

Nitrogen limitation of phytoplankton in a Spanish karst lake with a deep chlorophyll maximum: a nutrient enrichment bioassay approach

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An in vitro nutrient addition bioassay was performed to test the relative inorganic nitrogen (N) and phosphorus (P) limitation of phytoplankton in a Spanish karst lake (El Tejo) during the last part of the stratification period, when nutrient limitation is most pronounced. Nutrient deficiency was tested in samples from three different layers of the lake: the epilimnion, metalimnion and oxic hypolimnion. Nitrogen additions, either without or combined with P, increased phytoplankton growth in all three strata, compared with controls or P treatments. This showed that N was the nutrient limiting phytoplankton growth in late summer–early fall. Since both hypolimnetic diffusion and groundwater fluxes of N-rich waters into the lake are much reduced during summer, N becomes the limiting nutrient as stratification advances. We suggest that in this Mediterranean area with low atmospheric deposition of anthropogenic N and in lakes relatively free of surface run-off, nutrient supply by atmospheric deposition might be a key factor in controlling nutrient deficiency for phytoplankton growth.

INTRODUCTION

In order to better understand factors controlling production in aquatic ecosystems, it is useful to study nutrient limitation in geographically diverse areas, where the geology of the watersheds and the atmospheric deposition differ substantially (Perez *et al.*, 1998; Dore and Priscu, 2001). The relative flux of nitrogen (N) and phosphorus (P) into lakes is dependent on nutrient-rich discharges, the geology of the watershed, inputs from the airshed and *in situ* N fixation. The importance of nutrient inputs via the watershed and the impact of water pollution on the balance and magnitude of these have been recognized for decades. Only recently have we begun to recognize that atmospheric deposition may be an important nutrient source for freshwater lakes, rivers and estuaries, particularly where airsheds are heavily contaminated (Paerl, 1993). Human activities have doubled the supply of oxidized N and ammonia to global terrestrial ecosystems (Galloway, 1998; Matson *et al.*, 1999). It has been estimated that atmospheric N depo-

sition (either as wet- or dryfall) may contribute 10–50% to total external N load in aquatic systems (Loë-Pilot *et al.*, 1990; Paerl, 1993). Atmospheric deposition of nutrients, either as dissolved or particulate forms, can be an important source for phytoplankton growth in relatively pristine or oligotrophic waters (Psenner, 1984; Morris, 1991; Wolfe *et al.*, 2001). Although much work has focused on anthropogenic increases in N deposition (Jasby *et al.*, 1995), there is increasing evidence that nutrient-rich dust derived from arid regions can also increase P budgets in remote areas (Ahl, 1988; Psenner, 1999). For example, Rodá *et al.* reported that Saharan dust might impact southern European ecosystems (Rodá *et al.*, 1993). Phosphorus reportedly limits algal development in northern-temperate lakes (Schindler, 1977; Hecky and Kilham, 1988), whereas N limitation is more commonly found in subtropical and tropical lakes (Wurtsbaugh *et al.*, 1985; Pollinger *et al.*, 1988), which are usually less affected by atmospheric N deposition (Galloway, 1998). Nevertheless, Elser *et al.* pointed out that the role of N as a limiting nutrient in fresh water has probably been underestimated

