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ORIGINAL PAPER

Amendment of degraded desert soil with wastewater debris containing immobilized *Chlorella sorokiniana* and *Azospirillum brasilense* significantly modifies soil bacterial community structure, diversity, and richness

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Abstract The main goal of this study was to expand our knowledge of what happens to the soil bacterial community in an eroded desert soil when improvement of soil fertility is derived from the application of debris of tertiary wastewater treatment containing immobilized microalgae *Chlorella sorokiniana* and the plant growth-promoting bacterium (PGPB) *Azospirillum brasilense*. We hypothesized that an "improved" non-agricultural desert soil will exhibit substantial changes in the structure of the bacterial community in a relatively short time after amendment. To assess the effect of the amendments, microalgae and PGPB alone or combined, on the structure of the rhizosphere bacterial community, changes in species richness and bacterial diversity over time were based on sequence differences in the 16S rRNA gene, performed with PCR–denaturing gradient gel electrophoresis (DGGE) and then analyzed by similarity test and non-metric multidimensional scaling analysis. Root surface colonization and persistence in the rhizosphere of *A. brasilense* was monitored by fluorescent in situ hybridization and sequencing of DGGE bands. Application of waste debris significantly changed the rhizosphere bacterial population structure, whether comparisons were made over time, between inoculated and non-inoculated soil, and among different inoculated microorganisms. Species richness and diversity increased when the waste debris contained the microalgae–bacteria association and also over time. Even as its secondary role as an inoculant after wastewater treatment, *A. brasilense* colonized the root surface profusely and persisted within the rhizosphere bacterial community. This study demonstrated that small organic amendment to desert soil significantly changed soil bacterial community compared to the original soil and also 2 months after amendments were added.

Keywords *Azospirillum* · Bacterial community structure · Bacterial diversity and richness · *Chlorella* · Microalgae · Plant growth-promoting bacteria · PGPB · PGPR · Rhizosphere

This study is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC, Spain.

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Introduction

Deserts are frequently characterized by low productivity and large patches of bare surfaces. Most nutritional and water resources are concentrated in resource islands of three types: under the canopy of nurse plants, around pioneer plants in rocky environments, and in soil crusts. In these resource

islands, proliferation of numerous microorganisms occurs (Bashan and de-Bashan 2010). Nonetheless, compared to the literature on bacteria in agriculture, our understanding of arid zone soil microorganisms and microbial ecology of arid soils is miniscule.

Bacteria in arid lands were studied for decades, mostly related to agricultural crops. Bacteria in native desert habitats are far less studied. Mostly, studies were done in specific environments, such as the diversity of bacterial populations tolerant to desiccation and radiation in the Tataouine Desert in Tunisia (Chanal et al. 2006) or the limited bacterial population and diversity in the hyper-arid Atacama Desert in Chile (Drees et al. 2006; Warren-Rhodes et al. 2006; Gómez-Silva et al. 2008).

Studies of bacterial populations in eroded desert lands and factors controlling them, apart from arid mine tailings that are man-made "soils," are scarce. Restoration of these soils with plant growth-promoting bacteria (PGPB) that are well known in agriculture (Bashan and de-Bashan 2005; Lugtenberg and Kamilova 2009) was demonstrated only in recent years in vegetation restoration projects in abandoned, dry land habitats (for a review, see de-Bashan et al. 2012 and references therein).

The use of wastewater sludge for improving soil is very common (Smith 1995) and also tested in arid lands (Brendecke et al. 1993). A tertiary wastewater treatment, using immobilized microalgae and PGPB, was proposed, tested on an experimental scale, and is currently in a process of scaling up (de-Bashan et al. 2004; Hernandez et al. 2006). Once employed, this wastewater treatment produces leftover debris. This debris contains large populations of the microalgae *Chlorella* spp. and the PGPB *Azospirillum brasilense*. Recently, using this debris, small improvements in the fertility of highly eroded desert soil was detected, and its application significantly improved plant growth (Trejo et al. 2012). Consequently, the central hypothesis of this molecular study was that an "improved" non-agricultural desert soil will exhibit substantial changes in the profile of the bacterial community in a relatively short time after amendment. This will happen because changes in soil fertility, supported by various organic amendments in agricultural soils, are usually associated with effects on the microbial communities of the soil. The main goal of this study was to expand our knowledge of what happens to the soil bacterial community and to the PGPB in an eroded desert soil when improvement of soil fertility is derived from debris of tertiary wastewater treatment containing microalgae and a PGPB.

Denaturing gradient gel electrophoresis (DGGE) fingerprinting is a standard method of characterizing soil bacterial communities, together with T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments. Although each method assesses a different fragment, the general results regarding bacterial communities in the soil are very similar (Smalla et al. 2007). DGGE was employed in several studies of bacteria in deserts (Nagy et al. 2005; Drees et al. 2006; Gothwal et al. 2007; Campbell et al. 2009; Kenzaka et al. 2010).

