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Does the nutrient concentration of water ecosystems affect growth rates and maximum PSII quantum yield in calcium alginate-encapsulated *Scenedesmus ovalternus* and *Chlorella vulgaris*?

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ABSTRACT

Does the nutrient concentration of water ecosystems affect growth rates and maximum PSII quantum yield in calcium alginate-encapsulated *Scenedesmus ovalternus* and *Chlorella vulgaris*?

Aquatic ecosystems are susceptible to deterioration caused by eutrophication. Changes in the nutrient concentration may affect species physiology, making it a key factor in structuring communities. Phytoplankters have a short generation time and a fast response to environmental factors, which makes them a good model to address issues related to the effects of the trophic status on aquatic organisms. Our aim was to determine the changes in the growth and maximum PSII quantum yield of calcium alginate-encapsulated *Scenedesmus ovalternus* and *Chlorella vulgaris* incubated in aquatic ecosystems with different nutrient concentrations. We tested the following hypotheses: 1) alga with a greater capacity for nutrient absorption (*C. vulgaris*) would have the highest growth regardless of the environment nutrient concentration and 2) the concentration of nutrients positively affects the maximum PSII quantum yield in the two species. To test the hypotheses, *S. ovalternus* and *C. vulgaris* were immobilized in calcium alginate and cultured in wetlands with different nutrient concentrations. The growth of the two species differed between the wetlands; higher development was observed in the eutrophic environment. Significant differences were only recorded between the species in the eutrophic system, with greater growth in *C. vulgaris*. The hypereutrophic environment conditions were lethal for both species. The maximum quantum yield of PSII showed similar behaviour in both optimum nutrient conditions and limiting conditions. The nutrient concentration of the studied environments influenced the growth of the two species but not their maximum quantum yield of PSII, which seemed to be affected by factors other than nitrogen (N) and phosphorus (P) concentrations. *C. vulgaris* presented optimum growth only in the eutrophic system. The results showed that the growth rates of encapsulated algae could be a useful method for assessing changes, such as nutrient concentration, in the environmental conditions of the Sabana de Bogotá wetlands.

Key words: Algae immobilization, tropical high mountain wetland, biological monitoring, phytoplankton dynamic.

RESUMEN

¿La concentración de nutrientes de los ecosistemas acuáticos afecta la tasa de crecimiento y la eficiencia cuántica máxima del fotosistema II de* Scenedesmus ovalternus *y* Chlorella vulgaris *encapsuladas en alginato de calcio?

Los ecosistemas acuáticos son susceptibles al deterioro causado por los procesos de eutrofización. Los cambios en la concentración de nutrientes pueden afectar la fisiología de las especies y de esa forma ser un factor clave en la estructuración de las comunidades. Los organismos fitoplanctónicos presentan tiempos de generación cortos y respuestas rápidas a los factores ambientales, lo que los hace un modelo apropiado para abordar temas relacionados con el efecto del estado trófico sobre organismos acuáticos. Nuestro objetivo en este trabajo fue determinar los cambios en el crecimiento y en la eficiencia cuántica máxima del fotosistema II de Scenedesmus ovalternus *y* Chlorella vulgaris *encapsuladas en alginato de calcio e incubadas en ecosistemas acuáticos con diferente concentración de nutrientes. Las hipótesis a probar fueron 1) que el alga* *con una mayor capacidad de absorción de nutrientes (*C. vulgaris*) presenta el crecimiento más elevado sin importar la concentración de nutrientes del ambiente; y 2) que la concentración de nutrientes afecta de forma positiva la eficiencia cuántica máxima del PSII de las dos especies. Para probar estas hipótesis,* S. ovalternus *y* C. vulgaris *fueron inmovilizadas en alginato de calcio y expuestas a cuatro humedales de concentración de nutrientes. El crecimiento de las dos especies difirió entre humedales, con mayor crecimiento en el sistema eutrófico. Solo en ese sistema se registraron diferencias significativas entre las especies, con mayor crecimiento para* C. vulgaris. *Las condiciones del ambiente hipereutrófico (excesivamente rico en nutrientes) fueron letales para las dos especies. La eficiencia cuántica máxima del fotosistema II mostró un comportamiento similar tanto en situaciones óptimas de nutrientes como en condiciones limitantes. La concentración de nutrientes de los ambientes estudiados influenció el crecimiento de las dos especies pero no su eficiencia cuántica máxima del fotosistema II, que parece estar determinada por otros factores además de las cantidades de nitrógeno y fósforo.* C. vulgaris *tuvo un crecimiento óptimo solo en el sistema eutrófico. Los resultados mostraron que las tasas de crecimiento de las algas encapsuladas podrían ser un método útil para valorar los cambios en las condiciones ambientales de los humedales de la Sabana de Bogotá, como es el caso de la concentración de nutrientes.*

Palabras clave: *Inmovilización de algas, humedales tropicales de alta montaña, monitoreo biológico, dinámica del fitoplancton.*

INTRODUCTION

Inland aquatic ecosystems are susceptible to deterioration due to eutrophication processes that result from human activities. In aquatic environments, changes in nutrient concentrations can affect the structure of biological communities and ecosystems (Kulikova & Syarki, 2004; Ristau *et al*., 2012; Da Silva *et al*., 2014; Snickars *et al*., 2014). For phytoplankters, an increase in nutrient availability promotes the spread of some species but inhibits others (Tilman *et al*., 1982; Caputo *et al*., 2008; Zhu *et al*., 2010). The presence and abundance of particular phytoplankton species have been associated with the quantity of nutrients (Reynolds, 2006; Bellinger & Sigee, 2011). The abundance of a taxon in an ecosystem depends on its morphological and physiological characteristics (Reynolds, 2006), the availability of nutrients and light, the rate of nutrient uptake and the rate of biomass loss (i.e., sedimentation, washing, physiological death, herbivory, Tilman *et al*., 1982). The classical theory of succession determined by nutrients postulates that the growth of some microalgae species will be higher at low nutrient concentrations, while other taxa will present greater growth when the nutrient concentration is high (Margalef, 1978; Sommer, 1989; Reynolds, 2006); thus, the distribution of species and their abundance vary with the trophic gradient (Reynolds, 1998).

To determine the trophic characteristics of an aquatic environment based solely on nutrients can be difficult, especially when nutrients are limiting (Hudson *et al*., 2000; Rattan *et al*., 2012). Alternatively, it is possible to evaluate the direction of trophic changes in lakes indirectly via the physiological responses of autotrophic microorganisms, e.g., biomass production (Jaworska & Zdanowski, 2012) and growth rate (Chrzanowski & Grover, 2001); moreover, nutrient stress can be tested utilizing the maximum photochemical efficiency of photosystem II (F_v/F_m) (Parkhill *et al*., 2001), where F_v is the variable fluorescence of chlorophyll a (the difference between maximum and minimum fluorescence), and F_m is the maximum fluorescence. F_v/F_m is a measurement routinely used to determine the effects of environmental factors (e.g., nutrients and temperature) on phytoplankton photosynthetic efficiency (Cleveland & Perry, 1987; Kolber *et al*., 1988; Cullen *et al*., 1992; MacIntyre *et al*., 1997; Parkhill *et al*., 2001; Rattan *et al*., 2012; Wang *et al*., 2014). Laboratory studies have associated high and constant values of F_v/F_m to nutrient saturation conditions, while low values of F_v/F_m are linked to limiting conditions (Geider *et al*., 1993; Wang *et al*., 2014). *In situ* F_v/F_m readings reflect the total response of the phytoplankton community, and it is difficult to interpret the effects of nutrient concentration on the photosynthetic efficiency of individual species (Rattan *et al.*, 2012).

In this study, we used two microalgae species with different morphological and metabolic characteristics: *Chlorella vulgaris* (Beijerinck, 1890) and *Scenedesmus ovalternus* (Chodat, 1926). Because of its small size and rapid growth, the unicellular algae *C. vulgaris* can be characterized as an r-strategist (Pianka, 1970) with a high surface-volume ratio and is functionally classified in group I of the morpho-functional categorization proposed by Kruk *et al.* (2010). According to Reynolds (2006), *C. vulgaris* belongs to a group of algae that are characterized as organisms that have low biomass loss through sedimentation and have adapted to rapid nutrient acquisition. Organisms of this type are sensitive to nutrient deficiencies and are usually present in well-mixed shallow eutrophic lentic environments (Reynolds *et al.*, 2002). On the other hand, the cenobial algae *Scenedesmus ovalternus* is associated with eutrophic to hypereutrophic shallow lakes and rivers (Bellinger & Sigee, 2011). It is sensitive to low light intensity (Reynolds, 2006) and is functionally classified as a medium-sized species with a moderate tolerance to low nutrient concentrations (Group IV, Kruk *et al.*, 2010). *S. ovalternus* has a lower speed nutrient acquisition and a higher rate of biomass loss through sedimentation than *C. vulgaris* (Reynolds, 2006).

