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Macrophyte influence on the structure and productivity of photosynthetic picoplankton in wetlands

IRINA IZAGUIRRE[1]*, HAYDEE PIZARRO[1], PAULA DE TEZANOS PINTO[1], PATRICIA RODRÍGUEZ[1], INÉS O'FARRELL[1], FERNANDO UNREIN[2] AND JOSEP M. GASOL[3]

[1]FACULTAD DE CIENCIAS EXACTAS Y NATURALES—CONICET, UNIVERSIDAD DE BUENOS AIRES, ARGENTINA, [2]IIB-INTECH (INSTITUTO DE INVESTIGACIONES BIOTECNOLÓGICAS - INSTITUTO TECNOLÓGICO DE CHASCOMÚS)—CONICET, ARGENTINA AND [3]INSTITUT DE CIÈNCIES DEL MAR—CSIC, BARCELONA, SPAIN

*CORRESPONDING AUTHOR: iri@ege.fcen.uba.ar

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We used multiple approaches to analyze photosynthetic picoplankton (PPP) structure and production in a wetland in the Lower Paraná Basin (Argentina). A seasonal field survey was combined with an experimental study to analyze PPP variability under different light conditions. Flow cytometry analyses showed differences in PPP structure among the aquatic environments. Three main picoplankton populations were distinguished: phycocyanin-rich picocyanobacteria (Pcy), picoeukaryote (Peuk) and anaerobic anoxygenic photosynthetic bacteria (AnAnPB). The experimental study revealed important changes in PPP structure in relation to the light conditions imposed by floating plants: enclosures exposed to light showed a higher proportion of Peuk and Pcy over AnAnPB; in mesocosms covered by plants, just as in the highly vegetated relict lakes (ROLs), AnAnPB were dominant. Total picophytoplankton abundances varied from 1.7×10^4 to 4.6×10^5 cells mL^{-1} in the shallow lakes, and were lower (0.69×10^4 to 2.5×10^5 cells mL^{-1}) in the ROLs. Annual variations in temperature and hydrological conditions influenced the PPP abundances, observing maximum values during the warm dry phase. The photosynthetic rates per unit area of PPP (P_{APPP}) and algae >3 μm ($P_{A>3\ \mu m}$) were measured in the aquatic environments in winter and summer: $P_{A\ PPP}$ (1.5 to 100 mg C $m^{-2}h^{-1}$) was lower than $P_{A\ >3\ \mu m}$ and was directly affected by light, which was limiting under the dense floating plant cover.

INTRODUCTION

The importance of autotrophic picoplankton in lakes, and their role in pelagic food webs have been largely documented in the last two decades (Stockner and Porter, 1988; Weisse, 1993; Vörös *et al.*, 1998; Callieri and Stockner, 2002; Drakare, 2002). Picophytoplankton have been recognized as a significant fraction of freshwater phytoplankton in lakes of different latitudes, from polar and subpolar (Vincent, 2000; Izaguirre *et al.*, 2003) to tropical regions (Vuorio *et al.*, 2003; Sarmento *et al.*, 2008), including both deep and shallow water bodies, although most records refer to high latitude or temperate lakes (Bell and Kalff, 2001). Photosynthetic picoplankton (PPP) includes prokaryotic picocyanobacteria (Pcy) and picoeukaryotic phototrophs (Peuk). Despite many studies having revealed that the Peuk abundance is generally one order of magnitude lower than that of the Pcy, in some aquatic environments they can constitute an important fraction (Callieri, 2007 and cites therein). Anoxygenic anaerobic photosynthetic bacteria (AnAnPB) are another component of PPP, restricted to anoxic or poorly oxygenated waters (Pfennig and Trüper, 1989; Casamayor *et al.*, 2007; Zhang and Jiao, 2007). PPP are major players in carbon production in many aquatic systems, including

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extreme environments (Callieri and Stockner, 2002); their contribution to total carbon production ranges from 16 to 70% in most freshwater systems (Steitz and Velimirov, 1999; Callieri, 2007; Greisberger *et al.*, 2007) reaching 80% in Lake Baikal (Nagata *et al.*, 1994).

Different factors have been recognized as very important in the regulation of PPP structure, which were discussed by Callieri in her extensive review (Callieri, 2007): trophic state, lake morphometry thermal regime, hydrological retention time, nutrients, light conditions and biotic interactions.

Although picophytoplankton occurs all along the trophic gradient, it is widely accepted that their abundance and biomass increase with rising trophic state, whereas their contribution to total phytoplankton biomass and production decreases following the same gradient (Søndergaard, 1991; Stockner, 1991; Weisse, 1993; Sommaruga and Robarts, 1997; Bell and Kalff, 2001). However, a recent study on the PPP of the oligotrophic Lake Kivu (Africa) (Sarmento *et al.*, 2008) showed very high Pcy abundances, and suggested that the abundance of *Synechococcus*-like cyanobacteria in large oligotrophic lakes can also be very high. Moreover, in oligotrophic marine and freshwater systems, PPP are the major primary producers (Stockner and Antia, 1986; Callieri and Stockner, 2002). In eutrophic water bodies, their relative importance remains less clear (Camacho *et al.*, 2003); for example, this fraction exceeded 10^7 cells mL^{-1} in a hypereutrophic lake in Florida and contributed significantly to the phytoplankton biomass (Carrick and Schelske, 1997).

Among physical environmental factors, different studies have demonstrated that temperature influences the abundance and structure of picophytoplankton. In particular, a positive relationship between water temperature and PPP abundance was reported (Stockner and Porter, 1988; Agawin *et al.*, 2000). Considering the seasonal changes, in large deep lakes PPP maxima usually show a bimodal pattern, with a spring or early summer peak, and a second peak in autumn (Stockner *et al.*, 2000). In the shallow Lake Balaton, a pronounced seasonal pattern of picophytoplankton with peaks of Pcy in summer and Peuk in winter was observed (Mózes *et al.*, 2006). The cycles of mixing and stratification resulting from seasonal temperature changes also have a strong influence on the PPP distribution in the water column, and on their variations during the year (Padisák *et al.*, 1997; Gaedke and Weisse, 1998; Modenutti and Balseiro, 2002; Callieri *et al.*, 2007).

The influence of hydrological retention time of the lake on PPP has been comparatively less explored. Camacho *et al.* (Camacho *et al.*, 2003) found that the Pcy development was favoured by the stability of the water column and a high retention time.

Light quality and quantity constitute other key factors that influence PPP structure (Pick, 1991; Jasser and Arvola, 2003; Callieri, 2007). The variability in PPP structure among systems with different trophic status, particularly their pigment composition, has been related to differences in the light climate conditions. Phycocyanin (PC)-rich Pcy are usually dominant in eutrophic shallow lakes where red light prevails, whereas phycoerythrin (PE)- rich Pcy succeed in oligotrophic deep lakes dominated by green light (Vörös *et al.*, 1998; Camacho *et al.*, 2003; Callieri, 2007). Callieri *et al.* (Callieri *et al.*, 1996) experimentally demonstrated that PC-rich Pcy exploit red light better than PE-rich Pcy. In a recent study, Stomp *et al.* (Stomp *et al.*, 2007) analysed 70 aquatic ecosystems and confirmed that red Pcy (i.e. PE-rich) usually dominate in clear waters, whereas green Pcy (PC-rich) dominate in turbid waters; these authors also experimentally proved that the coexistence of red and green Pcy in lakes and seas may occur in waters of intermediate turbidity where niche differentiation is possible along the underwater light spectrum. However, there is evidence that in shallow eutrophic coastal waters, PE-rich Pcy can also be well represented (Jiao *et al.*, 2005; Vidal *et al.*, 2007). Regarding Peuk, some studies suggested that they seem to be favoured under severe light limitation conditions (Stockner and Antia, 1986; Craig, 1987; Søndergaard, 1991; Vörös *et al.*, 2009).

PPP may also be effectively controlled by grazing, mainly by heterotrophic nanoflagellates, small ciliates and mixotrophic algae (Porter *et al.*, 1985; Stockner and Antia, 1986; Sanders *et al.*, 1989; Šimek *et al*, 1995; Jones, 2000; Sherr and Sherr, 2002), and a high percentage of the carbon produced by PPP is taken up by protozoa and channelled to metazooplankton (Callieri, 2007 and cites therein).

In wetlands, the presence and relevance of PPP have not been well documented. Rahaingomanana *et al.* (Rahaingomanana *et al.*, 2002) reported a seasonal pattern in the contribution of PPP to total phytoplankton primary production in four lakes of different trophic status and with low nitrate concentrations from Rio Doce Valley in Brazil, some of them vegetated. They found that picoplanktonic contribution was higher in summer, accounting for up to 80% of total C uptake, and decreased in winter reaching between 10 and 25%. Angeler *et al.* (Angeler *et al.*, 2005) analysed the effect of water depth, macrophytes and fish on Pcy in a floodplain wetland from Spain, observing that the water depth affected the Pcy abundances. More recently, Vidal *et al.* (Vidal *et al.*, 2007) found that Peuk generally dominated over Pcy in subtropical coastal lagoons from Uruguay. There are few additional references to

picoplankton in wetlands from Argentina. A recent study described the autotrophic picoplankton structure in clear and turbid shallow lakes from the Pampa Plain (Allende *et al.*, 2009). In particular, for the wetland addressed in the present study, there is information obtained from two experimental studies, which revealed that picophytoplankton were dominated by Pcy (Sinistro *et al.*, 2006; de Tezanos Pinto *et al.*, 2007).

