et *al.*, 2013; Bar-Eya et *al.*, 2015), prolonged UV exposure (Garcia-Pichel & Castenholz, 1991), and high salt concentrations

(Chen et *al.*, 2003; Prasanna et *al.*, 2008). This capacity, along with the possibility of isolating them from soils and culturing them ex-situ, makes cyanobacterial inoculation a potentially successful soil restoration bioengineering tool.

Although inducing cyanobacteria colonization is a good candidate for ecological restoration of degraded environments, new advances for developing a field-ready technology are needed. Their potential for success is strongly affected by the species used, soil properties and climate conditions of the ecosystem to be restored (Zhao et *al.*, 2016). Soil texture is one of the soil properties described as a key driver affecting cyanobacterial colonization in natural (Williams et *al.*, 2013) and artificial biocrusts (Rozenstein et *al.*, 2014). However, while a large body of literature has addressed the application of cyanobacteria for restoring biocrust functioning on sandy soils (Chen et *al.*, 2006; Xie et *al.*, 2007; Wang et *al.*, 2009; Wu et *al.*, 2013; Zaady et *al.*, 2016; Park et *al.*, 2017), only a few researchers have focused on biocrust rehabilitation experiments involving fine-textured soils (Rogers & Burns, 1994; Falchini et al., 1996; Maqubela et *al.*, 2012; Ayuso et al., 2017). In addition, differences in soil conditions, such as different degrees of degradation or physicochemical properties might cause different responses to cyanobacterial application. Besides, their application to the restoration of soil functions in degraded soils such as reconstructed soils or mine tailing substrates has not yet been studied. Since the physicochemical properties of degraded soils in Mediterranean drylands vary widely, any application for soil restoration project should first examine inoculant behaviour under different soil conditions, such as differing soil texture or soil quality, to further maximize the achievement of its objectives under field conditions.

Selection of suitable inoculants is another critical issue to be solved before planning any cyanobacterial inoculation task (Rossi et *al.*, 2017). Cyanobacteria exhibit a wide variety of physiological adaptations in response to abiotic stress which could profoundly affect their soil colonizing capability. *Microcoleus vaginatus*, *Scytonema javanicum* and *Phormidium tenue* have been used as inoculants for soil improvement and stabilizing sandy soils (Xu et *al.* 2010; Zheng et *al.* 2011; Zhang et *al.* 2013; Liu et *al.* 2013). Although some of the strains used in the experiments reported can grow in very different environments (Tomaselli & Giovaneti, 1993; Malam-Issa et *al.*, 2007), native species would be desirable to avoid modification of the soil biota insofar as possible. Furthermore, native inoculants may have already generated specific adaptations to particular soil conditions, which could increase the success of biocrust rehabilitation. Therefore, biocrust rehabilitation would definitely benefit from a search for exploitation of new desert species with broader ecological aptitudes and functions. Indeed, *Scytonema hyalinum* and *Tolypothrix distorta* have been reported to be important components of the soil community in many nutrient-poor arid soils worldwide (García-Pichel et *al.*, 2001; Yeager et *al.*, 2007; Flechtner et *al.*, 2008; Dojani et *al.*, 2013), but their viability as inoculants for soil restoration has not yet been tested. These species have recently been genetically and morphologically identified as components of natural biocrust

communities in various ecosystems in the province of Almeria (SE Spain) (Roncero-Ramos et *al.*, 2017), and have therefore been proposed as promising candidates for inoculation in rehabilitation tasks in those areas. Furthermore, some laboratory studies have had successful results inoculating individual species (Falchini et *al.*, 1996; Malam-Issa et *al.*, 2007), while other studies on sand dunes suggest that a mixture of different cyanobacteria strains seems to be a more promising strategy than inoculation of single strains alone (Hu et *al.*, 2002; Xie et *al.*, 2007; Li et *al.*, 2013). Thus, before new inoculants can be used in real Mediterranean soil restoration projects, their viability with the characteristics of the soil to be restored must be previously tested in the laboratory. Hence, the purpose of this study was to test the effect of inoculating three native nitrogen-fixing cyanobacteria, *Nostoc commune,* and the previously untested *Scytonema hyalinum* and *Tolypothrix distorta,* alone and in a mixture, on new biocrust development and the properties of three different soils from the province of Almeria (SE Spain) with contrasting differences in soil development and physicochemical properties. Our specific objectives were to: 1) compare the potential of the three cyanobacteria species for inducing the colonization of a new biocrust on soil types with differing particle size distributions and fertility conditions, including a post-mine substrate; 2) evaluate the effect of cyanobacteria to significantly improve soil functioning, especially on soil fertility and soil surface albedo; and 3) compare the effect of the two inoculation procedures, individual and in a mixture in the same proportion of each (1:1:1), on soil properties. We hypothesized that cyanobacterial application would improve the fertility of soils with differing physicochemical properties, and that this improvement would be better in soils with initially high fertility. We also hypothesized that the application of a consortium would promote better results than the inoculation of each species alone, as synergistic action among species had been previously reported to improve inoculation performance.

Materials and Methods

Field soil collection

We used three soil types, from three different study areas in the province of Almeria (SE Spain) (Table 1). The selection of the study sites was made attending at their profound differences in soil conditions, such as different degrees of soil development and contrasting physicochemical properties, which might cause different responses to cyanobacterial application. Prior to field soil collection, the top crust of the soil (2 cm) was removed with a palette knife. Thereby, soil with 2-10 cm depth was collected, air-dried and sieved to 2 mm to remove small herbs and rock fragments. From higher to lower soil development level, the sites were: 1) Las Amoladeras experimental site, which is a Mediterranean grassland ecosystem located in the Cabo de Gata-Níjar Natural Park (N 36° 50’ 01” W 02° 15’ 08”). Vegetation is mainly dominated by *Macrochloa tenacissima (L.) Kunth (=Stipa tenacissima L.)* and biocrusts represent about 30% of total surface coverage. 2) El Cautivo

experimental site, which is a badland area subjected to intense water erosion processes and located in the Tabernas desert (N 37° 00' 37" W 02° 26' 30"). The area is characterized by a mosaic of discontinuous perennial plant cover, some annuals and very abundant physical crusts and biocrusts. 3) Gádor limestone quarry experimental area, which is located at the calcareous quarries from Sierra de Gádor (W 36° 55' 20'' 02° 30' 29'' W). As a result of the mining activity, the hillslope was formed by a mixed substrate of calcareous rock fragments and marls (Luna et *al.*, 2017).

Table 1 Mean annual temperature (°C) and precipitation (PP, mm), main use of soil, soil type, soil texture, pH, electrical conductivity (EC), total organic carbon (TOC) and total nitrogen (TN) of the three study sites: Amoladeras, El Cautivo and Gador quarry.

Study sites	T (°C)	PP (mm)	Main use	Soil type	Soil texture			pH	EC (mS cm^{-1})	TOC (g Kg^{-1})	TN (g Kg^{-1})
					Sand (%)	Silt (%)	Clay (%)				
Amoladeras	19	200	Grazing	Calcaric Leptosols or Haplic Calcisols	61.50 ± 5.10	28.40 ± 4.20	10.10 ± 2.10	8.03 ± 0.04	0.16 ± 0.01	21.41 ± 0.96	2.07 ± 0.11
El Cautivo	19	235	Research, hunting	Endoleptic or Calcaric Regosols and Eutric Gypsisols	29.20 ± 5.40	58.60 ± 5.80	12.20 ± 4.20	8.28 ± 0.12	0.13 ± 0.01	3.87 ± 0.09	0.57 ± 0.04
Gádor quarry	17.6	242	Mining	Endoleptic Leptosols, Epileptic Leptosols and Calcaric Regosols	31.20 ± 4.65	43.10 ± 2.34	25.70 ± 2.80	8.57 ± 0.03	1.98 ± 0.18	0.24 ± 0.21	0.17 ± 0.09

Cyanobacteria culture and inoculation

Three native filamentous nitrogen-fixing species which had been previously isolated from soil biocrust samples on the three study soils were selected and later identified by morphological characterization and 16S rRNA gene sequencing by Roncero-Ramos et *al.* (2017): *Nostoc commune* (CANT2 UAM 817), *Scytonema hyalinum* (CAU6 UAM 820) and *Tolypothrix distorta* (CANT7 UAM 825). Each species was cultured separately in Erlenmeyer flasks (250 mL) containing liquid $BG11_0$ medium, then scaled-up to larger recipients (5 L) when biomass concentration reached 2 g L^{-1}. The cultures were subjected to 16:8 hour light/dark cycles with 60 μmol photons m^{-2} s^{-1} irradiance in a temperature-controlled room (28 ± 1°C). Cyanobacterial biomass was harvested in the exponential phase by filtration, and the fresh biomass obtained was resuspended in distilled water for soil inoculation.

Cyanobacteria inoculation was carried out in 90-mm (diameter) x 15-mm (depth) Petri dishes. Each Petri dish was filled with 80 g of each substrate and watered with 20 mL of distilled water to form a

physical crust. The substrate was not sterilized in order to maintain indigenous microbiota communities and examine the effect of the cyanobacteria inoculants under closer conditions to natural ones. Once substrates dried, samples were inoculated at a biomass concentration of 6 g dry weight m^{-2} (40 mg per sample). The quantity of inoculum applied was determined according to a previous study (Maqubela et *al.,* 2009) and some preliminary tests with the study soils. Biomass was distributed around the Petri dish by spreading small volumes as uniformly as possible, and an equivalent amount of distilled water was applied to control samples. Five treatments were applied on the three different soils: 1) Uninoculated soils, 2) *Nostoc commune*, 3) *Scytonema hyalinum*, 4) *Tolypothrix distorta* and 5) Mixture of the three species with all in the same proportion (1:1:1). We set four replicates of each treatment and soil type for a total of 60 samples.

Inoculated soils were placed in a Fitotron Plant Growth Chamber (Air-Frio, Almeria, Spain) at a constant temperature and light intensity of 60 μmol photons m^{-2} s^{-1} and 16-h photoperiod for 90 days. The amount of water to be provided each sample was calculated based on the mean annual rainfall of the study sites during a wet year (380mm) taking into account the duration of the experiment. The resulting irrigation treatment consisted of drop-by-drop application of 30 mL of distilled water to the soil surface in each Petri dish with a 10-mL pippete every four days.

Soil measurements

Biocrust cover

To estimate cyanobacteria cover at the end of the study period, a vertical picture of each sample was taken with a CANON EOS 600D digital camera placed 25 cm above the sample surface. Using these pictures, we estimated the cover of 1) cyanobacteria and 2) bare soil, i.e., the uncolonized portion of the soil, by supervised maximum likelihood classification. Before starting classification, image analysis identified training and validation points, selecting 500 training points and 300 validation points for each class. Accuracy of maximum likelihood classification was assessed based on the validation points acquired by image anylisis using a confusion matrix for calculating the commission and omission errors for each class, the overall accuracy and Cohen's Kappa coefficient (K) (Congalton and Green, 2008). Image analysis was performed using ENVI 4.3 (ITT VIS, Boulder, CO, USA).

Spectral measurements of soil surface

At the end of the study period, surface reflectance measurements of each sample were acquired with an ASD FieldSpec® Hand Held portable spectroradiometer (ASD Inc., Boulder, 212 Colorado, USA) with a 3.5-nm optical resolution from 325 nm to 1075 nm. Spectral measurements were made under constant light conditions with an optical fiber placed 16 cm away in order to measure the entire

surface of the Petri dish. Three replicate measurements, each consisting of the average of three individual spectra, were collected for every sample. Before measuring each sample, a white reference was acquired using a Spectralon(r) panel to compute the reflectance factor. Data were subjected to a pre-treatment which consisted of removing noisy bands (350 to 400 nm and 950 nm upwards) applying a cubic polynomial smoothing filter with a 17 band-window (Savitzky & Golay, 1964). Visible surface albedo (from 400 to 700 nm) was calculated using the preprocessed spectra as the square root of the sum of the squares of reflectance at each wavelength (Escribano et *al.*, 2010).

Chlorophyll a, soil organic carbon and total nitrogen determination

At the end of the experiment, the biocrust along with the soil beneath it (3mm thick) was collected with a palette knife and air-dried at ambient temperature. When dry, it was crushed with a roller and ground with a mechanical agate mortar. Chlorophyll *a* was determined by the double extraction method with ethanol (Lan et *al.*, 2011; Castle et *al.*, 2011): 1 g of fine powder was mixed with 5 mL of ethanol, heated at 80°C for 5 min and then cooled at 4°C for 30 min. After that, samples were centrifuged at 4000 g for 10 min and the supernatant was recovered. This extraction method was applied twice to each sample. Chlorophyll *a* content was determined immediately after extraction by measuring absorbance at 665 nm and subtracting sample absorbance at 750 nm to discard any solution turbidity. Chlorophyll *a* concentration was then calculated by applying the ethanol solvent equations reported by Ritchie (2006) (Eq. 1):

$$\text{Eq.1: Chlorophyll } a \text{ } (\mu g \text{ g soil}^{-1}) = (11.9035 \times A(665\text{-}750) \times V) / \text{g soil}^{-1} \times L$$

where A is the absorbance value at the specific wavelength, V is the volume of the extract (L) and L is the optical path length of the spectrophotometer cuvette.

Total Organic Carbon (TOC) content was determined using the Walkley and Black method modified by Mingorance et *al.* (2007), consisting of organic matter oxidation by sulfuric acid and potassium dichromate and later measurement of absorbance in a spectrophotometer at 590 nm. Total nitrogen (TN) was determined using a LECO TRUSPEC C/N analyzer.

Statistical analysis

All variables were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's test. When needed, data were log transformed before performing parametric analysis. The effects of inoculation treatment, soil type and their interaction, on cyanobacteria cover, chlorophyll *a* gain, albedo, and TOC and TN gains were analyzed using generalized linear models (GLM). Comparisons between means (when differences were found) were performed with Fisher's Least Significant Difference (LSD) test. All statistical analyses were performed using SPSS 22.0 software (SPSS, USA). The quantitative gain of chlorophyll *a*, TOC and TN in each sample was calculated as

the difference between the value of the variable at the end of the experiment and the initial substrate value.

Results

GLM results showing the effect of soil and inoculation treatment on all the variables analyzed are shown in Table 2. Both soil type and inoculation treatment had a significant effect (p <0.01) on cyanobacteria cover, chlorophyll *a* gain, albedo, TOC and TN gains, but not soil type on TOC gain (p = 0.64). The effect of the inoculation treatment differed depending on soil type as shown by the significant interaction between inoculation and soil type for almost all variables except TN gain.

Table 2 Result of the General Lineal Model (GLM) showing the effect of the predictor factors on the variables studied. Response variables are represented by Soil Type (ST), Inoculation Treatment (IT) and the interaction of both (ST *IT). Values in bold represent significance at 95% confidence interval or p <0.05.

	Cover gain			Chlorophyll *a* gain			Albedo			TOC gain			N gain		
Response variable	F	p	Partial eta-square	F	p	Partial eta-square	F	p	Partial eta-square	F	p	Partial eta-square	F	p	Partial eta-square
ST	33.21	**0.00**	0.61	96.76	**0.00**	0.82	1792.90	**0.00**	0.99	0.27	0.64	0.02	12.25	**0.00**	0.36
IT	42.96	**0.00**	0.80	30.68	**0.00**	0.74	13.69	**0.00**	0.55	11.75	**0.00**	0.64	8.85	**0.00**	0.45
ST * IT	4.61	**0.00**	0.46	13.04	**0.00**	0.71	2.25	**0.04**	0.29	2.53	0.57	0.14	5.51	0.70	0.11

Cyanobacteria cover, biomass and changes in spectral response of soil surface

Cyanobacteria inoculation of soil promoted the development of a biocrust, with an average cover of up to 56%, 39%, and 25% in the most effective treatments in Amoladeras, El Cautivo and Gádor quarry, respectively (Fig. 1, Table 2). The most developed soil, Amoladeras, showed the highest cyanobacteria cover after three months from inoculation (Fig. 1). There were also significant differences between inoculation treatments and soils (Fig. 1). In general, soils inoculated with *Nostoc* and the mixture of the three species resulted in more cyanobacteria coverage than soils inoculated with *Scytonema* and *Tolypothrix.* For instance, cyanobacteria cover in Amoladeras soil inoculated with the mixture of the three species and *Nostoc* was almost two-fold higher than those inoculated with *Scytonema* and *Tolypothrix*, and yielded cover 44% and 259% higher than in El Cautivo and Gádor quarry soils, respectively (Fig. 1). Moreover, different inoculation treatments induced different biocrust development depending on the soil type. Whereas *Nostoc* promoted the most

development in Amoladeras and El Cautivo, the mixture showed better performance in Gádor quarry samples.

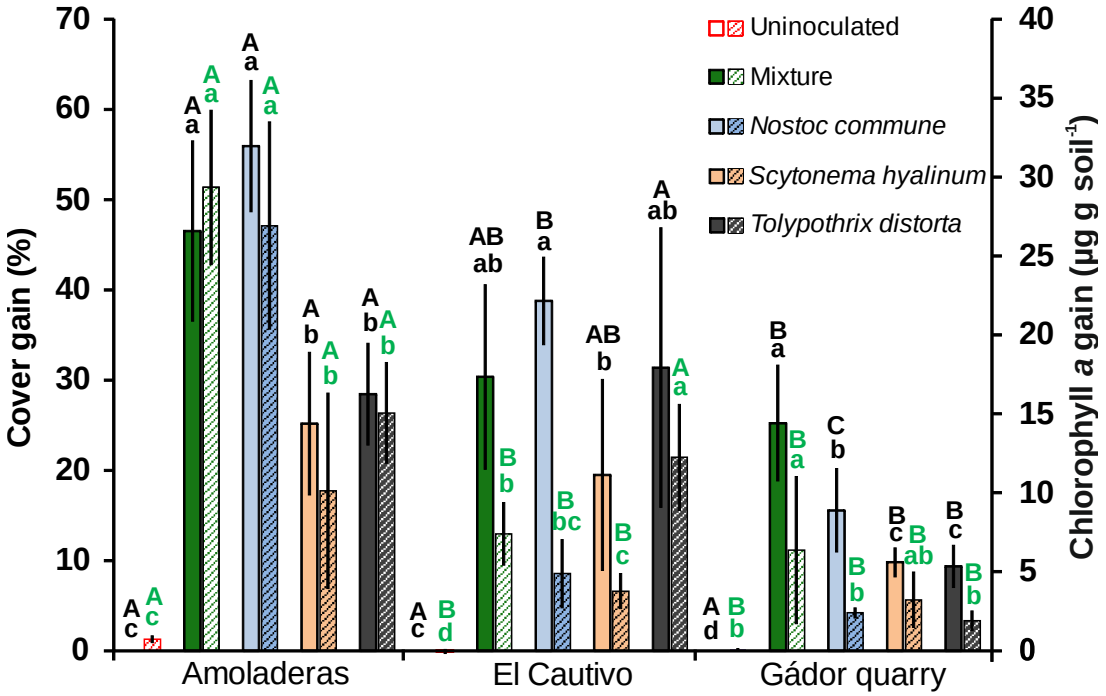

Figure 1. Mean (± SD, n=4) cyanobacteria coverage (plain bars) and chlorophyll a gain (striped bars) of the five treatments in Amoladeras, El Cautivo and Gádor quarry soils. Lower case letters mark significant differences ($p < 0.05$) in coverage gain (black) and chlorophyll *a* gain (green) among inoculation treatments for each soil. Capital letters mark significant differences among soils within each inoculation treatment.

As soil was colonized by cyanobacteria crust, chlorophyll *a* content also increased. Thus, similar to cyanobacteria coverage, chlorophyll *a* gain during the experiment depended on the inoculum and soil type (Fig. 1). Whereas in Amoladeras soils the mixture and *Nostoc* promoted the highest chlorophyll *a* gain, in Gádor quarry soils the mixture promoted higher chlorophyll *a* gain than the other inoculation treatments. On the contrary, soils from El Cautivo showed higher chlorophyll *a* gain when inoculated with *Tolypothrix* followed by *Nostoc* and the mixture.

All soils showed the typical soil spectral response with lower reflectance in the blue region and gradually increasing towards NIR. Uninoculated soils from Amoladeras, which had higher organic matter and Fe^{3+}, were characterized by the lowest albedo and showed a deeper absorption peak at 540 nm, whereas soils from Gádor quarry and El Cautivo showed the highest albedo (Fig. 2). Nevertheless, as soil was colonized by cyanobacteria, their overall reflectance decreased, resulting in lower albedo in all inoculated soils compared to uninoculated controls (Table 3, Fig. 2). In particular, soils inoculated with the mixture and *Nostoc*, which had the highest gains in cyanobacteria coverage and chlorophyll *a*, showed the most surface darkening at the end of the experiment (Table 3). Moreover, the chlorophyll *a* absorption peak (680 nm) was deeper in the inoculated soils, in agreement with the increase in chlorophyll *a* content observed (Fig. 2).

Effect of cyanobacteria inoculation on soil functions: TOC and TN contents

Cyanobacteria inoculation promoted a net increase in TOC content at the end of the experiment (Fig. 3a) with varying effects depending on inoculation treatment. Soil inoculation with the mixture and *Nostoc* showed higher TOC gains (up to 6 g Kg^{-1}) than those inoculated with *Scytonema* or *Tolypothrix* (Fig. 1). Notwithstanding absence of significant differences between inoculation treatments and soils (Table 2, Fig. 3a), the most important effect of cyanobacteria inoculation on TOC content was observed on soils from the Gádor quarry, which was characterized by very low initial TOC (0.24 g Kg^{-1}). The inoculation of this soil with the mixture and *Nostoc* showed the highest percentage increase in TOC from the initial substrate value (Fig. 4).

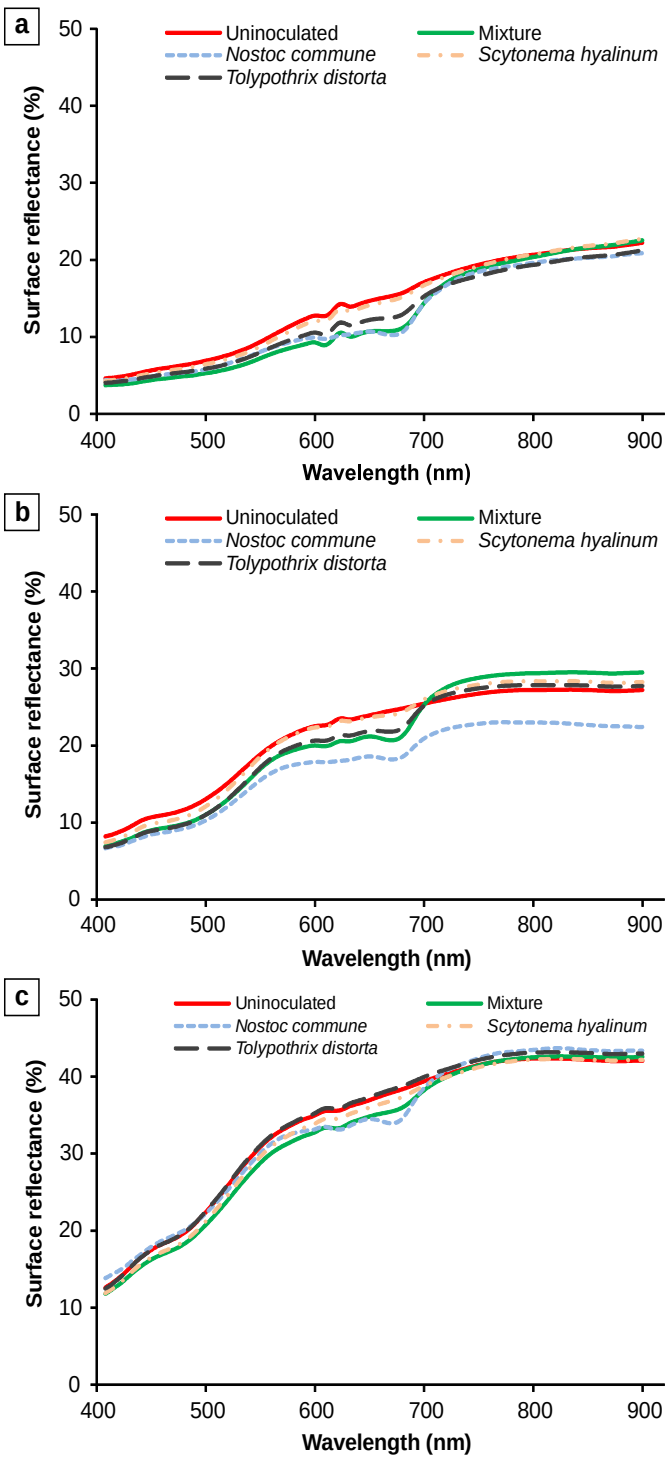

Figure 2. Surface reflectance spectra at 407 to 900 nm for soil inoculation treatments from A) Amoladeras, B) El Cautivo, and C) Gádor quarry. Values represent the mean reflectance at every band mean of the four samples per each treatment.

Cyanobacterial application on the soil substrates promoted an improvement in TN status in all soils at the end of the study, with differences among soil types and inoculation treatments. TN gains in Amoladeras soils, which were initially characterized by higher TN than the other soil types (2.07 vs 0.57 and 0.24 g Kg^{-1} TN in Amoladeras, El Cautivo and the Gádor quarry, respectively), were the highest at the end of the study period for all inoculation

treatments. However, no significant differences in TN gains were found between inoculated and uninoculated soils in Amoladeras (p = 0.12). On the other hand, inoculated Gádor quarry soils showed the greatest increases in TN percentage (Fig. 4) and also the widest differences compared to the uninoculated soils at the end of the experiment (Fig. 3b). Of the inoculation treatments, the cyanobacterial mixture and *Nostoc* were the most productive treatments in all soils, promoting TN gains of up to 0.90, 0.68 and 0.60 g Kg^{-1} in Amoladeras, El Cautivo and Gádor quarry soils, respectively (Fig. 3b).

Table 3 Mean (± SD, n=4) a) albedo in the 400 nm to 700 nm range of soil surface reflectance spectra for each inoculation treatment in the three soils studied. Lower-case letters mark significant differences among treatment s within each soil.

Inoculation treatment	Amoladeras	El Cautivo	Gádor quarry
Uninoculated	$1.88^{a} \pm 0.11$	$3.21^{a} \pm 0.22$	$5.08^{a} \pm 0.08$
Mixture	$1.38^{b} \pm 0.19$	$2.83^{bc} \pm 0.22$	$4.76^{b} \pm 0.27$
Nostoc commune	$1.43^{b} \pm 0.05$	$2.53^{c} \pm 0.09$	$4.85^{b} \pm 0.12$
Scytonema hyalinum	$1.77^{a} \pm 0.14$	$3.14^{ab} \pm 0.15$	$4.91^{ab} \pm 0.11$
Tolypothrix distorta	$1.55^{b} \pm 0.10$	$2.90^{ab} \pm 0.36$	$5.12^{a} \pm 0.10$

Discussion

Identifying suitable inoculants for differing characteristics of Mediterranean soils represents a challenge in developing new methodologies for restoring such zones. In this indoor experiment, we successfully promoted the development of a biocrust by inoculation of native cyanobacteria species in soils from three semiarid Mediterranean ecosystems with differing physicochemical properties, including fine-textured soils and mine tailings, under water availability conditions mimicking those found naturally in the selected semiarid zones. In 90 days, we were able to grow a cyanobacterial crust that colonized up to 50% of the soil surface in some cases (Fig. 1). A previous study reported similar cyanobacteria growth rates in desert field-settings (Wang et *al.*, 2009). Likewise, the entangled network formed by cyanobacteria and soil particles formed a stable organo-mineral layer which contributed to improving soil fertility, especially in those soils where it was initially low (Fig 3a, Fig. 3b, Fig. 4).

The survival and establishment of cyanobacteria inoculants in our study was closely related to the initial properties of the inoculated substrates (Fig. 1). Amoladeras, with the highest biocrust coverage in all treatments at the end of the experiment, provided the most suitable soil conditions for cyanobacteria soil colonization. This may be explained by differences in soil texture, but also by its higher initial organic carbon and nitrogen contents, as well as lower degradation and relatively low salt content (Table 1). On the contrary, the lowest colonization rates were observed on Gádor quarry substrates, the most degraded soil, characterised by high salinity and low fertility due to the inherent properties of the parent material (calcareous sandstone and calcitic-gypsiferous mudstone) and intense soil erosion processes (both water and wind erosion) (Luna et *al.*, 2017).

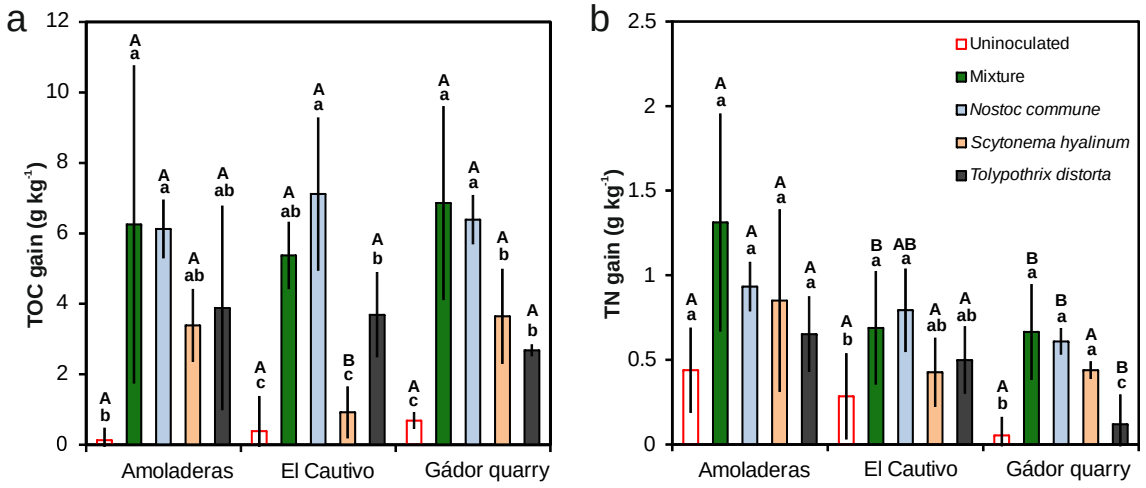

Figure 3. Mean (±SD, n = 4) total organic carbon (TOC) (a) and total nitrogen (TN) (b) gains for each soil and inoculation treatment. Different lower-case letters mark significant differences ($p < 0.05$) among inoculation treatments in each soil. Capital letters mark significant differences among soils in each inoculation treatment.

Inoculation of *Nostoc* alone and the mixture promoted the highest coverage in all soil types while *Scytonema* and *Tolypothrix* yielded the lowest. This is surprising, because previous inoculation experiments on sand dunes reported relatively poor coverage of *Nostoc* crusts on the surface compared to *Microcoleus vaginatus* and *Scytonema javanicum* crusts (Hu et *al.*, 2002). *S. hyalinum* and *T. distorta* showed branched trichomes several times larger than *N. commune* encased filaments, and their larger specific surface area would enable them to reach more soil grains, thus colonizing soil surface more rapidly than the thalli of *N. commune*. However, we also observed a major presence of exopolysaccharides (EPS) inside the *Nostoc* sheath (unpublished data) which could compensate for their smaller volume and contribute to soil proliferation, as the water-absorption characteristics of acidic sugars in the EPS synthetized by this cyanobacterium have been reported to contribute to establishing favorable living conditions (Tamaru et *al.*, 2005).

Cyanobacteria colonization during the experiment resulted in an increase in chlorophyll *a* content in all inoculation treatments and soils ($R^2 = 0.63$, $p < 0.0001$) reaching values similar to those observed in natural late-successional stage biocrusts (Lan et *al.*, 2011; Castle et *al.*, 2011) in the most

successful treatment (*Nostoc* and the mixture in Amoladeras). However, the chlorophyll *a* content in samples with low cyanobacteria cover was similar to natural light-dominated cyanobacterial crusts (Castle et *al.*, 2011). This increase in chlorophyll *a* promoted an increase in spectral absorption at 680 nm (Fig. 2), suggesting the possibility of using crust spectral features as a non-destructive indicator of biocrust biomass.

Unlike other frequently used inoculants like *M. vaginatus*, the species used in this experiment cannot migrate to the soil surface following rehydration and retreat in response to imminent desiccation. Instead, the selected cyanobacteria usually invest carbon and energy in light–harvesting pigments, such as scytonemin and carotenoids, which are involved in photosynthesis and protect them from photo-oxidative damage (Belnap et *al.*, 2004; Wada et *al.*, 2013). Accumulation of scytonemin from scytonemin-producing *Nostocales* (e.g. genera *Nosto*c, *Scytonema* and *Tolypothrix*) has been found to be the major force driving decrease in albedo in dryland soils (Couradeau et *al.*, 2016). These absorptions along with those produced by chlorophyll-related pigments could explain why soil surface albedo was markedly lower on treated than uninoculated soils (Table 3).

Inoculation of the N-fixing cyanobacteria species used in this study significantly improved the status of organic carbon and total nitrogen in all substrates (Fig. 3a, 3b). Higher TOC and TN found in inoculated samples can be attributed to the autotrophic and diazotrophic nature of the selected species, which are able to fix atmospheric C and N, incorporating them in the soil profile (Acea et *al.*, 2003; Wang et *al.*, 2009). Indeed, direct application of the cyanobacteria-based inoculum (1:1:1) was just 40 mg/petri dish at a time, which represented the addition of very small quantities of nutrients to the soil (TOC = 0.95 g kg^{-1}, TN = 0.15 g kg^{-1}) for the strong differences observed in cyanobacteria development response at the end of the experiment (gains up to 6 g Kg^{-1} of TOC and 0.60 g Kg^{-1} of TN, Fig. 3a, Fig. 3b). However, though Amoladeras soils showed higher TOC and TN contents at the end of the study, the highest TOC and TN increases were found on Gádor quarry soils, where TOC and TN contents three months after cyanobacteria inoculation were over 1000% of initial values found in this poor quarry substrate (Fig. 4). Thus, contrary to our initial hypothesis, cyanobacteria inoculation caused greater improvement in soil fertility in the least fertile soil. In organically poor post-mining operation substrates, this organic supply could play a significant role in improving soil structure and water retention (Six et *al.*, 2004), thus accelerating soil regeneration (Carlson et *al.*, 2015). From the different inoculation treatments, soils inoculated with *Nostoc* and the cyanobacterial mixture reached the maximum TOC and TN contents and also showed the highest TOC and TN gains (Fig. 3a, 3b). Differences among treatments may be partially explained by the different behavior of cyanobacteria in colonizing the soil surface. In fact, a positive relationship was observed between cyanobacteria coverage and TOC and TN gains, (R^2 = 0.76 and R^2 = 0.45, respectively), especially on Gádor quarry soils.

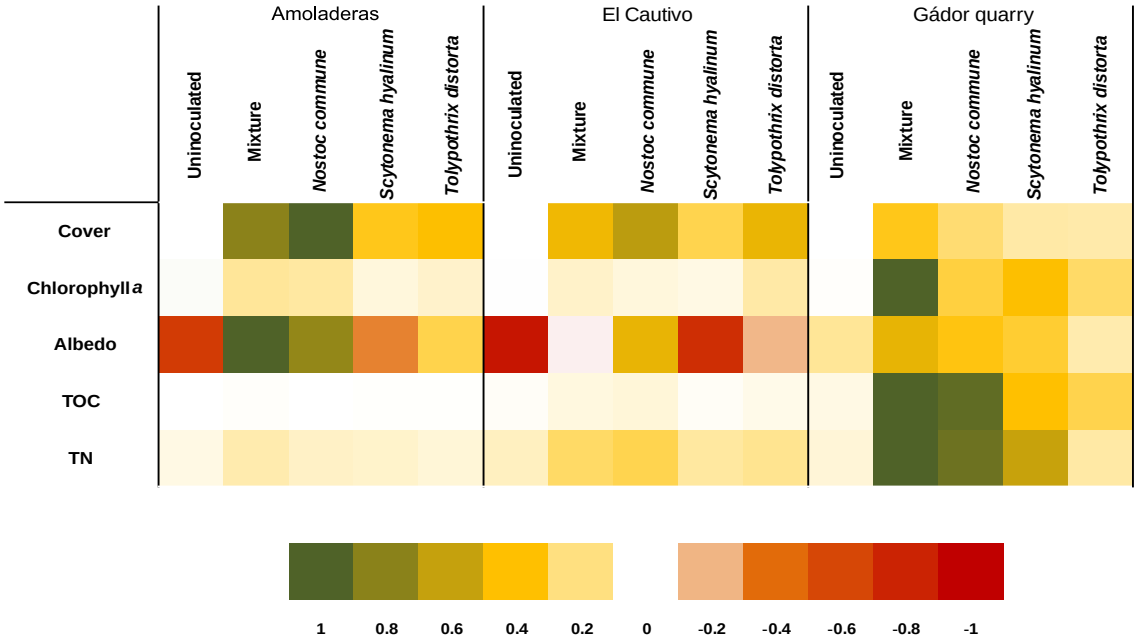

Figure 4. Heat map representing the relative increase in each variable from the initial substrate value for all treatments. Values of each variable were normalized with respect to the maximum value found in each variable. Values equal to + 1 and – 1% were classified as the maximum and minimum values on the scale, respectively. Negative decreases in albedo are shown as positive increases.

