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Recycling waste debris of immobilized microalgae and plant growth-promoting bacteria from wastewater treatment as a resource to improve fertility of eroded desert soil

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This study is dedicated to the memory of the Mexican nitrogen fixation researcher Dr. Jesus Caballero-Mellado (1953–2010) who pioneered *Azospirillum* research in Mexico.

Keywords:
Azospirillum
Biological recycling
Chlorella
Microalgae
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Soil amendment
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Wastewater treatment

ABSTRACT

This study attempted to demonstrate that biological residue from a new biological wastewater treatment is a resource for improving quality of arid soils and plant growth. After tertiary wastewater treatment, debris composed of alginate beads containing the microalgae *Chlorella sorokiniana* and the plant growth-promoting bacterium *Azospirillum brasilense* was used as an amendment for eroded, infertile desert soil having low levels of organic matter. *A. brasilense* survived in these used dried beads for at least one year. Three consecutive applications of the dry debris increased organic matter, organic carbon, and microbial carbon in the soil. Growth of sorghum in the amended soil was greater than plants grown in low organic matter, untreated soil or soil amended with beads containing other combinations of alginate, microalgae, or bacteria. The surface of plant roots growing in the amended soil was heavily colonized by *A. brasilense*, with no endophytic colonization; root tips were the preferred sites of colonization.

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1. Introduction

Urban primary and secondary wastewater treatment is a necessity and is currently done with a variety of physical, chemical, and biological treatments. Tertiary wastewater treatment (removal of nutrients) is done by chemical treatments to a lesser extent, and even far less by biological technologies (de-Bashan and Bashan, 2004, 2010). The common technologies used in large-scale wastewater treatment lead to massive quantities of secondary pollutants that mostly end in landfills. Some sludge debris from domestic wastewater and partially cleaned water are placed on fields as an amendment if they are free of hazardous contaminants, similar to application of compost to soils (Sæbø and Ferrini, 2006). Both organic amendments are commonly used to enhance soil fertility of arid lands (Graber et al., 2006) known for very low levels of organic matter (Bashan et al., 2000; Bronick and Lal, 2005). Because runoff and leaching of nutrient-rich amendments are responsible for large scale eutrophication of water bodies (Muñoz and Guieysse, 2006), a fundamental goal for developing a green technology for environmental application is to generate little or no secondary pollution (Henze et al., 2002). In the European Union, additional options are being considered: land reclamation and restoration, incineration, thermal processes (pyrolysis, wet oxidation, and gasification), and use of sludge in cement production as co-fuel (Fytili and Zabaniotou, 2008).

Biological agents (bacteria and microalgae) were proposed and later used for tertiary wastewater treatment and removal of nutrients for decades in various technologies; so far, not on a large operational scale because of cost and space (de-Bashan and Bashan, 2010; de la Noüe and de Pauw, 1988; Olguín, 2003). A major

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difficulty hampering these technologies is post-treatment collection and disposal of the biomass debris. One successful solution is immobilization of the biological agents in polymers prior to tertiary treatment, which are subsequently easy to remove by sedimentation after the treatment. So far, these technologies are hampered by the additional costs of the immobilizing material and the extra process (Chevalier and de la Noüe, 1985; de-Bashan and Bashan, 2010; Lau et al., 1997). So far, the disposable biomass has no further use.

A biologically efficient wastewater technology, using immobilized microalgae (*Chlorella* sp.) and plant growth-promoting bacteria (PGPB; *Azospirillum brasilense*), has been developed (de-Bashan et al., 2004; Hernandez et al., 2006) and is currently undergoing scaling up. *Chlorella* sp. is a common microorganism used for tertiary wastewater treatment (de la Noüe and de Pauw, 1988; Tam and Wong, 2000) and *Azospirillum* spp. is the most studied PGPB (Bashan et al., 2004). Details of this model were described (de-Bashan and Bashan, 2008). Similar to other biological technologies for wastewater, it yields large amounts of living biomass at the end of the wastewater treatment cycles. In earlier studies, we showed that the debris is composed of two components: (1) a large quantity of microalgae that harbor most of the nitrogen and phosphorus absorbed from wastewater and converted to proteins and other cell components, as well as fixed carbon from photosynthesis and (2) associated PGPB (de-Bashan et al., 2002, 2004).

The hypothesis of this study is that, as a whole, microalgae serve as supplemental organic matter and can be used to amend highly degraded soils in arid regions. Improved soil provides a suitable substrate for desert vegetation where it could not previously grow. Additionally, the amendment of debris serves as a PGPB-inoculant as its secondary role that enhances plant growth. As a result, debris from this wastewater technology, apart from cleaner wastewater, becomes a resource for improving soils and diverts a waste product from landfills. If implemented on a large scale, it creates an economically useful and non-contaminating application of a new wastewater technology. To demonstrate the feasibility and proof-of-concept of this approach, we documented three consecutive growth cycles of a model plant (sorghum) in a field where debris (beads containing microalgae and PGPB) were added to marginal arid soil.

2. Materials and methods

2.1. Microorganism immobilization in alginate beads and simulation of wastewater treatment

We used the unicellular microalga *Chlorella sorokiniana* Shih. et Krauss (UTEX 2805, Austin TX; de-Bashan et al., 2008) and the microalgae growth-promoting bacterium (MGPB) *A. brasilense* Cd (DSM 1843, Braunschweig Germany). *C. sorokiniana* was cultured on C30 medium and *A. brasilense* on TYG medium (Bashan et al., 2002, 2011; Gonzalez and Bashan, 2000).

