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ENVIRONMENTAL BIOTECHNOLOGY

Biological deterioration of alginate beads containing immobilized microalgae and bacteria during tertiary wastewater treatment

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Abstract Secondary treatment of municipal wastewater affects the mechanical stability of polymer Ca-alginate beads containing the microalgae *Chlorella vulgaris* that are jointly immobilized with *Azospirillum brasilense* as treating agents whose presence do not affect bead stability. Nine strains of potential alginate-degrading bacteria were isolated from wastewater and identified, based on their nearly complete 16S rDNA sequence. Still, their population was relatively low. Attempts to enhance the strength of the beads, using different concentrations of alginate and $CaCl_2$ or addition of either of three polymers (polyvinylpyrrolidone, polyvinyl alcohol, carboxymethylcellulose), $CaCO_3$, or $SrCl_2$, failed. Beads lost their mechanical strength after 24 h of incubation but not the integrity of their shape for at least 96 h, a fact that sustained successful tertiary wastewater treatment for 48 h. In small bioreactors, removal of phosphorus was low under sterile conditions but high in unsterile wastewater. Alginate beads did not absorb PO_4^{-3} in sterile wastewater, but in natural wastewater, they contained PO_4^{-3}. Consequently, PO_4^{-3} content declined in the wastewater. A supplement of 10 % beads (*w*/*v*) was significantly more efficient in removing nutrients than 4 %, especially in a jointly immobilized treatment where >90 % of PO_4^{-3} and >50 % ammonium were removed. Tertiary wastewater treatment in 25-L triangular, airlift, autotrophic bioreactors showed, as in small bioreactors, very similar nutrient removal patterns, decline in bead strength phenomena, and increase in total bacteria during the wastewater treatment only in the presence of the immobilized treatment agents. This study demonstrates that partial biological degradation of alginate beads occurred during tertiary wastewater treatment, but the beads survive long enough to permit efficient nutrient removal.

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

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-4703-6) contains supplementary material, which is available to authorized users.

Dedication This study is dedicated to the memory of the Italian microbiologist Prof. Franco Favilli (1933–2012) of the University of Florence, Italy, one of the pioneers of *Azospirillum* studies.

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Introduction

Numerous biotechnological processes and environmental and agricultural applications are using immobilization of microorganisms in alginate beads, among other polymers, when viable microbial cells are required (Nussinovitch 2010). Immobilizing microalgae is a common approach in several applications of bioremediation (de-Bashan and

Bashan 2010), of which, wastewater treatment is the oldest (de la Noüe and Proulx 1988).

One procedure for tertiary domestic wastewater treatment employed green, unicellular microalgae *Chlorella* spp. jointly immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense* (de-Bashan et al. 2004). Species of the aquatic microalgae *Chlorella* spp. (Chlorophyceae) are commonly used as models in studies of photosynthesis, respiration, and synthesis of carbohydrates and lipids in microalgae (de-Bashan et al. 2002a; Ilangovan et al. 1998). *Chlorella* spp. have many uses. They can produce low-volume, high-value compounds, including pigments and vitamins (Lebeau and Robert 2006), agents of wastewater treatment (de-Bashan and Bashan 2010; Oswald 1992), and as potential raw materials for biofuel production (Mata et al. 2010). Except for symbiotic rhizobia, *Azospirillum* is the most studied agricultural plant growth-promoting bacterium (PGPB; Bashan and de-Bashan 2005). It acts as a general PGPB for numerous crops (Bashan et al. 2004), including *Chlorella* (Gonzalez and Bashan 2000), via a multitude of growth-promoting mechanisms (Bashan and de-Bashan 2010). The natural alginate polymer is the most commonly used for immobilizing microorganisms (Smidsrød and Skjåk-Bræk 1990).

Our hypothesis was that deterioration of beads, immersed for a prolonged time in wastewater, result mainly from biological activity of naturally occurring heterotrophic bacteria that have a capacity to degrade alginate and not by phosphate cations that the wastewater contains after secondary wastewater treatment. Degradation of beads is relatively slow; hence, there is sufficient time to efficiently remove nutrients by microbial agents immobilized inside the beads.

Specific objectives were to (1) quantify the level of deterioration of alginate beads during tertiary wastewater treatment; (2) assay if naturally occurring bacteria in the wastewater are involved, and if yes, to identify potential alginate-degrading bacteria; (3) test several known immobilization-strengthening procedures to reduce deterioration; and (4) determine if the optimal dose of alginate beads that contain treating agents for small bioreactors is also suitable for tertiary wastewater treatment in 25-L bioreactors.

Materials and methods

Wastewater sampling and analyses

Municipal wastewater after secondary treatment and before chlorination was routinely collected from the wastewater treatment plant of the city of La Paz, Baja California Sur (BCS), Mexico (215,000 habitants; wastewater production of 26,000 $m^3 day^{-1}$ in 2010). The wastewater was immediately used in all quantitative experiments after only removing large particles by filtration with filter paper (Whatman No. 1). In one experiment, sterile controls (autoclaved for 15 min at 15 psi and 121 °C) were also included for comparison. Wastewater from La Paz is domestic wastewater because there is no industrial waste (Perez-Garcia et al. 2011). Although content varies with sampling time, general fluctuation and content were analyzed before and found to be consistent (Covarrubias et al. 2012; Perez-Garcia et al. 2011). Removal of nutrients in all experiments was assayed by standard water analyses (Eaton et al. 2005): PO_4^{-3} by the ascorbic acid technique PhosVer 3 (Hach, Loveland, CO) and NH_4^+ by the phenol-hypochlorite method (Solorzano 1969), as adapted for microplates by Hernández-López and Vargas-Albores (2003). Phosphate attached to the alginate beads was measured using bicarbonate extraction and perchloric acid digestion to measure total phosphorus (Olsen and Sommers 1982).