Among the environmental factors previously mentioned, light variability is particularly important in shallow lakes where macrophyte development affects directly and indirectly light quality and quantity. We hypothesize that in enriched vegetated floodplain shallow lakes, PPP structure and production are mainly affected by light climate in the water column, determining spatial differences among and within systems. Moreover, we test whether variations in PPP throughout the year are determined not only by seasonal temperature changes, but also by water level fluctuations. In this paper, we analyse PPP structure in a floodplain wetland of the Lower Paraná Basin, combining epifluorescence and flow cytometry (FC). We compare periods of contrasting temperatures and water levels, at sites differing in their underwater light climate conditions, mostly due to humic substances and to the degree of free-floating plant cover. We further examine the influence of light quantity by means of an experimental approach in field mesocosms, where we assess PPP changes in relation to light attenuation caused by floating macrophytes. We also estimate the photosynthetic rate per unit area (P_A) of the PPP and its relative contribution to the total P_A under different light conditions, analysing periods of contrasting temperatures.

METHOD

The aquatic environments studied are located in the Otamendi Natural Reserve, which is a natural floodplain wetland located in the Lower Paraná Basin (Buenos Aires Province, Argentina) delimited by the Paraná de las Palmas and Luján Rivers (34°14′S, 58°50′W) (Fig. 1). The area includes two permanent shallow lakes (Laguna Grande: 156 ha and El Pescado: 39 ha), which are connected by a channel, and several smaller semi-permanent water bodies, some of which are relict oxbow lakes. The two shallow lakes have a water depth mostly not exceeding 1.2 m, where more than 1% of the incident light reaches the bottom when the lakes are free of floating plants; the oxbow lakes are shallower with a maximum depth of 0.8 m. The dominant macrophytes in this wetland are the rooted *Schoenoplectus californicus* and *Scirpus giganteus* and several floating species such as *Ricciocarpus natans*, *Azolla filiculoides*, *Lemna minima*, *Wolffiella oblonga*, *Salvinia rotundifolia* and *Pistia stratiotes*. The climate of the region is defined as "temperate pampean humid" and precipitation occurs throughout the year, with a mean value of 950 mm. Drainage is poor and it is affected by groundwater fluctuations (Chichizola, 1993).

Following the classification by Williamson *et al.* (Williamson *et al.*, 1999), the aquatic systems in this wetland can be characterized as typical mixotrophic lake-ecosystems, which are characterized by high dissolved organic carbon (DOC) and total phosphorus contents. These water bodies differ in their underwater light conditions, as it has been described in our previous studies (O'Farrell *et al.*, 2003; Izaguirre *et al.*, 2004; Rodríguez and Pizarro, 2007) mainly due to the degree of development of free-floating plants, which reduce light penetration in the water column. Moreover, aquatic vegetation also affects the amount of dissolved organic substances, further contributing to the attenuation of underwater light.

Seasonal field survey

Sampling and physico-chemical analyses

Samples were collected from December 2004 to January 2006. Twelve sampling points were established (Fig. 1) in different types of aquatic environments within the wetland: Sites (S) 1, 2 and 3 (littoral, transitional and pelagial zones of Laguna Grande, respectively); sites 4 and 5 (channel connecting Laguna Grande with El Pescado); sites 6, 7 and 8 (pelagial, transitional and littoral zones of El Pescado, respectively); ROLs (R) 1, 2, 3 and 4 (sampling sites with contrasting macrophyte-cover in the relict oxbow lakes = ROLs). Sampling dates and characteristics were as follow: 6 December 2004 (late spring, dry period, abundant floating plants in littoral areas of the lakes and ROLs); 14 March 2005 (late summer, high waters, some floating vegetation at littoral zones and ROLs); 13 July 2005 (winter, high waters, floating plants restricted only to small patches of littoral areas); 24 January 2006 (summer, highest water period and lakes free of floating macrophytes). All samples were collected around noon.

Physical and chemical variables were measured *in situ* using portable electronic meters Hanna HI 9143, HI 991301 (Hanna Instruments, USA) for dissolved oxygen, temperature, pH and conductivity. Incident and underwater photosynthetically active radiation (PAR) were measured with a Li-Cor PAR spherical quantum sensor. Samples for nutrient and chlorophyll *a* analyses were collected at the subsurface in plastic bottles

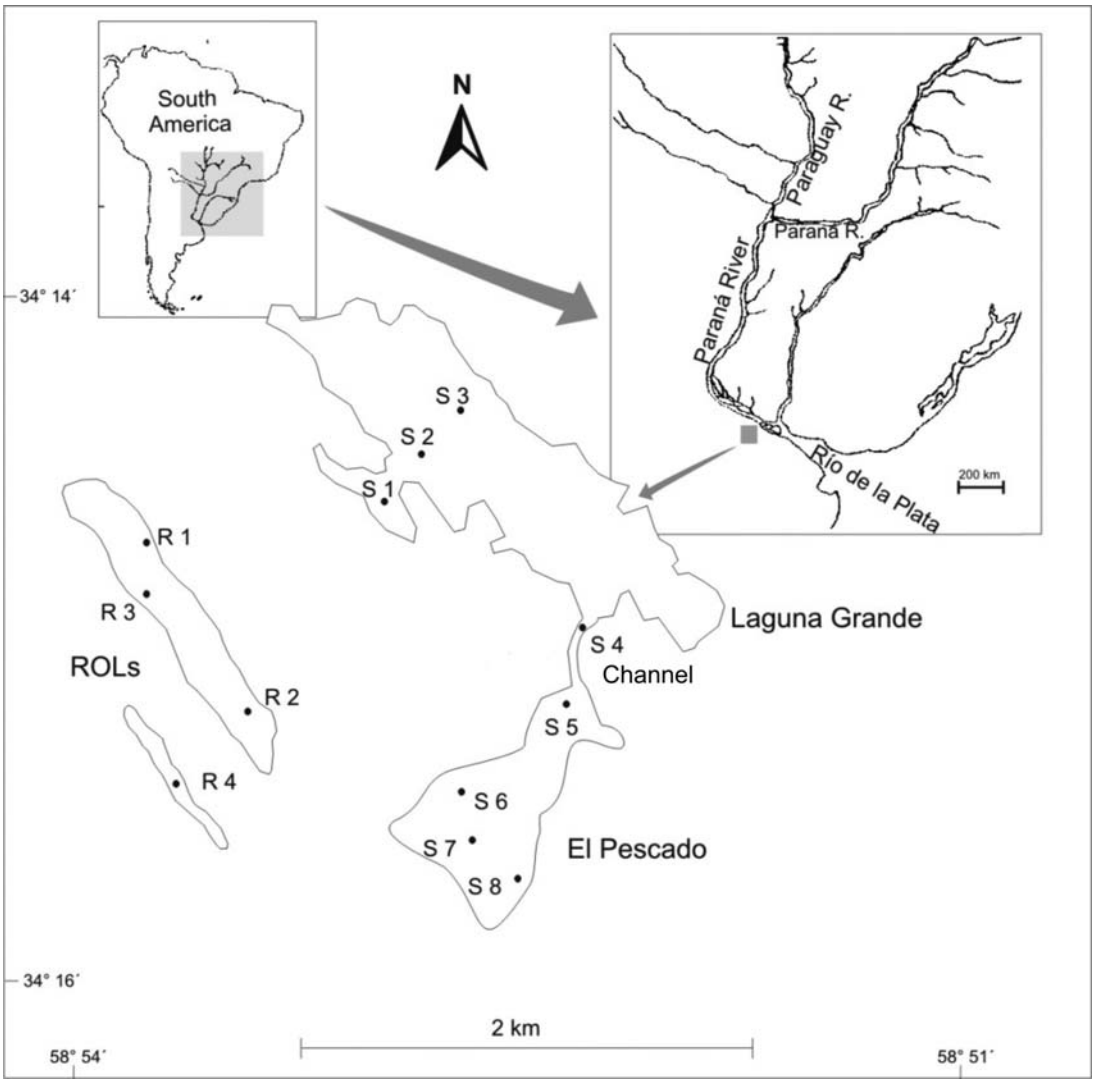

Fig. 1. Map showing the location of the water bodies studied and the sampling sites in the floodplain wetland: S, sampling sites in lakes and channel; *R*, sampling sites in the relict oxbow lakes (ROL*s*).

pre-rinsed in lake water and preserved in dark and cold conditions until their filtration in the laboratory (4 hours maximum) through fibreglass filters (Whatman GF/F). Phosphate and nitrate plus nitrite were analysed following the stannous chloride method, and cadmium reduction method respectively (APHA, 2005), with a Hach DR/2010 spectrophotometer and the corresponding kits of reagents Hach®. Ammonium (NH_4) was estimated by the phenate method (APHA, 2005). Humic substances were estimated by spectrophotometry reading the absorbance at 250 nm (Kronberg, 1999). Aliquots of filtered water (Whatman GF/F) were stored at 4°C for DOC analysis, which was determined in sonicated and acidified water samples using a high temperature Pt catalyst oxidation method (Shimadzu TOC-5000); this analysis was performed at the Université du Québec a Montréal. Suspended solids were determined drying the non-filterable (Whatman GF/C) residue at 103–105°C until constant weight (APHA, 2005).

The filters (Whatman GF/F) for the assessment of phytoplankton chlorophyll *a* were stored at −20°C for 24 h, and then pigments were extracted with hot ethanol (60–70°C). Chlorophyll *a* concentrations corrected for phaeopigments were measured with a spectrophotometer and calculated following Marker *et al.* (Marker *et al.*, 1980).

