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Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense*

Luz E. de-Bashan[a,b], Manuel Moreno[a], Juan-Pablo Hernandez[a], Yoav Bashan[a,*]

[a] *Environmental Biology Center for Biological Research of the Northwest (CIB), P.O. Box 128, La Paz, B.C.S. 23000, Mexico*
[b] *Department of Biology, Pontificia Universidad Javeriana, Santafe de Bogota, Colombia*

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Abstract

Coimmobilization of the freshwater microalga *Chlorella vulgaris* in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense* under semi-continuous synthetic wastewater culture conditions significantly increased the removal of ammonium and soluble phosphorus ions compared to immobilization of the microalgae alone. In continuous or batch cultures removal of these ions followed a similar trend but was less efficient than in semi-continuous culture. It is proposed that coimmobilization of a microalgae with microalgae growth-promoting bacteria can serve as a tool in devising novel wastewater treatments.

Keywords: Ammonium and phosphorus removal; Microbial immobilization; Microalgae; Plant growth-promoting bacteria; Wastewater treatment

1. Introduction

The fresh water unicellular microalga *Chlorella vulgaris* is used for tertiary wastewater treatment mainly for the removal of nitrogen and phosphorus compounds and heavy metals [1–4]. It is also used for several industrial processes unrelated to wastewater treatment [5,6].

The interactions between the microalgae and other microorganisms in its ecological niche or during wastewater treatment have not been well studied. *Pseudomonas diminuta* and *P. vesicularis*, two obligate aerobes isolated from laboratory algal cultures, stimulated the growth of the microalgae *Scenedesmus bicellularis* and *Chlorella* sp., without releasing any growth-promoting substance [7]. Recently, we have shown that coimmobilization of *C. vulgaris* with the plant growth-promoting bacterium (PGPB, [8]) *Azospirillum brasilense*, used as an inoculant in agriculture [9], significantly increased the growth and pigment production of the microalgae [10,11], analogous to the effect of the bacterium on the growth of numerous terrestrial plants [12]. However, coimmobilization of the microalgae with its associative bacterium from the wastewater treatment pond, *Phyllobacterium myrsinacearum*, changed the metabolism of the microalga, but did not enhance its growth; the microalgae senesced and died earlier when associated with *P. myrsinacearum* than when associated with *A. brasilense* [11,13].

The aim of this study was to determine whether the growth-promoting association of the PGPB *A. brasilense* with *C. vulgaris* will also improve ammonium and phosphorus ion removal by the microalgae from batch, continuous and semi-continuous synthetic wastewater cultures. This has been done as a first step toward

*Corresponding author. Fax: +52-112-54710.
E-mail address: bashan@cibnor.mx (Y. Bashan).

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developing treatments for agroindustrial wastewater rich in nitrogen and phosphorus but lacking in other major contaminants [3,14]. In this study, both microorganisms were confined by coimmobilization in small alginate beads, a practical means of using microorganisms for environmental applications [15,16,17].

2. Materials and methods

2.1. Microorganisms and axenic growth conditions

C. vulgaris (UTEX 2714) was isolated from a secondary effluent of a wastewater-treatment stabilization pond near Santafé de Bogotá, Colombia [3]. Before immobilization in alginate beads, *C. vulgaris* was cultivated in sterile mineral medium (C30) as previously described [3] for 5 days. *A. brasilense* was grown in liquid Nutrient Broth (Difco, Detroit, MI) at $30\pm2^{\circ}C$ for 48 h in a rotary shaker. Both organisms were harvested and prepared for coimmobilization as previously described [18,10].

2.2. Artificial wastewater

Artificial, sterile (by autoclaving) wastewater was prepared using the following (mg/L) [10]: NaCl, 7; $CaCl_2$, 4; $MgSO_4 \cdot 7H_2O$, 2; K_2HPO_4, 21.7; KH_2PO_4, 8.5; Na_2HPO_4, 33.4; and NH_4Cl, 3 (in some experiments 3.2–3.4 mg/L were used as specifically indicated). For continuous and semi-continuous cultures, KH_2PO_4, at levels in the range of 12–15 mg/L, was used as the sole phosphorus source.