Microorganisms were immobilized in alginate (de-Bashan et al., 2004). Briefly, axenic cultures (*C. sorokiniana* or *A. brasilense*) were mixed with 2% alginate solution. Initial cell concentrations were $1.0 \pm 0.5 \times 10^6$ (*C. sorokiniana*) and $0.25 \pm 0.05 \times 10^6$ (*A. brasilense*). Beads (2–3 mm diameter) were automatically produced in a 2% $CaCl_2$ solution for solidification (de-Bashan and Bashan, 2010). To immobilize the two microorganisms in the same bead, after washing the cultures, each was re-suspended in 10-mL 0.85% saline solution and then mixed with the alginate. Because immobilization normally reduces the number of cells of *Azospirillum* in the beads, a second overnight incubation of the beads in diluted nutrient broth (10% of full strength) was necessary.

Simulation of ammonia removal from wastewater was done in 300-mL bioreactors, each containing 12 g of beads for four days either with or without microorganisms. For simplicity and to avoid addition to the soil of unexpected toxic contaminants, which sometimes are part of real wastewater, the simulations were carried out in modified synthetic wastewater medium (de-Bashan et al., 2002), where the ammonium and phosphate concentrations were adjusted to the concentrations found in the city wastewater. Based on several previous comparative studies, using real or synthetic wastewater, results are consistent that removal of ammonium and phosphorus by this process is almost identical. Usually, all ammonium is removed (de-Bashan et al., 2004; Hernandez et al., 2006; Perez-Garcia et al., 2010). Hence, the variable of ammonium removal was chosen for the simulation of tertiary wastewater treatment. The synthetic wastewater contained the following ingredients (in mg L^{-1}): NaCl (7); $CaCl_2$ (4); $MgSO_4 \cdot 7H_2O$ (2); K_2HPO_4 (21.7); KH_2PO_4 (8.5); Na_2HPO_4 (25); and NH_4Cl (191: 50 mg L^{-1} NH_4^+) at pH 6.7, 28 °C ± 1 °C, constant 120-rpm shaking, and light intensity adjusted to continuous 60 μmol photon $m^2 s^{-1}$ with conventional fluorescent lamps that are used in similar bioreactors (de-Bashan et al., 2004, 2008).

2.2. Dehydration of beads after ammonium is removed

After all the ammonium is eliminated, beads were removed from each bioreactor, wastewater was discarded, and the beads were thoroughly washed three times in sterile saline solution (0.85% NaCl) and placed on sterile aluminum trays. No further analysis for potential leftover salts in the beads was done. Beads were dried either by incubation in an oven at 40–43 °C for 24 h (*A. brasilense* tolerates these temperatures) or in a laminar flow hood at 28–30 °C for 48 h. Once dried, each sample was placed in dry, hermetically-sealed glass containers at room temperature (23–28 °C) until used or analyzed.

2.3. Soil

The soil is a degraded alluvial haplic yermosol near La Paz, B.C.S., Mexico, previously described in detail. The relevant characteristics for this study are low total carbon (400 mg kg^{-1}), mostly inorganic carbon, low nitrogen (20 mg kg^{-1}), and low microbial population (Bashan et al., 2000). Most annual plants cannot grow normally in this soil, even after irrigation or rain (Bashan et al., 2009a,b). The samples come from a site that has been barren for over three decades.

2.4. Plant and growth condition

Sorghum (*Sorghum bicolor* (L.) Moench, cv. Honey Graze (Cal-Oro, Lubbock, TX)) was chosen because this robust species is one of the few annuals that can grow in this soil without any amendment. Seeds were disinfected for 10 min with 2% Tween®-20 (Sigma-Aldrich, St. Louis, MO), then for 5 min in 4% NaOCl. Verification of disinfection was done by putting seeds on plates containing nutrient agar (Sigma-Aldrich) for 72 h at 30 °C without bacterial growth associated with the seeds. Under aseptic conditions, seeds were then suspended in a large volume of sterile water for 2 h, decanted, and dried. Seeds were planted in 120 g of soil in black, plastic conical pots (2.5-cm inner diameter, 15.5 cm long) assembled in pre-fabricated trays commercially used for production of trees in nurseries (Polietilenos del Sur, Mexico City). We used, as inoculant, 0.2 g dry beads (containing 0.3×10^6 CFU $bead^{-1}$, corresponding to 1.5×10^6 CFU per pot) mixed with seed in each pot. All trays were transferred to a screen house at ambient temperature ~29 °C and light intensity of ~1000 μmol photons $m^2 s^{-1}$ for 20 days (one cycle). The potted seedlings were irrigated with 38.5 mL distilled water every three days to maintain field capacity. After 20 days, length and dry weight (dried at 70 °C for 48 h, shoots and