Picoplankton analyses

Samples for PPP analyses were collected avoiding the resuspension of bottom sediments from the 12 sampling sites on the four dates, and preserved in PVC flasks

with ice-cold filtered glutaraldehyde 2% final concentration. In the laboratory, subsamples (2 mL) were filtered on 0.2 μm pore-size black polycarbonate filters (Isopore GTPB). Filters were mounted on a microscope slide with immersion oil for fluorescence (Immersol 518 F). Using epifluorescence microscopy (Zeiss Axioplan), plastidic (assumed to be autotrophic) eukaryote picoplankton (Peuk) and pigmented (autotrophic) prokaryote picoplankton (Pcy) were identified by the fluorescence emitted by the photosynthetic pigments under blue and green light excitation (Callieri and Pinolini, 1995). The microscope was equipped with a HBO 50-W lamp, a plan-Apochromat x 100 objective and a filter set for blue light excitation (BP 450−490, FT 510 and LP 520 nm), green light excitation (BP 546, FT 580 and LP 590 nm) and UV excitation (BP 365, FT 395 and LP 397 nm).

To determine the relative proportion of picophytoplankton to other phytoplankton size fractions, we include in this paper the information of phytoplankton abundance and biomass corresponding to nano- and micro-phytoplankton counts, which were computed using the Utermöhl (Utermöhl, 1958) method. With this data set, we also analysed the seasonal variations of single and microcolonial pico-cyanobacteria.

An extra set of samples were collected only on January 2006 for flow cytometry analyses; 4 mL of lake water was immediately fixed with cold glutaraldehyde 10% (final concentration 1%), left in the dark for 10 min at room temperature and then stored at −70°C. We used a FacsCalibur (Becton and Dickinson) flow cytometer equipped with a standard blue laser 15 mW Argon-ion laser (488 nm emission) and a red laser (635 nm) diode. At least 30 000 events were acquired for each sample (usually 90 000 events). Fluorescent beads (1 μm, Fluoresbrite carboxylate microspheres, Polysciences Inc., Warrington, PA, USA) were added at a known density as internal standards. We used several parameters provided by the flow cytometer: 90° light scatter (SSC), which is considered to be related to the size and the internal granularity of the particles, the natural fluorescence of cyanobacterial phycoerythrines (FL2 channel at ca. 588 nm emission after blue light excitation) and phycocyanines (FL4 channel 670 nm emission after red light excitation), and chlorophyll and bacteriochlorophylls (red fluorescence: FL3 channel >650 nm emission after blue light excitation). The bead standard concentration was determined by epifluorescence microscopy. AnAnPB (anaerobic anoxygenic photosynthetic bacteria) were detected and differentiated from picocyanobacteria as explained previously (Casamayor *et al.*, 2007), based on their SSC signature, higher than that of other picoplanktonic organisms, and low >650 nm emission. This emission seems paradoxical since bacteriochlorophylls emit in the infrared (ca. 880 nm) after UV excitation. However, Casamayor *et al.* (Casamayor *et al.*, 2007) showed that they also emitted some fluorescence detectable by the >650 nm detector of a FacsCalibur when excited by blue (e.g. 488 nm) light. This is probably due to the fact that the per-cell pigment content of these organisms is large. Data analysis was performed with the Paint-A-Gate software (Becton and Dickinson).

Statistical analyses

The data obtained in the field survey were analysed by the ordination method RDA (redundancy analysis) after checking for the linearity of the data. Calculations were performed by the program CANOCO (Ter Braak, 1991). The analysis was based on Pcy singles, Pcy colonies and Peuk abundances, and on the environmental variables corresponding to each sampling site and date. Forward selection was used for adding environmental variables to the model. The significance of the ordination axes was assessed by Monte Carlo permutations.

Mesocosm experiments (contrasting light penetration due to free-floating macrophytes)

We also analysed by flow cytometry (FC) picoplankton samples collected during a mesocosm experimental study carried out in the littoral area of the Laguna Grande in spring 2003, whose main results were reported previously by de Tezanos Pinto *et al.* (de Tezanos Pinto *et al.*, 2007). The influence of free-floating plants on the structure of natural phytoplankton was assessed by duplicate in two treatments: enclosures completely covered by natural floating macrophytes and enclosures maintained cover-free. Cylindrical mesocosms (180 L, 46 cm depth) of transparent polycarbonate material were buried in the sediments of the littoral area to ensure isolation of the water column. Incoming lateral light was prevented due to a full coverage of floating plants outside the enclosures, mainly *Lemna minima*, *Wolffiella oblonga* and *Salvinia rotundifolia*. Temperature, conductivity, pH, dissolved oxygen and incident and underwater PAR were measured daily *in situ*; phytoplankton and nutrient samples were also collected daily. Here we analysed by FC samples collected at the initial (t_0) and final time (5 days: t_5) of the experiment. Samples were preserved with cold glutaraldehyde 10% (final concentration 1%). The procedure and details of the FC analyses are described earlier.

Primary productions

We compared areal photosynthetic rates (P_A) of the phytoplankton communities separated into the following size fractions: >3 μm and 3–0.2 μm (PPP). The analyses were done in Laguna Grande, El Pescado and in one ROL in August 2006 (winter) and January 2007 (summer), both during the wetland's dry periods. P–E curves were performed employing the ^{14}C assimilation technique as described in Holm-Hansen and Helbling (Holm-Hansen and Helbling, 1995). Incubations (2 h) were carried out under natural light on clear and sunny days near noon. Clear acrylic bottles (62.5 mL, sharp cut off at 400 nm) containing lake water were placed into a container (0.75 m^3) after the addition of 1 μCi of $NaH^{14}CO_3$ (Perkin Elmer Life Sciences, Inc., USA). The bottles were exposed in duplicate to 6–7 irradiances ranging between 0 and 100% of incident light, achieved using meshes of neutral attenuation. The samples were transported in cold and dark conditions to the laboratory.

Samples were filtered in darkness under low pressure filtration onto polycarbonate first through 3 μm and then through 0.2 μm pore filters. The filters were placed in scintillation vials in an atmosphere impregnated in HCl. Later, the scintillation cocktail (Opti-phase Hi Safe 3, Perkin Elmer, Life Sciences, Inc., USA) was added. The radioactivity was measured in a Beckman LS-6500 liquid scintillation counter with external quenching correction. To determine specific radioactivity in the sample, 1 mL of the incubated water was pipetted onto three drops of 0.1 N NaOH, mixed with the scintillation cocktail, and counted as described previously. Dissolved inorganic carbon (DIC) was determined from alkalinity, pH and temperature (Stumm and Morgan, 1996). Uptake rates of dark bottles were subtracted from those of the clear bottles. $P_{A\ PPP}$ and $P_{A\ >3\ \mu m}$ in the water column were calculated as the integral of the curve of production of each size fraction versus depth.

Chlorophyll *a* concentrations of each size fraction were estimated from water samples filtered sequentially through polycarbonate Millipore® filters of 3 and 0.2 μm pore size. After filtration, we followed the methodology described above for chlorophyll extraction. Physical and chemical variables of the water on each date were measured as described in the field sampling section. Incident and underwater PAR were measured every 5 cm in the water column to calculate the vertical attenuation coefficient (Kd) (Kirk, 1994). The mean irradiance (E_{mean}) in the water column was determined as the integral of the curve of PAR versus depth. The values of the parameter E_k (irradiance at the onset of light saturation) obtained from the P–E curves were compared with the E_{mean} intensities to estimate the potential light limitation. To estimate the humic content of the water, we measured absorbance at 440 nm (g440) on water filtered through polycarbonate filters of 0.2 μm pore size (Kirk, 1994).

RESULTS

Physico-chemical description

Mean values and ranges of the physical and chemical variables for the four aquatic systems during the seasonal survey are shown in Table I.

Water temperature followed the environmental seasonal fluctuations (Fig. 2), with slightly higher values at the ROLs. Water level fluctuations were mainly determined by total rainfall in the region ($r = 0.78$; $P < 0.05$); the lowest depth measured in the littoral zone of Laguna Grande was registered in December 2004 (22 cm), intermediate mean depths corresponded to March and July 2005 (58 and 45 cm, respectively). The highest water level coincided with the over-flowing among the different aquatic systems during January 2006 (85 cm). Dissolved oxygen showed marked variations among sites and dates that were mainly related to the presence of floating plants: the lowest concentrations (up to 2.88 mg L^{-1}) were observed under dense floating macrophyte-cover. In contrast, pH values were relatively constant, fluctuating around 8 in all systems, probably due to their high alkaline reserve (mean ranged between 5.2 and 8.8 mEq L^{-1}, Rodríguez, 2009). Conductivity variations were influenced by water level fluctuations: the highest conductivities (mean values 3348–6548 μS cm^{-1}) were recorded during the dry period and the lowest (mean values 884–1743 μS cm^{-1}) during the highest water period (Fig. 2). Comparing the different systems, highest mean values were registered at the ROLs. Suspended solid concentrations also varied according to water level: the highest mean values were observed in December 2004 (62–66 mg L^{-1}) and the lowest in January 2006 (11–20 mg L^{-1}).

Phosphate concentrations were high, with mean values varying between 14.2 and 77.1 μM; maximum values were observed at the ROLs. Dissolved inorganic nitrogen (DIN = ammonia + nitrate + nitrite) fluctuated strongly among sites and dates, and no definite spatial or temporal patterns were found; DIN was occasionally low in the shallow lakes, even below the limiting values reported for phytoplankton growth, *sensu* Reynolds (Reynolds, 2006).