Inoculation of individual *Nostoc*, *Scytonema* and *Tolypothrix* species alone has been reported to improve soil aggregation and structure in agricultural settings (Kaushik & Subhashini, 1985; Rogers & Burns, 1994) and soil reclamation projects (Falchini et *al.*, 1996; Malam-Issa et *al.*, 2007). However, other studies on cyanobacteria inoculation have reported the synergistic effect of using mixed cultures, mainly combining the advantages of nitrogen-fixing species with non-heterocystous genera. For example, in recent open field trials in China, researchers found that the co-inoculation (10:1) of the non-heterocystous *Microcoleus vaginatus* with *Scytonema javanicum* promoted successful results, increasing soil fertility and biocrust restoration success (Wang et *al.*, 2009). In another study in sand dunes, co-inoculation with *Microcoleus vaginatus* and *Phormidium tenue* was a suitable strategy for improving soil structure (Xie et *al.*, 2007). Hu et *al.* (2002) also reported that a blend of 80% *Microcoleus vaginatus* and 5% each of *Phormidium tenue*, *Scytonema javanicum*, *Nostoc* sp. and *Desmococcus olivaceus* was successful in increasing soil cohesion and reducing wind erosivity. Furthermore, Gueda et *al.* (2014) found that a mixture of *Nostoc kihlmani* and *Anabaena cylindrical* promoted higher TOC and nitrogen than inoculation of each of them alone. In agreement with our initial hypothesis, we found that *Nostoc commune* and the mixture promoted better results in all soils than inoculation of *Scytonema hyalinum* or *Tolypothrix distorta* alone (Fig. 4), leading to greater cyanobacteria coverage and biomass, chlorophyll spectral absorption and surface darkening, as well as TOC and TN gains in the soil (Fig. 4). To our knowledge, this is the first study where a blend of three N-fixing species, including the previously untested *Scytonema hyalinum* and *Tolypothrix distorta*, has been successfully employed to produce artificial biocrust leading to

improvements in soil properties, and suggesting that those inoculants can be employed to restore soil functions in further field experiments. Likewise, the selection of a suitable inoculation strategy depends on species pool access and biomass requirement. Cultivation of a single species reduces efforts by preventing contamination and accelerating biomass production. However, using a consortium of species reduces the required biomass for each species and maximizes the advantages of synergistic action. Based on these results, future long-term field experiments could allow us to confirm the suitability of both inoculants and identify the most suitable according to its performance on specific soil conditions.

So far, recent cyanobacteria application alone or in combination with other substances, such as biopolymers over large sand dune areas in Chinese Deserts, are the only successful attempt to translate this technology to the field (Chen et *al*., 2006; Wang et *al*., 2009; Park et al., 2017). However, other field trials involving cyanobacteria inoculation in desert environments of North America failed (Kubečková et al., 2003). Hence, our findings provide valuable insights for improving soil restoration based on the application of native N-fixing cyanobacteria combinations shown to improve soil functions on Mediterranean soils. Further experiments will examine the effectiveness of the study inoculants under field conditions in the tested sites, probably in combination of amendments for amelioration of abiotic stress.

Conclusion

This study suggests the feasibility of using individual native cyanobacteria species or a consortium of them to promote the formation and development of artificial biocrust communities and improve key soil properties related to the fertility of different soils from three Mediterranean ecosystems, including a post-mine substrate. Soil inoculation with *Nostoc commune* and the mixture of the three cyanobacteria species tested appeared to be the most effective inoculation strategy for promoting biocrust development and improving soil functions. This improvement in soil quality by cyanobacteria inoculation can be crucial in facilitating the outcome of rehabilitation. However, before they can be widely applied, more research is necessary to study the effectiveness of these species under field conditions.

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Chapter II:

Effect of water availability on induced cyanobacterial biocrust development

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Abstract

Cyanobacteria inoculation has recently become an innovative biotechnological tool for restoring degraded arid soils. A major challenge for researchers, however, is the search for suitable species able to cope with water stress under field conditions. The aim of this study was to test the effect of water availability on induced biocrust growth in three different degraded soils from semiarid areas of Almeria (Spain). Three native N-fixing cyanobacterial strains, *Nostoc commune*, *Scytonema hyalinum* and *Tolypothrix distorta*, were inoculated on soil samples from the study areas, individually and as a consortium. Two different irrigation treatments simulating the water availability in the selected areas, in a dry year (180 mm/year) and a wet year (380 mm/year), were applied for three months under laboratory conditions. Cyanobacteria cover, chlorophyll *a* spectral absorption (Chl*a* spectral absorption), soil organic carbon (SOC) and total exopolysaccharide (EPS) gains were measured as indicators of biocrust development. Cyanobacteria crust cover, SOC and EPS gains were higher in inoculated soils than in uninoculated soils. Even though the water regime had a generally significant effect on cyanobacteria cover, Chl*a* spectral absorption and EPS, similar biocrust development and improvement in soil edaphic conditions were observed under both water regimes for all treatments. Of the candidate inoculants, *N. commune* showed remarkably higher performance under dry conditions than the rest, providing evidence of high potential for growing under water-limited conditions and becoming a good candidate inoculant for restoration of arid degraded areas.

Keywords: Cyanobacteria inoculation; water availability, soil organic carbon, exopolysaccharides, biocrust restoration, semiarid

Introduction

Terrestrial cyanobacteria are early colonizers of soils in global drylands, where they coexist, in close association with soil particles and other organisms, such as bacteria, microalgae, microfungi, lichens and bryophytes, in what are known as biological soil crusts or biocrusts (Belnap et al., 2016). Biocrust-forming cyanobacteria play a crucial role in improving soil conditions by enhancing water availability (Chamizo et al., 2017), increasing soil fertility and enhancing soil stability (Chamizo et al., 2012; Chaudhary et al., 2009; Zhao et al., 2011), and facilitating the colonization of later-successional organisms (Dojani et al., 2011; Weber et al., 2016). In addition, cyanobacteria have developed a variety of protective mechanisms for dealing with desiccation (Potts, 1999), such as formation of buffer cells by secreting exopolysaccharides in response to stress conditions (Hill et al. 1997, Tamaru et al., 2005) or remaining temporarily dormant (Rajeev et al., 2013). To a certain extent, all these mechanisms protect cyanobacteria from damage from desiccation, and therefore, when cyanobacteria crusts are moistened, their photosynthetic activities rapidly recover (Harel et al. 2004). Due to both their essential contribution to ecosystem functioning and their capacity to survive

during long periods of drought, the use of cyanobacteria as a bioengineering tool for restoration of degraded arid soils is attracting growing interest (Rossi et al., 2017).

Indoor experiments have proved that inoculated cyanobacteria can promote the artificial formation of biocrust and improve the edaphic conditions of post-fire soils (Acea et al., 2003), mine substrates (Muñoz-Rojas et al., 2018), and a wide range of other degraded soils with differing properties and textures (Malam-Issa et al., 2007; Kheirfam et al., 2017; Chamizo et al., 2018; Sorochkina et al., 2018; Mugnai et al., 2019). However, laboratory conditions differ from the harsh conditions expected in the field, and therefore, real applications in dryland soils have achieved only limited success in cool deserts (Chen et al., 2006; Wang et al., 2009), whereas their application in hot deserts has shown high mortality (Kubečková et al., 2003). Despite its proven drought tolerance, water scarcity has been identified as a major abiotic stress affecting cyanobacteria survival and performance under natural conditions (Lange, 2001; Fernandes et al., 2018) and restoration settings (Bu et al., 2014). Indeed, the survival and development of cyanobacteria propagules in the field has only been ensured by application of additional water on the days following inoculation (Wang et al., 2009; Wu et al., 2013), and it has been suggested that field inoculation should be scheduled to occur in wet or mild seasons (Sorochkina et al., 2018; Giraldo-Silva et al., 2019a). Although the use of watering could be justified in the case of specific rehabilitation scenarios, providing enough supplemental water for large-scale biocrust restoration in arid environments is only minimally feasible. Thus, the application of technologies for the reduction of environmental stress, biocrust preacclimation or the search for drought resistant inoculants seem more reasonable approaches for large scale issues. Moreover, cyanobacterial species exhibit varying tolerance to abiotic stresses (Rossi et al., 2017), and the use of local inoculum sources makes it likely that they will be genetically preadapted to the site-specific conditions (Giraldo-Silva et al., 2019a), increasing probability of success. In addition, the sensitivity of different cyanobacterial species to water scarcity can vary depending on the soil physicochemical characteristics. For example, soil mineralogy composition, texture, porosity and soil organic matter content can noticeably influence water residence time in the soil, and thereby, the amount of water available for the inoculated crust. Thus, a major challenge for biocrust researchers is the search of appropriate native taxa able to cope with water limitation in a wide range of soils with differing physicochemical characteristics.

Water stress limitations are not restricted to survival and colonization, but also affect the functional behavior of biocrust communities. Maestre et al., (2006) found that water scarcity considerably decreased the capacity of artificially grown biocrusts to fix C, which in turn could affect the crust's capacity to produce and release useful organic compounds, such as exopolysaccharides. On the contrary, other studies have found higher total exopolysaccharides (EPS) in induced biocrusts in response to more restricted water regime conditions (Mugnai et al., 2018). Synthesis of these organic compounds by cyanobacteria has been found to improve soil fertility (Acea et al., 2001; Chamizo et

al., 2018; Román et al., 2018) and act as stabilizing agents that glue soil particles together, thus contributing to the formation of soil aggregates and increasing soil stability (Mazor et al., 1996; Zulpa de Caire et al., 1997). The EPS released by cyanobacteria also improve water retention and promote the creation of a moist atmosphere that protects them from desiccation (Colica et al., 2014; Adessi et al., 2018), increasing their likelihood of survival. Thus, in highly degraded arid zones with active erosion, the capacity of cyanobacteria to produce EPS and organic compounds under differing hydration regimes should also be explored in choosing the best candidate for rapid restoration of soil functions.

To identify the cyanobacterial inoculant able to improve edaphic conditions under water stress, we studied the effect of the hydration regime on induced biocrust development and on key soil properties after inoculating soil with three native nitrogen-fixing cyanobacteria, *Nostoc commune*, *Scytonema hyalinum* and *Tolypothrix distorta*, alone and in a consortium. As it was expected to be unlikely for inoculum response to differing water regimes to be uniform across soil types, the experiment was conducted on three different soils from the province of Almeria (SE Spain) with contrasting development and physicochemical properties. More precisely, we analyzed the effect of a simulated hydration regime corresponding to a dry and wet rainfall year and soil type on i) inoculated cyanobacteria colonization, (ii) EPS production, and (iii) soil organic carbon (SOC) content, in the laboratory.

Materials and Methods

Cyanobacteria and soils sampling and inoculum preparation

Cyanobacterial biocrust samples were collected from three study areas in the province of Almería (Southeast Spain) with marked differences in soil development and psychochemical properties. These areas, from less to higher degradation stage, were: i) *Las Amoladeras*, within the Cabo de Gata-Níjar Natural Park (N 36° 50’ 01” W 02° 15’ 08), is a Mediterranean grassland ecosystem where grazing is the main land use. The soil type is classified as Calcaric Leptosols and soil texture is sandy loam (64.50% sand, 28.40% silt and 10.10% clay). Soil organic carbon and nitrogen in the upper 0-2 cm is 21.41 and 2.07 g Kg^{-1}, respectively, and the water holding capacity is 17.55%. ii) *El Cautivo* experimental area, within the Tabernas Desert (N 37° 00’ 37” W 02° 26’ 30”), is a very eroded badlands landscape mainly dominated by gypsum-calcareous mudstones and calcaric sandstones where hunting and sporadic filming are the human activities. The soil type is classified as Endoleptic or Calcaric Leptosols and soil texture is silty loam (29.20% sand, 58.60% silt and 12.20% clay). Soil organic carbon and nitrogen in the upper 0-2 cm is 3.87 and 0.57 g Kg^{-1}, respectively, and the water holding capacity is 22.50%. iii) a calcareous quarry in Sierra de Gádor (hereafter: *Gádor quarry*, W 36° 55’ 20’’ 02° 30’ 29’’ W) which is currently under exploitation.

Soils consisted of a mixture of previously stockpiled calcareous fragments and calcitic-gypsiferous mudstones extracted from within the quarry pit. Soil texture is loamy (31.20% sand, 43.10 silt and 25.70% clay). Soil organic carbon and nitrogen in the upper 0-2 cm is 0.24 and 0.17 g Kg^{-1}, respectively, and the water holding capacity is 27.03%. The mean annual rainfall and temperature in the selected areas ranges from 200 – 242 mm and 17.2 – 19 °C, respectively. A detailed description of the study areas and soil types can be found in Roncero-Ramos et al. (2019a). At the same study sites, we collected soils substrates to conduct the inoculation experiments. Before soil field collecting, the top crust of the soil (2 cm) was cleared with a palette knife. Soil substrates were then air-dried and sieved to 2 mm.

A total of twenty-five cyanobacteria strains were isolated and identified from cyanobacterial-biocrusts propagules using a polyphasic approach based in morphological, molecular and ecological data (Roncero-Ramos et al., 2019a). Due to their higher growth rate, we selected three heterocystous strains for the inoculation experiments: *Nostoc commune* (CANT2 UAM 817), *Scytonema hyalinum* (CAU6 UAM 820) and *Tolypothrix distorta* (CANT7 UAM 825). Each strain was cultured separately in Erlenmeyer flasks (250 mL) containing liquid $BG11_0$ medium (Rippka et al. 1979). Cultures were scaled-up to larger recipients (5 L) when biomass concentration reached 2 g L^{-1} in order to maximize biomass production. The cultures were subjected to 16:8-hour light/dark cycles with 60 μmol photons m^{-2} s^{-1} irradiance in a temperature-controlled room (28 ± 1°C). The reactors were continuously aerated with air sterilized by filtering (0.22 μm, Millex EMD Millipore™) with an air-pump to ensure adequate mixing. Cyanobacterial biomass was harvested in the exponential phase (2.5 g L^{-1}) by filtration, and the fresh biomass obtained was used for soil inoculation.

Experimental set up

Cyanobacteria inoculation was conducted in Petri dishes (90 mm diameter x 15 mm depth) filled with 80 g of each unsterilized soil. Before cyanobacterial inoculation, each sample was watered with 20 mL of distilled water in order to form a physical crust similar to that found in the field. Then, samples were inoculated with 40 mg of biomass diluted in 20 mL of distilled water (2 g L^{-1}), resulting in a biomass concentration of 6 g dry weight m^{-2}. Inoculum was distributed uniformly with a sterilized test tube along the entire surface of the Petri dish. An equivalent amount of distilled water was applied to control samples. Five inoculation treatments were applied on the three different soils: 1) Uninoculated soil "control", 2) *Nostoc commune*, 3) *Scytonema hyalinum*, 4) *Tolypothrix distorta* and 5) Consortium of the three strains in the same proportion (1:1:1).

Two hydration treatments were selected based on local rainfall records after calculating average rainfall in the period 1991-2009: a) dry regime, consisting of the simulation of a dry rainfall year (180 mm) and, b) wet regime, consisting of the simulation of a wet rainfall year (380 mm). For the

simulation of the dry regime, samples received a total of 10 irrigation events distributed over 90 days (every 8 days), whereas samples subjected to the wet regime received 21 irrigation events in the same period (every 4 days). In each irrigation event, the samples received 30 mL (4.5 mm) of distilled water and were allowed to dry naturally thereafter. We set four replicates of each treatment and soil type for a total of 120 samples (3 soil types x 5 inoculation treatments x 2 irrigation treatments x 4 repetitions). Inoculated soils were placed in a Fitotron Plant Growth Chamber (Air-Frio, Almeria, Spain) at a constant temperature (28 ± 1 ºC), light intensity of 60 μmol photons m^{-2} s^{-1} and 16-h photoperiod for 90 days.

Biocrust cover and spectral response measurements

To estimate cyanobacteria cover, a zenithal picture of each sample was acquired at the end of the study period using a digital CANON EOS 600D placed at nadir, 25 cm above the sample surface. Then, the cyanobacteria coverage (%) on the soil substrates was calculated by applying a supervised maximum likelihood classification (see Román et al., 2018 for deeper description). The image analysis was performed using ENVI 4.3 (ITT VIS, Boulder, CO, USA).

In addition, as soil surface becomes darker with the growth of the cyanobacteria, we measured surface reflectance of each sample as an indicator of inoculum establishment (Román et al., 2018). Surface reflectance was measured at the end of the study period with an ASD FieldSpec® HandHeld portable spectroradiometer (ASD Inc., Boulder, 212 Colorado, USA) with a 3.5 nm optical resolution from 325 nm to 1075 nm. Prior to the spectral measurements, all samples were irrigated with 30 mL of distilled water to enhance the spectral response of the surface components. The optical fibre was placed at 16 cm above the sample surface, resulting in a field-of-view (FOV) that matched with the total area of the Petri dish (66 cm^2). For each sample three replicate measurements were acquired, each consisting of the average of three individual spectra, and then averaged to produce a single surface spectrum per sample. A Spectralon white reference sample was also measured before each set of three biocrust spectra measurements. All spectra were subjected to a pre-treatment that consisted of removing the noisy bands (350 – 400 nm and above 950 nm) and applying a cubic polynomial smoothing filter with a 17 bands-window size (Savitzky & Golay, 1964). Using the smoothed spectra, we calculated the continuum removal by applying the convex hull fit over the top of a spectrum using straight-line segments that connect the local spectra maxima so that values equal to 1 indicate the absence of absorption peaks (the spectrum matches the convex curve), while values lower than 1 indicate the presence of absorption traits (CR; Clark and Roush, 1984). From the CR spectra we estimated the maximum absorption at ~680 nm (CR_{680}), which has been found to be strongly correlated with chlorophyll *a* content in artificial cyanobacteria biocrusts (Román et al., 2019) and can be also used as a surrogate variable of cyanobacterial biocrust development. To improve data visualization and interpretation, we transform the CR_{680} values following Eq.1, thereby

higher values represent deeper chlorophyll *a* spectral absorption (hereafter named "Chl*a* spectral absorption").

Eq.1 Chl*a* spectral absorption = $1- CR_{680}$

Soil analyses

At the end of the study, the uppermost layer of the samples (2 mm) was scraped with a palette knife and the collected sample was ground in a mortar and pestle for EPS and SOC determination. The total exopolysaccharides content (EPS) was determined as the sum of the two main fractions: loosely bound (LB-EPS) and tightly bound (TB-EPS) exopolysaccharides. LB-EPS were recovered from 0.2 g of homogenized samples using three consecutive extractions with 5 mL of distilled water, following the procedures described in Chamizo et al., 2019. TB-EPS were recovered from the resulting pellet following the same procedure but using 0.1M Na_2EDTA as solvent. The exopolysaccharides content of the extracts was determined using the phenol-sulfuric acid assay, measuring the absorbance at 488 nm with a UV-VIS spectrophotometer (Dubois et al., 1956). A calibration curve was created using glucose as standard. Procedure was performed in triplicate for each crust sample and concentration values were averaged to produce a single EPS content value per sample. Soil Organic Carbon (SOC) content was determined by wet oxidation using the Walkley and Black method modified by Mingorance et al. (2007). The reaction was carried out by adding 3 mL of potassium dichromate and 6 mL of sulfuric acid into small heat-resistant vials that contain 0.2 g of homogenized sample. The vials are then placed inside an insulator block and vortexed for 10 min, after which they are kept in the block for 3 hours in order to retard cooling of the vials and increase the fraction of organic carbon recovered. Then, the block is left to settle from 24 to 48 h to allow solid particle deposition. The amount of Cr^{3+} resulting from organic carbon oxidation is measured using molecular absorption spectrophotometry at 590 nm in the supernatant solution. The quantitative gain of EPS and SOC in each sample was calculated as the difference between the value of the variable for each sample at the end of the experiment and the initial substrate value.

Statistical analysis

For each soil type, the effects of the inoculation treatment (Uninoculated soil, *N. commune*, *S. hyalinum*, *T. distorta* and a consortium of the three strains), hydration regime (dry and wet) and their interaction on response variables were analyzed using a permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distances. Comparisons between means (when differences were found) were performed with the PERMANOVA t-*statistic* and using a maximum of 9999 permutations to obtain the pseudo-*F* and p-values ($p < 0.05$). Monte Carlo correction was applied when necessary. All the analyses were performed using Primer 7 and Permanova + (PRIMER-E Ltd, Plymouth, UK).

Results

Influence of water availability on biocrust growth

Cyanobacteria inoculation led to the development of a stable biocrust that was visually evident at the end of the study period in all soil types and hydration regimes, whereas uninoculated soils remained uncovered three months after inoculation (Figure 1). Cyanobacteria coverage of induced biocrusts at the end of the study period was significantly affected by hydration regime (Table 1), and this effect varied depending on the soil type and inoculation treatment. The samples subjected to the wet regime showed cyanobacterial coverage values 5-10% higher than those grown under the dry regime at Las Amoladeras and El Cautivo soils, although those differences were not statistically significant (Figure 2A and 2B). On the contrary, equally or higher colonization rates were observed in the Gádor quarry soils grown under dry conditions (Figure 2C). For instance, *T. distorta* grown under dry regime showed significant higher biocrust cover than the one subjected to the wet regime (21.72% vs 9.35%) (Figure 2C). Furthermore, the most developed soil, Las Amoladeras, showed the highest cyanobacteria cover for all inoculation treatments and hydration regimes at the end of the study period (Figure 2).

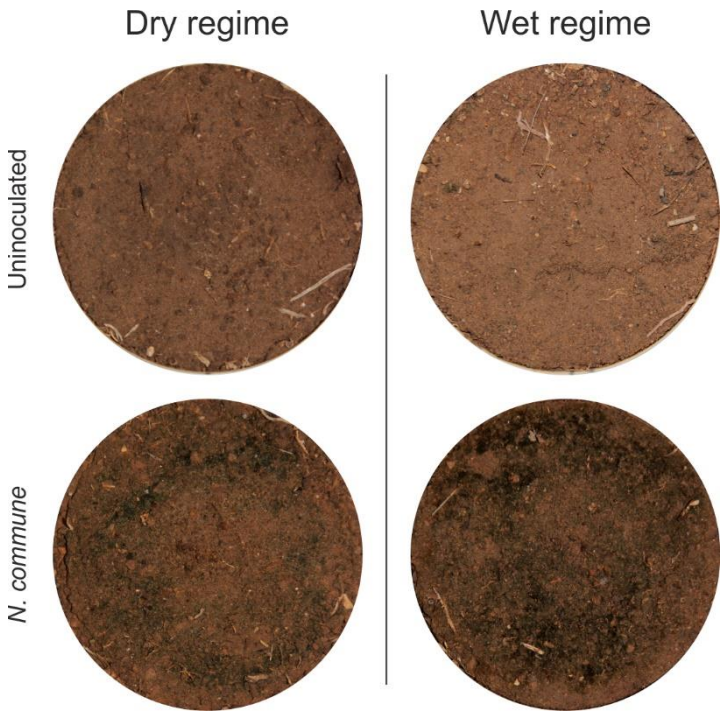

Figure 1. Pictures of an exemplary of uninoculated and Nostoc commune inoculated sample at Las Amoladeras for broth hydration regimes. Pictures were taken 90 days after inoculation.

Table 1. P values of the permutational multivariate analysis of variance performed to test the effect of the predictor factors on the studied variables. Predictor factors are represented by inoculation treatment (T), hydration regime (W) and soil (S) and the interaction of the three (T*W*S). Values in bold represent significance at 95% confidence interval or $p < 0.05$.

Predictor factors	Cyanobacteria cover	Chl*a* spectral absorption	EPS	SOC
T	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
W	**0.048**	**0.016**	**< 0.001**	0.170
S	**< 0.001**	**< 0.001**	**< 0.001**	0.161
T x W	0.282	**0.006**	**< 0.001**	0.439
T x S	**< 0.001**	**< 0.001**	**< 0.001**	0.179
W x S	0.057	**0.016**	**< 0.001**	0.602
T x W x S	0.276	0.185	**< 0.001**	0.825

A deeper analysis of the different inoculum reveals that the soils inoculated with *N. commune* had a greater cyanobacteria coverage than soils inoculated with any of the other inoculation treatments for both hydration regimes (Figure 2). The cyanobacterial colonization driven by this inoculum under the dry regime was even higher than that found in other inoculation treatments under the wet regime at Las Amoladeras and El Cautivo (Figure 2). For example, the soil inoculation with *N. commune* cultured under dry conditions in Las Amoladeras resulted in a cover 2.33%, 23.68% and 20.41% higher than those obtained after inoculation of the Consortium, *S. hyalinum* or *T. distorta* under wet conditions, respectively (Figure 2A).

Overall, as cyanobacteria coverage increased, there was a more accentuated spectral absorption in the red-edge region, showing a maximum in the CR curve at 680 nm due to the presence of a well-known chlorophyll- *a* absorption peak. In a similar way as cyanobacteria cover did, Chl*a* spectral absorption was affected by the hydration regime (Table 1), although this effect also depended on the soil and inoculum type (Table 1). At Las Amoladeras, the most developed soil, three of four inoculants showed deeper absorption peaks when grown under wet regime, albeit such differences were only significant in *N. commune* inoculum (Figure 2). On the other hand, the effect of hydration regime on Chl*a* spectral absorption showed mixed results at El Cautivo and Gádor quarry soils. For example, only the Consortium was positively affected by higher water availability at El Cautivo ($P<0.05$, Figure 2), whereas at Gádor quarry similar results were obtained.

Generally, the deepest Chl*a* spectral absorption values were found on *N. commune*- and Consortium-inoculated samples, which showed the best performance under all hydration regimes, following by *T. distorta*, *S. hyalinum* and uninoculated samples, respectively (Figure 2). Nevertheless, in the most degraded soil (Gádor quarry), the growth of *N. commune* showed deeper absorption peaks than other inoculum types (Figure 2). Remarkably, the Chl*a* spectral absorption driven by this inoculum

subjected to dry regime was even deeper than those obtained by the other inoculum cultured under wet conditions (Figure 2).

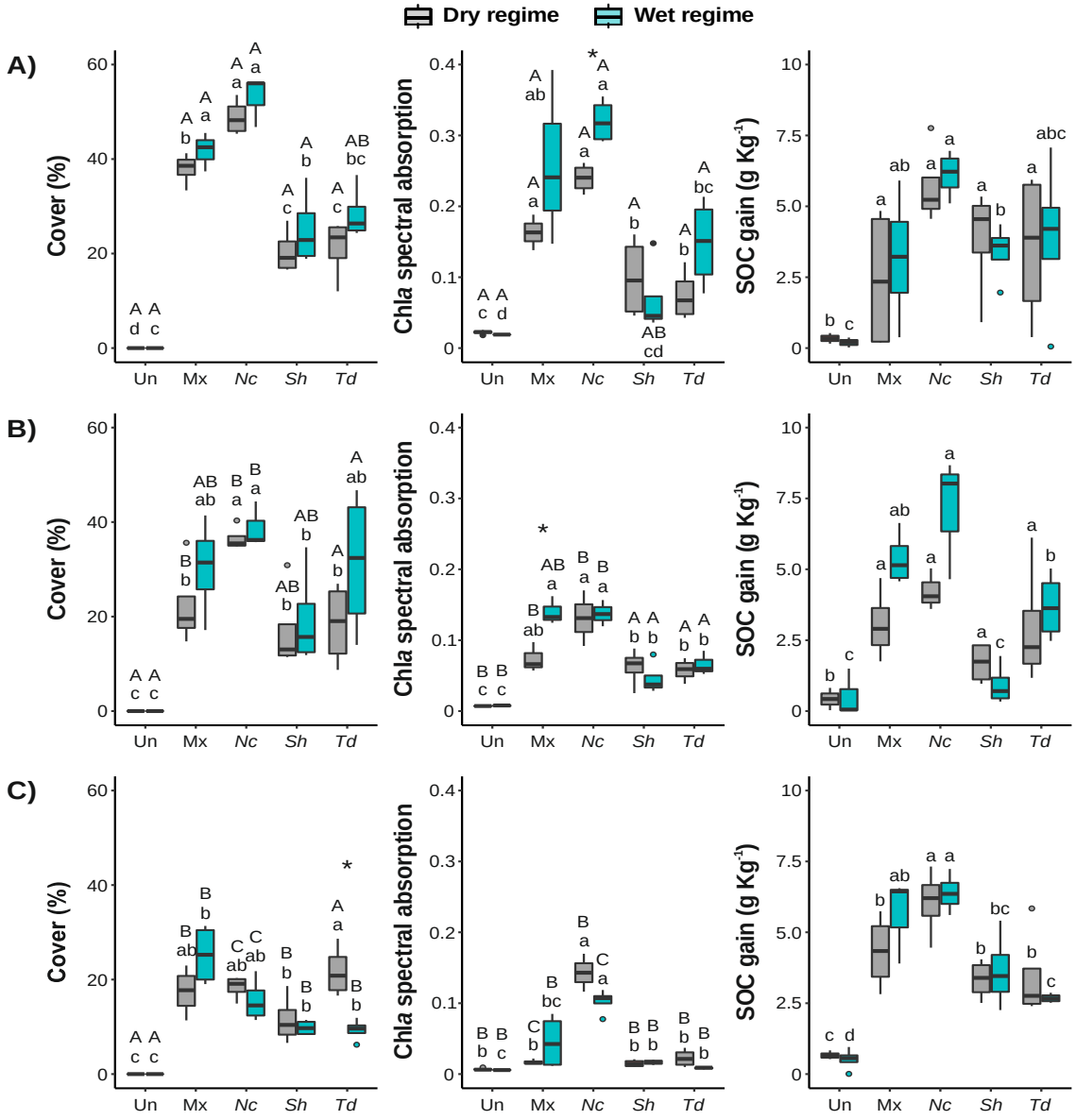

Figure 2. Mean (±SD, n = 4) cyanobacteria cover, Chl*a* spectral absorption and SOC gain of the five inoculation treatments in A) Las Amoladeras, B) El Cautivo, and C) Gádor quarry soils. Lower case letters mark significant differences among inoculation treatments within each hydration regime and soil ($p < 0.05$). Capital letters indicate significant differences ($p < 0.05$) between inoculation treatments across soils. The * marks significant differences between hydration regimes within each inoculation treatment and soil ($p < 0.05$). Un: Uninoculated, C: Consortium, Nc: *Nostoc commune*, Sh: *Scytonema hyalinum*, Td: *Tolypothrix distorta*.

Effect of hydration regime on exopolysaccharides and soil organic carbon production

The amount of EPS extracted from biocrusts was also affected by the hydration regime, soil type and inoculation treatment, as well as all the interaction among them. As observed in Table 1, PERMANOVA analyses revealed a general significant effect of the hydration regime on EPS

production, but similar EPS gains were observed under both hydration regimes for most of the inoculation treatments, except for *S. hyalinum* and *T. distorta* at El Cautivo and Gádor quarry, respectively, which showed significant higher EPS production under dry conditions ($P<0.05$, Figure 3B and 3C). EPS gains promoted by *N. commune* were much higher compared to other inoculation treatments. Those gains were especially remarkable at El Cautivo, where *N. commune* subjected to wet conditions produced almost 6-fold more EPS gains than the second most productive inoculum: the Consortium (Figure 3B). By contrast, small EPS gains were observed on *S. hyalinum*, *T. distorta* and uninoculated soils (Figure 3). For all treatments, EPS production occurred mainly in form of TB-EPS fraction at both Las Amoladeras and El Cautivo, whereas the lightly-bound fraction only represented a small fraction of EPS (Figure 3). In Gádor quarry soils, though, a great proportion (>50%) of LB- EPS were present in some of the most successful treatments, such as the Consortium and *N. commune* (Figure 3C). Moreover, when different soils were analyzed, we found that EPS gains were higher in Las Amoladeras soils than in the other study soils for all the inoculation treatments and hydration regimes, except for the huge EPS gains produced by *N. commune* at El Cautivo.

SOC gains were strongly correlated with EPS, with approximately 46-64% of the variation in SOC amounts explained by EPS content (R^2 = 0.54, 0.64 and 0.46 in Las Amoladeras, El Cautivo and Gádor quarry, respectively). However, contrary to EPS production, there was not an overall effect of hydration regime on SOC content (Table 1). Therefore, similar values of SOC gains were reached under dry and wet conditions in all treatments and soils (Figure 2). Thus, in a similar way to that observed on biocrust coverage and total EPS, the SOC gain was mostly controlled by the inoculation treatment ($p < 0.001$) in all soil types (Table 1). In addition, the soil type did not have a significant effect on SOC gains (Table 1). Regarding the effect of the inoculum treatment, soil inoculation with *N. commune* and the Consortium promoted higher SOC gains than the inoculation with *S. hyalinum* or *T. distorta* at the three study soils. The *N. commune* and Consortium inoculation promoted a net SOC gain of ~6 g Kg^{-1} soil in the three study soils. It is also noteworthy that the SOC content in the less fertile soil (Gádor quarry) inoculated with *N. commune* and cultured under the dry regime, increased by 25-fold compared to initial soil content during the 90-days incubation (Figure 2).

Discussion

Previous indoor studies have shown high rates of cyanobacteria colonization under daily irrigation with considerable amounts of water (≥ 80% of field capacity, Acea et al., 2003; Rozenstein et al., 2014) or equivalent to several times the average natural rainfall at origin (Antoninka et al., 2015; Ayuso et al., 2017). Our work confirmed that some cyanobacteria species can easily survive and colonize some soils under laboratory conditions with much less water and frequency, mimicking those found in the original field areas. Indeed, although the amount of water provided the samples

subjected to the dry regime (180 mm) was 20% less than the mean annual rainfall in the region (220 mm), it was still enough to achieve colonization rates, EPS and SOC gains similar to those cultured under wetter conditions (Figure 2 and 3).

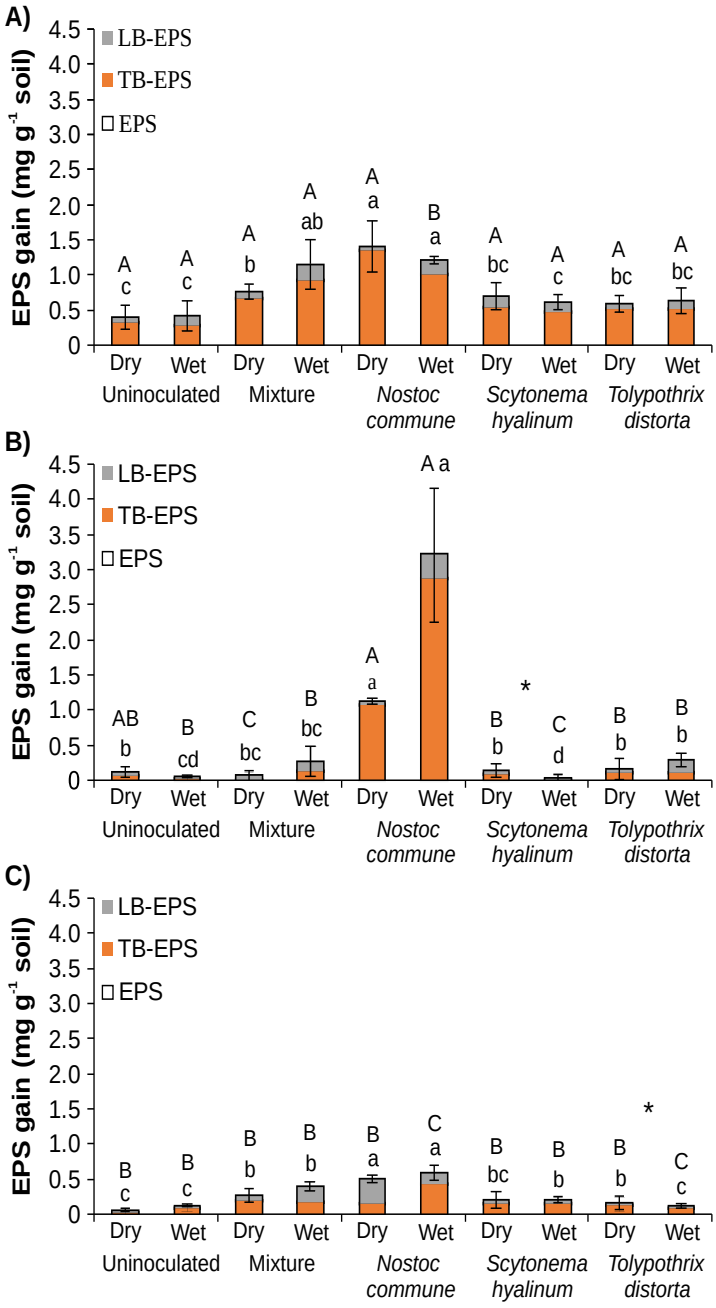

Figure 3. Mean (±SD, n = 4) total exopolysaccharides (EPS) of the five inoculation treatments in a) Las Amoladeras, B) El Cautivo, and C) Gádor quarry soils subjected to two hydration regimes (dry and wet). Grey indicates lightly-bound fraction of EPS and orange indicates tightly-bound fraction of EPS. Lower case letters mark significant differences among inoculation treatments within each hydration regime and soil ($p < 0.05$).