Microorganisms and initial growth conditions

The unicellular microalga *C. vulgaris* Beijerinck (UTEX 2714, University of Texas, Austin, TX, USA) was used. Before immobilization in alginate beads, the microalgae were cultured in sterile mineral medium (C30) for 5 days (Gonzalez et al. 1997). *A. brasilense* Cd (DSM 1843, Braunschweig, Germany) was grown in 50-mL BTB-1 medium (Bashan et al. 2011) at 34±2 °C for 24 h in a rotary shaker at 150 rpm, using standard techniques for this genus (Bashan et al. 1993).

Types of alginate, concentration used, and other polymers and solidification agents used in combination with alginate

For immobilization of microorganisms, three experiments were carried out. In the first experiment, we compared two types of alginate at 2 % (3,500 cps): (Sigma, St. Louis, MO; A7128 and A2033) and Algimar B (560 cps): (CICIMAR-IPN, La Paz, BCS, Mexico). In the second experiment, the same alginates were used but comparing the effect of preparing the alginate solution at three concentrations (2, 4, and 6 %). In each case, the gels were obtained with 2 and 4 % $CaCl_2$. In the third experiment, in addition to the $CaCO_3$, the following mixtures were used (separately): 2 % alginate and 2 % $CaCl_2$ with either 2 % polyvinylpyrrolidone (PVP, K-90), or 3 % polyvinyl alcohol (PVA) and 2 % H_3BO_3, or 2 % sodium carboxymethyl cellulose (CMC), or 2 % $SrCl_2$ instead of 2 % $CaCl_2$. (All chemicals were of analytical grade and obtained from Sigma. PVP, PVA, and CMC were obtained from Drogueria Cosmopolita, Mexico City, Mexico).

Immobilization of microorganisms in alginate beads

For most experiments and unless otherwise stated, microorganisms were immobilized, as described by de-Bashan et

al. (2004). Briefly, 20 mL of axenically grown cultures of *C. vulgaris* containing 6.0×10^6 cells per milliliter 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 alginate solution and stirred for 15 min; alginate was sterilized by autoclaving, with only slight reduction in viscosity. 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. Other variations of alginate, solidification agents, and hardening material used the same general technique. As controls, cultures of *A. brasilense* (10^9 CFU mL^{-1}) and cultures of *C. vulgaris* (6.0×10^6 cells per milliliter) 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). The second incubation in diluted nutrient broth (1:10, Sigma) lasted overnight. Where jointly immobilized cultures of *A. brasilense* and *C. vulgaris* 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.

Analyses of degradation of beads

Beads were analyzed for textural strength using a texture analyzer (TA.XT plus, Stable Micro Systems, Godalming, Surrey, UK) and for change in their diameter using a digital caliper (Traceable, Control Company, Friendswood, TX, USA). Data are presented as gram per square centimeter for strength and millimeter for diameter. To determine whether microorganisms residing in wastewater or the concentration of phosphates in the water or the pH are responsible for degradation, treatments included similar wastewater treatments (listed below) and compared with sterile wastewater. Levels of phosphorus and pH (Horiba, Kyoto, Japan) of the wastewater were measured.

Culture conditions in wastewater

Experiments in small volumes

(a) Microorganism species immobilized alone or jointly were grown under batch conditions for 96 h 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 or 10 g of beads) at 28±2 °C, agitated at 150 rpm under constant light at a density of 90 μmol photons $m^{-2}\cdot s^{-1}$ in an incubator (Innova 4340, New Brunswick Scientific, Edison, NJ, USA).

(b) Similar experiments designed for adsorption of phosphorus by alginate beads were performed in 2-L bioreactors containing 1 L medium of either sterilized wastewater or natural wastewater. Each bioreactor received 100 g beads, incubated at 28±2 °C under constant light intensity of 90 μmol photons $m^{-2}\cdot s^{-1}$ and supplied with aeration of 30 mL air mL^{-1}. For bioreactors containing sterilized wastewater, air was filtered with two 0.2-μm sterile filters (Acrodisc, Pall, Port Washington, NY, USA) and the air outlet with 1-μm pore bacterial air vent filter (Pall). Unfiltered air was provided to non-sterile bioreactors.

Experiments in 60-L bioreactor

Nutrients (PO_4^{-3} and NH_4^+) were removed from wastewater in 60-L airlifting, autotrophic, triangular bioreactor containing 25 L wastewater per run (Fig. 1). The wastewater contained 10 % beads (fresh weight/*v*), air 30 mL air min^{-1}, at 28±1 °C, at 90 μmol photons $m^{-2}\cdot s^{-1}$ light intensity.

Microbial 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 residing in the wastewater 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 fluorecein diacetate method (cells mL^{-1}; Chrzanowski et al. 1984). Counts of *C. vulgaris* were under a light microscope using a Neubauer hemocytometer. All counts were quantified with image-analyzing software (Image Pro-Plus 4.1, Media Cybernetics, Silver Spring, MD, USA).

Isolation, quantification, and identification of alginate-degrading bacteria and their effect on beads

The possibility that some heterotrophic bacteria are residing in the wastewater and are responsible for degradation of the beads was checked by plating 100 μL aliquots from wastewater on a solid medium (MA) composed of 10 gL^{-1} sodium alginate (Algimed) and the following ingredients (mgL^{-1}): NH_4Cl (50); KH_2PO_4 (14); $MgSO_4\cdot3H_2O$ (2); $CaCl_2$ (4); NaCl (7). The plates were incubated at 30±1 °C for 72 h. Bacterial colonies that developed were streaked two consecutive times on the same solid media to ensure that they

