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# Starvation enhances phosphorus removal from wastewater by the microalga *Chlorella* spp. co-immobilized with *Azospirillum brasilense*

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**Abstract**

In synthetic wastewater, growth and phosphorus absorption by two species of microalgae, *Chlorella sorokiniana* and *Chlorella vulgaris*, and in domestic wastewater by *C. sorokiniana* significantly enhanced after a starvation period of 3 days in saline solution, combined with co-immobilization with the microalgae growth-promoting bacterium (MGPB) *Azospirillum brasilense* Cd in alginate beads. Starvation of 5 days negatively affected the subsequent growth of *C. vulgaris*, but not of *C. sorokiniana* in fresh wastewater. Starvation of immobilized cultures of microalgae separately or microalgae with bacteria, followed by returning the immobilized cultures to the same wastewater did not enhance phosphorus absorption. However, a starvation period followed by subsequent submersion of the cultures in fresh wastewater allowed the continuation of phosphorus absorption. The best phosphorus removal treatment from a batch of synthetic or domestic wastewater was with tandem treatments of wastewater treatment with pre-starved, co-immobilized microalgae and replacement of this culture, after one cycle of phosphorus removal, with a new, similarly starved culture. This combination treatment with two cultures was capable of removing up to 72% of phosphorus from the wastewater. There was a direct correlation between the initial load of phosphorus in the domestic wastewater and the efficiency level of removal, being highest at higher phosphorus loads in co-immobilized cultures. This occurred for both immobilized and co-immobilized cultures. Further, the results showed that negative effects of starving the microalgae were mitigated by the application of the MGPB *A. brasilense* Cd. This is the first report of this capacity in *Azospirillum* sp. on a single-cell plant. This study showed that starvation periods, combined with co-immobilization with MGPB, have synergistic effects on absorption of phosphorus from wastewater and merits consideration in designing future biological treatments of wastewater.


*Keywords:* *Azospirillum*; *Chlorella*; Microalgae; Microbial immobilization; Phosphorus removal; Plant growth-promoting bacteria; Starvation; Wastewater treatment

## 1. Introduction

Microalgae *Chlorella* spp. are commonly used for tertiary wastewater biological treatment [1–4]. Although nitrogenous compounds are commonly removed, removal of phosphorus, the second most important nutrient in domestic wastewater, is far less efficient. Seldom is full removal of phosphorus possible by biological processes and usually only a small fraction of phosphorus compounds are removed [5,6].

In recent years, bacteria of the genus *Azospirillum* that are well known as plant growth-promoting bacteria (PGPB) in agriculture for numerous crop plants [7] are considered as a microalga growth-promoting bacterium (MGPB) [8,9]. One common strain, *Azospirillum brasilense* Cd, is also capable of promoting many growth parameters of the unicellular microalgae *Chlorella vulgaris* and *Chlorella sorokiniana* [10], and change the cytology and lipid and pigment production of the microalga [8,11,12]. Additionally, growth promo-

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tion improved capabilities of microalgae to remove nutrients from natural domestic wastewater has been demonstrated [13]. The microbial carrier chosen in these studies were alginate beads. Immobilization of microalgae in polysaccharide gels is an experimental way to use these microorganisms for wastewater treatment [14,15] because it reduces the major difficulty of collecting enormous populations of cells developed during the treatment, which hamper regular microalgae treatments [3,16]. Although the efficiency of removing phosphorus by the co-immobilized microorganisms is increased, it had not reached high values of removal. It was hypothesized that the cells became saturated with phosphorus before all the phosphorus in the wastewater was removed [13].

This study describes various attempts to enhance phosphorus removal from wastewater by adding a starvation period to normally grown cells and to phosphorus-saturated cultures, together with co-immobilization with a MGPB. It was hypothesized that starvation and co-immobilization will allow the microalgal culture to absorb more phosphorus and remove phosphorus from phosphorus-saturated cells. This allows the use of the same co-immobilized culture again. This procedure was demonstrated in domestic, municipal wastewater from the city of La Paz, B.C.S., Mexico and a synthetic wastewater.

## 2. Materials and methods

### 2.1. Microorganisms

Two species of unicellular microalgae, *C. vulgaris* Beijerinck (UTEX 2714, Austin TX) and *C. sorokiniana* Shih. et Krauss (UTEX 1602) were used. The microalgae growth-promoting bacterium *A. brasilense* Cd (DMS 1843, Braunschweig, Germany) was used for co-immobilization experiments with both microalgae species.

