# BIOMASS PRODUCTION AND NUTRIENT BUDGET IN OUTDOOR CULTURES OF SCENEDESMUS OBLIQUUS (CHLOROPHYCEAE) IN ARTIFICIAL WASTEWATER, UNDER THE WINTER AND SUMMER CONDITIONS OF MAZATLÁN, SINALOA, MEXICO

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# BIOMASS PRODUCTION AND NUTRIENT BUDGET IN OUTDOOR CULTURES OF *SCENEDESMUS OBLIQUUS* (CHLOROPHYCEAE) IN ARTIFICIAL WASTEWATER, UNDER THE WINTER AND SUMMER CONDITIONS OF MAZATLÁN, SINALOA, MEXICO

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*SCENEDESMUS OBLIQUUS*
OUTDOOR CULTURES
WASTEWATER
NUTRIENT BUDGET

ABSTRACT. – The biomass production of outdoor cultures of *Scenedesmus obliquus* in artificial wastewater maintained during three days under the winter and summer tropical conditions of Mazatlán, Sinaloa, Mexico, ranged from 26 to 43 $mg \cdot l^{-1}$, with protein contents of 33.5-38% and 51% in winter and summer, respectively. The final dissolved nitrogen concentrations were 53% of the initial value in winter and 21% in summer. In both seasonal situations, most of the removal was due to ammonia stripping caused by the high pH of the medium during light hours, and the final nitrogen content of the biomass showed that only 3.7% to 9.7% had been recycled. Phosphorus was removed only during the day, with a total abatement of 45% in winter and 73% in summer. Of this, only 41.5% to 51% was contained in the biomass, showing that the photosynthesis-induced high pH values of the medium play an important role in tertiary wastewater treatment and that, in addition, microalgae may be used only in outdoor systems with long retention times, which might make their use impractical for large or rapidly growing cities.

*SCENEDESMUS OBLIQUUS*
CULTURES EN PLEIN AIR
EAUX USÉES
BUDGET DES NUTRIMENTS

RÉSUMÉ. – La production de la biomasse des cultures de *Scenedesmus obliquus* en plein air, maintenu pendant 3 jours dans des eaux usées artificielles en été comme en hiver, dans des conditions tropicales de Mazatlan, Sinaloa, Mexique, varie entre 26 et 43 $mg.l^{-1}$, avec des contenus en protéines compris entre 33,5–38 % et 51 % en hiver et en été respectivement. La concentration finale de l'azote dissous est de 53 % de la valeur initiale en hiver, et de 21 % en été. Pendant les deux saisons, la plupart des changements proviennent de l'élimination de l'ammoniaque par le pH élevé du milieu pendant les heures du jour, et le contenu final en azote de la biomasse montre que 3,7 à 9,7 % seulement ont été recyclés. Le phosphore est supprimé pendant le jour seulement, avec une diminution totale de 45 % en hiver et de 73 % en été. De ces pourcentages, 41,5 % à 51 % seulement sont contenus dans la biomasse, montrant que la photosynthèse induite avec des valeurs de pH élevées du milieu, joue un rôle important dans le traitement tertiaire des eaux usées et que les microalgues, en outre, peuvent être utilisées seulement dans les systèmes extérieurs aux temps de rétention long, ce qui rend leur usage impraticable pour de grandes villes à croissance rapide.

## INTRODUCTION

Microalgae have different uses, as food for aquatic and terrestrial organisms, for agricultural soil improvement, for the production of chemicals of high commercial value and for domestic and industrial wastewater bioremediation (De la Noüe & De Pauw 1988, Becker 1994).

Their role in sewage oxidation ponds has been described more than 50 years ago by Caldwell (1946) and the use of unialgal cultures has been suggested for tertiary wastewater treatment, because it allows to recycle waste products into potentially valuable biomass (Sérodes *et al*. 1986, Talbot & De la Noüe 1993).