Our aim was to determine the growth and F_v/F_m responses of two algae species with different functional characteristics embedded in alginate beads and cultured under waters with different concentrations of nutrients. To achieve our goal, the cultures of each species were immobilized in sodium alginate beads and cultured in water bodies at different trophic states (oligotrophic to hypereutrophic). Immobilization allowed the respiratory and photosynthetic activities of the algae to occur and avoids cell loss through herbivory and sedimentation (VanDonk *et al.*, 1993; Faafeng *et al*, 1994). The alginate matrix does not affect light penetration and has very little effect on the self-diffusion of small molecules such as nutrients (Tanaka *et al.*, 1984). However, alginate beads with high cellular density would reduce the amount of light diffusion, which would affect the metabolism of algal cells (Hameed, 2013). Regarding the effects of encapsulation on algae, studies with *Scenedesmus obliquus* (Chevalier & de la Noüe, 1985) and *Chlorella vulgaris* (Lau *et al.*, 1998) have shown that the lag phase is longer for immobilized algae but that the maximum growth rate is similar for encapsulated and free algal cells. In addition, some studies have revealed that immobilization has little effect on the morphology of colonial, filamentous and unicellular algae taxa (Musgrave *et al.*, 1983; Bailliez *et al.*, 1985; Trevan & Mak, 1988).

We tested the following hypotheses: 1) the algae species with the greatest capacity to absorb nutrients (*C. vulgaris*) will exhibit the highest population growth regardless of the water nutrient content and 2) the concentration of nutrients will positively affect F_v/F_m in both unicellular and coenobium species, meaning that algae in the system with the highest concentration of nutrients will present higher F_v/F_m values.

MATERIALS AND METHODS

Study sites and limnological characterization

We conducted our experiments during the dry (February-March) and rainy (April-June) seasons of 2013. We selected three aquatic lentic ecosystems with different trophic statuses in the Bogotá River basin (between 2560 and 2900 masl), which was trophically characterized by Rodríguez (2012): the San Rafael reservoir (lower trophic level or oligo-mesotrophic, 4°42′10.6″N 73°59′26.4″W); the Santa María wetland (moderate trophic level or mesotrophic, 4°41′40.3″N 74°05′34.0″W); and the La Gaitana zone at the Juan Amarillo wetland (hypereutrophic, 4°44′13.1″N 74°06′47.5″W). To increase the environmental variability in our assays, during the rainy season, we included one more ecosystem with eutrophic status (La Conejera wetland, 4°45′42.8″N 74°06′13.7″W) (Acosta & Chivatá, 2016). A detailed description of the physical and morphological characteristics of these wet-

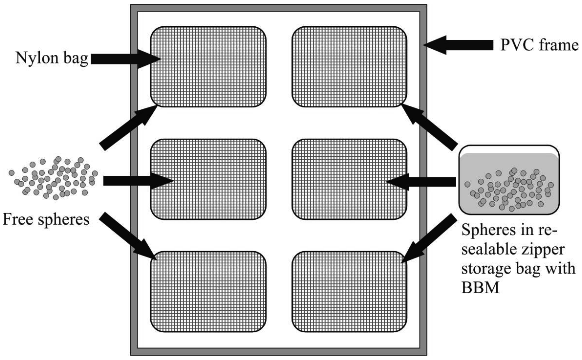

Figure 1. Diagram of the PVC structure for the experiments with immobilized algae carried out in aquatic ecosystems of the Sabana de Bogotá. *Esquema de la estructura en PVC para los experimentos con algas inmovilizadas llevados a cabo en los ecosistemas acuáticos de la Sabana de Bogotá.*

lands can be found in the studies of Consevación Internacional Colombia (2003) and Rodriguez (2012).

In each season and aquatic ecosystem, we measured the following environmental variables for ten days: photosynthetically active radiation (subsurface and at a 10 cm depth-PAR, μmol s^{-1} m^{-2}), electrical conductivity (Cond, μS/cm), dissolved solids (DS, mg/l), pH (units), oxygen (O_2, mg/l and saturation percentage, O_2%), temperature (°C), redox potential (RP, mV), and chlorophyll-*a* (mg/l). Additionally, on the first, fifth and tenth days of the experiments, we determined the concentration of ammonia (mg/l), total nitrogen (mg/l), total phosphorus (mg/l) and the chemical oxygen demand (COD, mg/l). All of these parameters were measured on the littoral of the ecosystems, where the encapsulated algae incubations were also made. For all procedures, the methodologies of APHA *et al.* (1995) were followed. We calculated the water light transmittance using the PAR measurements (Kirk, 2011) as follows:

$$Tr = \frac{l_z}{l_0} \times 100,$$

where l_z is the irradiance at z depth, and l_0 is the irradiance just below the water surface.

A principal component analysis (PCA) was performed to summarize the environmental variables (Legendre & Legendre, 1998) and determine the similarities of the environmental conditions between waterbodies and seasons. The variables were previously log-transformed (except pH) and standardized. The axis retention was evaluated using the Broken-Stick criterion (Jackson, 1993). For each season, the variation of the more important environmental variables evidenced in the PCA was tested using one-way analysis of variance (ANOVA), with waterbody as the factor.

We established the atomic N:P ratio and the trophic state of each environment using trophic indices for nitrogen (TSI_{TN}) (Kratzer & Brezonik, 1981), phosphorus (TSI_{TP}), and chlorophyll-*a* (TSI_{Chl}) (Carlson, 1977). The mean trophic state (TSI_{mean}) was calculated as follows:

$$\frac{[TSI_{TN} + TSI_{TP} + TSI_{Chl}]}{3}.$$

Preparation of immobilized algae beads

We used the species *Scenedesmus ovalternus* (LAUN 001 strain) and *Chlorella vulgaris* (LAUN 002 strain), which were both supplied by the Algal Culture Laboratory of the Departamento de Biología at the Universidad Nacional de Colombia. A solid inoculum of each species was added to 375 ml culture flasks with Bold Basic Medium (BBM) at a constant temperature (23 °C ± 2 °C). A light intensity of 60 μEm^{-2} s^{-1} was applied with a 16:8 h light-dark cycle. Cultures were grown to the stationary growth phase.

Subsequently, an aliquot of the culture was taken for each species, which was mixed with a 4% solution of sodium alginate at a 1:1 volume ratio in order to obtain a 2% solution of algae-alginate (the final cellular concentration is shown in Table 1). This new solution was placed in 5 ml syringes and allowed to drip into a solution of calcium chloride (4%). With this procedure, approximately 1200 spheres (diameter ≈ 3 mm) were produced per 50 ml of 2% algae-alginate solution. The beads were kept in 2% $CaCl_2$ at 6 °C for 24 hours to ensure hardening. This immobilization procedure has been used elsewhere on these algae genera with great results (Mallick, 2002).