2.3. Coimmobilization of microalgae and bacteria in alginate beads, bead solubilization, and count of microorganisms within beads

All were prepared as previously described [19,10].

2.4. Continuous, semi-continuous and batch cultures

A chemostat (Virtis, Gardiner, NY) was used for all continuous and semi-continuous culture experiments. Each run of the chemostat was composed of 20 g of microbe-containing alginate beads and 500 mL of solution. Continuous cultures were run for 6 days under the following conditions: $28\pm2^{\circ}C$, 90 rpm with 100% dissolved oxygen, a light intensity of 30 μ mole/m^2/s, and a medium exchange rate of 1.5 mL/h. Semi-continuous cultures were run under the same conditions, but the beads were incubated for 48 h in wastewater medium without replacement of the solution (one cycle). Then, the solution was decanted for analysis and new sterile wastewater solution was added for further incubation. This procedure was repeated for 6 consecutive runs. Batch cultures in Erlenmeyer flasks (500 mL of medium with 20 g of beads) were incubated as previously described [10].

2.5. Ammonium and phosphorus ions analyses

Ammonium and phosphorus ion content was measured using standard water analysis techniques [20] and a Hach DR/2000 spectrophotometer (Hach Co., Loveland, CO, USA). Ammonium was analyzed by the salicylate method, nitrate by the cadmium reduction method, and phosphorus (orthophosphate) by the molibdovanadate method. Kits developed by the Hach Company were used for the specific detection of ammonium and phosphorus ions.

2.6. Experimental design and statistical analysis

A single run of the chemostat (whether in continuous or semi-continuous mode) was considered a single replicate, and each experiment was performed in triplicate. For batch cultures, five replicates were analyzed where each Erlenmeyer flask served as one replicate. Each experiment was repeated at least twice, but usually each experiment was repeated, identically, three times. Controls were prepared similarly but without microorganisms in the beads. Controls having microalga alone immobilized in beads were routinely used. Three 50 mL samples were taken for each water analysis at each sampling time. Results of all repetitions were combined and analyzed together by one-way analysis of variance (ANOVA) at $P\leqslant0.05$ or by Student's *t*-test at $P\leqslant0.05$ using Statistica software (Statsoft, Tulsa, OK). Line fit was done by CurveExpert 1.2 software (Hyams, Central, SC).

3. Results

3.1. Growth of C. vulgaris coimmobilized with A. brasilense within alginate beads

Cultures of *C. vulgaris* coimmobilized with *A. brasilense* in alginate beads grew well, but to different extents, under batch, continuous and semi-continuous conditions (Fig. 1). The best growth was obtained under batch culture conditions, during which the population reached 4×10^6 cells/bead after 6 days of incubation. Under semi-continuous conditions, the growth of the population was somewhat less, reaching 3×10^6 cells/bead. Under continuous culture conditions, growth was the lowest, reaching a population density of only 1.3×10^6 cells/bead. In comparison, when *C. vulgaris* was immobilized alone in continuous culture, a population density of $<1.0\times10^6$ cells/bead was achieved [10].