However, the conventional treatment with microalgae lasts several days and requires wide ex-

tensions of land. These are rarely available at affordable costs close to the cities, especially in developing countries of the tropical and subtropical belt, where rapid urbanization and industrialization increase the demand of building sites and the real estate values of suburban areas. Previous laboratory-scale experiments showed that an effective short-term water treatment may be achieved in bioreactors with continuous light, which boosts also biomass production but affects nutrient recycling. Under continuous light, the biomass obtained daily with dense microalgae cultures was 120-150 $mg \cdot l^{-1}$ and 55 to 75% of the dissolved nitrogen was removed, but only 25 to 33% of this amount was recycled because of ammonia stripping due to the high pH of the medium (Nuñez *et al*. 2001). The same type of cultures maintained under an artificial night-day cycle gave organic productions of 34 to 40 $mg \cdot l^{-1} \cdot d^{-1}$ and only 17 to 22% of the total dissolved nitrogen was removed, but the efficiency of recycling varied between 42 and 44% (Voltolina *et al*. 2004).

However, the night and day temperatures used for the light-dark photoperiod experiments were 17 and 25.5 ºC, which are typical of the summer temperate climate of the city of Ensenada, Baja California, Mexico (approx. 32º Lat N) and the total irradiance was 18.4 $mol \cdot m^{-2} \cdot d^{-1}$, which is lower than the levels normally available outdoors, whereas tertiary treatment with microalgae should be especially efficient in tropical and subtropical climates, because of the joint effect of high temperature and high solar radiation.

The aim of this study was to obtain direct evidence of the efficiency of nitrogen and phosphorus removal and uptake in outdoor cultures of *Scenedesmus obliquus*, under the tropical summer and winter conditions of the city of Mazatlán, Sinaloa, Mexico.

## MATERIALS AND METHODS

The city of Mazatlán is located between 106º 24' and 106º 28' Long W and 23º 15' and 23º 10' Lat N, 40 Km south of the Tropic of Cancer. Its climate is hot, subhumid, with frequent storms and cloudy skies in summer and dry, cool days in winter. The mean annual temperature is close to 25ºC and ranges from 15-16ºC in winter to 30-32ºC during summer (Government of Sinaloa 1985). The solar radiation expected at this latitude may vary throughout the year from 300 to 700 $langleys \cdot d^{-1}$ (Oswald 1988), equivalent to 55-100 $mol \cdot m^{-2} \cdot d^{-1}$ (Lüning 1981).

The experiments were run between January 11 and 22 and from July 20 to 30, 2002, in an unshaded area of the courtyard of the Faculty of Marine Sci Univ Sinaloa, using triplicate, flat-bottom cylindrical containers with a surface of 0.75 $m^2$ and height of 35 cm. Stirring was by bubbling with three airstones spaced at 120º along the sides, and one in the center of each container. The water depth was 27 cm, the culture volume was 0.2 $m^3$ and the surface: volume ratio was 3.75.

As in the previous laboratory-scale experiments, the microalgae was the chlorophyte *Scenedesmus obliquus* (Turpin) Kützing, and the growth medium was artificial wastewater prepared with 1-µm filtered tap water and 11.83, 39.83 and 4.46 $mg \cdot l^{-1}$ of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$, which were the mean yearly concentrations of the effluent of the main treatment plant of the city of Ensenada, during 1992 (Voltolina *et al*. 1998).

Previous trials showed that between the third and the fourth day after inoculum the cultures had reached their maximum density and were close to decay. For this reason, the experiments started at the time of sunrise, lasted three complete day-night cycles and were repeated three times in each seasonal situation, giving a total of nine replicates in space and in time.

Light was measured immediately above the surface of each culture at three hour intervals, with at least two additional readings when the sky was not completely clear or covered, using a colour and cosine corrected exposimeter previously calibrated against a Biospherical Instruments QSL 100 quantum scalar irradiance meter. The total irradiance received by each culture in each light period was obtained by integration and these values were used to calculate the mean photon flux ($mol \cdot m^{-2} \cdot d^{-1}$) above the cultures in the two seasonal situations.