Four strategies were employed to address our hypothesis: (1) analysis of the structure of the bacterial community under various combinations of amendments. We used PCR-DGGE fingerprinting analyzed by a similarity test and a statistical multivariate analysis (non-metric multidimensional scaling analysis). (2) Partial assessment of bacterial richness and diversity, since the number of bands observed in the PCR-DGGE profile provides an estimate of species richness and that the relative intensity of each band provides an estimate of the relative abundance of each species (Iwamoto et al. 2000). We were also aware of the inherent limitation of this analysis (Bent and Forney 2008), which is incapable of detecting all of the microbial diversity in the soil of the Sonoran Desert. (3) Monitoring PGPB that was applied indirectly with the debris using the molecular detection technique of fluorescent in situ hybridization under confocal laser scanning microscope (FISH; Dazzo et al. 2007). (4) Identification of the applied *A. brasilense* within the general bacteria community of the rhizosphere by sequencing PCR products from excised bands of PCR-DGGE profiles.

Material and methods

Soil source, sampling, and soil fertility

Sorghum (*Sorghum bicolor* (L.) Moench, cv. Honey Graze, Cal-Oro, Lubbock, TX) plants were grown in highly degraded alluvial desert soil (haplic yermosol; described in Bashan et al. 2000). Artificially prepared domestic wastewater (Gonzalez and Bashan 2000) was used. The wastewater contained no heavy metals, antibiotics, human pathogens, or recalcitrant materials. Debris from a laboratory simulation of tertiary wastewater treatment was performed with the removal of nutrients using alginate beads containing the microalgae *Chlorella sorokiniana* Shih. et Krauss (UTEX 2805, Austin TX; de-Bashan et al. 2008) and the microalgae growth-promoting bacterium *A. brasilense* Cd (DSM 1843, Braunschweig, Germany), immobilized singly or together (de-Bashan et al. 2004). After wastewater treatment, alginate beads were recovered and oven-dried at 40–43 °C for 24 h, yielding debris of dry beads containing live *A. brasilense*. Then, 0.2 g of this debris was added to the soil at a level of 0.3×10^6 CFU bead^{-1} (*A. brasilense*), corresponding to 1.5×10^6 CFU pot^{-1} (120 ml). The debris of dry beads did not contain additional organic material. In this soil, sorghum plants were grown in a

shade house at ambient temperature ~29 °C and light intensity of ~1,000 μmol photons m^2 s^{-1} for 20 days (cycle 1). The potted seedlings were irrigated with 38.5 ml distilled water every 3 days to maintain field capacity and then harvested. A second application of new debris was added to the same soil and a new crop of sorghum was grown (cycle 2) for 20 days. The process was repeated one more time (cycle 3). Effects on plant growth (dry weight, shoot and root length) and increases in total C in the soil were measured after each cycle. Microbial C was measured after cycle 3 (Electronic supplementary material (ESM) Fig. S1 and Table S1). An increase in total C and microbial C served as an indicator of improved soil fertility. All of this was conducted in a previous study (Trejo et al. 2012). This experiment was repeated twice, once with tomato plants (unpublished data) and once with sorghum plants (Trejo et al. 2012). Both experiments yielded very similar results. In this study, soil samples from the experiment with sorghum plants were analyzed for changes in the microbial populations.

Extraction of DNA from soil and PCR amplification

Rhizosphere soil samples (soil adhered to root after mild agitation; Hartmann et al. 2008) from the treatment of alginate bead debris containing microalgae and bacteria were collected after each planting cycle. Samples from the remaining treatments were collected only at the third planting cycle. About 3 g of root-free rhizosphere soil was collected in 1.5-ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) and stored at −20 °C until analysis. Cold storage has no effect on the analysis of bacterial community by PCR-DGGE (Campbell et al. 2009). Extraction of DNA from soil was a modification of the method described by de-Bashan et al. (2010a, b), using a kit (Fast DNA SPIN for soils, MP Biomedicals, Santa Ana, CA), according to the manufacturer's instructions. To remove humic acids, the binding matrix–DNA complex was rinsed with saturated 5.5 M guanidine thiocyanate (Fluka Sigma-Aldrich, Buchs, Switzerland). Each DNA extraction was performed with a 0.6-g soil sample. Three replicates were analyzed for each treatment because of the technical limitation of the DGGE gels (see below).

Polymerase chain reaction

A modification of the PCR procedure described by de-Bashan et al. (2010a, b) was used. The V9 variable region of the 16S rRNA gene was amplified using the bacteria primers 1070F (5′-ATG GCT GTC GTC AGC T-3′) and 1406R (5′-ACG GGC GGT GTG TAC-3′) with a 40-bp GC clamp (Ferris et al. 1996). A modification of the PCR for DGGE by Colores et al. (2000) was used. Details of the modifications are presented in the ESM.

Denaturing gradient gel electrophoresis (PCR-DGGE) analysis

Initially, the community was analyzed at the end of the third cycle of inoculation, comparing inoculated plants with bead debris containing *A. brasilense* and C. *sorokiniana*, un-inoculated plants, unplanted soil, and a control of only beads. We also examined the time effect of inoculation with co-immobilized *A. brasilense* and *C. sorokiniana* on the rhizosphere bacterial community, comparing the three cycles of growth. A modification of the DGGE of the 16S rRNA gene products by de-Bashan et al. (2010a, b) was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Details of the modifications are presented in the ESM.