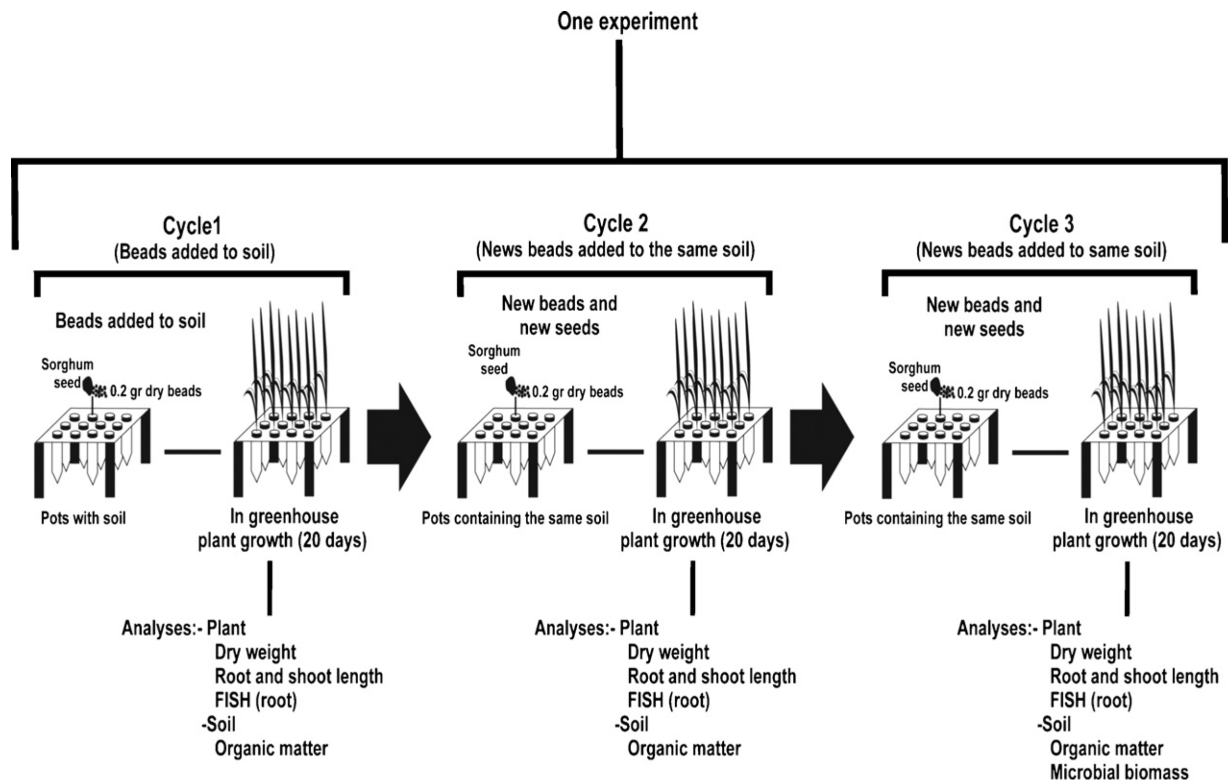

Fig. 1. Schematic representation of the setup for the greenhouse experiments and analyses performed at each growth cycle. Not to scale.

roots separately) was measured, soil carbon was assayed (listed below), and the degree of degradation of the beads was determined using the index developed by Bashan et al. (2002) and transformed into percent of degradation. Three growth cycles (20 days each) constituted one experiment where the same soil was used (Fig. 1).

2.5. Quantification of A. brasilense

2.5.1. Dried beads after ammonium removing treatment

Dried beads were dissolved in 2% sodium bicarbonate (one bead per milliliter) for 2 h under constant agitation. Viable bacteria were counted using a modification of the fluorescein diacetate method (Chrzanowski et al., 1984). From each sample of dissolved beads, 500 μL were further homogenized (vortexed, 2 min, three times) and centrifuged at 12,800 × *g* for 3 min. The pellet was suspended in 50 μL of fluorescein diacetate (20 mg in 10 mL acetone at 4 °C) in 0.002 M phosphate buffer pH 7.2 and then following the rest of the procedure. For counting bacteria, we used epi-fluorescent microscopy (Olympus BX41, Tokyo, Japan) with a green filter (excitation 460–490 nm with maximum emission of light at 520 nm). The microscope was connected to an image analyzing system (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). Confirmation of the results was done by the plate count method on nutrient agar medium (Sigma-Aldrich).

2.5.2. Detection and quantification of A. brasilense on sorghum roots

The three root zones (root tip, elongation zone, and lateral and root hair zone), all areas of *Azospirillum* sp. colonization (Bashan et al., 2004), were studied. Roots were prepared, as described by de-Bashan et al. (2010a). Briefly, roots were carefully separated from the soil, washed with 1× PBS, and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 2 h at 4 °C. After fixation, roots were washed with 1× PBS and stored in a mix of 1× PBS/96% ethanol (1:1, v/v) at −20 °C until hybridization.

Specific detection of *A. brasilense* on roots was done by fluorescence in situ hybridization (FISH) employing the following oligonucleotide probes: an equimolar mix of probes EUB-338-I 5′-GCTGCCTCCCGTAGGAGT-3′ (Amann et al., 1990) and II (5′-GCAGCCACC CGTAGG TGT-3′ and III 5′-GCTGCCACCCGTAGGTGT-3′ (Daims et al., 1999), specific for the domain bacteria. Probe AZO 440a 5′-GTCATCATCGTCGCGTGC-3′ (Stoffels et al., 2001) specific for *Azospirillum* spp., probe Abras-1420 5′-CCACCTTCGGGTAAAGCCA-3′ (Stoffels et al., 2001) specific for *A. brasilense*; and probe KO205 5′-GAACTGAAAGCCCGATCT-3′ (Franke-Whittle et al., 2005). Probes were labeled with either fluorochrome Cy3 or Cy5 (Interactive Division, Thermo Electron, Ulm, Germany). Hybridizations were done according to Stoffels et al. (2001) and de-Bashan et al. (2010b) at 35% formamide in the hybridization buffer for mix of EUB I, II, III, Abras 1420, and AZO 440a, and 15% stringency for KO205 at 46 °C for 2 h. The final concentration of the probes was 30 ng μL^{-1}. Samples were then washed at 48 °C for 15 min with 50 mL of pre-warmed washing buffer. The slides were rinsed for a few seconds with deionized water and then air-dried. For visualization, the slides were mounted in AF1 anti-fading reagent (Citifluor, London, UK). Visualization was done with epi-fluorescent microscopy (Olympus, Tokyo) employing Cy3 dye (maximum excitation at 552 nm with maximum emission of light at 565 nm, red fluorescence, Olympus America, Melville, NY), and confocal laser scanning microscopy (CLSM; LSM 510, Axiovert 100 M, Zeiss, Jena, Germany), as described by Rothballer et al. (2003). 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 auto-fluorescence of the root. This technique allowed overlapping of the images, resulting in differentiation among the observed objects: root surface were green, bacteria in general were red, and *Azospirillum* cells were magenta. Internal root colonization was checked by obtaining serial optical sections along the *z*-axis and analyzing them in 3D-video

reconstructions. The images were analyzed with specialized software (LSM 510 v4.2, Zeiss, Oberkochen, Germany).