Temperature and pH were measured with the same frequency in daylight, as well as three hours after sunset and before sunrise, with a precalibrated pH meter with temperature sensor. The cell and nutrient concentrations of each culture at the beginning and at the end of each light period were obtained by direct triplicate counts of the whole grid of a Neubauer chamber in the first case, and in cell-free triplicate subsamples in the second, using a Hach 4000 UV-VIS spectrophotometer and the reagents and methods suggested for wastewater analysis in the manufacturer's manual (Hach 1997): Nitrates: NitraVer 5, # 8171; Ammonia: Nessler # 8038; Phosphate: PhosVer 3, # 8048.

The ash-free biomass of each culture and the respective protein and phosphorus contents were obtained at the time of the inoculum, at the end of the third period of light (day 2.5) and after three complete day-night cycles, using triplicate samples of known volume concentrated on precalibrated Whatman GF-C glass fiber filters.

The total biomass and its inorganic content were determined using samples dried to constant weight at 65ºC and ashed later in a muffle furnace at 450ºC, and the organic biomass was calculated by difference between these values (Lora-Vilchis & Doktor 2001). The protein content was measured with the heated modified biuret method by Dorsey *et al*. (1977), which is particularly effective with green microalgae resistant to normal procedures of protein extraction such as *Scenedesmus*, and recovers approximately 90% of the Kjeldahl nitrogen of this type of samples (Dorsey *et al*. 1978).

For this reason, the total nitrogen content of the biomass was calculated dividing the protein concentration by the usual factor 6.25, and adding 10% to the resulting value. Total particulate phosphorus was determined directly, after acid persulfate digestion of the samples (Hach method #8190).

## RESULTS

### *Winter*

The temperature of the culture medium was 16.7 ± 1.4 ºC at 6:30, immediately before sunrise, and it increased to 18.8 ± 1.1 ºC at sunset, with a global daily mean of 17.6 ± 1.4 ºC. Light was unusually low, because of the unseasonable presence of persistent morning fogs and cloudy skies, and varied from 32.4 ± 18.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 6:30 to a maximum of 215.0 ± 66.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 12:30. By 17:30 it had decreased to 85.8 ± 38.9 $\mu mol \cdot m^{-1} \cdot s^{-1}$ and was close to nil between 10 to 15 minutes later.

The global irradiance received at the culture surface was one order of magnitude lower than that expected at this latitude, with a daily mean of 6.9 ± 1.3 $mol \cdot m^{-2} \cdot d^{-1}$. As a consequence of these low light levels pH showed limited variability, and ranged from 8.0 to 8.5 at sunrise to 8.8 - 9.0 throughout the rest of the light hours.

Cell concentrations increased from the initial $0.25 \cdot 10^6$ $cells \cdot ml^{-1}$ to approximately 1.4 ± $0.5 \cdot 10^6$ $cells \cdot ml^{-1}$ after the third light period and remained close to that value during the last night. During the last 24 hours variability was high because of contamination of some cultures, which for this reason had crashed or were close to crashing. Growth was observed at night during the first 48 hours, but the highest increase in cell numbers was recorded during the third period of daylight (Fig.1A). The total number of cell duplications was 2.62, with a daily average of 0.87.

Nitrate concentrations were close to 30% less than the initial value after the first 12 hours of light and remained approximately stable during the rest of the experiment, whereas ammonia decreased regularly, from 47.2 to 24.2 $mg \cdot l^{-1}$. Phosphates declined from 4.4 to 2.8 $mg \cdot l^{-1}$ (37% removal) during the first light period and continued to disappear at a slower rate, to a final mean value of 1.93 $mg \cdot l^{-1}$ (Fig. 1B).

The organic biomass increased from 13.7 ± 4.3 $mg \cdot l^{-1}$ to 39.7 ± 7.3 $mg \cdot l^{-1}$ at the end of the third light period. During the last night this value declined to 33.0 ± 13.8 $mg \cdot l^{-1}$, because of ciliate predation, and because some cultures had crashed. The initial protein content was 51.8% (7.08 $mg \cdot l^{-1}$), but the respective values after 2.5 and 3 days were 35.5 and 38% (Table IA).