### 2.2. Immobilization and starvation procedures

The procedure of co-immobilizing these microorganisms in alginate beads and then growing and counting them in cultures was previously reported [13]. Briefly, the experiments were performed in inverted, 1000 ml conical, glass bioreactors containing 600 ml wastewater, equipped with bottom aeration controlled by a peristaltic pump (3 l air $min^{-1}$ $l^{-1}$) at $26 \pm 2$ °C (equivalent to ambient temperature) with constant illumination of 60 μmol $m^{-2}$ $s^{-1}$ and pH of 6.8–7.0 [13]. *A. brasilense* was cultivated prior to immobilization by standard techniques for this species [17]. Starvation of cultures employed incubating the beads containing the microorganisms (after a single washing with 0.85% NaCl) in a new, sterile preparation of this saline solution at continuous light intensity of 60 μmol $m^{-2}$ $s^{-1}$ at $26 \pm 2$ °C for 3 days. Some experiments employed starvation for 5 days. The starvation periods and the scheme of each type of experiment are described in Table 1.

Table 1
Starvation periods imposed on co-immobilized cultures of *Chlorella* spp. and *A. brasilense*

| Initial treatment | | Starvation period (h) (0.85% NaCl) | Final treatment | |
|---|---|---|---|---|
| Type of water | Time of incubation (h) | | Type of water | Time of incubation (h) |
| | None | 120 | New SW | 240 |
| | None | 72 | New SW | 240 |
| SW | 240 | 72 | New SW | 240 |
| SW | 240 | 72 | The same SW | 240 |
| SW | 48 | 72 | New beads with the same SW | 48 |
| DW | 48 | 72 | New beads with the same DW | 48 |

SW, synthetic wastewater; DW, domestic wastewater.

### 2.3. Synthetic and municipal wastewater source

The composition of synthetic wastewater was previously described [10]. Municipal domestic wastewater was collected periodically for every separate run of the bioreactors at the municipal wastewater treatment plant of the city of La Paz, Baja California Sur, Mexico. Samples were collected from a stream of wastewater after the initial aerobic activated sludge treatment and immediately transferred to the laboratory. If necessary, debris in the wastewater was filtered through a cotton gauze filter in a funnel. Wastewater was used as it arrived from the treatment plant. The levels of NaCl and phosphorus in the wastewater were insufficient to damage the beads. Alternatively, when wastewater arrived from the treatment plant with a low level of phosphorus compounds, the level of P was increased to a level of 20 mg $l^{-1}$ by the addition of (mg $l^{-1}$): $KH_2PO_4$ (21.7), $K_2HPO_4$ (8.5), $Na_2HPO_4$ (33.4) and to 40 mg $l^{-1}$ by $KH_2PO_4$ (43.4), $K_2HPO_4$ (17), $Na_2HPO_4$ (66.8). The arriving wastewater was filtered and sterilized, a process that caused sedimentation of some P. Therefore, after adding the sources of phosphorus listed above, the mixture was sterilized again, losing part of the added P, but reaching the target P concentration in the solution. Wastewater was stored at 4 °C for several days as a precaution following the run of the bioreactors. The composition of the wastewater was previously described [13].

### 2.4. Water analysis of treated wastewater

Standard water analysis technique for phosphorus [18] was performed with a Hach DR/2000 spectrophotometer and Hach kits (Hach Co., Loveland, CO, USA).

### 2.5. Experimental design and statistical analysis

The experiments were performed in the bioreactors as described above. Each experiment was performed in triplicate, where one bioreactor served as a replicate. The experiment used batch cultures. Controls (beads without microorganisms, wastewater alone, and microalgae and bacteria

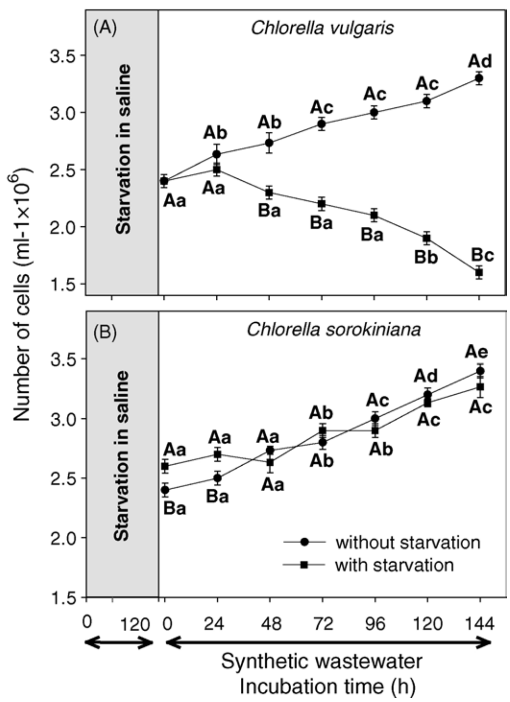


Fig. 1. Growth of *C. vulgaris* (A) and *C. sorokiniana* (B) immobilized separately in synthetic wastewater after starvation period in saline solution for 5 days. Points on curves denoted by a different lower case letter differ significantly by ANOVA at $P \le 0.05$. Points at each time interval denoted by a different capital letter differ significantly by Student's *t*-test at $P \le 0.05$. Bars represent standard error.

