Microbial Ecology

Watering, Fertilization, and Slurry Inoculation Promote Recovery of Biological Crust Function in Degraded Soils

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Abstract

Biological soil crusts are very sensitive to human-induced disturbances and are in a degraded state in many areas throughout their range. Given their importance in the functioning of arid and semiarid ecosystems, restoring these crusts may contribute to the recovery of ecosystem functionality in degraded areas. We conducted a factorial microcosm experiment to evaluate the effects of inoculation type (discrete fragments vs slurry), fertilization (control vs addition of composted sewage sludge), and watering frequency (two vs five times per week) on the cyanobacterial composition, nitrogen fixation, chlorophyll content, and net CO_2 exchange rate of biological soil crusts inoculated on a semiarid degraded soil from SE Spain. Six months after the inoculation, the highest rates of nitrogen fixation and chlorophyll *a* content were found when the biological crusts were inoculated as slurry, composted sewage sludge was added, and the microcosms were watered five times per week. Net CO_2 exchange rate increased when biological crusts were inoculated as slurry and the microcosms were watered five times per week. Denaturing gradient gel electrophoresis fingerprints and phylogenetic analyses indicated that most of the cyanobacterial species already present in the inoculated crust had the capability to spread and colonize the surface of the surrounding soil. These analyses showed that cyanobacterial communities were less diverse when the microcosms were watered five times per week, and that watering frequency (followed in importance by the addition of composted sewage sludge and inoculation type) was the treatment that most strongly influenced their composition. Our results suggest that the inoculation of biological soil crusts in the form of slurry combined with the addition of composted sewage sludge could be a suitable technique to accelerate the recovery of the composition and functioning of biological soil crusts in drylands.

Introduction

Arid and semiarid areas throughout the world are characterized by a sparse vegetation cover, in which vegetated patches are surrounded by a matrix devoid of vascular plants [58]. This matrix is often covered by biological soil crusts that, when well developed, are formed by bacteria, cyanobacteria, algae, mosses, liverworts, fungi, and lichens [61]. These crusts provide a number of key ecosystem services, including the fixation of atmospheric carbon and nitrogen, the increase in soil stability and resilience against erosion, and the control of hydrological processes through their effects on runoff and infiltration [12, 25, 30, 61]. In addition to indirect effects promoted by changes in soil stability, nutrient content, and hydrology, biological soil crusts can directly affect the establishment, survival, nutrient status, and water relations of vascular plants [22, 32, 47] and can be the habitat of a large number of arthropods and microorganisms [7, 53].

Given their critical role in ecosystem function, and the increasing awareness on their importance as a key component of natural ecosystems, it is not surprising that there is renewed interest in the response of biological soil crusts to anthropogenic and natural disturbances [11,

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15,21,33]. These crusts are very sensitive to disturbances such as trampling and grazing, and temporal estimates for their recovery under natural conditions in arid and semiarid areas typically are in the range of decades to millennia [11]. Such a slow recovery time is caused by factors such as climatic constraints, low growth rates of constituent species, lack of moisture availability, limitations to propagule dispersal after disturbances, and negative interactions with vascular plants [11, 33, 34]. To overcome some of these limitations, and to speed up recovery, *in situ* inoculation of soils with biological crust components, such as cyanobacteria, has been recommended in degraded arid and semiarid ecosystems [6, 17, 36, 54]. Studies conducted so far have shown that inoculation has potential to foster the natural recovery rates of biological soil crusts in these areas, as it enhances recovery rates of cyanobacterial and lichen biomass (reviewed by [11]). However, little is known on the factors limiting such recovery and on the effects that supplementary treatments, such as watering and fertilization, may have on the performance of inoculated biological soil crusts [11, 21, 36]. Such studies are of great interest to establish sound management approaches to restore biological soil crusts in degraded areas and to improve the effectiveness of inoculation as a technique to achieve this aim. Furthermore, most of the inoculation studies performed so far have been conducted in the USA [11], a fact that emphasizes the need for information gathered in other areas of the world.

Cyanobacteria constitute one of the main components of biological soil crusts in arid and semiarid soils [12]. These organisms contribute to the physical structure of the crust and provide fixed nitrogen and photosynthetically fixed carbon to other soil organisms [8]. Despite its importance, the cyanobacterial diversity in semiarid soil crusts remains poorly studied. Most studies conducted so far have focused on morphological analysis involving laboratory culture and/or microscopy [48]. Although these techniques are still suitable to obtain abundance or frequency data, their use may lead to underestimation of the diversity and inaccurate determination of cyanobacterial composition [10]. The use of polymerase chain reaction (PCR) targeting the 16S rRNA genes has proven to be a powerful culture-independent means for the characterization of microbial communities, revealing the presence of numerous populations that cannot easily be differentiated microscopically [26, 59, 60]. Recent studies have successfully employed this approach to characterize the microbial communities of biological soil crusts in arid environments [29, 48, 52, 62] and have demonstrated that this method is capable of revealing more extensive species diversity in these crusts than traditional techniques [28]. Fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene segments, provide valuable information on the composition and phylogeny of microbial communities [23, 43]. DGGE has also been recently used in diversity studies of cyanobacteria in desert soil crusts [28, 29].

In this study, we evaluated the effects of different treatments on the composition and functioning of inoculated biological soil crusts (a mixture of cyanobacteria and lichens) in a microcosm experiment, using soil and biological soil crust inoculum from a semiarid degraded area in SE Spain. Our main objectives were to: (1) evaluate the recovery of functions associated to the biological soil crusts after inoculation of a degraded soil; (2) assess the effects of inoculation type (discrete fragments vs slurry), fertilization (control vs addition of composted sewage sludge), and watering frequency (two vs five times per week) on such recovery; and (3) determine the effect of these treatments on the composition of the cyanobacterial assemblage by means of molecular techniques. Although the use of experimental microcosms does not allow us to mimic the full complexity of natural conditions, it permits us to elucidate potential responses to the different treatments applied and account for interactions that may occur in the field [38].