Measuring numerous bands on DGGE gels seldom allows reliable comparison among different gels. The effect of gel-to-gel variation is an inherent difficulty of DGGE analysis and is complicated to address (Tourlomousis et al. 2010). Practically, this limits the number of replicates of different treatments a single gel can handle. Only very recently, a simplified technical improvement to address this difficulty was proposed (Valentín-Vargas et al. 2013). This was not employed in this study which was carried out earlier. Consequently, a combination of several gels, each containing comparable treatments with their respective replicates, were used in this study to measure the effect of a specific treatment on the soil bacterial population (Lynch et al. 2004; Smalla et al. 2007). Each gel contained three or four soil treatments and an external reference ladder (total of 10–13 lanes per gel, as permitted by the equipment we used). Digital images of DGGE were analyzed to generate a densitometric profile of bands using imaging software (Quantity One 4.6.7, Bio-Rad Laboratories).

Identification of *A. brasilense* in PCR-DGGE profiles

Presumptive bands of *A. brasilense* Cd were excised from DGGE gels (45–60 % gradient) using sterile razor blades under UV illumination. The excised bands were eluted in 300 μl ultrapure water and incubated at 37 °C for 1 h. Aliquots were diluted 1:10 in ultrapure water; 2 μl of this dilution was used as a template to re-amplify the replicon using the same PCR conditions and DGGE primers described earlier. The size of the PCR product was confirmed on 2 % agarose gel after each round of amplification. Successive PCR-DGGE gels were run to verify the identity and purity of the excised bands by comparing the re-amplified PCR products to the profile of the external reference ladder

containing *A. brasilense*. PCR products that exhibited the highest identity to the *Azospirillum* band in the DGGE gel were purified using the QIAquick PCR purification kit protocol (Qiagen Sciences) and then submitted for commercial sequencing using primer 1070F (Genewiz, South Plainfield, NJ). The original PGPB inoculum and its corresponding band in the external reference ladder were also sequenced at the same time as the experimental samples. Representative sequences from experimental samples at each cycle of inoculation were deposited in the GenBank database (accession nos. JX133090, JX133091, JX133092, JX133093, JX133094).

Fluorescence in situ hybridization

These procedures followed Trejo et al. (2012). Briefly, the three root zones (root tip, elongation zone, and lateral and root hair zone), all areas colonized by *Azospirillum* spp. (Bashan et al. 2004), were prepared as described in de-Bashan et al. (2010a) and Trejo et al. (2012). Specific detection of *A. brasilense* on roots was done by FISH with the following oligonucleotide probes: an equimolar mix of probes EUB-338-I (5′-GCTGCCTCCCGTAGGAGT-3′; Amann et al. 1990), EUB-338-II (5′-GCAGCCACC CGTAGG TGT-3′), and EUB-338-III (5′-GCTGCCACCCGTAGGTGT-3′; Daims et al. 1999) that are specific for the domain bacteria and the probe Abras-1420 (5′-CCACCTTCGGG-TAAAGCCA-3′; Stoffels et al. 2001) specific for *A. brasilense*. Probes were labeled with either fluorochrome Cy3 or Cy5 (Interactive Division, Thermo Electron, Ulm, Germany). Hybridization was performed according to Stoffels et al. (2001) and de-Bashan et al. (2010b). Visualization was performed as described by Rothballer et al. (2003), with confocal laser scanning microscopy (LSM 510, Axiovert 100M, Zeiss, Jena, Germany). A three-channel observation technique was used at excitations of 543 and 633 nm, corresponding to the dyes Cy3 (red) and Cy5 (blue); a third color channel (488 nm, green) was used to visualize the autofluorescence of the root. This technique can use overlapping of images, resulting in differentiation among the observed objects: root surfaces were green, bacteria in general were red, and *Azospirillum* cells were magenta. The images were analyzed with specialized software (LSM 510 4.2, Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Each gel, with its respective treatments, was run several times; one representative gel was considered for each comparison of treatments. The band profiles obtained from DGGE gels were analyzed for similarity using the Dice coefficient. A dendrogram was built from the weighted pair group matching average. Similarity varies from 0 to 1, where 1 indicates 100 % similarity.

Additionally, the observed similarities between the profiles of DGGE were analyzed using Kruskal's non-metric multidimensional scaling (NMDS; Venables and Ripley 2002) using a computing software (Statistica 8.0, StatSoft, Tulsa, OK). Kruskal's stress coefficient was used to reflect the goodness of fit of the model. Values of Kruskal's stress <0.1 are considered a good fit.