Biocrust growth did not respond clearly to increasing watering frequency under any of the four inoculation treatments, and although biocrust cover and Chl*a* spectral absorption were slightly improved in wet samples, those differences were statistically nonsignificant (Figure 2). This was especially noticeable in the Gádor quarry soils, where biocrust development was the same or greater in dry samples as wet. The lower sensitivity to water availability observed on inoculated Gádor quarry samples can be attributed to the higher water-holding capacity characterizing this soil than the soil at El Cautivo, and particularly, at Las Amoladeras (see Section 2.1). This would allow the water to remain in the soil for longer periods, thereby reducing the differences between hydration regimes. Previous studies, however, have identified water availability as a critical positive factor for the development of cyanobacteria-dominated biocrusts. For instance, Bu et al. (2014) found that watering more frequently (every five days) produced significantly higher biomass, while Ayuso et al. (2017) found that a watering regime equivalent to double the average natural rainfall at origin resulted in higher growth rates than the watering regime simulating the mean annual rainfall. The main difference between these studies and our experimental approach is the inoculum. Whereas we used isolated cyanobacteria species or a consortium of different species, Bu et al. (2014) and Ayuso et al., (2017) employed natural biocrust propagules containing not only cyanobacteria, but also algae, mosses and lichens that interacted with cyanobacteria growth and impeded identification of the effect of watering. Thus, at least for some cyanobacteria, increasing the water input might not be as important as previously reported, and other factors, such as instability, UV radiation, high temperatures and interaction with original soil biota could be more important stressors limiting inoculation success (Kubečková et al., 2003; Roncero-Ramos et al., 2019b). Our results also revealed that the different strains tested as inoculants showed differing capacities to colonize and promote biocrust development. Therefore, the focus on species selection may also significantly improve biocrust restoration outcomes. Overall, *N. commune* and the Consortium clearly showed a stronger capacity for soil colonization than the other inoculum types (i.e. *S. hyalinum* and *T. distorta*). It is worth mentioning that only *N. commune* was successful when cultured under the dry regime in all the soils tested. Indeed, soil colonization driven by *N. commune* under dry conditions was even higher than other inoculation treatments under the wet regime, covering up to 45% of the soil surface in just 90 days (Figure 2). These colonization rates are similar to the colonization two years after inoculating with *Microcoleus vaginatus* and *Scytonema javanicum* in a cool desert in China (Wang et al., 2009), and over the natural colonization by light-colored cyanobacteria eight months after disturbance (Dojani et al., 2011). These findings are consistent with those found by Antoninka et al. (2015) who also observed that indoor moss cultivation, mostly unexpectedly, triggered colonization by *Nostoc* rather than *Scytonema* and *Microcoleus vaginatus* even with the minimum weekly hydration period.

In addition, it remains unknown whether culturing in liquid media could affect the cyanobacterial ability to tolerate dry-wet cycles ("dehardening") as observed in moss (Stark et al., 2012), or whether

the extent of this potential handicap differs by species. As a plausible solution, progressive preacclimation of the cultures to the conditions expected in the target area to be restored has been recommended (Antoninka et al., 2018), and the first experiments with cyanobacteria showed positive fitness test results (Giraldo-Silva et al., 2019b). Our results, then, open a wide range of opportunities for application of conditioning in *N. commune* cultures for biocrust restoration in very arid environments.

Several studies have shown increased EPS content after cyanobacteria inoculation (Mugnai et al., 2018; Chamizo et al., 2018). In the present study, EPS gains were closely related to cyanobacteria cover and Chl*a* spectral absorption in all soils. Therefore, although the hydration regime had a generally significant effect on EPS production, similar EPS gains were observed in all inoculation treatments whether subjected to dry or wet regimes. Furthermore, when the different strains were compared, we found that EPS responded in a way similar to coverage. *Nostoc commune* was more able to synthesize EPS than the other strains tested (Figure 3), and especially when cultured under dry conditions. *N. commune* is a well-known desiccation-tolerant species which has developed multi-protection mechanisms to cope with sudden dehydration (Potts, 1999; Sand-Jensen & Jespersen, 2012). Its photosynthetic recovery after desiccation is closely associated with the amount of water stored during rewetting (Satoh et al., 2002). Uronic acids, very common in the *N. commune* EPS matrix (Helm et al., 2000), are highly hydrophilic, contributing to the highly water-absorptive nature of EPS in *N. commune,* and therefore, to photosynthetic stress tolerance during desiccation (Tamaru et al., 2005). Such survival strategies in *N. commune* could explain the underlying mechanisms that enable it to grow faster than other cyanobacteria taxa, even in water-restricted scenarios. SEM images also revealed an entangled network of cyanobacterial trichomes and EPS, which formed organic bridges binding soil particles together and leading to a stable organo-mineral layer (Figure 4). EPS bonds have been found to improve aggregate stability and soil penetration resistance in cyanobacteria-inoculated soils (Chamizo et al., 2018). Thus, EPS generation by *N. commune* not only ameliorates stress conditions, but also improves soil stability, a factor identified as a potential constraint for artificial biocrust formation (Kubečková et al., 2003). On the contrary, we found that the tightly-bound fraction of EPS controlled the total EPS in the less degraded soils from Las Amoladeras and El Cautivo, even under dry conditions. In these soils, the TB-EPS production was higher than LB-EPS, especially, in the most successful treatments (Consortium and *N. commune*). On the contrary, the lightly-bound fraction had a higher contribution in some treatments in the less fertile soil from the Gádor quarry (Figure 4). A plausible explanation is that the TB-EPS are more chemically complex and condensed than LB-EPS, and therefore, TB-EPS synthesis seems to be more affected by harsh conditions than LB-EPS. This phenomenon, which has previously been reported for cyanobacterial inoculation over highly depleted sandy soils (Mugnai et al., 2018), does not seem to be dependent on water availability, and other factors, like strong differences in edaphic conditions

across soils could account for the different behaviors found between species. However, more research is needed to elucidate this behavior under field conditions.

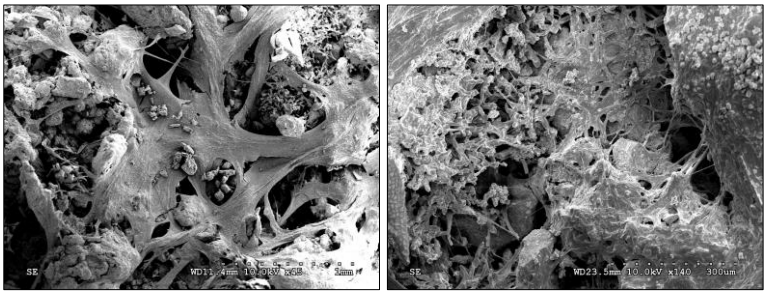

Figure 4. SEM images of Nostoc commune induced crust under the wet regime in Las Amoladeras soil.

Increased EPS content in treated soils was directly related to an increase in SOC gains in all soils. Again *N. commune* and the Consortium produced higher quantities of SOC than the other inoculation treatments under both hydration regimes (Figure 2). Especially relevant was the contribution of *N. commune* to SOC under the drier conditions in Las Amoladeras and Gádor quarry soils, which showed higher values compared to the other inoculation treatments even under wet conditions. Furthermore, the increase in SOC was more noteworthy in the Gádor quarry soil due to the much lower SOC content in that soil than in the El Cautivo or Las Amoladeras soils. For example, Nostoc inoculation on Gádor quarry soil increased SOC from 0.24 to 6.29 g Kg^{-1}, highlighting the ability of *N. commune* to increase SOC content of infertile substrates even under more limited water conditions. The soil organic carbon pool is a key indicator of soil quality as it affects essential biological, chemical and physical soil functions. In fact, soil organic carbon has been selected by the UNCCD as one of the three global land-based indicators for monitoring land degradation (Cowie et al., 2018). Therefore, improvement in the SOC pool found in the soils inoculated in our study could have important implications for soil water retention capacity, structure maintenance and fertility (Six et al., 2004; Abiven et al., 2009; Mager, 2010), thus helping to prevent soil degradation under water stress conditions.

Summarizing, our results revealed that an increase in the frequency of watering was not a crucial factor enhancing cyanobacterial growth or EPS and SOC production in any of the four inoculation treatments tested on three semiarid soils. However, some species responded better than others to a reduction in water availability. Of the inoculants used, *N. commune* showed the best capacity to colonize fine-textured and less fertile soils and improve crucial soil properties for soil stability and fertility, especially under water restrictive scenarios. It is also plausible that the good performance of the Consortium observed in this study may also have been at least partly attributable to the presence of *N. commune* in that inoculum blend. These findings make *N. commune* a valuable candidate as an inoculant for the artificial induction of biocrusts in such areas. Nonetheless, the good performance promoted by *N. commune* should not detract from the fact that the addition of the other heterocystous cyanobacteria, i.e. *Scytonema hyalinum* and *Tolypothrix distorta* in a consortium, would be likely to make for a more resilient inoculum. Although it has not been proved, the use of a

consortium may promote microbial interactions in the field that could help improve their ability to tolerate stressful conditions. For example, adding the warmth-adapted *Scytonema hyalinum* could enable higher temperature conditioning in areas where mean maximum temperatures rise above 30ºC in hot months (Muñoz-Martin et al., 2019). Moreover, using a consortium of species reduces the relative amount of biomass required of each, thereby reducing the time necessary to produce the biomass required for large-scale restoration. Nevertheless, according to our results, any future research exploring the use of a cyanobacterial consortium for restoring arid degraded soils should incorporate *N. commune* as an essential component.

Conclusions

This study confirms the feasibility of using native cyanobacteria inoculants to improve the conditions of soil subjected to hydration regimes similar to those found under natural conditions and on soils with differing physicochemical properties. Although the hydration regime had a generally significant effect on cyanobacteria cover, Chl*a* spectral absorption and EPS, similar colonization rates and improvement in soil edaphic conditions was achieved under both hydration regimes for all the treatments. Among the inoculated candidates, *N. commune* and the Consortium showed a stronger capacity for growth and modifying soil properties such as EPS and SOC contents than the other strains tested. *N. commune* promoted growth especially well under water-restrictive scenarios, highlighting its potential for use as an inoculant for soil restoration in arid environments.

Acknowledgements

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Chapter III:

Field inoculation of an indigenous cyanobacterial consortium for biocrust rehabilitation

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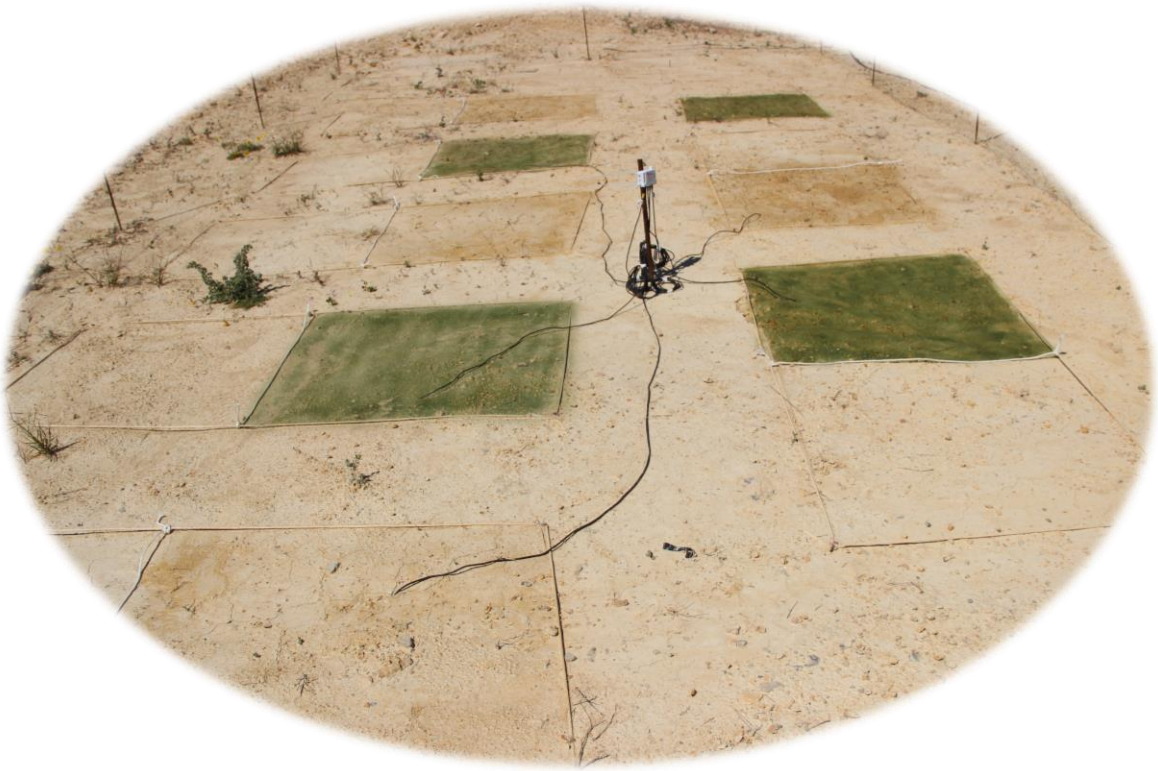

Abstract

Indoor studies have shown the feasibility of using cyanobacterial inoculation for promoting rapid biocrust formation and improving fertility in degraded arid soils. However, this technology has only been successfully employed in desert sand dunes in China. Therefore, further development of this technique is necessary to extend its applicability to other soil types and regions. In this study, we inoculated a consortium of three N-fixing native cyanobacteria (*Nostoc commune*, *Scytonema hyalinum* and *Tolypothrix distorta*) at three semiarid study sites in south-eastern Spain with differing soil properties and soil development. After two years, chlorophyll *a* spectral absorption and albedo in inoculated and control plots were similar. Consequently, a second experiment was conducted to test the effectiveness of progressive preacclimation of the cyanobacterial cultures to drought before inoculation and covering the inoculated soils with a vegetal fiber mesh or a plastic grid. Our results showed that: 1) hardening these cyanobacterial strains did not enhance their colonization capability, and 2) covering inoculated soils with a vegetal mesh did promote soil colonization by cyanobacteria, as shown by higher chlorophyll *a* soil content, Chl*a* spectral absorption and albedo than in the uncovered plots. Moreover, it promoted the presence of more condensed, tightly-bound EPS and higher MW molecules in loosely-bound EPS, which is related to soil aggregation. Finally, large quantities of xylose and galactose were also found in this treatment, indicating a more advanced stage of development of the induced biocrusts. The results of this study show that direct soil inoculation with cyanobacteria, whether after desiccation hardening or not, does not promote the artificial formation of biocrusts in the field. However, results were positive, although only slightly so, when inoculated soils were covered with a vegetal mesh to help cyanobacteria cope with abiotic stress and soil erosion. Therefore, more effort should be given to developing diversified efficient habitat amelioration strategies for applying this restoration technique in the field.

Keywords: N-fixing cyanobacteria, restoration, hardening, habitat amelioration, drylands

Introduction

Drylands, the largest biome on Earth, are being subjected to an alarming unprecedented pressure from human activities and climate change, which could potentially affect more than one third of the world's population and increase the ecosystem's vulnerability to desertification (Bestelmeyer et al., 2015). In these systems, human alteration of natural habitats has led to loss of biodiversity, altered hydrological cycles, soil erosion, and consequently, the loss of key ecosystem services, negatively affecting human well-being (MEA, 2005). Today, awareness of dryland degradation has given rise to international and regional policy initiatives for developing restoration frameworks to re-establish endangered ecosystem functions (UN, 2015). However, dryland restoration must deal with important constraints (Suding et al., 2004), such as poorly developed and infertile soils highly susceptible to

erosion, as well as low precipitation and high evapotranspiration rates (Noy-Meir, 1973). Therefore, traditional restoration approaches, which mainly focus on the rehabilitation of functional plant communities, are often unsuccessful, and other cost-effective restoration strategies specific to drylands are necessary.

Recently, the use of biocrust-forming cyanobacteria for counteracting soil degradation from intensified desertification in drylands has received a great deal of attention (Zhao et al., 2016, Rossi et al., 2017). Cyanobacteria, due to their capacity to cope with drought (Rajeev et al., 2013), high temperature (Lan et al., 2015) and prolonged UV radiation (Castenholz and Garcia-Pichel, 2013), are pioneer colonizers of poor, highly-degraded dryland soils. As key players in biocrust development, they provide multiple ecological benefits to the soils they inhabit (Román et al., 2018). Many of these benefits are related to their ability to synthetize and release large amounts of exopolysaccharides (EPS), thereby binding soil particles and contributing to increasing soil stability (Chamizo et al., 2018; Mazor et al., 1996) and reducing soil erosion (Chamizo et al., 2017). Indeed, EPS synthesized by cyanobacteria are essential in the formation of the extracellular polymeric matrix which has a fundamental protective and structural role contributing to initial biocrust formation (Rossi and De Philippis, 2015).

Cyanobacteria can be isolated from biocrust samples and cultured ex-situ in photobioreactors to produce large quantities of inoculum at a reasonable cost (Roncero-Ramos et al., 2019), enabling large-scale restoration projects to be undertaken. Their inoculation has been successfully applied in many laboratory studies, employing burned soils (Chamizo et al., 2020), mining substrates (Muñoz-Rojas et al., 2018) or agricultural soils (Maqubela et al., 2009). Our team recently demonstrated that the inoculation of a consortium of native N-fixing cyanobacteria improved several key soil properties in dryland soils differing in their state of degradation (Román et al., 2018), encouraging another step forward in testing this technology in the field. However, establishment of these laboratory-cultivated organisms in the field can be challenging. So far, successful field restoration by means of cyanobacterial inoculation has only been carried out on previously stabilized sand dune areas in China (Chen et al., 2006; Park et al., 2017; Wang et al., 2009; Xie et al., 2007). However, several field tests in deserts in the western USA using direct cyanobacterial inoculation (Faist et al., 2020) or hemp-cloth immobilized cyanobacteria (Kubeckova et al., 2003) were unsuccessful. It has been hypothesized that this lack of success could be attributed to a loss of cyanobacterial viability after inoculation, mainly due to UV radiation or photo-oxidation (Kubeckova et al., 2003). Following this hypothesis, two main approaches for improving establishment of inoculated cyanobacteria in the field have recently been put forward. The first consists of progressive preacclimation of the inoculum to the expected field conditions (hardening). This procedure was tested outdoors by Giraldo-Silva et al. (2019a), who found improved performance of most of the cyanobacterial species tested, especially the early-successional *Microcoleus* spp. The second approach consists of the application of habitat

amelioration treatments to reduce the abiotic stress of the inoculum after inoculation (e.g., providing shade). This technology has been applied in the field to enhance the establishment of nursery-grown biocrust communities including cyanobacteria, lichens and mosses, showing promising results (Antoninka et al., 2019; Bowker et al., 2019), but its application in laboratory cultivated cyanobacteria is yet to be tackled. Thus, in order to exploit this biotechnological tool in large-scale restoration, facilitating strategies for inoculated cyanobacteria colonization and establishment under natural conditions are necessary.

The purpose of this study was therefore to:

1) Test the feasibility of inoculating a consortium of three native N-fixing cyanobacteria for restoring soil biocrust and enhancing soil conditions in three semiarid ecosystems in the province of Almería (SE Spain) with different soil properties and subjected to different degradation degrees.
2) Analyse the effectiveness of cyanobacterial hardening and habitat amelioration in the establishment and development of artificially induced biocrusts.

As abiotic stresses may hinder induced biocrust development, we hypothesized that cyanobacterial hardening and habitat amelioration would improve cyanobacterial establishment, and thereby, soil conditions.

Materials and Methods

Study sites

The experiments were carried out at three sites located in the province of Almería (South-eastern Spain). The field sites were chosen due to the marked differences in soil development, and soil chemical and physical properties (Table 1). From lower to higher degradation degree these areas were: 1) *Las Amoladeras*, located in the Cabo de Gata-Níjar Natural Park (36°50′01″N, 02°15′08″W), is a dissected caliche where approximately one third of the soil surface is covered by dispersed shrubs, primarily *Macrochloa tenacissima* L. Kunth, being the rest of the surface covered by biocrusts at different successional stages (30%), calcaric outcrops and stones (Chamizo et al., 2012). 2) *El Cautivo*, is a badlands area located in the Tabernas desert (37°00′37″N, 02°26′30″W), where the parent material consists of gypsum-calcareous mudstones and calcaric sandstones. The area is subjected to intense water erosion processes (Cantón et al., 2003). Soil surface is covered by a mixture of discontinuous perennial plants, some annuals and abundant physical crusts (30% of the interplant soil) and biocrusts (50% of the interplant soil). 3) *Gádor limestone quarry experimental area* (hereafter named: "Gádor quarry"), is an active limestone quarry located at the south-eastern edge of the Gádor massif (36°55′20″N, 02°30′29″W). The hillslope was formed by a mixed substrate

as a result of mining activities and consists of calcareous rock fragments and calcitic-gypsiferous mudstones (Luna et al., 2018). The surface is covered by abundant physical soil crusts and incipient biocrusts.

Table 1. Mean annual temperature (T), precipitation (PP), main use of soil, soil type, soil texture, pH, electrical conductivity (EC, mS cm^{-1}), soil organic carbon (SOC, g Kg^{-1}), total nitrogen (TN, g Kg^{-1}) and water holding capacity (WHC) of the three study sites: Las Amoladeras, El Cautivo and Gádor quarry.

Study sites	T (°C)	PP (mm)	Main use	Soil type	Soil texture			pH	EC	SOC	TN	WHC (%)
					Sand (%)	Silt (%)	Clay (%)					
Las Amoladeras	19	200	Grazing	Calcaric Leptosols or Haplic Calcisols	61.50 ± 5.10	28.40 ± 4.20	10.10 ± 2.10	8.03 ± 0.04	0.16 ± 0.01	21.41 ± 0.96	2.07 ± 0.11	17.55 ± 0.30
El Cautivo	19	235	Research, hunting	Endoleptic or Calcaric Regosols and Eutric Gypsisols	29.20 ± 5.40	58.60 ± 5.80	12.20 ± 4.20	8.28 ± 0.12	0.13 ± 0.01	3.87 ± 0.09	0.57 ± 0.04	22.50 ± 0.19
Gádor quarry	17.6	242	Mining	Endoleptic Leptosols, Epileptic Leptosols and Calcaric Regosols	31.20 ± 4.65	43.10 ± 2.34	25.70 ± 2.80	8.57 ± 0.03	1.98 ± 0.18	0.24 ± 0.21	0.17 ± 0.09	27.03 ± 0.17

Cyanobacteria isolation and production

For soil inoculation, we selected three filamentous nitrogen-fixing cyanobacterial strains which had been previously isolated and identified from these study sites (Roncero-Ramos et al., 2019): *Nostoc commune* (CANT2 UAM 817), *Scytonema hyalinum* (CAU6 UAM 820) and *Tolypothrix distorta* (CANT7 UAM 825). Besides, these strains had also been tested in the same soil types under laboratory conditions showing a high capacity for fast colonization and improvement of soil conditions (see Román et al., 2018). The selected strains were cultured separately in individual flasks (500 mL) containing liquid BG11 medium (Rippka et al., 1979), and then scaled-up to larger flasks (10 L). The cultures were aerated with an air-pump and subjected to 16:8-hour light/dark cycles with 70 μmol photons m^{-2} s^{-1} irradiance at 25 ± 1°C. The final scaling-up stage consisted of inoculating 20% of the cultured biomass of each strain separately on 100L methacrylate bubble-column shaped photobioreactors, at Las Palmerillas Experimental Station. To reduce the overall cost of the biomass production, we employed the Mann and Myers medium (Mann and Myers 1968) made with fertilizers instead of chemicals (Roncero-Ramos et al., 2019). Cultures were continuously aerated by on-demand air inlet to ensure mixing. Cyanobacterial biomass was harvested in the exponential phase

(2.5 g dry weight L^{-1}) by centrifugation and the obtained fresh biomass was resuspended in distilled water for soil inoculation.

Cyanobacterial hardening

We inoculated each strain separately on open trays (54 x 40 cm) filled with a thin layer (0.2 cm) of each soil, which were previously ground to produce a fine powder (Fig. 1). Each strain was inoculated on the soil surface at a concentration of 6 g dry weight m^{-2} using a sprayer. In order to promote the progressive acclimation of the inoculants, the samples were incubated at 28 ± 1°C under 16:8-hour light/dark cycles irradiance and submitted to dry-wet cycles. It included an initial step where cyanobacteria were irrigated every 4 days for 3 months, following by another 3-months period where they received water every 8 days. After this treatment, the conditioned biomass was allowed to completely dry for one month before field inoculation.

Experiment 1: Direct cyanobacterial inoculation

The inoculation of non-hardened cyanobacterial biomass on soils (hereafter named: "Direct cyanobacteria inoculation") was carried out on April 2017 at the three study sites. At each location, 8 experimental 1 m^2 plots (1m x 1m) were delimited over bare soils (4 to be used as controls and 4 to be inoculated). Prior to soil inoculation, plots arrangement included removal of small herbs and small patches of incipient biocrusts that were scraped off to avoid their possible effect over the results found. Plots were also irrigated with distilled water to favour soil sealing and minimize particle displacement by water and wind erosion. Once soils were dried, a mixture of the three species with all in the same proportion (1:1:1) was uniformly inoculated with a sprayer at a biomass concentration of 6 g dry weight m^{-2} (Fig. 1). Each plot received a total quantity of 750 mL of inoculum at a concentration of 8 g L^{-1}. Non-inoculated plots received an equal quantity of distilled water. The optimal inoculum volume and concentration was set according to previous laboratory (Román et al., 2018) and field tests.

Experiment 2: Cyanobacterial hardening and habitat amelioration

This experiment was conducted at El Cautivo experimental site. We delimited experimental plots (0.25 m x 0.25 m) on December 2017 to test three inoculation treatments: non-inoculated (control), direct (CY) and hardened (HCY) cyanobacterial inoculation. In addition, after each inoculation, three habitat amelioration treatments were tested: uncovered, covered with a commercial plastic grid (mesh size: 5 mm, hereafter named: "plastic grid") and covered with a vegetal fiber mesh (mesh size: 2 mm, hereafter named: "vegetal mesh"). The plastic grid and the vegetal mesh were placed over the soil surface immediately after soil inoculation. Four replicates of each treatment were set for a total of 36 plots (3 inoculation treatments x 3 habitat amelioration x 4 replicates). Direct cyanobacteria

inoculation (CY) was performed following the same procedure than in Experiment 1. For the hardened cyanobacteria inoculation (HCY), the content of each tray containing single acclimated cyanobacteria inoculum was crumbled to produce small crust pieces (~ 0.2 cm diameter) and a mixture of the three species in the same proportion (1:1:1) was obtained. The inoculum was then sprinkled onto the soil surface at the same biomass concentration (6 g dry weight m^{-2}).

Soil measurements

Small soil cores of the soil surface were collected (0.5 cm depth x 1.6 cm diameter) on each plot to perform soil analyses. Before the analyses, samples were dried, crushed with a roller and ground with a mechanical agate mortar. Then, chlorophyll *a* content and soil organic carbon (SOC) were determined at different time spans.

To monitor biocrust growth, chlorophyll *a* content was determined at 10 days, 1 month, 3 months and 6 months after inoculation for the Experiment 1, and only at the end of the study period (6 months) for Experiment 2. Besides, as a significant correlation was found between chlorophyll *a* content and the spectral absorption at 680 nm due to the presence of this pigment (Román et al., 2019), biocrust development in Experiment 2 was assessed at the end of the study period (2 years) through spectral measurements (see section 2.7). The chlorophyll *a* content was determined by following the double extraction method with ethanol developed by Castle et al. (2011). 1 g of soil was suspended in 5 ml of ethanol, vortexed, heated at 80°C for 5 min and then incubated at 4°C for 30 min. Subsequently, samples were clarified by centrifugation at 4,000 g for 10 min and the supernatant was recovered. This procedure was applied twice to each sample. The absorbance at 665 and 750 nm was recorded with a spectrophotometer (Helios Zeta UVVIS, Thermo, UK), and chlorophyll *a* concentration was then calculated by applying the ethanol solvent equations reported by Ritchie (2006), considering the crust area (m^2) that was treated:

$$Chlorophyll\ a\ (mg\ m^{-2}) = \frac{11.9035 \times A(665 - 750) \times V}{sampling\ area(m^2) \times L}$$

where *A* is the absorbance value at the specific wavelength, *V* is the volume of the extract and *L* is the optical path length of the spectrophotometer cuvette.

At the end of the Experiment 1 (2 years), soil organic carbon (SOC) content was determined by wet oxidation using the Walkley and Black method modified by Mingorance et al. (2007). Briefly, 0.1 g of soil was mixed with 6 mL of $K_2Cr_2O_7$ 1N and 6 mL of H_2SO_4, then vortexed for 10 min and let stand for 3 hours, after which 10 mL of distilled water were added. After 48 hours, the resulting amount of Cr^{3+} from organic carbon oxidation in the supernatant solution was measured using absorption spectrophotometry at 590 nm. A calibration curve was built using sucrose standard solutions with increasing concentrations.

As a step forward, in order to understand the possible influence of the cyanobacteria-induced EPS matrix on biocrust performance, we conducted the quantification and characterization of EPS in natural cyanobacterial biocrusts, including two biocrust developmental stages (early cyanobacteria and well-developed cyanobacteria) and in our experimental treatments (Experiment 2), including the control and direct cyanobacteria inoculation treatments and the habitat amelioration treatment that provided better results in terms of biocrust growth. First, we measured the total amount of two differentiated soil EPS fractions: the loosely-bound EPS (LB-EPS), corresponding to the more soluble and less condensed EPS fraction, and the tightly-bound EPS (TB-EPS), corresponding to the more condensed EPS fraction. LB-EPS were recovered from 1 g of homogenized samples using three consecutive extractions with 5 mL of distilled water, following the procedures described in Chamizo et al. (2019) and Rossi et al. (2018). TB-EPS were recovered from the resulting pellet following the same procedure but using 0.1M Na_2EDTA extractant. The EPS content of the extracts was determined using the phenol-sulfuric acid assay, measuring the absorbance at 488 nm with a UV-VIS spectrophotometer (Dubois et al., 1956). A calibration curve was done using glucose as standard.

In addition, the monosaccharidic composition and molecular weight distribution of the two EPS fractions was performed. Apparent molecular weight (MW) distribution of LB-EPSs and TB-EPSs was determined by size exclusion chromatography (SEC). First, the excess of Na_2EDTA used for TB-EPS extraction was removed through dialysis with nitrocellulose tubular membranes (14 kDa MW cutoff, Medicell International, UK) followed by drying in a rotary evaporator. The dried extracts were recovered with 1 mL of deionized water and then analysed in a Varian Pro-Star liquid chromatograph (Varian Inc., USA). The chromatograph was equipped with a refractive index (RI) detector and two columns, PolySep-GFC-P 6000 and 4000 (Phenomenex, USA) with 700 mm length and 7.8 mm internal diameter and separation ranges of 100 kDa to 15 MDa and 0.3 to 400 kDa, respectively, both connected in series. The system operated at a temperature of 35ºC and using HPLC-grade water as eluent with a flow rate of 0.4 mL min-1. Dextran (Sigma-Aldrich, USA) 2000, 1100, 410, 150 and 50 kDa, and sucrose (0.36 kDa) were used as MW standards.

For monosaccharidic composition determination, the extracts of both LB-EPSs and TB-EPSs were first hydrolysed by mixing 1 mL of the extract with 4 N trifluoroacetic acid (TFA) and then heating at 120°C for 120 min. After this, the excess of TFA was removed by drying on a rotary evaporator and the dried extracts were re-solubilized in deionized water. Then, monosaccharide composition of the two EPS fractions was determined using a Dionex ICS-2500 ion exchange chromatograph (IEC, Dionex, USA) equipped with an ED-50 detector with a gold-working electrode and with a CarboPac PA1 column of 250-mm length and 4.6-mm internal diameter (Dionex). HPLC-grade water (A), 0.185 M Na hydroxide (B), and 0.488 M Na acetate (C) were used as eluents and the selected flow rate was 1 mL min^{-1}. The non-isocratic composition of the mobile phase was in accordance with

Mugnai et al. (2018). Peaks for each sugar were identified on the basis of the retention time of known standards.

Spectral response measurements

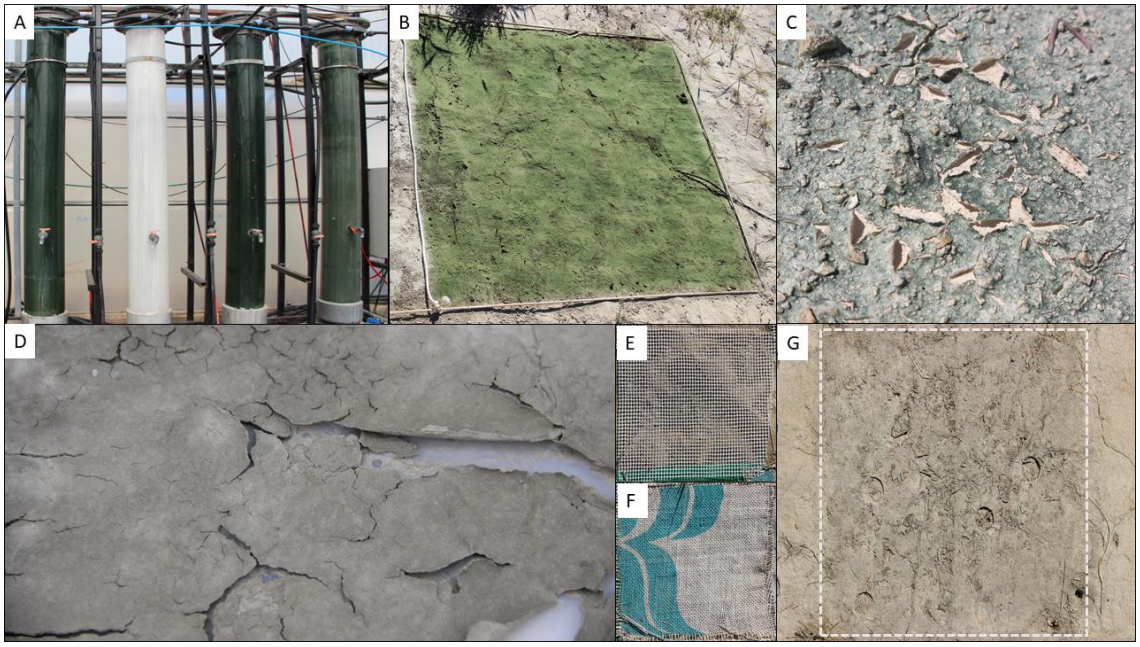

Figure 1. A) A view of the bubble-column photobioreactors (100 L) inoculated with the cyanobacterial strains. B) Inoculated plot with the cyanobacterial consortium at El Cautivo (Experiment 1). C) A close view of the induced biocrust flakes structures. D) A close view of the hardened cyanobacteria in the lab. E) Plots of the Experiment 2 covered with the plastic grid. F) Plots of the Experiment 2 covered with the vegetal fiber mesh. G) A close view of the cyanobacterial biocrust developed below the vegetal mesh 6 months after inoculation.

At the end of both experiments (2 years for Experiment 1 and 6 months for Experiment 2), the surface reflectance of each plot was measured using an ASD FieldSpec® Hand Held portable spectroradiometer (ASD Inc., Boulder, 212 Colorado, USA) with a 3.5 nm optical resolution from 325 nm to 1075 nm. Samples were irrigated with distilled water before data acquisition to enhance the spectral response of the surface components. For each plot five replicate measurements were acquired at different positions, each consisting of the average of three individual spectra, and then averaged to produce a single surface spectrum per plot. A Spectralon white reference sample was also measured before each set of three plot spectra measurements. Then, noisy bands (350 – 400 nm and above 950 nm) were removed and a cubic polynomial filter with a 17-band window size was applied (Savitzky and Golay, 1964). Afterwards, the spectral absorption of the chlorophyll *a* (Chl*a* spectral absorption) was calculated by applying the continuum removal procedure (CR; Clark and Roush, 1984). From the CR spectra we estimated the maximum absorption at ~680 nm (CR_{680}), which has been found to be strongly correlated with chlorophyll *a* content in artificial cyanobacterial biocrusts (Román et al., 2019). Chl*a* spectral absorption values were transformed to improve data visualization as follow:

Eq. 1 Chl*a* spectral absorption = $1 - CR_{680}$

thereby higher values of the variable represent deeper chlorophyll *a* spectral absorption. The visible surface albedo (from 400 to 700 nm), a variable that is strongly correlated with artificial cyanobacterial biocrust development (Román et al., 2018), was also calculated using the preprocessed spectra as the mean of the reflectance at each wavelength.