Sampling sites differed in their underwater light conditions (Fig. 3). In the two shallow lakes (Laguna

Table I: Maximum, minimum and mean values (in brackets) of the physical and chemical variables measured in the four studied systems during the seasonal survey

	Laguna Grande	Channel	El Pescado	ROLs
Temperature (°C)	13.7–28.1 (22.7)	14–30 (23.9)	14.6–29.8 (24.4)	15.9–32.6 (26.6)
pH	8.2–8.5 (8.3)	7.64–8.3 (8.1)	7.8–8.3 (8.1)	7.7–8.3 (8.1)
Conductivity ($\mu S\ cm^{-1}$)	887–3513 (2317)	890–3340 (2341)	880–3353 (2385)	1743–6548 (3833)
Dissolved O_2 ($mg\ L^{-1}$)	4.4–6.5 (5.4)	5.5–7.5 (6.6)	4.7–7.4 (5.8)	3.3–8.8 (6.5)
% subsurface light trasm.	30–51 (43.1)	42.5–63.5 (53.1)	39.3–48 (45.4)	24–31 (27.6)
PO_4 (μM)	8.7–50 (35.2)	16.1–33.2 (25.2)	12.9–48.8 (29.0)	18.7–77.1 (54.2)
DIN (mM)	5.5–15.4 (10.1)	4.77–53.9 (19.2)	4.3–33.6 (14.9)	9.6–29.7 (17.1)
DOC ($mg\ L^{-1}$)	22.3–33.4 (28.1)	27.1–28.5 (27.8)	28.6–29.5 (29.0)	48.4–56.6 (53.0)
Suspended solids ($mg\ L^{-1}$)	10.7–131 (57.6)	26.5–47.5 (35.7)	11.3–75.7 (35.7)	20–83 (52.4)
Humic substances (Abs. 250 nm)	1.08–2.32 (1.67)	1.15–1.81 (1.48)	1.17–1.8 (1.47)	1.76–5.18 (3.23)
Chl *a* ($\mu g\ L^{-1}$)	23–101.6 (77.7)	5–76.2 (46.1)	2.7–66 (40.6)	48.3–188.8 (115.1)
Free-floating plants cover	Littoral: 0–70%; pelagial: 0%	0%	Littoral: 0–50%; pelagial: 0%	20–80%

Laguna Grande (sites 1–3); connecting channel (sites 4 and 5); El Pescado (sites 6–8) and relict oxbow lakes = ROLs (sites 9–12).

Grande and El Pescado), average light penetration was lower in the littoral (24–28%) than in the pelagial zones (55–58%). ROLs exhibited relatively low percentages of light penetration (24–30%). The more pronounced differences at each site were mainly related to the degree of development of free-floating plants: under a profuse macrophyte-cover light penetration was about 5% of the incident light (e.g. sites S1, S8, R3, R4). Differences in colour were also important in order to determine light variations in the pelagial sites. DOC concentrations differed among the aquatic systems: lowest values were observed in both shallow lakes and in their connecting channel (22.3–33.4 mg L^{-1}), whereas concentrations were about 2-fold higher in the ROLs (48.4–56.6 mg L^{-1}) (Table I).

Temporal variations of picophytoplankton

Phycocyanin-rich picocyanobacteria (Pcy) dominated in almost all picophytoplankton samples (generally >80%); picoeukaryotes (Peuk) only dominated numerically in December 2004 in the ROLs (Fig. 4). Their abundances as determined by epifluorescence ranged from 5.7×10^3 to 4.6×10^5 cells mL^{-1}. The two shallow lakes exhibited higher average densities than the ROLs: abundances ranged from 1.7×10^4 to 2.3×10^5 cells mL^{-1} in Laguna Grande, from 3.3×10^4 to 4.6×10^5 cells mL^{-1} in El Pescado and from 0.6×10^4 to 2.5×10^5 cells mL^{-1} in the ROLs. The lowest values were recorded in the highly vegetated ROLs with abundant rooted emergent plants (R4), whereas the highest densities corresponded to the channel (S4 and S5) and lake El Pescado (S6, S7 and S8) (Fig. 4).

Seasonal variations of picophytoplankton were highly influenced by the hydrometric level; the highest densities were registered in December 2004 under dry conditions and the lowest in January 2006 when the highest water phase occurred. No correlation was found between total picophytoplankton density and temperature ($P > 0.05$). The seasonal pattern of total picophytoplankton biovolume coincided with that of cell density, and values varied from 6.9×10^3 to 5.4×10^5 $\mu m^3 mL^{-1}$.

The contribution of picophytoplankton to total phytoplankton density was very important, mainly in both shallow lakes (on average 60%); however, their share of total phytoplankton biovolume was negligible (generally <1%).

Colonial forms of *Synechococcus*-like cells were mainly observed in both shallow lakes. Their temporal fluctuations mirrored the pattern of single Pcy cells (Fig. 5), thus suggesting an inverse relationship between the abundance of free picoplankton single cells and the formation of microcolonies. The proportion colonial Pcy/total phytoplankton was inversely correlated with the proportion single Pcy/total phytoplankton ($r = -0.44$; $P < 0.05$).

The results of the ordination of PPP components with respect to environmental variables for axes 1 and 2 are shown in Fig. 6. Among the variables analysed in the RDA, temperature, dissolved inorganic nitrogen (DIN), phosphate, percentage of subsurface light transmittance, humic substances and conductivity were included in the model by forward selection. Variables significant in terms of the variance explained in the distribution of the PPP components are shown by solid dark arrows. The first two axes accounted for 96.6% of the variance (axis 1: 59.0%; axis 2: 37.6%). The Monte Carlo unrestricted permutation test on the first Eigen-value indicated that the abiotic factors were significantly correlated with the first axis ($P = 0.03$); the test of significance of all canonical axes was also significant ($P = 0.01$). The first axis is mainly correlated with conductivity (intra-set correlation coefficient 0.45); the

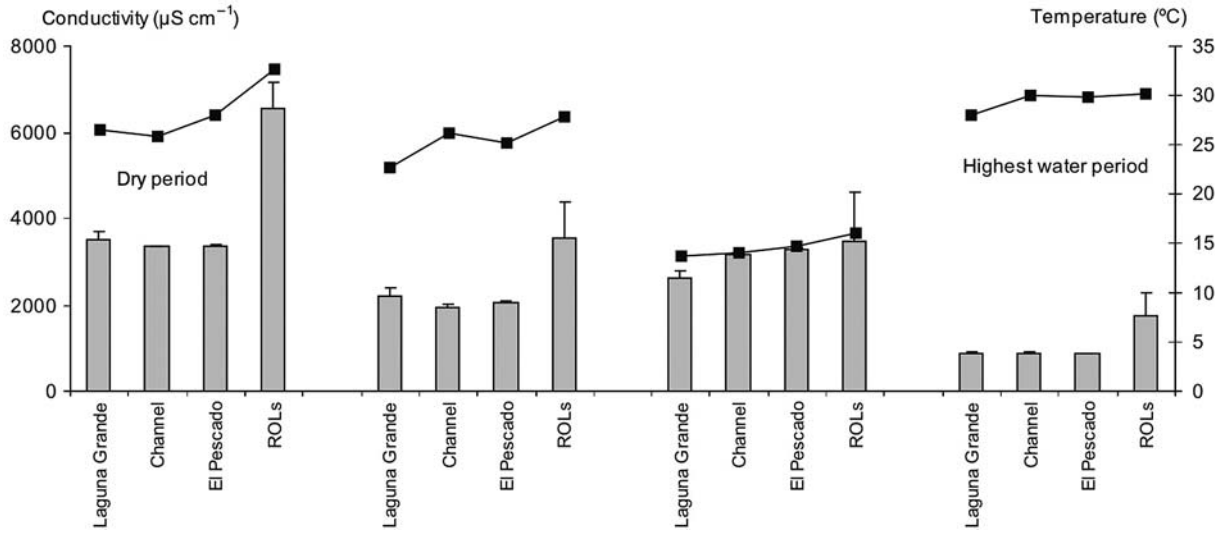

Fig. 2. Conductivity (bars) and temperature (line) values registered in the different water bodies during the seasonal survey.

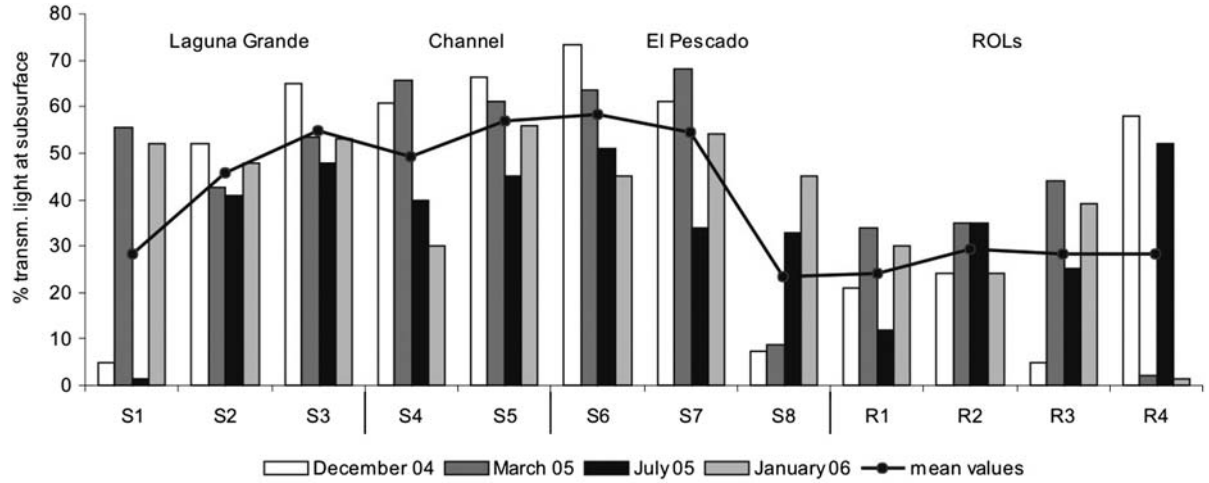

Fig. 3. Percentages and mean values of sub-superficial (10 cm) light (as a % of the incident light intensity) at each sampling site during the seasonal survey. The black circles indicate the average values for each site.