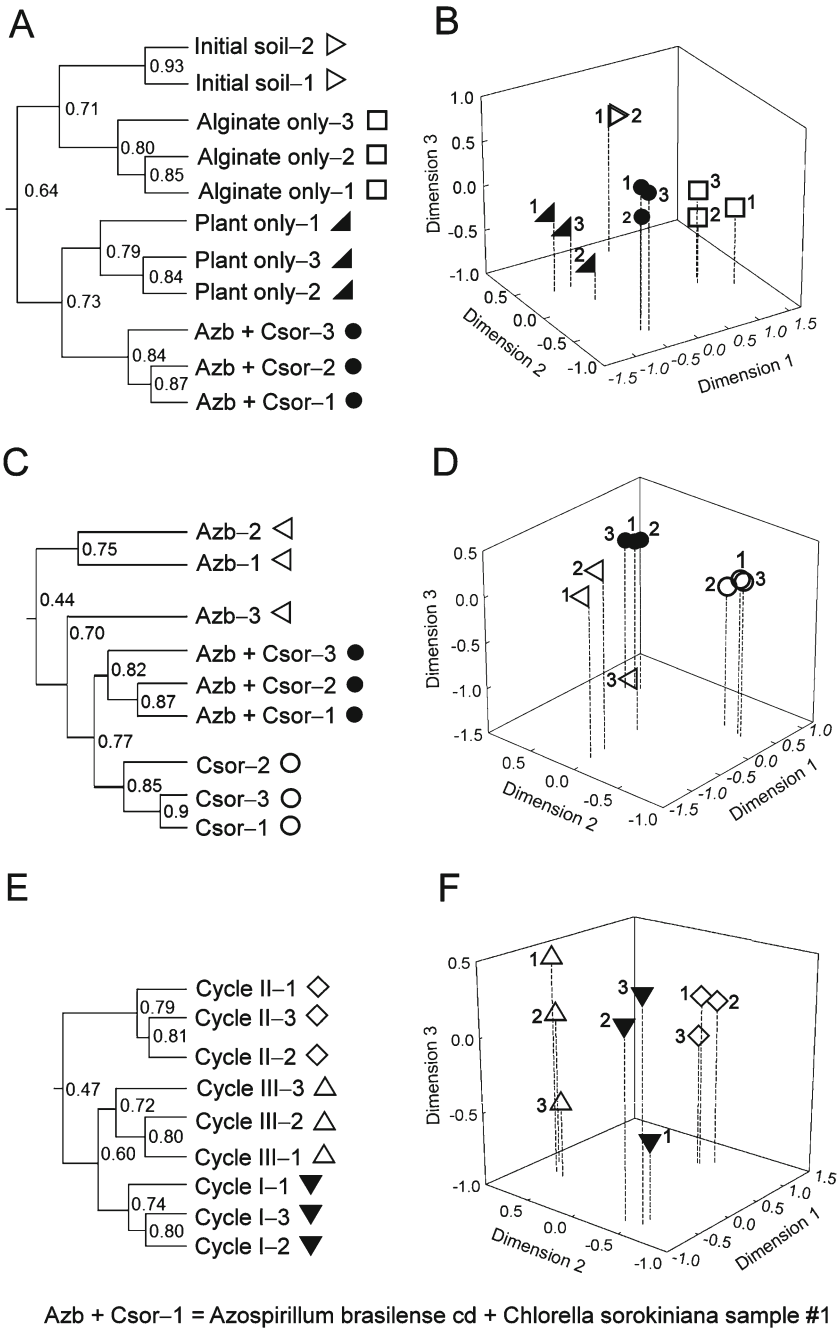
Bacterial richness considered each band as an individual operative taxonomic unit (Kisand and Wikner 2003). This was obtained from the Band Type Report of the Quantity One 4.6.7 imaging software (Bio-Rad Laboratories) that provides the number of bands detected in the DGGE profiles. Bacterial diversity was calculated by analyzing the relative intensity of each peak (corresponding to a defined band) in the densitometric profile with Shannon's diversity index (Iwamoto et al. 2000), calculated using the formula: $H=-\Sigma P_i \log_{10} P_i$, where P_i is the importance, probability of the bands in a gel lane and is calculated as $P_i=n_i/N$, where n_i is the intensity of a peak and N is the sum of all peak intensities of bands (Iwamoto et al. 2000). Data were analyzed using one-way ANOVA and then by Tukey's post hoc analysis at $P<0.05$ using statistical software (Statistica 6.0, StatSoft).

Results

Effect of the application of jointly immobilized *A. brasilense* and *C. sorokiniana* on the structure of the rhizosphere bacterial community

The bacterial rhizosphere community was monitored from plant samples harvested randomly from three pots in each treatment of the experiment with plants (Trejo et al. 2012; ESM Fig. S1). Figure 1 shows the results of the PCR-DGGE based on 16S rDNA fragments. The dendrogram built with the Dice coefficient measures the similarity among the banding patterns of replicate samples from each treatment. Four distinctive clusters (Fig. 1a) separate the community of inoculated plant from the controls that were not inoculated. Three-dimensional NMDS analysis of the PCR-DGGE profiles of the treatments confirmed that there was a significant effect of inoculation on the structure of the bacterial community. Similar to the results obtained from the dendrogram, each of the treatment replicates in the NMDS analysis were clustered, forming four distinctive groups that were significantly different from each other (stress factor $S=0.0027$; Fig. 1b). One group contained the untreated (control) soil samples. The second group contained the second control samples that were not inoculated. The third group contained the inoculated samples. The fourth group contained only empty beads.