Statistical analyses

We applied a factorial MANOVA analysis for both experiments using R VERSION 3.3.2 (R-project). For the Experiment 1, we first analysed the effect of time, inoculation treatment and soil type on chlorophyll *a* content. Afterwards, we analysed the effect of inoculation treatment and soil type on chlorophyll *a* content, Chl*a* spectral absorption, albedo and SOC at the end of the study period (2 years). For the Experiment 2, we analysed the effect of inoculation treatment and habitat amelioration on chlorophyll *a* content, Chl*a* spectral absorption and albedo at the end of the study period (6 months). All variables were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene's test. Data were log transformed before performing parametric analyses when necessary. Comparisons among means (when differences were found) were performed with the Fisher's Least Significant Difference test.

Results

Experiment 1: Direct cyanobacterial inoculation results

The direct soil inoculation with cyanobacteria led to a visually evident artificial biocrust the day immediately after inoculation (Fig. 1B). At day 1, most of the available plot area was greenish at the three study areas, probably due to the presence of cyanobacterial filaments. Although cyanobacterial paths were still visible 10 days after inoculation, the first signals of soil aggregation appeared, after cyanobacterial filaments started forming flakes with soil particles that produced discontinuous

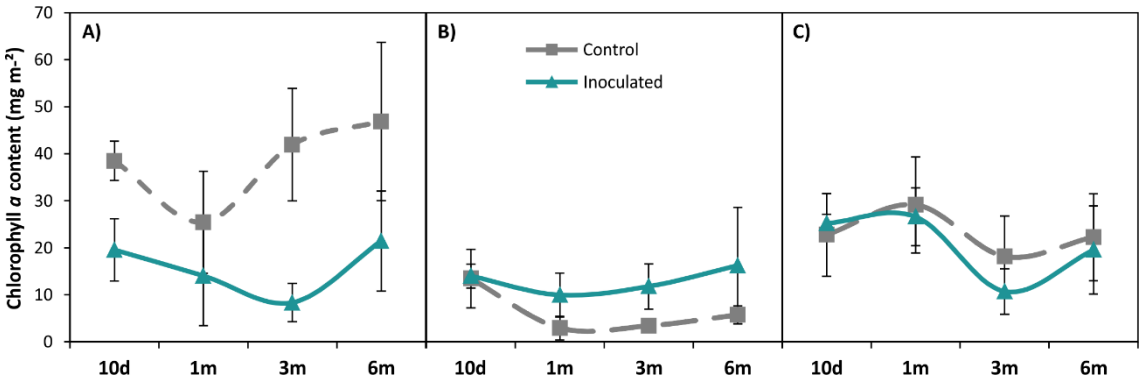

Figure 2. Chlorophyll *a* content (mg m^{-2}) mean (±SE, n=4) of the inoculated (green) and non-inoculated (grey) plots at Las Amoladeras (A), El Cautivo (B) and Gádor quarry experimental areas (C) at different time spans.

"cracks" of the artificial biocrust. This cracking effect increased with time, and a great portion of the artificial biocrusts disappeared towards the end of the study period, after wind and runoff events (Fig. 1C).

Direct soil inoculation with cyanobacteria had no effect on chlorophyll *a* content, as no differences were found between inoculated and control plots at none of the sampling times and study areas (Fig. 2). However, although non-significant, there was a slight decline in chlorophyll *a* content after 1 and 3 months in both treatments, coinciding with the summer period. Such content increased at month 6 in both inoculated and control soils in the three study areas due to soil recolonization by early successional-cyanobacteria following the first rainfalls after summer (Fig. 2). Soil type had a significant influence on chlorophyll *a* content ($P<0.05$) in the control plots. At Las Amoladeras, they had a significantly higher chlorophyll *a* content than at El Cautivo and the Gádor quarry. However, no significant differences were found among inoculated plots in the three sites.

Table 2. P values of the two-way ANOVA performed to test the effect of the predictor factors on the studied variables. Predictor factors are represented by inoculation treatment (T), soil (S) and the interaction of both (T*S). Values in bold represent the significance at 95% confidence interval or $p < 0.05$**.**

Predictor factors	Chlorophyll *a*	Chl*a* spectral absorption	Albedo	SOC
T	0.522	0.514	0.636	< **0.001**
S	0.136	**0.022**	< **0.001**	< **0.001**
T * S	0.281	0.877	0.396	0.248

Table 3. P values of the two-way ANOVA performed to test the effect of the predictor factors on the studied variables. Predictor factors are represented by conditioning treatment (CT), habitat amelioration (A) and the interaction of both (CT*A). Values in bold represent the significance at 95% confidence interval or $p < 0.05$.

Predictor factors	Chlorophyll *a*	Chl*a* spectral absorption	Albedo	SOC
CT	**0.021**	< **0.001**	0.863	0.211
A	**0.015**	< **0.001**	0.068	0.057
CT x A	0.429	< **0.001**	0.147	0.107

Measurements of the spectral response after 2 years showed no statistically significant differences in albedo and Chl*a* spectral absorption between inoculated and control plots (Fig. 3). However, contrary to chlorophyll *a* content and spectral response, cyanobacterial inoculation promoted a significant SOC increase in comparison to control plots at Las Amoladeras and El Cautivo 2 years

after inoculation (Fig. 3, Table 2). It increased 35% (13.40 vs 9.95 g Kg^{-1}) at Las Amoladeras and 42% (7.76 vs 5.44 g Kg^{-1}) at El Cautivo two years after inoculation compared to control plots (Fig. 3).

Experiment 2: Cyanobacterial hardening and habitat amelioration

Cyanobacterial hardening did not induce a significant improvement in biocrust formation compared to the direct soil inoculation with cyanobacteria, as the chlorophyll *a* content found in both uncovered CY and HCY plots 6 months after inoculation were similar (Fig. 4A). However, covering the plots surfaces with the plastic grid and the vegetal mesh exerted a protective effect on the cyanobacterial inoculum with time. Six months after inoculation, the artificial biocrust was visible in most of the covered plots, whereas it mostly disappeared in the non-covered plots. Remarkably, although the induced biocrusts remained until the end of the study, discontinuous "cracks" were also present, even after covering the plots (Fig. 1C).

The habitat amelioration had a largely positive effect on chlorophyll *a* content compared to uncovered plots, either after inoculating with CY or HCY (Table 3). All inoculated plots showed a significantly higher chlorophyll *a* content when covered with the plastic grid compared to control plots. The vegetal mesh had a significant positive effect only on CY inoculated plots

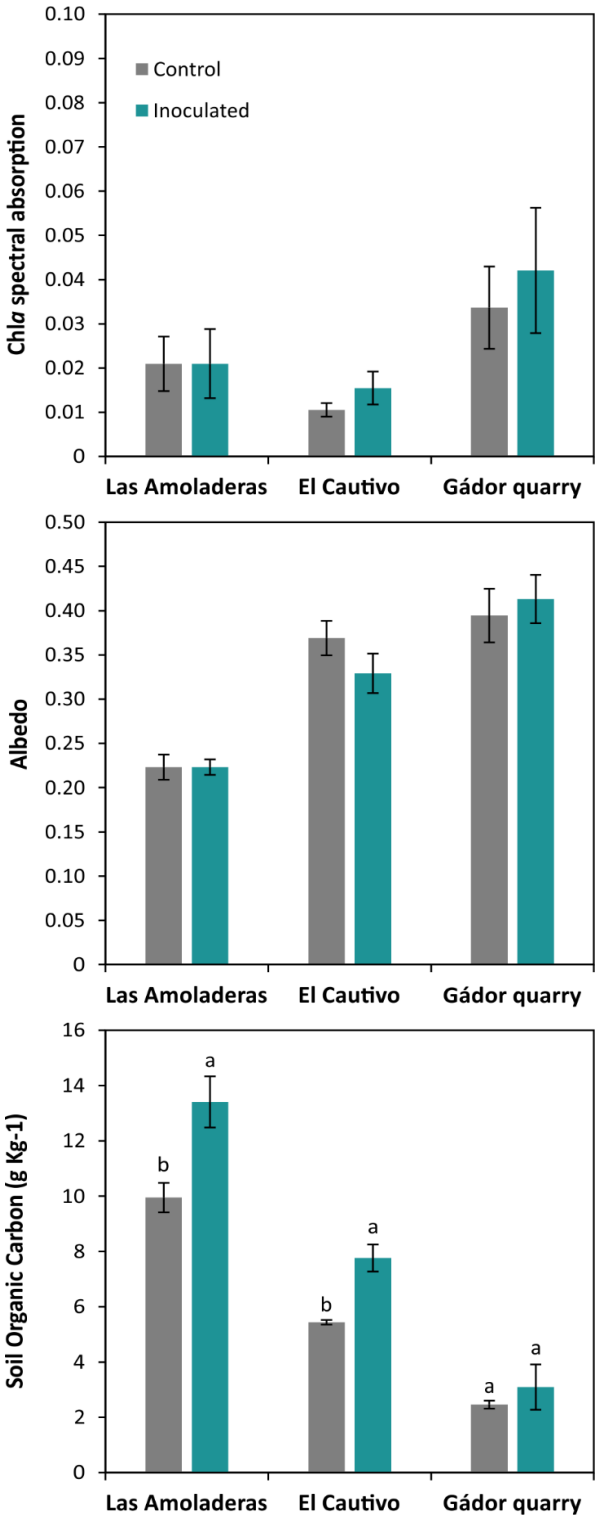

Figure 3. Mean (±SE, n = 4) A) Chl*a* spectral absorption, B) Albedo and C) Soil organic carbon (SOC) content of the inoculation and control treatments at Las Amoladeras, El Cautivo and Gádor quarry experimental areas 2 years after inoculation. Lower case letters indicate significant differences ($p < 0.05$) between treatments within each soil.

(Fig. 4A). HCY-inoculated plots showed lower chlorophyll *a* contents than CY-inoculated plots after both habitat ameliorations, although these differences were statistically remarkable only when covered by the vegetal mesh (Fig. 4A). The most successful treatments were those combining the vegetal mesh and the CY inoculation, which showed a chlorophyll *a* content over 80 mg m^{-2}. The Chl*a* spectral absorption had the same trend than the chlorophyll *a* content, and both the habitat amelioration and the inoculation treatment had a strong effect on this variable (Fig. 4B). The soils covered by the vegetal mesh and inoculated with CY showed the deepest Chl*a* spectral absorptions. On the other hand, although non-significant, albedo was also marginally affected by habitat modification (P = 0.06, Table 3), with CY + Vegetal mesh showing the lowest albedo (0.21 ± 0.04,

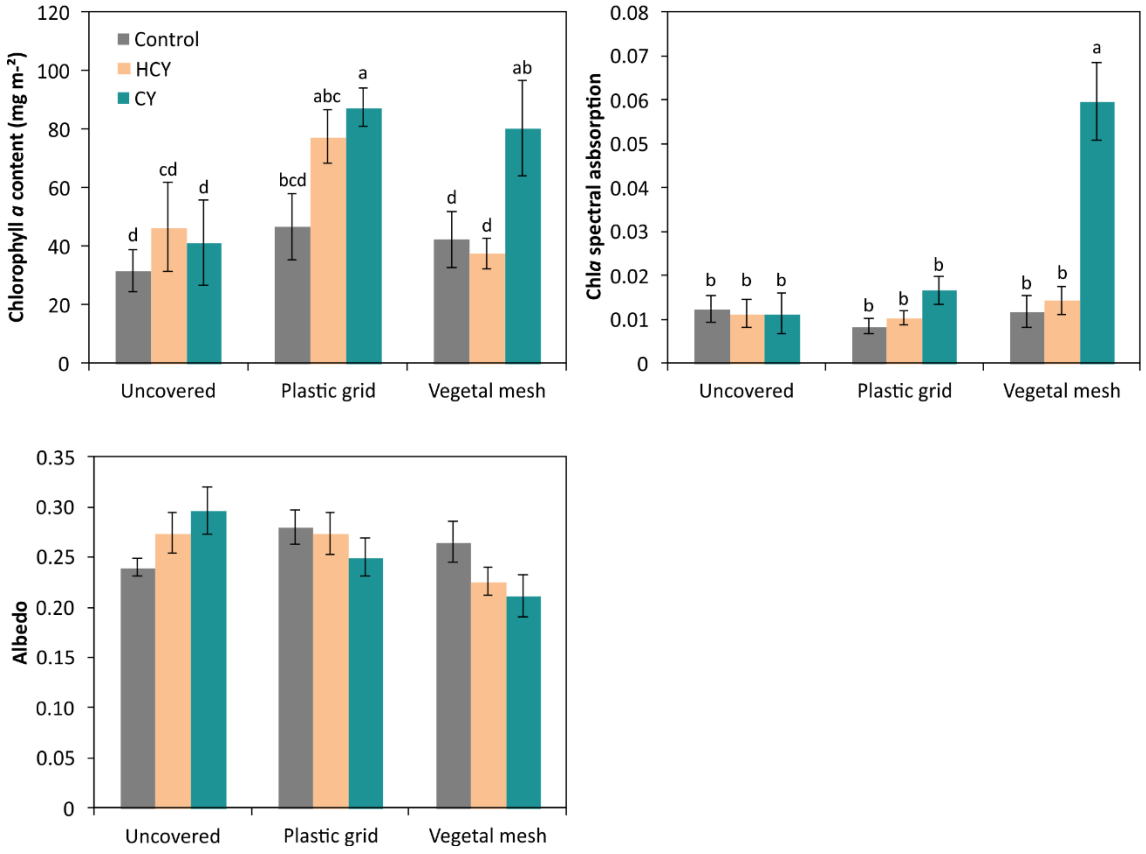

Figure 4. Mean (±SE, n = 4) of Chlorophyll *a* content (mg m^{-2}), Chl*a* spectral absorption and Albedo of the three inoculation treatments of Experiment 2 at El Cautivo 2 years after inoculation. Lower case letters indicate significant differences ($p < 0.05$) between treatments.

Fig. 4C).

We compared the EPS amount between uncovered and covered plots with the vegetal mesh, which was the habitat amelioration treatment that showed better results in control and CY plots in terms of biocrust growth (higher chlorophyll *a* content, deeper absorption peaks and lower albedo). The total amount of EPS was higher in the soils covered by the vegetal mesh, either with or without cyanobacterial inoculation (1.6 ± 0.5 mg/g soil), compared to the uncovered control (0.8 ± 0.2 mg/g soil) and uncovered CY inoculated (0.9 ± 0.1 mg/g soil). When the two EPS fractions are analyzed,

we observed comparable amounts of LB-EPS in all treatments (Figure 5). Comparing with natural cyanobacterial biocrusts, LB-EPS content of the experimental plots was always lower than in the well-developed (1.08 ± 0.55 mg/g soil) natural cyanobacterial biocrusts ($p<0.05$; One-way Anova, Tukey's post-test), while the incipient natural crust (0.44 ± 0.12 mg/g soil) was higher than the control soil and not different from the experimental treatments. The amount of TB-EPS was higher in the plots covered by the vegetal mesh, either with or without cyanobacteria inoculation, than in the direct cyanobacteria inoculation and control treatments. The natural cyanobacterial biocrusts showed TB-EPS contents comparable to the treatments covered with vegetal mesh (0.98 ± 0.51 and

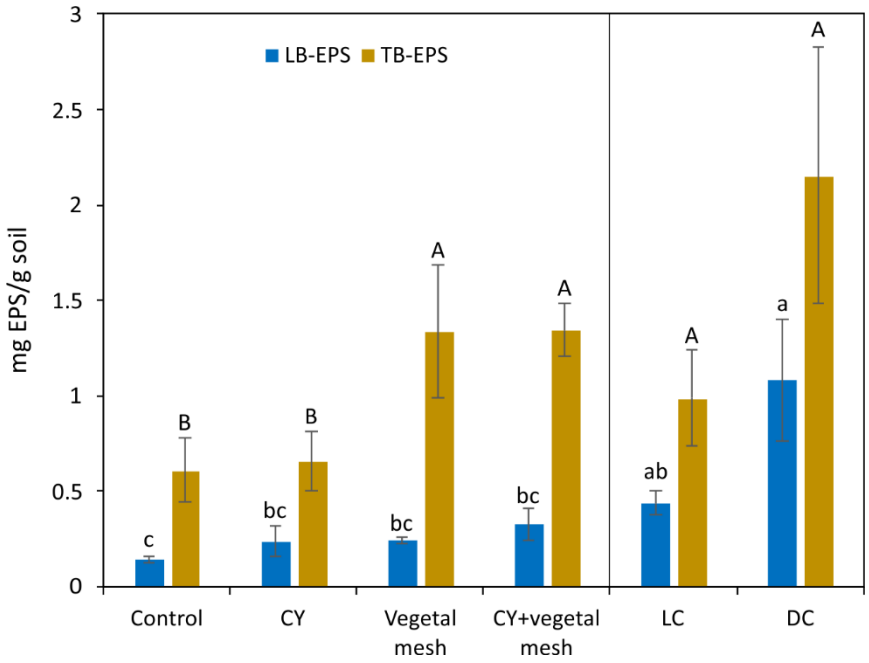

Figure 5. Mean (±SE, n = 4) of Loosely-bound and Tightly-bound EPS of the control, CY, control + vegetal mesh and CY + vegetal mesh treatments (experiment 2) at six months of development, and in natural light (LC) and dark (DC) cyanobacteria at El Cautivo. Lower case letters indicate significant differences ($p < 0.05$) in LB-EPSs content among inoculation treatments. Capital letters indicate significant differences ($p < 0.05$) in TB-EPSs content among inoculation treatments.

2.15 ± 0.95 mg/g soil, for light and well-developed natural cyanobacteria, respectively).

The monosaccharidic composition showed differed profiles in sugars' relative amounts between the two EPS fractions (LB an TB-EPS, Figures 6 and Supplementary material, respectively) and also between inoculation treatments for LB-EPS (Figure 6). Galactose and glucose were the most abundant monosaccharides in LB-EPS (Figure 6), while galactose was the most abundant monosaccharide in TB-EPS (Supplementary material). Main differences in monosaccharidic composition among treatments were observed in the LB-EPS fraction (Figure 6). The abundance of uronic acids, especially galacturonic acid, was significantly higher in the soil with direct cyanobacterial inoculation. The vegetal mesh significantly increased the abundance of glucose compared to the other treatments. The combined used of the vegetal mesh and the direct cyanobacteria inoculation significantly increased the abundance of xylose compared to the other

treatments. In the natural biocrust, the early cyanobacteria were mainly composed of uronic acids, rhamnose and galactosamine, while the well-developed cyanobacteria were mainly composed of galactose. The TB-EPS fraction had a similar monosaccharidic composition in all treatments, including that found in natural biocrusts, with the most abundant monosaccharides being galactose,

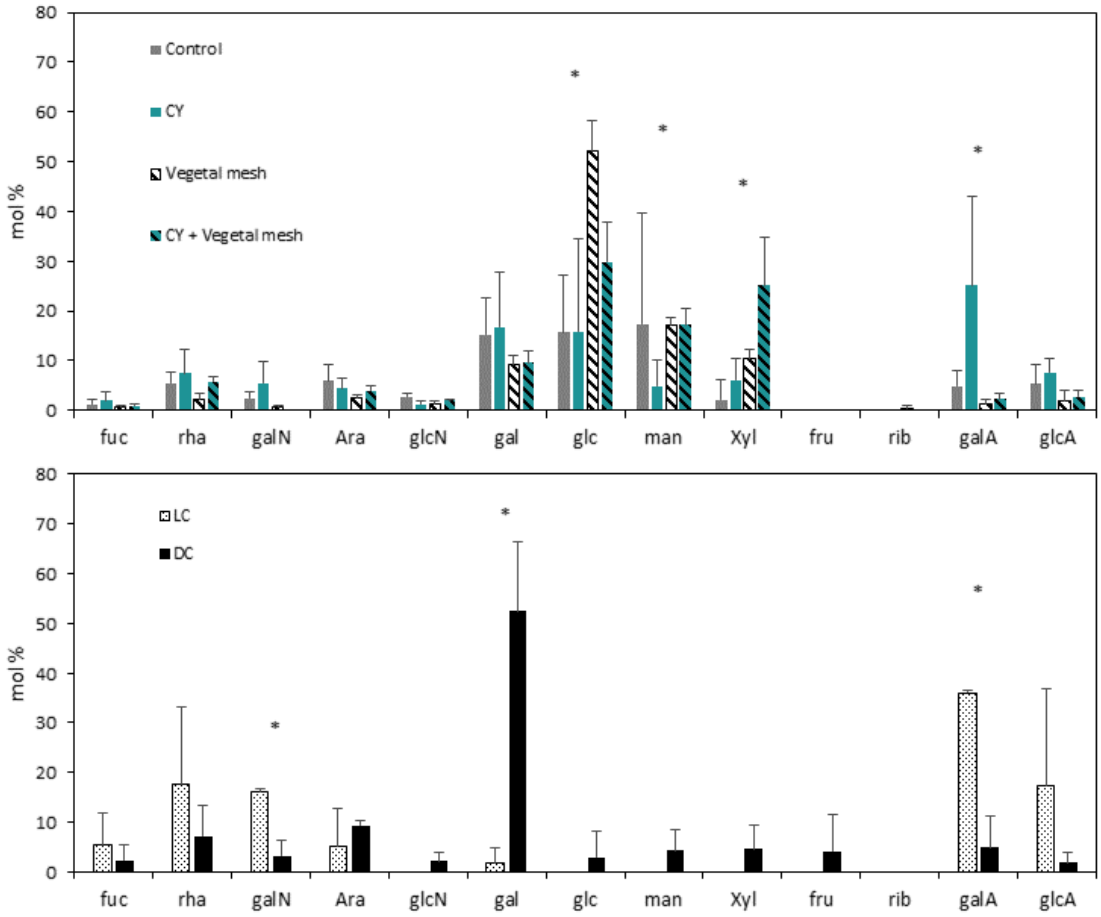

Figure 6. Monosaccharidic composition of LB-EPSs in the control, CY, control + vegetal mesh and CY + vegetal mesh treatments (Experiment 2) six months after inoculation (above), and in natural light (LC) and dark (DC) cyanobacterial biocrusts (below) at El Cautivo. Fuc: fucose, rha: rhamnose, galN: galactosamine, ara: arabinose, glcN: glucosamine, gal: galactose, glc: glucose, man: mannose, xyl: xylose, fru: fructose, rib: ribose, gala: galacturonic acid, glcA: glucuronic acid.

glucose, mannose and xylose (Figure S1).

The EPS molecular weight distribution differed between EPS fractions, with LB-EPS showing a higher abundance of low MW molecules (<0.38 kDa) and TB-EPS showing higher abundance of high MW molecules (1.1-2 MDa) (Figure 7). Differences between treatments were only observed in the LB-EPS fraction. In the control and CY treatments, there was a higher percentage of molecules < 0.38 kDa, while in the mesh treatments, either with or without cyanobacterial inoculation, there was a higher percentage of high MW molecules (1.1-2 MDa).

Discussion

Cyanobacteria inoculation alone or in combination with soil tackifiers and checkerboards over sand dunes in Chinese deserts have been the only successful attempts to date to transfer this technology to the field (Chen et al., 2006; Park et al., 2017; Wang et al., 2009). Identifying suitable inoculants, cyanobacterial hardening or habitat amelioration strategies to reduce the abiotic stress are essential for developing new restoration technologies in drylands. In this study, we applied several of these strategies in three semiarid ecosystems with strong differences in soil properties and developmental stages (Table 1; Román et al. (2018)). These soils were inoculated with a blend of native N-fixing cyanobacterial inoculum, which had already shown promising results in the laboratory (Román et al., 2018).

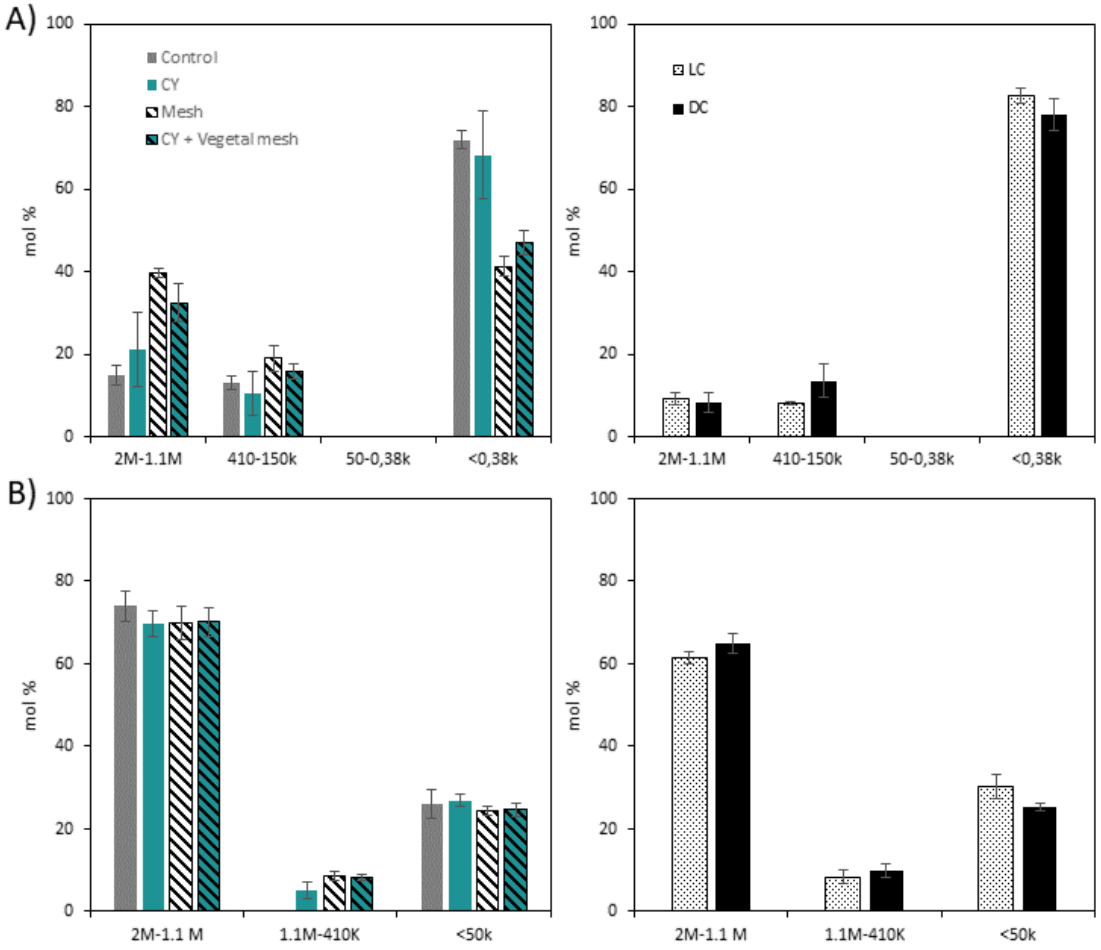

Figure 7. Apparent molecular weight (MW) distribution of LB-EPSs (A) and TB-EPSs (B) in the control, CY, control + vegetal mesh and CY + vegetal mesh treatments (Experiment 2) six months after inoculation, and in natural light (LC) and dark (DC) cyanobacterial biocrusts at El Cautivo. Fuc: fucose, rha: 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.

Our results showed that inoculating cyanobacteria directly from laboratory cultures (CY) did not promote the formation of a stable functional biocrust in any of the study areas. Moreover, the biocrust's natural recovery was as effective as the active restoration method. This general lack of

success was probably due to the biocrust depletion observed in most of the plots. Although cyanobacterial filaments rapidly formed aggregates with soil particles after inoculation, a thin discontinuous cracked layer detached from the surface appeared in less than one month and was partly washed away by a combination of raindrops, runoff and wind (Fig. 1). Consequently, the chlorophyll *a* content, Chl*a* spectral absorption and albedo in the inoculated plots did not differ from the uninoculated plots at the end of the study period (Table 2; Fig. 2). These results are surprising, as most of the available literature addressing cyanobacterial application on sandy substrates in China showed a high degree of biocrust development after a long period of time (Park et al., 2017; Wang et al., 2009), although it is known that inoculated cyanobacteria grow better in fine-textured soils (such as those in our study sites) than in coarse soils (Mugnai et al., 2020; Rozenstein et al., 2014). However, Wang et al. (2009) gave no details about the evolution of the uninoculated counterpart plots, and therefore the results cannot be compared with those found in control plots. Other studies applying hemp-immobilized cyanobacterial amendments in hot deserts in North America could not achieve significant biocrust recovery (Kubeckova et al., 2003). The lack of success was partly attributed to detachment of the inoculum material by wind and overland flow. To overcome this problem, a combination of cyanobacteria with soil fixing chemicals (e.g., sodium alginate) has been suggested for improving inoculated biocrust stabilization (Park et al., 2017; Peng et al., 2017). We therefore conducted a small test in the Gádor quarry plots, consisting of applying a slurry of a blend of cyanobacterial inoculum, a small amount of fine soil powder and sodium alginate (unpublished data). However, a few months after inoculation, this artificially induced biocrust also became detached from the soil surface, as shown in this study. Flaking and upward curling have been also described in unconsolidated substrates inoculated with *Schizothrix* cf. *delicatissima* (Mugnai et al., 2018) or with a consortium of *Nostoc* sp., *Phormidium* sp. and *Scytonema arcangeli* (Park et al., 2017), and in natural biocrusts in the Mojave Desert (Williams et al., 2012). According to Williams et al. (2012), this is caused by the expansion-contraction of clay particles and the viscoelastic properties of the entangled EPS network (Navarini et al., 1992). Even though it is a natural process in well-developed and stable biocrusts, this process may be less favorable for incipient artificially induced biocrusts, posing a barrier for soil colonization. However, the direct cyanobacterial inoculation improved the SOC content at Las Amoladeras and El Cautivo sites compared to non-inoculated plots, which may have been due to a portion of undetached inoculum having remained on the soil, thereby becoming a source of organic carbon.

These unsatisfactory results led us to explore new attempts for improving cyanobacterial establishment and development in the field. Two main constraints for artificial cyanobacterial colonization have been described: physiological stress and wind and water erosion (Bowker, 2007). Preacclimation, or hardening, of cyanobacteria to the conditions that are likely to be present in the field has been suggested (Antoninka et al., 2018; Giraldo-Silva et al., 2019a). During production in

the laboratory, cyanobacteria would not need to express the desiccation or UV radiation resistance mechanisms that enable them to survive in the field. Therefore, this hardening step would facilitate reactivation of the molecular pathways involved in these resistance mechanisms, such as scytonemin or EPS production. Giraldo-Silva et al. (2019b) found that fitness results for 13 out of 20 cyanobacterial strains, including *Tolypothrix* and *Scytonema* strains, subjected to preacclimation were positive. In our study, however, the hardening treatment did not significantly improve cyanobacterial establishment, and some variables were even lower than for the unhardened inoculants (Fig. 3; Table 3). Three main explanations for these results are possible. First, previous outdoor tests have proved that the effect of hardening is more effective with non-heterocystous cyanobacteria (e.g. *Microcoleus vaginatus*) than with heterocystous (e.g. *Nostoc*, *Scytonema* or *Tolypothrix* genera) (Giraldo-Silva et al., 2019b). Second, wind and water erosion events and the progressive detachment of the crusts observed in uncovered plots probably played a more important role in constraining biocrust development. Finally, the potential improvements provided by hardening could have been masked by a habitat amelioration treatment that reduced UV radiation and increased water availability for longer periods. Thus, our results are in agreement with previous field studies that have not shown any improvements either after applying a hardened inoculum compared to an unhardened nursery-grown biocrust inoculum (Bowker et al., 2019; Faist et al., 2020). Nevertheless, these results should not detract from the fact that hardening can provide some benefits, such as keeping the inoculum indoors for longer periods, avoiding the degradation that usually affects fresh cultures. This hardened cyanobacterial stock could then be used when needed to progressively restore large field areas.

Microsite modification for enhancing resource availability and reducing the impact of active erosion has shown promising results in nursery-grown communities (Bowker et al., 2019), but its effect on artificially induced cyanobacterial biocrusts is as yet unknown. Our results revealed that the application of habitat amelioration strategies on cyanobacterial inoculated soils promoted higher chlorophyll *a* content, deeper Chl*a* spectral absorption peaks and lower albedo (resulted from soil darkening with biocrust development) than in uncovered plots (Fig. 3). Of the three amelioration treatments applied, the vegetal mesh showed the best results, being the most effective combination its application on soil inoculated with unhardened cyanobacteria (CY). The application of the vegetal mesh was observed to significantly increased the amount of TB-EPS. However, the chemical composition and MW distribution of this fraction was very similar to that of the other treatments. Indeed, the characteristics of this EPS fraction appeared to be more strongly linked to soil type, which could explain the similarities in monosaccharidic composition and MW distribution among treatments, as well as between induced and natural biocrusts. Differences were found in the EPS characteristics of the LB-EPS fraction. The soil inoculated with cyanobacteria showed a higher abundance of uronic acids (especially galacturonic acid), similar to the pattern observed in incipient

natural cyanobacterial biocrusts. Uronic acids are unique in cyanobacteria (De Philippis and Vincenzini, 1998) and their synthesis has been described in *Nostoc* sp. as protection against stressful factors such as prolonged drought (Helm et al., 2000; Tamaru et al., 2005). The cover with the mesh increased the relative abundance of glucose, and in combination with direct cyanobacterial inoculation, an increase in the relative abundance of xylose. Greater abundance of glucose can be attributed to degradation of the mesh and release of this monosaccharide into the soil, and also to the creation of a less stressful microenvironment (less radiation, longer moisture retention) which could stimulate the synthesis of this sugar by the inoculated cyanobacteria. In the well-developed natural cyanobacterial biocrusts, galactose was the most abundant monosaccharide. In fact, a greater abundance of galactose, mannose and xylose has been reported in soils with higher OC content (Chamizo et al., 2019). Thus, higher xylose abundance in the cyanobacteria-inoculated soil covered with the mesh could be indicative of more biocrust development in this treatment. On the whole, the monosaccharidic composition has been revealed as a potential indicator of biocrust development, where strong abundance of uronic acids could be indicative of early biocrust developmental stages and increasing abundance of galactose and xylose could be indicative of greater biocrust development.

Similar to previous findings, LB-EPS were composed mainly of lower MW molecules, while TB-EPS were composed mostly of high MW molecules (Chamizo et al., 2019; Chen et al., 2014). It is worth noting that the application of the vegetal mesh combined with cyanobacteria soil inoculation increased the abundance of high MW molecules in LB-EPS, thus further favoring the effect of the mesh on the formation of heavier EPS, which could also be important in enhancing soil stability.

The vegetal mesh provided a reduction of the full-spectrum light and provided an additional supply of water due to the moisture accumulated in the vegetal fibers. Previous studies have shown that microclimatic modification of the habitat moderates extreme temperatures by reducing UV radiation and increasing water availability for longer periods, improving biocrust restoration (Antoninka et al., 2019; Ayuso et al., 2017; Fick et al., 2019). In addition to providing shade, the application of the vegetal mesh also reduced the negative effect of raindrop impact and overland flow, stabilizing the soil surface. Higher TB-EPS content as well as more MW molecules in the LB-EPS with this treatment could also have contributed to increasing crust stability (Chamizo et al., 2019). Therefore, cyanobacterial flakes may have remained attached to the soil surface during the experiment. Nevertheless, while the mesh covering could be a solution for local field settings, its application on larger scales would be economically unaffordable and technically unfeasible. Antoninka et al. (2019) suggested the use of mobile reusable shades as a solution for temporally promoting colonization cores.

Exploration of feasible strategies for ameliorating the harsh abiotic conditions in drylands constitutes a challenge for successful induce the formation of artificial biocrusts by cyanobacterial inoculation and restoration of soil functions in degraded ecosystems. Our study shows that covering cyanobacterial inoculated soils with a vegetal fiber mesh can contribute to improving biocrust establishment and colonization.

Conclusions

An attempt to restore soil functions in three semiarid habitats by means of direct cyanobacterial inoculation produced poor outcomes. The use of habitat amelioration strategies for the reduction of physiological stress and the negative impact of overland flow and raindrops showed promising results. Specifically, covering cyanobacterial inoculated soils with recycled vegetal fiber mesh resulted in better colonization, chlorophyll *a* content, deeper Chl*a* spectral absorption peaks and lower albedo than in uncovered plots. EPS characteristics in the inoculated soils, either left uncovered or covered by this vegetal cloth, and in natural cyanobacterial biocrusts showed that the mesh increased the amount of more condensed EPSs (TB-EPS fraction). An increase in abundance of higher MW molecules in the more soluble LB-EPS soil fraction was also observed. Both fractions probably contributed to enhancing soil aggregation and biocrust stability. The higher abundance of xylose and galactose in the inoculated soils covered by the vegetal mesh could also be indicative of greater biocrust development. Although important advances have been achieved and the application of this technique can provide promising results on a local scale, further investigation is necessary for the development of amelioration strategies that can be applied on larger scales.