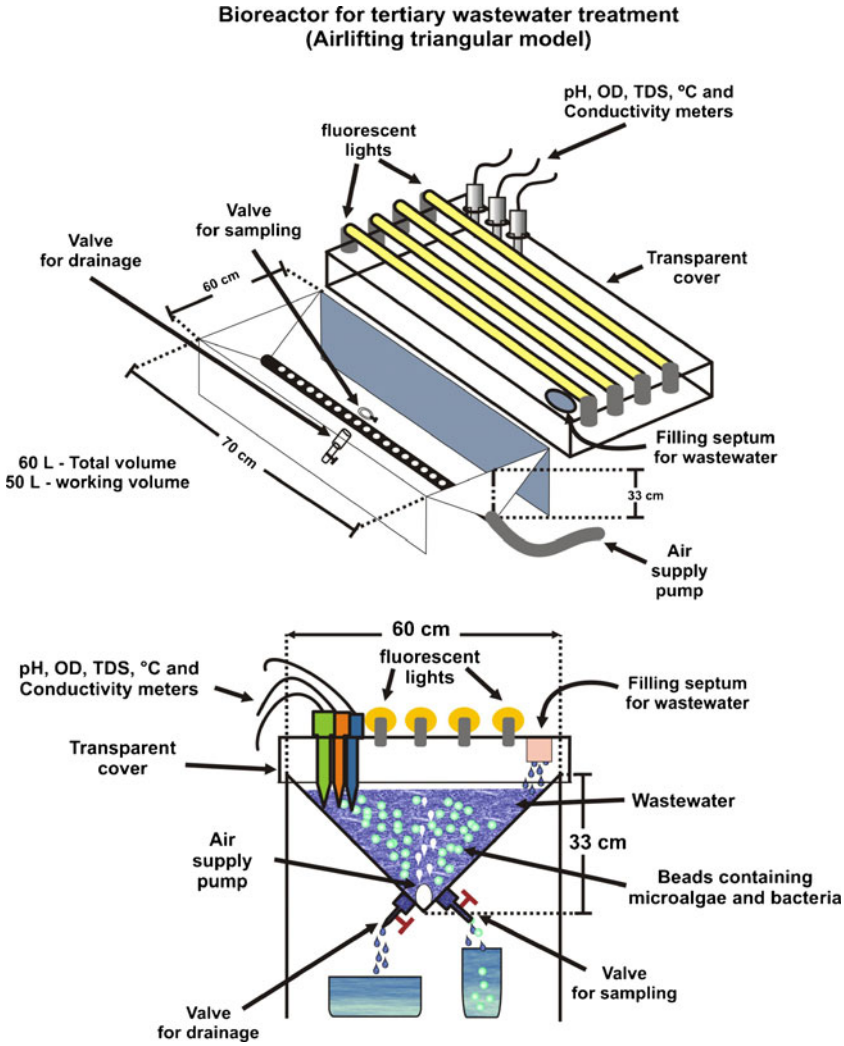

Fig. 1 Schematic diagram of 60-L, triangular, airlifting autotrophic bioreactor used in this study. This bioreactor was filled with 25 L of secondary-treated wastewater for each run

can grow on alginate as the sole carbon source. Nine different morphotypes were grown on 60 % Trypticase soy broth (TSB) medium (BD, Franklin Lakes, NJ, USA), and their entire 16S rDNA genes were sequenced by a commercial service (Genewiz, South Plainfield, NJ, USA). The range of length of the amplificates was 1354 to 1418 bp. Isolates were compared with sequences deposited in the GenBank database using the BLAST tool (www.ncbi.nlm.nih.gov/Blast.cgi). All sequences were deposited in the GenBank database.

The capacity of these isolates to degrade alginate beads were tested in four combinations of media.

(1) Alginate beads (4 g) without microorganisms were placed in 250-mL Erlenmeyer flasks containing 100 mL MA medium without an additional carbon source, apart from the polymerized alginate beads. Each of the isolated strains was first cultivated in TSB medium at 30±2 °C for 72 h; cells were extracted by centrifugation (1,717×*g*); washed three times in 0.85 % saline solution; and their number adjusted to 10^9 CFU mL^{-1}. One milliliter of the washed suspension was inserted into each flask and incubated at 30±2 °C for 96 h at 120 rpm. Every 48 h, 30 beads and medium were sampled. Bead diameter was measured as described earlier, and the bacterial population in the medium was recorded at OD_{540} (DR-2000, Hach).

(2) The capacity of the strains to survive on dissolved alginate monomers was tested similarly in MA medium, where salt-only MA medium, without alginate monomers, served as the control.

(3) Alginate beads (4 g) were incubated either in 50 or 100 % TSB medium and tested as described in assay 1.

(4) High concentrations of each strain (12×10^9 CFU mL^{-1}) were initially cultivated in TSB, washed as described earlier and added to MA medium containing 4 g of alginate beads. Increasing changes (losing bead mechanical strength) or decreasing changes (direct degradation) in bead size over time concomitant with changes in bacterial population in the medium served as indicators of alginate-degrading capacity of the strains.

Experimental design and statistical analysis

Cultures using small quantities of wastewater (250–1,000 mL per bioreactor or Erlenmeyer flask) were prepared in five replicates, where a single flask served as a replicate. Gel strength and diameter were performed in five replicates, using ten independent beads as a replicate. Addition of different polymers and solidifying substances and alginate-degrading capacity of the strains were done in three replicates. Each of these experiments was repeated two to four times. Cultures in the 60-L bioreactor were repeated twice. Several independent runs of the bioreactor, each assaying a single treatment under identical environmental conditions, were considered and presented as a single experiment. Data were first analyzed by one-way ANOVA and then by Tukey's post hoc analysis or by Student's *t* test. Both analyses set confidence at $P < 0.05$. All statistical analyses were performed with software (Statistica 8.0, StatSoft, Tulsa, OK, USA).

GenBank accession numbers of alginate-degrading bacteria

The following nine sequences were deposited in the GenBank with the accession numbers as follows: JQ995474 to JQ995482.

Results

Degrading effects of wastewater microorganisms on alginate beads

After secondary wastewater treatment, natural bacterial populations, which were the source of bacteria affecting the stability of the beads in this study, were present at a density of $4.8 \pm 0.2 \times 10^4$ cells per milliliter.