second axis is defined by a combination of humic substances and percentage of subsurface light transmittance (intra-set correlation coefficients: 0.51 and −0.39, respectively). The results of this analysis showed that most of the samples of the ROLs are ordinated in the upper part of the triplot where the percentage of light transmittance is lower, and the concentration of humic substances and DIN are higher. On the other hand, most of the samples of the two shallow lakes are plotted at the lower part of the figure with higher values of light penetration. Regarding the different sampling periods, most of the samples corresponding to January 2006 (high water period) are ordinated in the lower-left quadrant, where conductivity values are lower. As for the PPP components, the proportion of colonial Pcy and Peuk increases towards the upper-right side of the figure where most of the samples of ROLs R1, R2 and R3 are plotted. The proportion of Pcy is higher to the lower-right side with higher percentage of light penetration and temperatures, where all the samples of the two shallow lakes corresponding to December 2004 (dry period) are plotted.

Detailed analysis of PPP structure by flow cytometry

The flow cytometry (FC) analyses revealed the presence of four main groups of PPP in the aquatic systems of

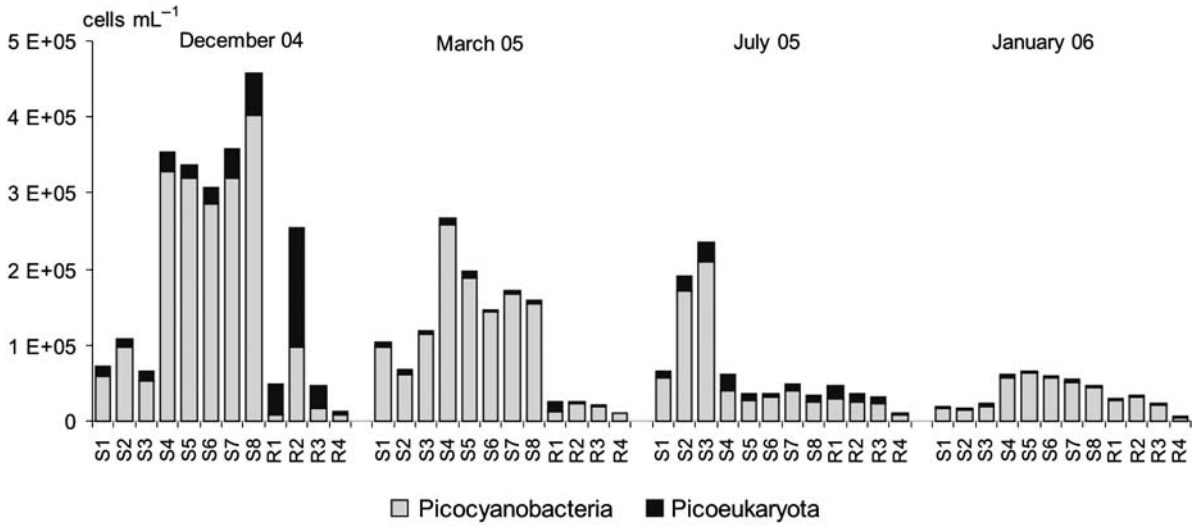

Fig. 4. Total picophytoplankton abundances of the Pcy and Peuk in the studied sites throughout the sampling period.

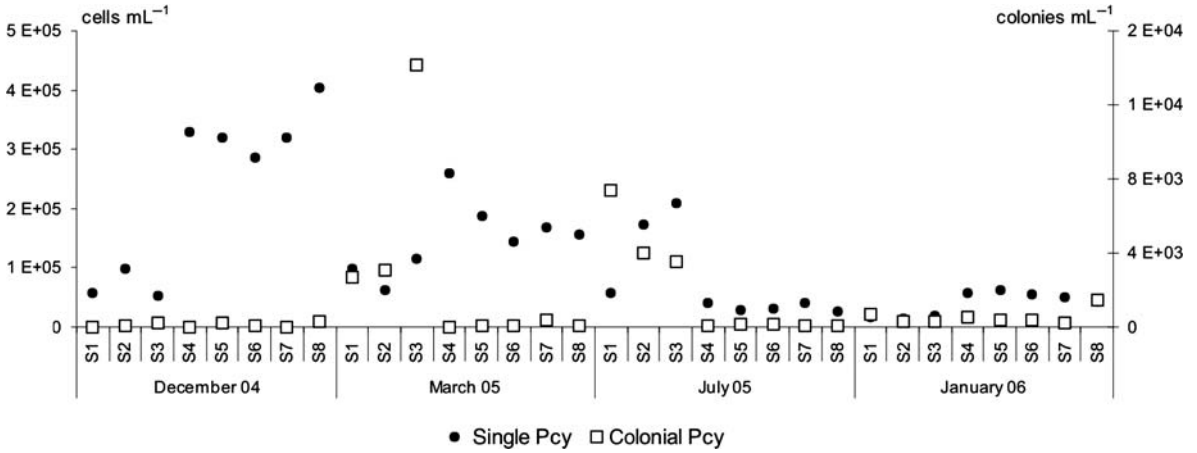

Fig. 5. Temporal fluctuations of the colonial forms of *Synechococcus*-like cells and single Pcy cells in Laguna Grande, the channel and El Pescado.

this wetland: Pcy (phycocianine-rich picocyanobacteria), Peuk1 (picoeukaryote population 1), Peuk2 (picoeukaryote population 2) and AnAnPB (anaerobic anoxygenic photosynthetic bacteria). Other Peuk populations were detected in some samples but with lower abundances. Figure 7 illustrates the four main populations retrieved in the experimental mesocosm study.

The FC analyses performed with the samples collected in January 2006 showed interesting differences in PPP structure among the aquatic environments of this wetland. In the two lakes Pcy and Peuk1 dominated over AnAnPB abundances that were very low; the proportion of AnAnPB was higher at the ROLs sites (Fig. 8A). Pcy and Peuk1 abundances were lower in Laguna Grande than in El Pescado: Pcy ranged from 1.5 to 8.8×10^4 cells mL^{-1} in Laguna Grande and from 1.2 to -1.7×10^5 cells mL^{-1} in El Pescado; Peuk1 ranged from 1.5 to 9.7×10^4 cells mL^{-1} in Laguna Grande, and from 7.8×10^4 to 1.1×10^5 cells mL^{-1} in El Pescado. In the ROLs, both Pcy and Peuk1 were less abundant (Pcy: undetectable to 2.63×10^4 cells mL^{-1}; Peuk 1: 8.07×10^3 to 1.38×10^4 cells mL^{-1}). AnAnPB were abundant in the ROLs, where anoxic conditions usually occur, varying between 4.3×10^4 and 1.6×10^5 cells mL^{-1}. Conversely, in the shallow lakes, AnAnPB densities were generally much lower; oxygen levels in these lakes are usually high.

The results obtained by FC and by epifluorescence for the same samples showed, in general, a good fit. The correlation between the two methods was

