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ENVIRONMENTAL BIOTECHNOLOGY

Alginate beads provide a beneficial physical barrier against native microorganisms in wastewater treated with immobilized bacteria and microalgae

Sergio A. Covarrubias · Luz E. de-Bashan · Manuel Moreno · Yoav Bashan

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Abstract When the freshwater microalga *Chlorella sorokiniana* and the plant growth-promoting bacterium *Azospirillum brasilense* were deployed as free suspensions in unsterile, municipal wastewater for tertiary wastewater treatment, their population was significantly lower compared with their populations in sterile wastewater. At the same time, the numbers of natural microfauna and wastewater bacteria increased. Immobilization of *C. sorokiniana* and *A. brasilense* in small (2–4 mm in diameter), polymer Ca-alginate beads significantly enhanced their populations when these beads were suspended in normal wastewater. All microbial populations within and on the surface of the beads were evaluated by quantitative fluorescence in situ hybridization combined with scanning electron microscopy and direct measurements. Submerging immobilizing beads in wastewater created the following sequence of events: (a) a biofilm composed of wastewater bacteria and *A. brasilense* was created on the surface of the beads, (b) the bead inhibited penetration of outside organisms into the beads, (c) the bead inhibited liberation of the immobilized microorganisms into the wastewater, and (d) permitted an uninterrupted reduction of ammonium and phosphorus from the wastewater. This study demonstrated that wastewater microbial populations are responsible for decreasing populations of biological agents used for wastewater treatment and immobilization in alginate beads provided a protective environment for these agents to carry out uninterrupted tertiary wastewater treatment.

Keywords Alginate · *Azospirillum* · *Chlorella* · Microalgae · Plant growth-promoting bacteria · Protection · Wastewater treatment

Dedication This study is dedicated for the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1960–2011) of CSIC, Spain

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S. A. Covarrubias · L. E. de-Bashan · M. Moreno · Y. Bashan (✉)
Environmental Microbiology Group,
Northwestern Center for Biological Research (CIBNOR),
Mar Bermejo 195, Col. Playa Palo de Santa Rita,
La Paz B.C.S. 23090, Mexico
e-mail: bashan@cals.arizona.edu

Y. Bashan
e-mail: bashan@cibnor.mx

L. E. de-Bashan · Y. Bashan
The Bashan Foundation,
3740 NW Harrison Blvd.,
Corvallis, OR 97330, USA

Introduction

Immobilization of microorganisms in polymers and in beads prepared from polymers for production of various products and environmental and agricultural applications is well-known and has increasing applications in the last two decades (Cassidy et al. 1996; Lebeau and Robert 2006; Mallick 2003; Moreno-Garrido 2008). Immobilizing microalgae is a common approach in several bioremediation applications (de-Bashan and Bashan 2010). Immobilization in various substances provides microorganisms several major advantages over free-living suspensions. These include: (1) an uninterrupted supply of nutrients without competing with other microorganisms (de-Bashan et al. 2004) and (2) protection against environmental stress (de-Bashan and Bashan 2010; Moreno-Garrido et al. 2002), bacteriophages (Steenson et al. 1987), toxins, and UV irradiation (Tanaka et al. 1994; Zohar-Perez et al. 2003),

field soil (Hall et al. 1998), soils contaminated by hydrocarbons (Weir et al. 1995), and possible grazing by zooplankton (Faafeng et al. 1994). All these potential benefits notwithstanding, only rare experimental evidence exists of direct physical protection of the immobilized microorganisms against grazing microfauna, particularly protozoa and metazoa (Leung et al. 2000), and no evidence for protection of microorganisms used in treating wastewater treatment against competition and predation by the many protozoa and metazoa residing in wastewaters (Amaral et al. 2004; Martín-Cereceda et al. 2001).

A recently developed tertiary domestic wastewater treatment uses green microalgae *Chlorella* spp. and the plant growth-promoting bacterium (PGPB) *Azospirillum brasilense* that are jointly immobilized in alginate beads (de-Bashan et al. 2004). Every unit in this technological model, a single polymeric bead, contains within its matrix cavities holding together the microalgae and bacteria (de-Bashan and Bashan 2008, de-Bashan et al. 2011). Entrapment of microorganisms can also be within the solid matrix of the polymeric bead. In some cases, the microbial cells are on the surface or partially within and partially outside of the gel matrix. From contraction of the alginate bead during formation and curing, close to its surface, the number of microorganisms is higher than in its interior (Zohar-Perez et al. 2004a).

Chlorella spp. (Chlorophyceae) are simple, non-motile, unicellular, aquatic green microalgae. *Chlorella* has been used in studies of photosynthesis, respiration, and synthesis of carbohydrates in microalgae (Ilangovan et al. 1998). From a biotechnological standpoint, the potential for mass cultivation of this microalga for producing high-value, low-volume compounds includes pigments for the food industry, including the health food market in industrialized countries (Lebeau and Robert 2006), wastewater treatment (Oswald 1992; de-Bashan and Bashan 2010), and biofuel (Mata et al. 2010).

Except for symbiotic rhizobia, *Azospirillum* is the most studied agricultural PGPB (Bashan and de-Bashan 2005). It is a highly competent, rhizosphere-dwelling diazotroph that is versatile in its nitrogen transformations and carbon consumption, acts as a general PGPB for numerous plants (Bashan et al. 2004), including *Chlorella* (Gonzalez and Bashan 2000), and uses a multitude of growth-promoting mechanisms (Bashan and de-Bashan 2010). Alginate is the most commonly used polymer for immobilizing microorganisms within small cavities within its matrix (Smidsrød and Skjåk-Bræk 1990). Immobilization of microorganisms in alginate beads is widely used when viable microbial cells are required in numerous biotechnological processes (Prasad and Kadokawa 2009).

Our working hypothesis was that immobilization in alginate beads of the microalgae *Chlorella sorokiniana* with the PGPB *Azospirillum brasilense* protects the microalgae when they perform wastewater treatment. The beads act as a physical barrier between the two environments. This maintains the treatment agents within the bead and keeps the native wastewater organisms out, thus allowing uninterrupted tertiary wastewater treatment.