The presence or absence of *C. vulgaris* and *A. brasilense* inside the bead in sterile wastewater had no effect on the dimensions or the strength of the gel that form the beads during incubation for 96 h (Fig. 2a, b; analysis indicated with lower case letters). In natural, secondary-treated wastewater, reduction of ~70 % in gel strength and of ~10 % in diameter occurred during incubation for 48 h. After incubation for 96 h, the strength of the gel was below the lower measurement capacity of the test equipment, and the diameter of each bead was diminished by ~35 % (Fig. 2a, b; analysis indicated with capital letters).

Alginate-degrading bacteria in wastewater

The concentration of potential alginate-degrading bacteria in natural wastewater was $1.62 \pm 0.09 \times 10^3$ CFU mL^{-1}, which was 3.36 % of the bacterial population residing in the wastewater. Of these, nine morphotypes were selected, purified, their entire 16S rDNA gene was sequenced, and the bacteria were identified (Table 1). These strains were tested for their alginate-degrading capacity. None of them survived well for 96 h in minimal medium without a carbon source. After application of alginate monomers or alginate beads, all strains survived well, and several multiplied. Culturing the strains in rich TSB medium at 50 or 100 % strength yielded dissolution of all the beads within 48 h. Addition of a high concentration of each strain to the minimal medium supplemented with alginate beads increased the populations of two strains, maintained the same population of five other strains, and reduced the population of one strain. Although beads were not degraded, they had greatly enlarged (depending on the strain), indicating the loss of mechanical strength of beads (Table S1, Fig S1, supplemental material).

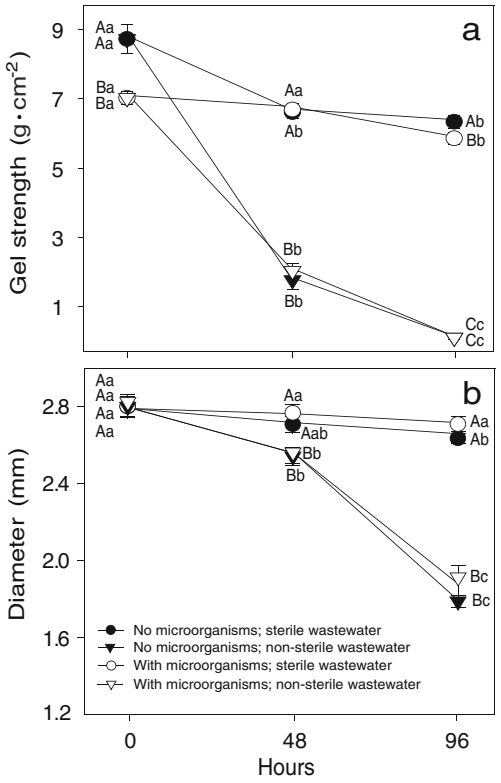

Fig. 2 Degrading effects on alginate beads from microorganisms in wastewater. **a** Effect on gel strength, **b** effect on bead diameter. Points on each curve in each subfigure, denoted by a *different lower case letter*, differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. Points denoted by a *different capital letter* at each time of incubation differ significantly at $P<0.05$ in one-way ANOVA. *Bars* represent the SE. *Absence of bar* means negligible SE

Enhancing the strength of alginate beads against biological degradation

Two different strategies were used to increase the strength of beads against biodegradation: (1) manipulation of the

Table 1 Potential alginate-degrading bacteria present in secondary municipal wastewater. All isolates exhibited 99 % 16S rDNA sequence homology to isolates from the GenBank

Phylum	16S rDNA-based identification	Strain	Sequence length[a]	GenBank accession number
α-Proteobacteria	*Xanthobacter flavus* (99 %)[b]	ICS1	1354	JQ995474
α-Proteobacteria	*Ancylobacter polymorphus* (99 %)	ICS3	1367	JQ995476
β-Proteobacteria	*Zoogloea resiniphila* (99 %)	ICS2	1413	JQ995475
γ-Proteobacteria	*Pseudomonas stutzeri* (99 %)	ICS4	1414	JQ995477
γ-Poteobacteria	*Pseudomonas plecoglossicida* (99 %)	ICS5	1413	JQ995478
γ-Proteobacteria	*Acinetobacter junii* (99 %)	ICS6	1415	JQ995479
γ-Proteobacteria	*Pseudomonas* sp.1 (99 %)	ICS7	1411	JQ995480
γ-Proteobacteria	*Pseudomonas putida* (99 %)	ICS8	1407	JQ995481
γ-Proteobacteria	*Pseudomonas* sp.2 (99 %)	ICS9	1418	JQ995482

[a] Actual number of bases sequenced

[b] All isolates exhibited 99 % 16S rDNA sequence homology to isolates from the GenBank

concentration of alginate and $CaCl_2$ (source of Ca^{+2}) (2, 4, and 6 % each) or (2) adding separately three polymers or Sr^{+2} to the alginate mixture.

Initially, the higher the concentrations of alginate and Ca^{+2} in the mixture, the stronger was the gel (Fig. 3a, capital letter analysis). At 48 h of incubation in wastewater, gel strength had diminished to <5 % of the original strength and stayed low until the end of the test at 96 h (Fig. 3a). No statistical difference among treatments was found during these periods of incubation (Fig. 3a, lower case analysis).

Addition of any of the three polymers or Sr^{+2} to the alginate mixture yielded a gel weaker than the control gel with 2 % Ca^{+2}. PVP produced the relatively stronger gel of the four additives and Sr^{+2} produced the weakest gel (Fig. 3b, capital letter analysis). The gel strength was significantly reduced in all treatments after 48 h, maintaining the relative difference in strength among additives (Fig. 3b, lower case analysis). After 96 h of incubation in wastewater, all gels were very weak ~2–6 % of their original strength (Fig. 3b, detailed analysis not shown).

Removal of phosphorus by microbially immobilized and empty alginate beads in small bioreactors

Under sterile conditions, no significant removal of phosphorus was observed in empty beads or beads containing immobilized microorganisms (Fig. 4a, lower case analysis), where only slightly more removal of P occurred. This was not statistically significant in microbially immobilized beads after 96 h (Fig. 4a, capital letter analysis). In natural wastewater, significant phosphorus removal occurred in both empty and microbially immobilized beads (Fig. 4a, lower case analysis), where microbially immobilized beads removed more (Fig. 4a, capital letter analysis).