Table 1. Initial concentration of cells for each species. The number of cells per ml of inoculum + calcium alginate is provided. Values are given in cells × 10^3. *Concentración inicial de células para cada especie. Se presenta el número de células por ml de inóculo + alginato de calcio. Los valores están dados en células × 10^3.*

Waterbody	San Rafael		Santa María		Juan Amarillo		La Conejera
Season	Dry	Rainy	Dry	Rainy	Dry	Rainy	Rainy
S. ovalternus	232	410	440	380	370	380	315
C. vulgaris	115	325	305	355	380	355	320

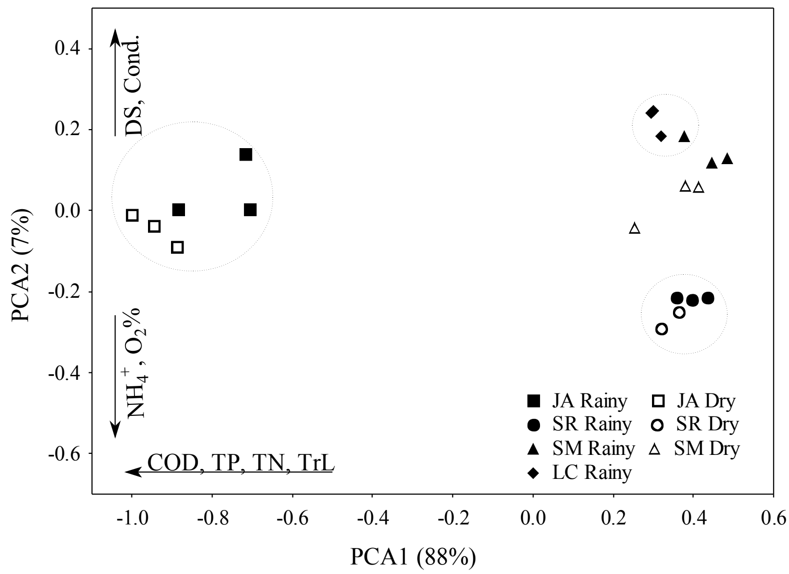

Figure 2. Dispersion of the scores on the first two axes of the principal components analysis performed with the environmental variables of the studied aquatic ecosystems in two climatic seasons (JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera, COD: Chemical oxygen demand, TP: Total phosphorus, TN: Total nitrogen, TrL: transmittance of light, DS: dissolved solids, and Cond: Conductivity). *Dispersión de los puntajes en los dos primeros ejes del análisis de componentes principales, llevado a cabo con las variables ambientales de los ecosistemas acuáticos estudiados, en las dos temporadas climáticas (JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera, COD: Demanda química de oxígeno, TP: fósforo total, TN: nitrógeno total, TrL: transmitancia de la luz, DS: sólidos disueltos, Cond: Conductividad).*

Experimental design

For each species, groups of 200 beads were formed and arranged in white nylon bags (mesh size ≈ 0.7 mm). We call these sets bags of “free spheres”. A control was made with the same number of spheres placed in re-sealable zipper transparent storage bags containing nutritive Basic Bold (BBM). In turn, these re-sealable bags were put in nylon bags. For each species, a polyvinyl chloride (PVC) frame was built in which replicates of three bags of “free spheres” and three controls were placed (Fig. 1). The bags were placed 10 cm below the surface to ensure the adequate availability of sunlight. The percentage of light reduction by nylon, which was measured in a laboratory, was approximately 12%. The controls allowed for the determination of whether the available light and temperature in the water ecosystems affected the growth and chlorophyll fluorescence of algae in conditions of optimum nutrient availability. To facilitate access to the PVC devices, they were maintained in the littoral zone of each site for ten days every season.

Chlorophyll fluorescence measurements and cell counts

Chlorophyll fluorescence, which was based on the maximum quantum yield of PSII (F_v/F_m), was monitored daily. First, 15 spheres were extracted from each nylon bag every day around the same time in the morning (≈ 8:00 h) and stored in vials at 4 °C and in darkness for one hour until chlorophyll-*a* fluorescence readings. Readings were taken with an unmodulated Handy PEA fluorometer coupled to a liquid-phase adapter (HPEA/LPA2) that provided a beam of actinic light (photosynthetic) using a 637 nm red LED. The measurement of this parameter in the immobilized algae was performed according to Kruskopf & Flynn (2006) with the modifications of Delgadillo (2014). The F_v/F_m was recorded as an estimate of photosynthetic efficiency (Cosgrove & Borowitzka, 2010; Maxwell & Johnson, 2000).

Subsequently, five of the fifteen collected spheres were diluted with 1 ml of a 4% calcium bicarbonate solution. To ensure a complete dilution, the beads were refrigerated for 24 hours (4 °C) with intermittent agitation. Cell counts were performed for each sample in a Neubauer chamber. Then, growth curves were constructed, and growth rates (μ) were calculated as follows:

$$\mu = \frac{\mathrm{Ln}\, N_{t1} - \mathrm{Ln}\, N_{t0}}{t_1 - t_0}$$

where N_{t1} is the cell number at the final time (t_1, last day of the experiments), and N_{t_0} is the cell number at the initial time (t_0, beginning day of the experiments).

Statistical analysis

Means and standard deviations of cell number, growth rate, and F_v/F_m were calculated for each treatment ($n = 3$) using box plots. Two-way ANOVA was employed to determine the effects of the species and water bodies on the growth rate and F_v/F_m values and to test the differences in the growth rate between the species and seasons in each environment. A *post hoc* Tukey's test was employed to test significant differences between the treatments. The assumption of homogeneity of variance was attained with the data. The analyses were carried out using R software (R Development Core Team, 2014).

Table 2. Means and coefficient of variation (CV%) of the physical and chemical factors measured in the water ecosystems studied in the two seasons. Ammonium (NH_4^+ mg/l); chemical oxygen demand (COD mg/l); total phosphorus (TP mg/l); total nitrogen (TN mg/l); water temperature (T° C); pH; electrical conductivity (Cond μS/cm); dissolved solids (DS mg/l); dissolved oxygen (DO mg/l); photosynthetically active radiation (PAR μmol s^{-1} m^{-2}); percentage of oxygen saturation (O_2%); redox potential (RP mV); transmittance of light (TrL %); and chlorophyll (Chl mg/l). *Promedios y coeficientes de variación (CV%) de los factores físicos y químicos medidos en los ecosistemas acuáticos estudiados en las dos temporadas climáticas. Amonio (NH_4^+ mg/l); demanda química de oxígeno (COD mg/l); fósforo total (TP mg/l); nitrógeno total (TN mg/l); temperatura del agua (T °C); pH; conductividad eléctrica (Cond μS/cm); sólidos disueltos (DS mg/l); oxígeno disuelto (DO mg/l); radiación fotosintéticamente activa (PAR μmol $s^{-1}m^{-2}$); porcentaje de saturación de oxígeno (O_2%); potencial rédox (RP mV); transmitancia de la luz (TrL %); clorofila (Chl mg/l).*

Variable	San Rafael		Santa María		La Conejera	Juan Amarillo	
	Dry	Rainy	Dry	Rainy	Rainy	Dry	Rainy
T°C	17.1 ± 0.3	17.9 ± 0.6	17.7 ± 0.9	18.1 ± 0.8	16 ± 1.1	20.9 ± 3.3	18 ± 1.8
Cond	59.3 ± 0.5	55 ± 2.3	195.1 ± 1.4	165.5 ± 4.7	264.4 ± 27.2	879.8 ± 220	654.7 ± 204.1
DS	29.6 ± 0.3	29.5 ± 0.9	97.5 ± 0.7	91.1 ± 1.6	154.5 ± 16	465.3 ± 97	370.4 ± 121
pH	7.9 ± 0.2	7.4 ± 0.1	7.5 ± 0.1	7.4 ± 0.2	7.1 ± 0.1	7.2 ± 0.1	7.3 ± 0.1
DO	6.8 ± 0.1	6.9 ± 0.2	4.5 ± 0.7	3.8 ± 1	2.1 ± 0.6	0.4 ± 0.1	0.4 ± 0.4
%O_2	99 ± 2	104 ± 3	64.1 ± 12	53.3 ± 17.2	28.5 ± 9.3	5.5 ± 2	6.4 ± 5.5
PAR	545.9 ± 465	1051 ± 754	645.8 ± 525	553.2 ± 400	270 ± 124	420 ± 312	243 ± 232
TrL	80.4 ± 13	85.5 ± 8	80.3 ± 5.3	76.2 ± 13.6	86 ± 6	48.1 ± 9.6	35.4 ± 16
RP	225 ± 13.3	76.8 ± 21	174.9 ± 53	158.2 ± 30	55.3 ± 27.8	−284.4 ± 25	−205.8 ± 55.2
Chl	1.7 ± 0.3	1.3 ± 0.3	4.2 ± 1.3	4.4 ± 1.5	3.4 ± 0.5	22.2 ± 22	40.5 ± 79.4
NH_4^+	0.9 ± 0.25	0.6 ± 0.07	0.3 ± 0.28	0.08 ± 0.02	0.12 ± 0.02	64.3 ± 9	26.5 ± 17.4
COD	6.5 ± 0.7	12 ± 6.9	13 ± 4.6	11.7 ± 5.1	20.7 ± 2.9	227 ± 78.7	270.7 ± 104.7
TP	0.1 ± 0.01	< 0.10	0.13 ± 0.05	< 0.10	0.33	17.6 ± 4.1	7.4
TN	1.17 ± 0.33	0.78	0.63 ± 0.16	< 0.54	< 0.54	82.7 ± 11.8	33.97