alone) were routinely used. Three 50 ml samples were taken for each water analysis at each sampling time. Each experiment was repeated three times. Results were analyzed by ANOVA and Student's *t*-test, with significance at $P \le 0.05$ using software package STATISTICA, Version 6 (StatSoft, Inc., Tulsa, OK, USA). As results were similar, only one representative experiment is presented.

## 3. Results

### 3.1. Effect of starvation period on the population of C. vulgaris and C. sorokiniana and their capacity to remove phosphorus from synthetic wastewater

A starvation period of 5 days irreversibly and negatively affected the population of *C. vulgaris*, which declined for 144 h into the experiment even though it was transferred into a fresh wastewater medium (Fig. 1A). The population of *C. sorokiniana* was unaffected and continued to grow similarly to non-starved culture (Fig. 1B, capital letters in statistical analysis). A starvation period of 3 days allowed *C. vulgaris* to recover part of its population size (Fig. 2A). However, co-immobilization with *A. brasilense* negatively affected the population level similar to a starvation period of 5 days (compare Fig. 2B with Fig. 1A). Three days starvation had only marginal effects on the population of *C. sorokiniana*. The immobilized and co-immobilized populations continued to grow after starvation but at a lower level (Fig. 2C and D).

The pattern of decline of phosphorus in synthetic wastewater differed from the growth pattern of the microalgae. Starved cells of *C. vulgaris* absorbed more phosphorus, but in smaller quantities in a gradual manner than non-starved cells after 48 and 144 of incubation (Fig. 3A). Starved cells of *C. sorokiniana,* on the other hand, absorbed significantly more phosphorus than non-starved cells during the experiment (Fig. 3B). Co-immobilization with *A. brasilense* changed this pattern for *C. vulgaris*. In co-immobilized cultures with *C. vulgaris*, significantly more phosphorus was removed after the first 48 h. The level of removal stabilized thereafter and no additional phosphorus was removed. Co-immobilized, non-starved cultures, although removing less phosphorus during the first 96 h, continued to remove it during the entire 240 h trial and eventually removed more phosphorus than starved cells (Fig. 3C). Non-starved co-immobilized *C. sorokiniana* continuously removed phosphorus similar to *C. vulgaris*, but in lesser quantities (compare Fig. 3C and D). Starved co-immobilized *C. sorokiniana* responded in a similar pattern as starved *C. vulgaris*, but absorbed more phosphorus (Fig. 3D).

Calculating the maximum removal of P from the wastewater showed that immobilization with the MGPB, *A. brasilense*, significantly increased P absorption for both species of microalgae, both as a whole culture and absorption per single cell (Table 2). Starvation increased the P absorption of the culture further, but only for *C. sorokiniana* (Table 2). Starvation significantly increased efficiency of the single cells to absorb P and was in a range of 71–93% regardless of co-immobilization with *A. brasilense* (*C. sorokiniana*) and 12–109% (*C. vulgaris*) where co-immobilization was the major factor (Table 2). Adding beads without microorganisms (control) did not affect phosphorus removal (Fig. 3A and B), while control of *A. brasilense* Cd alone did not remove measurable quantities of phosphorus (data not shown).

### 3.2. Effect of changing the wastewater or the microbial culture on growth and removal of phosphorus by immobilized and co-immobilized cultures of C. sorokiniana subjected to starvation periods

Immobilized cultures of *C. sorokiniana*, after normal growth (Fig. 4A) and subjected to 3 days starvation, declined in population (Fig. 4A). Co-immobilized cultures with *A. brasilense* Cd initially produced a larger microalgal population (Fig. 4A). When these cultures were starved, their populations did not decline further, but rather did not grow, and stayed constant (Fig. 4A). Cultures that were not starved continuously removed phosphorus for 240 h, and co-immobilized cultures removed more (Fig. 4B). Starvation period and returning the cultures to the same wastewater blocked removal of phosphorus by immobilized or co-immobilized *C. sorokiniana* (Fig. 4B).