Methods

Site and Sampling Characteristics. The soil for this experiment was collected from a degraded shrubland located near Relleu, in SE Spain (UTM coordinates: 735591 m E, 4269506 m N; Zone 30S; 395-m altitude, 11°SE orientation). Climate is Mediterranean semiarid with annual rainfall and temperature values of 378 mm and 16°C, respectively. The natural vegetation of the area has been degraded by human activities that, such as livestock grazing and wood and fiber harvesting, have been conducted for centuries [40]. Nowadays, it is dominated by the shrub *Rosmarinus officinalis* L. (Rosemary) and the tussock grass *Stipa tenacissima* L. (Alpha grass). Soil was collected from two points (0- to 20-cm depth) located in shrub interspaces devoid of vascular plants and of the visible components of biological crusts. Well-developed biological crusts were collected from a *S. tenacissima* steppe located 1 km away from the place where the soil was collected. Lichens (mainly *Collema* sp., *Placidium* sp., *Psora decipiens*, and *Psora crenata*) and cyanobacteria constitute the main macroscopic component of these crusts (Calatayud, personal communication). Crust samples containing both lichens and cyanobacteria were collected from tussock interspaces devoid of plant litter and organic matter debris. These samples, about 5 × 5 cm, 1 cm deep each, were taken from the soil surface and transported in plastic petri dishes to the laboratory. The sampling of both the soil and the biological crusts took place in early July 2003, when the soil was dry

because of summer drought. Once in the laboratory, the soil was homogenized by sieving through a 5-mm mesh, and stones, roots, litter, and organic debris were removed.

Experimental Setup. A factorial microcosm experiment was carried out in the University of Alicante between July 2003 and February 2004. The design of the experiment consisted of two levels of watering frequency (two *vs* five times per week), two levels of fertilization (control vs addition of organic residue), and two types of inoculation (discrete fragments *vs* slurry), resulting in eight treatment combinations. The organic residue used in this experiment consisted of composted sewage sludge, coming from Aspe wastewater treatment plant (Alicante province, SE Spain, Table 1). Prior to the onset of the experiment, the soil and the composted sewage sludge were sterilized by autoclaving at 121°C twice for 1 h.

The experimental microcosms consisted of 22.06-cm^2 sterile petri dishes (5.3-cm diameter, 1.2-cm height) filled with 18 g of sterilized soil. Half of these dishes received 2 g of composted sewage sludge, which was thoroughly mixed with the soil. Inoculation was performed in two ways: by transplanting discrete fragments and by spreading a slurry. In both cases, six circular biological crust fragments containing both lichens and cyanobacteria (0.7-cm diameter, 1 cm deep), equivalent to a surface area of 2.31 cm^2, were added. In the fragment treatment, the circular crust fragments were placed in a regular fashion and were buried in the soil until their surface was at the same height as the surface of the surrounding soil. In the slurry treatment, the six fragments were crushed and homogenized with the help of 4 mL of distilled water in a mortar, and the resulting slurry was evenly spread on the soil surface of the petri dishes. Two watering treatments were also applied: low and high watering frequency. In the high watering frequency, the microcosms were watered five times a week (everyday from Monday until Friday); in the low watering frequency, the microcosm were watered twice a week (on Monday and Friday). In every watering event, distilled water was added to get a moisture equivalent to 80% of field capacity in both watering treatments. The amount of water added each time was adjusted by weighing the microcosms.

Table 1. Analytical characteristics of the composted sewage sludge used in the experiment

Soil moisture (%)	49
Total organic carbon ($mg \cdot g^{-1}$)	319
pH	7
electrical conductivity (1:5 water extracts; $dS \cdot m^{-1}$)	5.86
Total N ($mg \cdot g^{-1}$)	34
Total P ($mg \cdot g^{-1}$)	23
Total K ($mg \cdot g^{-1}$)	4
Total Ca ($mg \cdot g^{-1}$)	88
Total Mg ($mg \cdot g^{-1}$)	6
Fe ($\mu g \cdot g^{-1}$)	9722
Mn ($\mu g \cdot g^{-1}$)	139
Cd ($\mu g \cdot g^{-1}$)	5
Cr ($\mu g \cdot g^{-1}$)	29
Pb ($\mu g \cdot g^{-1}$)	132
Zn ($\mu g \cdot g^{-1}$)	1012
Ni ($\mu g \cdot g^{-1}$)	30
Hg ($\mu g \cdot g^{-1}$)	1.1

Twenty-eight microcosms for each of the eight treatment combinations were established, resulting in 224 microcosms in total (2 fertilization levels × 2 inoculation levels × 2 watering frequency levels × 28 replicates). In addition, eight noninoculated (control) microcosms for each fertilization level in the low frequency watering treatment were established, with the aim to check for spontaneous recolonization by microorganisms. After the application of the composted sewage sludge and inoculation treatments, all microcosms were brought to field capacity and introduced in a growth chamber maintained at temperatures ranging from 22 (night) to 34°C (day) and photosynthetic active radiation of 75 μmol $m^{-2}s^{-1}$ with a 12-h photoperiod. The average relative humidity ranged from 29 to 70%. Microcosms were maintained in the chamber for 6 months, and their positions were randomly interchanged every 2 weeks.

Crust Nitrogenase Activity. Nitrogen fixation was measured at the end of the experiment by using the acetylene (C_2H_2) reduction assay [55]. Eight microcosms for each combination of inoculation, fertilization, and watering frequency treatments and four control microcosms (with and without composted sewage sludge added) were randomly selected for these measurements. Prior to measurements, all samples were irrigated with 2 mL distilled water, which was carefully added with a pipette. Crust samples were introduced in an airtight, nonreactive, plastic bags; after that, C_2H_2 was added to each bag to create an atmosphere of 10% C_2H_2. The bags were later incubated for 4 h in a growth chamber (maintained at 300 μmol m^{-2} s^{-1} irradiance and 25°C temperature). After incubation, a 3-mL gas sample was taken from each bag; gas samples were stored in airtight vials until analysis. The ethylene (C_2H_4) concentration was measured on a gas chromatograph equipped with a flame ionization detector (Hewlett Packard 6890, Hewlett Packard, Palo Alto, CA). Data are presented as the rates of C_2H_4 accumulation over time, accounting for headspace and crust area.

Chlorophyll Content. The content of chlorophylls *a* and *b* was evaluated at the end of the experiment on five randomly selected microcosms per each combination of treatments. It was also evaluated in four additional control samples (with and without composted sewage sludge). The biological crust was carefully scraped with a

scalpel, and the chlorophyll was extracted in 80% acetone. These pigments were quantified after spectrophotometric determination according to Barnes *et al.* [5]. In the treatments where the crust was inoculated as discrete fragments, two measurements per replicated microcosm were taken (one for the inoculated crust fragment area and another for the noninoculated area). For each measured microcosm belonging to this treatment, a weighted average value was obtained taking into account the proportion of the microcosm's surface area occupied by both sections.