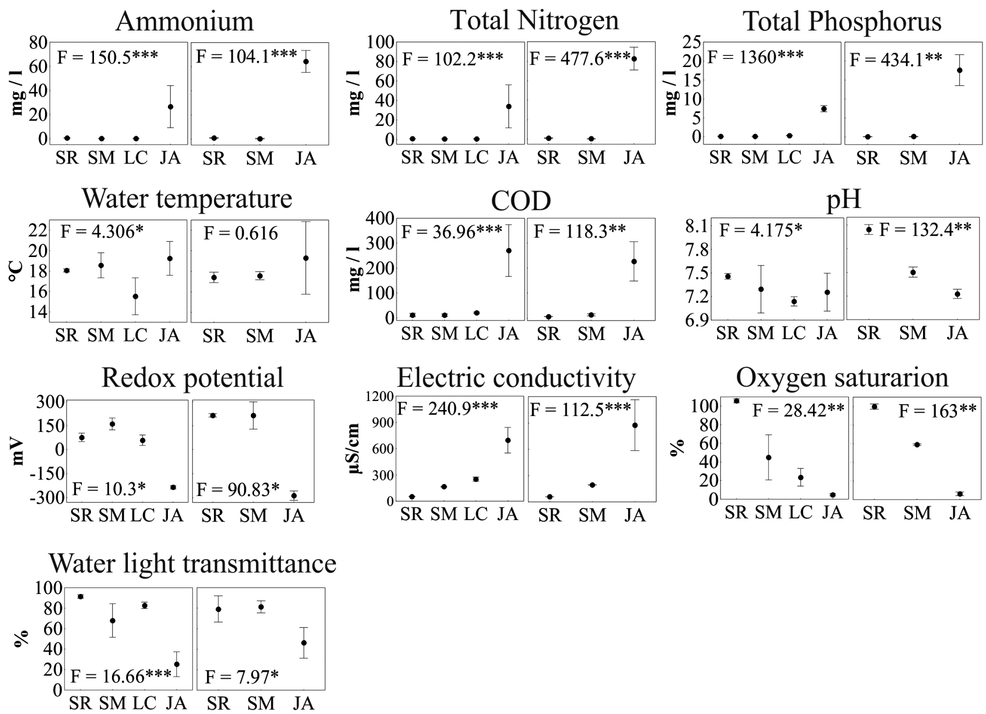

Figure 3. Variation of environmental variables (mean values and standard deviation) in the water bodies in the two seasons (for each variable, left: rainy season; right: dry season). Differences were tested using one-way ANOVA. Degrees of freedom: 3 (Rainy season) and 2 (Dry season). $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera, and COD: Chemical oxygen demand. *Variación de las variables ambientales (valores promedio y desviación estándar) en los ecosistemas acuáticos en las dos temporadas (para cada variable, izquierda: temporada lluviosa; derecha: temporada seca). Las diferencias fueron probadas usando ANOVA de una vía. Grados de libertad: 3 (temporada lluviosa) y 2 (temporada seca).* p *> 0.05,* *p *< 0.05,* **p *< 0.01,* ***p *< 0.001. JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera, COD: Demanda química de oxígeno.*

RESULTS

Physical and chemical characterization

The measured parameters showed low seasonal variation in all lentic environments (Table 2). The wetlands showed differences in nutrient concentration (NH_4^+, TP, TN) and in variables related to organic matter load (COD, O_2%, Cond, DS) (Fig. 2). Juan Amarillo presented extreme values of those variables.

Only the first PCA axis of Figure 2 was significant, which explained 88% of the total limnological data and had total phosphorus ($r = -0.47$), total nitrogen ($r = -0.46$) and chemical oxygen demand ($r = -0.31$) as the most-related chemical variables. The scores showed high dispersion. The first axis separated the Juan Amarillo wetland from the other water ecosystems. San Rafael, Santa María and La Conejera were related to the second axis. The environments did not show seasonal variation. Instead, all environmental variables showed significant differences between waterbodies (Fig. 3). Extreme values of the environmental variables were registered in the Juan Amarillo wetland (Fig. 3). Electrical conductivity and oxygen displayed a clear gradient between the waterbodies.

Trophic and biological results

The trophic categories of the wetlands differed between the indices (Table 3). The phosphorus-based index categorized all environments as either hypereutrophic or eutrophic. In relation to the other indices, the Juan Amarillo wetland

Table 3. Trophic classification of the water ecosystems using different approaches. N:P, nitrogen to phosphorus ratio (Lampert & Sommer, 2007); TSI_{Chl}, trophic state index based on chlorophyll (Carlson, 1977); TSI_{TP}, trophic state index based on phosphorus (Carlson, 1977); TSI_{TN}, trophic state index based on nitrogen (Kratzer & Brezonik, 1981). Oligo, oligotrophic; Meso, mesotrophic; Eu, eutrophic; Hyper, hypereutrophic. *Clasificación trófica de los ecosistemas acuáticos desde diferentes enfoques. N:P, relación nitrógeno fósforo (Lampert & Sommer, 2007);* TSI_{Chl}, *índice de estado trófico basado en la clorofila (Carlson, 1977);* TSI_{TP}, *índice de estado trófico basado en el fósforo (Carlson, 1977);* TSI_{TN}, *índice de estado trófico basado en el nitrógeno (Kratzer & Brezonik, 1981). Oligo, oligotrófico; Meso, mesotrófico; Eu, eutrófico; Hyper, hipereutrófico.*

Waterbody	N:P		TSI_{Chl}		TSI_{TP}		TSI_{TN}		TSI_{mean}	
	Value	Trophic State	Value	Trophic State	Value	Trophic State	Value	Trophic State	Value	Trophic State
Juan Amarillo	450	Hyper	57.45	Eu	132.6	Hyper	105.3	Hyper	98.45	Hyper
La Conejera	22.3	Eu	42.57	Meso	87.9	Eu	45.6	Meso	58.69	Meso-Eu
Santa María	29.6	Meso	44.1	Meso	70.6	Eu	45.6	Meso	53.43	Meso
San Rafael	42.4	Meso	33.17	Oligo-Meso	70.6	Eu	50.9	Meso	51.55	Meso

had the highest trophic category (hypereutrophic according to the TSI_{mean}). La Conejera was included among the eutrophic and mesotrophic categories. San Rafael and Santa María were included in the oligo-mesotrophic groups based on the N:P relation and the nitrogen and chlorophyll indices. San Rafael was included as eutrophic with regards to phosphorus.

The values of the trophic indices based on phosphorus, chlorophyll and nitrogen, as well as the mean trophic index, showed significant differences between some waterbodies (Fig. 4). A higher mean trophic index value was verified in Juan Amarillo, followed by La Conejera (Fig. 4). Between the San Rafael and Santa María wetlands, only the chlorophyll based index exhibited differences.

For the growth rate, the ANOVA analysis showed a significant interaction between the species and season for each aquatic ecosystem

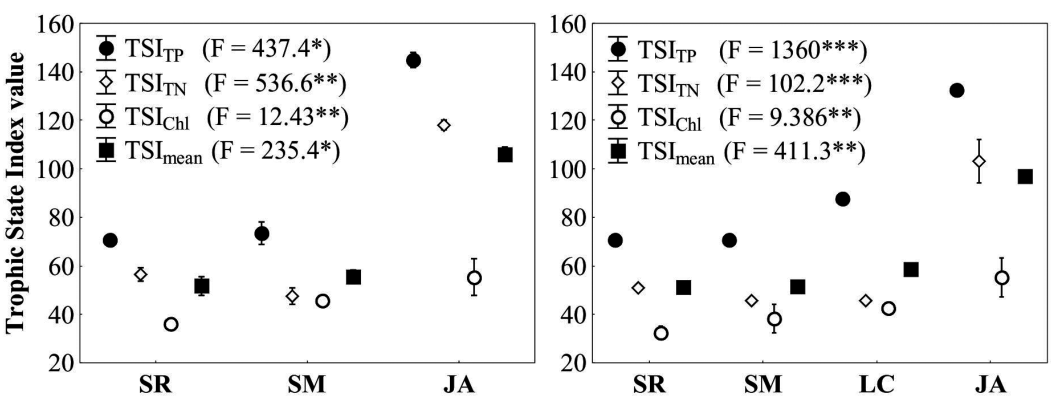

Figure 4. Variation of the trophic indices values (mean and standard deviation) in the water bodies in the two seasons (left: dry season; right: rainy season). Differences were tested using one-way ANOVA. Degrees of freedom: 3 (Rainy season) and 2 (Dry season). $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera, TSI_{Chl}: trophic state index based on chlorophyll; TSI_{TP}: trophic state index based on phosphorus, TSI_{N}: trophic state index based on nitrogen, and TSI_{mean}: mean trophic state index. *Variación de los valores de los índices tróficos (promedio y desviación estándar) en los cuerpos de agua en las dos temporadas (izquierda: temporada seca; derecha: temporada lluviosa). Las diferencias fueron probadas usando ANOVA de una vía. Grados de libertad: 3 (temporada lluviosa) y 2 (temporada seca).* $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *JA: Juan Amarillo, SR: San Rafael, SM: Santa María, LC: La Conejera,* TSI_{Chl}*: índice de estado trófico basado en la clorofila,* TSI_{TP}*: índice de estado trófico basado en el fósforo,* TSI_{N}*: índice de estado trófico basado en el nitrógeno,* TSI_{mean}*: índice de estado trófico promedio.*