*C. sorokiniana* cultures (immobilized and co-immobilized, separately) that removed phosphorus in the usual man-

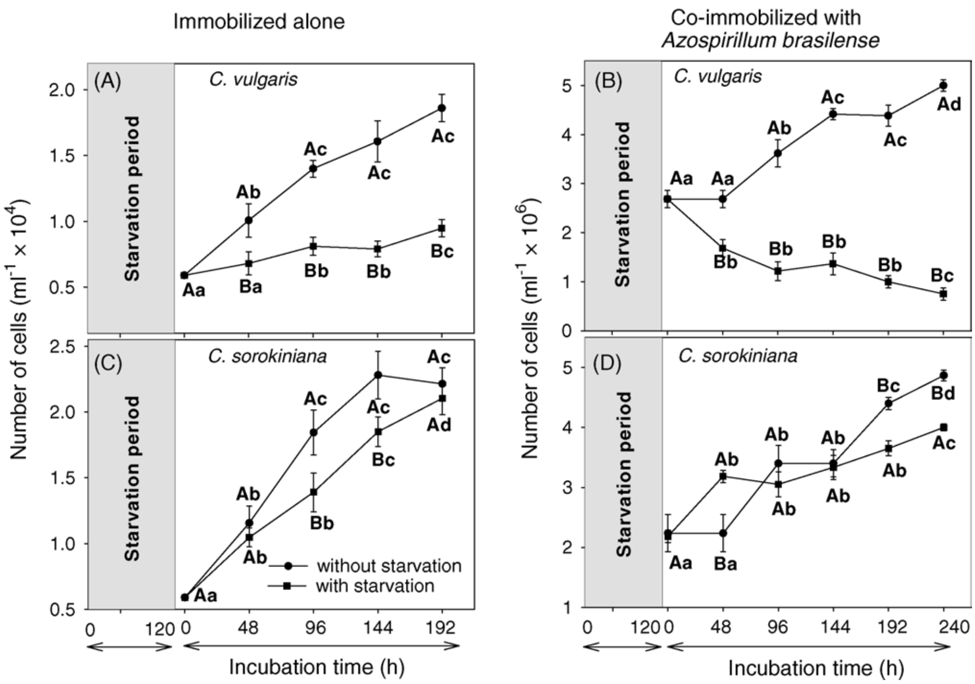


Fig. 2. Growth of *C. vulgaris* and *C. sorokiniana* immobilized separately (A and C) and co-immobilized with *A. brasilense* (B and D) in synthetic wastewater after starvation period in saline solution for 3 days. Points on curves denoted by a different lower case letter differ significantly by ANOVA at $P \leq 0.05$. Points at each time interval denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. Bars represent standard error.

ner (Fig. 5A) were subjected to starvation for 72 h in saline and then were submerged in fresh wastewater. The two types of culture reacted differently to this condition. Immobilized culture continued to remove phosphorus from the new wastewater similar to cultures that were not starved, while co-immobilized cultures remove phosphorus for additional 48 h and then stopped (Fig. 5B). Yet, this removal was greater than removal of phosphorus by immobilized cultures.