CO_2 Exchange Measurements. Net CO_2 uptake and dark CO_2 losses were used to estimate the net carbon exchange rate of the microcosms at the end of the experiment. Eight randomly selected microcosms per each combination of treatments and five additional control microcosms (with and without composted sewage sludge) were selected for these measurements. These were conducted with a LiCor LI-6400 Portable Photosynthesis System (LiCor, Lincoln, NE, USA) and a conifer cuvette (LI-6400-05, LiCor), with a flow rate set to 350 μmol s^{-1}. Prior to measurements, all samples were irrigated with 2 mL distilled water, which was carefully added with a pipette. Net CO_2 uptake and dark CO_2 losses were taken by consecutively adjusting the light levels within the cuvette. Photosynthetically active radiation was 850.7 ± 52.5 and 0.6 ± 0.1 μmol m^{-2} s^{-1} (mean ± SD, $n = 73$) for net CO_2 uptake and dark losses, respectively. Inflow CO_2 concentration was maintained at 400 μmol mol^{-1}. Temperatures in the cuvette ranged from 28 to 32°C. The net CO_2 exchange rate for each microcosm was obtained by subtracting net CO_2 losses from net CO_2 uptake values, which were previously adjusted to the surface area of the microcosm introduced in the cuvette.

Molecular Community Characterization. For the extraction of nucleic acids, the crust was physically excised from the rest of the soil with the aid of a sterile metallic spatula. Extraction was initiated by freezing the crust in liquid nitrogen and grinding it in a mortar while frozen. A 3 mL extraction buffer (50 mM Tris pH 8, 0.5% sodium dodecyl sulfate) and 4 mL of phenol were added to the samples. The aqueous phase was recovered and followed a second extraction with phenol, an extraction with phenol/chloroform (1:1), and a final extraction with chloroform. Nucleic acids in the aqueous phase were precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of cold ethanol. Pellets containing the nucleic acids were washed with 70% ethanol, dried, and resuspended in H_2O. The integrity of the total DNA obtained was checked by agarose gel electrophoresis. Nucleic acid extracts were stored at −70°C until analysis.

DGGE was used to compare the cyanobacterial communities from 10 samples, 2 samples belonging to the original soil crust (OC1 and OC2) used for the inoculation of the microcosm experiment and 8 representative samples from this experiment subjected to different inoculation strategies or growth conditions. To get an insight on the capabilities of cyanobacteria to colonize adjacent areas in those samples that had been inoculated with fragments of the soil crust, DGGE analysis was performed with DNA from the exact point where the original crust fragments were inserted and from adjacent surfaces. DNA extracted from the 10 samples was used as template in a PCR performed using hot start Taq DNA polymerase (Hot Star, Qiagen, Hilden, Germany) and cyanobacterial-specific 16S rDNA oligonucleotide primers CYA106F (with 40 nucleotide GC clamp at the 5′ end) and CYA781R [45]. Amplified products were of 675 bp. The PCR conditions have been previously described by Nübel *et al.* [45]. DGGE was carried out with a Dcode system (Bio-Rad, Hercules, CA, USA) as described by Díez *et al.* [23]. Electrophoresis conditions were 75 V for 16 h in a linear gradient of denaturing agents from 45 to 80% (where 100% denaturing agent was 7 M urea and 40% deionized formamide). After electrophoresis, the gel was stained in 1× Tris–acetate–EDTA buffer containing SyberGold Nucleic Acid Stain (1:10,000 dilution; Molecular Probes) to reveal the banding patterns, and the results were recorded using a fluorescent scanner (Typhoon 8600 Variable Mode Imager, Amersham Biosciences, Freiburg, Germany). The presence of DGGE bands was estimated by image analysis using the QuantityOne software (Bio-Rad). The number of DGGE bands was considered to be the number of phylotypes in each sample. A matrix was constructed with the number and position for individual DGGE bands in all samples, taking into account the presence or absence of individual bands in all lanes (binary matrix). The identification of the species within the populations revealed by DGGE was addressed by sequencing most of the major bands of the gels (19 in total) as described by Díez *et al.* [23]. The nucleotide sequences of the DGGE bands were determined using the Big Dye Terminator Cycle Sequencing kit and an ABI PRISM® 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained are available in GenBank under accession numbers DQ146317–DQ146335.

Sequence Analysis. Partial DGGE 16S rRNA sequences were compared initially with reference sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using basic local alignment and searching tool (BLAST) [1] and subsequently aligned with 16S rRNA reference sequences in the ARB package ([39], http://www.arb-home.de). A dendrogram (data not shown) was generated using nearly complete

16S rRNA sequence representatives of the cyanobacteria, using the neighbor-joining algorithm [51], after calculation of evolutionary distances using the Jukes and Cantor correction [35]. The sequences of the DGGE bands obtained in this study were added to the tree using the parsimony tool implemented in the ARB package (sequences are introduced according to parsimony criteria without allowing changes in the tree topology). Apart from those found in the ARB package, the following sequences were included as reference (GenBank accession numbers are indicated in parentheses): *Nostoc calcicola* III (AJ630447), *Nostoc edaphicum* X (AJ630449), *Tolypothrix* sp. IAM M-259 (AB093486), *Nostoc ellipsosporum* V (AJ630450), *Nostoc muscorum* I (AJ630451), *Nostoc* sp. 8916 (AY742447), *Trichormus variabilis* HINDAK 2001/4 (AJ630456), *Aphanizomenon issatschenkoi* LEMCYA31 (AY196088), *Chlorogloeopsis* sp. PCC 7518 (X68780), *Scytonema* sp. IAM M-262 (AB093483), *Oscillatoria* sp. MPI990BR03 (AF284810), *Oscillatoria spongeliae* isolate 504bg (AF534688), *O. spongeliae* 513bg (AF534693), *Microcoleus steenstrupii* 173-1A (AF355388), *M. steenstrupii* 177-5A (AF355389), *Microcoleus* sp. PCC 8701 (AY768403), *M. steenstrupii* 148-2A (AF355379), *Microcoleus* chthonoplastes EDB (AJ272598), *Symploca* sp. HPC-9 (AY430153), *Symploca atlantica* PCC 8002 (AB039021), *Microcoleus vaginatus* isolate MOA4-1 (AF355374), *M. vaginatus* isolate OTA3-2 (AF355373), Lyngbya hieronymusii (AF337650), *Lyngbya majuscula* CCAP 1446/4 (AY668394), *Phormidium* sp. ETS-05 (AJ548503), *Pseudoanabaena tremula* UTCC471 (AF218371), uncultured Antarctic cyanobacterium clone Fr246 (AY151757), uncultured soil bacterium clone 349 (AY493984), and uncultured Crater Lake bacterium CL120-14 (AF316710).