The specific objectives of this study were to: (1) determine if immobilization halts reduction in the populations of wastewater treating agents when exposed to natural wastewater and its organisms, (2) determine the nature of protection that the immobilization process offers, and (3) determine if efficient tertiary wastewater treatment continues after providing protection to the microorganisms in the bead matrix.

To understand the details of such protection, we used a combination of microbial and quantification techniques, which are as follows: (1) direct microbial counts in all environments of all microorganisms and microfauna and (2) fluorescence in situ hybridization (FISH) measured by epifluorescence microscopy. FISH is widely used to investigate cultivation-independent bacterial communities in a range of ecosystems (Bertaux et al. 2007; Daims et al. 2001; Dazzo et al. 2007). This technique combines molecular identification, enumeration, and localization of physiologically active bacteria. FISH detects nucleic acid sequences by a fluorescently labeled probe that hybridizes with its complementary target sequence within the fixed cells (Moter and Göbel 2000). It has been used in PGPB research to assay colonization of wheat roots by *A. brasilense* (Assmus et al. 1995), *Azospirillum amazonense*, and other diazotrophic PGPB in sugarcane plantlets (Oliveira et al. 2009) and measure the basic physical interaction between *Chlorella vulgaris* and *A. brasilense* (de-Bashan et al. 2011). (3) Our FISH images were compared with images obtained by scanning electron microscopy of beads from the same batch of wastewater, (4) specialized image-analysis quantification software, and (5) quantification of tertiary wastewater treatment using standard water techniques.

Materials and methods

Microorganisms and initial growth conditions

The unicellular microalga *C. sorokiniana* Shih. and Krauss (UTEX 2805 University of Texas, Austin, TX) was used. Before immobilization in alginate beads, the microalga was cultured in sterile mineral medium (C30) for 5 days (Gonzalez et al. 1997). *A. brasilense* Cd (DMS 1843, Braunschweig, Germany) was grown in nutrient broth (Sigma) at 35±2 °C for 18 h in a rotary shaker at 120 rpm, using standard techniques for this genus (Bashan et al. 1993).

Immobilization of microorganisms in alginate beads

Microorganisms were immobilized, as described by de-Bashan et al. (2004). Briefly stated, 20 mL of axenically grown cultures of *C. sorokiniana* containing 6.0×10^{6} cells mL^{-1} was harvested by centrifugation at 2,000×*g* and washed twice with sterile saline solution (0.85% NaCl). The cells were then mixed with 80 mL sterile (by autoclaving, only slight reduction in viscosity), 6,000 cP 2% alginate solution (a solution made of alginate mixed at 14,000 and 3,500 cP, Sigma, St. Louis, MO. Catalog nos. A7128 and A2033, respectively) and stirred for 15 min. Beads (2–3 mm in diameter) were automatically produced in a 2% $CaCl_2$ solidification solution, as described by de-Bashan and Bashan (2010). The beads were left for 1 h at 22±2 °C for curing and then washed in sterile saline solution. As controls, cultures of *A. brasilense* (approximately 10^{9} CFU mL^{-1}) and cultures of *C. sorokiniana* (6.0×10^{6} cells mL^{-1}) were immobilized similarly. Because immobilization normally reduces the number of *Azospirillum* in the beads, a second incubation step was necessary for cultures of *A. brasilense* after initial curing and washing, a process that restores the size of the population of bacteria in the beads (Bashan 1986). This second incubation in diluted nutrient broth (1:10) lasted overnight. Where jointly immobilized cultures of *A. brasilense* and *C. sorokiniana* were used, the same concentration of each microorganism, as used in pure cultures, was mixed prior to mixing with alginate solution, but the volume of each microbial culture was reduced to 10 mL before adding alginate.

Culture conditions in wastewater

Experiments with immobilized cultures

Most experiments used immobilization cultures. Microorganism species immobilized alone or jointly were grown under batch conditions for 10 days in natural wastewater that was filtered to remove large suspended solids (>1 mm). The cultures were incubated in 250-mL, unbaffled Erlenmeyer flasks (100 mL medium containing 4 g of beads) at 28±2 °C, agitated at 120 rpm under constant light at a density of 60 μmol photons·m^{-2}·s^{-1} (Innova 4340, New Brunswick Scientific, Edison, NJ).

Experiments with microbial suspension

Experiments in suspension were conducted to measure the risk to *C. sorokiniana* and *A. brasilense* when exposed in wastewater in free suspension (not immobilized in alginate beads). Flasks containing 40 mL natural wastewater from the wastewater plant's oxidation pool either sterile (by filtration, 0.45 μm) or non-sterile were used. The flasks were inoculated separately with free suspensions of *C. sorokiniana* ($8.1\pm71\times10^{8}$ cells mL^{-1}) or *A. brasilense* ($7.2\pm0.2\times10^{6}$ CFU) and incubated under the same conditions as the experiments described for immobilized cultures. Numbers of *A. brasilense* were counted at 12 h and *C. sorokiniana* at 96 h.

Microbial and microfauna counts

For counting cells in each experiment with immobilized microorganisms, the beads were dissolved by immersing them in 4% $NaHCO_3$ solution for 30 min. Samples of microorganisms in free suspension experiments were used without additional treatment. Routine counts of *A. brasilense* Cd (distinctive pink colonies) and culturable heterotrophic bacteria in wastewater or within the beads were counted by the plate count method (CFU mL^{-1}) in a series of dilutions (in 0.85% saline) on nutrient agar plates (Sigma). Counts of total bacteria in wastewater or beads were done by the fluorescein diacetate method (cells mL^{-1}; Chrzanowski et al. 1984). Counts of *C. sorokiniana* were under a light microscope using a Neubauer hemocytometer. *A. brasilense* residing on and within beads was additionally counted by FISH analysis as described later. All counts were quantified with image-analyzing software (Image Pro-Plus 4.1, Media Cybernetics, Silver Spring, MD).