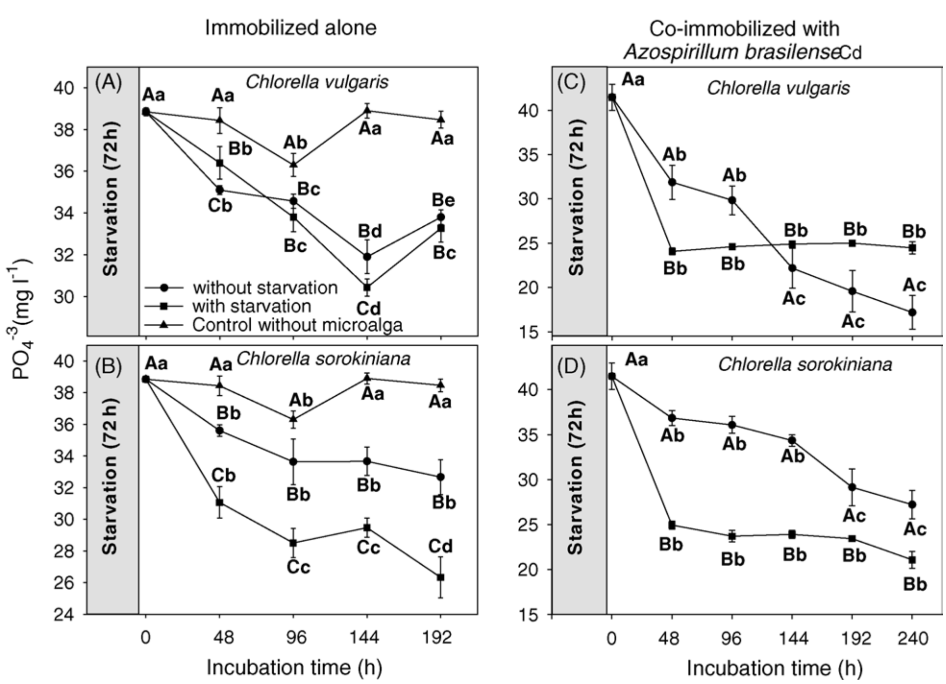


Fig. 3. Removal of phosphorus from synthetic wastewater by *Chlorella* spp. immobilized separately and co-immobilized with *A. brasilense* Cd. *C. vulgaris* (A and C) and *C. sorokiniana* (B and D). Points on curves denoted by a different lower case letter or italics lower case letter, separately, differ significantly by ANOVA at $P \leq 0.05$. Points at each time period denoted by a different capital letter differ significantly by ANOVA at $P \leq 0.05$ (A and B) and Student's *t*-test at $P \leq 0.05$ (C and D). Bars represent standard error. Absence of S.E. means a negligible S.E. *Y*-axis is different in each subfigure to enhance clarity.

Table 2
Calculated maximum removal of phosphorus from synthetic wastewater by *C. sorokiniana* and *C. vulgaris* immobilized alone and co-immobilized with *A. brasilense* Cd under starvation

| Microalgae | Co-immobilization with *A. brasilense* | Time of incubation (h) | Starvation of 72 h in saline | Removal of P from wastewater per | |
|---|---|---|---|---|---|
| | | | | Culture ($mg\,l^{-1}$)[a] | Cell ($\mu g\,cell^{-1}$)[a] |
| *C. sorokiniana* | None | 192 | None | 6.18 ± 0.91 a | 0.03 ± 0.06 a |
| | + | | | 12.33 ± 2.54 b | 2.8 ± 1.8 b |
| | None | | + | 12.52 ± 1.5 b | 0.07 ± 0.06 a |
| | + | | | 18.30 ± 1.51 c | 4.94 ± 0.7 c |
| *C. vulgaris* | None | 144 | None | 6.95 ± 1.02 a | 0.04 ± 0.004 a |
| | + | | | 19.30 ± 2.37 b | 4.37 ± 0.14 c |
| | None | | + | 8.42 ± 0.6 a | 0.01 ± 0.002 a |
| | + | | | 16.57 ± 1.24 b | 0.12 ± 0.069 b |

±, S.E.
[a] Columns denoted by a different (a–c) lower case letter, for each microalgal species, separately, differ significantly by ANOVA at $P \leq 0.05$.

An attempt to enhance phosphorus removal by two successive treatments: first by initial starvation period of the co-immobilized culture, and then by subjecting the same wastewater to a new culture that also passed through a starvation period, significantly enhanced phosphorus removal from synthetic wastewater using co-immobilized cultures. Final phosphorus levels reached $12 \pm 2\,mg\,l^{-1}$ from initial concentration of $38\,mg\,l^{-1}$ in the synthetic wastewater, removal of $69 \pm 2\%$ (Fig. 6A). This double treatment served as a template for evaluating the system with domestic wastewater at three phosphorus loads, low, medium and high. When the initial level of phosphorus in the wastewater was low ($7.2\,mg\,l^{-1}$), the level of phosphorus after 96 h declined to $4.5\,mg\,l^{-1}$, correspond to a removal of 37.5% (Fig. 6B). Increased level of phosphorus in the wastewater change the removal to 45% for the wastewater with $22\,mg\,l^{-1}$ phosphorus ($22–12.3\,mg\,l^{-1}$) (Fig. 6C) and 72% ($45–12.3\,mg\,l^{-1}$) for the wastewater with $45\,mg\,l^{-1}$ phosphorus (Fig. 6D). Although immobilization with *C. sorokiniana* also removed P efficiently, the removal values were always smaller than with co-immobilization (Fig. 6A–D). *C. sorokiniana*, alone and co-immobilized with *A. brasilense*, increased during each cycle, but was significantly higher in co-immobilized culture (Fig. 6E, data shown only for high phosphorus load). There