There were also differences in community structure when comparing the effect of inoculation with debris containing: (1) jointly immobilized microorganisms; (2)



Azb + Csor–1 = Azospirillum brasilense cd + Chlorella sorokiniana sample #1

Fig. 1 Comparison of the rhizosphere bacterial community structure of six treatments: soil supplemented with immobilized *C. sorokiniana* and planted; soil supplemented with immobilized *A. brasilense* and planted; soil supplemented with jointly immobilized *C. sorokiniana–A. brasilense* and planted; no inoculated plant (plant only); no planted soil (initial soil); and soil supplemented with empty alginate beads (alginate only). **a**–**d** Comparison at the third cycle of inoculation. **e**, **f** Comparison of the three cycles of growth for soils supplemented with co-immobilized *C. sorokiniana–A. brasilense* and planted. **a**, **b** Comparison among soils treated with jointly immobilized microorganisms and soils without supplementation of microorganisms. **c**, **d** Comparison among soils treated with microorganisms, alone or jointly immobilized. **a**, **c**, **e** Dendrograms from cluster analyses by Dice coefficient. **b**, **d**, **f** 3D configuration derived from Kruskal's NMDS of bands. Data with *the same symbols* represent the replicates within each treatment

only the bacteria, and (3) only the microalgae. The overall results of the similarity analysis show three distinctive clusters of PCR-DGGE samples (Fig. 1c). These clusters were similar to the results obtained from the NMDS analysis (S=0.000036; Fig. 1d): one group corresponds only to *A. brasilense* immobilized alone, the second group corresponds only to *C. sorokiniana* immobilized alone, and the third group corresponds to the two microorganisms jointly immobilized.

We also examined the time effect of inoculation with leftover debris containing *A. brasilense* and *C. sorokiniana* on the rhizosphere bacterial community, comparing the three cycles of growth. Once again, the similarity analysis (Fig. 1e) and NMDS (S=0.0027; Fig. 1f) indicated three distinctive groups in each cycle that revealed a significant effect of treatment during the three cycles. These results suggest a shift in the community structure over time.

Effect of adding debris containing jointly immobilized *A. brasilense* and *C. sorokiniana* on rhizosphere bacterial richness and diversity

Richness of bacterial species in the rhizosphere community of plants inoculated with bead debris containing *A. brasilense*

and *C. sorokiniana* at the end of the third cycle (60 days after the first inoculation) was significantly higher than the controls which were not inoculated (P=0.0006; Fig. 2a). Species richness after the application of bead debris with jointly immobilized microorganisms or bead debris containing only *C. sorokiniana* was also higher than on plants inoculated only with bead debris containing *A. brasilense* (P=0.002; Fig. 2c). The effect of time of inoculation with bead debris containing *A. brasilense* and *C. sorokiniana* was indicated by the increased richness of species. A significant (P=0.03) increase in the number of species occurred in the second and third cycles compared to the first cycle (Fig. 2e).

The Shannon diversity index (H') was calculated to assess changes in bacterial diversity on the rhizosphere of the plants based on the intensity of the DGGE banding patterns. The diversity index of the bacteria in plants inoculated with bead debris containing *A. brasilense* and *C. sorokiniana* is significantly higher (P=0.0007) than the values obtained for the three controls: (1) plants that were not inoculated, (2) soil that was not treated, and (3) soil containing beads without immobilized microorganisms (Fig. 2b). The diversity index is significantly higher (P=0.002) in rhizosphere bacteria of plants inoculated with jointly immobilized microorganisms or only with *C. sorokiniana* than the diversity index of the bacterial community on plant roots inoculated only with *A. brasilense* (Fig. 2d). Similar to the richness of species, the Shannon diversity index indicates that the diversity of bacteria increased with time when plants were inoculated; diversity in cycle 3 was higher than in cycles 1 and 2 (P=0.02; Fig. 2f).

Identification of *A. brasilense* within the rhizosphere bacterial community

Purified bands of putative *A. brasilense* were obtained after the second purification step of the bands of PCR-DGGE in treatments involving the application of debris with this PGPB. When comparing the sequences of these bands with those of the positive control in the external reference ladder or the sequence of pure culture of *A. brasilense* Cd, we found 100 % homology among all tested sequences (ESM Table S2). This confirmed the identity for the recovered bands (representing the PGPB) with the original inoculum. Further analysis compared each of the excised band sequences with the GenBank database and showed 99 % identity with *A. brasilense* Cd.