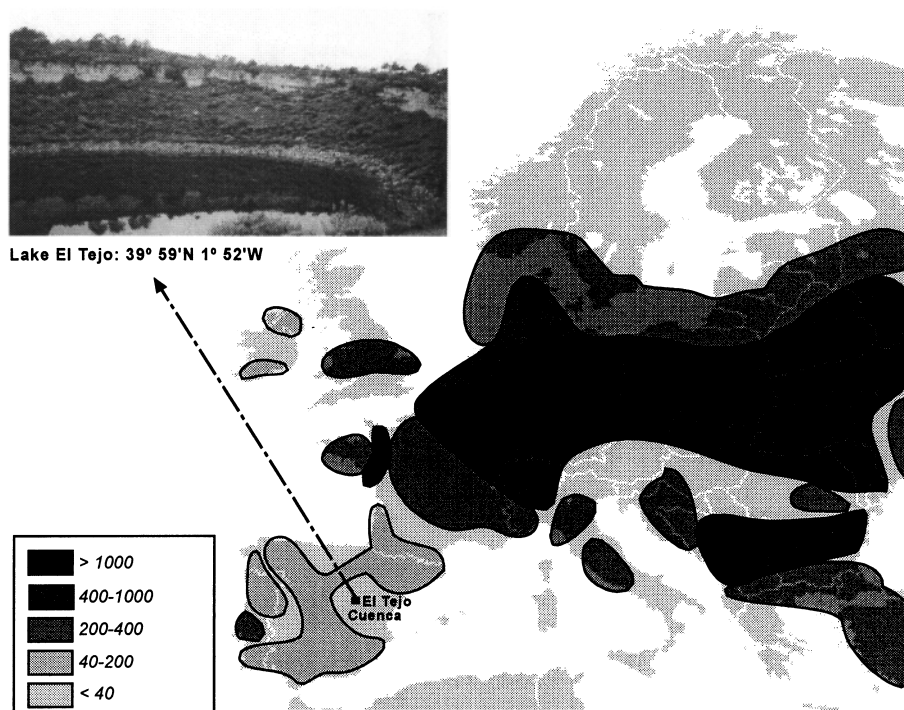


Fig. 1. Map of Europe showing atmospheric N deposition in $\text{eq ha}^{-1} \text{year}^{-1}$ (redrawn from data by European Environmental Agency, 1998) and the location of Lake El Tejo in the Iberian Peninsula, with a photograph of the lake.

in north-temperate lakes, particularly those in western North America (Elser *et al.*, 1990).

Here we report analyses of Lake El Tejo, a karst lake in east-central Spain, where both the watershed and the airshed are relatively clean compared with many areas in central and northern Europe (Figure 1). Enrichment bioassays performed on a time scale of days were used to evaluate the separate and interactive roles of N and P as limiting nutrients for phytoplankton growth. Additionally, we investigated whether nutrient additions would differentially influence phytoplankton in the epilimnion and in two strata of the deep chlorophyll (Chl) maximum in the lake.

METHOD

Study site

Lake El Tejo is located (Figure 1) in the karstic area of Cuenca (central-eastern Spain), 1000 m above sea level, formed in the dolomites of the upper Cretaceous. It is a closed, hard-water lake located at the lower part of a circular 300-m-diameter and 80-m-deep doline. Lake level is determined by a perched local water table. At the time of the study, respective mean and maximum lake depths were 11.9 and 26.4 m, and the median lake

diameter was 145 m. The watershed, covered primarily by pine forest, lies in one of the least populated regions in Spain, where the air quality is among the highest in Europe (European Environmental Agency, 1998). Recreational use of the lake is very limited because access is via a 0.4-km-long footpath.

Limnological variables

Lake El Tejo was sampled weekly from September to mid-October 1997 and monthly until the end of the year. Temperature, dissolved oxygen, conductivity, pH and Eh were measured at 0.5- to 1-m intervals with the appropriate calibrated sensors. Vertical profiles of photosynthetically active radiation (PAR; 400–700 nm) were measured with a 4π scalar irradiance sensor. Water samples for chemical analyses were filtered through Whatman GF/F filters on our boat, poured into glassware and plastic bottles that had previously been acid washed, and preserved immediately after collection. The samples were held on ice and inorganic nutrients were analysed within 36 h. Nitrate plus nitrite, ammonia and orthophosphate were determined according to Golterman *et al.* (Golterman *et al.*, 1978). A capped spring with a tap, perched half way up the doline, was also sampled periodically for nutrient concentrations.