Colonization by *Azospirillum* was counted from images of CLSM with imaging software (Image Pro Plus 6.3.1.542, Media Cybernetics, Silver Spring, MD), which is described elsewhere (Shopov et al., 2000; Treiser et al., 2007). Using the software RGB color code definitions, the specific magenta color of *Azospirillum* detected qualitatively by FISH in these images was composed R-255, G-000, and B255. The software measured the number of pixels that harbor this specific fluorescence and ignored other colors. The coverage (in %) of this fluorescence per area of root (in μm^2) was measured; this reflects the presence and level of colonization of each of the 10 segments measured for each root part. These 10 segments covered the entire root tip.

2.6. Analytical methods

Ammonium content of the synthetic wastewater was analyzed from 5-mL samples obtained from the bottom outlet of each bioreactor by using the phenate colorimetric method, a standard water analysis techniques (Eaton et al., 2005) adapted to microplate measurements (Versa Max tunable microplate reader, Molecular Devices, Sunnyvale, CA) described elsewhere (Hernández-López and Vargas-Albores, 2003). We used the standard potassium dichromate–sulfuric acid method to measure organic matter and organic carbon in soil (Walkley and Black, 1934). Microbial biomass (microbial carbon) of soil was determined with a combination of the fumigation–extraction–oxidation of dichromate techniques described elsewhere (Joergensen and Brookes, 2005; Vance et al., 1987).

2.7. Experimental design and statistical analysis

Simulation of ammonium removal from synthetic wastewater was done in four replicates, where a single bioreactor served as a replicate. The experiment was repeated three times. Viable counts of *Azospirillum* from beads were done in two beads taken at random per replicate and in 10 microscopic fields of each bead. Results are expressed as cells $bead^{-1}$. These counts had backup counts done by the plate count method on nutrient agar medium and expressed as CFU $bead^{-1}$. Counts from dry beads were done after 1, 5, 15, 60, 90,180, 270, and 360 days of storage.

Experiments used 10 replicates of the following treatments (one pot served as a replicate): Treatments with potted plants: (1) bead debris of the jointly immobilized system consisted of microalgae and *Azospirillum* in alginate; (2) immobilized *A. brasilense*; (3) immobilized *C. sorokiniana*; (4) plants without microorganisms; (5) alginate beads without microorganisms; and a treatment with only (6) untreated soil. Each experiment used the same soil during the three planting cycles and inoculating stages (Fig. 1). Visualization of root colonization by *Azospirillum* was done by FISH (two microscopic methods) in 10 replicates, where one root served as a replicate. A total of 225 images were taken (100 from epi-fluorescence microscopy, 125 from CLSM; and 14 3D-video reconstructions) for root colonization by the two microscopic methods of FISH at the end of each cycle. All analytical analyses were done in five replicates per treatment and each analysis was repeated twice, using independent samples. Analysis of microbial carbon was done only at the end of the three cycles because the analyses required large samples of soil. This analysis was done in six replicates per treatment, including soil that was not fumigated. This analysis was repeated three times.

Data was analyzed by one-way ANOVA and then by Tukey's post hoc analysis or Student's *t*-test, set at $P < 0.05$, using statistical software (Statistica v6.0, StatSoft, Tulsa, OK).

3. Results

3.1. Long-term survival of A. brasilense in dry alginate debris after wastewater treatment

The beads containing the microalgae and *A. brasilense* were subjected to the wastewater treatment for four days, during which time all ammonium was removed from the water. After drying, the beads contained large populations of living bacteria ($5.89 \pm 0.46 \times 10^5$ cells $beads^{-1}$, FDA count, Fig. 2a). With dehydration, each bead lost up to 90% of its initial volume and shrunk from an average 3 mm diameter to 1.02 ± 0.02 mm and formed irregular shapes. Viable cells of *A. brasilense* further decreased with time; yet, over 10^4 cells $beads^{-1}$ survived after one year under dry conditions (Fig. 2a). Because *C. sorokiniana* is not known as a plant growth promoter and need not be alive for our purposes, it only served as a source of organic matter. Survival of these cells was not determined. Formation of the microbial complex within beads was in micro-colony aggregates. Each aggregate contained both species. Aggregates maintained their shape, but were reduced in size, even after dissolving the alginate matrix required for counting cells (Fig. 2b and c). No differences in populations of *A. brasilense* were detected between the two drying procedures of the beads and the two counting techniques (data not shown).