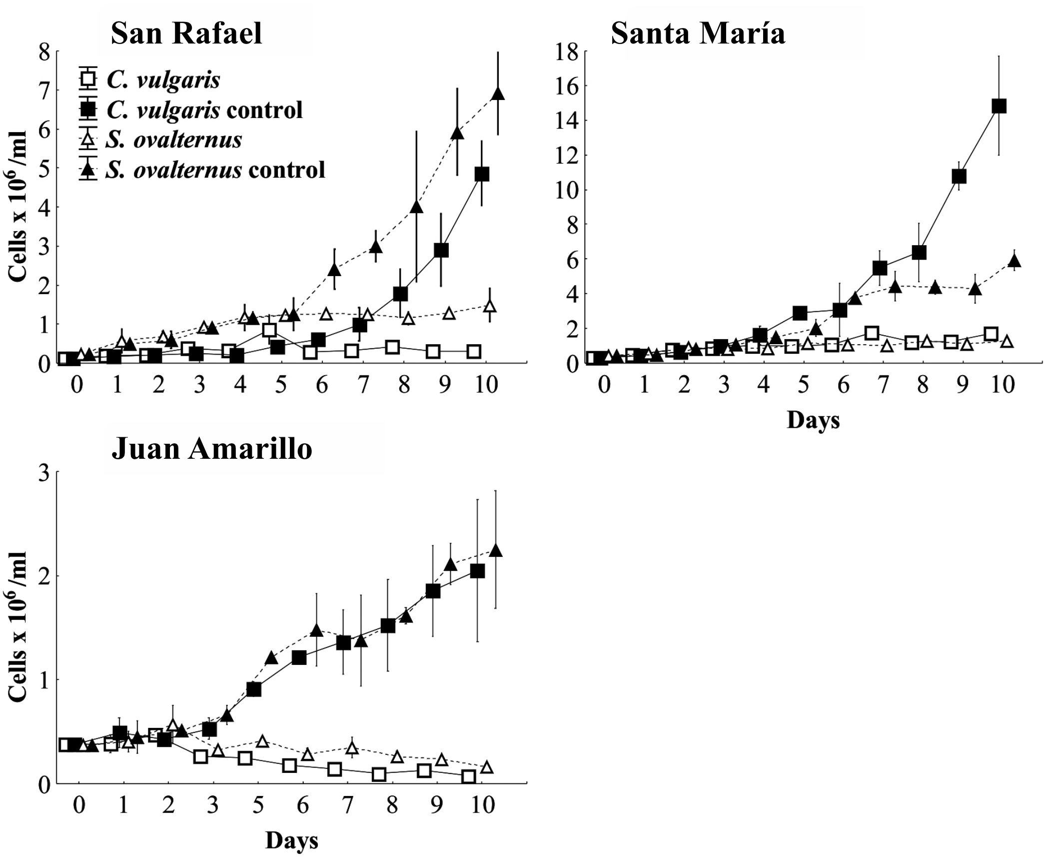

Figure 5. Growth curves of the species and controls during the dry season. The control treatments are shown with the symbols filled with black colour. *Curvas de crecimiento de las especies y los controles durante la estación seca. Los tratamientos control se muestran con los símbolos rellenados con color negro.*

and a significant interaction between the waterbody and species for each season (Table 4 and Table 5). In the dry season, differences were observed in the growth between the species in San Rafael and Santa María (Fig. 5 and Fig. 7). *S. ovalternus* had higher growth and exceeded the growth rate of *C. vulgaris* in San Rafael (Tukey's test $p = 0.005$) (Fig. 5 and Fig. 7), whereas the *C. vulgaris* growth rate was higher in the Santa María wetland (Tukey's test $p < 0.001$).

Table 4. Results from the analysis of variance (Two-way ANOVA) for the growth rates and F_v/F_m values in the two sampling seasons. The factors are species and water bodies. *Df*, degrees of freedom; *F* value, statistic *F*; *p* value, probability. *Resultado del análisis de varianza (ANDEVA de dos vías) de las tasas de crecimiento en los dos periodos de muestreo. Los factores son las especies y los cuerpos de agua.* Df, *grados de libertad;* F *value, estadístico* F*;* P *value, probabilidad.*

	Season	Species			Waterbody			Species*Waterbody		
		Df	*F* value	*P* value	*Df*	*F* value	*P* value	*Df*	*F* value	*P* value
Growth Rate	Rainy	3	606.6	< 0.001	3	474.6	< 0.001	9	91.6	< 0.001
	Dry	3	292.8	< 0.001	2	319.8	< 0.001	6	15.9	< 0.001
F_v/F_m	Rainy	3	32.87	< 0.001	3	63.87	< 0.001	9	27.94	< 0.001
	Dry	3	93.14	< 0.001	2	76.96	< 0.001	6	49.92	< 0.001

The highest species growth rates were recorded in La Conejera, where *C. vulgaris* did not show significant differences from the control (Fig. 6 and Fig. 7) but exhibited a higher growth than *S. ovalternus* (Tukey's test $p < 0.001$). Juan Amarillo presented negative values in the growth rate of the two species due to cell death (Fig. 5, Fig. 6 and Fig. 7).

For F_v/F_m, the ANOVA analysis showed a significant interaction between the species and season for each wetland and a significant interaction between water bodies and species for each season (Table 4 and Table 5). The mean F_v/F_m values were between 0.184-0.695 for *C. vulgaris* and 0.207-0.659 for *S. ovalternus*. The lowest F_v/F_m values were recorded at the Juan Amarillo wetland (mean values 0.184-0.350), while the highest ones were observed at La Conejera (0.635-0.695) (Fig. 8, Fig 9. and Fig. 10). For all cases, the F_v/F_m values of *C. vulgaris* were lower than that of *S. ovalternus* in the early days of the experiment (Fig. 8 and Fig. 9). A greater F_v/F_m stability (lower coefficient of variation) was exhibited by *C. vulgaris* at La Conejera. The species presented seasonal variations that were similar to those of their respective controls (Fig. 8, Fig. 9, Fig. 10 and Table S1, available at www.limnetica.net/es/limnetica/36), except at the Juan Amarillo and Santa María wetlands for *S. ovalternus* (Fig. 10).