Chlorophyll *a* was measured spectrophotometrically

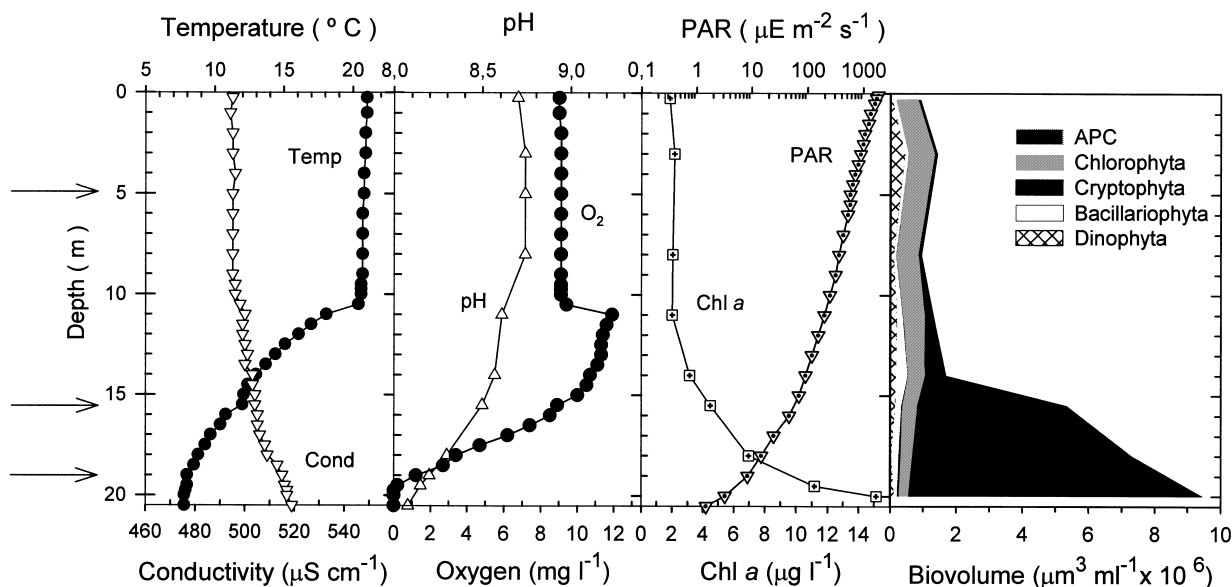


Fig. 2. Vertical profiles of several physical and chemical variables and phytoplankton abundances (as biovolume) in Lake El Tejo on September 30, 1997, when samples were obtained from three depths (arrows) for the bioassay experiment.

and calculated according to Jeffrey and Humphrey (Jeffrey and Humphrey, 1975). *In vivo* absorption spectra of the samples (Trüper and Yentsch, 1967) were used to confirm the distribution of phycoerythrin-containing picocyanobacteria. Algal numbers were determined with an inverted microscope at $\times 400$ and $\times 1000$ from Lugol-fixed samples using the Utermöhl sedimentation method (Utermöhl, 1958). A number of individual cells were measured and geometric shapes were used to determine biovolumes (Rott, 1981). Autotrophic picoplankton were counted on $0.2\ \mu\text{m}$ black polycarbonate filters (Isopore GTBP; Millipore) with a fluorescence microscope at $\times 1250$ (MacIsaac and Stockner, 1993).

Bioassay design

Whole-water samples, collected at the centre of the lake on 30 September 1997, from three depth strata (epilimnion, 5 m; metalimnion, 15.5 m; oxie hypolimnion, 19 m) were used for a microcosm experiment. Subsamples of 900 ml were placed in acid-washed, 1 l glass bottles. Four treatments with three replicates were tested for each depth: control without nutrient additions (C); NH_4NO_3 added to increment the N concentration by $30\ \mu\text{mol l}^{-1}$ (N); Na_2HPO_4 to increment the P concentration by $2\ \mu\text{mol l}^{-1}$ (P); and both N and P enrichments together (N+P). Nutrients were added every 2 days. Microcosms were incubated for 8 days in thermostatic light chambers with a 16/8 h light/dark photoperiod, under the following conditions, which mimicked those in the lake: (i) epilimnetic at 21°C and $490\ \mu\text{mol quanta m}^{-2}\text{ s}^{-1}$;

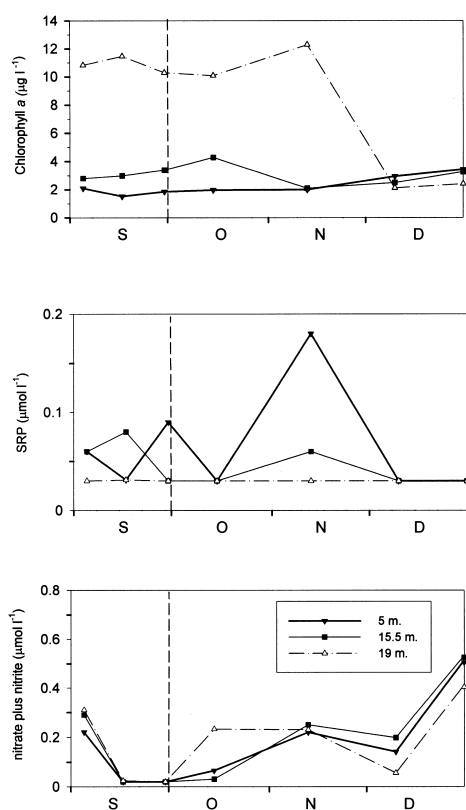
(ii) metalimnetic at 12°C and $130\ \mu\text{mol quanta m}^{-2}\text{ s}^{-1}$; and (iii) hypolimnetic at 10°C and $14\ \mu\text{mol quanta m}^{-2}\text{ s}^{-1}$. The flasks were swirled twice daily to resuspend the plankton. The Chl *a* concentration was determined for each microcosm at the beginning of the experiment, on day 4, and at the end of the experiment (day 8), whereas phyto- and picoplanktonic densities and biovolumes were determined at the beginning (day 0) and at the end (day 8) of the experiment. For each depth, one-way univariate ANOVAs and *post hoc* analyses (Duncan test) were used to test for statistically significant differences between nutrient treatments using SPSS 10.0 statistical software.