3.2. Effect of three cycles of incorporating bead debris into the soil on organic matter and organic carbon in the soil

Application of any treatment containing alginate debris (with or without microorganisms) always significantly increased organic matter and organic carbon in the soil over untreated, eroded soil or soil where plants were growing without a microbial treatment from cycle 1 to cycle 3 (Fig. 3). Because data on organic matter reflected the results for organic carbon, the latter was not presented. Beads that contain microorganisms, especially those containing only *A. brasilense* or *A. brasilense* with *C. sorokiniana* led to higher, but not statistically significant, organic matter and organic carbon content than beads containing only alginate (Fig. 3). Separately for each treatment, there was a lack of statistically significant changes across cycles. For clarity, statistics for comparisons of cycles was not directly presented on the graphs. Sixty days after beads were added to the soil, microbial carbon significantly increased in treatments with beads containing both microorganisms or the beads containing only *A. brasilense*. When only the microalga *C. sorokiniana* was added, less microbial carbon was present in the soil. When alginate beads without microorganisms were added, there was no increase microbial carbon in the soil (Table 1). Partial degradation of beads occurred in soil during the 20-day incubation of each cycle and was very similar for all treatments in each cycle. Degradation rates of beads reached an average of 58% during cycle 1, 53% during cycle 2, and 57% during cycle 3.

3.3. Effect of three cycles of amendments containing bead debris on development of plants

Any addition of dry alginate beads for 60 days enhanced the growth of the plants compared to plants growing on untreated, degraded soil (Fig. 4). Depending on the microbial content of the bead, effects varied. As a positive control, application of alginate bead without microorganisms had, in several analyses (4 of 12; Fig. 4), the smallest, statistically significant, positive impact on shoot and root development. For beads containing the microalga *C. sorokiniana*, growth increased (7 of 12 analyses). For beads containing the PGPB *A. brasilense*, growth increased (8 of 12 analyses). Beads containing both microorganisms had the best results (12 of 12 analyses). On average, this treatment has the

Table 1
Total microbial carbon content in soil treated with waste debris from wastewater treatment after 60 days of sorghum plant growth.

Soil treatment	Microbial carbon mg kg^{-1} soil		
	Fumigated soil	Non-fumigated soil	*Ec*/*KEc*
A. brasilense + *C. sorokiniana*	1.95 ± 0.011 a	0.52 ± 0.004 a	4.15 ± 0.002 a
A. brasilense	1.81 ± 0.006 a	0.52 ± 0.004 a	3.91 ± 0.002 a
C. sorokiniana	1.30 ± 0.004 b	0.52 ± 0.004 a	2.73 ± 0.008 b
Plant only	1.26 ± 0.005 bc	0.52 ± 0.004 a	2.65 ± 0.009 bc
Alginate only	1.17 ± 0.003 cd	0.52 ± 0.004 a	2.48 ± 0.008 cd
Untreated soil	1.12 ± 0.005 d	0.52 ± 0.004 a	2.33 ± 0.007 d

Ec/*KEc* = microbial carbon, where: *Ec* = values of fumigated soil minus values of soils that were not fumigated; *KEc* = a 0.38 constant (Vance et al., 1987). To clarify how microbial carbon was calculated, values of fumigated and unfumigated soils, the raw source of the data, are provided.
Values in each column with different letters differ significantly by one-way ANOVA and Tukey's post hoc analysis at $P < 0.05$.
± = Standard error.

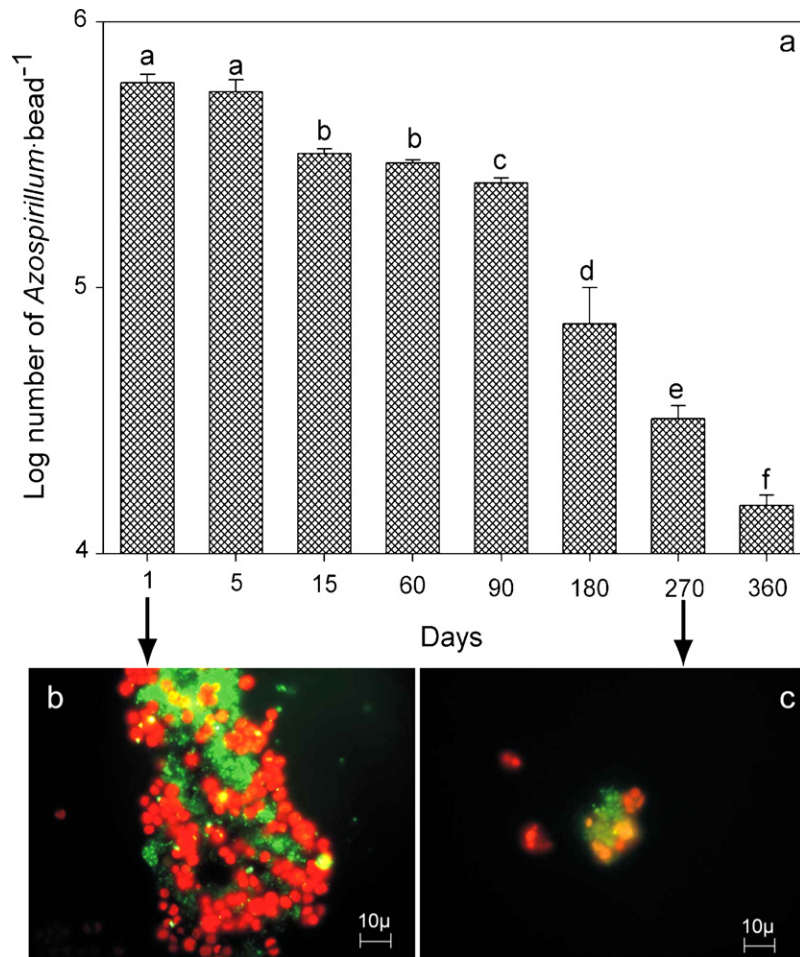

Fig. 2. (a) Long-term survival (1–360 days) of *A. brasilense* Cd in waste of dry alginate beads after wastewater treatment. (b, c) Fluorescent microphotographs of microbial aggregates within the beads after one day (b) and 270 days (c). Green fluorescence = *A. brasilense* Cd; red fluorescence = *C. sorokiniana*. Columns denoted with a different lower case letter differ significantly by one-way ANOVA and by Tukey's post hoc analysis at $P < 0.05$. Bars represent standard error. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