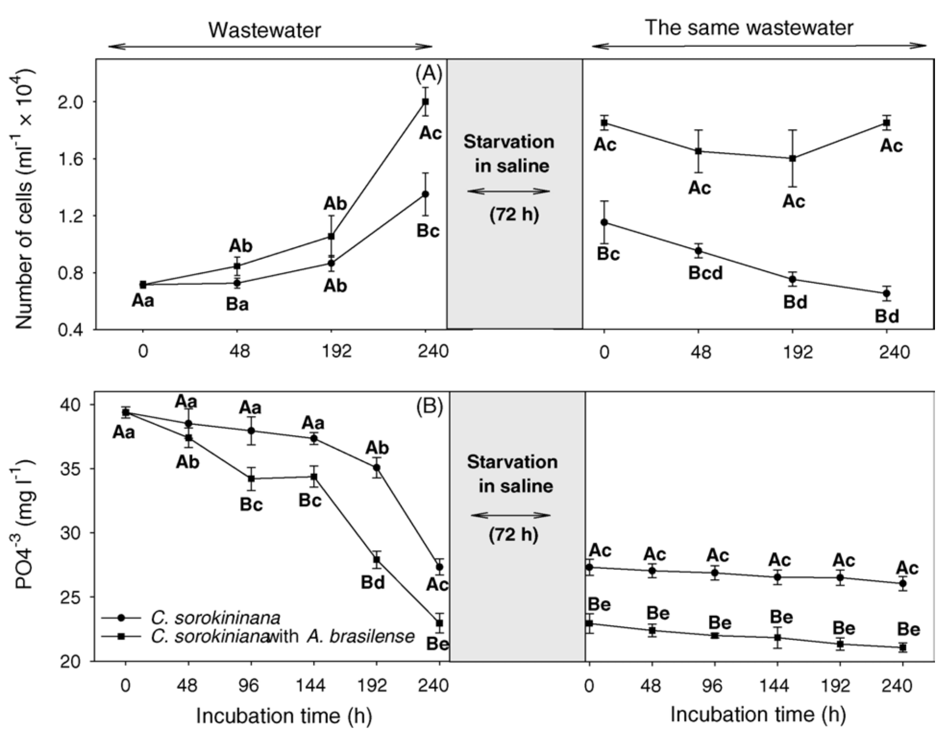


Fig. 4. (A) Growth and (B) removal of phosphorus from synthetic wastewater by *C. sorokiniana* separately and co-immobilized with *A. brasilense* Cd, after a starvation period in saline solution and returning the cultures to the same synthetic wastewater. Points on curves denoted by a different lower case letter differ significantly by ANOVA at $P \leq 0.05$. Points at each time interval denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. Bars represent standard error.

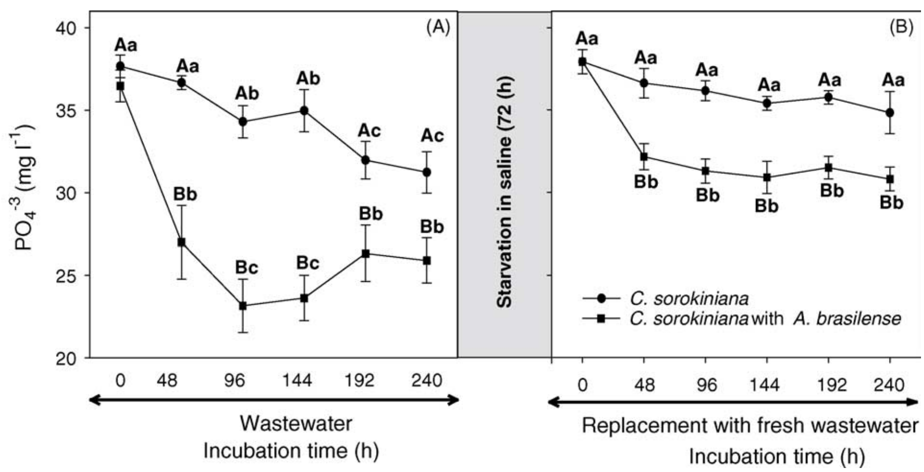


Fig. 5. Removal of phosphorus from synthetic wastewater, by *C. sorokiniana* separately and co-immobilized with *A. brasilense* Cd before (A) and after (B) a starvation period in saline solution and returning the cultures to a fresh synthetic wastewater. Points on curves denoted by a different lower case letter differ significantly by ANOVA at $P \leq 0.05$. Points at each time interval denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. Bars represent standard error.

was a direct correlation between the initial load of P in the domestic wastewater and the level of removal, being higher at higher P load in co-immobilized cultures (Fig. 6F). This occurred for both immobilized and co-immobilized cultures ($r^2$ = 0.93–0.99).

## 4. Discussion

Nitrogen and phosphorus starvation induced significant metabolic and physiological changes on microalgal cell. Starvation of nitrogen nutrients changed the photosynthetic efficiency of *Dunaliella tertiolecta* [19], enhanced adsorption of the cells to polymeric foams and increased N-uptake in *Scenedesmus obliquus* [20], and reduced biomass and growth rates together with increased crude lipid content in *Olva pertusa* [21]. Starvation of phosphorus nutrients increased the population upon re-exposure to wastewater in *Scenedesmus bicellularis* [22], changed the composition of fatty acids in the cells in *O. pertusa* [21] and temporarily enhanced P-uptake in *Scenedesmus quadricauda* [23]. During a period of phosphate starvation, the phosphate content of cells of *C. vulgaris* decreased especially inorganic polyphosphate [24], and several metabolic parameters were changed during phosphorus starvation in *Chlorella kessleri* [25].