Statistical Analyses. The effects of the different treatments on rates of C_2H_2 reduction, chlorophyll content, and net CO_2 exchange rate were evaluated using analysis of variance (ANOVA) models. Some variables (C_2H_2 reduction, chlorophyll *b*, and net CO_2 exchange rate) did not follow a normal distribution, nor did they show homogeneity of variances, even after data transformation. Thus, the semiparametric ANOVA approach developed by Anderson [2] was used. This method is based on the use of permutation tests to obtain *P* values, does not rely on the assumptions of traditional parametric ANOVA, and can handle complex experimental designs such as those used here. To investigate higher-order interactions, data were divided into subsets based on one of the factors of the interaction, and then were subject to ANOVA. For these analyses, the Euclidean distance and 10,000 permutations (permutation of raw data, [4]) were used to analyze our data. As suggested by Gotelli and Ellison [31], the experiment-wide error rate was not adjusted, and all the interpretations of the effects of the different treatments were performed by evaluating the raw *P* values. All ANOVA analyses were performed using the program PERMANOVA 1.6 [3].

The binary matrix obtained with the number and position of individual DGGE bands was used to generate a similarity matrix with the Jaccard coefficient of similarity. A dendrogram showing the relationships among samples was obtained by cluster analysis. This analysis was performed using the similarity matrix and the unweighted pair-group method algorithm (UPGMA), as implemented in the UPGMA program of the PAUP* software package, version 4.0 [56]. Estimates of statistical support for the resulting clusters were obtained in a UPGMA bootstrap analysis over 10,000 replicates.

Results

At the end of the experiment, average rates of C_2H_2 reduction in the noninoculated microcosms were negligible, regardless of the addition of composted sewage sludge (<0.001 nmol $C_2H_4 \cdot cm^{-2}$ h^{-1} in both cases, data not shown). The highest reduction rates in inoculated microcosms were found when the crust was inoculated as slurry, composted sewage sludge was added, and the microcosms were watered five times per week (Fig. 1A). However, ANOVA analyses revealed significant three- and two-way interactions for this variable (Table 2). Separate analyses conducted at each fertilization level revealed a negative effect of watering frequency (two-way ANOVA, $F_{1,28} = 5.69$, $P = 0.025$), but no significant effect of inoculation technique ($F_{1,28} = 1.00$, $P = 0.327$) or the watering × inoculation technique interaction ($F_{1,28} = 0.33$, $P = 0.572$) on C_2H_2 reduction rates in the absence of composted sewage sludge. In amended microcosms, significant effects of watering frequency ($F_{1,28} = 17.68$, $P < 0.001$) and inoculation technique ($F_{1,28} = 10.65$, $P < 0.001$), as well as a significant interaction ($F_{1,28} = 6.50$, $P = 0.012$), were found. A detailed analysis of this interaction showed that C_2H_2 reduction rates were higher when the biological soil crusts were inoculated as discrete fragments in the high watering treatment (one-way ANOVA, $F_{1,14} = 11.19$, $P = 0.005$). In the low watering treatment, the effect of inoculation was only marginally significant ($F_{1,14} = 4.43$, $P = 0.054$).

The content of chlorophyll *a* was significantly affected by all the treatments evaluated (Fig. 1B, Table 2). Higher values of this variable were found when the crust was inoculated as slurry. The addition of composted sewage sludge and the increase in watering frequency also increased the content of chlorophyll *a*. The content of chlorophyll *a* in the noninoculated microcosms (6.05 ± 2.47 and 3.72 ± 1.23 $mg \cdot mg^{-2}$ with and without composted sewage sludge, respectively; mean ± SE, $n = 5$) was significantly lower than that found in the inoculated

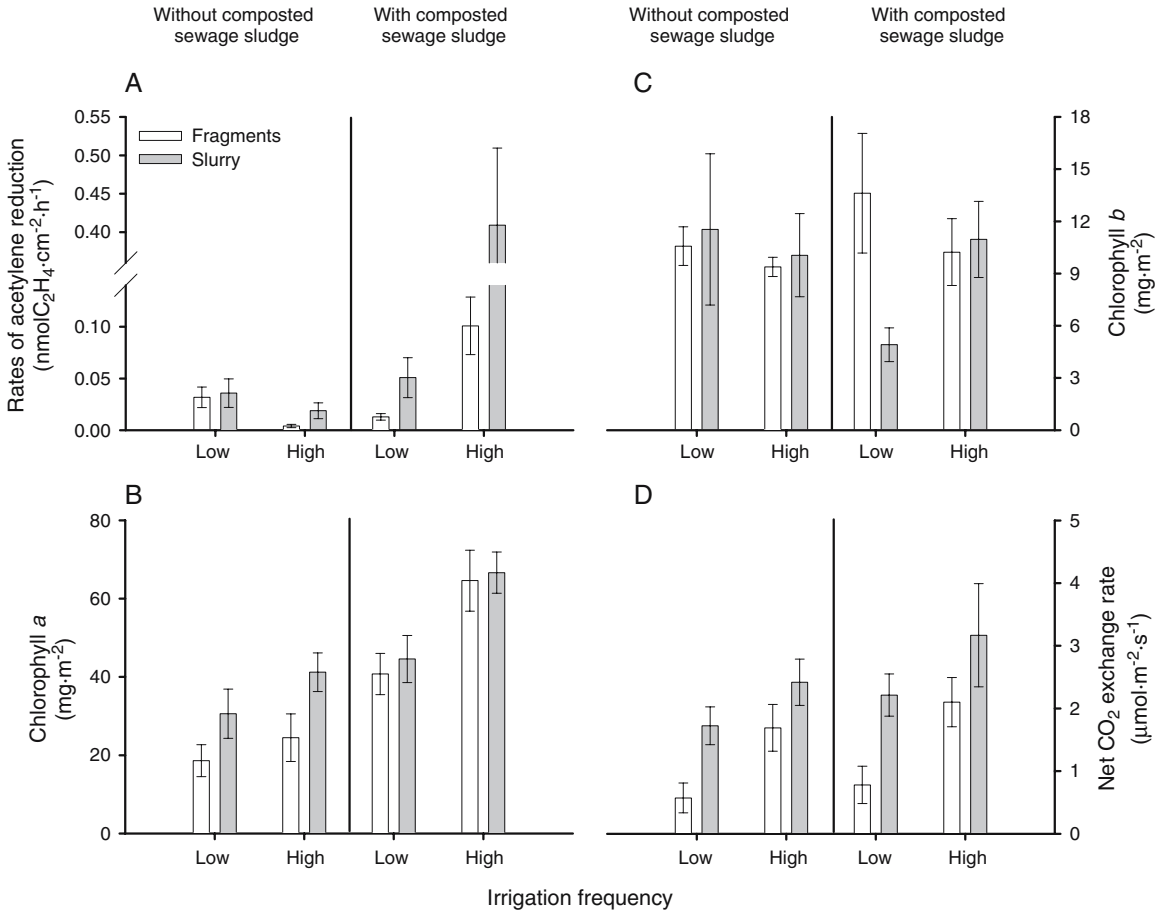


Figure 1. Rates of acetylene reduction, expressed as ethylene production (A), content of chlorophylls *a* (B) and *b* (C), and net carbon exchange rate (D) compared among treatments 6 months after the inoculation of the microcosms. Data represent mean ± SE (n = 8 for acetylene reduction and net CO_2 exchange rate and n = 5 for chlorophyll measurements).