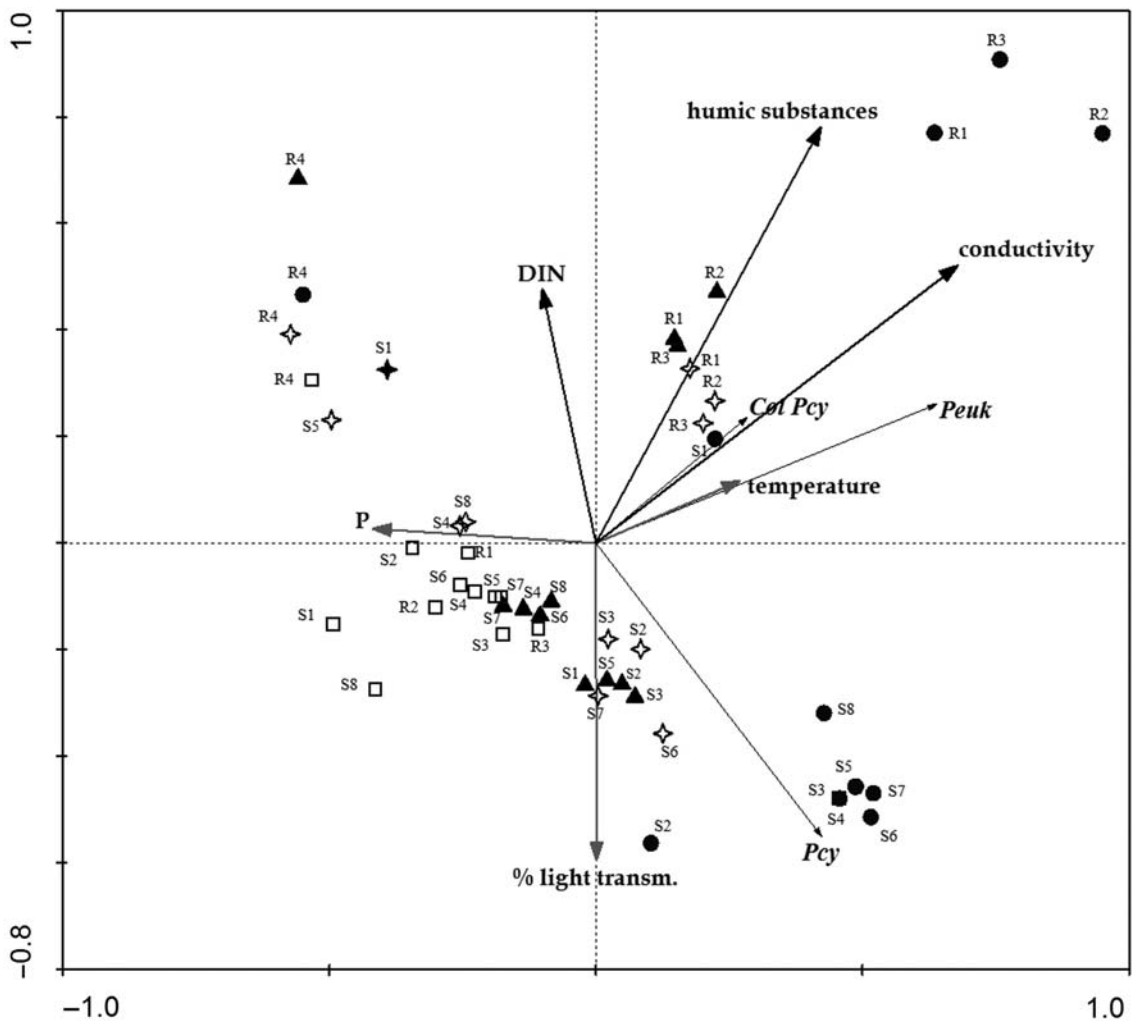

Fig. 6. Triplot on the redundancy analysis (RDA) of the abundances of PPP components from the 12 sampling sites surveyed in the Otamendi wetland. Environmental variables are indicated by arrows: solid lines correspond to significant variables. circles: December 2004 (dry period); triangles: March 2005; stars: July 2005; squares: January 2006 (high waters).

significant both for Pcy and Peuk1 ($r = 0.51$, $P < 0.05$, $n = 22$, with a 1.30 slope for Pcy; $r = 0.96$, $P < 0.05$, $n = 22$, with a 1.45 slope for Peuk1).

On the other hand, the results of the mesocosm experiments reflected the differences in PPP structure between contrasting light scenarios owing to free-floating plant cover. At the onset of the mesocosm experiment, the shallow lake was entirely covered by floating plants (100%), thus producing initial anoxic conditions for both treatments. The treatment without plant removal remained dark, and anoxic conditions prevailed throughout the experiment. Conversely, the removal of the free-floating macrophyte cover implied an improvement of the underwater light condition, a reversion of initial anoxia, a pH increase and a strong dissolved nutrient decrease at the end of the experiment (Table II). These environmental differences resulted in marked differences in PPP structure and abundance (Fig. 8B). AnAnPB completely dominated in all the anoxic scenarios established under the profuse free-floating plant cover. At day 0, AnAnPB, Pcy, Peuk1 and Peuk2 average abundances in the lake were 7.6×10^5, 1.8×10^4, 5.8×10^4 and 2.7×10^4 cells mL^{-1}, respectively. At this time, both treatments showed a picoplankton structure similar to that of the shallow lake. By the end of the experiment (day 5), a marked increase of the oxygenic picoplankton populations occurred in the enclosures without macrophyte-cover, and no AnAnPB were detected. The average densities for Pcy, Peuk1 and Peuk2 were 6.9×10^5, 6.2×10^5 and 1.4×10^4 cells mL^{-1}, respectively. In contrast, the treatment with floating plants exhibited a high AnAnPB abundance

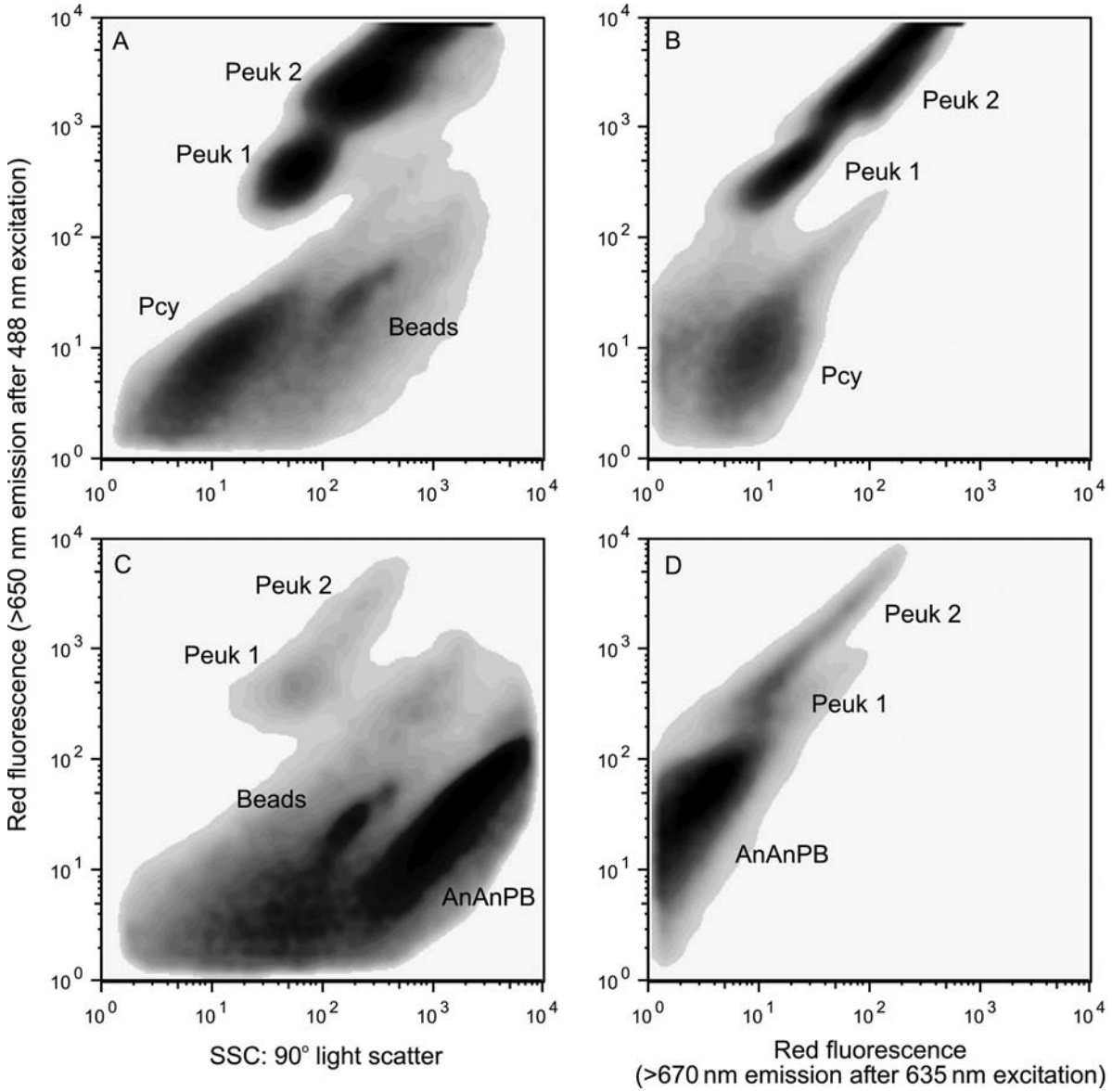

Fig. 7. Cytograms of the mesocosm experiment showing the photosynthetic picoplankton populations present in the treatment without floating macrophytes (**A** and **B**), and with floating macrophytes (**C** and **D**), at the end of the incubation period (5 days). Pcy: picocyanobacteria; Peuk1 and Peuk2: picoeukaryote; AnAnPB: anaerobic anoxygenic photosynthetic bacteria. The beads are yellow-green 1 μm (Polysciences). The "Y"-axis is the red fluorescence after blue-light excitation, while the "X" axis are side scatter (a surrogate of cell size) in (A) and (C), and the far red fluorescence after red-light excitation in (B) and (D).

(5.7×10^5 cells mL^{-1}) and low densities of the other oxygenic PPP populations. The total density of oxygenic and anoxygenic PPP for the two treatments is shown in Fig. 8B.

When integrating the data from the field survey and the mesocosm experiment, a direct significant relation between all the oxygenic picoplanktonic components (Pcy, Peuk1 and Peuk2) and percentage of light penetration ($r = 0.56$, $r = 0.56$, $r = 0.53$ respectively; $P < 0.05$) and dissolved oxygen concentrations ($r = 0.56$, $r = 0.56$, $r = 0.58$ respectively; $P < 0.05$) is revealed. On the other hand, AnAnPB were inversely correlated with light ($r = -0.72$; $P < 0.05$), dissolved oxygen ($r = -0.71$; $P < 0.05$) and directly with DIN ($r = 0.94$; $P < 0.05$) and conductivity ($r = 0.66$; $P < 0.05$).

Primary production

Water level in the wetland was low on the two sampling dates corresponding to primary production studies.