At the end of 72 hours, the concentration of total dissolved nitrogen was 46.6% of the initial (30.43 $mg \cdot l^{-1}$), with a 36.3% decrease of nitrates and 48.7% of ammonia; particulate nitrogen increased from 1.26 to 2.23 $mg \cdot l^{-1}$, indicating that only 3.65% of the nitrogen removed had been recycled by the microalgae.

The final concentration of dissolved phosphorus was approximately 45% of the initial value. The total amount removed was 2.48 $mg \cdot l^{-1}$, and 41.5% of this (1.03 mg) was contained in the biomass (Table 1B).

Table I. – Winter experiments. A, mean values and standard deviation of cell concentrations of *Scenedesmus obliquus* outdoor cultures (N, in $10^6$ $cells \cdot ml^{-1}$), and of dry organic biomass (AFDW) and protein content (in $mg \cdot l^{-1}$) at the time of inoculum and after 2.5 and 3.0 light-dark periods. B, initial and final concentrations of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, total dissolved nitrogen (ΣN), particulate nitrogen, and of reactive and particulate $P\text{-}PO_4^{3-}$. △: difference between initial and final values. All data in $mg \cdot l^{-1}$, n=9 in all cases.

A

| Days | 0.0 | 2.5 | 3.0 |
|---|---|---|---|
| N | 0.25 ± 0.08 | 1.39 ± 0.51 | 1.55 ± 0.76 |
| AFDW | 13.67 ± 4.30 | 39.73 ± 7.29 | 32.98 ± 13.81 |
| Protein | 7.08 ± 2.57 | 14.12 ± 4.76 | 12.54 ± 3.82 |

B

| | Initial | Final | Δ |
|---|---|---|---|
| $N\text{-}NO_3^-$ | 9.80 ± 1.35 | 6.24 ± 1.37 | -3.56 ± 0.34 |
| $N\text{-}NH_4^+$ | 47.17 ± 3.59 | 24.19 ± 2.90 | -22.98 ± 6.22 |
| ΣN | 56.97 | 30.43 | -26.54 |
| N part. | 1.26 ± 0.46 | 2.23 ± 0.68 | 0.97 ± 0.69 |
| $P\text{-}PO_4^{3-}$ | 4.41 ± 0.24 | 1.93 ± 0.24 | -2.48 ± 1.03 |
| P part. | 0.55 ± 0.11 | 1.58 ± 0.39 | 1.03 ± 0.46 |

### *Summer*

The mean water temperature was 30.2 ± 3.0ºC and varied from 26.0 at sunrise to 31-32ºC from 12:30 until 19:30. After sunset it declined regularly throughout the night, to the initial 26ºC. The mean light level at 06:30 was 143 $\mu mol \cdot m^{-2} \cdot s^{-1}$. By 12:30 it had increased to 2215 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and remained close to this value until 15:30. After this time it declined to 190 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 19:30 and total darkness was 15-20 minutes later, with a mean irradiance of 82.9 ± 9.6 $mol \cdot m^{-2} \cdot d^{-1}$.

As a consequence of these light levels, during the day pH was notably higher than in winter, and ranged from close to 9.0 between 1 and 2 hours after sunrise to 9.3-9.5 between 09:30 to 19:30. During the night it decreased continuously, reaching a minimum of 7.5 at the time of sunrise.

The tendency of the cells to divide mainly during the dark hours was more evident than in the winter experiments, and the high variability of the last 24 hours was the result of individual differences in growth, rather than because of crashes. From initial inocula with $0.33 \cdot 10^6$ $cells \cdot ml^{-1}$, the