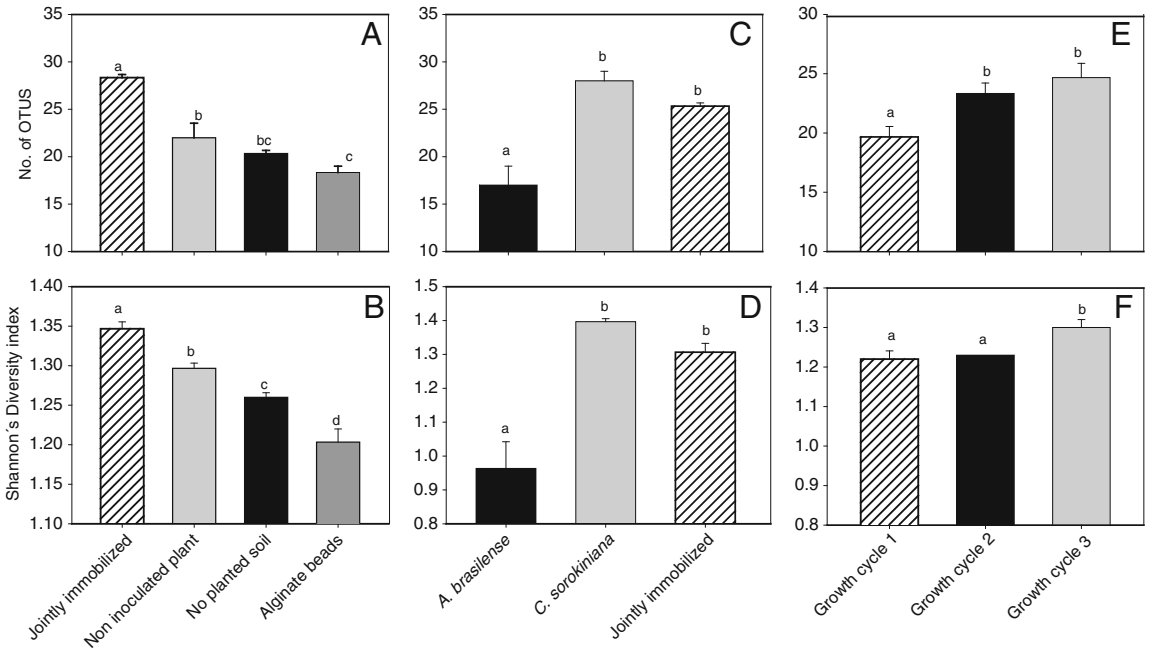


Fig. 2 Comparison of the species richness and diversity of the bacterial community of the six treatments: soil supplemented with immobilized *C. sorokiniana* and planted; soil supplemented with immobilized *A. brasilense* and planted; soil supplemented with jointly immobilized *C. sorokiniana–A. brasilense* and planted; non-inoculated plant; no planted soil; and soil supplemented with empty alginate beads. Species richness (**a**) and diversity (**b**) comparison among soils treated with jointly immobilized microorganisms and soils without supplementation of microorganisms. Species richness (**c**) and diversity (**d**) comparison among soils treated with microorganisms, alone or jointly immobilized. Species richness (**e**) and diversity (**f**) comparison among soil supplemented with jointly immobilized microorganisms during a time course of three cycles of growth. In each subfigure, *columns denoted with different lowercase letter* differ significantly at P<0.05 by one-way ANOVA and Tukey's post hoc analyses. *Bars* represent SE. *Absence of a bar*, negligible SE

Persistence of the original inoculum within the bacterial community of the rhizosphere of sorghum plants was detected at the end of each cycle after the application of debris containing *A. brasilense* and *C. sorokiniana*. Similarly, the PGPB was identified after cycle 3 application of debris only containing *A. brasilense*.

Colonization of sorghum root by *A. brasilense* derived from bead debris

FISH analysis assayed the colonization of *A. brasilense* on the roots of sorghum growing in soil amended with bead debris. Colonized roots were detected during the three growth cycles in all three zones of most root segments (Fig. 3). Colonization was primarily found in the apical root zone (Fig. 3b, c, f, arrows) and the root elongation zone (Fig. 3d, e); if plants had not been inoculated, only unidentified bacteria were detected (Fig. 3a).

Discussion

Earlier plant experiments demonstrated that there was a small improvement in soil fertility from the application of small amounts of debris containing microorganisms immobilized in alginate beads derived from simulation of tertiary wastewater treatment. Small increases in total C and microbial C and significant improvement in plant growth were observed. These effects were significantly greater compared to effects from untreated, eroded soil (Trejo et al. 2012). This molecular study focuses on the structure of the bacterial community in this amended desert soil.

The main goal of this study was to expand our knowledge of what happens to the soil bacterial community and to the PGPB in an eroded desert soil when improvement of soil fertility is derived from bead debris of tertiary wastewater treatment containing microalgae and a PGPB. Application (=an indirect inoculation) of leftover dry bead debris to the desert soil planted with sorghum significantly shifted the