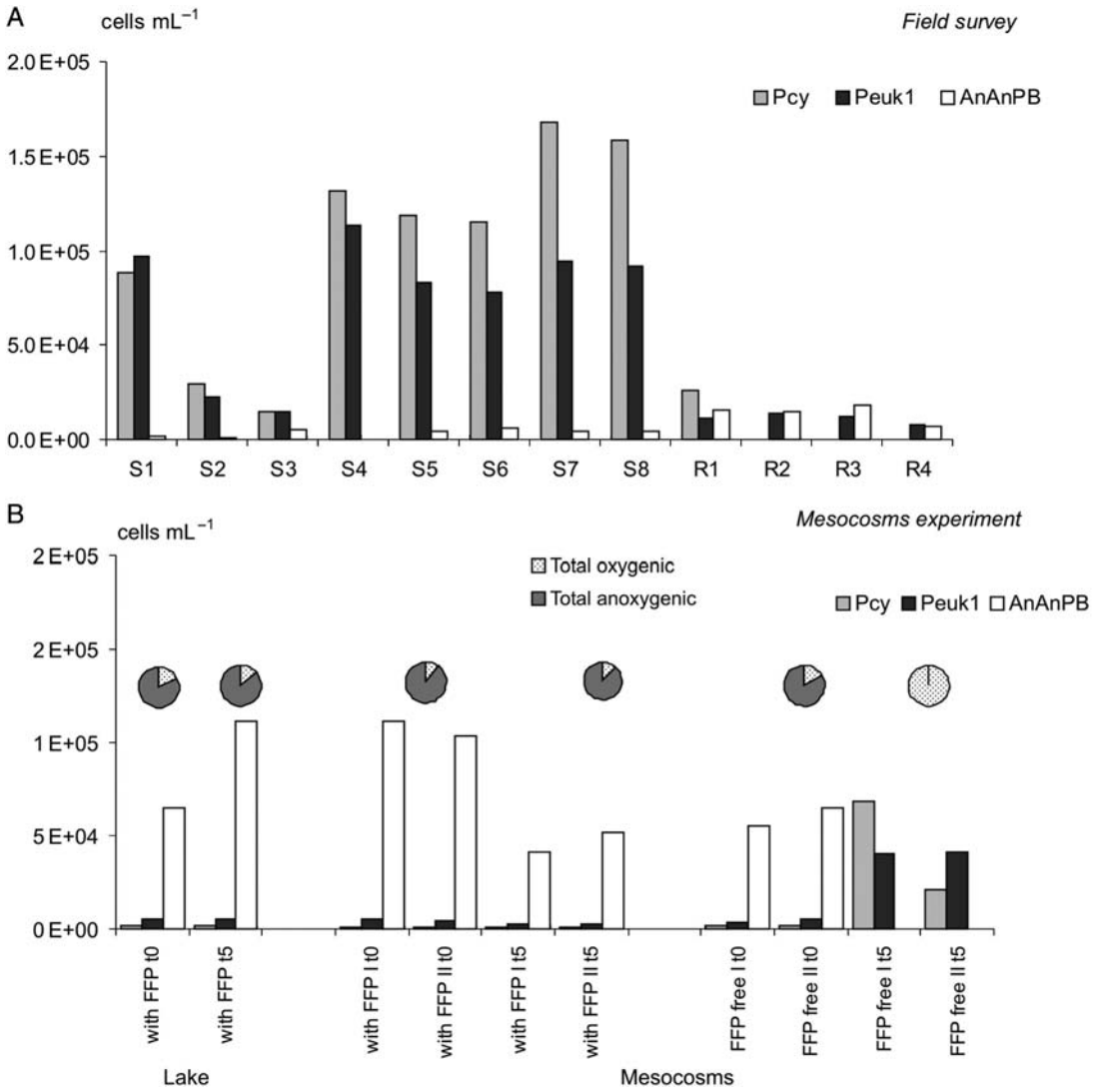

Fig. 8. (**A**) Concentrations of the main picoplankton populations identified by FC in the field survey in January 2006. (**B**) PPP structure and abundance in the mesocosm experiment; Pcy, picocyanobacteria; Peuk1, picoeukaryotes 1; AnAnPB, anaerobic anoxygenic photosynthetic bacteria; FFP, free-floating plants, t0, initial time; t5, day 5. Circles show the percentage of total oxygenic photosynthetic picoplankton in relation to total anoxygenic photosynthetic picoplankton.

The ROL was completely covered by free-floating plants on both sampling dates, whereas the opposite occurred in both shallow lakes. Moreover, the ROL showed higher concentration of humic substances (high Kd and g440) than the shallow lakes. In this water body, the E_k values both for PPP and phytoplankton >3 μm were higher than the E_{mean} on both sampling dates, thus implying light limitation (Table III).

Total production per unit area in the water column ($P_{A\ total}$, including both size fractions) fluctuated from 1.6 to 314 mg C m^{-2} h^{-1} (Fig. 9) and was highest in summer (Mann–Whitney test; $P < 0.05$). $P_{A\ PPP}$ ranged from 1.5 to 100 mg C m^{-2} h^{-1}, and $P_{A\ >3\ \mu m}$ from 0.1 to 214 mg C m^{-2} h^{-1}. Production per unit area for PPP and for algae >3 μm showed higher values in summer than in winter (Mann–Whitney test; $P < 0.05$). There was a trend to a higher contribution of PPP to total phytoplankton production in winter (45–93%) than in summer (22–49%) (Fig. 9). A strong seasonal shift in the PPP contribution occurred in the ROL, with a higher percentage in winter and lower in summer. $P_{A\ total}$, $P_{A\ PPP}$ and $P_{A>3\ \mu m}$ were significantly related to water temperature ($\log P_{A\ total} = -1.3 + 0.12$ temp; $r^2 = 0.83$; $\log P_{A\ PPP} = -1.12 + 0.1$ temp; $r^2 = 0.69$; $\log P_{A>3\ \mu m} = -2.54 + 0.17$ temp; $r^2 = 0.86$; $P < 0.05$). Furthermore, $P_{A\ total}$ and $P_{A\ PPP}$ were related to subsurface irradiance (E_0) ($\log P_{A\ total} = 0.54 + 0.0009\ E_0$; $r^2 = 0.61$; $\log P_{A\ PPP} = 0.21 + 0.0009\ E_0$;

Table II: Physical and chemical variables registered during the mesocosm experiment carried out in the littoral area of Laguna Grande in spring 2003 with contrasting light penetration due to floating plants (fp)

	t_o	t_5 (free waters)	t_5 (floating macrophytes)
Kd (m^{-1})	5	4.86	4.95
E_{mean} (μmol photons m^{-2} s^{-1})	33.6 (without fp); −0.04 (with fp)	314.6	0.46
% subsurface light transmittance	30 (without fp); −0.1 (with fp)	30	0.1
pH	7.3–7.5 (7.45)	8.5–8.7 (8.6)	7.3–7.4 (7.3)
Dissolved oxygen (mg L^{-1})	0	12.8–14.4 (13.8)	0
Temperature (°C)	26.2–26.7 (26.4)	24.9–25.5 (25.2)	24.1–25.5 (24.7)
PO_4 (μM)	13.5–16.5 (14.8)	6.45–7.09 (6.77)	13.5–15.5 (14.8)
DIN (mM)	41.7–55.4 (47.8)	1.78–4.26 (2.77)	20.6–60.6 (38.6)

t_0 (initial time) and t_5 (final time = 5 days)

Table III: Environmental variables estimated during winter 2006 (win 06) and summer 2007 (sum 07) in the aquatic systems studied when P–E curves were performed

	Laguna Grande		El Pescado		ROL	
	Win 06	Sum 07	Win 06	Sum 07	Win 06	Sum 07
Floating plants cover (%)	5	5	0	0	100	100
Water temperature (°C)	14.5	27.5	19	27	12.1	25.6
E_0 (μmol photon m^{-2} s^{-1})	764	1790	787	1745	70	16
E_{mean} (μmol photon m^{-2} s^{-1})	381	577	508	870	2.6	3
E_k phytoplankton >3 μm (μmol photons m^{-2} s^{-1})	101	345	35	450	258	298
E_k PPP (μmol photons m^{-2} s^{-1})	241	224	192	56	48	222
Humic substances g440 (m^{-1})	7.1	19	7.4	29	16	42
Kd (PAR) (m^{-1})	4	7	2	3.2	12	15

$r^2 = 0.74$; $P < 0.05$). However, $P_{A\ >3\ \mu m}$ was not affected by incident light intensity.

DISCUSSION

Picophytoplankton contributed a significant fraction to both phytoplankton abundance and productivity in the vegetated shallow lakes of the Otamendi Natural Reserve. Although, PC-rich Pcy were in general numerically dominant in this wetland, light quality and quantity determined the differences in the PPP structure among the systems: dark and anoxic environments had significant abundances of AnAnPB, and much lower aerobic Pcy. Regarding the seasonal variations, temperature stimulated the PPP abundance as there were more of them in summer, whereas overflowing of the aquatic system appeared to flush and dilute them.