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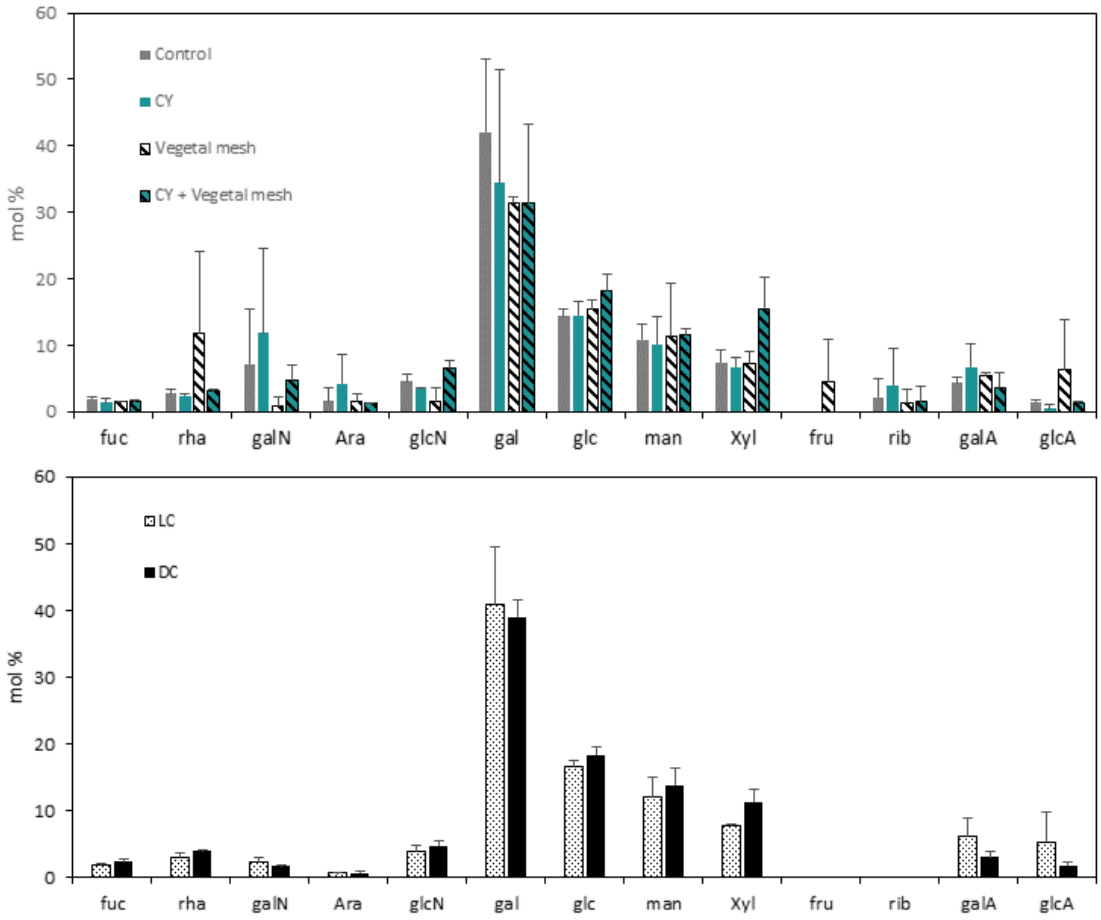
Supplementary Material



Supplementary Figure 1. Monosaccharidic composition of TB-EPSs in the control, CY, control + vegetal mesh and CY + vegetal mesh treatments (experiment 2) six months after inoculation (above), and in natural light and dark cyanobacterial biocrusts (below) at El Cautivo. 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.

Chapter IV:

Assessing the viability of cyanobacteria pellets for application in arid land restoration

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Abstract

The role of cyanobacteria from soil biocrusts in restoring degraded land is gaining interest in recent years because of their critical role in enhancing soil fertility and preventing erosion. However, soil restoration through cyanobacteria application remains a challenge for large-scale restoration efforts and new methodologies for effective cyanobacterial application need to be developed. Here, we propose a bioenvironmental approach to inoculate soils with pelletized cyanobacteria from soil biocrusts. Fresh cultures of three soil native cyanobacteria strains from two representative N-fixing genera (*Nostoc* and *Scytonema*) and a non-heterocystous filamentous genus (*Leptolyngbya*) were added into a substrate composed of commercial bentonite powder and sand (1:10 weight ratio) and extruded into pellets. Then, in a multifactorial microcosm experiments under glasshouse conditions, we evaluated (i) the survival and establishment over time of the cyanobacteria encapsulated in pellets, and ii) the viability of pelletized cyanobacteria after drying and storing for 30d, on soils from three arid regions in Australia. Our results showed that pellets can dissolve completely and spread out in all treatments. *Scytonema* and the consortium of the three cyanobacteria species showed significant ($P < 0.001$) deeper Chl*a* spectral absorption, higher chlorophyll *a* contents and lower albedo compared to the other inoculation treatments. Storing the pellets for 30 d significantly affected the viability of the cyanobacteria inoculum with reductions of approximately 50% in chlorophyll *a* content (a proxy for cyanobacteria biomass). Overall, our results showed that some cyanobacteria species can be successfully incorporated into extruded pellets and survive on degraded soils. This technology opens a wide range of opportunities for application in large scale restoration programs although further testing and refining through field trials is recommended.

Keywords: Biocrust; Cyanobacteria inoculation; land degradation; ecosystem restoration; drylands

Introduction

Biological soil crusts, or biocrusts, are communities of microscopic and macroscopic organisms that include bacteria, cyanobacteria, algae, lichens, fungi and mosses (Chilton et al. 2107). These communities inhabit the first few millimetres of the soil in open spaces in drylands (Belnap, 2007) and are key components of these ecosystems, covering about 12% of the global terrestrial surface and providing multiple ecosystems services (Rodríguez-Caballero et al., 2018). Among biocrust components, cyanobacteria are often the first colonizers of poor, degraded soils due to the extraordinary ability to survive and grow under high temperatures (Lan et al., 2015), prolonged UV radiation exposures (Garcia-Pichel and Castenholz, 1991) and water scarcity (Rajeev et al., 2013). Several methods have been tested to recover degraded soils, e.g. inoculum composed by exogenous

soil microorganisms (Moreira-Grez et al., 2019). However, soil inoculation with cyanobacteria from soil biocrusts for re-establishing soil functionality of degraded land is gaining interest in recent years (Rossi et al., 2017) because of their potential for restoring soil fertility (Chamizo et al., 2012) and preventing erosion (Chamizo et al., 2017).

Cyanobacterial strains can be isolated from soils, cultured ex-situ in liquid media and re-inoculated on donor soils to produce artificial biocrusts, enhancing soil conditions and promoting faster succession dynamics (Román et al., 2018; Giraldo-Silva et al., 2019). Cyanobacteria inoculation has been successfully applied under laboratory conditions (Malam-Issa et al., 2007; Muñoz-Rojas et al., 2018a) and in local field settings (Chen et al., 2006; Wang et al., 2009). However, despite important advances, soil restoration through cyanobacterial applications remains a challenge for large-scale restoration activities (Chua et al., 2019) as large and viable volumes of cyanobacteria inoculum need to be produced and transferred in a timely manner to the field to avoid degradation and contamination (Roncero-Ramos et al., 2018; Giraldo-Silva et al., 2019). To fully exploit the application of cyanobacteria inoculum in large-scale restoration, the development of approaches that facilitate effective cyanobacterial application and distribution must be explored and developed.

Here, we propose a bioenvironmental approach that consists of incorporating cyanobacteria cultures within extruded soil pellets. Film coating and pelleting techniques are commonly used technologies in the seed-based restoration of vascular plants (Brown et al., 2019; Madsen et al.,2016; 2019). These technologies allow to preserve the encapsulated material for an extended period of time, enhancing its survival and delivery, and facilitating its application into the field. This approach is of special interest in large degraded zones like the Australian arid zone, where restoration efforts involve thousands of hectares (Erickson et al., 2017). Our hypothesis is that through pelletization, cyanobacteria inoculum could overcome application challenges in field- scale restoration programs. In previous studies, cyanobacteria incorporation into pellets was tested using alginate as binder, showing promising results with the non-heterocystous cyanobacteria *Microcoleus vaginatus* (Buttars et al., 1997). However, its incorporation in starch pellets resulted in high mortality (Howard and Warren, 1998). Different pellet compositions, cyanobacterial species, and their performance under various substrates are yet unexplored. In this study, we ran two experiments in order to: 1) test the feasibility of using cyanobacteria-inoculated extruded pellets to promote biocrust development and, 2) evaluate the pellet viability after drying and storing for 30 days. We carried out these experiments on three types of degraded soils from three different Australian arid regions.

Materials and Methods

Soil collection

Soils used in this study were collected from degraded sites from three areas: i) the Cobar Peneplain Bioregion (central New South Wales, -31.511129, 145.935964, hereafter named: 'Cobar') ii) the Pilbara biogeographical region (north-west Western Australia, -23.365624, 119.732407, hereafter named 'Pilbara' and, iii) the Simpson Strzelecki Dunefields (South Australia, -27.511556, 138.449678, hereafter named: 'Dunefields'). Soils from the Pilbara consisted of topsoil removed from the upper 20 cm of the soil profile and stockpiled for subsequent use in rehabilitation (Muñoz-Rojas et al., 2018a). Soils from Cobar and Dunefields were poorly structured soils from sites undergoing degradation processed as a consequence of overgrazing and erosion. Site characteristics of the three regions are summarized in Table S1.

Cyanobacteria isolation, culturing and pelleting process

We used three cyanobacterial species from two representative heterocystous genera (*Nostoc sp.* and *Scytonema sp.*) and a non-heterocystous filamentous genus (*Leptolyngbya sp.*), previously isolated and characterised from biocrusts sampled at Gallery Hill, Western Australia (Muñoz-Rojas et al., 2018b). These species were selected based on their broad presence in soils from Australian arid lands and worldwide (Flechtner et al., 2009; Dojani et al., 2014; Aboal et al., 2016; Chilton et al., 2017). Cyanobacterial isolates were cultured in BG11- media (Cyanobacteria BG-11 Freshwater Solution, Merck) under light:dark cycles (16:8h at 28/20°C) and constant light conditions (80 μmol $m^{-2}s^{-1}$) in a plant growth chamber (Thermoline Scientific Climatron 2400-TH-CO_2) at the School of Biological, Earth and Environmental Sciences (UNSW, Sydney) (Chua et al., 2019). Cultures were filtered at the exponential phase to harvest biomass for pelleting, and chlorophyll *a* and carotenoid contents for each cyanobacteria culture were analyzed as in Zavřel et al. (2015) (Table S2). Fresh biomass of each strain alone and an equal mix of all strains (hereafter: 'Consortium') were added into a substrate composed of commercial bentonite powder and sand (1:10 weight ratio). The solution was mixed to reach a final concentration of 0.13 g wet biomass/g pellet. An equivalent solution mixed with distilled water without cyanobacteria was composed to produce control pellets (uninoculated). The composed solutions for each inoculum treatment (*Leptolyngbya*, *Nostoc*, *Scytonema*, Consortium and control) were separately extruded through a jerky gun with an extruder nozzle into sets of pellets (1 cm diameter x 2 cm length, 2.0±0.1 g). Pellets were dried at 30°C for 24 h (Fig.1).

Experimental design

After drying, a subset of the dried pellets (hereafter 'DP') was used in a microcosms experiment, and a second subset of pellets was stored at room temperature (25°C ± 1) after drying (dry and store treatment, hereafter 'DSP') and maintained without irrigation for 30 d. The microcosms experiment consisted of individual pellets from each inoculation treatment (*Leptolyngbya*, *Nostoc*, *Scytonema*, Consortium and control) that were placed on 90 mm Petri dishes filled with 40 g of autoclaved soil

from each study site (Cobar, Pilbara and Dunefields). Treatment combinations for each inoculum type were arranged in a completely randomised order of individual microcosms (n=4 per treatment and soil type, total n =60). Samples were incubated in the plant growth chamber described above at 25±1°C for 30 d. After this period, we set up new microcosms with the DSP pellets including the same inoculation treatments (*Leptolyngbya*, *Nostoc*, *Scytonema*, Consortium and control; n=60) and maintained in the growth chamber for 15 d. For the duration of the experiment (45 d since the start of the experiment), each microcosm was irrigated with a simulated precipitation regime of 9 mm consisting of three watering pulses of 3 mm (20 mL) every 24 h, followed by dry down events of 5 days (Muñoz-Rojas et al., 2018a).

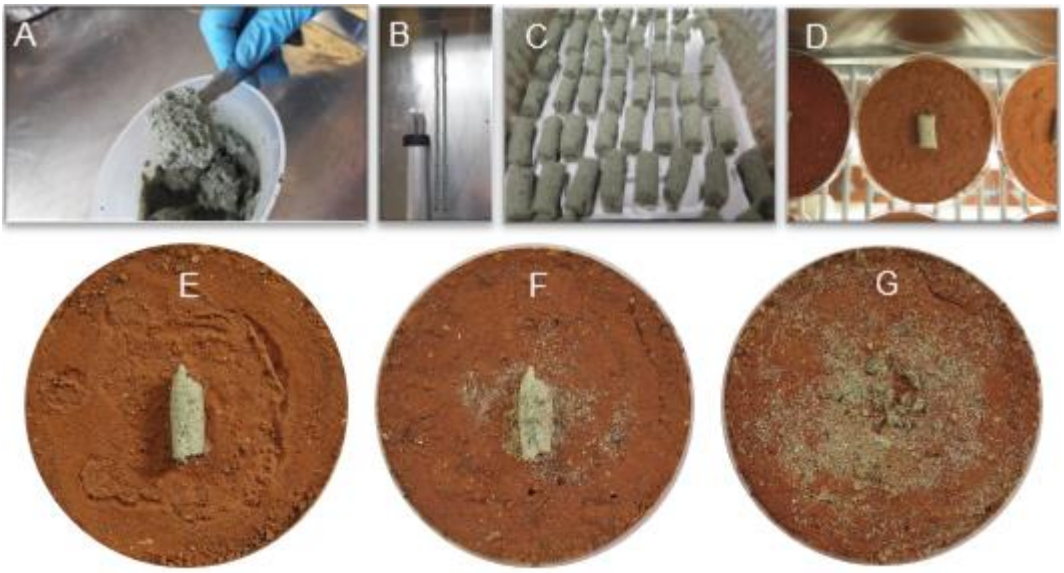

Figure 1 A) Pellet solution resulting from mixing cyanobacteria cultures and bentonite and sand. B) Extruded material using a commercial jerky gun. C) Close view of dried inoculated pellets after 24 h drying (DP). D) Microcosms containing an inoculated pellet and Cobar soil substrate. *Scytonema* inoculated pellet over the Cobar soil at incubation time 0 (E), 15 (F) and 45 (G) days.

Cyanobacteria development measurements

Cyanobacteria survival was evaluated by measuring chlorophyll *a* concentration (μg g^{-1}) as in Castle et al., (2011) at 0, 15 and 45 days for DP, and at 15 d for the DSP. Samples were collected and ground using a mortar and pestle, and the absorbance at 665 nm was measured in the spectrophotometer (Aqualog, HORIBA Scientific). Additionally, cyanobacteria development for DP was monitored at each time period using non-destructive techniques, e.g. visual observation, surface albedo and the chlorophyll spectral absorption at 680 nm. Cyanobacteria coverage of each samples was visually assessed by taking photos 25 cm above each microcosm with a digital camera Canon EOS 600D 18 Megapixels resolution (Canon Inc., Tokyo, Japan). Surface reflectance of each sample was measured with an ASD FieldSpec® Handheld portable spectroradiometer and pre-treated to remove noisy bands and smooth the spectra. Due to the presence of a well-known chlorophyll *a* spectral absorption

at 680 nm, the smoothed spectra were used to calculate continuum removal at 680 nm (Clark and Roush, 1984), an index that has been found to be strongly correlated with chlorophyll *a* content in restored cyanobacteria biocrusts (Román et al., 2019). In order to improve data visualization, the original continuum removed values at 680 nm were used to calculate chlorophyll *a* spectral absorption (hereafter named: "Chl*a* spectral absorption") as follow:

$$\text{Eq. 1} \qquad \text{Chl}a \text{ spectral absorption} = 1 - CR_{680}$$

where CR_{680} = continuum removal value at 680 nm.

Hence, higher values of the variable correspond to deeper chlorophyll *a* spectral absorption. Soil surface albedo in the visible region (from 400 to 700 nm) was calculated as the square root of the sum of the squares of reflectance at each wavelength (Escribano et al., 2010).

Data analyses

None of the data met assumptions of normality required for parametric statistical tests and were not able to be transformed. Therefore, a permutational multivariate analysis of variance (PERMANOVA) was performed to analyse: a) the effect of incubation time and inoculation treatment on Chl*a* spectral absorption, b) the effect of inoculation treatment and soil type on Chl*a* spectral absorption, albedo, chlorophyll *a* at the end of the study period for the DP (45 days) and, c) the effect of pellet storage on chlorophyll *a* at 15 d of incubation. All PERMANOVA were based on Euclidean distances. Comparisons between means (when differences were found) were performed with post-hoc comparisons with the PERMANOVA t-*statistic* and using a maximum of 9999 permutations to obtain the pseudo-*F* and P-values ($p < 0.05$). Monte Carlo correction was applied when necessary. All the analyses were performed using Primer 7 and Permanova + (PRIMER-E Ltd, Plymouth, UK).

Results and discussion

Soil inoculated with dried cyanobacteria pellets (DP) developed an artificial biocrust that underwent considerable modification during the inoculation period (Fig. 1). In the most successful treatments, inoculated with *Scytonema*, pellet material was progressively redistributed from the centre of the microcosm and spread out to the surrounding areas, covering about 40% of the available microcosm surface 45 days after incubation (Fig. 1). As the soil surface was gradually covered by cyanobacteria, substantial changes were found in the spectral response of the samples. Overall, inoculated samples showed significant ($P < 0.001$) deeper absorption peaks compared to the control samples, as indicated by the higher Chl*a* spectral absorption values at all incubation times, which reflect higher chlorophyll *a* concentration (Fig. 2). Moreover, inoculation with *Scytonema* alone and the Consortium promoted

higher absorption peaks in all three study soils compared to the other inoculation treatments (Fig. 2). These results are in contrast with previous inoculation trials using cyanobacteria strains from arid soils in Spain, which showed higher colonization rates when soils were inoculated with *Nostoc* compared to *Scytonema* strains (Román et al., 2018). Incubation time had a significant ($P < 0.001$) effect on Chl*a* spectral absorption (Fig. 2) but it varied with the inoculation treatment (Table 1). Although Chl*a* spectral absorption values did not show significant changes on *Leptolyngbya* and *Nostoc* samples towards the end of the study period, these values increased for the Consortium and *Scytonema* samples in the three study soils (Fig. 2). This response suggests that the cyanobacteria inoculum in these treatments not only survived the pelleting process but were successfully redistributed to adjacent areas. Cyanobacteria colonization resulted in a substantial decrease of the soil surface albedo, showing lower albedo in all inoculated soils compared to uninoculated samples ($P < 0.001$, Table 2). Moreover, albedo was lower in *Scytonema* and *Nostoc* samples than in *Leptolyngbya* and uninoculated treatments. This result may be explained by the accumulation of scytonemin, a sunscreen pigment produced by *Nostocales* (e.g., genera *Nostoc*, *Scytonema*), that has been reported to reduce soil surface reflectance in dryland soils (Couradeau et al., 2016).

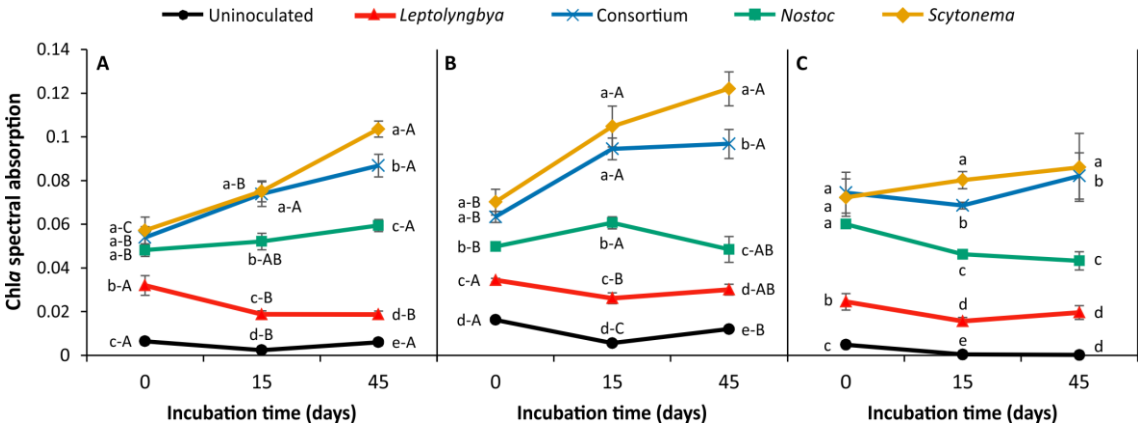

Figure 2 Mean (± SE, n=4) Chl*a* spectral absorption for each inoculation treatment at 0, 15 and 45 days after pellet inoculation (dried pellets) in A) Cobar, B) Pilbara and C) Dunefields soils. Lower-case letters mark significant differences ($P < 0.05$) among inoculation treatments within each incubation time. Capital letters indicate significant differences ($P < 0.05$) between incubation times within each inoculation treatment.

Direct measurements of chlorophyll *a* concentration at 45 days of incubation showed significant ($P < 0.001$) differences in pelleting survival among inoculation treatments (Table 1). Chlorophyll *a* concentration was significantly higher in the *Scytonema* and Consortium inoculum compared to the other inoculation treatments for all soil types (Fig. 3). Among all treatments, highest concentrations of chlorophyll *a* (1.19 ± 0.07 µg g^{-1}) were obtained for Cobar soil inoculated with *Scytonema* pellets. Those concentrations are in the range of values obtained by Wang et al., (2009) one year after inoculating with *Microcoleus* and *Scytonema* genera and by Muñoz-Rojas et al., (2018a) with a blend of *Nostoc*, *Scytonema* and *Tolypothrix* inoculated in a mine substrate. *Leptolyngbya* treatments

showed a negative trend where biomass progressively declined and no traces of cyanobacteria were found at the end of the study period (Fig. 3). The contrasting behavior shown by the inoculum treatments can be partially attributed to a different tolerance to the desiccation stress imposed by the pelleting process and pellet composition. Previous studies involving cyanobacteria encapsulation in clay pellets reported a high mortality because clay particles seemed to desiccate cyanobacteria beyond their capacity to recover (Warren, 2017). Therefore, in this study we aimed to develop a pellet that remained rigid enough to avoid crumbling during shipping and handling but reducing as much as possible the content of clay to avoid cell mortality and facilitate rapid dissolution following inoculation.

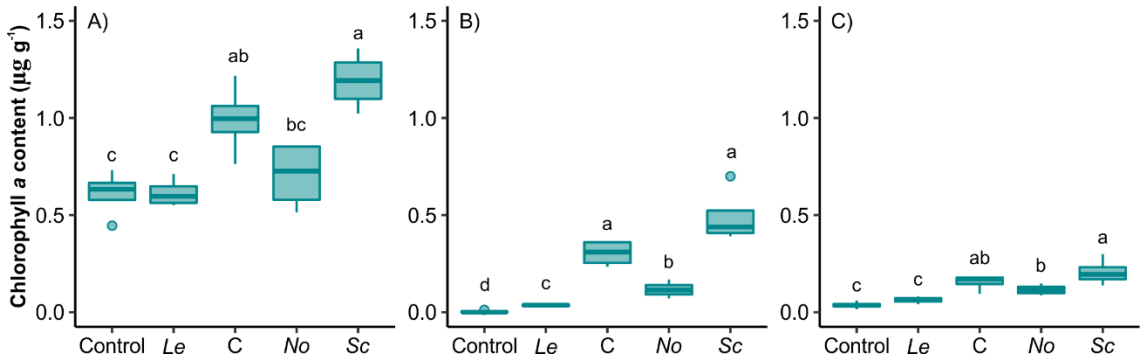

Figure 3 Boxplot of chlorophyll *a* concentration of the five pellet inoculation treatments for the dried pellets at A) Cobar, B) Topsoil and C) Dunefields, at the end of the study period (45 days). Lower case letters mark significant differences among inoculation treatments ($P < 0.05$). *Le*: *Leptolyngbya*, C: Consortium, *No*: *Nostoc*, *Sc*: *Scytonema*.

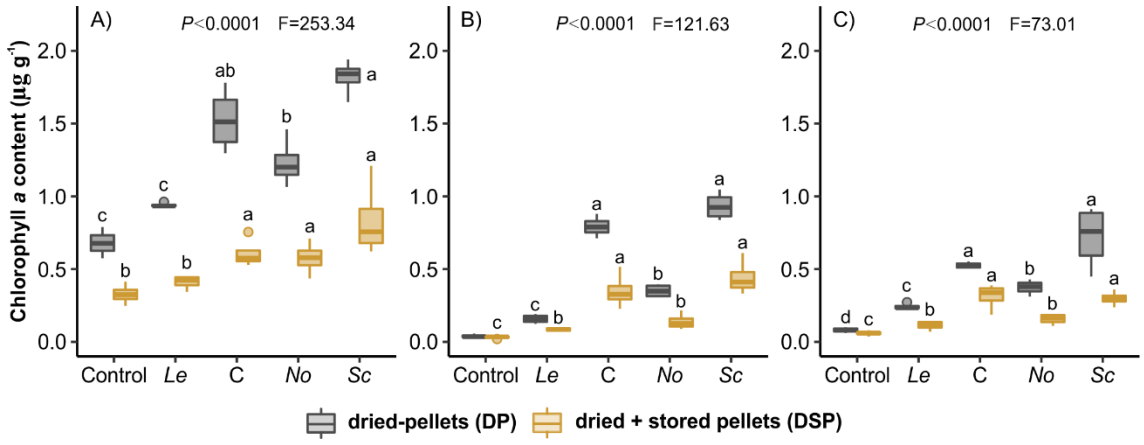

Figure 4 Boxplot of chlorophyll *a* concentration of the five pellet inoculation treatments at A) Cobar, B) Topsoil and C) Dunefields, after 15 d incubation for dried pellets (DP) and dried + stored pellets (DSP). Lower case letters mark significant differences among inoculation treatments within each pellet experiment ($P < 0.05$). *Le*: *Leptolyngbya*, C: Consortium, *No*: *Nostoc*, *Sc*: *Scytonema*.

The 30 d storage of the pellets (DSP) resulted in a significant decrease in chlorophyll *a* content in comparison to non-stored pellets (DP) 15 d after incubation (Fig. 4). These reductions were consistent across inoculation treatments and soil types, showing a reduction of 50% in the survival compared to the DP results. Regarding inoculation treatments, the same survival trends were found,

and *Scytonema* and the Consortium showed higher chlorophyll *a* content than other inoculants for both DP and DSP experiments (Fig. 4). Giraldo-Silva et al., (2019) showed that cyanobacteria inoculum can be partially desiccated by applying dry/wet cycles for 12 d in order to harden cultures. Dodi and Zaady (2006) also suggested the possibility of drying the inoculum for long-term storage of the propagules. Our results agree with previous studies and suggest that some cyanobacteria species can survive to the inoculum pelletization and short-term storage. Nevertheless, further research is needed to enhance the cyanobacteria survival to the pelletization process.

Table 1. PERMANOVA test at 45 days of incubation of DP experiment to assess the effects of inoculation treatment (IT), soil type (S) and interactive effects of these factors on Chl*a* spectral absorption, albedo, chlorophyll *a* (Chl*a*, μg g^{-1}) and total organic content (TOC, g kg^{-1}). Statistical significance levels: ***P < 0.001, **P < 0.01, * P < 0.05, ns- not significant.

Response		**Chl**a **spectral absorption**		**Albedo**		**Chlorophyll *a***		**TOC**	
	df	**Pseudo-F**	***P* value**	**Pseudo-F**	***P* value**	**Pseudo-F**	***P* value**	**Pseudo-F**	***P* value**
IT	4	134.96	***	13.75	***	39.89	***	1.28	ns
S	2	7.93	***	220.76	***	322.42	***	540.62	***
IT x S	8	1.39	ns	3.10	***	4.28	***	0.21	ns

Table 2. Mean (± SD, n=4) a) albedo in the 400 nm to 700 nm range of soil surface reflectance spectra for each inoculation treatment in the three soils studied at end of the study period (45 days). Lower-case letters mark significant differences (P < 0.05) among inoculation treatments within each soil.

Inoculation treatment	Cobar	Pilbara	Dunefields
Uninoculated	$2.51^{a} \pm 0.08$	$2.24^{abc} \pm 0.04$	$2.94^{a} \pm 0.04$
Leptolyngbya	$2.34^{abc} \pm 0.05$	$2.34^{a} \pm 0.04$	$2.74^{bc} \pm 0.06$
Consortium	$2.34^{ab} \pm 0.03$	$2.22^{b} \pm 0.02$	$2.71^{b} \pm 0.01$
Nostoc	$2.34^{b} \pm 0.02$	$2.23^{b} \pm 0.03$	$2.64^{c} \pm 0.03$
Scytonema	$2.26^{c} \pm 0.01$	$2.10^{c} \pm 0.00$	$2.59^{bc} \pm 0.06$

Several methods have been developed to recover degraded soils using biocrust inoculation, including the direct use of field-collected native inoculum (Chandler et al., 2019), the greenhouse cultivation from small amounts of native taxa and later reallocation in the field (Ayuso et al., 2017; Antoninka et al., 2019), or the isolation of cyanobacteria and culturing ex-situ in liquid media to produce enough

biomass for direct inoculation in the field (Wang et al., 2009). However, the production of sufficient inoculum and the transport and delivery to the field at a reasonable cost is still a major challenge for researchers in large-scale restoration efforts. The pelleting technology presented here would enable first, the inoculum storage for longer periods in a dry state allowing its delivery to remote areas and second, the use of available machinery for seed enhancement technologies (Erickson et al., 2017) for dispersal and the coverage of larger areas (Sears and Prithiviraj, 2012). Although promising, the response of the different species under field conditions, mixed in different concentrations, and in a higher temporal scale needs to be further investigated before application in field restoration programs.

Conclusions

Overall, our results showed that cyanobacteria can be successfully incorporated into extruded soil pellets and redistributed via watering to modify properties of degraded soil substrates. Among inoculation treatments, *Scytonema* and the Consortium showed the best results in terms of chlorophyll *a*, Chl*a* spectral absorption and albedo at the end of the study period, whereas *Nostoc* and *Leptolyngbya* showed a high mortality. This technology is ready for further testing and refining through field trials, opening a wide range of opportunities for application in large scale soil restoration programs.

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Supplementary Material

Table S1. Site characterization of studied soils.

Field collection areas[1]	Climate[2]	MAT (ºC)[3]	Annual rainfall (mm)	Vegetation type	Soil type
Cobar Pineplain 'Cobar' (NSW)	Hot semi-arid	15 - 19	258 - 537	Open woodlands of poplar and red box	Sandy loam
Pilbara biogeographical region 'Pilbara'(WA)	Hot semi-arid	19.4 - 33.2	250 - 400	Mulga low woodlands and hummock grasslands	Loamy sand
Simpson Strzelecki Dunefields 'Dunefields' (SA)	Hot desert	19 - 21	130 - 249	Perennial woodland and shrubland	Sand

[1] State between brackets. NSW: New South Wales, WA: Western Australia, SA: South Australia.

[2] Köppen climate classification

[3] MAT: Mean annual temperature

Table S2. Characteristics (chlorphyll *a* and carotenoids contents) of the cyanobacteria cultures forming the pellets inoculant.

Cyanobacteria species	Culture		Dry biomass	
	Chlorophyll *a* (µg· ml^{-1})	Carotenoids (µg· ml^{-1})	Chlorophyll *a* (µg· ml^{-1})	Carotenoids (µg· ml^{-1})
Leptolyngbya sp.	0.53±0.05	0.26±0.03	26.73±2.70	12.87±1.57
Nostoc sp.	1.32±0.18	0.38±0.12	22.05±3.02	6.35±1.94
Scytonema sp.	0.45±0.01	0.63±0.44	8.99±0.13	12.55±8.80

Chapter V:

Spectral response analysis: an indirect and non-destructive methodology for the chlorophyll quantification in biocruts

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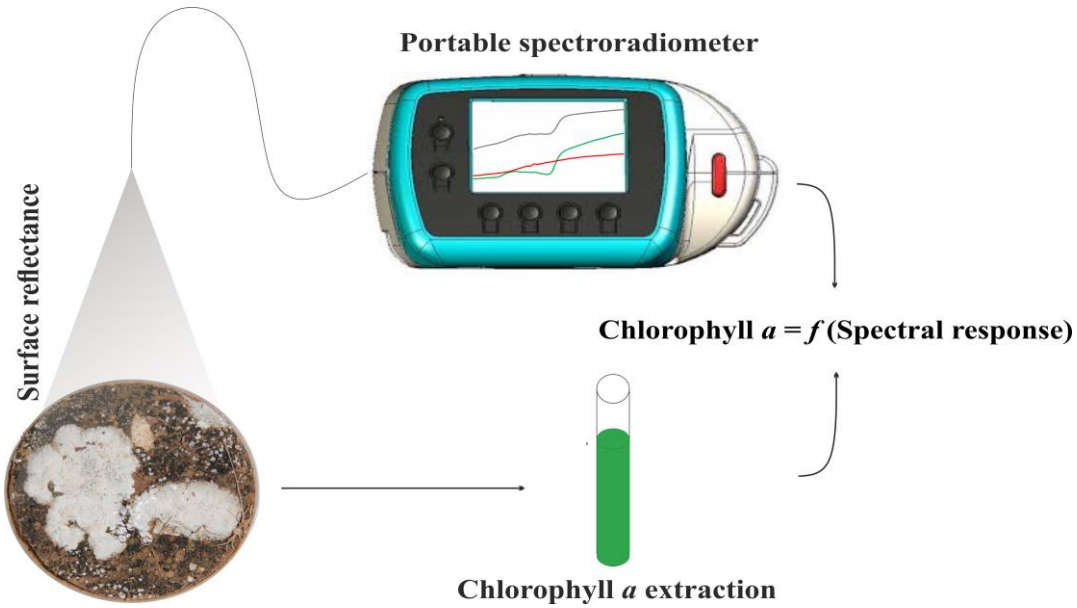

Abstract

Chlorophyll *a* concentration (Chl*a*) is a well-proven proxy of biocrust development, photosynthetic organisms' status, and recovery monitoring after environmental disturbances. However, laboratory methods for the analysis of chlorophyll require destructive sampling and are expensive and time consuming. Indirect estimation of chlorophyll a by means of soil surface reflectance analysis has been demonstrated to be an accurate, cheap, and quick alternative for chlorophyll retrieval information, especially in plants. However, its application to biocrusts has yet to be harnessed. In this study we evaluated the potential of soil surface reflectance measurements for non-destructive Chl*a* quantification over a range of biocrust types and soils. Our results revealed that from the different spectral transformation methods and techniques, the first derivative of the reflectance and the continuum removal were the most accurate for Chl*a* retrieval. Normalized difference values in the red-edge region and common broadband indexes (e.g. normalized difference vegetation index (NDVI)) were also sensitive to changes in Chl*a*. However, such approaches should be carefully adapted to each specific biocrust type. On the other hand, the combination of spectral measurements with non-linear random forest (RF) models provided very good fits ($R^2 > 0.94$) with a mean root mean square error (RMSE) of about 6.5 µg/g soil, and alleviated the need for a specific calibration for each crust type, opening a wide range of opportunities to advance our knowledge of biocrust responses to ongoing global change and degradation processes from anthropogenic disturbance.

Keywords: Biocrusts; biological soil crust; chlorophyll quantification; hyperspectral; random forest; remote sensing

Introduction

Water scarcity restricts vegetation growth and productivity in most desert regions around the world. Under these hostile conditions, other life forms, such as biological soil crusts (biocrusts) have been described to play a key role in ecosystem primary productivity (Pointing and Belnap, 2012). Biocrusts are inconspicuous communities, mainly dominated by photosynthesizing cyanobacteria, algae, lichens, or bryophytes that can survive during long drought periods in a dormant state and rapidly become active after small water pulses (Weber et al., 2016). These traits allow biocrusts to fix a large amount of atmospheric carbon and nitrogen (Williams et al., 2017; Büdel et al., 2018), which becomes incorporated within the upper layer of the soil to be used by heterotrophic fungi, bacteria, archaea (Maier et al., 2016), and other soil surface inhabitants (Bamforth, 2008; Darby et al., 2016). As a result, they improve soil fertility (Chamizo et al., 2012) and other soil properties, such as surface stability and soil water retention capacity (Belnap et al., 2014; Rodríguez-Caballero et al., 2015a; Chamizo et al., 2017; Adessi et al., 2018), preventing degradation processes and

maintaining the ecosystem capacities to provide multiple services to society (Rodríguez-Caballero et al., 2018).