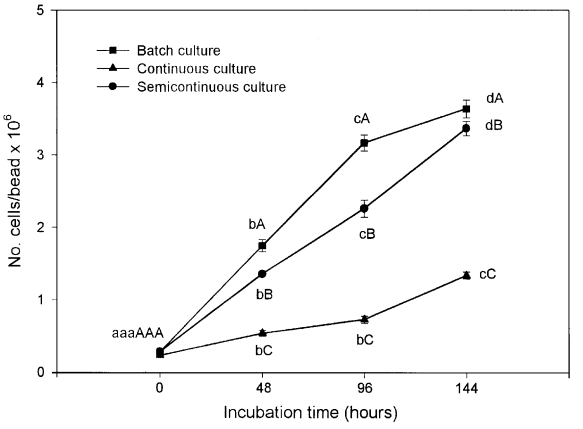

Fig. 1. Growth in synthetic wastewater of *C. vulgaris* coimmobilized with the microalgae growth-promoting bacteria *A. brasilense* in alginate beads, under batch, continuous and semi-continuous conditions. Points on each curve denoted by a different lower case letter differ significantly at $P \leqslant 0.05$ in one-way ANOVA. Points denoted by a different capital letters at each incubation time differ significantly at $P \leqslant 0.05$ in one-way ANOVA. Bars represent the SE. When the SE bar is absent, the SE is smaller than the point.

3.2. Removal of ammonium and phosphorus by C. vulgaris coimmobilized with A. brasilense or immobilized alone under batch culture conditions

Under batch culture conditions, inoculation with coimmobilized microorganisms resulted in the removal of more ammonium and phosphorus from the wastewater than inoculation with immobilized microalgae alone. After 2 days of incubation, 93% of the ammonium was eliminated (Fig. 2A), (from 3 to 0.2 mg/L NH_4^+) in the coimmobilized culture compared to only 53% in the immobilized culture (from 3 to 1.4 mg/L NH_4^+). After 6 days of incubation, 99% of the ammonium was eliminated (Fig. 2A), (from 3 to 0.1 mg/L NH_4^+) in the coimmobilized culture and only 86% was eliminated in the cultures containing microalgae alone (from 3 to 0.4 mg/L NH_4^+).

No phosphorus was removed when the concentration of PO_4^{-3} in the water exceeded 20 mg/L; therefore, this study employed PO_4^{-3} concentrations of 15 mg/L or less. Seventy five percent of the phosphorus was eliminated after 48 h of incubation in the coimmobilized culture (from 12 to 3 mg/L PO_4^{-3}); no further removal was observed after this time. *C. vulgaris* immobilized alone did not remove phosphorus from the wastewater. On the contrary, there was a small accumulation of phosphorus during the incubation period (Fig. 2B).

3.3. Removal of ammonium and phosphorus by C. vulgaris coimmobilized with A. brasilense or immobilized alone in continuous culture

After 48 h of incubation, 91% of the ammonium was removed from the wastewater inoculated with coimmobilized cultures (from 3.2 to 0.3 mg/L NH_4^+) (Fig. 3A) but only 59% of the ammonium was removed from the culture containing microalgae alone (from 3.2 to 1.3 mg/L NH_4^+). Prolonged incubation (more than 48 h) did not result in additional removal of ammonium ions; rather, a small accumulation of ammonium ions was noted in both cultures. There was no depletion of phosphorus from either culture during 72 h of incubation used in this section of the study. The level of phosphorus fluctuated but did not drop below the initial phosphorus level (Fig. 3B).

3.4. Removal of ammonium and phosphorus by C. vulgaris coimmobilized with A. brasilense or immobilized alone in semi-continuous culture

As a result of the previous experiments that showed optimal removal of ions from the wastewater after 48 h of incubation with beads containing microorganisms, the semi-continuous system was designed with cycles of 48 h each. In the microalgae-bacteria coimmobilized cultures, after the first 48 h incubation (cycle no. 1), 100% of the ammonium was removed. This removal rate was maintained for three more cycles of 48 h each. Then, the efficiency of the system decreased, with less ammonium removed during each subsequent cycle, and finally, at the end of the sixth cycle (286 h total incubation time), only 67% of the ammonium was removed (from 3.4 to 0.9 mg/L NH_4^+). Cultures containing the microalgae immobilized alone were less efficient, removing only 85% of the ammonium during the first cycle. In each of the following cycles, even less ammonium was removed—up to 35% of the ammonium added at the beginning of each cycle (from 3.4 to 2.2 mg/L NH_4^+) (Fig. 4A).

