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## Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater

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### ABSTRACT

Two species of microalgae growing as immobilized and free-cells were compared to test its ability to remove N and P in batch cultures of urban wastewater. The best microalgae-cell growth configuration was selected to be tested in bioreactor operated in semi-continuous mode. *Scenedesmus obliquus* showed a higher N and P uptake rate in urban wastewater than *Chlorella vulgaris*. When tested in semi-continuous mode and with the re-calcification of beads, *S. obliquus* was more effective in removing N and P for longer periods (181 h) than batch cultures; fecal coliforms removal was good (95%) although the final concentration was still unsuitable for discharge to natural water bodies. Protein and lipids content analysis suggest that, from a practical point of view, immobilized systems could facilitate the separation of the biomass from the treated wastewater although in terms of nutritional value of the biomass, immobilized systems do not represent an advantage over free-cell systems.

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### 1. Introduction

Secondary effluents from wastewater treatment plants contain nutrients ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ ) which have been identified as the main causes leading to eutrophication in natural waters. Therefore, the wastewater must receive suitable treatment before being discharged into water bodies. Several types of unit processes exist for the removal of nutrients from wastewater but these are costly and produce high sludge content. Microalgae have been proposed as an alternative biological treatment to remove nutrients (Mallick, 2002). One of the limitations for the development of wastewater treatment systems based on microalgae is the harvest of the biomass at the end of the treatment process.

However, the immobilization of cells can represent an alternative for solving the problem as well as providing advantages such as an increase in the cell retention time within bioreactors and higher metabolic activity (Tam et al., 1994). Calcium alginate is commonly used for immobilizing microalgae and maintains the high viability of cells for extended periods of time. However, the matrix is vulnerable to the presence of chelating agents present in wastewater, such as phosphate and citrate, which affect the strength of the gel matrix and, ultimately, dissolves it (Jimenez-Perez et al., 2004). Nevertheless, this problem can be resolved with

the re-calcification of alginate beads (Smidsrod and Skjak-Braek, 1990).

Studies on nutrient removal from urban wastewater by immobilized microalgae are limited and include studies on *Chlorella* immobilized in alginate (Lau et al., 1997); *Scenedesmus obliquus* immobilized in k-carrageenan (Chevalier and De la Noüe, 1985a) and *Scenedesmus intermedius* immobilized in calcium alginate (Jimenez-Perez et al., 2004). These studies have evaluated only the quality of the final effluent, and few have determined the nitrogen incorporation efficiency as protein, as well as the lipid content. Immobilized cells could be used as animal feed or as source of high-value chemicals if the biomass is harvest, along with nutrient removal from wastewater (Nuñez et al., 2001).

Therefore, in the present study the growth rates of *Chlorella vulgaris* and *S. obliquus* on urban wastewater were determined; their N- $\text{NH}_4$  and P- $\text{PO}_4$  removal capacity in free-cells and immobilized cells reactors and the protein and lipid content of each species was analyzed. The feasibility of semi-continuous cultures using immobilized cells in alginate beads with re-calcification to prolong the stability of the matrix was also evaluated.

### 2. Methods

#### 2.1. Routine algal culture and acclimatization

Two species of microalgae were used: *S. obliquus*, isolated from a hypereutrophic soil and *C. vulgaris*, isolated from agricultural soil,

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both maintained as strains in the culture collection of the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE). For initial experiments, artificial wastewater with the following composition was prepared ( $\text{mg L}^{-1}$ ): NaCl, 7 mg;  $\text{CaCl}_2$ , 4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2;  $\text{KH}_2\text{PO}_4$ , 15 and  $\text{NH}_4\text{Cl}$ , 115. These concentrations were used simulating the mean values of the secondary effluent from the Universidad Autónoma de Baja California (UABC) wastewater treatment plant:  $\text{N-NH}_4^+$ :  $32.5 \text{ mg L}^{-1}$ ;  $\text{N-NO}_3^-$ :  $2.0 \text{ mg L}^{-1}$  and  $\text{P-PO}_4^{3-}$ :  $2.5 \text{ mg L}^{-1}$ . Trace metals and vitamins were added following guidelines for “f/2” medium preparation (Guillard and Ryther, 1962). During acclimatization (1 month), the microalgae were transferred to fresh artificial wastewater every seven days. The artificial wastewater was only used for the acclimatization of cells and for direct comparison with real urban wastewater. For all other experiments, real secondary effluent from the UABC's wastewater treatment was used; due to its nature, there was large variation in the concentration of nutrients so percentages of removal were used to determine removal efficiency.