The net effect of alginate beads without immobilized microorganisms in sterile and natural wastewater showed that, under sterile conditions, the beads did not absorb phosphorus during wastewater treatment (Fig. 4, lower case

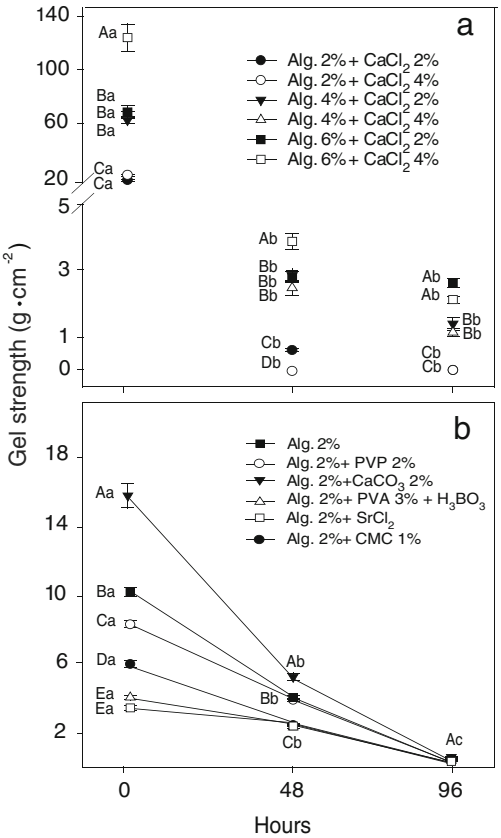

Fig. 3 Effect of enhancing the strength of alginate beads on gel strength. **a** Combinations of different concentrations of alginate and $CaCl_2$; **b** addition of hardening agents to alginate. Points on each curve, or with time, in each subfigure, denoted by a *different lower case letter*, differ 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 time of incubation differ significantly at $P<0.05$ in one-way ANOVA. *Bars* represent the SE. *Absence of bar* means negligible SE

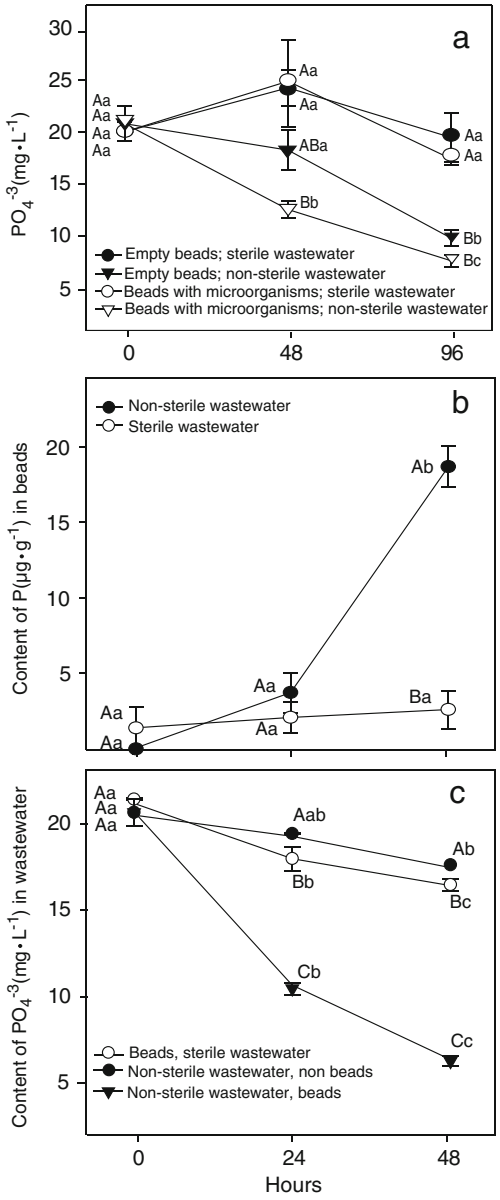

Fig. 4 **a** Removal of PO_4^{-3} by immobilized and empty alginate beads in sterile and not sterile wastewater in small bioreactors, **b** accumulation of phosphorus in beads during the wastewater treatments performed in (**a**), **c** reduction in PO_4^{-3} content of the same wastewater. Points on each curve, in each subfigure, 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 time of incubation differ significantly at $P<0.05$ in one-way ANOVA. *Bars* represent the SE. *Absence of bar* means negligible SE

analysis), while in natural wastewater, there was a significant increase in absorption of P (Fig. 4b, lower case analysis) after incubation for 96 h (Fig. 4b, capital letter analysis). In the same bioreactors, natural wastewater without beads or when beads were added to sterile wastewater, the level of P dissolved in wastewater was only slightly reduced over time, even though it was statistically different (Fig. 4c). In natural wastewater containing beads, the level of dissolved P significantly diminished (Fig. 4c, lower case analysis) and was significantly different from the first two treatments (Fig. 4c, capital letter analysis).

Evaluation of performance of 4 and 10 % (*w*/*v*) alginate beads for tertiary wastewater (P and N removal) in small bioreactors

Removal of P after incubation of wastewater for 24 h was close to zero at both concentrations of empty beads. Adding *C. vulgaris* significantly enhanced removal. Joint immobilization further enhanced removal, where the 10 % bead concentration containing microorganisms significantly removed more P than the 4 % bead concentration (Fig. 5a, capital letter analysis). Compared to incubation after 24 h,