Biocrusts' capacity to fix atmospheric carbon and nitrogen, as well as their role in all other key soil surface properties and processes, is mainly driven by the biomass of photosynthetically active organisms within the biocrust community (Lange, 2001) that have been demonstrated to be one of the best indexes of biocrust development (Zaady et al., 2010; Lan et al., 2017). Moreover, it is known that chlorophyll can be used to assess biocrust states in response to seasonal dynamics (Bowket al., 2002; Belnap et al., 2007), anthropogenic disturbances (Kidron et al., 2008; Dojani et al., 2011), ongoing climate changes (Ferrenberg et al., 2015; Rutherford et al., 2017), and restoration activities (Chamizo et al., 2018; Muñoz-Rojas et al., 2018). For all these reasons, large efforts have been devoted during the past decades to obtain reliable measurements of biocrust chlorophyll concentrations (Castle et al., 2011; Lan et al., 2011; Caesar et al., 2018). In a similar way to wet chemical methods used for the extraction of chlorophyll *a* from plant tissue, most of these techniques require extraction in a solvent followed by spectrophotometric determinations of the chlorophyll-related absorbance peaks in the supernatant, and then conversion from absorbance to concentrations using standard published equations (Castle et al., 2011; Lan et al., 2011; Caesar et al., 2018). Though accurate, these laboratory methods require destructive field sampling and are expensive and time consuming. This thereby limits the applicability for punctual measurements that may not represent the inherent spatial and temporal variability of biocrust communities within the landscape (Büdel et al., 2009; Rodríguez-Caballero et al., 2015b). For this reason, although biocrusts have been described as one of the main photosynthetic communities in dryland regions around the world (Rodríguez-Caballero et al., 2018), there exist only a few rough estimations of their contribution to the total photosynthetic biomass pool at regional (Rodríguez-Caballero et al., 2017a) or global scales (García-Pichel et al., 2003; Elbert et al., 2012; Porada et al., 2014) and these estimates rarely account for seasonal dynamics in biocrust biomass.

Chlorophyll *a*, as well as other pigments found in phototrophic organisms, absorbs incoming solar radiation at specific wavelengths. These specific spectral absorptions can be detected by means of field spectroscopy techniques that have been proven as an alternative for the non-destructive estimation of leaf and canopy chlorophyll concentrations (Gitelson et al., 2003; Le Maire et al., 2004; Zhao et al., 2005). Moreover, the combination of field spectroscopy techniques with airborne and satellite images, which provide spatially explicit information at high temporal resolutions, represents an ideal solution for the derivation of chlorophyll spatial distributions within the landscape (mapping and scaled-up, site-specific measurements for a whole ecosystem or region), and for large-scale monitoring of chlorophyll dynamics at a reasonable cost (Milton et al., 2009). Indeed, during the last few decades, there have been numerous studies using remote sensing methods for vegetation and phytoplankton chlorophyll analysis at different spatial and temporal scales (Louchard et al., 2002;

Stephens et al., 2003; Tong and He, 2017). Vastly different methodologies and indexes have been developed for these analyses using both multi- and hyper-spectral information (Yoder et al., 1995; Haboudane et al., 2002; Zhang et al., 2008; Croft et al., 2013; Elarab et al., 2015; Clevers et al., 2017). Contrastingly, though there are numerous studies demonstrating that biocrusts have spectral traits related to the presence of photosynthetic pigments (Weber et al., 2016; Weber et al., 2008; Rodríguez-Caballero et al., 2017b; Chamizo et al., 2012; Alonso et al., 2014; Rodríguez-Caballero et al., 2014), remote sensing applications for biocrust chlorophyll *a* quantification are mostly limited to a few local analyses using the normalized difference vegetation index (NDVI; e.g., Karnieli et al., 2001) or surface reflectance at 680 nm (Weber et al., 2016). This occurs for two main reasons. First, monitoring drylands and biocrusts from remote sensing platforms has been quite challenging because the spectral signal of these sites is often the results of the interaction between different components with different spectral properties and life cycles such as soil, biocrusts, and vascular plants (Weber et al., 2016; Rodríguez-Caballero et al., 2014; Karnieli et al., 2001). Second, when dry, the biocrust spectral signal is very subtle (Rodríguez-Caballero et al., 2014), which increases the difficulty in detecting chlorophyll-related absorption peaks, and therefore, the biocrust's chlorophyll quantification. Therefore, any application of spectroscopy for the retrieval of Chl*a* in the biocrust should first examine the spectral response of different biocrust communities colonizing soils with different biocrust coverages and soil conditions, such as differing soil texture, color, or soil quality, before this technology can be directly transferred to produce reliable Chl*a* estimations in the field.

The goals of this study were: i) to analyze the effect of Chl*a* on the spectral response of different natural and artificially induced biocrust communities in different soils, and with different biocrust cover; and ii) to explore whether this information, the spectral transformations, and the most widely used spectral indexes proposed in the literature are appropriate for non-destructive chlorophyll *a* quantification in biocrusts. Finally, we discuss the applicability of the different methods for non-destructive monitoring applicable to long-term studies aimed at analyzing the effects of ongoing climate change on the biocrust communities, as well as their usefulness to monitor biocrust recovery after disturbances under natural conditions and on assisted restoration activities, depending on the available information and requirements.

Materials and Methods

Sample collection

Selected with contrasting differences in soil composition and color (Supplementary Table S1), a total set of 93 samples of natural biocrust communities were collected from three semiarid experimental areas within the province of Almeria (Spain) (Figure 1): i) Las Amoladeras, within the Cabo de Gata-Níjar Natural Park (N 36° 50' 01" W 02° 15' 08); ii) El Cautivo experimental area, within the

Tabernas Desert (N 37° 00’ 37” W 02° 26’ 30”); and iii) a calcareous quarry in Sierra de Gádor (W 36° 55’ 20’’ 02° 30’ 29’’ W). The mean annual rainfall and temperature in the selected areas ranges from 200–242 mm and from 17.2–19 °C, respectively.

Las Amoladeras is a semiarid steppe dominated by the alpha grass species *Macrochloa tenacissima* (L.) Kunth (= *Stipa tenacissima* L.) and sandy loam soils with a 10YR 4/6 color (dry). Most of the open spaces between plants are covered by three main biocrust types that together represent more than 30% of the total area: a) cyanobacteria-dominated biocrust, composed of well-developed communities of cyanobacteria along with some dark lichens; b) lichen-dominated biocrusts, mostly *Diploschistes diacapasis* (Ach.) Lumbsch and *Squamarina lentigera* (Weber) Poelt; and c) moss-dominated biocrusts that also contain a small proportion of cyanobacteria (≈15%) (see Chamizo et al., 2012 for further details).

El Cautivo is a badlands landscape developed on Tortonian gypsiferous mudstones with silty loam textured soils (Cantón et al., 2003) that have a 2.5Y 6/3 color (dry). The vegetation is mainly *M. tenacissima* that grows along with other dwarf shrubs and a dense layer of biocrust mainly dominated by two different types: a) cyanobacteria-dominated biocrusts that also contained numerous pioneer lichens, and b) lichen-dominated biocrusts mainly composed of the light-colored *Diploschistes diacapasis* and *Squamarina lentigera* (Lázaro et al., 2008). Moss-dominated biocrusts are also representative in some topographical positions, such as in the steep northern aspect hillslopes where low incoming solar radiation levels maximize water availability. A detailed description of the area can be found in Cantón et al. (2003).

The third study site (Gádor limestone quarry experimental area), described in Luna et al. (2018), was chosen as an example of a highly degraded ecosystem where biocrusts are present in an early successional stage. The soil texture in the area is clay loam with a 2.5Y 8/3 color (dry). Two main biocrust types were identified at this site: a) cyanobacteria-dominated biocrusts, mostly incipient cyanobacteria mixed with small, dark pioneer lichens including *Collema* sp.; and b) moss-dominated biocrusts in spaces close to the shrub patches in undisturbed areas.

Figure 1 (A) Study area location. (B) Exemplars of different natural biocrust types found in the study zones: cyanobacteria-dominated, lichen-dominated, and moss-dominated biocrusts.

From each biocrust community described at the three different sites (cyanobacteria-dominated biocrust from Las Amoladeras, El Cautivo, and Gádor quarry; lichen-dominated biocrusts from Las Amoladeras and El Cautivo; and mosses-dominated biocrust from Las Amoladeras, El Cautivo, and Gádor quarry), a minimum of 12 replicates, representing different compositions and coverage values, were collected between December 5–7, 2017. Before sampling, the crust coverage and composition were visually estimated and samples were selected representing different compositions and coverage values. For sampling, a 90 mm (diameter) per 15 mm (depth) Petri dish was used. The Petri dish was pressed 15 mm deep into the soil, a spatula was pushed underneath, and then both were pulled out and the Petri dish was sealed with the cap. Then, samples were transported to the University of Almería, air dried, and stored at room temperature at low light intensities for less than one week until the surface spectra and chlorophyll *a* extraction measurements were conducted.

At the same experimental areas, soil substrates were collected at a 2 cm depth, air-dried, and sieved to 2 mm in order to remove the small herbs and rock fragments. Three native filamentous nitrogen-fixing cyanobacteria species, *Nostoc commune* (CANT2 UAM 817), *Scytonema hyalinum* (CAU6

UAM 820), and *Tolypothrix distorta* (CANT7 UAM 825) that had been previously isolated from undisturbed biocrusts (Roncero-Ramos et al., 2019) and cultured ex situ were inoculated separately and in a mixture (1:1:1) onto Petri dishes containing soils from the three different study sites. Furthermore, non-inoculated samples from each soil type were set to serve as a control (hereafter "bare soil"). A total set of 119 inoculated soils were placed in a Fitotron Plant Growth Chamber (University of Almería, Almería, Spain) at a constant temperature (25 ± 1 °C) and a light intensity of 60 μmol photons m^{-2} s^{-1} with a 16 h photoperiod for 90 days before the spectral and chlorophyll a measurements were conducted (see details in Román et al., 2018).

Combining all these samples, we obtained a final dataset of 212 samples (93 natural and 119 artificially induced) that represented a wide range of biocrust coverage and developmental stages in different soil types with contrasting spectral properties.

Spectral Measurements and Biocrust Coverage Estimation

Thirty minutes before the spectral measurements, all samples were irrigated with 30 mL of distilled water. Next, the surface reflectance of each sample was measured using an ASD FieldSpec® Handheld portable spectroradiometer (ASD Inc., Boulder, CO, USA) with a 3.5 nm optical resolution from 325 nm to 1075 nm. All measurements were conducted in stable light conditions provided by two 14.5 V, 50 W ASD Pro Lamp (ASD Inc., Boulder, CO, USA) halogen lamps, with the optical fiber placed 16 cm above the sample surface, resulting in a field-of-view (FOV) area of 66 cm^2 corresponding to the entire surface of the Petri dish. A total of three replicate measurements, each consisting of the average of the three individual spectra, were collected for each sample and then averaged to produce the sample surface spectrum. A Spectralon (ASD Inc., Boulder, CO, USA) white reference sample was also measured before each set of three biocrust spectra.

In addition, after the spectral measurements, a zenithal picture of each sample was acquired using a digital Canon EOS 600D (Canon Inc., Tokyo, Japan) placed at nadir, 25 cm above the sample surface. Then, the biocrust coverage (%) on the soil substrates was calculated using the supervised maximum likelihood classification in the ENVI 4.7 software (ITT VIS, Boulder, CO, USA).

Chlorophyll *a* extraction

After the spectral measurements, a 3-mm thick biocrust subsample was collected from each Petri dish for determining the biocrust chlorophyll a concentrations (Chl*a*). Samples were air-dried at ambient temperature, crushed with a roller, and then ground with a mechanical agate mortar. Chlorophyll *a* extraction from soil samples was conducted by following the ethanol double-extraction method developed by Castle et al. (2011). The supernatants from both extractions were combined and the absorbance was measured at 649, 665, and 750 nm. As the inoculated soils were

only comprised of cyanobacteria-inoculated strains, a specific equation developed by Ritchie (2006) for these organisms was applied in order to calculate Chl*a*, as follows:

$$\text{Chl}a\ (\mu\text{g/g soil}) = \frac{(11.9035 \times \text{A}(665 - 750) \times \text{V})}{(\text{g soil} \times \text{L})} \quad (1)$$

where the absorbance coefficients have the dimensions μg·cm/mL·A, A is the absorbance value at the specific wavelength, V is the volume of the extract (mL), and L is the optical path length of the spectrophotometer cuvette (cm).

For natural biocrust communities, comprised of a mix of different photoautotrophic organisms, Chl*a* was calculated by applying the equation for chlorophytes and bryophytes reported by Ritchie (2006) (Equation 2):

$$\text{Chla}\ (\mu\text{g/g soil}) = \frac{(13.5275 \times \text{A}(665 - 750) - 5.201 \times \text{A}(649 - 750)) \times \text{V})}{(\text{g soil} \times \text{L})} \quad (2)$$

where the absorbance coefficients have the dimensions μg·cm/mL·A, A is the absorbance value at the specific wavelength, V is the volume of the extract (mL), and L is the optical path length of the spectrophotometer cuvette (cm).

Spectral Data Analysis

Before the spectral analyses, all data were subjected to a pre-treatment that consisted of removing the noisy bands (350–400 nm and above 950 nm) and applying a cubic polynomial smoothing filter with a 17-nm window size [60]. Moreover, all spectra were resampled to a Sentinel-2 multispectral resolution, which included broad bands at the blue (490 nm), green (560 nm), red (665 nm) and near-infrared (NIR, 842 nm) regions of the spectra, as well as some narrower bands within the red edge region (740, 783, and 865 nm). This procedure was done by applying the spectral resampling functions included within the "hsdar" package in R version 3.3.3 (R-project; Lehnert et al., 2018).

Data transformation

As the reflectance at a single determined wavelength (R) is very sensitive to variable irradiance, background effects, and the geometric arrangement of the sensor and surface (Munden et al., 1994), we explored the usefulness of well-known spectra transformations that have proven to be very useful for isolating the particular absorption features of different pigments, such as the continuum removal (CR; Clark and Roush, 1984; Mutanga et al., 2004) and the first derivative of the spectra (ρ; Filella

and Peñuelas, 1994; Curran et al., 2001; Xue et al., 2009). The CR and ρ were calculated for each biocrust type using the pre-treated spectra from 400 to 950 nm by 1 nm. The CR was calculated by applying the convex hull fit over the top of a spectrum using straight-line segments that connect the local spectra maxima so that values equal to 1 indicate the absence of absorption peaks (the spectrum matches the convex curve), while values lower than 1 indicate the presence of absorption peaks (Clark and Roush, 1984). The ρ was calculated using the Savitzky and Golay (Savitzky and Golay, 1964) method with third-order polynomials and a 17-nm window size, based on previous exploratory analysis. We also estimated the band depth (1- CR; BD) for the complete 212-sample dataset and from this we calculated several well-known spectral properties related to two main chlorophyll spectral absorptions at 460 nm and 680 nm including: i) the area under the absorption peak (area), ii) the maximum absorption value (max), iii) the wavelength at which maximum absorption is observed (maxwl), iv) the wavelength positions of upper and lower half-max values (up-wlhm and lo-wlhm), v) the difference between wavelength positions of upper and lower half-max values (width wlhm), and vi) the root mean square error (RMSE) between a Gaussian distribution function and the feature values between the lower half-max and the maximum position and between the upper half-max and the maximum position (gaus-low wlhm and gaus-up wlhm). All analyses were done using the "hsdar" package in R (R-project; Lehnert et al., 2018).

Band Ratios and Standard Spectral Indexes

Biocrust spectra at hyper- and Sentinel-2 multispectral resolutions were also used to calculate the normalized difference indexes (ND) for all possible band combinations between 450–900 nm, following Equation 3:

$$\mathrm{ND} = \frac{(\mathrm{RB1} - \mathrm{RB2})}{(\mathrm{RB1} + \mathrm{RB2})} \tag{3}$$

where ND is the normalized difference index between bands 1 and 2, and RB1 and RB2 are the reflectance values at spectral bands B1 and B2, respectively.

Finally, a set of 38 existing published Chla-related narrow- and broad-band vegetation indexes were also calculated for each sample (Supplementary Table S2). The selected indexes were chosen from previous studies that reviewed leaf chlorophyll quantification from spectral information (Le Maire et al., 2004; Xue et al., 2009; Main et al., 2011). Specific details about the formulations and reference sources are summarized in Supplementary Table S2.

Statistical Analysis

The differences in Chla between the different biocrust types and sample sites were analyzed using the two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc test. Moreover, a linear regression analysis was performed to explore the relationships between Chl*a* concentration and R, CR, and ρ values of different bands of the spectrum, as well as the relationships between Chl*a* and all possible ND and standard vegetation indexes. This was repeated for the complete 212 sample dataset and for each crust type separately: i) artificially induced cyanobacteria biocrusts ("inoculated") on soils from Las Amoladeras, El Cautivo and Gádor quarry; ii) natural cyanobacteria biocrust communities ("cyanobacteria-dominated") from Las Amoladeras, El Cautivo, and Gádor quarry; iii) lichen biocrusts ("lichen-dominated") from Las Amoladeras and El Cautivo; and iv) mosses biocrust ("moss-dominated") from Las Amoladeras, El Cautivo, and Gádor quarry. For all regression models, the differences between predicted values and observed values was assessed using the root mean squared error (RMSE) and the coefficient of determination (R^2) of the chlorophyll quantifications were estimated.

Finally, we developed a random forest (RF) multivariate model for Chl*a* estimation, which is applicable to a wide range of biocrust coverage and compositions using all spectral properties derived from the two main Chla absorption features corresponding to the first two peaks of the BD values (at 460 nm and 680 nm). To do this, we used the complete dataset in a 20-fold cross-validation strategy. Moreover, in order to test the influence of biocrust coverage on our Chla estimations, we repeated this model also considering the coverage of incipient cyanobacteria, well-developed cyanobacteria and some pioneer lichens such as Collema cristatum (L.) F.H. Wigg, Endocarpon pusillum Hedw, Fulgensia fulgida (Nyl.) Szat, and Fulgensia desertorum (Tomin), lichens, and mosses. The number of predictor variables performing the data partitioning at each node and the total number of trees to be grown in the model run were set to 20 and 100, based on a previous tuning experiment. Model fit was tested using the mean RMSE obtained during the cross-validation process, and importance of predictor variables was measured using the Gini decrease in node impurity measure, which is computed by permuting the predictor variables with the out-of-bag data in the RF validation approach (details in Liaw and Weiner, 2002). Finally, in order to get an estimation of linear fit between estimated versus measured Chl*a* concentrations that were comparable with this obtained by linear regression, we applied the obtained RF model to the complete dataset.

Results

Chla of Different Biocrust Types

From the different natural biocrust types considered in this study, the moss-dominated biocrusts showed the highest Chl*a* amongst the three different study sites. The values ranged from 8 µg/g soil in the moss samples with a surface coverage below 28%, to 54 µg/g soil on fully covered samples

from Las Amoladeras (Figure 2). The lichen-dominated biocrusts showed intermediate values (from 5.28–30.31 µg/g soil), whereas cyanobacteria showed the lowest values amongst the three different sites (from 1.22–38.94 µg/g soil). However, the differences between cyanobacteria- and lichen-dominated biocrusts were not significant as a consequence of the large variability in coverage and chlorophyll concentrations amongst the different samples (Figure 2). The Chl*a* of the 119 artificially inoculated cyanobacteria (three months old) samples ranged from 0–34.05 µg/g soil. The highest values corresponded to cyanobacteria inoculations on soils from Las Amoladeras that showed very similar values to those found in natural cyanobacterial biocrust communities, whereas the artificially induced cyanobacterial biocrusts on the quarry substrates showed the lowest Chl*a* amongst all the samples analyzed in this study (Figure 2).

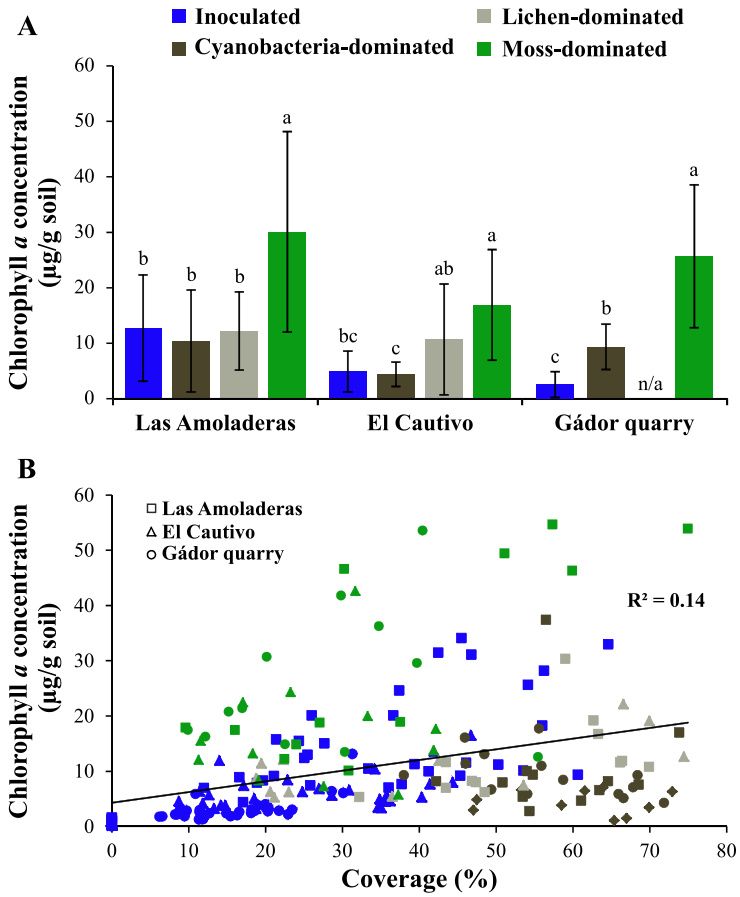

Figure 2 (A) Mean (± SD) of the chlorophyll *a* concentration (µg/g soil) in different crust types in Las Amoladeras, El Cautivo, and Gádor quarry study sites. (B) Linear regression of the chlorophyll *a* concentration and biocrust coverage (%).

Effect of Biocrust and Chla on the Soil Surface Spectra

Figure 3 shows the mean reflectance, CR, and ρ of the different natural biocrust communities from the three different study sites, as well as the values of the spectral variables for the artificially induced cyanobacterial biocrusts on the soils from the three sites. Overall, the biocrust samples dominated by

white lichens showed higher reflectance values than the bare soils, whereas the moss- and natural cyanobacteria-dominated crusts showed the opposite effect (Figure 3). The artificially inoculated cyanobacteria biocrusts also produced a slight decrease in the overall surface reflectance of the three different reference soils, but this effect was lower than the effect from the natural communities (Figure 3). There were also some important differences in the spectral shape between the bare soils and biocrusts. For example, all biocrust types showed an important absorption peak at about 670 nm that was not pronounced in the reflectance spectrum but could be easily identified in the CR, ρ, and BD (Figure 3). Overall, samples with higher biocrust coverage or Chl*a* had a more accentuated spectral absorption with a minimum in the CR curve at 670 nm and a spike in the ρ (Figure 3), with some exceptions occurring on the lichen and natural cyanobacteria samples (Figure 4b). Subsequently, this spectral absorption was more pronounced on the moss-dominated biocrusts than on the lichen, cyanobacteria, and artificially inoculated cyanobacteria biocrusts (Figure 3), exhibiting a similar pattern to the Chl*a*. Another important spectral absorption at about 500 nm was identified from the CR and ρ of the moss, cyanobacteria, and bare soil samples that was masked in lichen-dominated biocrusts. When the spectral signatures of the different biocrusts were resampled to Sentinel-2 multispectral resolution, the spectral curves showed a similar shape, but differences were subtler than those observed at a hyperspectral resolution (Figure 5).

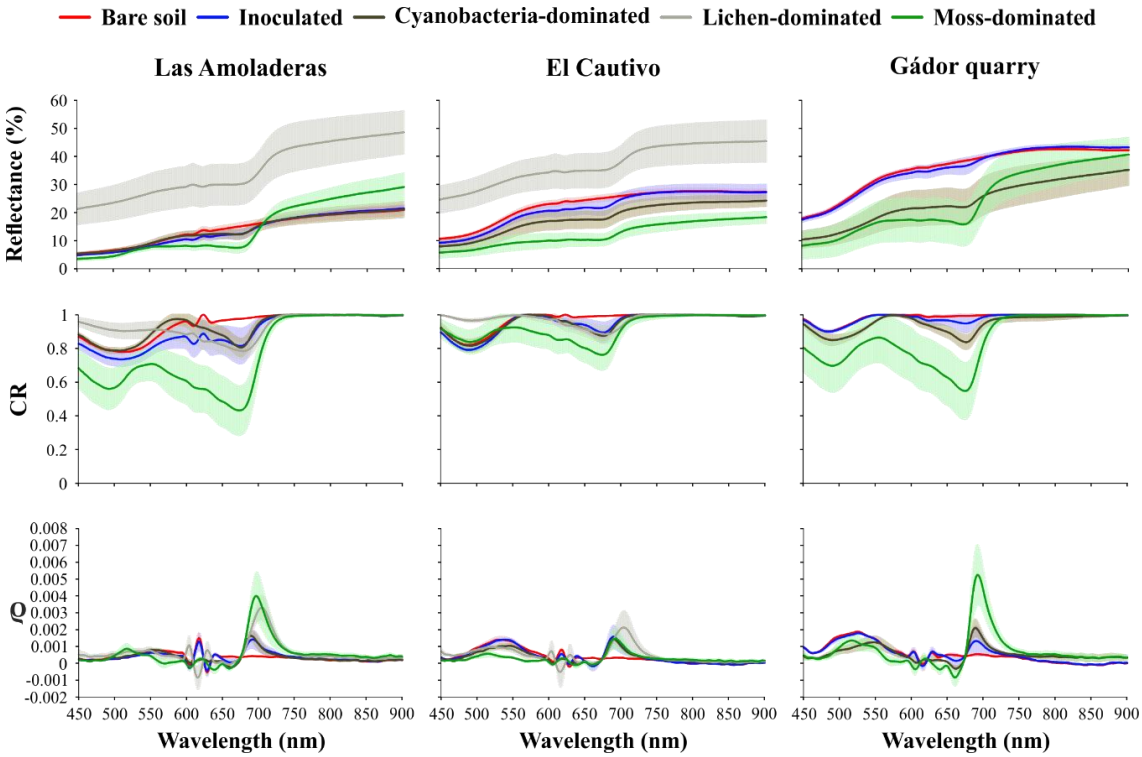

Figure 3 Mean (± SD represented as the shaded area) of the hyperspectral reflectance (R), continuum removal (CR), and first derivate of reflectance (ρ) of different natural biocrust types, as well as artificial biocrust (inoculated) and non-inoculated control plots samples (bare soil), in Las Amoladeras, El Cautivo, and Gádor quarry study sites.

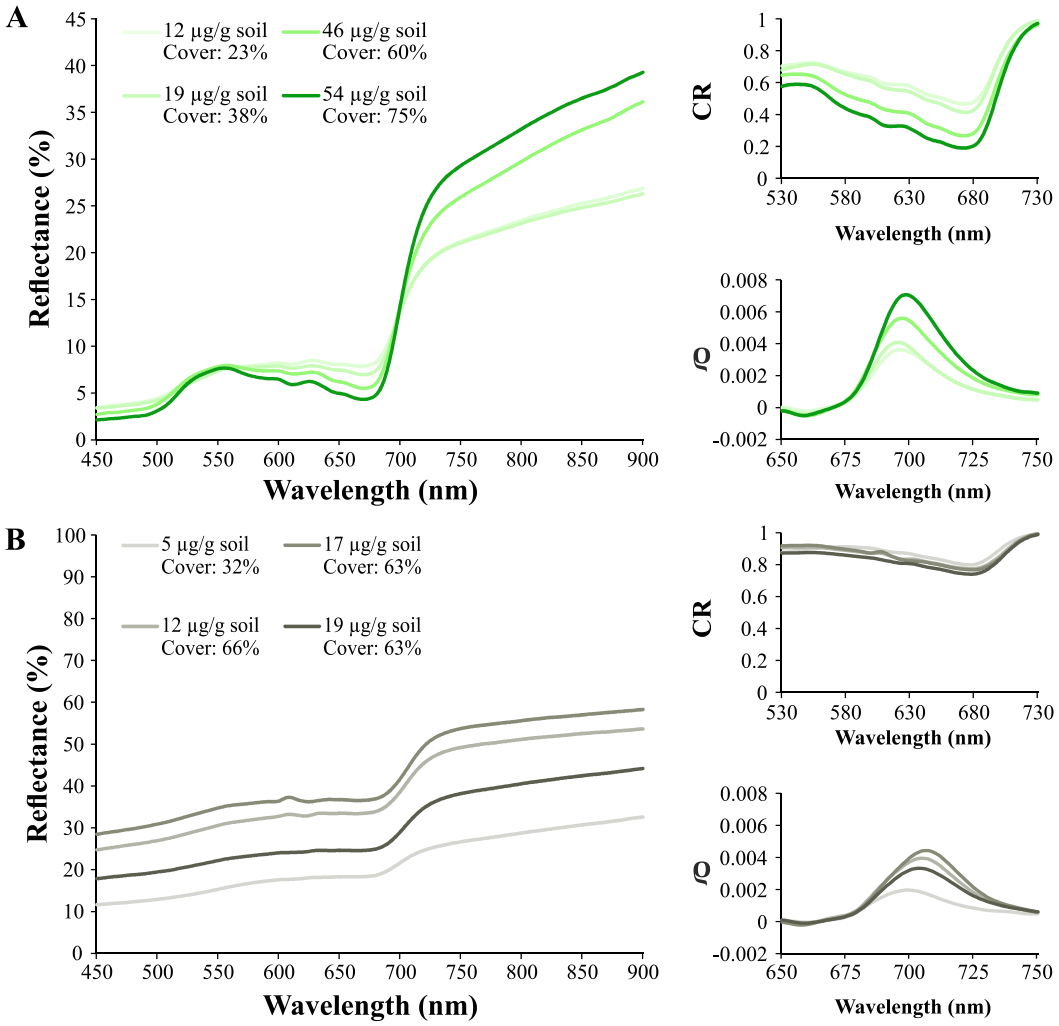

Figure 4 Reflectance, continuum removal (CR), and first derivate of reflectance (ρ) for selected samples of (A) moss- and (B) lichen-dominated crust with different cover and Chla concentration from the Las Amoladeras study site.

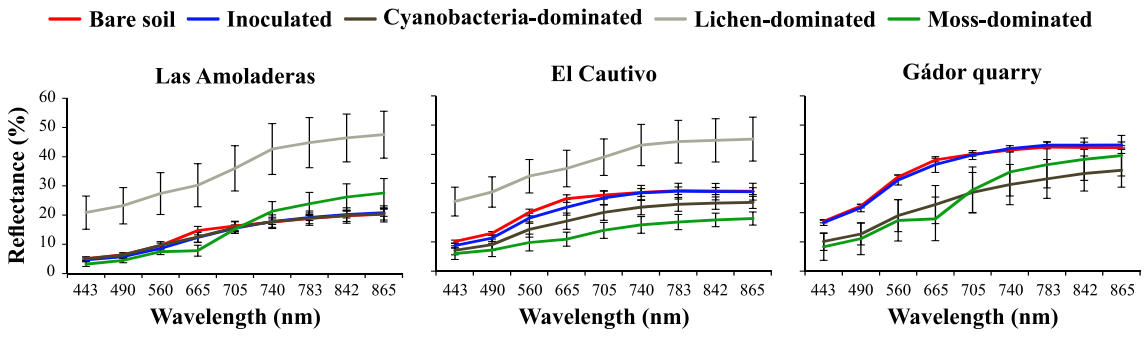

Figure 5 Sentinel-2 resampled reflectance for different biocrust types in Las Amoladeras, El Cautivo, and Gádor quarry study sites.

Table 1 Performance of the reflectance (R), continuum removal (CR), first derivative of the reflectance (ρ), normalized difference (ND), and standard vegetation indexes for predicting chlorophyll a concentration in different biocrust types. The regression model for the band, band combination, or standard vegetation index (within parenthesis) with the highest coefficient of determination (R^2) and the lowest root mean square error (RMSE) is shown for each biocrust type. Bold values represent significance at 95% confidence interval or $p < 0.05$.

		R		CR		ρ	
	Biocrust Type	**Model**	**R^2 (RMSE)**	**Model**	**R^2 (RMSE)**	**Model**	**R^2 (RMSE)**
Hyperspectral data	Inoculated	**−43.91x+ 17.25 (673)**	**0.37 (5.85)**	**−86.71x + 85.51 (646)**	**0.71 (3.95)**	**−32278x + 31.75 (565)**	**0.53 (5.04)**
	Cyanobacteria	21.89x + 2.29 (900)	0.07 (6.03)	**1114x + 1101.50 (894)**	**0.2 (5.59)**	**20439x − 4.03 (720)**	**0.27 (5.33)**
	Lichen	**38.50x − 6.30 (853)**	**0.23 (5.3)**	**23062x + 23048 (765)**	**0.18 (5.47)**	**28969x − 0.13 (581)**	**0.41 (4.65)**
	Moss	69.87x + 3.94 (900)	0.25 (23.62)	**−58.79x + 60.50 (663)**	**0.60 (9.19)**	**19148x − 3.22 (723)**	**0.65 (8.60)**
	All	**−45.66x + 20.21 (674)**	**0.21 (9.49)**	**−62.23x + 64.24 (655)**	**0.67 (6.11)**	**6084.40x + 0.49 (698)**	**0.51 (7.43)**
Multispectral data	Inoculated	**−43.98x + 17.28 (665)**	**0.36 (5.88)**	**−221.66x + 224.83 (705)**	**0.67 (4.22)**	**−17296x + 19.23 (560)**	**0.41 (5.69)**
	Cyanobacteria	9.30x + 6.67 (665)	0.01 (6.23)	−36.45x + 40.61 (665)	0.08 (5.99)	**20297x − 1.81 (740)**	**0.22 (5.52)**
	Lichen	**39.50x − 1.44 (665)**	**0.21 (5.30)**	−15.54x − 1.87 (665)	0.03 (5.96)	**26617x − 2.24 (560)**	**0.26 (5.21)**
	Moss	−53.95x + 31.05 (665)	0.05 (14.09)	**−61.54x + 64.05 (665)**	**0.6 (9.16)**	**23194x + 2.47 (740)**	**0.64 (8.71)**
	All	**−44.85x + 20.20 (665)**	**0.19 (9.57)**	**−61.09x + 63.57 (665)**	**0.67 (6.12)**	**13415x − 1.40 (705)**	**0.47 (7.75)**

Table 1. Continuation.

	Biocrust Type	Normalized Difference		Standard Vegetation Indexes	
		Model	R^2 (RMSE)	Model	R^2 (RMSE)
Hyperspectral data	Inoculated	**320.91x − 3.50**	**0.72**	**6.35x − 0.99**	**0.73**
		($ND_{730-704}$)	**(3.90)**	**(EGFR)**	**(3.84)**
	Cyanobacteria	**1182.20x − 9.36**	**0.26**	**154.82x − 2.68**	**0.21**
		($ND_{466-455}$)	**(5.37)**	**(∑dRE)**	**(5.56)**
	Lichen	**−9702.90x + 19.02**	**0.19**	**74.14x + 32.80**	**0.46**
		($ND_{679-678}$)	**(5.45)**	**(BND)**	**(4.42)**
	Moss	**4757.60x − 0.95**	**0.65**	**1094.10x + 6.51**	**0.69**
		($ND_{519-518}$)	**(8.57)**	**($MCARI_{[705,750]}$)**	**(8.09)**
	All	**91.32x − 0.53**	**0.68**	**312.42x − 316.61**	**0.60**
		($ND_{734-687}$)	**(6.02)**	**(Vogelman3)**	**(6.73)**
Multispectral data	Inoculated	**273.25x − 4.02**	**0.71**	**25.42x − 27.18**	**0.71**
		($ND_{740-705}$)	**(3.96)**	**(SR)**	**(3.98)**
	Cyanobacteria	**152.57x + 1.23**	**0.11**	**69.99x − 0.71**	**0.18**
		($ND_{740-705}$)	**(6.10)**	**(EVI)**	**(5.68)**
	Lichen	−55.45x + 16.45	0.05	**−4.79x + 24.52**	**0.21**
		($ND_{705-665}$)	(6.20)	**(BSCI)**	**(5.36)**
	Moss	**104.27x + 2.68**	**0.62**	**95.27x − 0.07**	**0.64**
		($ND_{705-665}$)	**(9.57)**	**(EVI)**	**(8.67)**
	All	**85.44x − 2.06**	**0.69**	**85.09x − 3.10**	**0.66**
		($ND_{740-665}$)	**(6.28)**	**(OSAVI)**	**(6.21)**

Chlorophyll a Predictions

Direct Chl*a* Predictions from the Surface Reflectance, CR, and ρ

The relationships between the biocrust spectra and Chl*a* are shown in Figure 6. Overall, hyperspectral information provided more accurate results in Chl*a* predictions than multispectral data, as shown by slightly higher values of the coefficient of determination and lower RMSE (Figure 6, Table 1). The soil surface reflectance within the Vis-NIR region decreased as Chl*a* increased, with the best regression coefficient found at about 674 nm (Figure 6, Table 1). However, the regression fits were very low at both multi- and hyperspectral resolutions (R^2 = 0.21 and 0.19, respectively). The CR in the red region and ρ transformation showed a better relationship with Chl*a* than surface reflectance, yielding a coefficient of determination of up to 0.68 and 0.51, respectively. Overall, the CR values decreased as Chl*a* increased, with the best regression fits between CR and chlorophyll observed at 655 nm (Figure 6, Table 1). On the other hand, ρ showed the best regression fits with spectral bands at the inflection point of the red-edge (about 700 nm).