## 2.2. Preparation of immobilized algal beads

Once the microalgae were acclimatized, algal cells were harvested by centrifugation at 3500 rpm for 10 min. The cells were resuspended in 50 mL of distilled water to form a concentrated algal suspension with a cell density of  $10 \times 10^7 \text{ cells mL}^{-1}$ . The algal suspension was then mixed with a 4% sodium alginate solution in 1:1 volume ratio to obtain a mixture of 2% algae–alginate suspension. The mixture was transferred to a 50 mL burette and drops were formed when “titrated” into a calcium chloride solution (2%). This method produced approximately 6500 uniform algal beads of approximately 2.5 mm diameter with an initial cells number of  $3.5 \times 10^5 \text{ cells bead}^{-1}$  for every 100 mL of the algae–alginate mixture. The beads were kept for hardening in the  $\text{CaCl}_2$  solution for 4 h at  $25 \pm 2 \text{ }^\circ\text{C}$ , then rinsed with sterile saline solution (0.85% NaCl) and subsequently with distilled water. A concentration of 2.6 beads per ml of wastewater (equivalent to 1:25 bead:wastewater v/v) were placed in bioreactors made of transparent polyethylene terephthalate (PETE) containing 2.5 L of artificial wastewater or urban wastewater. The approximate volume of each bead was 0.01538 mL.

## 2.3. Batch cultures

Stock suspensions of *C. vulgaris* and *S. obliquus* were cultivated in 3 L bioreactors containing 2.5 L of artificial wastewater at  $25 \pm 1 \text{ }^\circ\text{C}$  and light intensity of  $135 \mu\text{E m}^{-2} \text{ s}^{-1}$ . The bioreactors were aerated to keep free-cells and immobilized beads in suspension and in completely mixed conditions. The following configurations were set up: free-cells in artificial wastewater (AWF); free-cells in urban wastewater (UWF); immobilized cells in artificial wastewater (AWI); immobilized cells in urban wastewater (UWI); a control (urban wastewater without microalgae). All experiments were run three times.

At 6 h intervals, 150 mL of wastewater were collected for analysis during 48 h runs.  $\text{N-NH}_4$ ,  $\text{N-NO}_3$ ,  $\text{P-PO}_4$  and pH concentrations were determined according to standard methods (APHA, 1995). The number of algal cells in the beads were determined with particle counter (Beckman Coulter Multisizer 3) after dissolving one bead in (0.25 M)  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  solution (pH 7.0) and 0.5 mL of lugol. The determination of chlorophyll *a* in free and immobilized cells was carried out according to Parsons et al. (1984). The cultures were filtered through a glass fiber filter (Osmonics GF/C) of 2.4 cm in diameter. Filters were plunged into tubes containing 5 mL of 90% acetone and kept in darkness at  $4 \text{ }^\circ\text{C}$  during 24 h for pigment extraction; subsequently, chlorophyll *a* concentration was determined spectrophotometrically.

For the lipids and protein analysis (expressed in percentage based on the dry organic weight), five alginate beads were dissolved and subsequently filtered through a GF-C glass fiber filter (2.5 cm diameter), previously rinsed with distilled water, and incinerated at  $470 \text{ }^\circ\text{C}$  for 4 h. After filtering, the samples were stored at  $-20 \text{ }^\circ\text{C}$  as recommend by Cordero-Esquivel et al. (1993) and proteins and lipids extraction was done directly on this algal concentrate obtained from filtration. Proteins were extracted with NaOH, as proposed by Correa-Reyes (1996) and then analyzed according to Lowry et al. (1951) with bovine albumin (98%) as standard. Lipids were extracted following the methodology of Bligh and Dyer (1959) and their quantification was estimated by the method of Pande et al. (1963) using tripalmitin (99%) as standard. For the determination of ash-free dry weights, five beads were dissolved and filtered through a GF-C glass fiber filter as previously described. The samples were dried at  $120 \text{ }^\circ\text{C}$  and put to constant weight for 2 h in a conventional oven and then in a muffle furnace at  $450 \text{ }^\circ\text{C}$  for 3 h.

## 2.4. Semi-continuous cultures

Once the best microalgae-growth configuration for urban wastewater treatment was established, this was chosen to be used in bioreactors running under the same conditions as batch cultures but in semi-continuous mode. Moreover, photosynthesis–irradiance curves suggested an optimum light intensity of  $200 \mu\text{E m}^{-2} \text{ s}^{-1}$  to be used. The beads were initially incubated for 48 h in wastewater without solution replacement (pre-cycle). Later the solution was decanted for analysis and fresh wastewater was added for further incubation for 35 h (subsequent cycles). This procedure was repeated for six consecutive runs and samples were collected every 24 h and at the end of each cycle (35 h) and analyzed for nutrients, chlorophyll, protein and lipids content as explained earlier. Two series of reactors were set, one with artificial wastewater (AWI) and another with urban wastewater (UWI). Experiments were run three times.