So far, there is almost no report on complete removal of $PO_4^{3-}$, the main form of inorganic phosphorus used by algae, from domestic wastewater by biological wastewater treatment with in vitro small-scale experiments [5,26]. The microalgal cells apparently became saturated fast with $PO_4^{3-}$ [4,13,26], probably due to accumulation of polyphosphates [27]. Furthermore, the preparation of co-immobilized cultures has a cost. Therefore, our working hypothesis to overcome these deficiencies were to subject the co-immobilized cultures to starvation periods aiming to increase $PO_4^{3-}$ absorption by the cells, and re-use of the same culture on a fresh wastewater upon "discharging" phosphorus from saturated cultures by starvation periods.

Starvation of immobilized microalga cultures differed in its effect between the two species of microalgae used concerning growth and their phosphorus absorption. In general, any starvation period declined the population of *C. vulgaris*. The 5 days starvation period had a detrimental and irreversible effect on the population. This resembled the effect of starvation on *S. bicellularis* [16]. However, the populations of both *Chlorella* spp. recovered after a shorter starvation period of 3 days, where *S. bicellularis* could not. On the other hand, the population growth of *C. sorokiniana* was less affected by starvation. Co-immobilization with the MGPB *A. brasilense,* that usually enhances growth of *Chlorella* spp. under normal growth conditions [10], had no positive effect on microalgae growth when the microalgae was treated to a short starvation period. For *C. vulgaris,* even a negative effect was present in the developing populations.

Starvation changed the pattern of $PO_4^{3-}$ absorption. In microalgae that were immobilized alone, $PO_4^{3-}$ absorption resembled known $PO_4^{3-}$ absorption of these cells [13,28]. However, starvation combined with co-immobilization with the MGPB *A. brasilense* Cd significantly increased $PO_4^{3-}$ absorption, being strongest during the first cycle of growth in the wastewater. This pattern resembled $PO_4^{3-}$ uptake by *S. quadricauda* grown alone in batch cultures [23].

Manipulating starvation periods, changes of wastewater, and changes of immobilized cultures treating the same wastewater all had effects on $PO_4^{3-}$ absorption and eventual removal from the wastewater. This study demonstrates that there is a synergism in $PO_4^{3-}$ absorption, at the culture and the single cell level, between co-immobilization of microalgae and imposed starvation. This combination significantly increased $PO_4^{3-}$ absorption in *C. sorokiniana*. The most successful treatment, in both synthetic wastewater and domestic wastewater, was enhanced phosphorus removal by subjecting