Microfauna were counted with a Neubauer hemocytometer, following the method described by Tso and Taghon (1997). Briefly, 35 mL of raw wastewater was concentrated by centrifugation at 1,600×*g* for 10 min. The pellet was resuspended in 1 mL 0.85% sterile saline solution and fixed with 2% glutaraldehyde solution in 1 M HEPES buffer at pH 7.2 and then counted.

Wastewater samplings and analyses

Municipal wastewater after secondary treatment and before chlorination was routinely collected from the wastewater treatment plant of the city of La Paz, B.C.S., Mexico, and immediately used in all quantitative experiments after removing large particles by filtration by Whatman no. 1 filter paper. Wastewater from an oxidation pool at the plant was used to determine the potential competitiveness of microfauna and microbial populations residing in this wastewater. Municipal wastewater from La Paz is domestic wastewater because there is no industrial waste, only domestic ingredients (Table 1; Perez-Garcia et al. 2011). Because wastewater varied with sampling time, the wastewater was analyzed before the experiments. Wastewater analyses were performed by the analytical service laboratory of CIBNOR using standard water analyses (Eaton et al. 2005) for the following parameters: NH_4^{+} (μM), NO_3^{-} (μM), NO_2^{-} (μM), PO_4^{3+} (μM), pH, conductivity (mS m^{-1}), salinity (‰), silicates (μM), total hardness (mg L^{-1}, $CaCO_3$), Cl (mg L^{-1}), SO_4^{2-} (mg L^{-1}), acidity (mg L^{-1}), total

Table 1 Analysis of domestic wastewater obtained from the municipal wastewater treatment plant of the city of La Paz, Baja California Sur, Mexico

Parameter	Concentration
pH	7.59
Electrical conductivity	236.33 ($mS{\cdot}m^{-1}$)
Salinity	1.1 (‰)
Ammonium	2,630 μM
Nitrites	1.18 μM
Nitrates	3.01 μM
Phosphates	100.38 μM
Total phosphorus	101.40 μM
Silicates	1,205 μM
Total hardness ($CaCO_3$)	669.59 mg L^{-1}
Alkalinity	422.37 mg L^{-1}
Acidity	63.92 mg L^{-1}
Chloride	391.40 mg L^{-1}
Sulfate	92.42 mg L^{-1}
Total suspended solids	12.64 mg L^{-1}
Dissolved solids	944 mg L^{-1}
Sediment solids	<0.1 mg L^{-1}

suspended solids (mg L^{-1}), dissolved solids, and sedimented solids (mg L^{-1}).

Scanning electronic microscope

Five beads were fixed with glutaraldehyde using the method described by Bashan et al. (1986), with some modifications. Briefly, beads were fixed for 5 h in a 5% (*v*/*v*) glutaraldehyde solution in 1 M HEPES buffer at pH 7.2. After fixation, the beads were washed twice in 1 M HEPES and then dehydrated with increasing concentrations of ethanol, which are as follows: 25% for 10 min, 50% for 30 min, 70% for 10 h, and 100% for 60 min at 4 °C. Samples were dried with CO_2 in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD). The beads were mounted on a stub and coated with palladium foil for 35 min at 40 mA in a sputter coater (Vacuum Desk II, Denton, Scotia, NY).

Visualization was done with a scanning electronic microscope (SEM; S-3000N, Hitachi High-Technologies, Tokyo, Japan) at 15 kV, using a 45° angle of the slide to the electron beams. Microphotographs were obtained with software (Quartz PCI 5.5, Quartz Imaging, Vancouver, BC, Canada). The diameter of the pores on the surface of alginate beads was measured directly from 100 random pores on the microphotographs.

Fixation and preparation of samples for FISH

Because preliminary experiments showed that increasing incubation time leads to a thick layer of bacteria on the surface of submerged beads in wastewater and this reduces visualization of the immobilized cells of bacteria and microalgae (see the "Results" section), the surface of the beads were gently washed three times with 4% sterile Tween® 20 (Sigma-Aldrich, St. Louis, MO) followed by several rinsing with 0.85% NaCl. Washed and unwashed beads were used for FISH analyses.

Five beads were carefully sliced with a new, sterile scalpel under a stereoscope; slices were mounted on gelatin (0.1% *w*/*v*, pre-washed with 0.01% *w*/*v* chromium potassium sulfate)-coated microscope slides, attached to the slide by adding one drop of warm, low-melt agarose solution (0.25% *w*/*v*, Sigma), and dried at 37 °C for 45 min. The samples were then fixed with 50 μL 4% paraformaldehyde and incubated at 4 °C for 1 h. Then, the paraformaldehyde was removed by pipet; the samples were washed with 0.85% saline solution, dehydrated by successive 50%, 80%, and 96% ethanol washes (3 min each), air-dried, and stored at 4 °C until hybridization (de-Bashan et al. 2011).

In situ hybridization

This assay was based on the technique described by Assmus et al. (1995), with numerous small modifications. Hybridization was performed at 35% formamide stringency at 46 °C for 2 h. The final concentration of the probe was 3 ng μL^{-1}. Samples were then washed for 5 min with 50 ml pre-warmed washing buffer at 48 °C. The slides were rinsed for a few seconds with ice-cold, deionized water and then air dried. Slides were stored at −20 °C in the dark until visualization. An equimolar mixture of EUB338 I (Amann et al. 1990) and EUB338 II and III (Daims et al. 1999) probes, when combined, detected almost all bacteria. For *A. brasilense*, we used the specific probe Abras 1420 (Stoffels et al. 2001). EUB338 I, II, and III probes were labeled with the fluorochrome Cy3; Abras 1420 was labeled with fluorescein isothiocyanate (fluorochrome FITC). All fluorescent-labeled probes were purchased from Integrated DNA Technologies, Coraville, IA. Before visualization, the slides were mounted in an anti-fading reagent (AF1, Citifluor, London, UK).