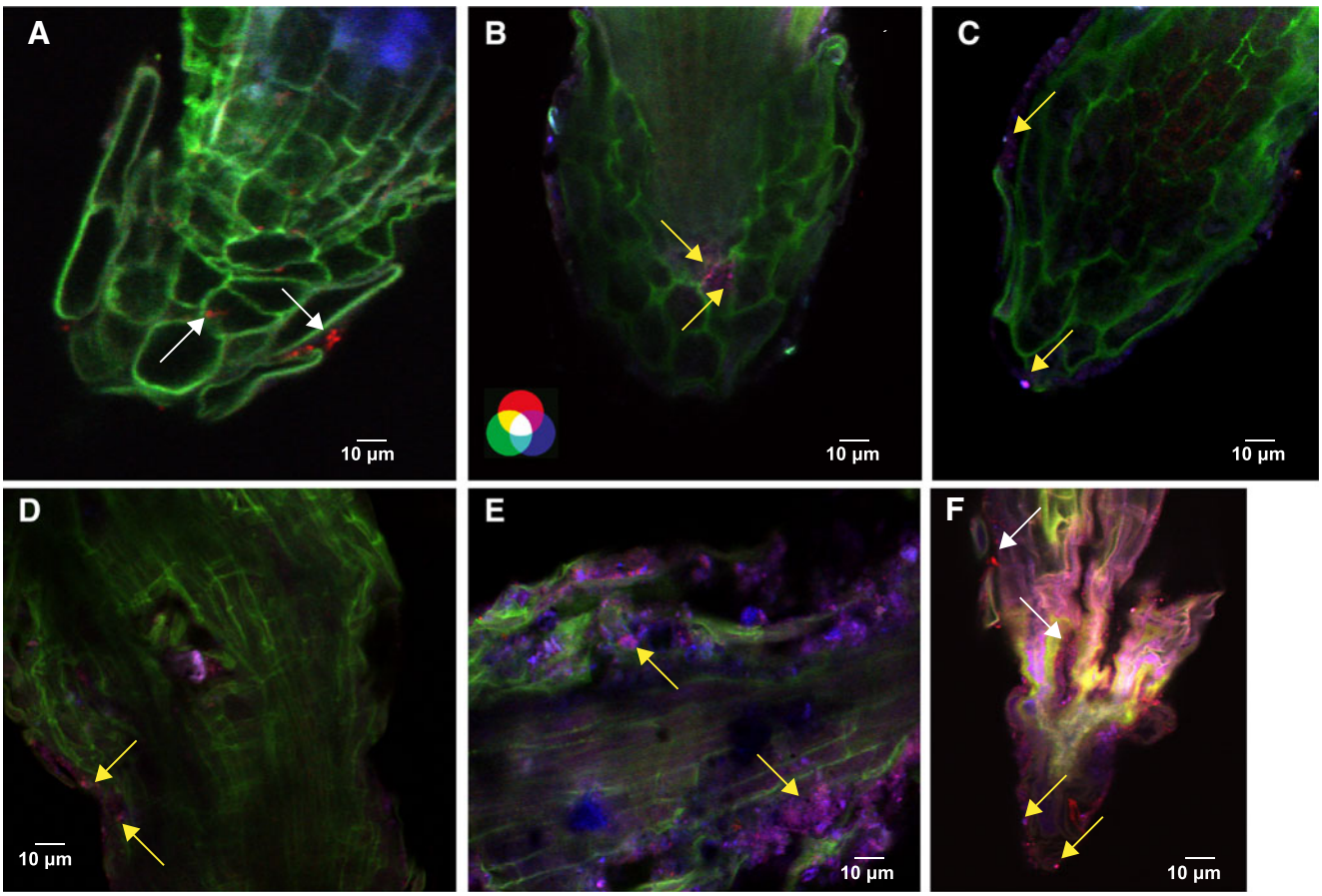


Fig. 3 Colonization of roots of sorghum by *A. brasilense* Cd detected by FISH probes and observed under confocal laser scanning microscopy. **a** Non-inoculated control. **b**–**c**, **f** Root tip. **d**, **e** Elongation zone. FISH experiments were performed with Abras-1420-Cy5 (*blue*) specific for *A. brasilense* and with the probe made of a mix of EUB-338-I, II, III-Cy3 (*red*) specific for the domain bacteria. The composed RGB images result in a *magenta color* for *A. brasilense* Cd cells (marked with *yellow arrows*), which indicates co-labeling by both probes. The third color channel (*green*) was used to visualize autofluorescence and the structure of roots. *Red labeling* corresponds to cells of unidentified bacteria (indicated with *white arrows*)

rhizobacterial community after three successive applications compared to untreated soil, soil with plants that were not inoculated, or soil having plants receiving only one microorganism. Similar detectable change of bacterial community structure based on sequence differences in the 16S rRNA gene and analyzed by PCR-DGGE was observed when seedlings of the desert quail bush (*Atriplex lentiformis*) were inoculated with *A. brasilense* Sp6 or *Bacillus pumilus* ES4 during experiments in the phytostabilization of two toxic mine tailings (de-Bashan et al. 2010a, b). Baudoin et al. (2009) document a transient, but statistically significant, change in the structure of the rhizobacteria communities when *Azospirillum lipoferum* CRT1 was inoculated on maize using automated ribosomal intergenic spacer analysis (ARISA). Yet, two other studies using *A. brasilense* examining maize grown in pots in a controlled greenhouse showed the contrary. Analysis of the bacterial community structure in these experiments, using the fingerprinting methods DGGE and ARISA, showed that inoculation with *A. brasilense* strains Cd and Sp 245 had no effect or very marginal effect on the size or structure of the bacterial communities. Furthermore, the age of the plants was far more significant in affecting bacterial communities (Herschkovitz et al. 2005; Lerner et al. 2006). Other parameters of importance affecting the structure of the bacterial community are the microbial species and their genomic traits (Barret et al. 2011). Using DGGE, another study showed similar trends (no effect) with tomatoes inoculated with *A. brasilense* combined with *B. subtilis* in a peat-based substrate (Felici et al. 2008).

Our study further measured DGGE patterns to partially assess bacterial richness and diversity as intrinsic descriptors of the rhizosphere community. Richness assessment in complex microbial communities, such as soil, required extensive sampling using diversity indices through the analysis of clone libraries (Bent and Forney 2008). However, because our main aim was to measure whether there is any effect of the application of PGPB on the soil bacterial community and not measure the total diversity and richness of a soil of the Sonoran Desert per se, partial analyses that detect dominant populations in the soil, as performed in this study, are meaningful. In the soil, the structural and functional diversity of bacterial populations is affected by root exudates, flavonids, rhizodeposition, soil amendments, and the characteristics of the soils (Gomes et al. 2001; Nannipieri et al. 2008a, b; Cesco et al. 2012). Our results demonstrated that three successive applications of small amounts of bead debris containing the mix of *A. brasilense* and *C. sorokiniana* or only *C. sorokiniana* favored bacterial richness, which may have occurred by growth of the bacterial population after the first application of debris. This first cycle represents the highest bacterial richness, while no significant changes occurred in the second or the third cycle of inoculation. The increase in bacterial richness could be explained by the increase of organic matter, as indicated by higher organic C and higher bacterial C in this poor soil (Trejo et al. 2012).