Only in the first cycle (48 h) was there significant phosphorus removal (83%) by the coimmobilized cultures (from 15 to 2.5 mg/L PO_4^{-3}) (Fig. 4B). In the second cycle, there was an accumulation of phosphorus for some unknown reason. In later cycles, the system had a limited removal capacity: 33% in the fourth cycle but usually $<14\%$ in other cycles. Cultures of microalgae immobilized alone showed lower phosphorus removal: 33% in the fourth cycle but usually $<20\%$ in other cycles (from 15 to 12 mg/L PO_4^{-3}) (Fig. 4B).

4. Discussion

The use of several species of microalgae as a tertiary wastewater treatment was proposed well over a decade

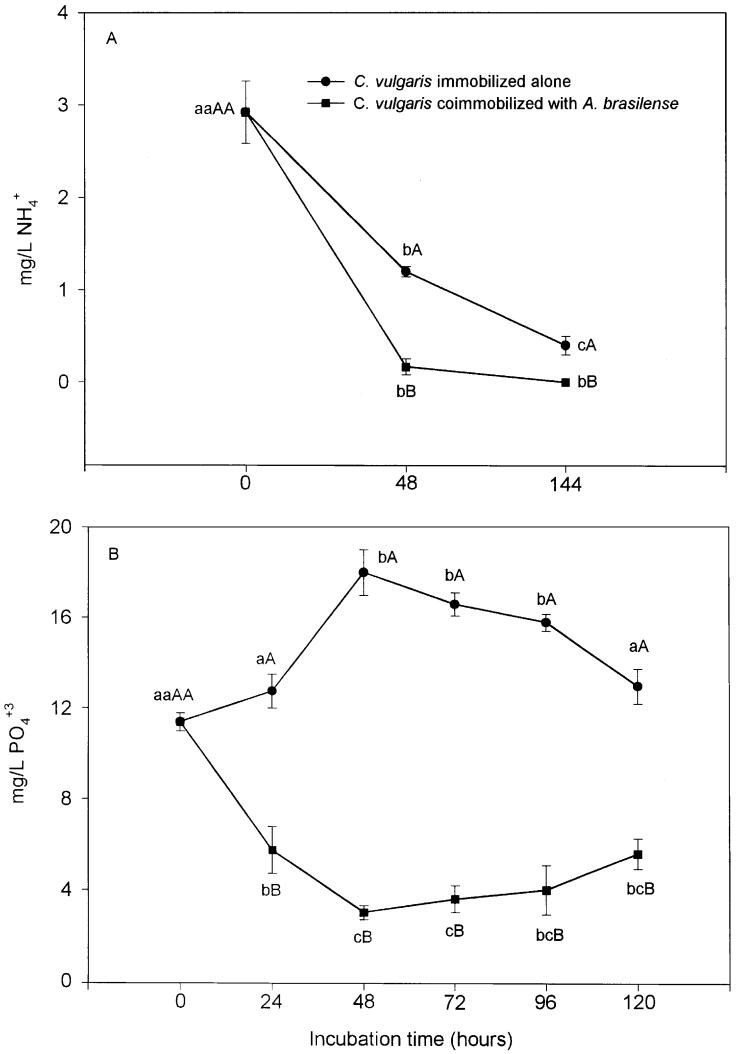

Fig. 2. Ammonium and phosphorus removal by *C. vulgaris* immobilized alone and coimmobilized with *A. brasilense* in batch cultures. (A) Ammonium content of culture (mg/L NH_4^+), (B) phosphorus content of culture (mg/L PO_4^{-3}). Points on each curve denoted by different lowercase letters differ significantly at $P \leqslant 0.05$ in one-way ANOVA. Points denoted by different capital letters after each incubation time differ significantly at $P \leqslant 0.05$ in Student's t test. Bars represent the SE. When the SE bar is absent, the SE is smaller than the point.

ago [21], and various potential treatments continue to be evaluated today [3,22]. The underlying assumption is that the microalgae will transform some of the contaminants to non-hazardous materials and the treated water can then be reused or safely discharged [23]. *C. vulgaris*, immobilized in a polymer matrix, has been proposed for wastewater treatment [24–30,17]. The use of a combination of microalgae with microalgae growth-promoting bacteria has not previously been documented.