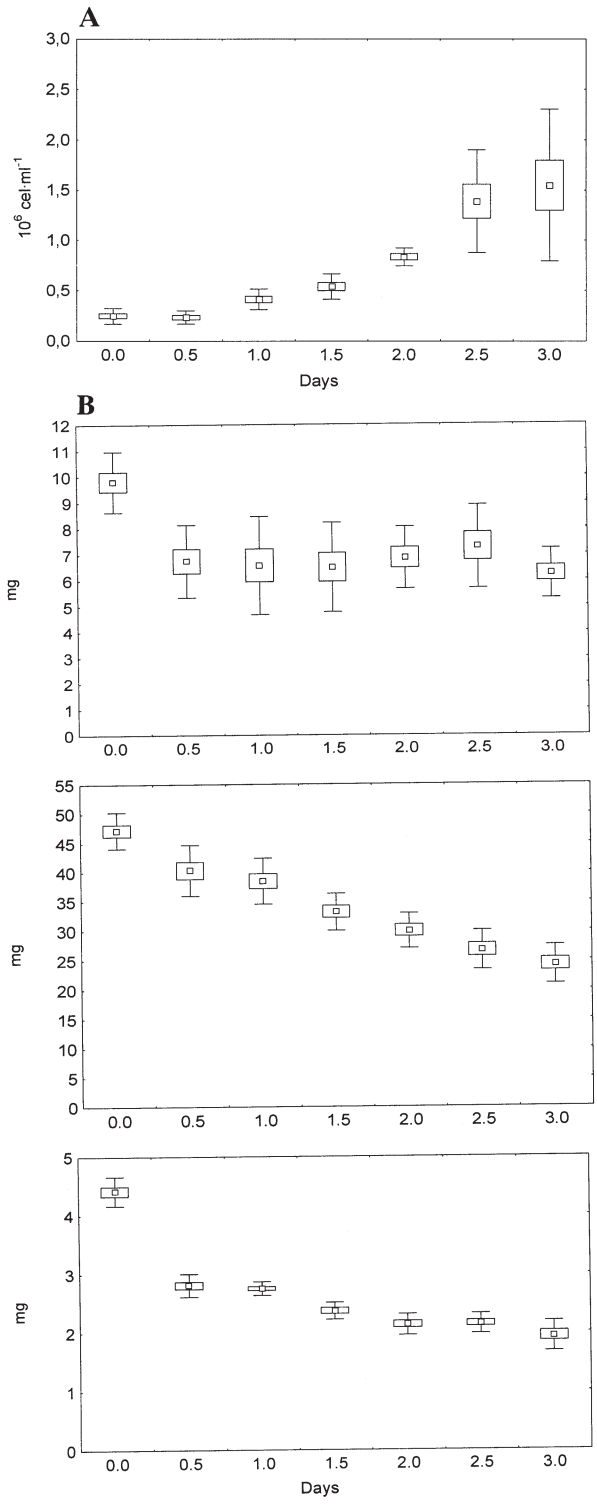


Fig. 1. – A, Mean (□), standard error (boxes) and standard deviation (whiskers) of *Scenedesmus obliquus* cell concentrations (in $10^6$ cells·ml$^{-1}$) in outdoor cultures during winter 2001, n=9 in all cases. B, Mean concentrations (□), standard error (box) and standard deviation (whiskers) of N-$NO_3^-$ (top), N-$NH_4^+$ (middle) and P-$PO_4^{3-}$ (bottom) in *Scenedesmus obliquus* outdoor cultures during winter 2001. All data in mg·l$^{-1}$, n=9 in all cases.