DISCUSSION

The growth of *C. vulgaris* and *S. ovalternus* was different between the wetlands in this study,

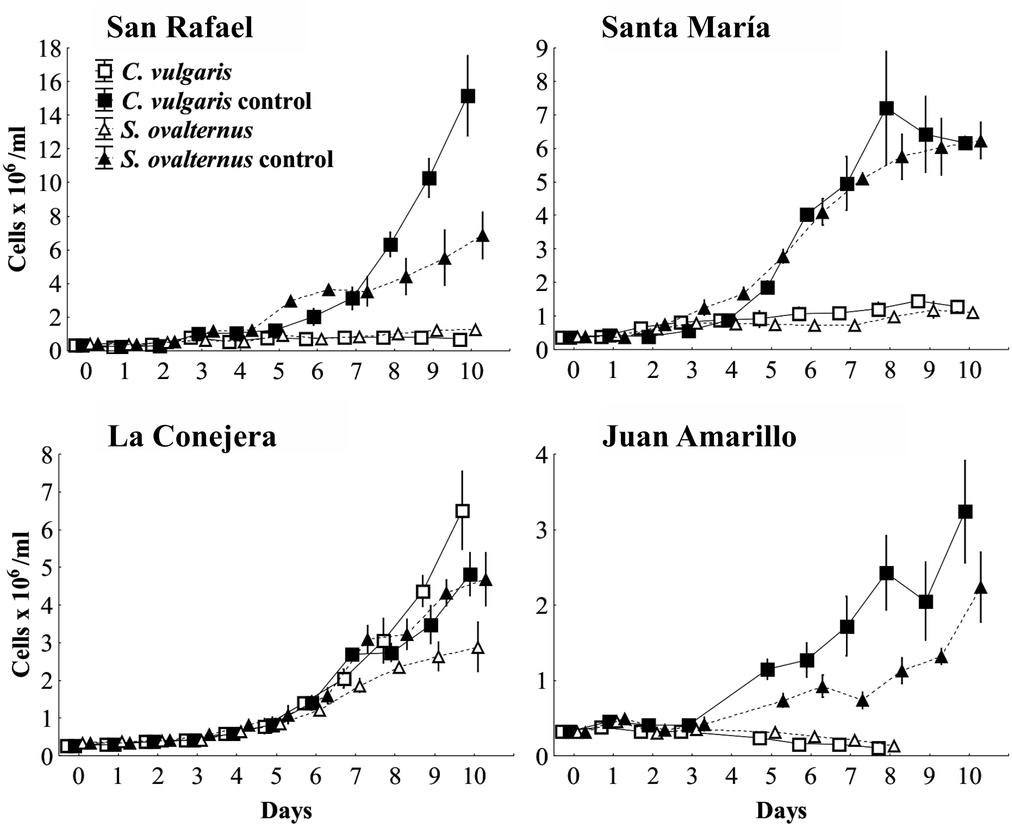

Figure 6. Growth curves of the species and controls during the rainy season. The control treatments are shown with the symbols filled with black colour. *Curvas de crecimiento de las especies y los controles durante la temporada de lluvias. Los tratamientos control se muestran con los símbolos rellenados con color negro.*

Table 5. Results from the analysis of variance (Two-way ANOVA) for the growth rates and F_v/F_m values in each waterbody. The factors are species and sampling season. *Df*, degrees of freedom; *F* value, statistic *F*; *p* value, probability. *Resultado del análisis de varianza (ANDEVA de dos vías) de las tasas de crecimiento y de los valores de* F_v/F_m *en cada cuerpo de agua. Los factores son las especies y los periodos de muestreo*. Df, *grados de libertad;* F *value, estadístico* F*;* P *value, probabilidad.*

	Waterbody	Species			Season			Species*Season		
		Df	*F* value	*P* value	*Df*	*F* value	*P* value	*Df*	*F* value	*P* value
Growth Rate	San Rafael	3	223.3	< 0.001	1	13.4	0.002	3	4.1	0.024
	Santa María	3	449.7	< 0.001	1	39.4	< 0.001	3	28.7	< 0.001
	Juan Amarillo	3	393.1	< 0.001	1	2.21	0.15	3	7.1	0.003
	La Conejera	3	18.47	0.001	—	—	—	—	—	—
F_v/F_m	San Rafael	3	31.3	< 0.001	1	9.5	0.003	3	6.4	< 0.001
	Santa María	3	21.0	< 0.001	1	0.18	0.68	3	4.01	0.008
	Juan Amarillo	3	292.8	< 0.001	1	31.1	< 0.001	3	6.0	< 0.001
	La Conejera	3	1.4	0.25	—	—	—	—	—	—

suggesting that the population dynamics of individual species may be an indirect but useful way to evaluate the nutrient conditions of aquatic environments. Alginate spheres allow light penetration, water and nutrient exchange and minimize biomass losses through sedimentation and herbivory (Van Donk *et al*., 1993; Faafeng *et al*, 1994). Spheres also guarantee an isolating effect against physical factors such as abrasion and water washout. Thus, the use of encapsulated microalgae placed directly in the water ecosystems permits for a better understanding of the key factors that affect the growth, development, and establishment of phytoplankton species in natural conditions.

The growth of *C. vulgaris* and *S. ovalternus* was related to nutrient availability. Their growth was low in environments with less nutrients (San Rafael and Santa María) and higher in the eutrophic environment (La Conejera). An acute organic load and excessive nutrient conditions, which occurred in the Juan Amarillo wetland (hypereutrophic), proved to be adverse for the two species.

In the La Conejera wetland (eutrophic), the species showed the highest growth rates with-

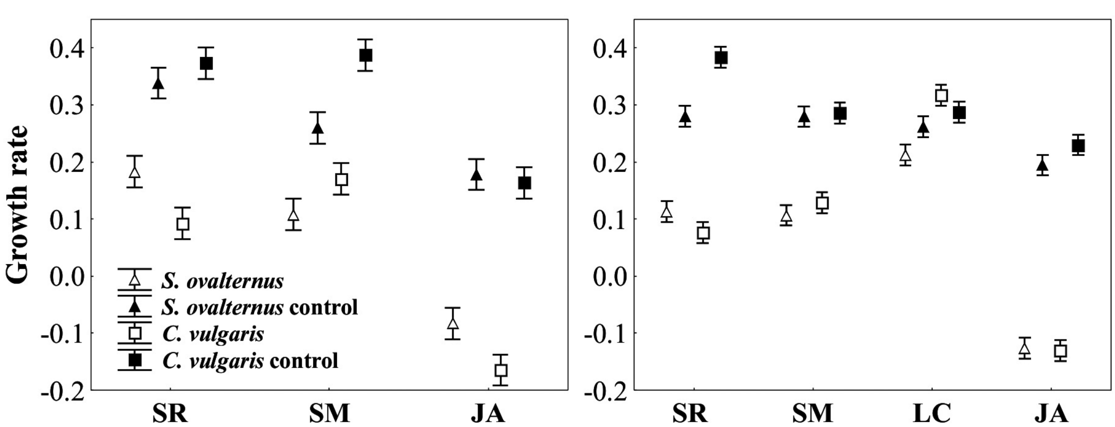

Figure 7. Changes in the growth rates of the species and controls in the studied water ecosystems in the two climatic seasons (left: dry season; right: rainy season). The control treatments are shown with the symbols filled with black colour. *Cambios en las tasas de crecimiento de las especies y de los controles en los ecosistemas acuáticos estudiados en las dos temporadas climáticas (izquierda: temporada seca; derecha: temporada lluviosa). Los controles se muestran con los símbolos rellenados con color negro.*

out differences to the controls. This response appears to have been advanced by high but not saturating nutrient concentrations and probably moderate light intensity as the experimental area had partial shade from riparian trees. There is evidence that light intensities greater than 240 μmol m^{-2} s^{-1} may affect the photosynthesis of *Chlorella* sp., which would be reflected in a low cell concentration (Guo *et al.*, 2015).

The fact that in La Conejera, there were no differences in the growth rate between the species and its controls indicate that the nutritional conditions were optimal for the growth of these species (high trophic status, without reaching hypereutrophy). On the other hand, the highest growth rate of *C. vulgaris* in this eutrophic environment, compared to that of *S. ovalternus*, could indicate that in a natural system with nutrient availability and minimized biomass loss conditions, a small cellular size provided an adaptive advantage (Acevedo-Trejos *et al.*, 2015). New experiments with species of different sizes must be carried out to confirm this hypothesis, but in principle, it seems that small cell size is associated with an increased rate of the diffusion of nutrients and greater light gathering, which accelerated cell division and was reflected in both a higher growth rate and a high cell concentration (Eppley *et al.*, 1969; Margalef, 1978; Padisák *et al.*, 2003; Reynolds, 2006). It has been recorded under laboratory conditions with the optimal concentrations of nutrients that the growth of *C. vulgaris* is higher than that of *S. ovalternus* (González, 2010; Del-