Even though richness of the bacterial community increased after the application of amendments, our study raised further questions regarding changes in the composition of the bacterial community. These will require further refinements of microbial ecology analysis, such as determination of the 16S rRNA gene copy numbers that might elucidate increased numbers for the amendment treatments and replacement of the commonly used Sybr Green staining that may not be sensitive enough for a soil environment with possibly $AgNO_3$ or ethidium bromide as stains.

Small increases in bacterial diversity after the three applications of *A. brasilense* and *C. sorokiniana* indicate that the abundance of bacterial species is becoming more evenly distributed, adapting to improved organic matter in the soil. The bead debris may have led to higher bacterial diversity in the rhizobacterial community of inoculated plants, considering that heterogeneity of microenvironments in the rhizosphere presumably results in the formation of many different in situ niches supporting an abundant and diverse bacterial assemblage in these root microenvironments (Wang et al. 2007). Additionally, increased diversity and richness after the third application suggests that the soil environment becomes less restrictive than the initial eroded soil. Debris with microalgae and/or PGPB has a stronger effect on the community than expected because it promotes microbial succession more efficiently than applications of debris without microorganisms or when the plants were grown without amendments.

Apart from compost (not part of this study), soils receiving large amounts of organic waste of various sources or pioneer plants that add large amounts of C support greater biomass of the microbial populations (Van Veen and Kuikman 1990; Luna-Suárez et al. 2000; Bastida et al. 2008, 2009). Microalgae are excellent for transporting nutrients because they contain important quantities of essential nutrients, especially N and P (about 10 and 1 %, respectively, on a dry weight basis), quantities that are several-fold higher than most terrestrial plants (Benemann et al. 2003). Nutrients recovered from wastewater treatment in the residues of the algal biomass could have premium value as organic fertilizers (Benemann et al. 2003). *Chlorella* spp. are not known to promote plant growth via active production of substances affecting plant metabolisms. Even though *Chlorella* sp. can survive, in small numbers, in the soil for prolonged periods (Trainor and Gladych 1995), the population of this aquatic microalga diminished rapidly when applied to soil. Thus, we theoretically assumed that the effect recorded on the microbial community is through the addition of organic matter by the decomposing cells.

Azospirillum spp., on the other hand, is known to survive for limited time in the bulk soil (Bashan 1999) and far longer in the rhizosphere of many plants and in different soils (Bashan et al. 1995). Consequently, we had followed its incorporation into the rhizosphere community of the plant in this study. Although the use of microalgae as a soil amendment is not widespread, microalgal biomass as a soil conditioner is a promising biotechnological venue (Pulz and Gross 2004).

In general, to verify that the effects on plant growth are derived from inoculation with PGPB, it is necessary to demonstrate that plants inoculated with a PGPB strain show root colonization by the specific strain (Lugtenberg and Kamilova 2009). Different methods for identifying root colonization, specifically by *Azospirillum*, have been developed, including traditional microbiology, immunology, molecular insertion of specific marker genes (Jacoud et al. 1998), insertion of the *gfp* green fluorescent protein (Ramos et al. 2002; Bacilio et al. 2004), *lacZ* markers (Arsene et al. 1994), and FISH (Rothballer et al. 2003). FISH monitored with confocal laser microscopy is a powerful tool to detect PGPB in soils and rhizosphere samples (Assmus et al. 1995). In our study, *A. brasilense* cells were specifically detected by the FISH technique on colonized root tips and elongation zone areas, which are known locations for colonization by *Azospirillum* (Bashan and Levanony 1989a, b; Bashan et al. 2004).

Additional evidence of the persistence of *A. brasilense* within the rhizosphere bacterial community was provided by the sequencing and identification of putative bands of *Azospirillum* in DGGE gels. The identity of presumptive bands was a 100 % match to the original inoculum, which was present after the third application of inoculation. As was reported by de-Bashan et al. (2010a, b), sequences of the V9 variable region of the 16S rDNA is useful in identifying *A. brasilense* in the rhizosphere bacterial community.

In summary, this study demonstrated that small improvements in soil fertility from the application of small amounts of waste bead debris from experimental, simulated tertiary wastewater treatment significantly changed the bacterial soil community compared to the original soil and continued for more than 2 months. This amendment improved biological quality by promoting a richer soil bacterial community, which supports the hypothesis of this study. A limitation of this study is the relative short-term growth period of plants (60 days) that was employed. We cannot rule out that the measured effects on the bacterial community are transient and will not have long-term effects on the fertility of the soil.

Taking this molecular study and its predecessor plant study (Trejo et al. 2012) together, several issues are pending. Since our specific objective was to demonstrate the proof of concept of a new idea, scaling up, use of different soils, potential secondary soil contamination when the waste beads came from heavily contaminated industrial wastewater, a strain-specific approach for analyzing the different rhizosphere communities, comparison to application of compost or fertilizers, issues of costs and obstacles to implementation, potential savings, and potential suitability in non-degraded soils have not yet been investigated.

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