Our previous investigations in this floodplain wetland showed the structuring role of the floating macrophytes on micro- and nano-phytoplankton (Izaguirre *et al.*, 2001a; O'Farrell *et al.*, 2003, 2007, 2009; Izaguirre *et al.*, 2004). Changes in the macrophyte cover led to shifts in the light availability that influenced the phytoplankton composition and structure (de Tezanos Pinto *et al.*, 2007), and the proportion of strictly autotrophic/heterotrophic + mixotrophic nanoplanktonic taxa (Sinistro *et al.*, 2006). In a similar way, the different approaches used in the present study confirm that free-floating plants also play a crucial role in structuring PPP. The changes in the macrophyte cover, which strongly affected the light availability and in turn modified the redox conditions in the water column, regulated the relative contribution of the PPP components. Flow cytometry analyses revealed that anaerobic anoxygenic photosynthetic bacteria (AnAnPB) were dominant in the anoxic dark experimental mesocosms with dense macrophyte cover and in the vegetated natural environments from the wetland which were frequently suboxic/anoxic due to the persistence of macrophyte cover. According to the flow cytometric signatures (high SSC 90° light scatter versus low FL3: red fluorescence), the AnAnPB identified would correspond to purple sulphur bacteria following the observations of Casamayor *et al.* (Casamayor *et al.*, 2007). These authors underlined the presence of phototrophic sulphur bacteria in a great variety of anoxic environments and also in water bodies with episodic oxygen depletion. Under well-illuminated water column conditions, different oxygenic picoplankton populations (Pcy and Peuk) reached higher

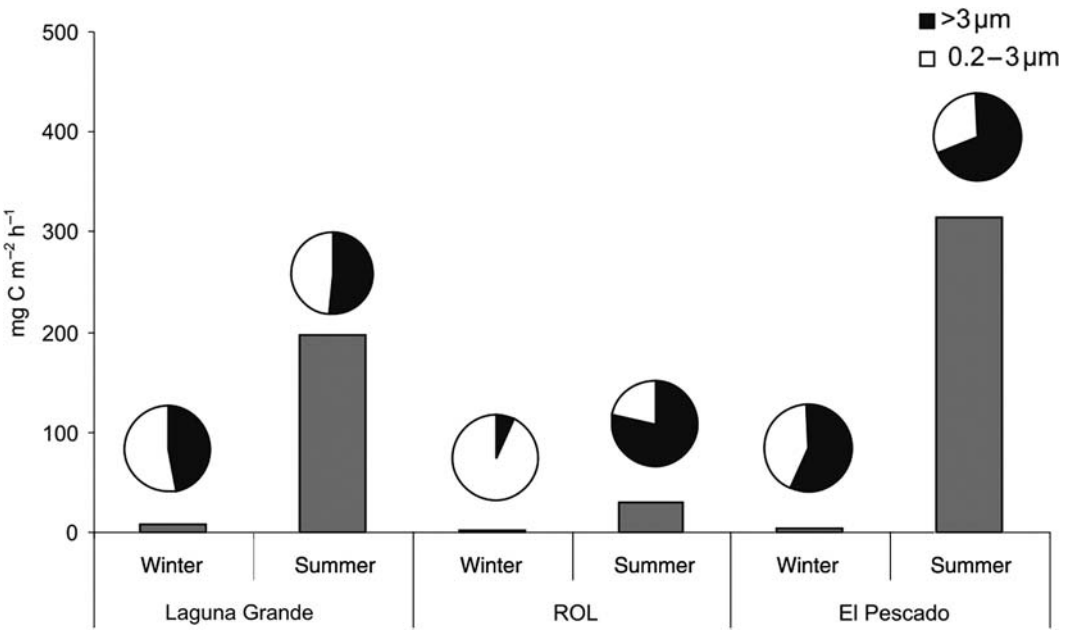

Fig. 9. Total phytoplankton production per unit area (P_A) (bars) and relative contribution of each size fraction to total P_A (circles).

abundances. Moreover, both the correlation analyses based on flow cytometry data and the RDA results showed that light enhanced oxygenic PPP abundances. The fact that picophytoplankton seem to be favoured by good light conditions has been mentioned by Drakare (Drakare, 2002) in a comparative study among lakes. Nevertheless, other studies have reported the success of picophytoplankton populations at low light intensities (Glover *et al.*, 1986; Padisák *et al.*, 1997; Callieri *et al.*, 2007). The differing results are probably associated with light quality rather than quantity, which also has a great importance for the PPP structure, as was observed by Vöros *et al.* (Vörös *et al.*, 1998). Callieri and Stockner (Callieri and Stockner, 2002) stressed the importance of light quality in the selection of different strains of picocyanobacteria, thus explaining why PPP maxima have been found at a variety of light intensities in different lakes. In our study, the influence of light quality on PPP is also shown. The dominance of phycocyanin-rich Pcy in the shallow lakes of the Otamendi wetland coincides with observations reporting that PC-rich Pcy are dominant in eutrophic and coloured shallow lakes (Vörös *et al.*, 1998; Camacho *et al.*, 2003; Jasser and Arvola, 2003), since they are chromatically better adapted to harvest the longer PAR wavelengths than phycoerythrin-rich cells, which dominate in large oligotrophic lakes (i.e. Sarmento *et al.*, 2008).

Among the different sites of this wetland, the lower abundances of PPP found in the relict lakes (ROLs) compared to the two shallow lakes may be related to the higher DOC concentrations derived from the abundant rooted and floating vegetation in the ROLs. Moreover, in the ROLs, there exists a permanent strong light attenuation due to the high content in humic substances, summed to the occasional presence of free-floating macrophytes. There is evidence that under poor light conditions and high DOC supply (typical of coloured humic lakes), bacterial production is more independent of DOC phytoplankton production, and bacteria have a competitive advantage for nutrients over algae (del Giorgio and Peters, 1993; Drakare, 2002). In this sense, Drakare *et al.* (Drakare *et al.*, 2003) observed a decreasing proportion of picophytoplankton with increasing DOC concentrations when comparing different lakes in Sweden.

Temporal fluctuations of picophytoplankton can be explained by a combination of changes in temperature and water level, as depicted in the RDA. The highest abundances of PPP (mainly Pcy) were observed during the warm dry period, whereas the lowest ones corresponded to the summer period of high hydrometric level and higher water flow between lakes. These results are comparable with those obtained by Camacho *et al.* (Camacho *et al.*, 2003) who found very scarce Pcy in lakes with low retention times during periods of high surface runoff. The typical seasonal pattern of picophytoplankton described for temperate lakes has usually a spring or early summer peak (Søndergaard, 1991; Gaedke and Weisse, 1998; Crosbie *et al.*, 2003).

Nevertheless, in our study, the hydrological changes overlapped the seasonal temperature regime, as it usually occurs in temperate floodplain lakes. Although high temperatures are known to promote enhanced PPP abundances (Stockner and Porter, 1988; Agawin *et al.*, 2000), in this system we observed highest abundances only in the summer 2004 with dry conditions, whereas in the summer 2006 a high hydrometric level accounted for low PPP densities. The overflow triggered a high connectivity that was shown by a relatively more homogeneous PPP structure. In tropical and subtropical regions, temporal fluctuations of phytoplankton communities from floodplain shallow lakes are driven by the hydrological regimes (de M. Huszar and Reynolds, 1997). In floodplain lakes located in temperate regions, both hydrological and temperature regimes regulate changes in biological communities throughout the year (Izaguirre *et al.*, 2001b).

Interestingly, we found that the temporal variation of single Pcy mirrored the pattern of cyanobacterial colonies potentially belonging to PPP; these correspond to microcolonies of four or more Pcy cells of similar or apparently identical morphology (Crosbie *et al.*, 2003). The grouping of picoplankton cells into colonies has been recorded by several authors (Caron *et al.*, 1985; Padisák *et al.*, 1997; Passoni and Callieri, 2000). The adaptive function of the Pcy colony-forming strategy has been understood in terms of a higher efficiency in nutrient recycling, protection against predators and/or protection against high irradiation (Stockner, 1991; Koblížek *et al.*, 2000; Callieri and Stockner, 2002; Komárková, 2002; Crosbie *et al.*, 2003; Callieri, 2007). Experimental studies performed by Jezberová and Komárková (Jezberová and Komárková, 2007) showed that the presence of mixotrophic flagellates (*Ochromonas* sp.) promoted the formation of colonies in Pcy. Schallenberg and Burns (Schallenberg and Burns, 2001) suggested that the aggregation could facilitate nitrogen fixation due to the formation of anoxic microsites within the cells, which would be necessary for the functioning of the nitrogenase enzyme. The grazing-avoidance hypothesis seems the more likely explanation in this vegetated system, since a high grazing pressure on Pcy was observed in an experimental study (Sinistro *et al.*, 2006), and recent analyses (unpublished data) have revealed that under the floating macrophytes the abundance of heterotrophic nanoflagellates (HNF) is three times higher than in open waters in Laguna Grande. The RDA performed confirms this idea because in this ordination the colonies are mainly located in the vegetated ROLS where HNF are very abundant.

Regarding PPP production, temperature and light were also recognized as key factors affecting temporal and spatial variations in our study, as higher production was observed in summer and in the better illuminated environments. Seasonal fluctuations in PPP production due to higher temperatures have also been shown before for different ecosystems (Joint *et al.*, 1986; Barkmann, 2000; Rahaingomanana *et al.*, 2002). The $P_{A\ total}$ in the ROL was markedly lower than in the shallow lakes probably due to the prevailing light limitation; this relict system presents light restrictions owing to the high concentration of coloured humic substances reflected in the high Kd and g440 values, and a dense free-floating macrophyte cover. Moreover, productivities in Laguna Grande and El Pescado were more affected than the ROL by temperature changes, due to the more constant ecological conditions provided by the persistent macrophyte cover of the ROLs (O'Farrell *et al.*, 2003). As for the effect of the water level on PPP production, this issue could not be addressed since the observations were only performed during low water level periods.

The recognition of key factors driving the ecology of picoplankton in vegetated shallow lakes is a challenge to correctly outline the theoretical framework of the functioning of these systems. In this paper, we contribute with novel data to the characterization of the structure and production of natural photosynthetic picoplankton in relation to the environmental driving parameters in vegetated floodplain wetlands from warm temperate climates, namely underwater light conditions strongly affected by floating plants and humic substances, temperature and hydrological regime. We conclude that free-floating plants play a crucial role in structuring PPP, regulating the relative contribution of their components. The combination of epifluorescence and flow cytometry allowed an accurate quantification and qualification of PPP populations.

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