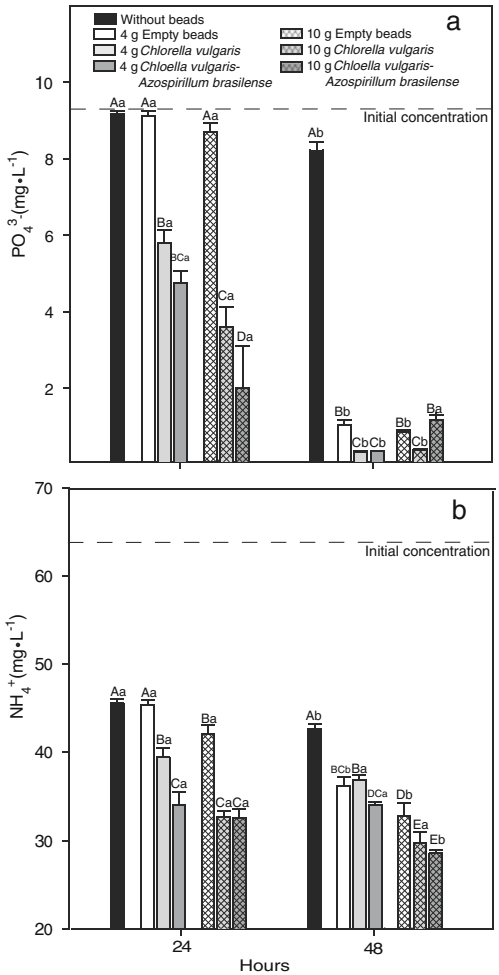

Fig. 5 Performance of 4 and 10 % (*w*/*v*) alginate beads for tertiary wastewater on **a** depleted PO_4^{-3} and **b** depleted NH_4^+ in small bioreactors. Columns for each time interval and in each subfigure, denoted by a *different capital letter*, differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. Columns denoted by a *different lower case letter* comparing each time of incubation (24 vs. 48 h) in each subfigure differ significantly at $P<0.05$ in one-way ANOVA. *Bars* represent the SE. *Absence of bar* means negligible SE

incubation after 48 h of all treatments with beads containing both microorganisms significantly enhanced removal of P over wastewater incubated without any treatment (control), which was up to 90 % removal vs. 15 % removal, respectively (Fig. 5a, lower case analysis).

Removal of ammonium after 24 h was similar to the removal of P, where joint immobilization, either at dosage of 4 or 10 % performed the same significantly greater removal (Fig. 5b, capital letter analysis). After incubation for 48 h, all immobilized treatments, and also empty beads, performed significantly better than wastewater incubated without additional treatment and dosage of 10 % beads performed the best (Fig. 5b, capital letter analysis). Only small differences were observed between *C. vulgaris* immobilized alone or immobilized with *A. brasilense* in either the 4 or 10 % dosage, when each concentration was analyzed separately (Fig. 5b). The amount of ammonium removed was ~50 % with the best treatment.

Tertiary wastewater treatment in triangular airlifting bioreactor

Similar treatments and parameters that were evaluated in small bioreactors were evaluated in 60-L bioreactors containing 25 L of secondary-treated wastewater. Incubating wastewater under aeration removed a low percentage of P after 24 h that somewhat increased after 48 h but was still low (<15 %; Fig. 6a, lower case analysis). After incubation for 24 h, adding empty beads significantly enhanced removal of P, and adding *C. vulgaris* enhanced removal of P even more. Adding *C. vulgaris* and *A. brasilense* was the most efficient treatment (Fig. 6a, capital letter analysis). After incubation for 48 h, all treatments that contained alginate beads were very efficient in removing P, ranging from 85 to 95 % (Fig. 6a, capital letter analysis). Similar phenomenon occurred in removing ammonium, with the exceptions of treatments with *C. vulgaris* alone or jointly immobilized performed at the same level (Fig. 6b, capital letter analysis). Removal by treatments with *C. vulgaris* removed almost all ammonium after 48 h.

The initial bacterial population in secondary-treated domestic wastewater was $4.8 \pm 0.2 \times 10^4$, which slightly increased with incubation time (Fig. 6c, lower case analysis). Adding empty beads significantly increased the population after 24 h but was later reduced to the same level as untreated wastewater (Fig. 6c, capital letter analysis). Adding immobilized *C. vulgaris* and especially the treatment of microalgae with *A. brasilense* continuously and significantly increased the number of bacteria residing in the wastewater (Fig. 6c, capital letter analysis). Analysis of the strength of beads during incubation for 48 h showed a significant decrease in strength after 24 h. The weak beads kept their low strength until the end of the wastewater treatment and did not disintegrate further (Fig. 6d).

Discussion

Biodegradable alginate is the most common polymeric matrix to immobilize microorganisms for many uses (de-Bashan and Bashan 2010). Alginate properties (structure, mechanical strength, porosity, and gelling force) are important variables to consider in selecting alginate for an application (McHugh 1987). It was long suggested in the literature that the frequent degradation of alginate beads during some applications, including wastewater treatment, and the immanent release of the immobilized microorganisms, rendering the process less effective, are because of chemical dissolution of the spheres. This instability of alginate happens when the gels are in contact with cations that serve as chelating agents and anti-gelling cations, such as dissolved phosphorus, EDTA citrates, sodium bicarbonate, and several more (Dainty et al. 1986; Kierstan and Coughlan 1985; Martinsen et al. 1989; Moreira et al. 2006). Intended degradation by microorganisms of alginate beads was demonstrated in soils where they serve as inoculants for plant growth-promoting bacteria (Bashan 1986; Bashan et al. 2002; Power et al. 2011) or in wastewater after prolonged periods or after addition of alginate-degrading bacteria (Vogelsang and Østgaard 1996). Furthermore, it is expected that damage to the polymeric alginate spheres increases in in situ experiments mainly because of the elimination or replacement of the common solidifying cation Ca^{2+} with other cations or anions, such as P^{-3} and Na^{+}, which cause the alginate to have a lower mechanical strength, is a form that is more susceptible to leakage of entrapped cells (Moreira et al. 2006).