## 3. Statistical analyses

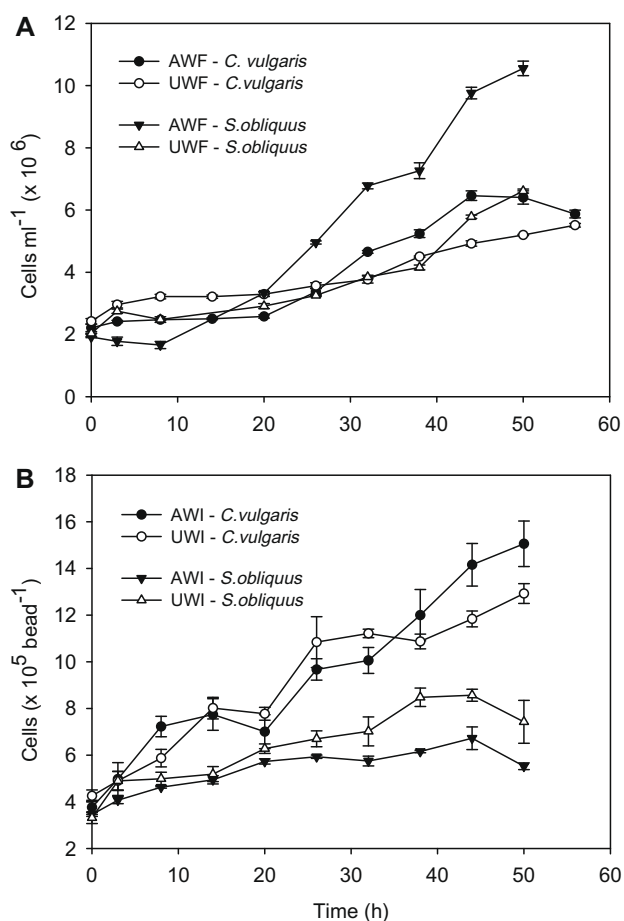
For all statistical analyses, STATISTICA 5.0 software was used. The mean, confidence interval and standard deviation values of the triplicates for each treatment were calculated. The effects caused by two substrates (artificial wastewater and urban wastewater) on the growth of both types of microalgae and nutrients removal cultivated in free and immobilized state were evaluated by analysis of covariance (ANCOVA) at  $P \leq 0.05$ . The Tukey Test at  $P \leq 0.05$  was applied when results showed significant differences.

## 4. Results and discussion

### 4.1. Algal growth under batch culture conditions

Free and immobilized cells growth for both types of microalgae followed an exponential model (Fig. 1A and B). The lag phase in free-cell cultures was shorter for *S. obliquus* (8 h) than *C. vulgaris* (20 h) for both AWF and UWF (Fig. 1A), suggesting that *S. obliquus* had a greater adaptation and viability than *C. vulgaris* in urban wastewater, as reported by Martinez et al. (2000). In the present study, both species of microalgae immobilized in alginate showed growth immediately after the beads were added in the medium (Fig. 1B) unlike other studies where immobilized cells showed a longer lag period compared with free-cells (Chevalier and De la Noüe, 1985a; Lau et al., 1997).

Growth rates for free-cell cultures of *C. vulgaris* cultivated in AWF and UWF did not show significant differences ( $P = 0.975$ )



**Fig. 1.** Growth curves (mean  $\pm$  standard error) of free (A) and immobilized (B) batch cultures of *C. vulgaris* and *S. obliquus* (AWF, artificial wastewater free-cells; UWF, urban wastewater free-cells; AWI, artificial wastewater immobilized cells; UWI, urban wastewater immobilized cells).

nor did *S. obliquus* ( $P = 0.731$ ). *C. vulgaris* and *S. obliquus* presented higher growth rates in AWF ( $0.377 \text{ d}^{-1}$  and  $0.401 \text{ d}^{-1}$ , respectively) compared to UWF ( $0.186 \text{ d}^{-1}$  and  $0.285 \text{ d}^{-1}$ , respectively). The growth rate can vary depending on culture conditions (temperature, medium composition) which seemed to be less favorable in UWF than in AWF. Similar results observed by Lau et al. (1995) suggested that the presence of indigenous bacteria and protozoa in the wastewater and the different chemical composition between urban wastewater and artificial wastewater interferes with microalgae growth (Martinez et al., 2000).

The concentration of *C. vulgaris* cells obtained in AWF and UWF cultures did not show significant differences ( $P = 0.583$ ), being similar for both cultures and reaching a maximum concentration of  $6.4 \times 10^6$  and  $5.3 \times 10^6$  cells  $\text{ml}^{-1}$ , respectively. The increase in cells concentration for *S. obliquus* did show significant differences ( $P = 0.0001$ ) when cultivated in both types of cultures, reaching a concentration of  $9.76 \times 10^6$  cells  $\text{ml}^{-1}$  in AWF in comparison to  $5.8 \times 10^6$  cells  $\text{ml}^{-1}$  in UWF after 48 h. The increase in cell numbers indicated that the algal cells were able to undergo cell division when cultivated in urban wastewater (Fig. 1A) and that *S. obliquus* had a higher capacity to grow in urban wastewater than *C. vulgaris*.