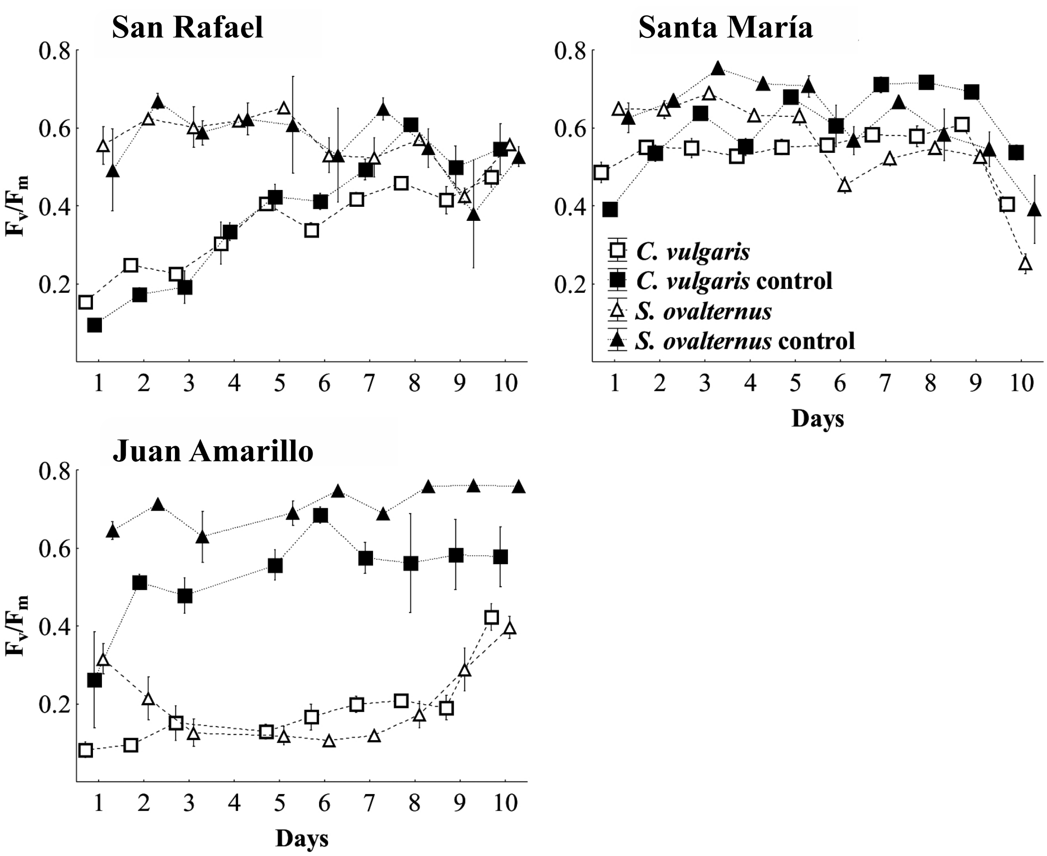

Figure 8. Daily variation of the F_v/F_m values of each species in the studied water bodies during the dry season. The control treatments are shown with the symbols filled with black colour. *Variación diaria de los valores de* F_v/F_m *de cada especie en los cuerpos de agua estudiados durante la temporada seca. Los controles se muestran con los símbolos rellenados con color negro.*

gadillo, 2014). Thus, in the eutrophic system (La Conejera), the availability of nutrients and the morpho-functional characteristics of *C. vulgaris* resulted in growth that exceeded that of the other tested taxon.

San Rafael and Santa María had similar trophic characteristics (Fig. 2 and Table 3) that were reflected by the species growth. In a laboratory experiment with *S. ovalternus* and *C. vulgaris* immobilized in calcium alginate and grown in water from San Rafael and Santa María, Delgadillo (2014) observed that there were no differences in growth between the treatments. While there were no growth differences between these wetlands, the two species did show seasonal discrepancies in the speed of cell increase, which was probably influenced by variations in light intensity and the algae number of the initial inoculum. Seasonal differences in the growth were seen in the species and controls, and the fact that the PCA analysis showed no separation between the seasons supported these possible explanations. Other studies have shown that the initial inoculum concentration, the light intensity and the functional characteristics may influence the establishment and development of microalgae (Forehead & O'Kelly, 2013; Lau *et al*., 1995; Margalef, 1978).

Extreme conditions for *C. vulgaris* and *S. ovalternus*

The Juan Amarillo wetland (hypereutrophic) presented extreme conditions, both for the species and the calcium alginate spheres. Although *C. vulgaris* and *S. ovalternus* have been associated with high pollution and high nutrient concentrations (Palmer, 1969; Pearson, 2003; Bellinger & Sigee, 2011), the harsh physicochemical characteristics of Juan Amarillo apparently exceeded the tolerance of these organisms, which caused a decrease in F_v/F_m and cell death. The low light availability, minimum oxygen concentration, excessive quantity of ammonium, elevated conductivity and high organic load registered in Juan Amarillo (Table 2) were probably the cause of the extremely adverse effects observed in the species (Hynes, 1960; Abelovich & Azov, 1976; Walsh, 1978; Konig, 1984; Konig *et al*., 1987; Mills, 1987; Pearson *et al*., 1987; Athayde, 2001; Pearson, 2003).

Ammonium concentrations over 2 mM have been reported as adverse to the photosynthetic process in *Scenedesmus* and *Chlorella* genera (Abeliovich & Azov, 1976) and may limit the absorption of nitrates (Parker *et al*., 2010). Although we did not analyse hydrogen sulphide (H_2S), the strong "rotten eggs" smell that was detected in Juan Amarillo indicated its presence (Camargo & Alonso, 2006). H_2S is toxic to eukaryotes (Admiraal & Peletier, 1979; Cohen *et al*., 1986), affecting oxygenic photosynthesis by blocking the electron transport chain (Fenchel & Finlay, 1995).

For light availability, Juan Amarillo had the highest turbidity and lowest light transmittance, which could explain the lower growth observed in the controls in this wetland compared to the other studied water bodies. Although light could be a limiting factor, it did not appear to affect the F_v/F_m of the *S. ovalternus* control, as evidenced by the values near 0.70 for F_v/F_m (Fig. 8 and Fig. 9). F_v/F_m readings at approximately 0.65 have been suggested as high for the healthy cells of some microalgae taxa (Kolber *et al*., 1988; Parkhill *et al*., 2001; Wang *et al*., 2014). Specifically for *S. ovalternus,* F_v/F_m values as high as 0.70 have been reported under laboratory nutrient-replete conditions and in light controlled circumstances (Delgadillo, 2014). The fluorescence of the *C. vulgaris* control in Juan Amarillo was also high. It was clear that the re-sealable bags protected the spheres from the inhospitable waters of this wetland.

The Juan Amarillo conditions also had a destabilizing effect on the calcium alginate matrix. In the first days of culture, the volume of the free spheres increased (nearly three times), causing a loss in the matrix compaction. Subsequently, the viscosity increased and fragmentation occurred in some spheres. By culturing alginate spheres in wastewater, Cruz *et al*. (2013) observed similar effects. In the rainy season, when the highest concentration of phosphate was observed, the effects on the alginate were so intense that by the ninth day of the experiment, the