Bacteria exclusively associated with unicellular aquatic plants are not well studied [7,13,11]. However, the plant growth-promoting bacterium *A. brasilense*, normally associated with terrestrial plants, had a significant positive effect on the growth of the microalgae when coimmobilized in alginate beads [10]. Therefore, this bacterium can be labeled as a "microalgae growth-promoting bacterium" (MGPB). The aim of this study was to determine whether growth promotion correlates with improved water biotreatment capacity of the microalga, specifically, an improved ability to remove ammonium and phosphorus ions from the wastewater. These ions are major contaminants of agroindustrial wastewater, and are present in large quantities from the dairy industry and from cattle and pig farming [3,14].

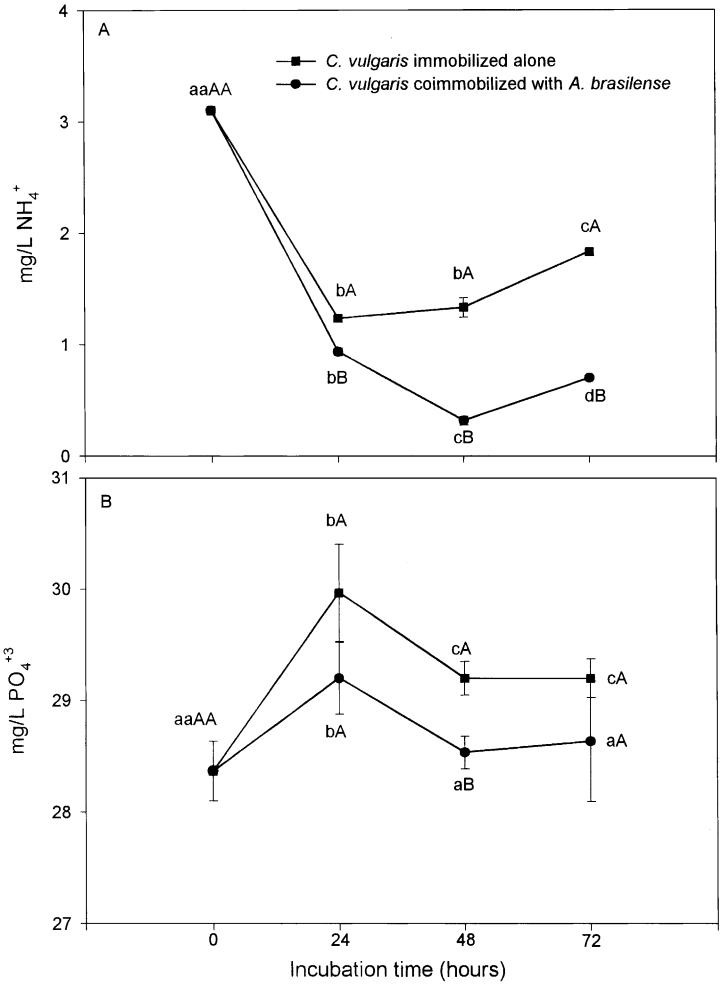

Fig. 3. Ammonium and phosphorus removal by *C. vulgaris* immobilized alone and coimmobilized with *A. brasilense* in continuous cultures for 72 h. (A) Ammonium content of culture (mg/L NH_4^+), (B) phosphorus content of culture (mg/L PO_4^{-3}). Points on each curve denoted by different lower case letters differ significantly at $P \leqslant 0.05$ in one-way ANOVA. Points denoted by different capital letters after each incubation time differ significantly at $P \leqslant 0.05$ in Student's *t* test. Bars represent the SE. When the SE bar is absent, the SE is smaller than the point.