Visualization

Under a fluorescent microscope (BX41, Olympus, Tokyo, Japan) at magnification ×1,000, fluorescence was detected in two separate channels using detection filter in red color (552 nm wavelength, Cy3, Olympus America, Melville, NY) and in green color (495 nm, FITC, Olympus America). Separate images of each photomicrograph were recorded by the digital camera of the microscope (Evolution VF Cooled Color, Media Cybernetics, Silver Spring, MD) and further processed with Adobe Photoshop 8.0 (Adobe Systems,

Mountain View, CA). All bacterial cells were labeled in red color and tentative *A. brasilense* cells in green color. The two signals were digitally superimposed by the software (Image ProPlus 6.3.1.542, Media Cybernetics), yielding highly specific identification of *A. brasilense* as yellow cells. Cells of *C. sorokiniana* were not labeled because the cells have strong autofluorescence and can be easily distinguished from bacterial cells by size (de-Bashan et al. 2011).

Quantification of *A. brasilense*

Percentage of *A. brasilense* cells of total bacteria within each image was detected by specialized software that recorded the number of pixels of each colors in each image, resulting in a percentage of color coverage (Orrala, A. and Hernandez, J.P., unpublished).

Experimental design and statistical analysis

The following treatments were conducted in each experiment with municipal wastewater: (1) control without beads or microorganisms, wastewater only; (2) control with beads alone; (3) beads containing *C. sorokiniana*; (4) beads containing *A. brasilense*; and (5) beads containing *C. sorokiniana* and *A. brasilense*. Two 48-h cycles were performed for each experiment, using the same beads but changing the wastewater after the first 48 h. Cultures were prepared in five replicates, where a single flask served as a replicate. Each experiment was repeated four times. FISH analyses of 10 replicates were done; 442 images of FISH were recorded. We quantified microorganisms in 30 microphotographs. A total of 42 SEM microphotographs were analyzed. Data were first analyzed by one-way ANOVA and then by Tukey's post hoc analysis or Student's t test at $P<0.05$. All statistical analyses were performed with computer software (Statistica v6.0, StatSoft, Tulsa, OK).

Results

Survival of *C. sorokiniana* and *A. brasilense* as free cells in municipal wastewater

With time, the number of culturable bacteria and general microfauna in wastewater significantly increased (Fig. 1a). No attempt was made to identify the species of microfauna. Under sterile conditions, the population of *C. sorokiniana* significantly increased during treatment, similar to what is known for growth *C. vulgaris* in sterile wastewater (Perez-Garcia et al. 2011) (Fig. 1b, lower case letter analysis). However, in raw, natural wastewater, the population of *C. vulgaris* was significantly smaller. It took 96 h of cultivation for the population to reached ~33% of the population size under sterile conditions (Fig. 1b, capital letter analysis). The population of *A. brasilense* declined during treatment under sterile and non-sterile conditions (Fig. 1c, lower case letter analysis), but was significantly less under non-sterile conditions (Fig. 1c, capital letter analysis).

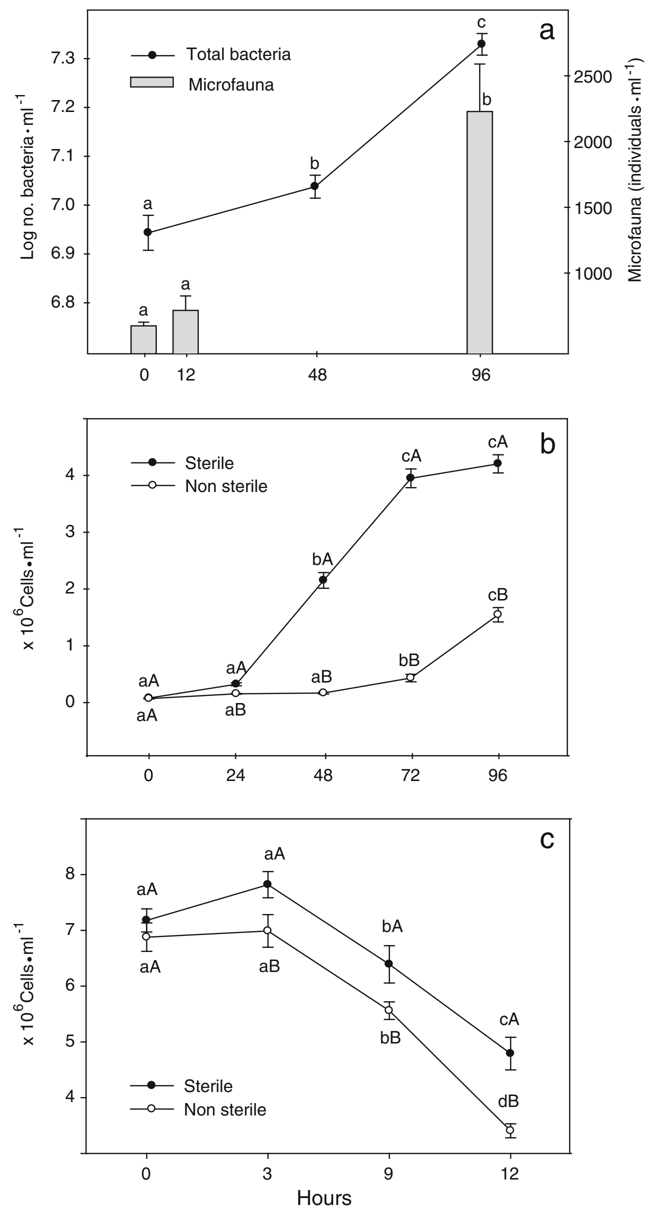

Fig. 1 Populations of free-living microorganisms and microfauna in municipal wastewater after several periods of incubation. **a** Total numbers of general wastewater bacteria and microfauna. **b** Multiplication of *Chlorella sorokiniana* in sterile and non-sterile wastewater. **c** Survival of *Azospirillum brasilense* in sterile and non-sterile wastewater. *Points on each curve* or *each set of columns*, in each subfigure separately, *denoted by a different lower case letter* differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. *Points denoted by a different capital letters* at each time of incubation differ significantly at $P<0.05$ in Student's t test. *Bars* represent the SE. Absence of bar means negligible SE

Quantification of colonization of microorganisms inside and outside immobilized alginate beads submerged in wastewater

Whether *C. sorokiniana* and *A. brasilense* were present inside the beads, heterotrophic bacteria heavily colonized the surface of the beads (Fig. 2a, b, lower case letter analysis). Depending on the counting method, there were a significant difference larger number of bacteria on the surface in favor of the fluorescein diacetate method. Probably, this method also counts non-culturable bacteria, while the plate count method counts a bacterial aggregate (presented later) as a single colony. Washing the surface of the bead was essential for visualization (described later); this had only a small effect on the total of bacteria residing on the surface (Fig. 2a, b, capital letter analysis). At the same time, the level of colonization of the immobilized microorganisms within the beads, increased for *C. sorokiniana* and decreased for *A. brasilense* (Fig. 2c, d, lower case letter analysis). Enhanced survival of cells of both species occurred when jointly immobilized (Fig. 2c, d, capital letter analysis), as was previously demonstrated for this interaction (de-Bashan et al. 2008).