Growth rates in experiments with alginate-immobilized *S. obliquus* did not show significant differences ( $P = 0.836$ ). The growth rates ( $0.110$  and  $0.157 \text{ d}^{-1}$ ) were similar for AWI and UWI, respectively, and smaller than those reported by Jimenez-Perez et al. (2004) for immobilized *S. obliquus* ( $0.264 \text{ d}^{-1}$ ). Similarly, *C. vulgaris* cultures did not show significant differences ( $P = 0.404$ ) in UWI and AWI ( $0.183 \text{ d}^{-1}$  and  $0.195 \text{ d}^{-1}$ , respectively), also smaller than re-

sults presented by Jeanfils et al. (1993) for *C. vulgaris* in artificial wastewater ( $0.360 \text{ d}^{-1}$ ).

The concentration of *C. vulgaris* cells within beads growing in AWI and UWI did not show significant differences ( $P = 0.916$ ). The maximum concentration reached after 48 h was  $15 \times 10^5$  cells  $\text{bead}^{-1}$  and  $13 \times 10^5$  cells  $\text{bead}^{-1}$  for AWI and UWI, respectively. Similar observation were made for *S. obliquus* where cells concentration showed no significant difference ( $P = 0.953$ ) and reached a maximum at 48 h of  $6.7 \times 10^5$  and  $8.5 \times 10^5$  cells  $\text{bead}^{-1}$  in AWI and UWI, respectively. Thus, cell densities for *S. obliquus* were smaller than densities reached for *C. vulgaris* (Fig. 1B). The disparity in cell size could explain such difference. Both types of microalgae presented a smaller cell size ( $2.7 \mu\text{m}$  for *C. vulgaris* and  $4.2 \mu\text{m}$  for *S. obliquus*) after immobilization suggesting that smaller size cells could have a greater chance to occupy homogeneously the space within the gel matrix (Tam et al., 1994; Lau et al., 1997) and, thus, present a greater cell density per bead. These results are in disagreement with Bailliez et al. (1985) who reported that immobilized *Botryococcus* in alginate had a size 2.5 larger than free-cells. Trevan and Mak (1988) also reported larger cells of *Chlorella* when immobilized compared to its size as free-cells.

The smaller size meant that *C. vulgaris* and *S. obliquus* could still grow within the matrix as greater growth near the surface of the beads were detected by direct microscopic observation of beads, as reported also by Pane et al. (1998) and Jimenez-Perez et al. (2004). Studies by Chevalier and De la Noüe (1985b) and Uemoto and Saiki (2000) have suggested that cells near the surface of the beads have a high activity due to a better contact with the substrates and light than cells located in the centre of beads limiting, thus, their normal physiological functions.

Overall, the larger number of cells at the surface of the beads caused a greater self-shading effect to the cells inside the beads, limiting its growth but not pigment production, as observed by Chevalier and de la Noüe (1985a) and Lau et al., 1997. Higher chlorophyll *a* content for immobilized *C. vulgaris* ( $0.45 \text{ pg cell}^{-1}$ ) and *S. obliquus* ( $2.21 \text{ pg cell}^{-1}$ ) were observed than those for free cells ( $0.0056$ – $0.0057 \text{ pg cells}^{-1}$ ) suggesting that immobilized cells were physiologically active in compensation to self-shading effect.

The increase of cells and chlorophyll *a* content showed that both algae after immobilization were still able to undergo cell division and carry out photosynthesis, as previously reported for alginate-immobilized *C. vulgaris* (Tam and Wong, 2000) and confirming that alginate-immobilized cells retained the same catalytic activity, viability and growth rate as the free cells (Robinson et al., 1986).

#### 4.2. Removal of N under batch culture conditions

As a result of the nature of the urban wastewater used, there were large variations in the concentration of  $\text{NH}_4^+\text{-N}$  ( $34$ – $48 \text{ mg L}^{-1}$ ) in the wastewater used for the experiments. Therefore, removal percentages were used to determine removal efficiency. The removal of  $\text{NH}_4^+\text{-N}$  in the control (no microalgae) was 40% and all bioreactors with microalgae achieved higher removal than in the control. Experiments with free and immobilized cells bioreactors showed that *S. obliquus* was more effective in removing ammonia than *C. vulgaris* after 48 h of treatment (Table 1). Chevalier and De la Noüe (1985b) reported similar  $\text{NH}_4^+\text{-N}$  removal (100%) for carrageenan-immobilized *S. obliquus* while Travieso et al. (1996) reported N removal of 82% for alginate-immobilized *C. vulgaris* cultivated in urban wastewater and Lau et al. (1997) reported 95%  $\text{N-NH}_4$  removal for alginate-immobilized *C. vulgaris* growing in artificial wastewater.