Several methods to treat wastewater with microalgae are possible [21]. These include: (i) batch cultures, where added microbes treat a fixed, but limited, volume of wastewater whether in a fermentor or in a wastewater pond [31,29,30]; (ii) continuous cultures, where a continuous flow of the wastewater is passed through a compartment (fermentor, chemostat, bioreactor, or as part of a constructed wetland) containing the microalgae. Theoretically, this will treat an unlimited volume of wastewater [31,32,27,28]; however, in practice, we found (in this study) that the system becomes saturated after a while depending on the level of contamination and the inherent capacity of the microalgae species to adsorb or transform the contaminants; (iii) semi-continuous cultures, where a volume of wastewater is treated under batch culture conditions for a limited period of time, then the wastewater is replaced but the same microbial culture is used to treat the new volume of wastewater. This can be repeated until the system is saturated; and (iv) re-circulation of wastewater through a number of different treatments with biotreatment agents until the water attains the required cleanliness.

The efficiency of NH_4^+ and PO_4^{-3} removal by the first three techniques using the novel combination of microalgae and bacteria was compared in this study. We chose to use sterile wastewater to avoid possible interference by indigenous bacteria [13]. Natural wastewater contains numerous nitrogen and phosphorus compounds [23,26], which can obscure the measurement of certain compounds during water analysis, thus, defined synthetic