Visualization of colonization by FISH and SEM of microorganisms inside and outside alginate beads submerged in wastewater

Beads from the same batch of cultivation were used for SEM and FISH analyses (Figs. 3 and 4). When submerged in wastewater, beads remained intact (Fig. 3a); many protuberances were present on the surface (Fig. 3b, black arrows) and pores (Fig. 3b, white arrows). When sliced open, the alginate beads revealed the typical internal structure of cavities (de-Bashan et al. 2011; Zohar-Perez et al. 2003, 2004a, b; Fig. 3c). After incubation for 48 h,

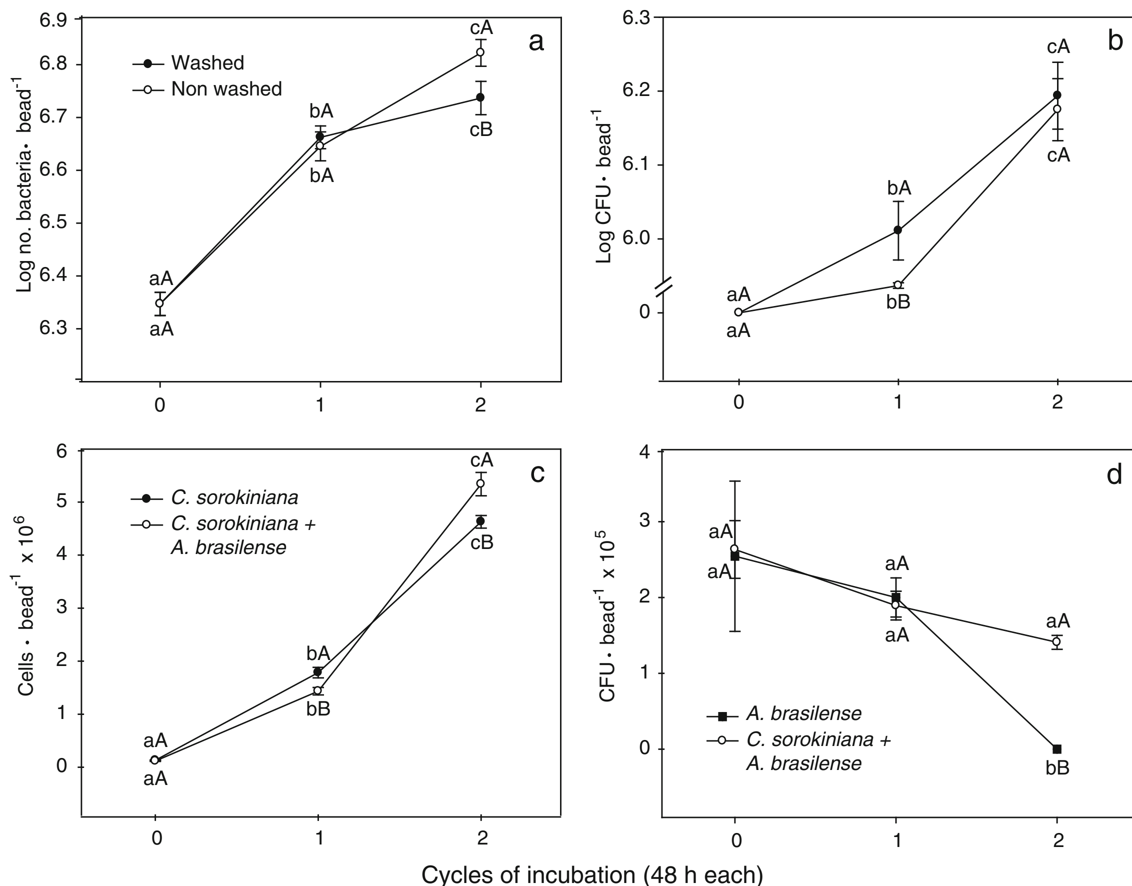

Fig. 2 Colonization of alginate beads by bacteria and microalgae immobilized in wastewater during two cycles of growth (48 h each). **a** Total heterotrophic bacterial colonization of bead surface measured by the fluorescein diacetate technique (FDA) and expressed as bacterial cells per bead. **b** Total culturable bacteria measured by plate count method and expressed as CFU per bead. **c** Colonization of the interior of the bead by *Chlorella sorokiniana* immobilized alone or together with *Azospirillum brasilense*. **d** Colonization of the interior of the bead by *A. brasilense* immobilized alone or together with *C. sorokiniana*. *Points on each curve denoted by a different lower case letter* differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. *Points denoted by a different capital letter* at each cycle of incubation differ significantly at $P<0.05$ in Student's *t* test. *Bars* represent the SE. Absence of bar means negligible SE