RESULTS

Characterization of Lake El Tejo at the end of the stratification

Lake El Tejo is a highly stratified lake. At the end of September 1997 (Figure 2), when the bioassay experiment was performed, the epilimnion extended to 11 m and the metalimnion was located between 11 and 18 m. After a metalimnetic oxygen maximum, oxygen began to decline from 16 m depth, becoming undersaturated below that depth and an oxie-anoxic interface was established at 19.5–20 m. Light availability at the selected depths of the bioassay, i.e. 5, 15.5 and 19 m, were 30, 7 and 0.5% of surface irradiance, respectively. The 0.5% level represented a midday PAR of $10\ \mu\text{mol quanta m}^{-2}\text{ s}^{-1}$. At these

A



B

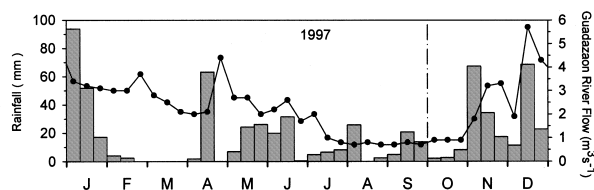


Fig. 3. (A) Temporal changes in concentrations of Chl *a*, nitrate plus nitrite and SRP in Lake El Tejo at the depths selected for the bioassay (5, 15.5 and 19 m) from September to December 1997. A dashed line indicates the day of sample collection for the bioassay (detection levels were $0.02 \mu\text{mol l}^{-1}$ for nitrate plus nitrite and $0.03 \mu\text{mol l}^{-1}$ for P). (B) Hydrograph of the River Guadazaon (line; $\text{m}^3 \text{s}^{-1}$, averaged for periods of 10 days) and rainfall from a meteorological station nearby Lake El Tejo (bars; in mm accumulated for 10 days).

depths (5, 15.5 and 19 m), pH was 8.7, 8.5 and 8.2, respectively.

Soluble inorganic N and P were very scarce in Lake El Tejo during the last part of stratification. During September and October, nitrate plus nitrite were always $<0.3 \mu\text{mol l}^{-1}$ and soluble reactive phosphorus (SRP) concentrations were $<0.2 \mu\text{mol l}^{-1}$ (Figure 3). Ammonia concentrations during these months were below the detection level ($0.2 \mu\text{mol}$), therefore they are not plotted in Figure 3. During the same period, the perched spring

in the doline had nitrate concentrations between 55 and $82 \mu\text{mol l}^{-1}$, and SRP concentrations below detection ($<0.03 \mu\text{mol l}^{-1}$), which are indicative of the groundwater nutrient content, although the spring flowed from a very small aquifer located above the water table connected with the lake.

Since the lake is a closed water body, nutrient input is highly related to groundwater inflow. During summer and early autumn, rainfall was very low. Figure 3 also shows precipitation measured 1 km from the lake, as well as the water flow of a nearby small river, whose discharge may be used to indicate recharge periods in the aquifer feeding Lake El Tejo. These data indicate that inflow during summer decreases markedly. From May to October, the water level in Lake El Tejo fell, indicating that inflows were too low to compensate for lake evaporation.