Nitrogen removal in the present study was achieved relatively fast, while the time required to remove N in other studies have been longer (8 days) for *C. vulgaris* (De la Noue and Basseres,

**Table 1**

Removal (%) and uptake rates of N-NH<sub>4</sub> and P-PO<sub>4</sub> for *C. vulgaris* and *S. obliquus* cells growing in batch cultures of artificial wastewater and urban wastewater ( $\pm$  confidence interval, 95%).

Species	Cultures	Removal (%)		Uptake rate ( $\mu\text{g h}^{-1} 10^{-6}$ cells)	
		N-NH <sub>4</sub>	P-PO <sub>4</sub>	N-NH <sub>4</sub>	P-PO <sub>4</sub>
<i>C. vulgaris</i>	AWF	74.3 $\pm$ 13.9	70.2 $\pm$ 6.1	0.134 $\pm$ 0.01	0.006 $\pm$ 0.001
	UWF	60.1 $\pm$ 13.7	80.3 $\pm$ 5.5	0.139 $\pm$ 0.03	0.021 $\pm$ 0.004
	AWI	65.6 $\pm$ 11.7	84.6 $\pm$ 3.61	0.226 $\pm$ 0.12	0.016 $\pm$ 0.006
	UWI	80.0 $\pm$ 1.8	53.3 $\pm$ 4.8	0.512 $\pm$ 0.11	0.041 $\pm$ 0.002
<i>S. obliquus</i>	AWF	100 $\pm$ 0.01	60.0 $\pm$ 1.7	0.180 $\pm$ 0.01	0.036 $\pm$ 0.002
	UWF	100 $\pm$ 0.01	83.3 $\pm$ 2.7	0.177 $\pm$ 0.01	0.052 $\pm$ 0.003
	AWI	95.4 $\pm$ 3.1	85.1 $\pm$ 5.6	0.365 $\pm$ 0.02	0.033 $\pm$ 0.006
	UWI	96.6 $\pm$ 1.7	55.2 $\pm$ 3.1	0.621 $\pm$ 0.04	0.041 $\pm$ 0.01

1989; Lau et al., 1995). This shorter period of treatment by immobilized cells might be an indication that the concentration of 2.6 beads per mL of wastewater was enough to provide adequate nutrients removal and is lower than the concentration reported by Tam and Wong (2000) of 3.8 beads mL<sup>-1</sup> wastewater. However, not only is the number of beads important but also the cellular density per bead and the number of active cells per bead. This effect has previously been reported by Tam et al. (1994) who found that immobilized cells of *C. vulgaris* at low cellular density had higher physiological activity and Robinson et al. (1985) who reported that cellular metabolic activity (indicated by respiratory rate per cell) of immobilized cells of *Chlorella emersonii* decreased as cellular density increased. Therefore, a denser concentration of beads and a high cellular density per beads would reduce the amount of light penetrating the bioreactor and potentially enhance the self-shading effects which would then limit the growth and metabolic activities of the algal cells. Moreover, it could affect the amount of aeration required necessary to suspend the beads and provide for the required mixing conditions within the bioreactor.

The N uptake rates obtained for free-cells of *S. obliquus* did not show significant differences ( $P = 0.653$ ) in AWF and UWF nor did *C. vulgaris* ( $P = 0.999$ ) after 48 h of treatment (Table 1), suggesting that the N removal capacity of *S. obliquus* and *C. vulgaris* was not affected when growing in urban wastewater. The N uptake rates for alginate-immobilized *C. vulgaris* and *S. obliquus* were significantly higher than in free-cells. In general, immobilized *S. obliquus* presented higher N uptake rate than *C. vulgaris* cultures after 48 h for both AWI and UWI (Table 1).

The high N uptake rate detected in UWI for both microalgae might have been triggered by the higher ammonia concentration in urban wastewater, causing cells to be more stimulated to remove more nitrogen. Studies by Carpenter and Guillar (1971) indicate that populations of phytoplankton in oligotrophic regions, adapted to low nutrient concentrations, are able to assimilate nitrate and ammonia at a faster rate when higher nitrogen concentrations are available thus, showing a larger half-saturation constant. As an example, estuarine phytoplankton subject to relatively high concentrations of nitrate (1.5  $\mu\text{M NO}_3$ ) presented larger half-saturation constants than phytoplankton adapted at concentrations of 0.75  $\mu\text{M NO}_3$ . In the current study, the average concentration of ammonia in artificial wastewater was 30 mg L<sup>-1</sup>, lower than the average concentration in urban wastewater. Therefore, in terms of ammonia availability, cells passed from a least concentrated to a more concentrated medium which could have caused a greater uptake rate and adaptability to nutrients variation. Both types of microalgae adapted well to high levels of nitrogen and presented high levels of nutrient uptake.