microcosms (*post hoc* results not shown). ANOVA analyses revealed a marginally significant inoculation type × watering frequency interaction when evaluating chlorophyll *b* data (Table 2). This interaction was mainly driven by the low chlorophyll *b* values found when the crust was inoculated as a slurry, sewage composted sludge was added, and the microcosms were irrigated twice a week (Fig. 1C). When composted sewage sludge was added to the noninoculated microcosms, the content of chlorophyll *b* (1.87 ± 0.36 mg·mg^{-2}; mean ± SE, n = 5) was significantly lower than that found in most of the inoculated microcosms (*post hoc* results not shown). When this sludge was not added to the noninoculated microcosms, the content of chlorophyll *b*

Table 2. Summary of ANOVA analyses conducted with the rates of acetylene reduction, the content of chlorophyll, and the net CO_2 exchange data obtained six months after the inoculation of the microcosms

	Rates of acetylene reduction			*Chlorophyll a*			*Chlorophyll b*[a]			*Net CO_2 exchange rate*		
Source of variation	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Inoculation type (I)	11.47	1,56	***<0.001***	4.43	1,32	***0.046***	3.34	1,32	0.076	13.14	1,56	***<0.001***
Water frequency (W)	13.89	1,56	***<0.001***	14.43	1,32	***0.001***	0.68	1,32	0.407	11.47	1,56	***<0.001***
Fertilization (FE)	20.09	1,56	***<0.001***	38.26	1,32	***<0.001***	0.37	1,32	0.546	2.37	1,56	0.126
I × W	6.81	1,56	***0.005***	0.03	1,32	0.855	3.82	1,32	0.062	0.43	1,56	0.517
I x FE	9.23	1,56	***<0.001***	1.94	1,32	0.178	1.42	1,32	0.237	0.26	1,56	0.619
W × FE	20.75	1,56	***<0.001***	3.20	1,32	0.082	1.00	1,32	0.321	0.15	1,56	0.701
I × W × FE	5.81	1,56	***0.011***	0.16	1,32	0.694	2.09	1,32	0.159	<0.01	1,56	0.964

Values below $P < 0.05$ are in bold.
[a] ANOVA results are shown for $\log_{10}$- transformed data.

(4.84 ± 1.91 mg·mg^{-2}; mean ± SE, $n = 5$) did not differ with that found in the inoculated microcosms (*post hoc* results not shown).

The net CO_2 exchange rate measured at the end of the experiment was positive in all cases, indicating that photosynthetic rates were greater than respiration rates (Fig. 1D). The effects of inoculation technique and watering frequency on this variable were significant (Table 2). Higher values of net CO_2 exchange rate were found when the crust was inoculated as slurry and under the high watering frequency treatment. The net CO_2 exchange rate values measured in the control microcosms were negative, both with (−1.15 ± 1.11; mean ± SE, $n = 5$) and without (−0.24 ± 0.49; mean ± SE, $n = 4$) composted sewage sludge. The differences between these treatments were not significant (one-way ANOVA; $F_{1,7} = 2.34$, $P = 0.170$).

DGGE analysis allowed the identification of a total of 32 different band positions (Fig. 2A). Each sample produced a complex fingerprint composed of a large number of bands: from 16 to 22 bands in the two control samples from the original soil crust (OC1 and OC2 in Fig. 2A), and from 6 to 21 bands in the remaining samples. Mostly identical DGGE patterns were observed in both OC replicates, whereas the other samples analyzed exhibited varied banding patterns. The data obtained in the DGGE gels were subjected to image analysis, which resulted in the binary matrix (presence or absence of bands in different samples) shown in Fig. 2B. This analysis revealed that samples under different treatments shared several common phylotypes but displayed distinct fingerprints and band richness. Only one phylotype was shared between all the samples analyzed including the OC samples (band 14 in Fig. 2A and B), whereas four phylotypes were unique to the OC samples (bands 2, 3, 26, and 27 in Fig. 2A and B), and eight new phylotypes appeared only in inoculated samples (bands 10, 11, 13, 18, 20, 29, 30, and 31 in Fig. 2A and B).