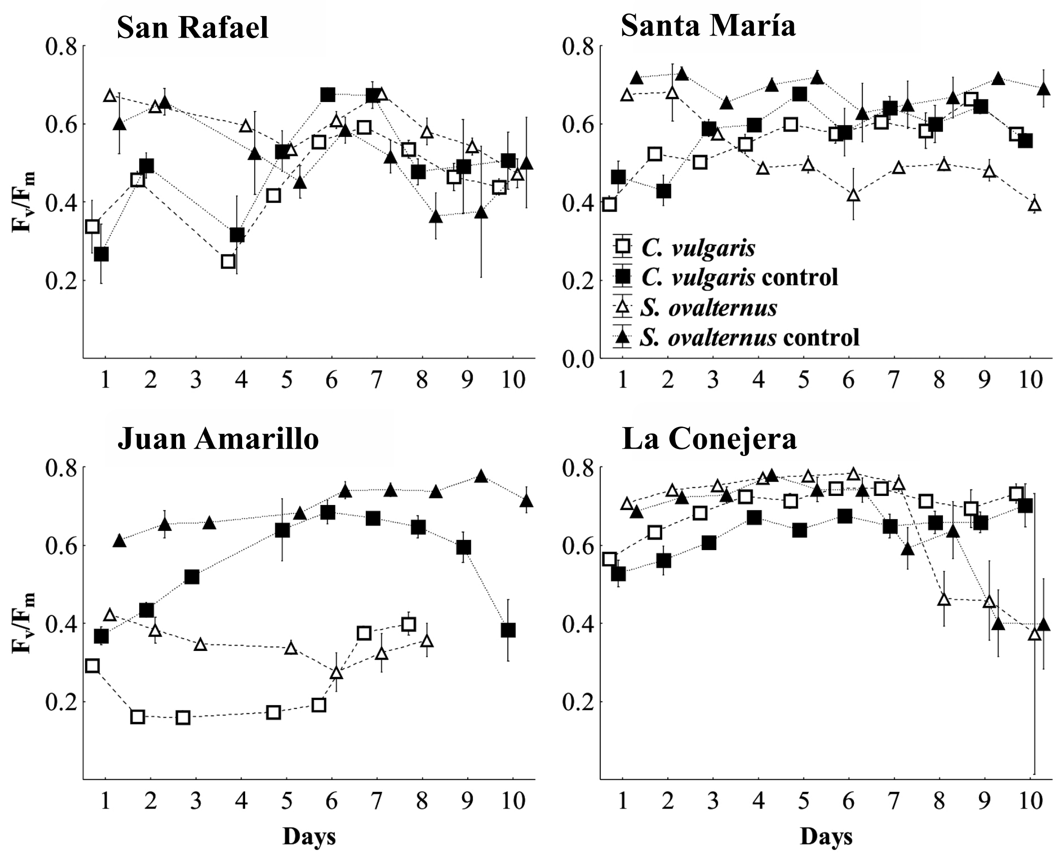

Figure 9. Daily variation of the F_v/F_m values of each species in the studied water bodies during the rainy season. The control treatments are shown with the symbols filled with black colour. *Variación diaria de los valores de* F_v/F_m *de cada especie en los cuerpos de agua estudiados durante la temporada lluviosa. Los controles se muestran con los símbolos rellenados con color negro.*

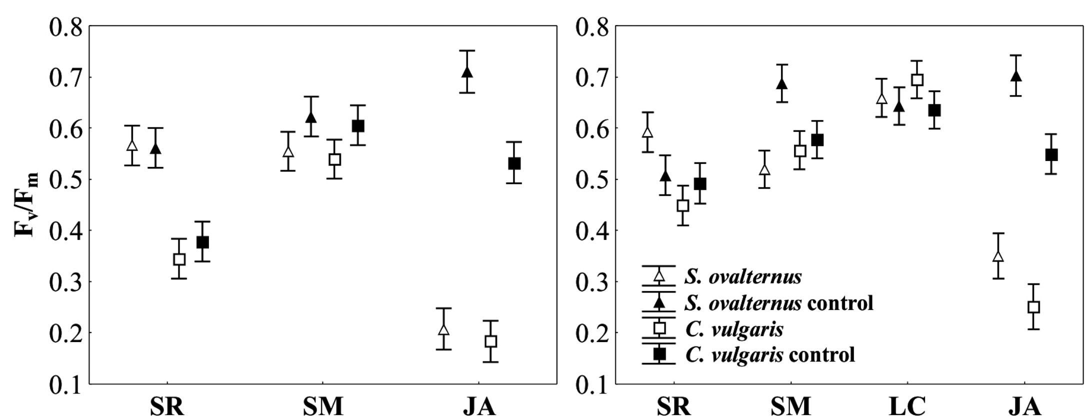

Figure 10. Variation of F_v/F_m values of the species and controls in the studied water bodies in the two climatic seasons (left: dry season; right: rainy season). The control treatments are shown with the symbols filled with black colour. *Variación de los valores de* F_v/F_m *de las especies y de los controles en los cuerpos de agua estudiados en las dos temporadas climáticas (izquierda: temporada seca; derecha: temporada lluviosa). Los controles se muestran con los símbolos rellenados con color negro.*

spheres were fully dissolved. Phosphate acts as a chelating agent and can remove Ca^{2+} cations (responsible for maintaining the structure of calcium alginate), leading to an initial increase in viscosity, a decrease in the elastic force of the gel, and further dilution (Draget *et al.*, 2004; Barsanti & Gualtieri, 2006; Donati & Paoletti, 2009). The loss of mechanical strength of the alginate probably favoured cell loss and influenced the population decline of the immobilized microalgae (Dainty *et al.*, 1986; Serp *et al.*, 2000; Moreira *et al.*, 2006).

Maximum quantum yield of PSII (F_v/F_m)

There were differences in the F_v/F_m measurements among the seasons and species. However, the variations of the F_v/F_m values were similar between the species and their respective controls (Fig. 10), indicating that photosynthetic efficiency was probably not affected by divergences in the nutrient concentration. The insensitivity of the F_v/F_m ratio to nutrient limitations have been reported before (Cullen *et al.*, 1992; MacIntyre *et al.*, 1997; Parkhill *et al.*, 2001), especially in studies where the nutrient concentration was constant and the cells were previously acclimated (balanced growth) (Bannister & Laws, 1980; Cullen *et al.*, 1992). Parkhill *et al.* (2001) indicated that if the limitation of a nutrient is constant in natural environments, it is likely to have some degree of balanced growth, which would limit the power of F_v/F_m as an indicator of nutrient stress. However, F_v/F_m values vary in situations where the availability of a limiting nutrient decays with cell growth (Cleveland & Perry, 1987; Kolber *et al.*, 1988). Thus, it is likely that the Santa María and San Rafael wetlands have a stable nutrient limitation and allowed balanced growth with little variation in the F_v/F_m values.

In San Rafael, Santa María and La Conejera, the F_v/F_m values of *C. vulgaris* and their controls varied similarly over time (Fig. 8 and Fig. 9), with low values at the beginning of the experiment and high ones at the end. This result suggested that the F_v/F_m parameter was influenced by factors unrelated to nutrient concentration. Other factors, such as light and temperature, also affect PSII and may modify the F_v/F_m response (Greene *et al.*, 1992; Matorin, 2000; Wozniak *et al.,* 2002). However, microalgae can be adapted, as it seemed to occur in our study, to situations of low temperature and high light intensity. Such adaptations may be reflected in increased F_v/F_m values (Matorin, 2000). The F_v/F_m readings suggest that *C. vulgaris* is more sensitive than *S. ovalternus* to environmental factors other than nutrients and needs more time to adapt to low temperatures and different light intensities. In many cases, the species and controls showed a decrease in the F_v/F_m data towards the end of the experiments, which was probably due to self-shading within the beads that was induced by increased cell density (Lau *et al.*, 1995).

Trophic categories

The trophic state of tropical aquatic ecosystems is routinely assessed by methods originally designed for environments of different latitudes. As noted before, our results indicate that trophic categorization may vary between methodologies because each approach prioritizes different factors (Lind *et al.*, 1992). Trophic indices based on phosphorus are used the most because it is assumed that this element limits primary production and is key in controlling eutrophication (Schindler, 1974; Correll, 1999; Sheela *et al.*, 2011). However, in this study, the phosphorus-based index was ineffective and placed all of the environments in hypereutrophy or eutrophy categories probably because the original categorization was created for environments with lower concentrations and ranges of phosphorus (Salas & Martino, 1991). It is also possible that phosphorus in the sediments had been resuspended because the samples were taken in the littoral zone. This could increase the concentration of this element.

Indices based on chlorophyll, nitrogen and the N:P ratio classified the four study wetlands into three trophic categories, but the classification was not consistent in all cases. This inconsistency between the indices indicates the need to develop an adequate trophic categorization for these tropical aquatic environments. Although there are methods for the evaluation of limnological characteristics in tropical high mountain wetlands (Davis & Rolls, 1987; Ehrenfeld, 2000; Pinilla, 2010; Alakananda *et al.*, 2011), there is no a particular trophic ranking for these aquatic environments. If possible, a new categorization for tropical wetlands should include new ranges of nutrient concentration and primary productivity data. As an initial approximation, in the Sabana de Bogotá wetlands, growth rates of encapsulated *C. vulgaris* and *S. ovalternus* species less than 0.15 would reflect a low concentration of nutrients, growth rates between 0.15-0.2 would reflect a moderate concentration of nutrients, and growth rates greater than 0.2 would reflect a high quantity of nutrients. Of course, more research will be needed to confirm and refine these preliminary values.

In conclusion, the different nutrient characteristics of the four studied environments seem to affect the growth of the two species; meanwhile, the maximum quantum yield of PSII was not apparently influenced by the nutrient supply. Thus, the growth dynamics of *C. vulgaris* and *S. ovalternus* immobilized in calcium alginate could be a tool with high potential for the characterization and monitoring of wetlands nutrient conditions. To optimize this tool, more data will be required to improve the preliminary proposed ranges of growth rates in order to reflect the nutrient concentrations.

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