In terms of nitrate concentrations, these did not present significant differences ( $P = 0.102$ ) throughout the study (Table 2), which suggests that nitrification was limited and that *C. vulgaris* and *S. obliquus* showed preferences to ammonium before any other form

of nitrogen present in the water. Moreover, ammonium could have been removed by other means in immobilized cultures. The reactors containing alginate beads without microalgae registered approximately 15% (2.7 mg N L<sup>-1</sup>) reduction of ammonium-N in 48 h. This process can be related to ionic interactions between the carboxyl groups of the gel and the ammonium ions. It has previously been reported that the gel has the ability to concentrate N which can be later used by the microalgae and hence increase the ammonium removal (Tam and Wong, 2000). In addition to adsorption, some ammonium was found to be removed by ammonia volatilization. The high pH observed in the bioreactors (9.0–9.5) was caused by a shortage of inorganic carbon after being rapidly consumed by algae. Although atmospheric air was supplied by air bubbling, this was not sufficient to provide enough CO<sub>2</sub> to avoid a large rise in pH. The high pH observed did not seem to interfere with algal growth (Fig. 1A and B), but could have contributed to NH<sub>3</sub> stripping from the medium. Elimination by desorption has been discussed by Talbot and De la Noüe (1993) in cultures of the cyanobacterium *Phormidium bohneri* in secondary effluent, where desorption appeared to represent 38–100% of the total removal of N.

In the present study the protein and lipids content of both species of immobilized microalgae was 16–17% and 10–11%, respectively, in batch cultures after 48 h (Fig. 3). These results were lower than the 45% protein contents reported by Nuñez et al. (2001) for free-cells of *C. vulgaris* y *S. obliquus*. Pane et al. (1998) suggests that this lower protein content in immobilized cells could be directly associated to the light limitation caused by the immobilization.

#### 4.3. Removal of P under batch culture conditions

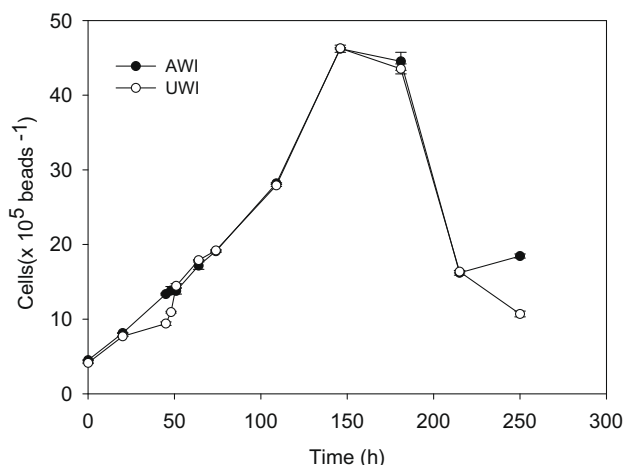
The percentage of phosphorus removal for free-cells cultures of *S. obliquus* and *C. vulgaris* did not present significant differences ( $P = 0.213$ ) when treating urban and artificial wastewater (Table 1). However, during experiments with immobilized cells, larger phosphorus removal was reached during the treatment of artificial wastewater than for urban wastewater (Table 1).

The phosphorus uptake ( $\mu\text{g h}^{-1} 10^{-6}$  Cells) for free-cells experiments was significantly greater for *S. obliquus* incubated in artificial and urban wastewater compared with *C. vulgaris*. However, immobilized *S. obliquus* culture showed a significantly higher P uptake rate than *C. vulgaris* when cultivated in AWI but similar on UWI (Table 1).

Although studies have been reported that immobilization carries the disadvantage of restricted nutrient diffusion (Van et al., 1993), the present study found no P and N restrictions. Chevalier and De la Noüe (1985a) and Lau et al. (1997) supported the idea that simple inorganic ions such as nitrate, ammonium and phosphate would be as freely available to immobilized algae as to their free counterparts, because nutrients must diffuse through the alginate pores to reach the algal cells. The higher phosphorus removal in immobilized cells may be directly related to the initial concen-

**Table 2**  
Mean concentration (mg L<sup>-1</sup>) and  $\pm$  standard deviation of nitrate for *C. vulgaris* and *S. obliquus* cells growing in batch cultures of artificial wastewater and urban wastewater.