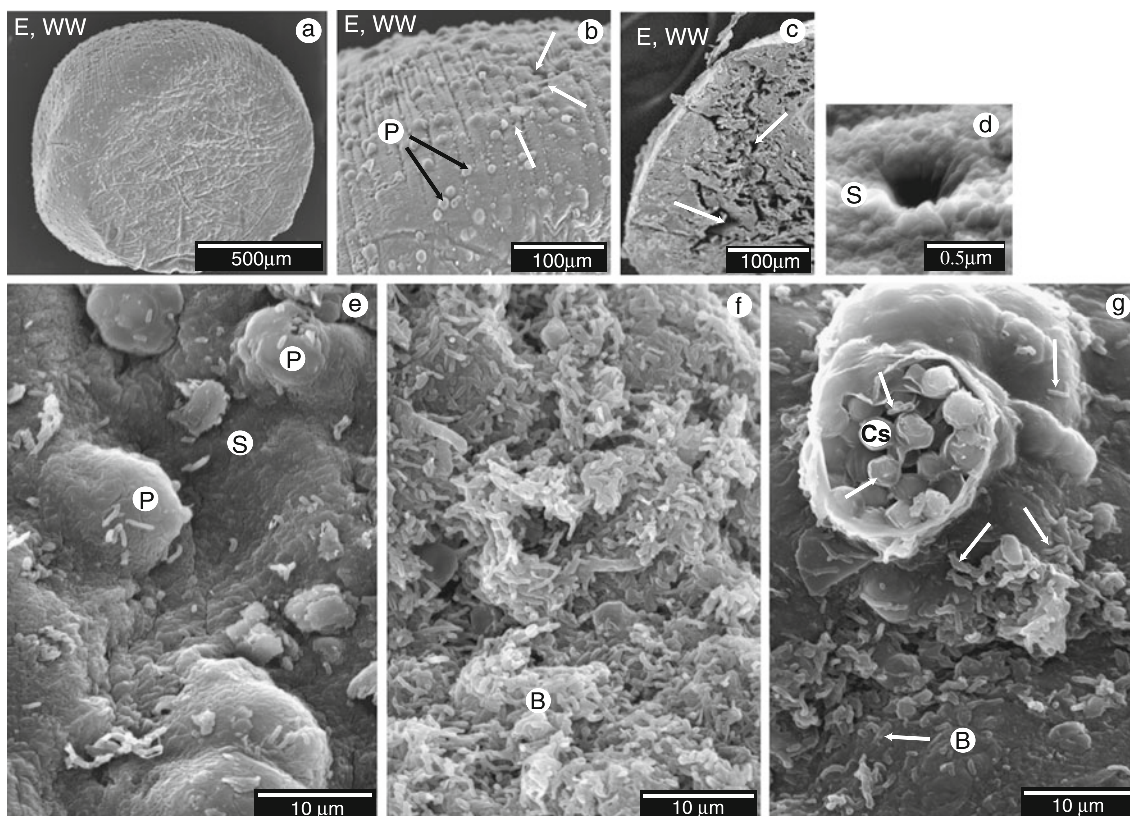

Fig. 3 **a** Microphotograph by scanning electron microscopy of the surface of an alginate bead (containing jointly immobilized *Chlorella sorokiniana* and *Azospirillum brasilense*) after incubation in regular wastewater after 48 h. **b** Magnification of subfigure **a**. *Black arrows* indicate protuberances and *white arrow* indicates pores. **c** Sliced bead showing the internal cavity structure (*arrows*) typical of alginate beads. **d** Typical pore cavity of the surface of the bead. **e** Colonization of surface of beads by wastewater bacteria submerged in wastewater for 48 h, and **f** after 96 h, forming a massive biofilm on the surface. **g** Surface of the bead with biofilm and single bacteria (*arrows*) after removal of the alginate cover of one protuberance on the surface of the bead. This shows the internal structure of the bead containing *Chlorella*-type cells and bacteria (*arrows* in the vicinity of the microalgae). *WW* wastewater, *E* exterior of the bead, *B* bacterial biofilm, *P* protuberance formation (containing the immobilized microorganisms) on the outer surface of the bead, *S* the alginate surface, *Cs C. sorokiniana*

many indigenous bacterial cells from the wastewater, and possibly cells *A. brasilense* immobilized on the surface, started to accumulate and formed aggregates (Fig. 3e). After 96 h, a thick biofilm completely covered the surface of the bead (Fig. 3f). No *Chlorella*-type cells (by size and shape) were embedded in the biofilm. Bacterial analysis of this biofilm was provided by FISH. When beads were washed, it was possible to eliminate part of the biofilm and observe the surface where numerous pores were located (Fig. 3b, d). The average size of pores was 0.514± 0.132 μm in diameter. When the outer layer of alginate was mechanically removed from a protuberance on the bead, the interior population of *C. sorokiniana* and *A. brasilense*, later specifically identified by FISH analysis, was revealed (Fig. 3g).

Immobilized alginate beads that were submerged for 96 h in wastewater were washed or left unwashed; beads from both procedures were then sliced (Fig. 4a). Colonization by wastewater bacteria and immobilized *A. brasilense* were observed by FISH. A thick biofilm of wastewater bacteria (visualized solely in red) covered the exterior of beads (>95% of the surface) and almost blocked observation of the alginate surface (Fig. 4b). Coverage with *A. brasilense* was only 1.04±0.65% of the surface. When the beads were washed, which probably released some bacteria loosely adhering to the surface, a mix of unidentified wastewater bacteria and *A. brasilense* (detected as yellow cells) directly adhered to the surface (Fig. 4c, d). The percentage of colonization by *A. brasilense* was 19.27±4.85% and that of the wastewater bacteria was 80.73±4.3%. When interior slices of washed bead were examined, only *A. brasilense* (Fig. 4e) or a mixture of *A. brasilense* and *C. sorokiniana* (Fig. 4f,) were identified by the FISH procedure. There was no need for FISH labeling of microalgae because of the large difference in size of bacteria.