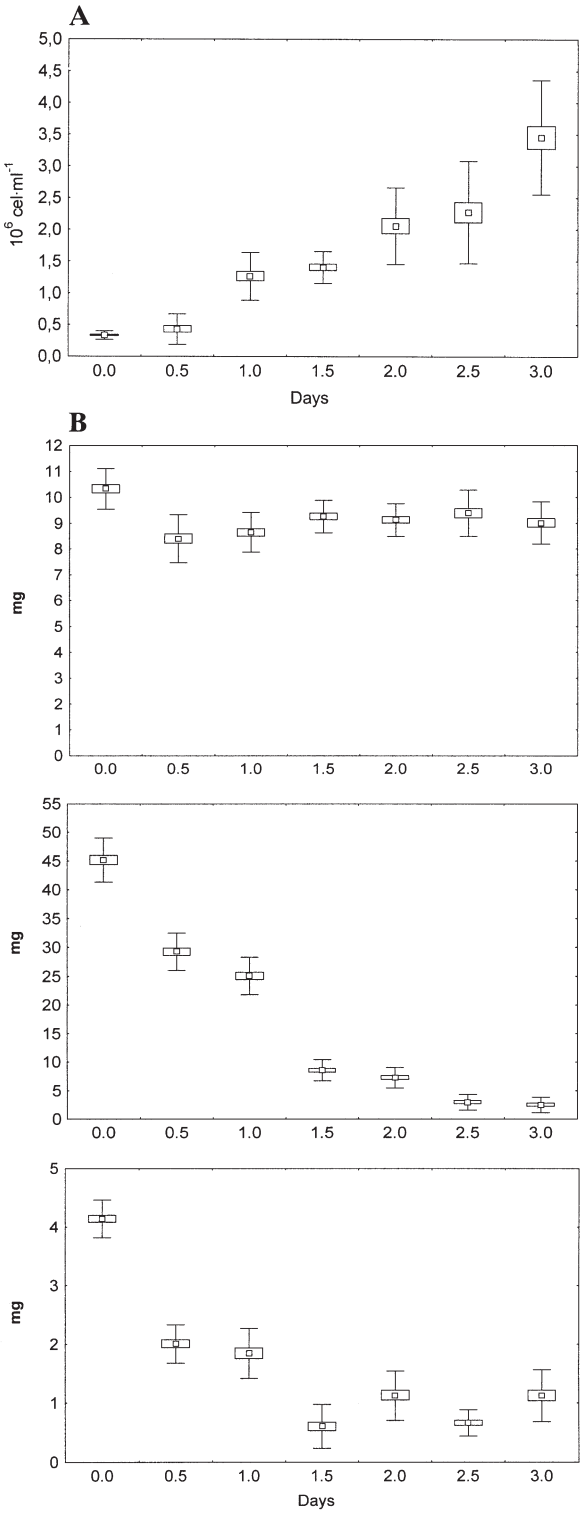


Fig. 2. – A, Mean (□), standard error (boxes) and standard deviation (whiskers) of *Scenedesmus obliquus* cell concentrations (N, in $10^6$ cells·ml$^{-1}$) in outdoor cultures during summer 2002, n=9 in all cases. B, Mean concentrations (□), standard error (box) and standard deviation (whiskers) of N-$NO_3^-$ (top), N-$NH_4^+$ (middle) and P-$PO_4^{3-}$ (bottom) in *Scenedesmus obliquus* outdoor cultures during summer 2002. All data in mg·l$^{-1}$, n=9 in all cases.

respective mean cell densities at the end of the last light and dark periods were $2.27 \cdot 10^6$ and $3.46 \cdot 10^6$ cells·ml$^{-1}$, and the total number of cell duplications was close to 3.4 (Fig. 2A).

Nitrates decreased only after the first light period, from 10.3 to 8.4 mg·l$^{-1}$ and varied irregularly during the rest of the experiment, with a final concentration of 9.0 mg·l$^{-1}$. Ammonia declined mainly in daylight, with an overall abatement of 42.5 mg·l$^{-1}$ in the three days of the experiment. During the first period of light, phosphates decreased by 50% (4.2 to 2.1 mg·l$^{-1}$) and this rate of removal continued during daylight but their concentrations increased again during the last two dark periods. The final value was 1.1 mg·l$^{-1}$, equivalent to a removal of 73% (Fig. 2B).

The initial and final organic biomass were 23 mg·l$^{-1}$ and 66.15 mg·l$^{-1}$, without any difference between the values obtained at the beginning and at the end of the last night of the experiments. The total organic production was 43 mg·l$^{-1}$, equivalent to 14.3 mg·l$^{-1}$·d$^{-1}$. Proteins increased with a similar trend, from 10.2 to 34.1 - 34.3 mg·l$^{-1}$. In this case, their relative concentrations were 44.3% at the beginning of the experiment and 51.5 - 51.7% after 60 to 72 hours in culture (Table IIA).

The total amount of dissolved nitrogen removed was 44.0 mg·l$^{-1}$ (79.3% of the initial value) and the increase in particulate nitrogen was 4.25 mg·l$^{-1}$, with an efficiency of recycling of 9.7%. Dissolved phosphorus decreased by 73%, equivalent to 3.02 mg·l$^{-1}$, of which 50.7% (1.53 mg) were recycled into biomass (Table IIB).