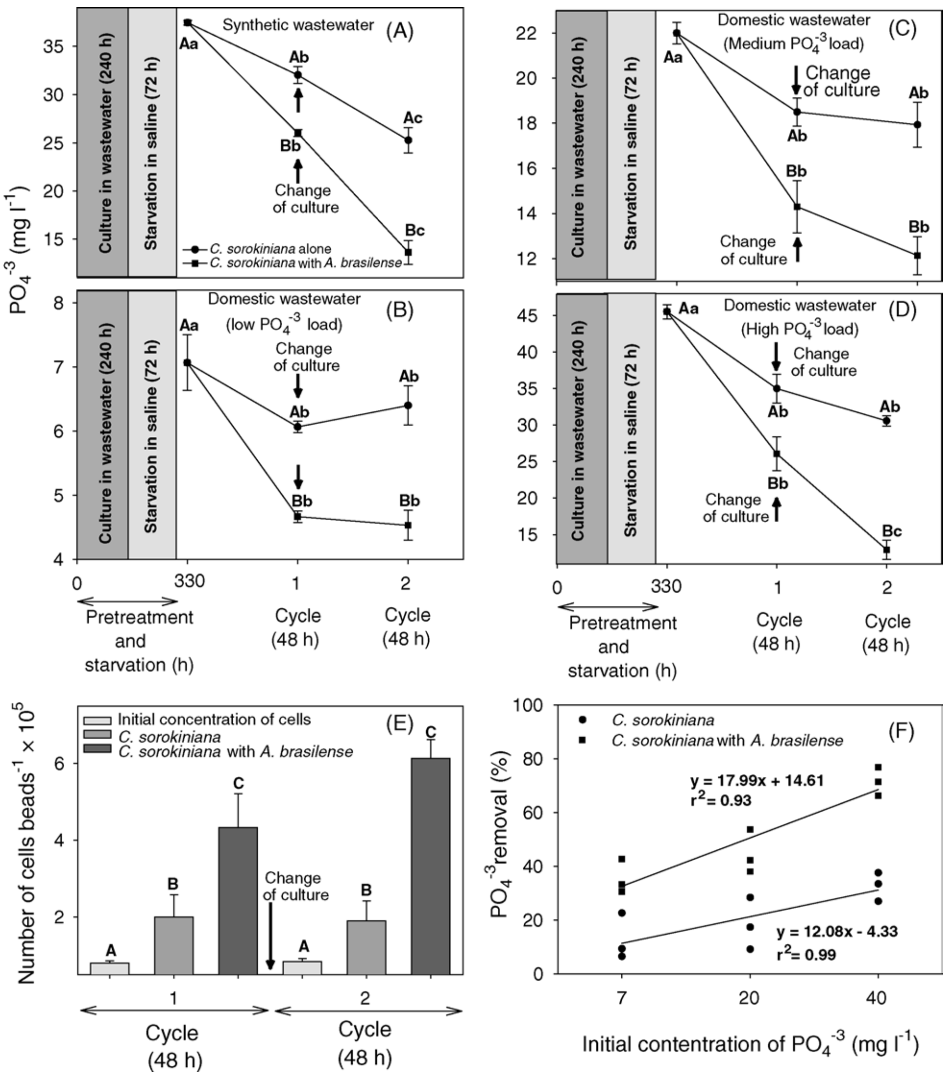


Fig. 6. Removal of phosphorus from synthetic (A) and three different P concentrations in municipal wastewater, low (B), medium (C) and high (D) by *C. sorokiniana* separately and co-immobilized with *A. brasilense* Cd. The treatment followed the incubation scheme: culturing in wastewater, starvation, change to fresh wastewater for one cycle, and then subjecting the same wastewater to a different immobilized starved culture. Points on curves denoted by a different lower case letter differ significantly by ANOVA at $P \leq 0.05$. Points at each time interval denoted by a different capital letter differ significantly with Student's *t*-test at $P \leq 0.05$. (E) Population levels of *C. sorokiniana* in the two cycles described in (D). Columns in each cycle denoted by a different capital letter differ significantly by ANOVA at $P \leq 0.05$. Bars represent standard error. (F) Linear regression analyses between the initial P concentration in the wastewater and the percentage of removal achieved with this treatment. Absence of S.E. means a negligible S.E.

the same wastewater to two different co-immobilized cultures of *C. sorokiniana*, each culture subjected to a starvation period in tandem. This removed 69% of $PO_4^{3-}$ from the synthetic wastewater and 37.5% from domestic wastewater with low $PO_4^{3-}$ loads, and up to 72% from domestic wastewater with high $PO_4^{3-}$ concentrations. These results demonstrate significant improvement of absorption of phosphorus from authentic wastewater, comparable to or better than previous studies that demonstrated uptake efficiency of 44 [26] and 22% for *C. vulgaris*, and 36% for *C. sorokiniana* [13]. Additionally, this study demonstrated that phosphorus is removed from wastewater in a linear way based on the initial concentration of phosphorus in the domestic wastewater. Removal declines with time and with the reduction of phosphorus in the wastewater. The low efficiency of phosphorus uptake at low phosphorus concentration was demonstrated in earlier in vitro trials, but on a very small-scale [26].

Indigenous microflora of the domestic wastewater was not analyzed in detail in this study. There are always a large number of bacteria in wastewater samples. However, it was previously shown that several other semi-continuous treatment schemes did not yield significant removal of nutrients by the native microflora [13]. Additionally, the short time of the experiments (only two cycles of 48 h each) was insufficient to induce destabilization of the alginate beads by the presence of phosphorus ions.

It appears that the negative effects of starvation on growth and phosphorus absorption were mitigated by the application of the MGPB *A. brasilense* Cd, except in severe starvation episodes lasting 5 days. This agrees with the known capacity of *Azospirillum* spp. to mitigated several other environmental stresses on higher plants, such as drought [29,30], salt [31–33] and humic acids [34]. This is the first report that this beneficial capacity of *Azospirillum* sp. was demonstrated on single-cell microalgae.

## 5. Conclusions

Starvation periods (3 or 5 days in saline solution) significantly affected the growth and phosphorus absorbing capabilities of two species of microalgae, *C. vulgaris* and *C. sorokiniana*. Starvation enhanced $PO_4^{-3}$ absorption by *C. sorokiniana* and co-immobilization with the MGPB *A. brasilense* further enhanced $PO_4^{3-}$ absorption. Increased efficiency in removing $PO_4^{3-}$ from wastewater can be accomplished by a combination of starvation, co-immobilization with MGPB, and changing the cultures that treat wastewater.

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