largest impact on plant development for the three cycles: (1) root length increased 20.8 ± 3.3%, 15.4 ± 3.7%, and 17.3 ± 2.1% and its dry weight increased 216.9 ± 43.8%, 115.6 ± 77.8%, and 151 ± 49.0%; (2) shoot length increased 29.9 ± 11.9%, 27.9 ± 7.0%, and 24 ± 6.7% and its dry weight 64.6 ± 15.5%, 138.4 ± 29.5%, and 59.7 ± 14.0%. The most pronounced effect was on shoot development, where both length and dry weight increased with each cycle of growth (Fig. 4c and d). The effect was more variable for root development, even though the effect was always positive (Fig. 4a and b). Shoot-to-root ratio is an acceptable way to measure the effect of *Azospirillum* at the whole-plant level (Bashan and Dubrovsky, 1996). Dry weight of the shoot-to-root ratio was variable for the treatment of the two microorganisms. For the treatment with *A. brasilense*, it increases with each growth cycle; whereas, the other treatments produced variable ratios (Fig. S1, supplementary materials). Variable shoot-to-root ratios occurred for length of shoots and length of roots (Fig. S1).

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

To explain promotion of growth by *A. brasilense* of any plant species, a study of root colonization should accompany the main study (Bashan et al., 2004). We measured colonized roots from the

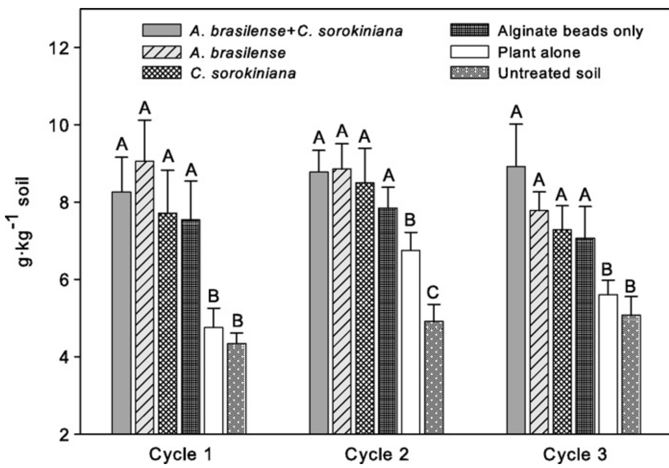

Fig. 3. Changes in content of organic matter in eroded soil following treatment with alginate beads with or without microorganisms during three cycles (20 days each) of growth of sorghum. Columns denoted with different capital letter for each cycle of growth (groups of 6 columns) differ significantly at $P < 0.05$ for one-way ANOVA and Tukey's analyses. Bars represent standard error.

same samples that were used for measuring promotion of growth. With specific oligonucleotides of *A. brasilense* for FISH analysis and detecting cells by epifluorescence microscopy or CLSM, we found colonization in all three zones of most root segments. Colonization occurred primarily in the root apical zone (Fig. 5a), but was also present in the root elongation zone (Fig. 5b) and the root hair zone (Fig. 5c). Measurement of colonization in the root apical zone ranged from 7.3 to 19.8% coverage of the surface area of the root tip. Optical sections along the *z*-axis of the same roots, when prepared for CLSM and analyzed by 3D-video reconstruction, revealed no internal colonization of sorghum roots by *A. brasilense* (Video S1, supplementary material).

4. Discussion

This study addresses, on a small scale, two major environmental problems of arid zones: (1) soils in arid areas are continuously degraded by human activities (Wang et al., 2004) in a process called desertification and (2) recycled wastewater produce large amounts of residue that can become a major source of pollution. Although these are common problems worldwide, in the Americas, they are prevalent in the southwestern USA, northwestern Mexico, and high Andean deserts. Various strategies have addressed these two issues. These include reforestation with native and imported plants (Miyakawa, 1999), urban, agricultural, and pastoral