Cultures	Nitrate (mg L <sup>-1</sup> )					
	<i>Chlorella vulgaris</i>			<i>Scenedesmus obliquus</i>		
	8 h	26 h	50 h	8 h	26 h	50 h
AWF	4.2 $\pm$ 0.6	4.1 $\pm$ 0.07	2.8 $\pm$ 0.4	3.0 $\pm$ 0.6	3.0 $\pm$ 0.2	2.8 $\pm$ 0.2
UWF	3.8 $\pm$ 0.3	4.0 $\pm$ 0.1	4.0 $\pm$ 0.3	1.4 $\pm$ 0.1	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2
AWI	1.4 $\pm$ 0.1	1.0 $\pm$ 0.02	1.0 $\pm$ 0.05	1.4 $\pm$ 0.1	1.3 $\pm$ 0.05	1.3 $\pm$ 0.07
UWI	0.5 $\pm$ 0.03	0.4 $\pm$ 0.01	0.6 $\pm$ 0.04	1.6 $\pm$ 1.8	1.6 $\pm$ 1.9	1.7 $\pm$ 1.8



**Fig. 2.** Mean growth (bars = standard error) for immobilized *S. obliquus* in the semi-continuous system cultivated in artificial wastewater (AWI) and urban wastewater (UWI).

tration of cells per ml of culture medium where immobilized and free-cells had an initial concentration of  $1 \times 10^6$  cell ml<sup>-1</sup> and  $2 \times 10^6$  cells ml<sup>-1</sup>, respectively. As discussed previously, the lower cells density may have caused a greater light diffusion and, thus, lower self-shading effect within cells. This could have allowed for greater cellular activity and consequently reach larger nutrients removal in comparison to free-cells experiments.

High P uptake rates in urban wastewater suggested that bacteria and algae were capable of removing phosphorus from the urban wastewater. The removal by algal and bacteria activity, combined with phosphates precipitation caused by the pH increment (9–9.5) probably contributed to the decrease of phosphorus levels (Lau et al., 1997).

#### 4.4. Growth under semi-continuous culture conditions

Overall, immobilized cells of *S. obliquus* treating urban wastewater were more efficient for the removal of ammonia and grew faster than *C. vulgaris* treating urban and/or artificial wastewater (Table 1) suggesting, that *S. obliquus* could be a better choice to be further tested in an immobilized cell semi-continuous bioreactor. Batch cultures of immobilized *S. obliquus* appeared to suffer growth limitation due to nutrients depletion. In semi-continuous mode, when fresh medium was added, higher cell concentrations of *S. obliquus* could be reached within the matrix for 4 consecutive cycles and total operation time of 181 h (Fig. 2). A similar trend was observed in the concentration of chlorophyll *a* with a maximum concentration of 1.6 pg Chl*a* cell<sup>-1</sup> for AWI and UWI in the fourth cycle. After 181 h (cycle 5), the concentration of chlorophyll *a* decreased to 0.28 pg Chl*a* cell<sup>-1</sup> and after 250 h the concentration was  $9.0 \times 10^{-3}$  pg Chl*a* cell<sup>-1</sup>.

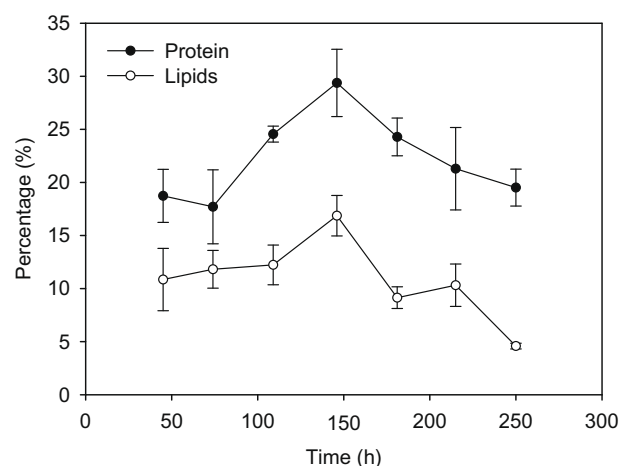
The high production of chlorophyll *a* can be related to an adaptation to the self-shading affect which has been found out to hap-

pen in immobilized cells (Trevan and Mak, 1988). The high chlorophyll *a* content is related to the growing energy requirement (light) for higher production of biochemical compounds like proteins. However, in the experiments with batch cultures of immobilized *S. obliquus* the chlorophyll *a* and protein content decreased after 48 h of treatment, probably as a result of nitrogen and light limitation within the alginate matrix. On the other hand, in the semi-continuous runs of immobilized *S. obliquus* showed protein and lipid content of 30% and 16%, respectively, after 146 h of treatment (Fig. 3), thus higher to those obtained in batch cultures (16–17%). The increase of protein suggests that the cultures in these cycles were maintained in the exponential phase of growth, phase in which the protein content is higher. In the following cycles, the protein concentration decreased at the end of the treatment (250 h), similarly to the lipids content of 3.4%. The results suggested that the collapse of protein and chlorophyll *a* after four cycles could have been caused by a protein synthesis limiting step which can cause a decreasing amount of protein cell<sup>-1</sup> for every increasing number of cycles in semi-continuous systems (Picard Gaston, 1976).