Joint immobilization (also known as co-immobilization) of bacteria and microalgae in alginate beads was proposed and demonstrated for tertiary wastewater treatment (de-Bashan et al. 2004; Hernandez et al. 2006; Perez-Garcia et al. 2010). The end goal of our study was to produce more stable alginate beads for application in situ during tertiary wastewater treatment, allowing a demonstration that this proposed technology is feasible and functions in natural wastewater. Proposals for materials for hardening of alginate beads involve adding polyvinyl alcohol (Wang et al. 1995; Chen and Lin 1994), polyvinylpyrrolidone (Doria-Serrano et al. 2001), carboxymethylcellulose (Joo et al. 2001), calcium carbonate ($CaCO_3$), lead, copper, cadmium, barium, and strontium (Gaserød et al. 1999; Smidsrød and Skjåk-Bræk 1990); all, with the exceptions of $CaCO_3$ and strontium, have their use for immobilizing microorganisms largely restricted because of their relative toxicity to the cells (Widerøe and Danielsen 2001). All these additives were previously successful in increasing bead stability. Still, most studies employed sterile or synthetic wastewater for testing. Furthermore, only a few have investigated quantitatively the mechanical stability of alginate (Dainty et al. 1986; Draget et

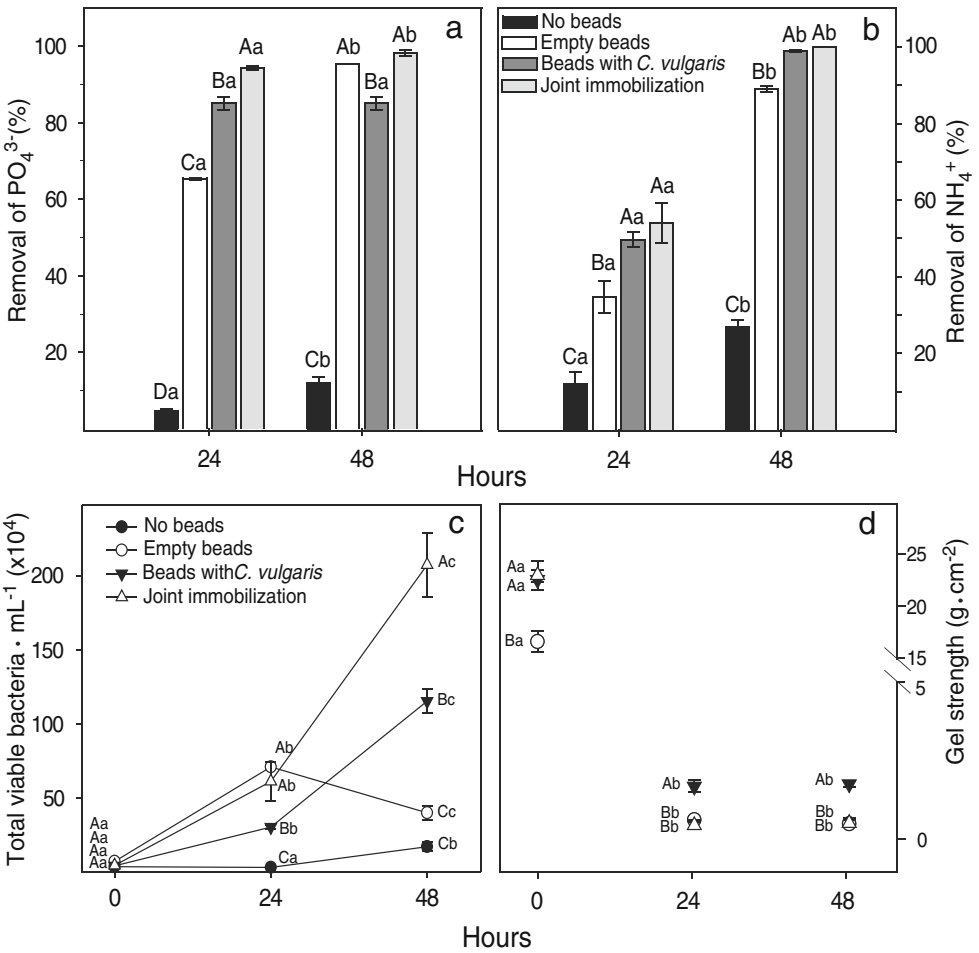

Fig. 6 Tertiary wastewater treatment in 60-L, triangular, airlifting bioreactor containing 25 L wastewater. **a** Depletion of PO_4^{-3}, **b** depletion of NH_4^+, **c** total number of bacteria residing in the wastewater during the wastewater treatment, and **d** gel strength during the wastewater treatment. For **a** and **b**, columns for each time period and in each subfigure, denoted by a *different capital letter*, differ significantly at $P<0.05$ in one-way ANOVA, according to Tukey's post hoc analysis. Columns denoted by a *different lower case letter* comparing each time interval of incubation (24 vs. 48 h) in each subfigure, differ significantly at $P<0.05$ in one-way ANOVA. For **c** and **d**, points on each curve, in each subfigure, 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 interval of incubation differ significantly at $P<0.05$ in one-way ANOVA. *Bars* represent the SE. *Absence of bar* means negligible SE

al. 2006; Moreira et al. 2006). As far as we know, none have studied the stability of the beads during standard hydraulic retention time in real wastewater, and the effect of the native microorganisms residing in the wastewater on stability of the matrix, while evaluating the nutrient removal capacity of the treating agents entrapped within. When six of the above supplements and an additional treatment of increasing alginate concentrations, also recommended as a hardening solution (Martinsen et al. 1989), were tested in natural wastewater, they initially increased gel mechanical strength, but without exception, all failed. The reduction of the gel strength was over 95 % at all concentrations tested, during the retention time needed for standard tertiary wastewater treatment of 24–48 h in this system (de-Bashan et al. 2002b, 2004).