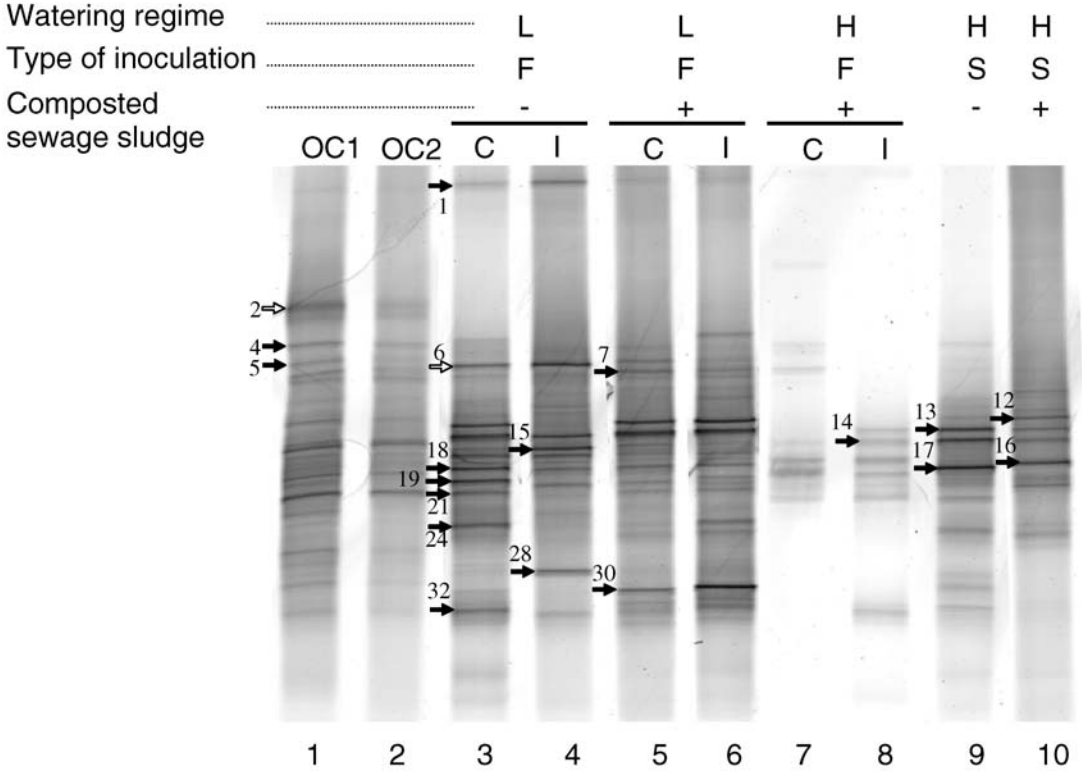
Phylogenetic analyses of sequenced bands using the ARB package indicated that all DGGE bands were of cyanobacterial (17 bands) or chloroplast (2 bands) origin (Fig. 2B). From these analyses, it was apparent that the diversity among cyanobacteria was pronounced and encompassed both unicellular and filamentous genera. Sequence similarity levels in BLAST analyses were above 98% in only two cases: band 15, which was 98% similar to the 16S sequence of an uncultured soil bacterium from the Imperial Valley, CA (AY493984, Kim, personal communication), and band 28, which was 99% similar to the 16S sequence of *Oscillatoria* sp. MPI990BR03 (AF284810), an isolate from a carbonaceous silt crust in Spain [28]. Sequence of band 14 shared a 97% similarity with the 16S sequence of *Microcoleus* sp. PCC 8701 (AY768403), which was consistent with its position within the *Microcoleus* clade in our tree. Similarities of all other bands to sequences in the databases were all below 96%. However, clustering information from our phylogenetic analyses allowed us to associate the remaining sequences with previously characterized genera (Fig. 2B).

The binary matrix was used to build a dendrogram based on the comparison of the cyanobacterial assemblage patterns by cluster analysis (Fig. 3). This analysis revealed that those samples that were treated in a similar way (e.g., watering frequency) exhibited more similar genetic fingerprints. In contrast, a remarkable difference was observed when high and low watering frequency fingerprints were compared. Samples with low watering frequency (lanes 3–6 in Fig. 2A) yielded the highest band richness (17–21) and were grouped together into the same cluster as the closest to the OC samples (16–24 bands). Only a few bands present in the OC samples were apparent in samples from the high watering frequency treatment (lanes 7–10 in Fig. 2A). The addition of composted sewage sludge also generated fingerprint differences between samples with the same watering frequency. For instance, low watering frequency samples (lanes 3 and 5 in Fig. 2A) were placed in two separate subclusters, depending on the addition of composted sewage sludge. A similar phenomenon appeared in samples subjected to frequent watering, where the addition of composted sewage sludge and the inoculation technique separated the samples into several clusters. In those samples where inoculation was performed by inserting intact crust fragments, cyanobacterial populations from the original crust fragment or from the regions between fragments clustered together in the dendrogram (Fig. 3), indicating a similar composition in cyanobacterial species at the point of inoculation and in the surrounding soil surface.

Discussion

Responses of the Inoculated Biological Crusts to Inoculation and Fertilization. The inoculation of a degraded soil with biological soil crusts significantly improved the nitrogen fixation and carbon sequestration capability of the soil. Our results agree with those of Buttars *et al.* [17], who found higher N fixation rates 3 months after the inoculation of a degraded soil under laboratory conditions. Complementary field investigations showed an enhanced recovery of lichen and cyanobacterial biomass and species diversity after the inoculation of degraded soils with cyanobacterial inoculants and intact crust fragments in arid environments ([6, 21, 54], but see [36]). Information on cyanobacteria recovery rates is scarce. However, it seems that, under appropriate environmental conditions, cyanobacteria colonization may be relatively fast [6, 11]. The treatments evaluated had, however, important effects on the way that the

A



B

	1	2	3	4	5	6	7	8	9	10	Clusters with in phylogenetic analyses	Length
Band 1											*Band 5/ Spirulina subsalsa/ Synechococcus sp. PCC 7002/ Pleurocapsa sp. PCC 7516*	317 nt
Band 2											*Chlorella vulgaris plastid*	579 nt
Band 3												
Band 4											*Band24/ Band28/ Band32/ Oscillatoria sp. MPI990BR03/ Chroococcidiopsis thermalis*	289 nt
Band 5											*Band 1/ Spirulina subsalsa/ Synechococcus PCC 7002/ Pleurocapsa sp. PCC 7516*	328 nt
Band 6											*Chlorella vulgaris plastid*	343 nt
Band 7											*Band 18/ Trichodesmium erythraeum /Microcoleus vaginatus MOA4/ Lyngbya majuscula/ Phormidium sp. ETS-05*	376 nt
Band 8												
Band 9												
Band 10												
Band 11												
Band 12											*Band 16/ Leptolyngbya sp. VRUC 135/ Uncultured Antarctic cyanobacterium clone Fr246*	384 nt
Band 13											*Band 14/ Band 19/ Band 30/ Microcoleus steenstrupii clone 148-2A/ Microcoleus sp. PCC 8701/ Microcoleus chthonoplastes/ Symploca atlantica PCC 8002*	408 nt
Band 14											*Band 13/ Band 19/ Band 30/ Microcoleus steenstrupii clone 148-2A/ Microcoleus sp. PCC 8701/ Microcoleus chthonoplastes/ Symploca atlantica PCC 8002*	358 nt
Band 15											*Uncultured soil bacterium clone 349*	395 nt
Band 16											*Band 16/ Leptolyngbya sp. VRUC 135/uncultured Antarctic cyanobacterium clone FR246*	393 nt
Band 17											*Phormidium mucicola M-221/ Pseudoanabaena sp. PCC 7367/ Oscillatoria limnetica MR1*	355 nt
Band 18											*Band 07/ Trichodesmium erythraeum /Microcoleus vaginatus MOA4/ Lyngbya majuscula/ Phormidium sp. ETS-05*	386 nt
Band 19											*Band 13/ Band 14/ Band 30/ Microcoleus steenstrupii clone 148-2A/ Microcoleus sp. PCC 8701/ Microcoleus chthonoplastes/ Symploca atlantica PCC 8002*	329 nt
Band 20												
Band 21											*Oscillatoria sp. M-117/ Phormidium sp. M-99/ Leptolyngbya boryanum PCC73110/ Leptolyngbya foveolarum Komarek 1964/112*	355 nt
Band 22												
Band 23												
Band 24											*Band04/ Band28/ Band32/ Oscillatoria sp. MPI990BR03/ Chroococcidiopsis thermalis*	344 nt
Band 25												
Band 26												
Band 27												
Band 28											*Band04/ Band24/ Band32/ Oscillatoria sp. MPI990BR03/ Chroococcidiopsis thermalis*	410 nt
Band 29												
Band 30											*Band 13/ Band 14/ Band 19/ Microcoleus steenstrupii clone 148-2A/ Microcoleus sp. PCC 8701/ Microcoleus chthonoplastes/ Symploca atlantica PCC 8002*	410 nt
Band 31												
Band 32											*Band04/ Band24/ Band28/ Oscillatoria sp. MPI990BR03/ Chroococcidiopsis thermalis*	323 nt