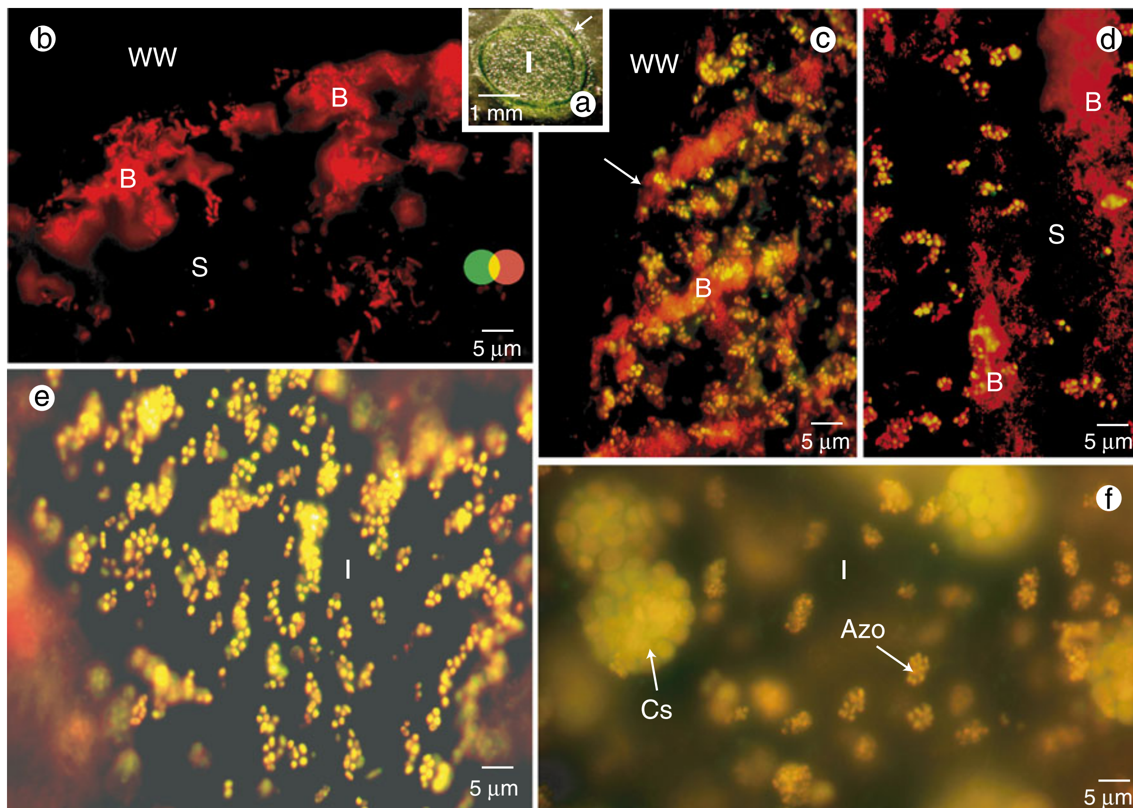

Fig. 4 FISH analysis of colonization of the surface and the interior of jointly immobilized *Chlorella sorokiniana* and *Azospillum brasilense* Cd in alginate beads. **a** Cross-section of a bead. *Arrow* indicates the surface of the bead. **b** Segment of the exterior of the bead revealing formation of the bacterial biofilm (*red labeling*). **c** The same area after light washing, showing that the biofilm on the surface is made of a mixture of indigenous bacteria (red-labeling Eubacteria) and *A. brasilense* Cd (*yellow*). *Arrow* indicates the surface of the bead. **d** Close view of subfigure **c**. **e** Segment of the interior of the bead, showing exclusive colonization by *A. brasilense* (*yellow*) or **f** *C. sorokiniana* and *A. brasilense* Cd. *Yellow-colored bacteria* indicate specifically detected micro-colonies and individual cells of *A. brasilense* Cd by FISH. FISH experiments were performed with the probe Abras 1420 (*green*), specific for *A. brasilense*, and with the probe mix of EUB338-I, II, III-Cy3 (*red*), specific for the domain bacteria. The composed RGB images result in a *yellow color* for cells of *A. brasilense* Cd, which indicates co-labeling by both probes and *red color* for all other bacteria in the wastewater. *WW* wastewater, *B* biofilm, *I* interior of the bead, *S* surface of the bead, *Cs C. sorokiniana*, *Azo A. brasilense* Cd

Tertiary wastewater treatment by *C. sorokiniana* and *A. brasilense* immobilized in alginate beads

The same wastewater containing beads with the two microorganisms was also tested for its capacity to remove ammonium and phosphorus during tertiary wastewater treatment. The bead cultures removed ammonium (Fig. 5a) and phosphorus (Fig. 5b) at various levels of efficiency during two treatment cycles, each lasting 48 h.

Discussion

For a long time, literature reviews have taken for granted that immobilization of beneficial microorganisms in polymers or in beads prepared from various polymers, from inoculants in agriculture to bioremediation of contaminated environments, provide some physical or chemical protection against competitors, predators, and adverse conditions (Trevors et al. 1992; McLoughlin 1994; Cassidy et al. 1996; Bashan 1999; de-Bashan and Bashan 2010; Moreno-Garrido 2008; Prasad and Kadokawa 2009). Yet, meager experimental evidence is available that this protection exists (Leung et al. 2000). Our main objective was to determine the level of physical protection that immobilization in alginate provides wastewater treating agents against predation and competition by populations of microorganisms that naturally reside in wastewater.

Wastewater is a habitat populated by numerous bacteria and microfauna (protozoa and metazoa). The latter are the top predators in wastewater treatment (Kinner and Curds 1987; Luna-Pabello et al. 1990; Madoni et al. 1993; Martín-Cereceda et al. 1996), fresh and salt water (Sherr and Sherr 1987; Novitsky 1990; Berninger et al. 1991), and soils (Habte and Alexander 1977). Consequently, when a biotreatment agent is added to wastewater, it faces