The high cellular density ( $4.6 \times 10^6$  cells beads<sup>-1</sup>) observed within the alginate-beads suggest that the re-calcification of alginate beads made possible to keep the cells within the matrix and extend its stability. However, the high cellular density into alginate beads does not necessarily increase nutrient removal as observed by Chevalier and De la Noüe (1985b) since the metabolism of these cells is much less active, compared to those of the first and second cycles.

#### 4.5. Removal of N and P under semi-continuous culture conditions

Immobilized *S. obliquus* removed 97% and 90% of ammonium in AWI and UWI, respectively, during the first 48 h (pre-cycle). After



**Fig. 3.** Protein and lipid content (%) for *Scenedesmus obliquus* in semi-continuous culture growing in artificial wastewater (bars = standard deviation).

**Table 3**

Removal (%) of ammonium and phosphate from artificial and urban wastewater with alginate-immobilized *S. obliquus* cells in semi-continuous culture ( $\pm$ confidence interval, 95%).

Cultures	$NH_4-N$ (% removal)						
	48 h	74 h	109 h	146 h	181 h	215 h	250 h
AWI	97 $\pm$ 2.5	80 $\pm$ 4.6	80 $\pm$ 4.8	80 $\pm$ 4.8	80 $\pm$ 5.7	30 $\pm$ 7.1	30 $\pm$ 4.2
UWI	90 $\pm$ 1.9	87 $\pm$ 1.7	86 $\pm$ 3.1	87 $\pm$ 3.4	88 $\pm$ 3.4	42 $\pm$ 3.1	10 $\pm$ 0.3
Cultures	$PO_4-P$ (% removal)						
	48 h	74 h	109 h	146 h	181 h	215 h	250 h
AWI	82 $\pm$ 3.1	86 $\pm$ 3.7	83 $\pm$ 3.1	84 $\pm$ 0.5	85 $\pm$ 3.6	32 $\pm$ 2.3	30 $\pm$ 2.8
UWI	63 $\pm$ 0.4	63 $\pm$ 1.3	62 $\pm$ 3.9	64 $\pm$ 3.5	63 $\pm$ 1.3	53 $\pm$ 5.8	18 $\pm$ 3.1

**Table 4**

Removal (%) of fecal coliforms in the semi-continuous system with alginate-immobilized *S. obliquus* cells ( $\pm$  confidence interval).

Cycles	pH	MPN coliforms ( $\times 10^6$ 100 ml $^{-1}$ )		Removal (%)
		Influent	Effluent	
1	10.1	2.4 $\pm$ 0.97	0.05 $\pm$ 0.0116	98
2	10.0	2.4 $\pm$ 1.21	0.05 $\pm$ 0.0187	98
3	10.1	1.6 $\pm$ 0.83	0.08 $\pm$ 0.0071	95
4	10.1	1.6 $\pm$ 1.04	0.08 $\pm$ 0.0352	95
5	10.1	1.6 $\pm$ 1.58	0.8 $\pm$ 0.252	50
6	8.5	1.6 $\pm$ 0.52	1.6 $\pm$ 2.25	0.0

74 h, ammonium removal decreased to 80 and 87% in AWI and UWI, respectively, and maintained for four more cycles (181 h). After 250 h the removal decreased to 30 and 10%, respectively for AWI and UWI (Table 3).

A similar pattern was observed for phosphorous removal where 85% removal in AWI was maintained for four cycles, after which the system had a removal capacity of only 30%. The cultures in UWI showed a lower removal capacity (64%) sustained for four consecutive cycles and 20% at the end of treatment (250 h) (Table 3). A proposed method to improve the removal of phosphorus in bioreactors is the use of starved cultures, which might accumulate more phosphate than the level obtained in this study. Although our cultures were not starved prior to the application of fresh wastewater, they showed a high phosphorus affinity and good phosphorus uptake during four cycles considering that the removal of P from wastewater depends on its bioavailability (Ekholm and Krogerus, 1998).

From a practical point of view, the semi-continuous culture of *S. obliquus* does not appear to allow a sustainable culture to treat wastewater. The main reason being that the culture collapse after a few cycles (four cycles). These results also suggest that the immobilized systems could facilitate the separation of the biomass from the treated wastewater although in terms of nutritional value the by-product biomass is fairly poor.

#### 4.6. Fecal coliforms removal

In the semi-continuous bioreactor, fecal coliforms were monitored in order to determine their survival during wastewater treatment with microalgae. High-values of pH were observed at the end of each cycle (35 h) causing 95% removal of fecal coliforms for four cycles (Table 4). Swati and Nair (1984) reported that at pH 10, 99% removal of fecal coliforms and *E. coli* was achieved in batch and semi-continuous cultures. This confirms that the high pH produced as a result of microalgae activity is unfavorable for the survival of fecal coliforms. However, the final concentration of fecal coliforms in the present study was still within the range of 5.0–8.0  $\times 10^4$  MPN/100 mL therefore, unsuitable for discharge to natural water bodies so a method of disinfection would have to be added.

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