Table II. – Summer experiments. A, mean values and standard deviation of cell concentrations of *Scenedesmus obliquus* outdoor cultures (N, in $10^6$ cells·ml$^{-1}$), and of dry organic biomass (AFDW) and protein content (in mg·l$^{-1}$) at the time of inoculum and after 2.5 and 3.0 light-dark periods. B, initial and final concentrations of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, total dissolved nitrogen (ΣN), particulate nitrogen, and of reactive and particulate $P\text{-}PO_4^{3-}$. △: difference between initial and final values. All data in mg·l$^{-1}$, n=9 in all cases.

A

| Days | 0.0 | 2.5 | 3.0 |
|---|---|---|---|
| N | 0.33 ± 0.07 | 2.27 ± 0.81 | 3.46 ± 0.90 |
| AFDW | 23.01 ± 2.82 | 66.13 ± 10.14 | 66.15 ± 10.17 |
| Protein | 10.20 ± 2.18 | 34.20 ± 5.52 | 34.09 ± 4.01 |

B

| | Initial | Final | Δ |
|---|---|---|---|
| $N\text{-}NO_3^-$ | 10.33 ± 0.82 | 9.01 ± 0.54 | -1.32 ± 1.04 |
| $N\text{-}NH_4^+$ | 45.15 ± 4.01 | 2.50 ± 1.17 | -42.65 ± 3.57 |
| ΣN | 55.48 | 11.51 | -43.97 |
| N part. | 1.81 ± 0.39 | 6.06 ± 0.71 | 4.25 ± 0.84 |
| $P\text{-}PO_4^{3-}$ | 4.14 ± 0.32 | 1.13 ± 0.44 | -3.02 ± 0.95 |
| P part. | 0.63 ± 0.35 | 2.16 ± 0.57 | 1.53 ± 0.68 |

## DISCUSSION

These results, obtained under natural conditions, confirm those of earlier laboratory-scale experiments with an artificial day-night cycle. In both situations, approximately 30 to 50% of the dissolved phosphorus was removed in 24 hours, and the efficiency of recycling was between 35 and 50%, whereas more than 50% was removed through a different mechanism, possibly because of a pH-driven change of state, from the ionic species to insoluble salts, or by sequestration and coprecipitation with carbonates and sulphates.

In agreement with our previous results (Voltolina *et al.* 2004), phosphorus was removed mainly during the day, when light energy is available for photosynthesis, with consequent high pH and P precipitation, as well as for active nutrient uptake because of the need to reestablish the individual cell quota depleted the previous night by reproduction, and presumably by dark excretion which would explain the night increase in dissolved P concentration (Soeder *et al.* 1971, Brown & Harris 1978).

In the case of nitrogen, the data show that the efficiency of microalgae in nutrient recycling has been overestimated, and that it is lower under natural conditions than in laboratory-scale bioreactors. In the laboratory, total nitrogen removal in 24 hours varied from 17 to 22% and 42 to 44% of this was recycled. Under natural conditions, the percentage removed in the same time was slightly higher (at least in summer), but the respective amounts recycled in winter and summer into single-cell nitrogen structures were only 3.7% and 9.7%.

This confirms that the photosynthesis-induced pH changes are the main factors in tertiary wastewater bioremediation and that the existing solar technology cannot be used for short term treatment, because even after three days the residual nitrogen concentrations are too high for direct disposal into the environment.

Whereas the final phosphorus concentrations would be between 43 and 27% of the initial value, and the rest would not constitute an immediate environmental threat because it is usually present in excess in coastal areas (Goldman 1976), these experiments show that even after three days of treatment the effluent would still contain 53% of the initial total dissolved nitrogen during winter months and close to 21% in summer conditions. These might be still too high, considering that nitrogen enrichment has been related to cultural

eutrophication of marine coastal areas (Ryther & Dunstan 1971, Karydis *et al*. 1983) and to the increasingly frequent toxic or potentially toxic red tides of Mazatlán Bay (Alonso Rodriguez *et al*. 2000).

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