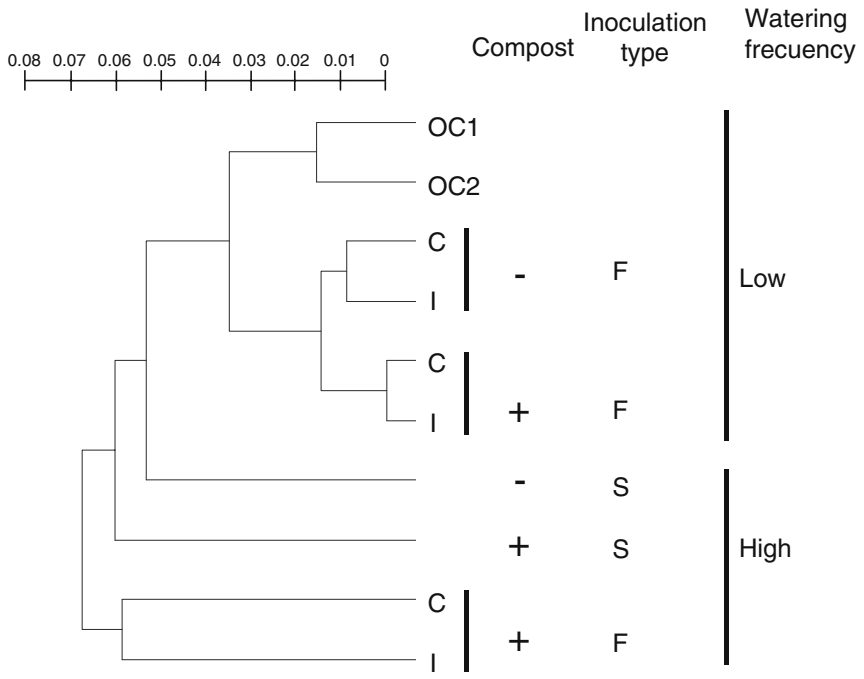


Figure 3. Dendrogram showing the degree of similarity between the cyanobacterial communities from selected samples 6 months after the inoculation of the microcosms. For explanation of codes, see Fig. 2 caption. *Scale bar* indicates dissimilarity: 0.01 is equivalent to 1 difference in 10 bands.

different functions evaluated recovered 6 months after the initial inoculation.

None of the sequences in our samples is related to heterocyst-forming cyanobacteria, which are common inhabitants of desert biological crust [62]. Although nitrogen fixers are found among all cyanobacterial subsections, nonheterocystous cyanobacteria generally separate the incompatible processes of photosynthesis and nitrogen fixation temporarily, so that nitrogen fixation only occurs in the dark. The fact that we can detect acetylene reduction under illumination may be caused by the presence of heterocyst-forming cyanobacteria that have not been detected in our sequences (i.e., the corresponding bands were not picked from the gel) or by the presence of nonheterocystous genera such as *Chroococcidiopsis* or *Symploca*, which include some species able to fix nitrogen in oxic conditions under constant illumination [13, 46].

The remarkable differences in N fixation rates between our samples most likely resulted from differences in the density of diazotrophs and/or from differences in the regulation of the activity of the nitrogenase complex caused by the different treatments applied. It has been shown that cyanobacteria are physiologically active only when wet, and that the rates of N fixation by the biological soil crust constituents are ultimately controlled by soil moisture [8, 9]. The effect of soil moisture on the rates of N fixation by biological soil crusts is most likely mediated by its effects on cyanobacterial carbon fixation [37]; as N fixation requires the products of photosynthesis, water availability ultimately determines its extent [8]. Interestingly, the rates of C_2H_2 reduction in our study increased with increasing watering frequency only in the presence of composted sewage sludge and decreased with increasing watering frequency in unamended samples. These results were unexpected initially, as generally N additions have been shown to reduce or eliminate N fixation activity by crust organisms ([8], but see [44]). There are, at least, two plausible hypotheses that could explain the results obtained. It is

Figure 2. (A) DGGE profiles of PCR-amplified 16S rRNA gene fragments obtained from the different treatments 6 months after the inoculation of the microcosms. L = low frequency of watering, H = high frequency of watering, F = inoculation as discrete crust fragments (in this treatment, C and I indicate samples from the inoculated crust fragments and the interspaces between them, respectively), and S = inoculation as slurry. OC1 and OC2 are samples coming from the original soil crust used to inoculate the microcosms. The addition of composted sewage sludge is indicated by a "+" symbol. Bands that were sequenced are pinpointed with an *arrow*. Lanes of the gel are indicated at the *bottom*. (B) Binary matrix indicating presence (*black boxes*) or absence (*empty boxes*) of bands in the DGGE gel. *Dashed boxes* indicate bands that are not present in the original crust samples. *Shaded boxes* indicate bands that are present only in the original crust samples. *Band numbers on the left* correspond to numbers assigned to each band in the DGGE gel. *Numbers 1–10* represent the different lanes displayed in (A). The best hits in BLAST analyses and the similarity percentage are indicated for those sequences with similarity above 96%. Selected genera from the same clade in phylogenetic analyses are shown, and the length of the sequences is indicated on the *right*.

possible that the fixation of atmospheric N is limited by resources other than carbon (e.g., phosphorus and micronutrients such as Cu and Mn). Acetylene reduction activity is frequently P-limited [8]. Cu deficiency has been linked to suppressed N fixation in vascular plant–rhizobium associations [20] and could limit N fixation for N-fixing lichens as well. Bowker *et al.* [16] reported that Mn deficiency controls the distribution of the common N-fixing lichen *Collema tenax* in western USA. Therefore, as with Cu, limitation by Mn may reduce N fixation by biological soil crusts. The results obtained may also be explained by the higher soil moisture availability in the treatments with composted sewage sludge because of increased water retention caused by the addition of organic matter [42]. Our experimental design and measurements cannot provide consistent evidence to discern among these alternatives, and future studies are needed to elucidate the mechanisms underlying the responses observed.