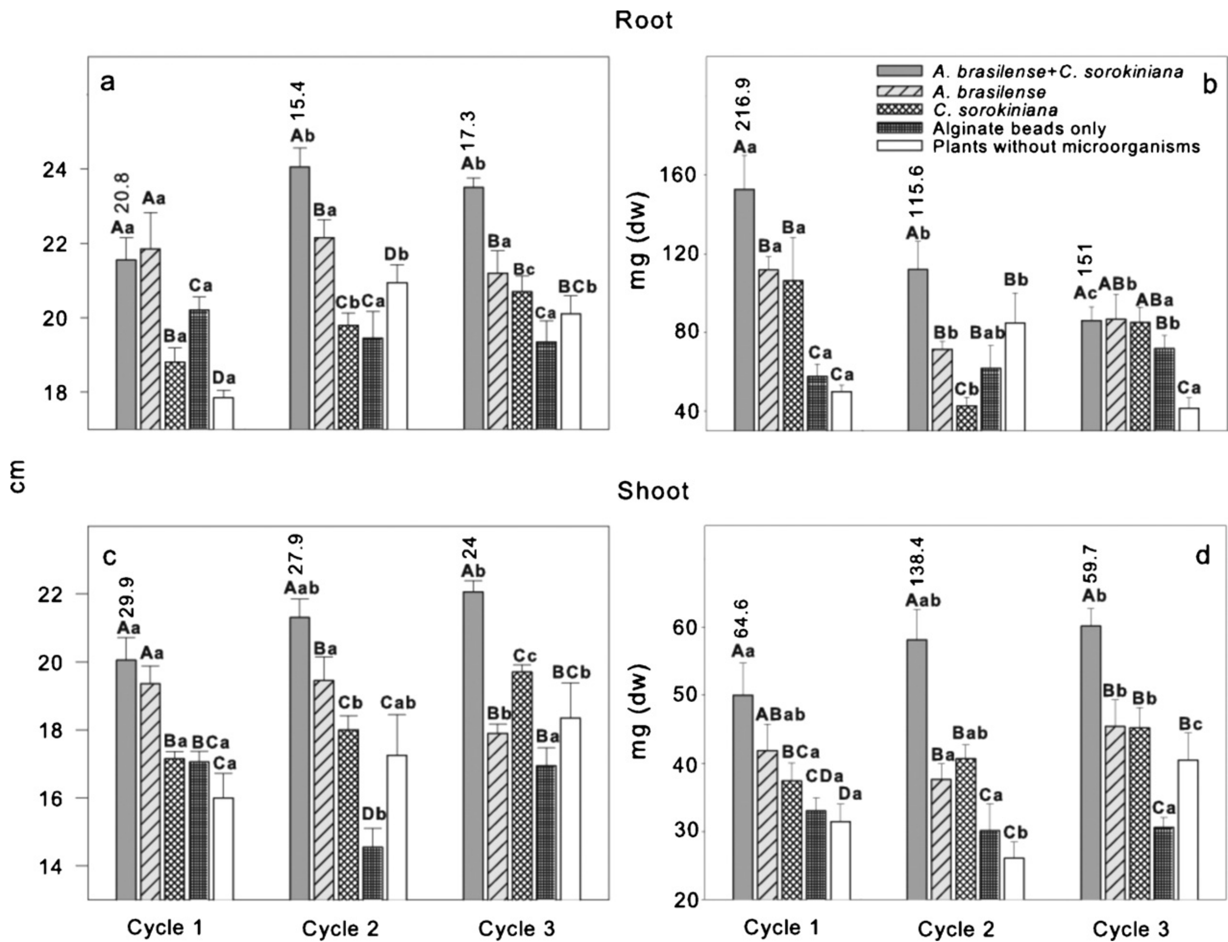

Fig. 4. Effects on growth of roots (a, b) and shoots (c, d) of sorghum after three 20-day cycles of growth following treatment of eroded soil with alginate beads with or without microorganisms. In each subfigure, columns denoted with different lower case letter presents the results of between-cycle comparisons, where each treatment is considered separately, differ significantly at $P < 0.05$ by one-way ANOVA and Tukey's analyses. Columns denoted with different capital letter for each cycle of growth (groups of 5 columns) present results of between-treatment comparisons (and each cycle considered independent of the two other cycles), differ significantly at $P < 0.05$ by one-way ANOVA and Tukey's analyses. Bars represent standard error. Vertical numbers above the treatment with two jointly-immobilized microorganisms (■) indicate relative increase (in percentage) over untreated plants (□).