When the different biocrust communities were analyzed separately, we found that the correlation patterns between the surface reflectance, CR, and ρ and Chl*a* strongly varied amongst the biocrust communities (Figure 7a,b). Whereas the CR within the red region showed the best correlation with Chla on the artificially induced cyanobacteria biocrusts (646 nm, R^2 = 0.71), and ρ at the inflection point of the red edge showed the best results on the moss and natural cyanobacteria dominated biocrust communities (723 nm and R^2 =0.65, and 720 nm and R^2 = 0.27, respectively) (Figure 7b, Table 1). Chla within the lichen-dominated biocrusts best fit to the ρ values within the green region of the spectra (581 nm, R^2 = 0.41).

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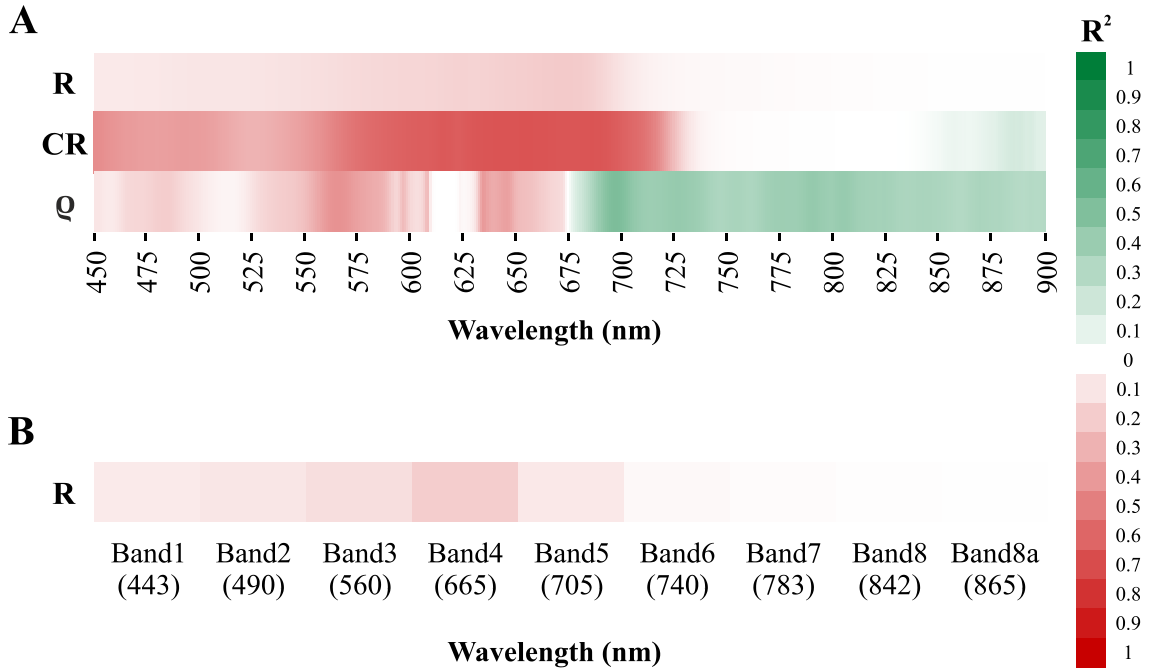

Figure 6 Comparing the coefficient of determination (R^2) between reflectance (R), continuum removal (CR), and first derivate of reflectance (ρ) and chlorophyll *a* concentration for all the studied communities calculated with the hyperspectral (A) and Sentinel-2 resampled (B) data. I: incipient, C: cyanobacteria-dominated, L: lichen-dominated, and M: moss-dominated. Green scale values of the coefficient of determination indicate a positive relationship between response variable (Chla) and predictor factor (spectral information), whereas red scale values indicate a negative relationship.

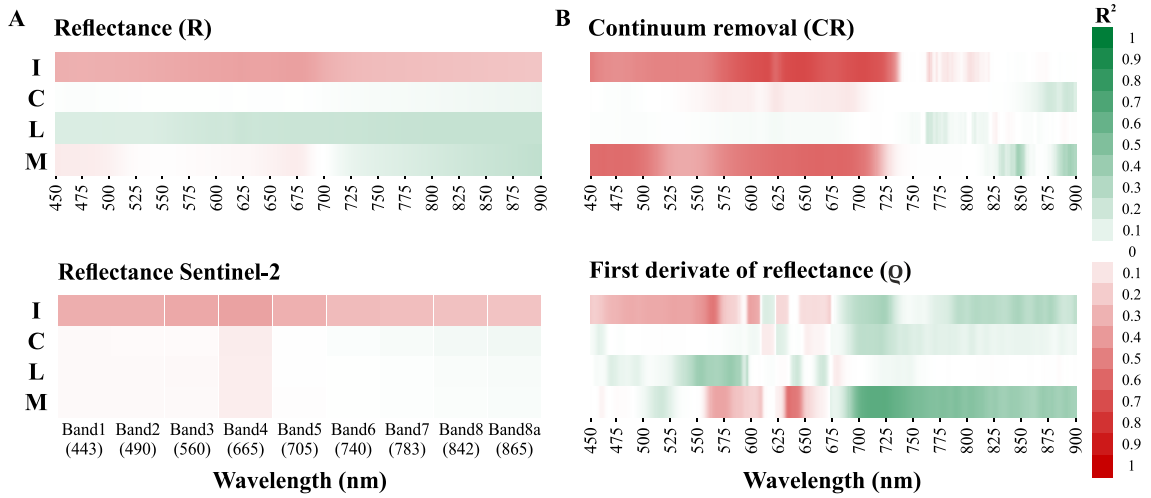

Figure 7 Comparing the coefficient of determination (R^2) between: (A) reflectance calculated with the hyperspectral data (R) and reflectance calculated with Sentinel-2 resampled data and chlorophyll *a* concentrations for each biocrust type; and (B) continuum removal (CR), and first derivate of reflectance (ρ) and chlorophyll a concentrations for each biocrust type calculated with the hyperspectral data. I: incipient, C: cyanobacteria-dominated, L: lichen-dominated, and M: moss-dominated. Green scale values of the coefficient of determination indicate a positive relationship between the response variable (Chl*a*) and predictor factor (spectral information), whereas red scale values indicate a negative relationship.

Chlorophyll *a* Predictions from the Normalized Band Ratios

The normalized band combination ratios were as sensitive to chlorophyll changes as CR and ρ at both the hyperspectral and Sentinel-2 multispectral resolutions (Table 1). For example, when all biocrust samples were analyzed together, the best fits were obtained by combining the chlorophyll absorption band at 687 nm and the reflectance from 731–736 nm for the hyperspectral data, and band 4 and band 5–6 for the Sentinel-2 spectral resolution, yielding a coefficient of determination of 0.69 (Table 1; Supplementary Figures S5 and S10). However, similar to the results found when the Chl*a*-R, CR, and ρ regressions were analyzed, the best band selection and overall fit strongly varied between the crust types. For artificially inoculated cyanobacteria, the best results were obtained using the combination of the surface reflectance at 704 nm (band 5 of Sentinel-2) and the surface reflectance at 730 nm (band 6 of Sentinel-2), yielding a coefficient of determination of about 0.72 (Table 1; Supplementary Figures S1 and S6). The moss chlorophyll showed a better correlation with band ratios based on spectral reflectance at 518 and 519 nm, yielding a coefficient of determination of 0.65. However, these subtle spectral traits were not correctly characterized at the Sentinel-2 spectral resolution, at which the best correlation was found using band 4 and bands 5–6 ($R^2 = 0.62$) (Table 1; Supplementary Figure S9). Band ratios within the red or NIR regions were not sensitive to changes in Chl*a* in the natural cyanobacteria-dominated crusts that showed the best relationship with band ratios combining the surface reflectance at 466 nm and 455 nm (Table 1; Supplementary Figure S2). As previously shown for R, CR, and ρ, the absolute values were again very low ($R^2 = 0.26$). On the lichen-dominated biocrust samples, no significant relationship was found between the normalized bands and Chl*a* for most bands. In these crusts, only the region around 680 nm showed a poor relationship with Chl*a* ($R^2 = 0.19$; Table 1; Supplementary Figure S3).

Sensitivity of the Standard Spectral Indexes to the Chlorophyll *a* Determination

Similar to the normalized band ratios, the standard spectral indexes also were sensitive to changes in the biocrust chlorophyll concentration. Of the 28 narrow band and 10 broad band spectral indexes (Supplementary Table S2), 6 were robustly sensitive to the biocrust Chl*a* ($R^2 > 0.6$; Figure 8). The best retrievals for the general biocrust communities (all samples) were provided by the broad band greenness indexes using red and NIR information, such as the NDVI, simple ratio (SR), modified simple ratio (MSR), soil adjusted vegetation index (SAVI), and the optimized soil-adjusted vegetation index (OSAVI), yielding a coefficient of determination above 0.6 at both the hyper and Sentinel-2 spectral resolutions (Figure 8, Table 1). Biocrust specific indexes crust index (CI) and biological soil crust index (BSCI), on the other hand, did not provide good results.

The broad band greenness indexes also appeared to be good predictors of Chl*a* for the artificially induced cyanobacteria and the natural moss biocrust samples. However, when these biocrust types were analyzed separately, some narrow band pigment-related indexes, such as the modified

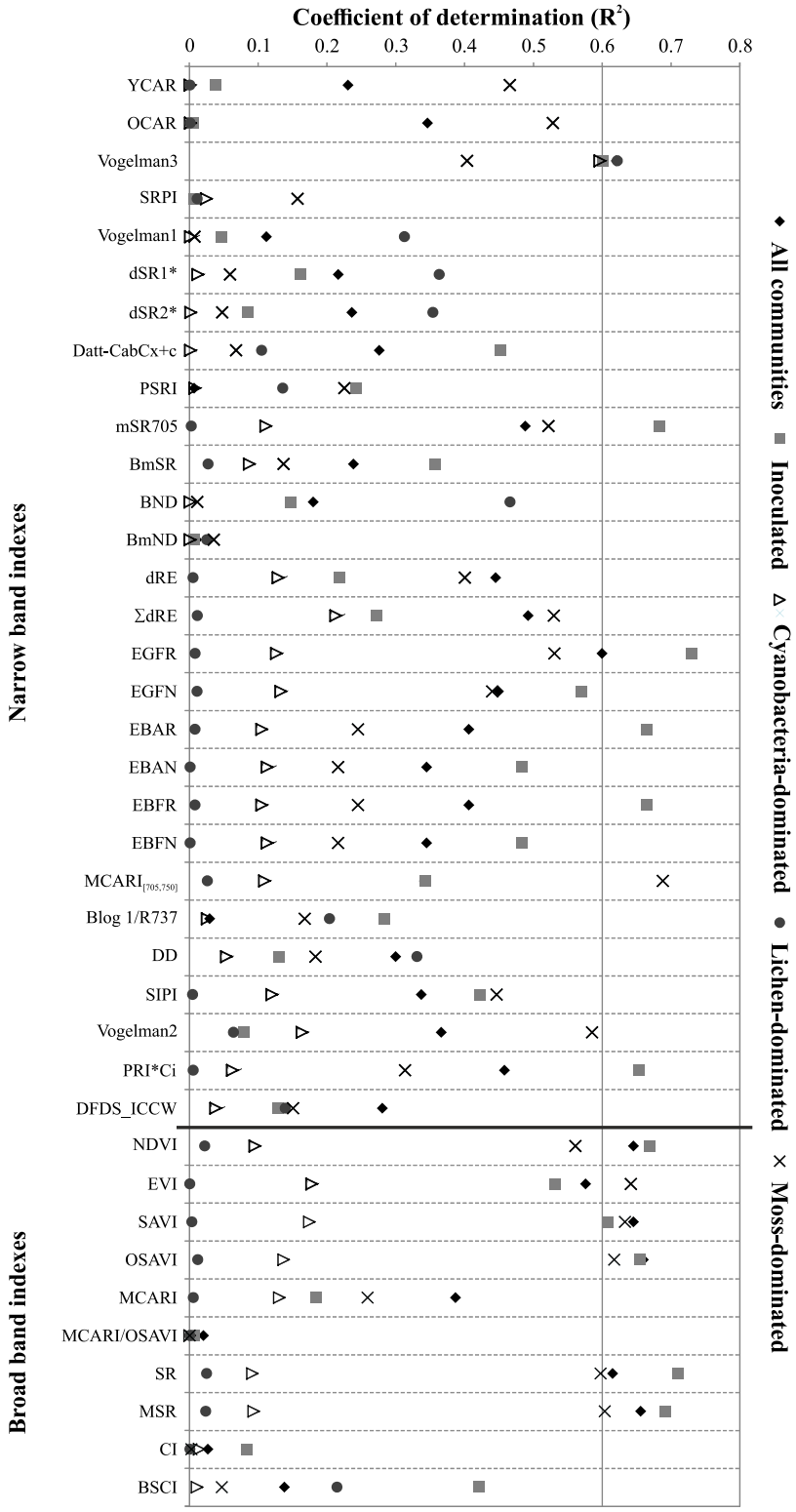

Figure 7 Coefficient of determination (R^2) between the chlorophyll a concentration and the standard vegetation indexes for each biocrust type.

chlorophyll absorption in reflectance index ($MCARI_{[705,750]}$) (for mosses) and the ratio of first derivative maxima in red-edge region and green region (EGFR) (for artificially induced cyanobacteria) showed better relationships with the chlorophyll concentrations than the broad band indexes, yielding coefficients of determination up to 0.69 and 0.72, respectively. In the case of the

lichen-dominated biocrusts, only the narrow band normalized band ratio between 700–722 nm (BND; Xue et al., 2009) showed a good fit, whereas none of the narrow or broad band ratios fit well with Chl*a* on the natural cyanobacteria-dominated biocrusts.

General Random Forest Model for Chla Prediction over a Wide Range of Biocrust Coverage and Composition Values

Figure 9 summarized the results of the random forest model performance. Overall, both RF models (the model that exclusively considered spectral properties derived from the two main absorption peaks and the RF model also including biocrust coverage) provided a very good fit with a mean RMSE about 6.5 µg/g soil. Moreover, when they are applied to the complete dataset, in order to get an accuracy estimation comparable with that obtained using linear regression, the prediction accuracy increased (coefficient of determination between predicted and observed Chl*a* values = 0.95; Figure 9). The three most important predictor variables for Chl*a* estimation in both models were maximum depth within the 680 nm absorption peak, gaus-low wlhm, and gaus-up wlhm, whereas the coverage of the different biocrust components played only a secondary role (Supplementary Figure S11).

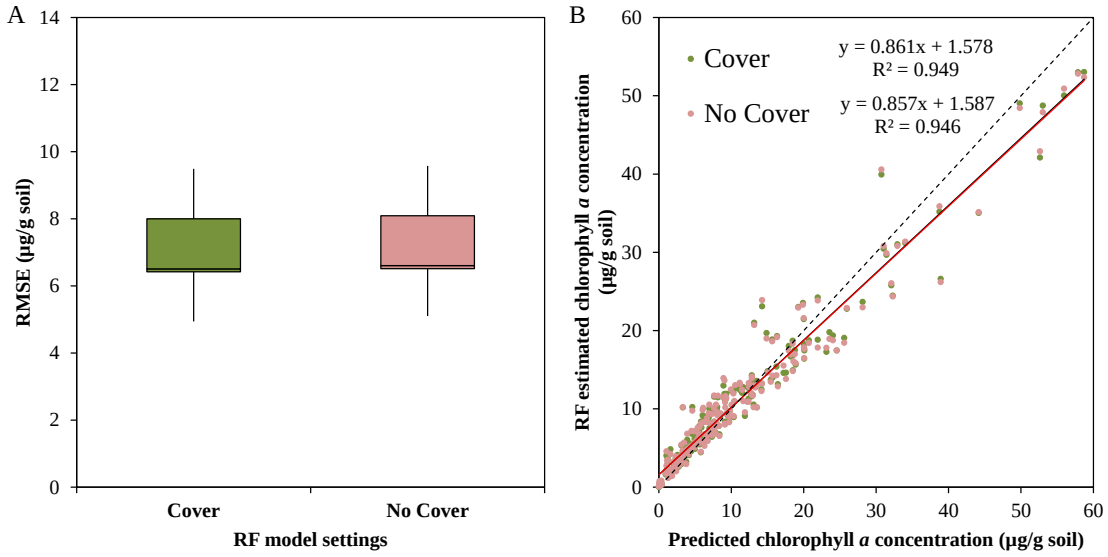

Figure 9 Random forest models (RF) fit: (A) Box plots showing mean (line), the lower and upper quartile (box), and the minimum and maximum values (whiskers) of the mean root-mean-square error (RMSE; µg/g soil) obtained by the 20-fold cross-validations of the model that exclusively considered spectral properties derived from the two main absorption peaks (red) and the RF model also including biocrust coverage (green). (B) Linear regression between measured the chlorophyll a concentration (µg/g soil) and estimations obtained when the RF model that exclusively considered spectral properties derived from the two main absorption peaks (red) and the RF model also including biocrust coverage (green) were applied to the complete dataset.

Discussion

Chl*a* provides valuable information about the photosynthetic capacity of biocrusts (Caesar et al., 2018) and have been widely used as a surrogate for biocrust development and growth of both natural and artificially induced biocrust communities (Büdel et al., 2009; Wang et al., 2009; Zaady et al., 2010; Ayuso et al., 2017; Román et al., 2018). However, until now, most analyses were based on punctual measurements and required destructive sampling that hindered the analysis of seasonal dynamics and growth rates in these spatially heterogeneous communities during long term monitoring experiments. The present study demonstrates that natural and artificially induced biocrust communities present certain common spectral traits related to the presence of chlorophyll that, although less accurate than traditional laboratory analyses, represent an alternative non-destructive and reliable method to quickly quantify Chl*a* and biocrust development.

The spectral analysis of our experimental dataset corroborated that, according to several studies analyzing different biocrust communities around the world (Weber et al., 2008; Chamizo et al., 2012; Alonso et al., 2014; Rodríguez-Caballero et al., 2014; Weber et al., 2016; Rodríguez-Caballero 2017b), biocrusts modify soil surface reflectance with a main absorption detected in the reflectance red peak at about 680 nm, and along the red edge (Figure 3) that corresponds to the well-known chlorophyll *a* spectral absorption (Rock et al., 1988; Vogelmann et al., 1993; Carter, 1994; Gitelson et al., 1996). However, contrary to previous studies (Darvishzadeh et al., 2008; Weber et al., 2016), we found that the use of single band reflectance values at this wavelength were not able to accurately estimate Chl*a* of biocrust communities, as shown by the lower coefficient of determination and higher RMSE values obtained compared to those obtained with the ρ, CR, ND, and standard spectral indexes (Table 1). This may be due to the fact that our dataset was composed of different biocrust communities dominated by a variety of phototrophic organisms with different spectral traits (Chamizo et al., 2012; Weber et al., 2016) that, as previously reported by Escribano et al., 2017 (Escribano et al., 2010), interact with the soil reflectance in multiple ways, modifying the final reflectance values independently of the biocrust chlorophyll concentration. For example, whereas dark cyanobacteria- and green moss-dominated biocrusts reduced the overall soil surface reflectance when compared to the bare soil spectra, white lichens that occur in the experimental zones caused the opposite effect, especially in the dark-colored soils from Las Amoladeras (Figure 3). A chlorophyll-*a*-related absorption peak at about 680 nm was enhanced by the biocrust spectra normalization using the CR and ρ spectral transformations. In this study, all biocrust types showed a deep absorption peak in the CR spectra at about 670 nm and a subsequent spike at 700 nm in the ρ spectra (Figure 3). CR and ρ are commonly used to isolate particular absorption features because they are less sensitive to differences in the overall reflectance and color between different samples (Curran et al., 2001; Le Maire et al., 2004; Mutanga et al., 2004) and they have been found to be a

good predictor of Chl*a* in plants and leaves (Filella and Peñuelas, 1994; Curran et al., 2001; Mutanga et al., 2004; Malenovský et al., 2013; Malenovský et al., 2015). However, previous studies on natural biocrust communities that described this chlorophyll-related spectral feature were focused on identification and mapping (see Weber and Hill, 2016 and references within) and thereby did not explore the potential of the CR and ρ to quantify biocrust Chl*a*. We found that absorption values within the red and the red-edge spectral region extracted from the CR and ρ normalization, respectively, improved chlorophyll *a* estimations when compared with reflectance values, yielding a coefficient of determination above 0.6 (Figure 6). Moreover, the depth of the absorption peaks increased from the very incipient artificially induced cyanobacteria-dominated biocrust to the well-developed, moss-dominated biocrusts as Chl*a* increased (Figure 3), highlighting the potential of spectral transformations to be used as an alternative biocrust development index.

Normalized difference band ratios including the red and NIR spectral bands, as well as the standardized broad- and narrow-band spectral indexes using the same spectral information (e.g., MSR, OSAVI, NDVI, SAVI), were also strongly correlated with the biocrust Chl*a*, giving a similar prediction accuracy as the CR and ρ spectral transformations (Table 1). Thus, results obtained two decades ago by Karnieli et al. (2001) who found a good relationship between the NDVI value of biocrust communities representing different levels of development and chlorophyll, are now confirmed for a dataset composed of 212 samples from different ecosystems that comprised a wide range of biocrust coverage and compositions with chlorophyll *a* values that varied from 0–54.63 µg/g soil on different soils with contrasting soil textures and physicochemical properties, and thus, spectral properties. This approach may reduce the obtained accuracy in comparison with local applications that only considered one soil or biocrust type, but it increases the transferability of the results to a wider range of applications that consider different soil types and biocrust compositions (including monitoring, analysis and comparison of natural biocrust dynamics, and artificially induced biocrust communities).

The good relationships found between the hyperspectral data and Chl*a* are promising and suggest that the best indexes found in this study (Table 1) can be used for reliable estimations of biocrust Chl*a*. Moreover, in a similar way as Chl*a*, spectral absorption at 670 nm and the value of the different spectral indexes values increased as biocrust development did. This positive relationship allowed us to propose biocrust reflectance as a quick, cheap, and non-destructive sampling method to monitor biocrust development over long-time spans, with several potential applications for field and laboratory studies. Furthermore, the good results obtained with the use of hyperspectral information denote a high potential of space-based hyperspectral imagery, as the already launched DESIS or PRISMA hyperspectral missions [80] or several upcoming missions, such as EnMAP and HySPIRI, with hundreds of spectral channels covering the RED and NIR regions with a high potential for biocrust monitoring and biomass retrieval over a wide variety of locations and scenarios. Of note are

also the good results obtained by the application of broad band greenness indexes, yielding models that were as reliable and accurate at predicting Chl*a* as those obtained at hyperspectral resolution (Figure 8). The good fits obtained via the application of broad band greenness indexes at the Sentinel-2 multispectral resolution, combined with the high temporal and spatial resolution that characterize this satellite, open a wide range of opportunities for biocrust monitoring at regional scales that would allow for their use in monitoring programs aimed at understanding biocrust responses to ongoing global change and degradation processes. However, before imaging spectroscopy can be applied in the quantification of chlorophyll *a* in biocrusts, more effort is needed in order to deal with additional difficulties related with atmospheric effects, shadows effects, effect of soil water content on spectral response, or interactions with other surface components such as vegetation that absorb solar radiation at similar wavelengths to biocrusts. Moreover, a specific calibration would be necessary as marked differences are found when the biocrust behaviors are analyzed separately (Figure 7a). Chl*a* for the mosses and artificially induced cyanobacteria showed good correlations with most indexes that fit well for the whole dataset, whereas worse results were obtained for lichen-dominated biocrusts and natural cyanobacteria communities. This can be explained by several factors. To cope with high UV levels, natural cyanobacteria communities produce a wide variety of sunscreen pigments, such as scytonemins and carotenoids (Belnap et al., 2004; Wada et al., 2013), that also absorb solar radiation and interact with the chlorophyll spectral absorption profile (Couradeau et al., 2016). Other species, such as *Microcoleus vaginatus* or *Oscillatoria* sp., reside below the soil surface during droughts and glide upwards only during long favorable periods when soil pore water is available (Pringault et al., 2004; Rajeev et al., 2013), a trait that reduces detection accuracy. Moreover, in dryland soils with numerous vesicular pores where solar radiation can penetrate the soil profile, recent studies revealed that high levels of activity beneath the soil surface would not be observable using remote sensing technologies (Raanan et al., 2016). In the case of lichen-dominated biocrusts, the photobiont is usually sheltered in tight fungal structures that may be difficult to detect in chlorophyll-*a*-related spectra. Moreover, the lichen species analyzed in this study, such as *Diploschistes* sp., have a rugged surface that can create an additional habitat between the thalli and the soil for other organisms, such as cyanobacteria, that cannot be observed using optical techniques. In these cases, when the chlorophyll variability is below the detection limit, accurate laboratory analyses are necessary, and other approaches such as the use of coverage values, surface albedo, or roughness could be an alternative.

As an efficient machine learning algorithm, our RF model successfully sorted out all difficulties related with spectral dissimilarities between different biocrust types and provided the most accurate estimation of all methodologies tested in this study without the need for a specific calibration for each biocrust type (Figure 9). This can be explained by the capacity of RF to model non-linear relationships (Breiman, 1996), but also because the RF model implies the combination of Chl*a*

absorption values at a specific wavelength with other properties related with the shape and specific position of Chl*a* absorptions that are specific to the different biocrust components (Figure 3). This represents a step toward the usability of spatially-explicit spectral information with coarser spatial resolution, such as that obtained from future satellite hyperspectral images, as they showed good transferability during the cross-validation process (Figure 9a). However, as in the case of the application of specific absorption features or vegetation indexes, more effort is needed in order to deal with the additional difficulties presented when airborne or satellite images are analyzed.

Conclusions

Our results demonstrate that biocrust reflectance represents a valuable alternative for non-destructive and reliable retrieval of general biocrust chlorophyll *a* concentration and biocrust development that could be incorporated into monitoring programs. From the different methods analyzed, spectral transformation, such as CR and ρ, as well as normalized band ratios and standard hyperspectral and broad band indexes, provided accurate results. However, such approaches should be carefully adapted to each specific case as these methods were not able to correctly predict subtle differences in chlorophyll *a* concentration in lichen-dominated biocrusts and natural cyanobacteria communities, and therefore, specific calibrations prior to broader application are recommended. These marked differences in the biocrust spectral behaviors can be solved by the application of RF models based on different spectral properties derived from Chl*a* or by a combination of absorption spectral properties and coverage values.

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Supplementary Material

Table S1. Soil texture, pH, electrical conductivity, total organic carbon (TOC) and total nitrogen (TN) of the of the three soils employed in this study: Las Amoladeras, El Cautivo and Gádor quarry (from Román et al., 2018).

Soil types	Soil texture			pH	Electrical Conductivity (mS/cm)	TOC (g/Kg)	TN (g/Kg)
	Sand (%)	Silt (%)	Clay (%)				
Las Amoladeras	61.50 ± 5.10	28.40 ± 4.20	10.10 ± 2.10	8.03 ± 0.04	0.16 ± 0.01	21.41 ± 0.96	2.07 ± 0.11
El Cautivo	29.20 ± 5.40	58.60 ± 5.80	12.20 ± 4.20	8.28 ± 0.12	0.13 ± 0.01	3.87 ± 0.09	0.57 ± 0.04
Gádor quarry	31.20 ± 4.65	43.10 ± 2.34	25.70 ± 2.80	8.57 ± 0.03	1.98 ± 0.18	0.24 ± 0.21	0.17 ± 0.09

Table S2. Summary of the different spectral indices used in this study. R: reflectance; ρ: the first derivative of reflectance; RBLUE: reflectance in the blue region, RGREEN: reflectance in the green region, RNIR: reflectance in near-infrared region.

Index	Formulation	Reference
Simple ratio or modified simple ratio of reflectance or derivatives		
YCAR	R_{600}/R_{680}	Schlemmer et al. (2005)
OCAR	R_{630}/R_{680}	Schlemmer et al. (2005)
Vogelman3	R_{740}/R_{720}	Vogelman et al. (1993)
SRPI (Simple Ratio Pigment Index)	R_{430}/R_{680}	Peñuelas et al. (1994)
Vogelman1	ρ_{715}/ρ_{705}	Vogelman et al. (1993)
dSR1*	ρ_{725}/ρ_{702}	Kochubey and Kazantsev (2007)
dSR2*	ρ_{705}/ρ_{722}	Zarco-Tejada et al. (2002)
Datt-CabCx+c	$R_{860}/(R_{550} * R_{708})$	Datt (1998)
PSRI	$(R_{680} - R_{500})/R_{750}$	Merzlyak et al. (1999)
mSR705	$(R_{750} - R_{445})/(R_{705} - R_{445})$	Sims and Gamon (2002)
BmSR	$(\rho_{722} - \rho_{502})/(\rho_{700} - \rho_{502})$	le Maire et al. (2004)

Table S2. (Continuation)

Index	Formulation	Reference
	Normalized difference of derivatives	
BND	$(\rho_{722} - \rho_{700})/(\rho_{722} + \rho_{700})$	le Maire et al. (2004)
	Modified normalized difference of derivatives	
BmND	$(\rho_{722} - \rho_{700})/(\rho_{722} + \rho_{700} - 2\rho_{502})$	le Maire et al. (2004)
	Indices related with red edge derived with derivatives	
dRE	First derivative maxima in red-edge region (680-780 nm)	Filella and Peñuelas (1994)
∑dRE	Sum of first derivative reflectance in red-edge region (680-780 nm)	Filella and Peñuelas (1994)
EGFR (Ratio of first derivative maxima in red-edge region and green region (530-570 nm))	dRE/dG	Penuelas et al. (1994)
EGFN	(dRE − dG)/(dRE + dG)	Penuelas et al. (1994)
EBAR (Ratio of Sum of first derivative reflectance in red-edge region (680-780 nm) and blue region (490-530 nm))	∑dRE/∑dB	Xue et al. (2009)
EBAN	(∑dRE − ∑dB)/(∑dRE + ∑dB)	Xue et al. (2009)
EBFR	dRE/dB	Xue et al. (2009)
EBFN	(dRE − dB)/(dRE + dB)	Xue et al. (2009)

Table S2. (Continuation)

Index	Formulation	Reference
	Broad band indices	
Normalized Difference Vegetation Index(NDVI)	$(R_{NIR} - R_{RED} / R_{NIR} + R_{RED})$	Rouse et al. (1973)
Enhanced Vegetation Index (EVI)	$2.5 * (R_{NIR} - R_{RED}) / (R_{NIR} + 6R_{RED} - 7.5R_{BLUE} + 1)$	Huete et al. (2002)
Soil-adjusted vegetation index (SAVI)	$(R_{NIR} - R_{RED} / R_{NIR} + R_{RED} + 0.5) * (1+ 0.5)$	Huete (1988)
Optimized soil-adjusted vegetation index (OSAVI)	$(R_{NIR} - R_{RED} / R_{NIR} + R_{RED} + 0.16) * (1 + 0.16)$	Rondeaux et al. (1996)
Modified Chlorophyll Absorption in Reflectance Index (MCARI)	$[(R_{NIR} - R_{RED}) - 0.2(R_{NIR} + R_{GREEN})] * (R_{NIR} / R_{RED})$	Daughtry et al. (2000)
MCARI/OSAVI	MCARI/OSAVI	Daughtry et al. (2000)
Simple Ratio Index (SR)	(R_{NIR} / R_{RED})	Jordan (1969)
Modified Simple Ratio Index (MSR)	$[(R_{NIR} / R_{RED}) - 1] / [(R_{NIR} / R_{RED}) - 1]^{1/2}$	Chen (1996)
Crust Index (CI)	$1 - (R_{RED} - R_{BLUE}) / (R_{RED} - R_{BLUE})$	Karnieli, 1997
Biological Soil Crust Index (BSCI)	$\frac{1 - 2 \times \lvert R_{RED} - R_{GREEN} \rvert}{R_{GRNIR}^{mean}}$	Chen et al. (2005)

Table S2. (Continuation)

Index	Formulation	Reference
	Others	
$MCARI_{[705,750]}$	$[(R_{750} - R_{705}) - 0.2(R_{750} - R_{550})] * (R_{750}/R_{705})$	Wu et al. (2008)
Blog 1/R737	the first derivative of logarithm $1/R_{737}$	Yoder and Pettigrew-Crosby (1995)
DD	$(R_{749} - R_{720})/(R_{701} - R_{672})$	le Maire et al. (2004)
SIPI (Structure Insensitive Pigment Index)	$(R_{800} - R_{445})/(R_{800} - R_{680})$	Peñuelas et al. (1995)
Vogelman2	$(R_{734} - R_{747})/(R_{715} + 726)$	Vogelman et al. (1993)
PRI (Photochemical reflectance index) * Ci (chlorophyll ratio index)	$[(R_{570} - R_{530})/(R_{570} + R_{530})] * [(R_{760}/R_{700}) - 1]$	Garrity et al., (2011)
DFDS_ICCW	sum of $\rho_{675\text{-}680}$ − sum of $\rho_{640\text{-}674}$	Zhang et al. (2014)

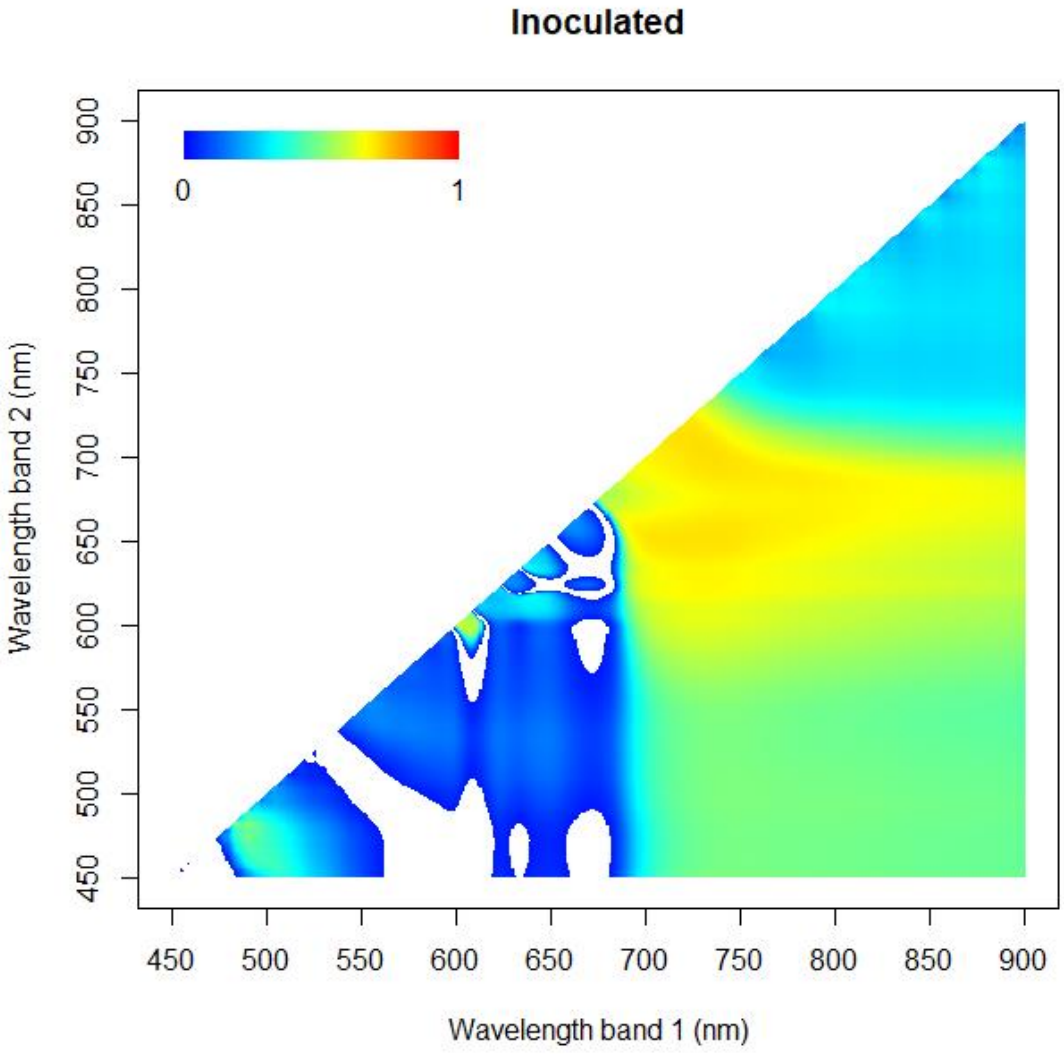

Figure S1 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at hyperspectral resolution, for cyanobacteria artificially inoculated. Only the significant values ($P < 0.05$) are represented.