Because all commonly known hardening procedures failed to a great extent, we investigated the reasons for these failures. This was built upon a previous study that showed that, during normal retention time of 48 h, a buildup of layers of bacteria on the outer surface of the alginate beads occurred when these beads are submerged in wastewater (Covarrubias et al. 2012). This study found strong evidence that the microorganisms naturally residing in the wastewater significantly contributed to the failure of all previously proven hardened alginate beads and not the concentration of phosphorus in the wastewater. The evidences that this study provided for this assertions are as follows: (1) no degradation occurred when the wastewater was sterilized, (2) the level of P in wastewater was not enough to dissolve the beads, and (3) relatively significant population of bacteria (>3 %) with capacity to degrade alginate beads, albeit only in the presence of more nutritionally available carbon sources, as frequently occurred in wastewater, were detected. Although degradation of alginate, and especially polymerized alginate, by microorganisms in general, is not easy because of the complexity of the molecule (Tang et al. 2009), these bacteria can degrade the building blocks of the polymer itself by their enzymes when the major growth of their population is supported by other carbon sources. Therefore, the hardening agents that provide extra binding sites to the alginate strands of mannuronic and guluronic acids were probably largely ineffective. Still, despite the significant loss of gel strength and part of the outer layers of the beads, the beads did not lose their general integrity and did not release most of the entrapped microorganisms for at least 48 h, which was enough time for wastewater treatment (discussed later). Similar phenomenon was

observed where Ba^{2+} alginate beads lost their mechanical strength after intentional addition of alginate G-lyase-producing bacterium *Klebsiella pneumoniae* (Vogelsang and Østgaard 1996).

The nine strains of potentially alginate-degrading bacteria that we identified in our study did not have a previous record as alginate-degrading bacteria or were documented to possess alginate lyase, the key enzyme responsible for alginate degradation. Their closest features relevant to wastewater treatment are that members of the genus *Acinetobacter* commonly occur in wastewater treatment, and based on culture media growth, they are the predominant bacteria in the industrial Enhanced Biological Phosphorus Removal (EBPR) process (de-Bashan and Bashan 2004; Mino et al. 1998). *Acinetobacter junii*, for example, is known as a phosphorus-accumulating bacteria in wastewater treatment (Hrenovic et al. 2011), *Pseudomonas* sp., and *Xanthobacter flavus* have been tested for several bioremediation procedures (Song et al. 2003), and *Zoogloea resiniphila* is known to degrade resin in activated sludge and has the ability to degrade and remove contaminants from aerated lagoons treating pulp and paper mill effluents, such as phenols, phosphates, chlorinated solvents, and aromatic hydrocarbons (Yu and Mohn 2002). In our study, one has also to consider that the incubation time of 96 h during which alginate solubilization activity was tested is perhaps too short to observe complete dissolution of the beads, compared to dissolution over a period of 3.5 to 6 weeks of incubation in wastewater (Vogelsang and Østgaard 1996). Still, all bacterial strains survived and maintained their populations, which they could not do without the presence of alginate monomers or alginate beads.

Hydraulic retention time is a primary factor in wastewater treatment and 24 to 48 h of treatment is considered acceptable (Henze et al. 2002; de-Bashan et al. 2004). Consequently, evaluation of tertiary wastewater treatment in increasing volumes of wastewater, but within the time frame of 48 h, was investigated using the original alginate and $CaCl_2$ concentration (2 %) but with increasing quantities of beads (150 % increase, 4 to 10 % *v*/*v*). This technical change significantly improved removal of nutrients. Within only 24 h, the removal of phosphorus and ammonium was higher than removal after 5 days when only 4 % of beads was used (Perez-Garcia et al. 2010) and where the jointly immobilized treatment generally removed more nutrients. The level of removal of phosphorus was exceptionally high, compared to previous studies (de-Bashan et al. 2002b, 2004; Hernandez et al. 2006). All these happened even when the mechanical strength of the gel was reduced to very low levels. This can be explained that even in case of degradation of the outer surface of the beads, demonstrated in this study as a reduction of the diameter of the beads with time, the effect of the microflora in the wastewater on the treating agents was limited. This is related to the porous inner cavity-type structure of alginate beads. Most of the internal cavities are not connected, restricting mobility within the beads (de-Bashan et al. 2011; Lebsky et al. 2001; Nussinovitch 2010). Even if the natural microflora of the wastewater degrades the outer layers of alginate, mobility of the invading organisms inside deeper layers of the bead is still low, allowing sufficient time for the bio-treatment agents to complete tertiary water treatment within 24 h. This theory is strongly supported by evidence of efficient nutrients removal in this study.

When tested in either 1- or 25-L bioreactors, alginate beads were able to remove phosphorus even in the absence of immobilized treatment agents; more phosphorus was detected in the beads themselves, and the concentration of phosphorus in the surrounding water decreased. This can be explained by the nature of wastewater and the way alginate beads treat wastewater. Wastewater, especially after secondary treatment, contains a large assortment of microorganisms potentially capable of degrading and absorbing phosphorus compounds (Bond et al. 1999; Onuki et al. 2002; Seviour et al. 2003; this study). When these microorganisms are freely suspended in the wastewater, their efficiency for nutrient removal is low, as demonstrated in this study and in earlier studies (de-Bashan et al. 2004; Hernandez et al. 2006). This feature notwithstanding, when alginate beads are suspended in wastewater, a thick film of microorganisms is always formed on the surface of the beads (Covarrubias et al. 2012). Biofilms in wastewater are known to improve efficiency of many functional groups of microorganisms and are the fundamental design element of many bioreactors (Bitton 2010). Consequently, it is feasible that the initial low efficiency of phosphorus removal by microorganisms is improved, as shown in this study, when they form biofilms on the surface of the beads. Those biofilms are possibly responsible for this phenomenon. A support for biological removal of phosphorus, not by a direct absorption of phosphorus by the alginate, is that when the microorganisms in wastewater were eliminated by sterilization, the alginate beads remove insignificant amounts of phosphorus from this wastewater.

In summary, this study demonstrated that microorganisms, naturally residing in secondary wastewater effluent, during tertiary wastewater treatment are involved in reducing the mechanical strength of alginate beads containing immobilized treating agents, even after several hardening procedures. Still, the beads survived long enough to allow completion of the removal of phosphorus and ammonium from this municipal wastewater, where higher dosage of beads was more efficient. The contaminants in the wastewater significantly reduced the mechanical strength of the beads. Several potential alginate-degrading bacteria were identified in these wastewaters.

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