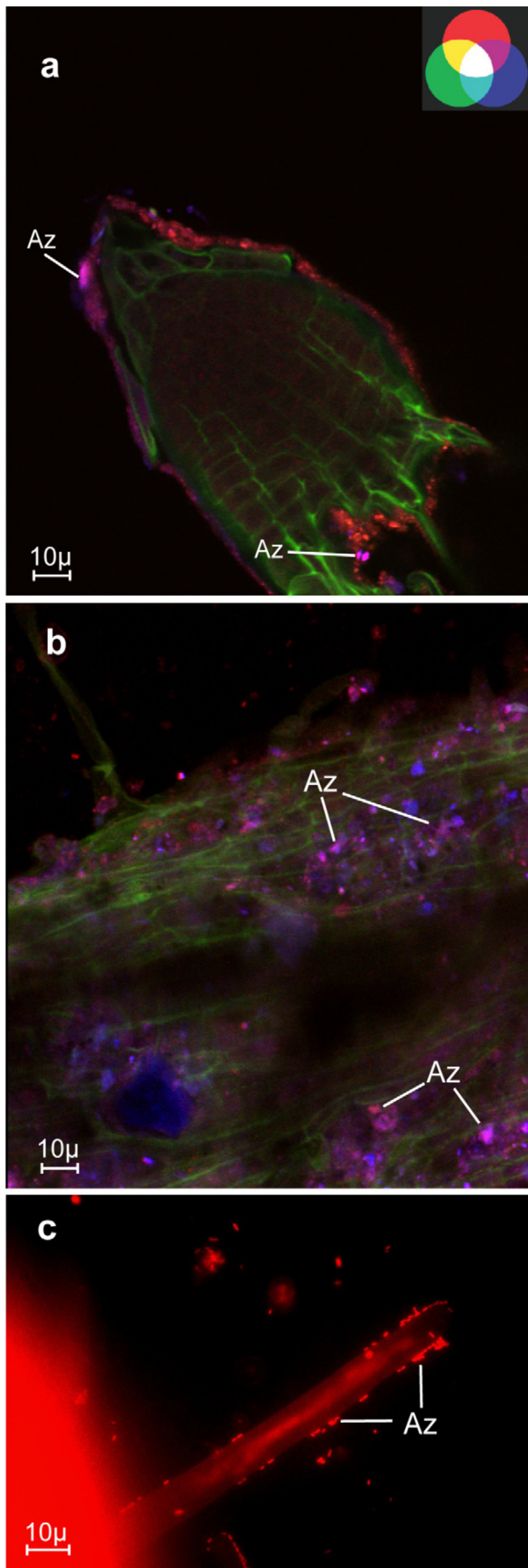

Fig. 5. Detection of *A. brasilense* Cd on roots of sorghum using specific fluorescence in situ hybridization (FISH) probes and observed under confocal laser scanning microscopy (a, b) and epifluorescence microscopy (c). (a) root tip; (b) root elongation zone; (c) root hair area. Magenta-stained bacteria (in a and b) and red bacteria (in c) marked with an arrow and AZ label indicates typical micro-colonies and individual cells of *A. brasilense* Cd. FISH experiments were performed with Abras-1420-Cy5 (blue), KO205 (red used only for epifluorescent microscopy) specific for *A. brasilense* and with the probe mix of EUB-338-I, II, III-Cy3 (red) specific for the domain bacteria. The composed RGB images (visual index in Fig. 4a) result in a magenta color for *A. brasilense* Cd cells, which indicates co-labeling by both probes. The third color channel (green) was used to visualize auto-fluorescence and the structure of roots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

coverage (Portnov and Safriel, 2004), compost or sludge amendments to increase organic matter and water-holding capacity (Mendez et al., 2007), and inoculation with plant growth-promoting microorganism (Grandlic et al., 2008, 2009). Although some of these approaches are performed on large scale, most are not used as widely as they can be.

While wastewater treatment is obligatory in most communities, reuse of these waters is more prevalent in arid areas, where domestic sludge is used as a soil amendment and industrial sludge is buried in landfills (Henze et al., 2002). Consequently, development of any wastewater technology for arid areas requires the least amount of debris discharged in landfills. Tertiary domestic wastewater technology employing microalgae and microalgal growth-promoting bacteria was developed to address these current problems (de-Bashan et al., 2004). The end-products are ammonium-free water and alginate beads containing large amounts of immobilized microalgae and bacteria identical to the waste debris used in this study. This proof-of-concept study measured the debris and assessed its potential as a biological resource to enhance soil quality (organic matter and microbial activity) and as an efficient inoculant of PGPB to enhance the usually limited plant growth normally occurring in these soils (Bashan et al., 2009a,b). This would demonstrate a wastewater technology that does not produce a waste burden.

Applying waste debris on soil organic matter and its carbon content was greater than applying alginate (0.2 g in 120 g of soil), even with its internal microbial populations. Therefore, this study cannot rule out the possibility that there is a fertilization effect from applying alginate plus salts from the treated wastewater. Also, we emphasize that the weight of the microorganisms within the beads is small (de-Bashan et al., 2005), compared to the weight of the alginate and that it may take more than the three cycles performed in this study to show significant increases in organic matter as a specific result of applying microbial material associated with alginate beads.

The degraded soil that we used barely allows any annual plant to grow, even if irrigated (Bashan et al., 2000). Inoculation with purposely-produced inoculants of PGPB and mycorrhizal fungi plus limited compost enhanced growth of several desert shrubs and cacti (Bashan et al., 2009a,b). In this report, we quantitatively demonstrated that the PGPB *A. brasilense*, initially employed in wastewater treatment, retained its growth-promoting capacity in the bead debris, creating a practical and useful inoculant for plants, as a secondary role.

Alginate is not known to inhibit plant growth, one reason why it is widely used in synthetic inoculants for PGPB (Bashan, 1998; Bashan et al., 2002; Yabur et al., 2007). Yet, in some cases in our study, although not consistently, a small inhibitory effect of alginate on root and shoot growth was observed. This inhibitory effect was more than counteracted by *A. brasilense*. Several species of *Azospirillum* are well known for mitigating many environmental stressors of plants (Bashan et al., 2004; Bashan and de-Bashan, 2010).

As mentioned earlier, adding commercial compost and wastewater sludge to degraded desert soils improve organic matter content and enhance microbial activity (Iverson and Maier, 2009; Mendez

and Maier, 2008). This waste product serves the same purpose. Since our feasibility experiments were performed on a small scale and used small amounts of debris, which contributed limited amounts of organic matter as microalgae, bacteria, and alginate, improvement in soil quality was modest, compared to a massive application of compost. Yet, these small amounts of debris were sufficient for sorghum to grow well at a level that is similar to the results of inoculation of sorghum with a purposely-constructed *A. brasilense* inoculant used in arid land agriculture (Sarig et al., 1984) and similar to enhanced growth of quailbush (*Atriplex lentiformis*) in arid mine tailings inoculated with several PGPB of *Azospirillum* and *Bacillus* species (de-Bashan et al., 2010a,b). Similar trends in plant response were also obtained when tomato seedlings were grown in a similar system to the one described in this study (O. Perez-Garcia and Y. Bashan, unpublished data).

Root colonization by any PGPB, *Azospirillum* included, is a mandatory requirement when assessing potential effects of inoculation on plant growth (Lugtenberg and Kamilova, 2009). *Azospirillum* colonizes root tips (Bashan and Levanony, 1989), the root elongation zone, and the root hair zone of numerous plant species (Bashan et al., 2004) and this was observed and quantified on sorghum plants growing in degraded soil and amended with waste debris using three microscopy techniques employing highly specific fluorescently labeled phylogenetic oligonucleotide probes (FISH).

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, 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.

5. Conclusions

This study demonstrates that waste debris from a new technology of biological wastewater treatment can be used as an amendment to improve soil quality and promote plant growth in degraded arid soils.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.08.007.

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