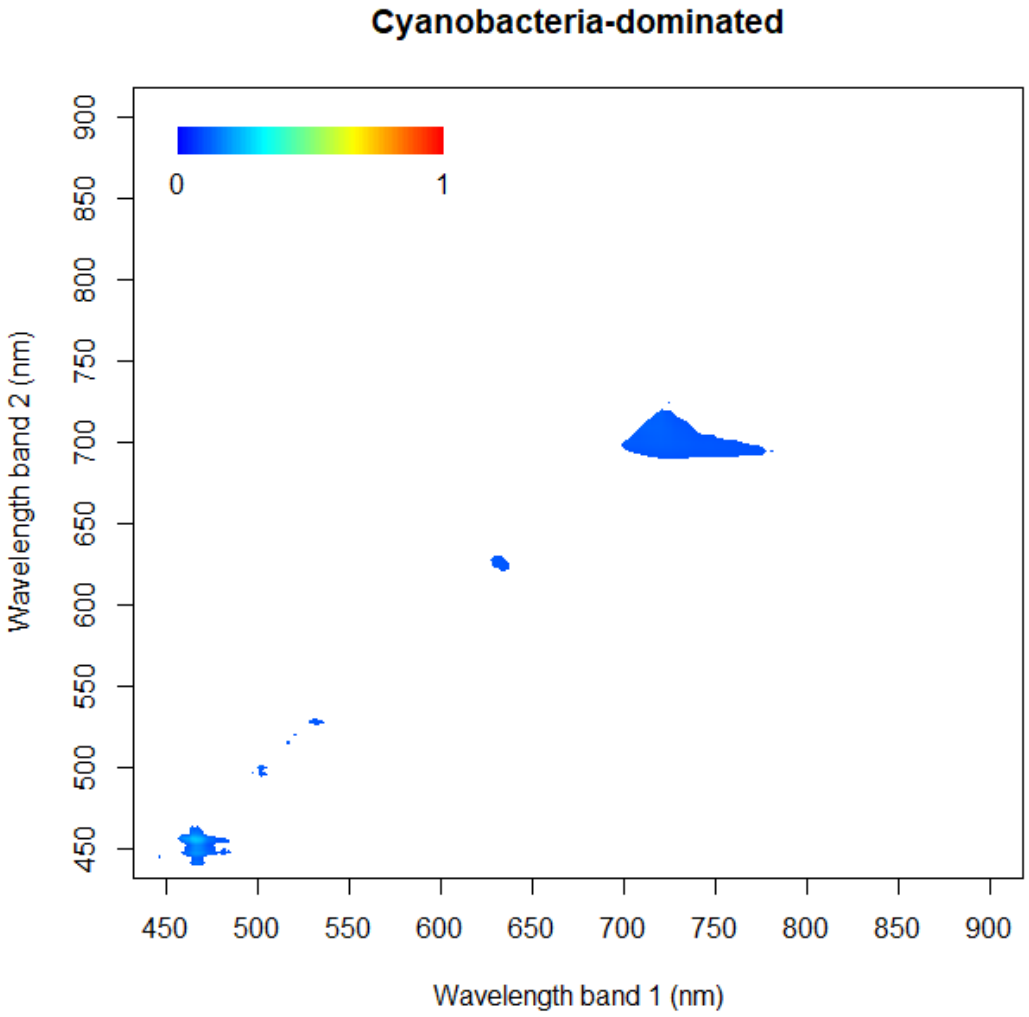

Figure S2 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at hyperspectral resolution, for natural cyanobacteria-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

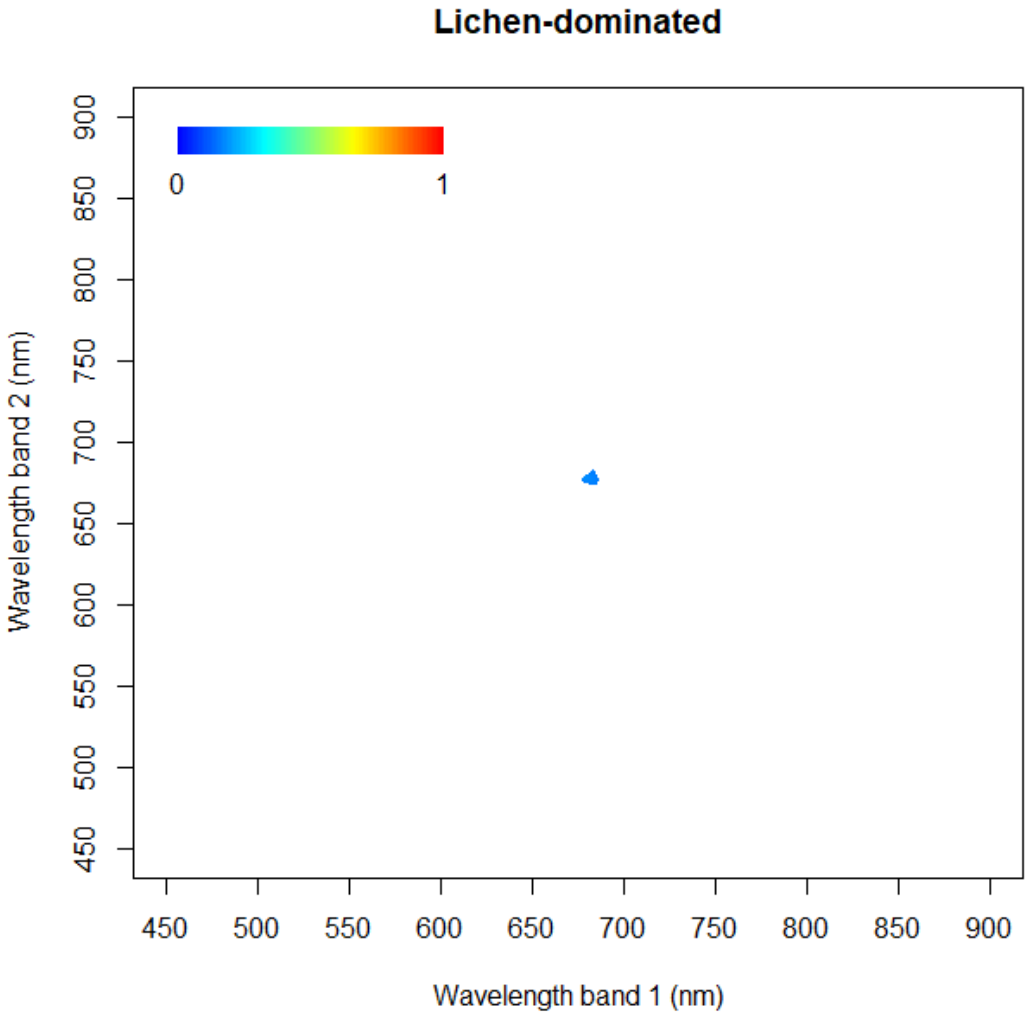

Figure S3 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at hyperspectral resolution, for natural lichen-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

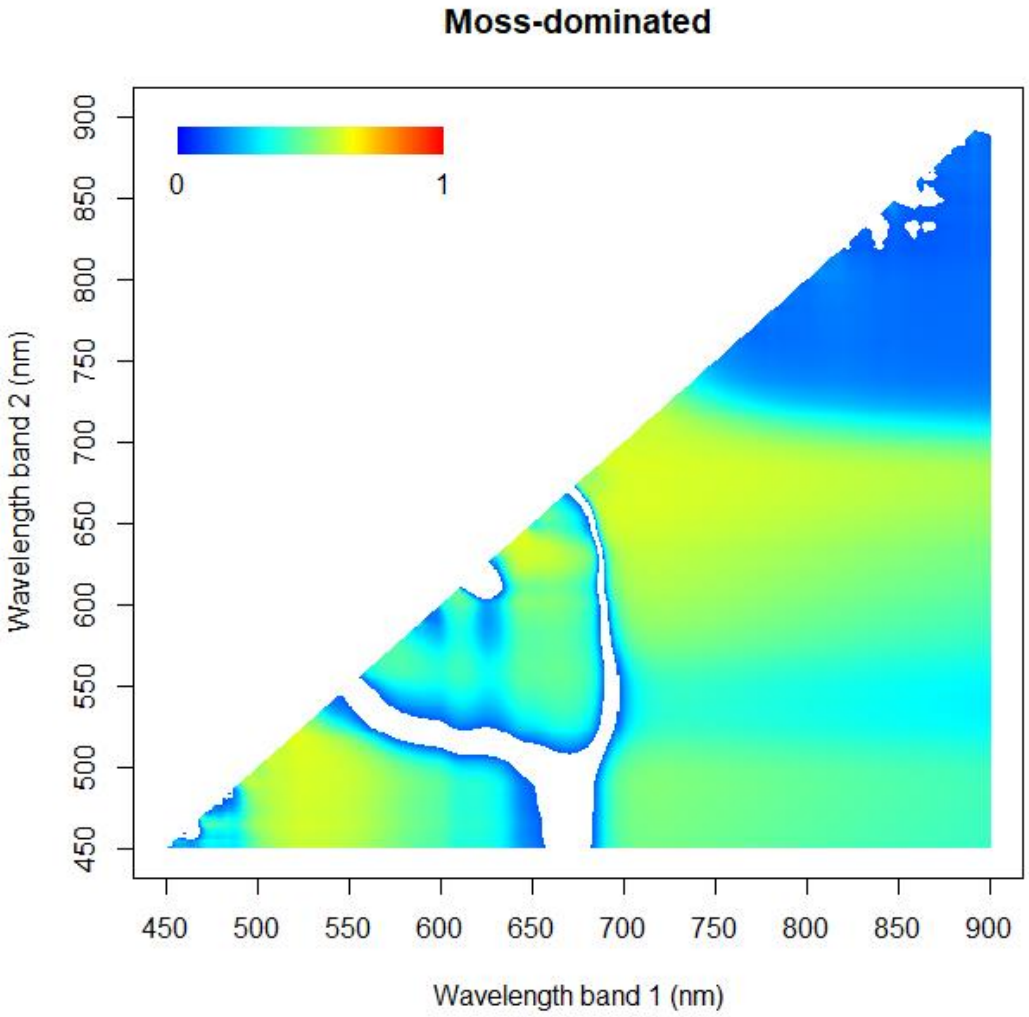

Figure S4 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at hyperspectral resolution, for moss-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

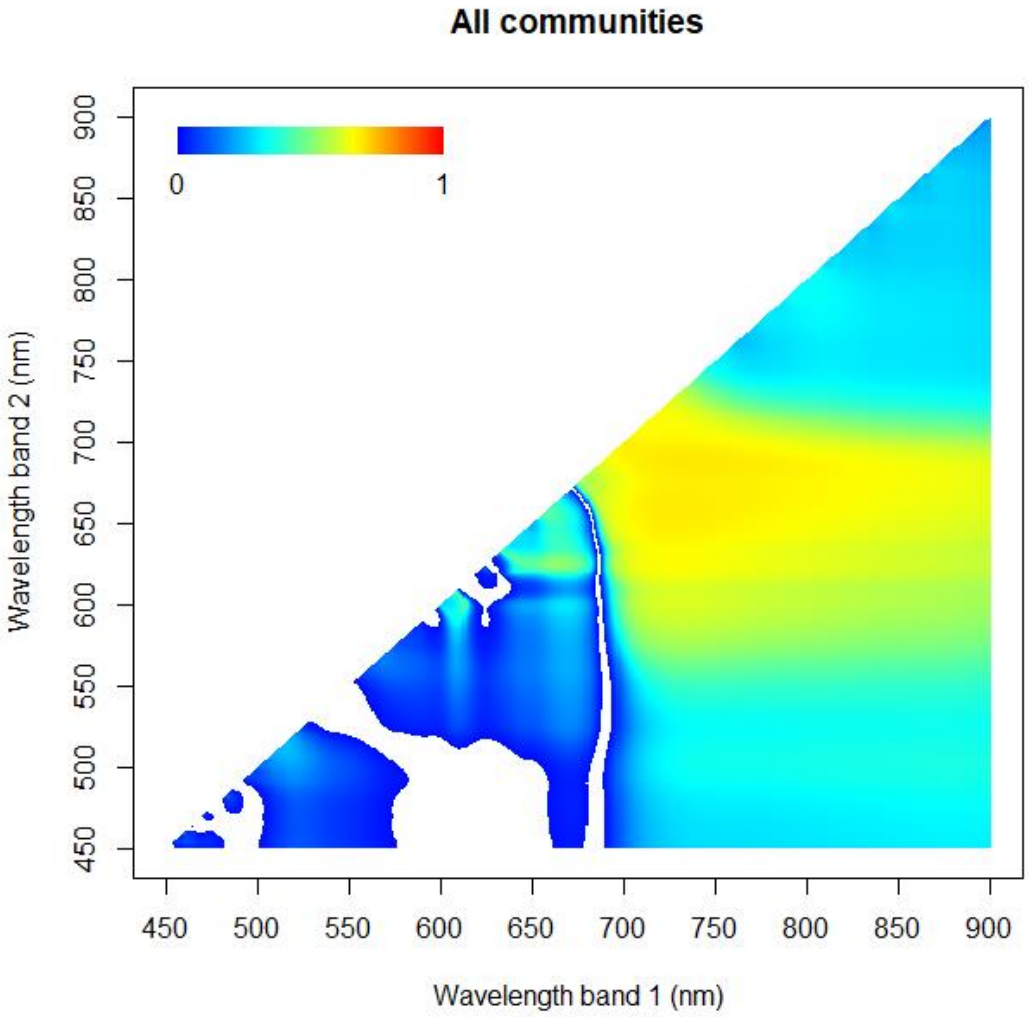

Figure S5 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at hyperspectral resolution, for the entire dataset. Only the significant values ($P < 0.05$) are represented.

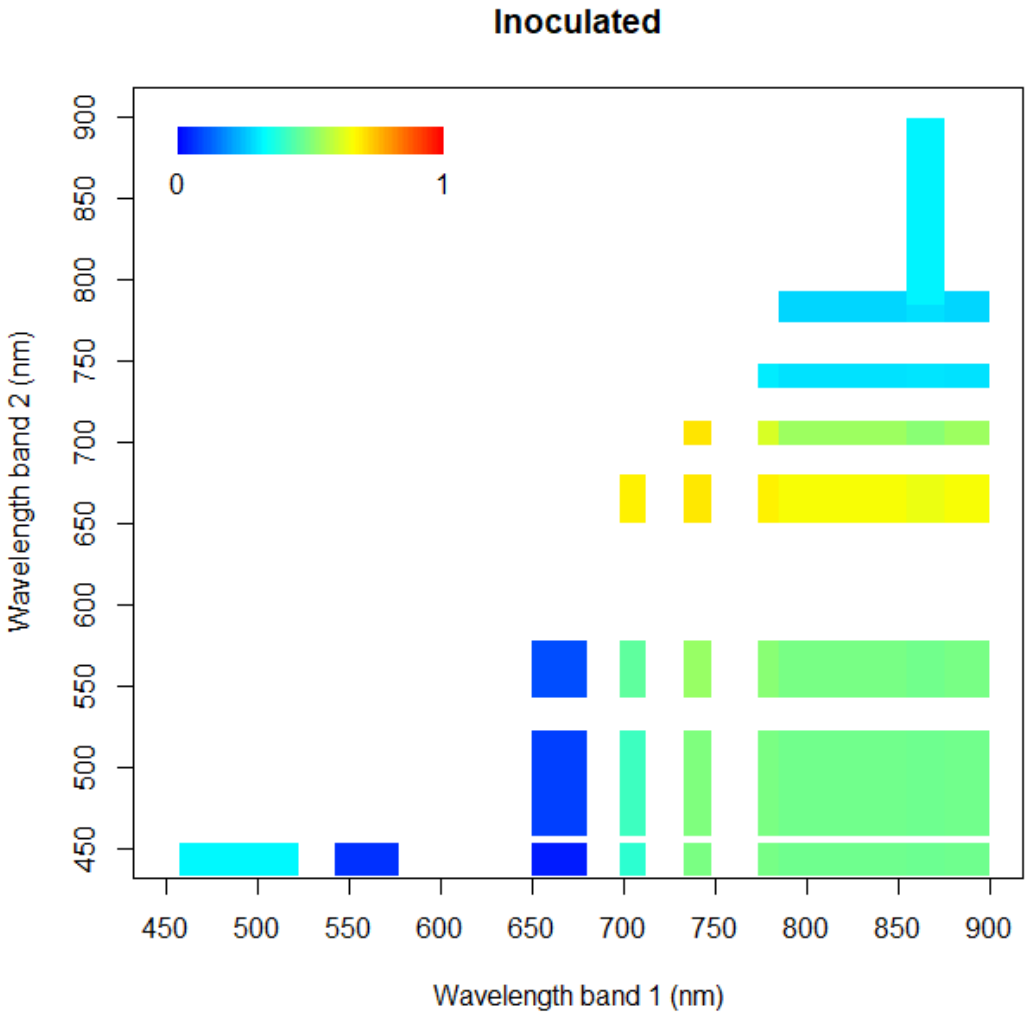

Figure S6 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at Sentinel-2 spectral resolution, for artificially inoculated cyanobacteria subsamples. Only the significant values are represented ($P < 0.05$).

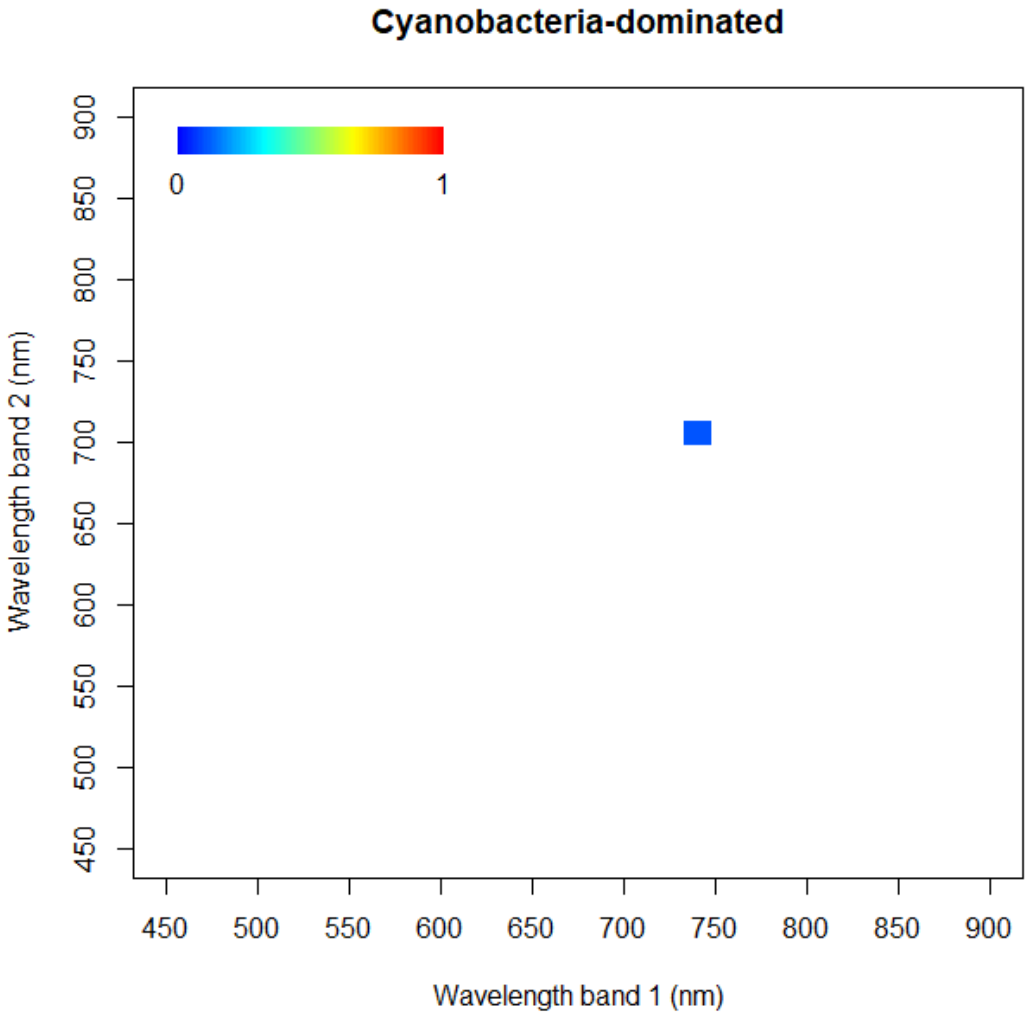

Figure S7 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at Sentinel-2 spectral resolution, for natural cyanobacteria-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

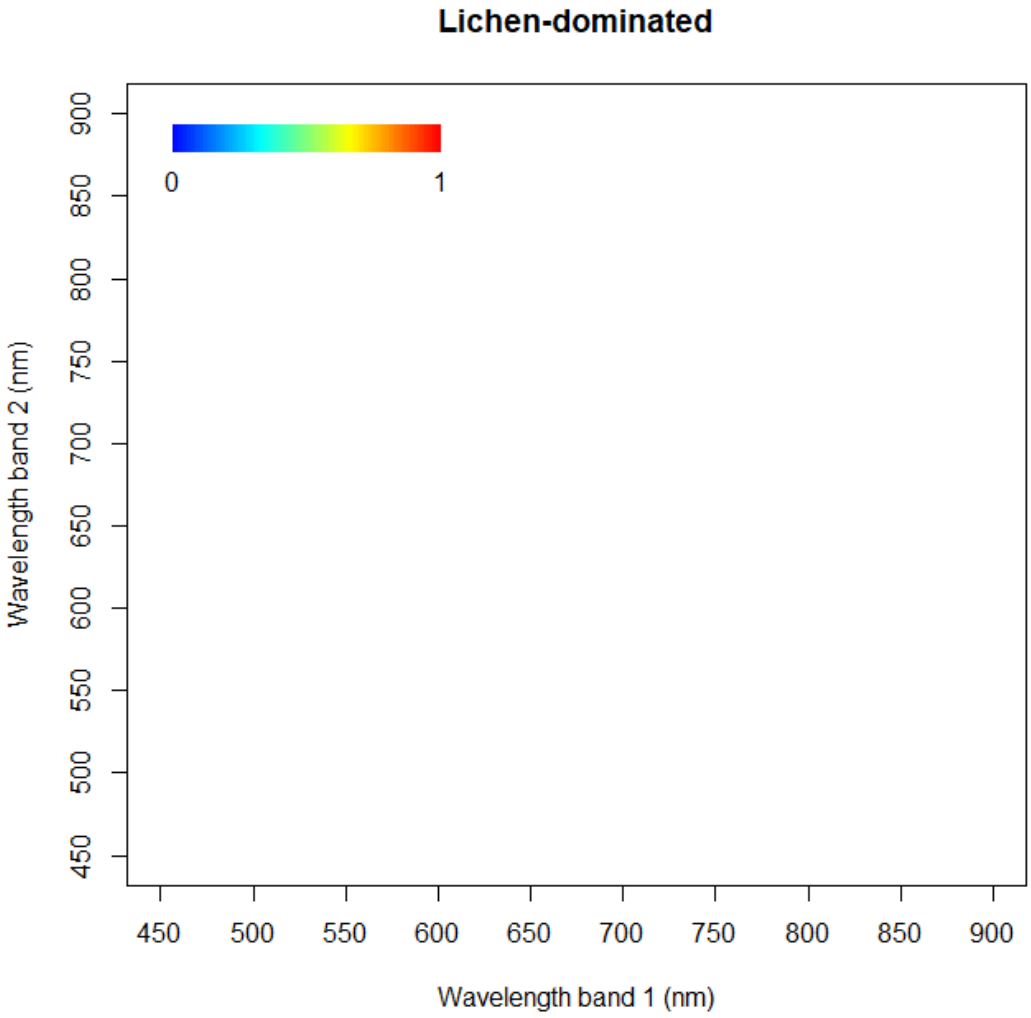

Figure S8 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at Sentinel-2 spectral resolution, for lichen-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

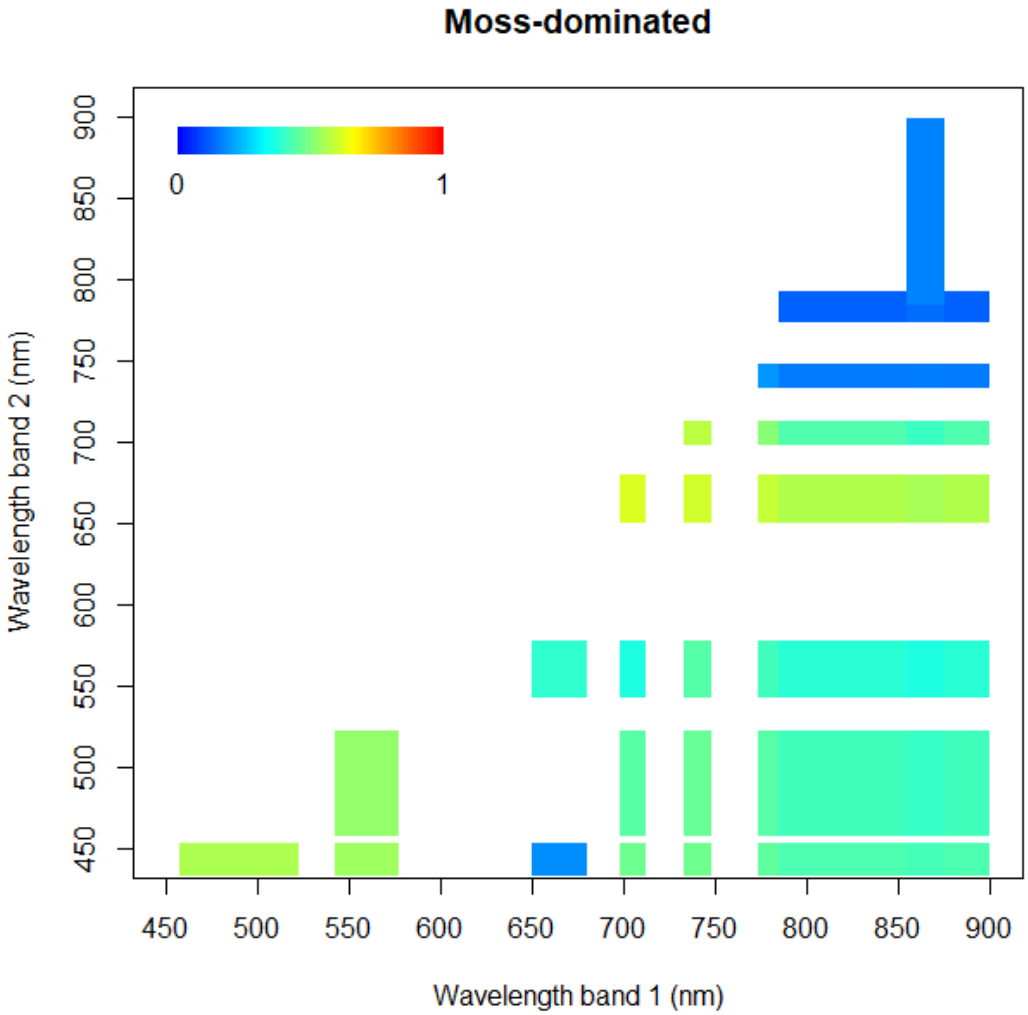

Figure S9 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at Sentinel-2 spectral resolution, for moss-dominated subsamples. Only the significant values ($P < 0.05$) are represented.

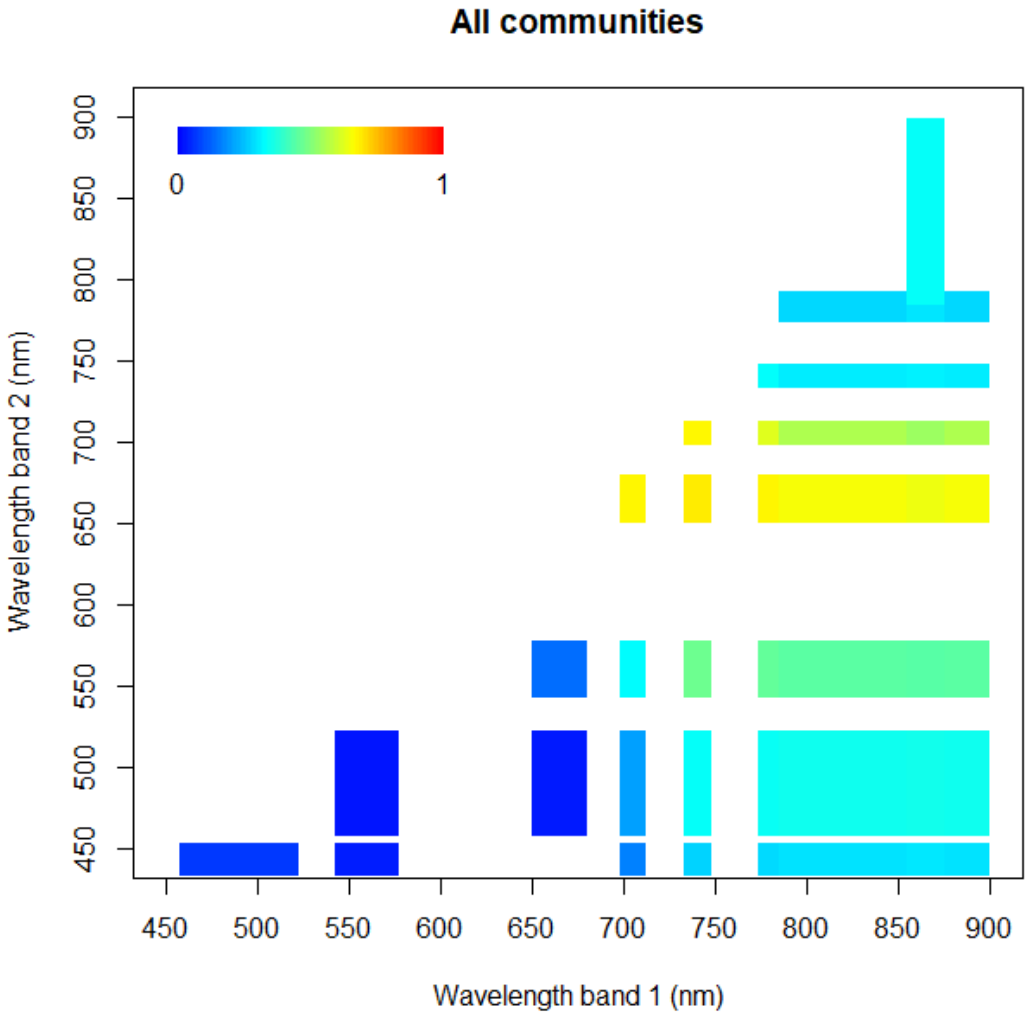

Figure S10 2-D correlation plot illustrating the coefficient of determination (R^2) of the normalised difference indices for all possible band combinations between 450 – 900 nm at Sentinel-2 spectral resolution for the entire dataset. Only significant values ($P < 0.05$) are represented.

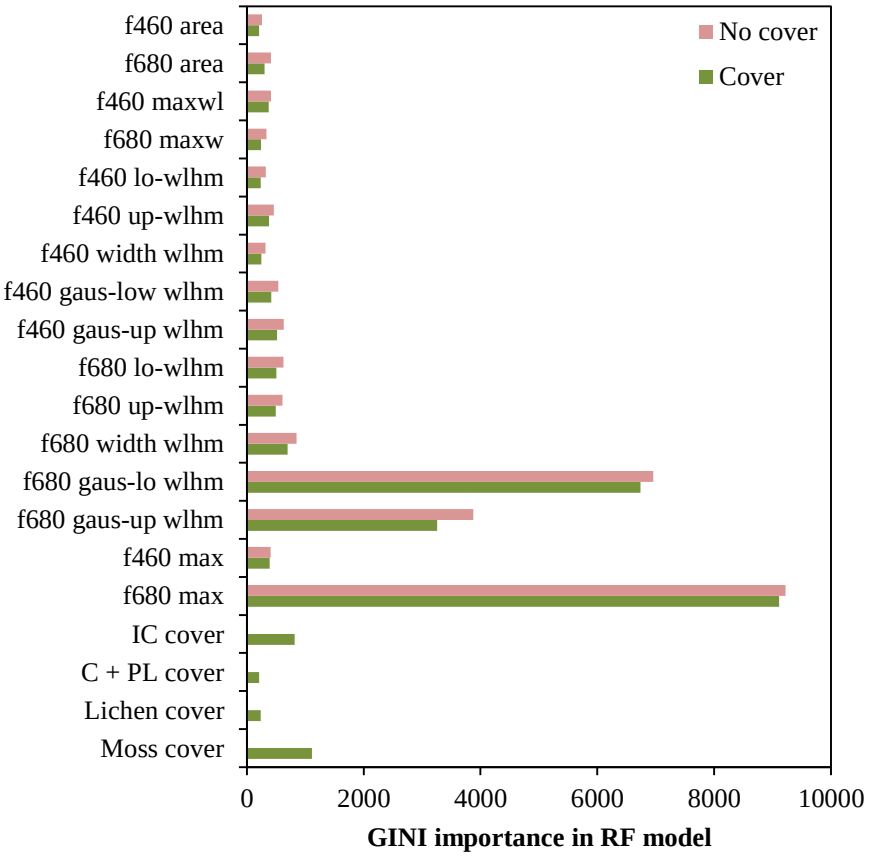

Figure S11 GINI importance of each variable in Random Forest model. Two options were tested: a) with cover (green bars) and, b) without cover (pink bars). IC: Incipient cyanobacteria, C + PL: mix of cyanobacteria and pioneer lichens.

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General conclusions

1. The inoculation of three native N-fixing cyanobacterial strains, *Nostoc commune*, *Scytonema hyalinum* and *Tolypothrix distorta*, alone and in a consortium, induced the formation and development of a functional biocrust in soils from three degraded Mediterranean ecosystems of the province of Almería (Spain) under laboratory conditions. The new biocrust significantly improved key properties related to soil fertility in all soils, with special relevance in very poor post-mine substrates. Of the inoculants tested, *N. commune* and the consortium promoted the highest colonization and enhancement of edaphic conditions, supporting their use as suitable candidates for land restoration in degraded drylands.
2. The inoculation of these three N-fixing cyanobacteria, alone and in a consortium promoted similar biocrust development and improvement of soil properties when subjected to a hydration regime simulating the water availability in the selected areas in a dry and a wet hydrological year. *N. commune* was the strain that showed a better performance under dry conditions as it promoted higher cyanobacteria cover, exopolysaccharides production and soil organic carbon content than the other tested strains, being a potential candidate for soil restoration in water-limited ecosystems.
3. Though laboratory results were very promising, the attempt to restore degraded soils in the field by means of direct soil inoculation with an indigenous cyanobacterial consortium showed limited results. At the three studied ecosystems, similar biocrust growth was found either in the inoculated or control plots. However, two years after inoculation, organic carbon content was higher in inoculated than in than control plots. The progressive pre-acclimation of the cultures before inoculation did not lead to significant improvement in biocrust survival and establishment, as similar cyanobacterial biomass and surface albedo were found in control, pre-conditioned and non-conditioned inoculated plots, six months after inoculation. In contrast, application of habitat amelioration techniques led to improvements in cyanobacterial performance. Covering the inoculated crust with a vegetal fiber mesh promoted biocrust growth, as demonstrated by the higher chlorophyll *a* content, deeper chlorophyll *a* spectral absorption peaks and lower albedo values observed on these plots respect to uncovered ones. Also, a screening of the exopolysaccharides content (EPS) in the induced biocrust demonstrated that the use of the mesh significantly increased the amount of the more condensed EPS as well as the abundance of high molecular weight molecules in the more soluble EPS fraction, both effects likely contributing to enhancing soil aggregation and stability. Moreover, higher abundance of xylose and galactose was found in covered inoculated soils, resembling the EPS composition of natural developed cyanobacterial crusts and possibly indicating a higher biocrust growth.

4. The incorporation of different cyanobacteria species into extruded pellets revealed that some strains can survive the pelleting process and spread out to surrounding areas via watering. Of the encapsulated inoculants, *Scytonema* spp. and the consortium promoted higher chlorophyll *a* contents and lower albedo than the other tested strains. Storage of the pellets for 30 days significantly affected cyanobacterial biomass as showed by the 50% reduction in chlorophyll *a* content observed in comparison to non-stored pellets. The biomass reduction due to storage was consistent across inoculation treatments and soil types. Although promising, this technology should be tested and refined before it can be applied under real field conditions.
5. Our results demonstrate that biocrust surface reflectance measurements can be effectively used for non-destructive monitoring of biocrust dynamics in natural and restored soils. Chlorophyll *a*, which is the most widely used measurement for monitoring biocrust development, was accurately predicted by spectral features obtained by the first derivative of the reflectance and the continuum removal of the biocrust spectra. Moreover, normalized difference band ratios in the red-edge region and common hyperspectral and broad band indexes were also accurate in chlorophyll *a* quantification. Nevertheless, this method should be carefully adapted to each specific biocrust component as the proposed indices were not able to correctly detect subtle differences in chlorophyll *a* concentration of lichen-dominated and cyanobacteria-dominated biocrusts. Interestingly, the use of non-linear random forests based on the combination of spectral properties and biocrust coverage sorted out the necessity for a specific calibration for each biocrust type.

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