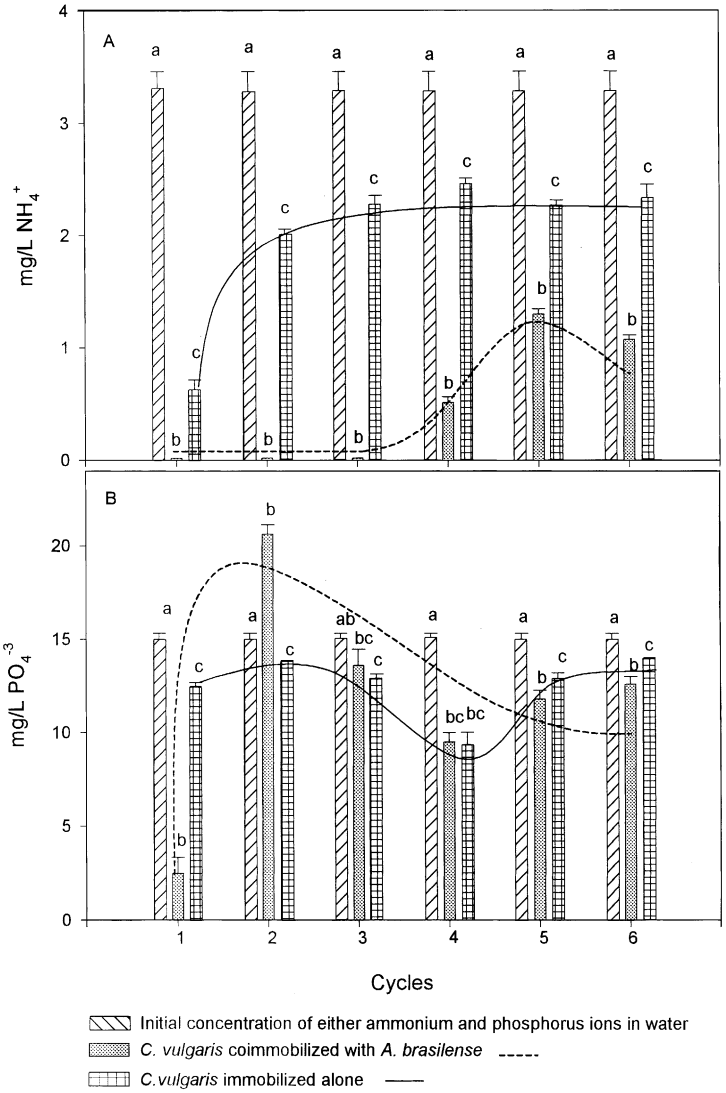

Fig. 4. Ammonium and phosphorus removal by *C. vulgaris* immobilized alone and coimmobilized with *A. brasilense* in semi-continuous cultures for six cycles of 48 h each for a total 286 h. (A) Ammonium content of the culture (mg/L NH_4^+), (B) phosphorus content of the culture (mg/L PO_4^{-3}). Columns in each cycle denoted by different lowercase letters differ significantly at $P \leqslant 0.05$ in one-way ANOVA. Bars represent the SE. When the SE bar is absent, the SE is smaller than the point. Line fit in A—Gompertz relation, $r = 0.995$ (*C. vulgaris* immobilized alone); rational function, $r = 0.99$ (coimmobilization). Line fit in B—Sinusoidal fit, $R = 0.933$ (*C. vulgaris* immobilized alone); rational function, $r = 0.90$ (coimmobilization).

wastewater was used to allow us to follow the disappearance of specific compounds.

This study shows that, in addition to increasing microalgal growth [10], coimmobilization of MGPB and microalga (where both microorganisms are confined to cavities of limited space inside alginate beads, microbial numbers are high, and there is no interference from other microorganisms), can also improve wastewater treatment. The bioremoval of nitrogen and phosphorus was significantly improved when both microorganisms were present compared to removal by the microalgae alone (e.g., for NH_4^+, removal after the fourth cycle of 100% vs. 35%, and for PO_4^{-3}, removal after the first cycle of 83% vs. $<20\%$). Thus, this combination can be useful as a potential tool to develop novel wastewater treatments.

Semi-continuous coimmobilized cultures were superior in removing the nutrients from wastewater compared to continuous or batch cultures. A high level of ammonium removal could be maintained by these cultures for six consecutive cycles; in the initial four cycles all ammonium was removed. The microalgae

immobilized alone removed the nutrients for only three consecutive cycles and at a lower initial removal rate in the first cycle of 85%. Later, the removal rate declined even further to 35%. After these cycles, both systems became saturated and removal of ammonium ions stopped. Phosphorus ions were also removed from the wastewater but to a lesser extent. At high phosphorus levels in the wastewater or under continuous culture conditions, with or without accompanying MGPB in the bead, no phosphorus removal by the microalgae was detected. In batch and semi-continuous cultures, the MGPB–microalga combination was more efficient at removing phosphorus than the microalgae alone but removal was substantial only after the first 48 h period of incubation (the first of six wastewater replacements treated by the microorganisms), reaching 83% removal. In subsequent cycles, the removal rate was reduced. After 96 h of incubation, there was usually an unexplained increase in the phosphorus content of the wastewater, which declined in later cycles. None of the later cycles had the capacity of the first cycle in removing phosphorus.

It is worth mentioning that, while the removal of PO_4^{-3} was significant, the amount remaining in all the performed tests was still high. A future development in the coimmobilized system described in this study should lower phosphate concentration to about 2–3 orders of magnitude before wastewater can be safely discharged into the environment. Microalgae accumulate orthophosphate in the form of polyphosphates. This mechanism increases upon starvation of the cells [33]. Our cultures were not starved before being applied to the wastewater. Therefore, it is feasible that starved cultures may accumulate more phosphate than the level demonstrated in this study. To enhance phosphate removal, one may suggest the use of phosphate-starved microalgae combined with a phosphate starvation period for microalgae that have become *P*-saturated.

It seems that semi-continuous culture conditions offer an advantage over continuous or batch cultures perhaps because of the longer exposure time of the wastewater to the transforming microorganisms. In continuous cultures, replacement of the effluent was apparently too fast to ensure sufficient assimilation of the nutrients; the system, because of the high medium exchange rate, became saturated quickly.

5. Conclusion

This study demonstrates that artificial associations in alginate beads between the microalga *C. vulgaris* and the microalgae growth-promoting bacterium *A. brasilense* (a.k.a. a general plant growth-promoting bacterium) has a potential in treating wastewater containing ammonium and phosphorus as major contaminants.

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