The content of chlorophyll *a* was affected by the three treatments evaluated, as suggested by the lack of significant interactions between them. Higher contents of chlorophyll *a* were found with increasing watering frequency, when composted sewage sludge was added and when the biological soil crusts were inoculated as slurry, indicating that these treatments accelerated the recolonization of the inoculated soil by photosynthetic organisms. Among these treatments, the strongest effect was promoted by the addition of composted sewage sludge; this agrees with studies showing increases in the content of chlorophyll *a* after fertilization [11]. It must be noted that the soil used for this experiment was highly degraded, and nutrient availability was probably very low. The content of chlorophyll *a* was significantly related to both net CO_2 exchange rate (Spearman correlation coefficient = 0.391, $P = 0.013$, $n = 40$) and C_2H_2 reduction rates (Spearman correlation coefficient = 0.665, $P < 0.001$, $n = 40$), indicating that this variable may act as a good surrogate for potential carbon and nitrogen fixation. However, it must be noted that chlorophyll content within a cell changes throughout time, depending on conditions that affect carbon fixation or the need for carbon products for other compounds (e.g., ultraviolet pigments during periods of high light intensity) [27]. Thus, chlorophyll *a* would be a surrogate for potential nitrogen fixation only during certain conditions/seasons. Chlorophyll *b* is synthesized in crusts by chlorophytes, whose presence in our samples has been evidenced by sequence analysis (see above), but not by cyanobacteria [15]. It is interesting to point out that the content of chlorophyll *b* did not differ among the non-inoculated microcosms and some of the inoculated treatments after 6 months, suggesting that phototrophs other than cyanobacteria were able to spread and colonize surrounding areas of soil in our experiment. This interpretation should, however, be taken cautiously. Although we did not measure the abundance of chlorophytes in our samples, they usually have a low abundance in biological soil crusts [15], something that do not match with the relatively high content of chlorophyll *b* we found. Thus, it is possible that some contamination with moss tissue or lichen photobionts occurred.

Both the inoculation technique and the watering frequency significantly affected the net CO_2 exchange rate, which showed higher values when the crust was inoculated as slurry and under the highest watering frequency. The effect of watering was expected, as the photosynthetic activity of the organisms forming the biological soil crusts, and thus their growth, is primarily limited by water [37]. The significant effect of inoculation type on this variable indicates that the inoculation of the crust as slurry promoted the growth of its photosynthetic components during the course of our study.

Some of the 16S sequences obtained from our samples (band 24) are highly similar to known crust inhabitants, such as *Oscillatoria* MPI 990BR03, or to cyanobacteria from agricultural soil (band 15). Others cluster within cyanobacterial clades of crust-associated cyanobacteria, such as *Microcoleus steentrupii* (bands 13, 14, 19, and 30) [14], *Phormidium* sp. M-99, *Oscillatoria* sp. M-117, *Leptolyngbya boryanum* and *Leptolyngbya foveolarum* (band 21), or *Chroococcidiopsis* sp. and *Oscillatoria* MPI 990BR03 (bands 4, 28, and 32) [24, 28]. The other sequences cluster with diverse cyanobacteria not necessarily associated to crust or soil ecosystems. Band richness in DGGE gels suggests that cyanobacterial populations recovered by DGGE are consistently less diverse under high-frequency watering than under low watering frequency and less diverse than the original soil crust. These results contrast with those observed by Rothrock and García-Pichel [50] in benthic microbial mats, who found that the richness and diversity of cyanobacteria decreased as desiccation frequency increased. The decrease in cyanobacterial diversity with watering frequency observed in our study may result from the proliferation of species with faster growth rates, which may outcompete other cyanobacteria from the original community.

The subclustering of samples shown in Fig. 3 is indeed consistent with the different treatments applied and indicated that they promoted clear changes in the composition of the communities according to the way the biological crust was inoculated. Watering frequency was the factor with the most drastic effect on the composition of the cyanobacterial community, separating our samples in two higher clusters, followed by fertilization, and inoculation type as the least important factor for selection. Thus, all treatments used in our study were important to select the composition and distribution (or colonization) of species at different levels. It is worth noting that

cyanobacterial composition in inoculated crust fragments and in the interspaces between crust fragments was very similar (Fig. 3), indicating that most of the species already present in the core had the capability to spread and colonize the surface of the surrounding soil.

Implications for the Restoration of Degraded Drylands. Given their sensitivity to human-induced disturbances, biological soil crusts are in a degraded state in many areas throughout their range [11]. As these organisms play a critical role in the functioning of arid and semiarid ecosystems, restoring the crusts of degraded areas may contribute to the recovery of their functionality [41]. This study represents one of the first attempts to evaluate the potential of inoculation techniques to accelerate the recovery of the composition and functioning of biological soil crusts in degraded soils from semiarid Mediterranean areas. Our results suggest that the inoculation of biological soil crusts in the form of slurry combined with the addition of composted sewage sludge could be a suitable technique to foster the recovery of biological soil crust in these areas. The use of organic residues such as composted sewage sludge to aid in the recovery of biological soil crusts in Mediterranean semiarid areas is especially appealing. It has been shown that the addition of composted sewage sludge enhances soil fertility and microbial activity in these regions [49], as well as the performance of vascular plants commonly used in restoration programs [19, 57]. The use of composted sewage sludge to enhance the recovery of biological soil crusts would complement such uses.

Our experimental approach has some limitations that need to be acknowledged. For example, the standardized environmental conditions within the chambers (i.e., low light and up to 70% RH, regular irrigation regimes) do not reflect those existing in the field and may amplify (high humidity and regular irrigation regimes) or diminish (low light conditions) responses to watering and fertilization over those that may be observed in the field. This limitation is applicable to many controlled-environment studies. Owing to these limitations, the extrapolation of our results to the natural world should be performed with caution. Rather, they must be considered as a first step in the development of a strategy to recover the biological soil crusts of these areas. The effectiveness of the treatments evaluated here must be confirmed by field studies conducted under natural environmental conditions and over larger areas and time frames. These could be complemented with studies devoted to develop suitable application techniques at a management scale and to isolate native cyanobacteria suitable for *ex situ* mass culturing methods [17, 36]. Such a development would minimize the collection of intact biological crusts from nondisturbed areas to obtain inoculum, one of the main drawbacks of using inoculation techniques to restore biological soil crusts in degraded areas [18].

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