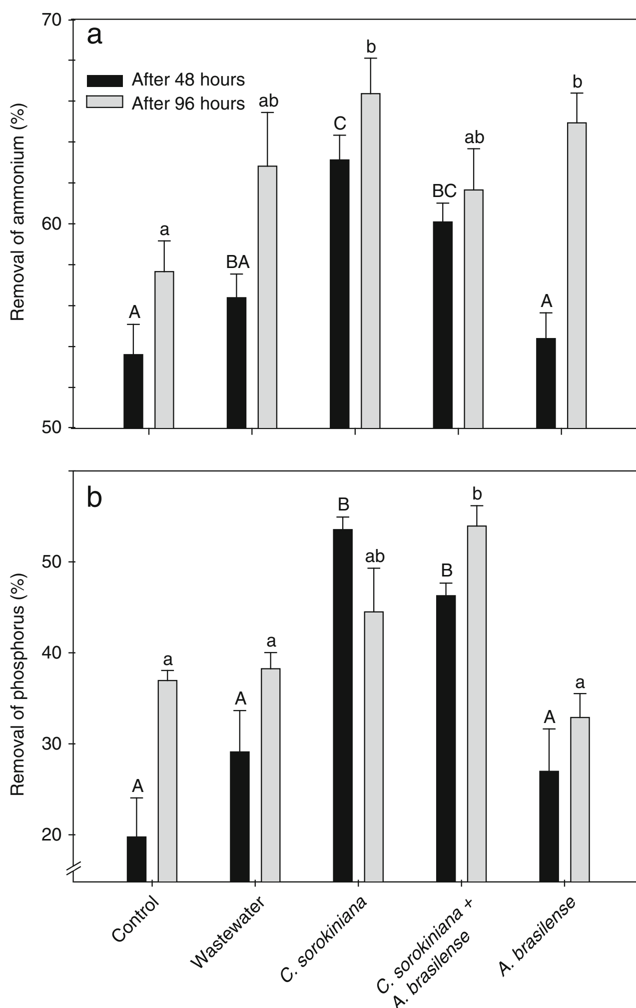

Fig. 5 Removal of ammonium (**a**) and phosphorus (**b**) by *Chlorella sorokiniana* from domestic wastewater, immobilized alone or with *Azospirillum brasilense* Cd in alginate beads after two cycles of 48 h each. *Columns* for each cycle (48 h or 96 h, separately) in each separate subfigure, *denoted by a different letter*, differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. *Bars* represent SE

competition by the local bacterial population, at best. At worst, the agent serves as an additional source of nutrients for predatory microfauna. Both scenarios reduce the population and efficiency of the agent. Under these adverse circumstances, any protection for the agent, even for a limited time, is an advantage.

A possible major factor in providing protection against predators or competitors is physical separation between competitor/predator and their prey. This was provided by the nature of the alginate bead. After immobilization of the microorganisms in the bead, the bead is internally composed of many cavities where the microalgae and bacteria reside and multiply. These cavities are located in every part of the bead, from just below the surface where the microorganisms produce protuberances on the outer surface of the bead to the center of the bead (de-Bashan et al. 2011; Lebsky et al. 2001; this study). Cells of *A. brasilense* that were immobilized randomly on the surface of the bead can multiply there and create a mixed biofilm with the indigenous wastewater bacteria (Bashan 1986; this study). In this study, *C. sorokiniana* cells were not found on the surface of the bead and were probably prey to the microfauna in the wastewater.

The most distinct feature and possibly the main protection barrier is the surface of the bead. This rigid surface is a solid layer having only a few relatively large pores. This type of surface is produced instantly when a drop of liquid alginate falls into a solidifying solution of cations (such as $CaCl_2$, as in this study, or several other cations) during polymerization, creating a layer of hard polymerized alginate (Smidsrød and Skjåk-Bræk 1990). The curing process and contraction after cross-linking that the bead undergoes after initial surface polymerization probably produces the cavities below the surface (Zohar-Perez et al. 2004a, b). The surface allows wastewater molecules to diffuse (discussed later) and acts as a physical barrier between the internal microbial agents and the external microfauna and bacteria residing in the wastewater. This happens because the size of the pores (~0.5 µm) on the surface is smaller than most bacteria and microalgae. *Chlorella* spp. are ~10 µm in diameter (de-Bashan et al. 2005) and *Azospirillum* spp. range from 0.6 to 1.7 µm (Bergey's Manual of Systematic Bacteriology 2005). Their size keeps them trapped inside the bead. The size of wastewater indigenous bacteria observed in this study was 1–4 µm and the microfaunas were far larger organisms. Hence, all indigenous microorganisms cannot penetrate the surface of the beads to reach the inner cavities. Although it is theoretically possible that very small bacteria may penetrate these pores, they were not detected by FISH in this study.

Alginate properties (structure, mechanical strength, porosity, and gelling force) are important variables to consider in selecting alginate for an application (McHugh 1987). Alginate produced from the Pacific ocean kelp *Macrocystis pyrifera* is most frequently used for immobilization of cells and most wastewater treatment immobilization studies (for review: de-Bashan and Bashan 2010), including this study. This source yields gels with lower strength and stability than gels from other alginates (Skjåk-Bræk and Martinsen 1991). Alginate is also biodegradable, especially by soil microorganisms (Bashan 1986; Bashan et al. 2002). Many of these soil microorganisms also occur in wastewater. The surface layer may degrade with longer retention time in wastewater than was the case in this study (Faafeng et al. 1994; Cruz et al., unpublished data). Even in cases of degradation of the outer surface competition/predation of the wastewater agents will be limited because of the inner structure of the cavities in the beads. Although

some internal cavities are interconnected, most are not (de-Bashan et al. 2011; Lebsky et al. 2001; Zohar-Perez et al. 2004a,b; this study). If microbes breach the solid surface by degrading the outer layer of alginate, mobility of the invading organisms within the bead is still low, allowing sufficient time for the biotreatment agents to complete tertiary water treatment. This proposal needs additional experimental evidence.

This study shows that production of a heavy biofilm on the surface of beads, a phenomenon common in wastewater treatment (Henze et al. 2002), is clearly demonstrated by FISH and SEM analyses and does not restrict tertiary biological wastewater treatment performed by immobilized microorganisms. Because the calcium alginate gel allows diffusion of small molecules with upper limit of ~140 kDa, molecules of nitrogen and phosphorus compounds easily diffuse into the beads (de-Bashan et al. 2002, 2004; Yabur et al. 2007). Compared with removal of nutrients under sterile conditions (de-Bashan et al. 2002), removal of ammonium and phosphorus in this study is lower. However, when compared to other studies using non-sterile wastewater (de-Bashan et al. 2004; Hernandez et al. 2006), the percentage of removal of these compounds is similar.

In summary, this study demonstrates that wastewater microbial populations are responsible for reducing populations of biological agents added for wastewater treatment. Immobilization in alginate beads provides a protective environment for these agents for sufficient time to allow uninterrupted tertiary wastewater treatment.

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