At the end of stratification, Chl *a* concentrations (Figures 2 and 3) in the epilimnion were relatively low, increased through the metalimnion and peaked in the oxic hypolimnion. However, during autumn mixis (December 1997), Chl *a* concentrations in surface waters were higher than in deeper waters. At the time of the bioassay, the abundance of the different algal groups in Lake El Tejo changed markedly with depth (Figure 2). Epilimnetic phytoplankton were dominated by eukaryotic algae, mainly Chlorophyta (*Lagerheimia quadriseta*, *Cosmarium* spp. and *Tetradron minimum*), diatoms (*Fragilaria acus* and *Cyclotella wuethrichiana*) and dinophytes (*Peridinium* spp.). In contrast, phycoerythrin-containing unicellular picocyanobacteria were the dominant phototrophs in the metalimnetic waters, although all of the mentioned taxa were also present. In the bottom part of the oxic hypolimnion, where the highest Chl *a* concentrations were found (Figure 2), picocyanobacteria accounted for most of the algal biovolume and formed a deep Chl maximum. Cryptophyta (*Cryptomonas phaseolus*) and Euglenophyta (*Euglena agilis*), although not very abundant, increased their relative importance in this layer with respect to upper layers.

Bioassay

Nutrient concentrations were very low at the time of the bioassay (Figure 3). Nitrate, ammonia and SRP were below the detection level in the water used for the bioassay, except for P in the epilimnetic water (which was $0.08 \mu\text{mol P l}^{-1}$). Nitrogen additions (N treatment) significantly increased Chl *a* concentrations and algal abundance (as biovolume) with respect to both controls (C) and P additions (P treatments) in all three strata (Table I; Figure 4). In the epi- and metalimnion, N treatments approximately doubled Chl levels compared with controls, but in the hypolimnion the response to N treatment was reduced, although still significantly higher. No

Table I: ANOVA results from the bioassay with water samples from the different strata of Lake El Tejo

	Chl <i>a</i>				Total phytoplankton/APC/chlorophytes		
	C	N	P		C	N	P
C				C			
N	E,M,H			N	E, M, H		
P		E,M,H		P		E, M, H	
N+P	E,M,H	E,M,H	E,M,H	N+P	E, M, H	E, M	E, M, H
Diatoms				Cryptophytes			
	C	N	P		C	N	P
C				C			
N	H			N	H		
P		E, H		P		H	
N+P	E, M, H	E, M, H	E, M, H	N+P	H	H	H

Significant differences ($P < 0.05$) of the treatments: nitrogen additions (N), phosphorus additions (P) and additions of both nutrients (N+P) to control (C) and between treatments, for Chl *a* concentrations ($t = 4$ and $t = 8$ days) and for final ($t = 8$ days) biovolume of total phytoplankton (nanophytoplankton and autotrophic picocyanobacteria; APC) as well as for biovolumes of the different groups separately (APC, chlorophytes, diatoms and cryptophytes) are indicated by the initial of the corresponding strata (E, epilimnion; M, metalimnion; H, hypolimnion). The analyses with total phytoplankton, APC or chlorophytes yielded the same results, thus only one hemimatrix with the three headings is shown together for the three groups.

significant differences were found between controls and the P treatments. However, when P was added concurrently with N (N+P treatments), growth was far higher than when N was added alone, except in the hypolimnion, where Chl *a* still increased, but not so algal biovolume. Autotrophic picocyanobacteria and Chlorophyta significantly increased in all depths when N (either N or N+P) was added compared with controls or P treatments. In contrast, diatom growth was primarily stimulated by N+P treatments (all three strata) and only in the hypolimnetic strata did the N treatment have a significant effect on this algal group. Cryptophyta were very scarce, showing only some abundance in the hypolimnion; in that strata they were also stimulated by N or N+P additions.

DISCUSSION

Nitrogen additions to the lake water increased Chl *a* and the abundance of all the major phytoplankton taxa, but P additions alone had no effect (Figure 4). This indicates that low N availability in Lake El Tejo constrained phytoplankton growth during the last part of stratification, when there was a reduction in internal nutrient fluxes to the upper strata as a consequence of density gradients. The higher response when both nutrients were added was expected since the addition of one in excess would further promote the limitation by the other

nutrient with time in the enclosure, as reported from other bioassay experiments (Elser *et al.*, 1990). The lack of response with P enrichment indicates that another factor was primarily limiting, although in this lake the high pH and calcium concentration would probably cause P to become biologically unavailable (Murphy *et al.*, 1983; Golterman, 1988). However, limestone and dolomite deposits sometimes contain P-rich deposits (Wurtsbaugh, 1988), and these may provide phosphate for the system. Nevertheless, SRP levels in the perched hard- water spring were always low, but the pH and other conditions in this spring outflow are different from those of the underwater spring inflows near the bottom of the lake (lower pH, redox and oxygen).

The growth of the deep Chl maximum in Lake El Tejo is controlled by a balance of nutrients and light. This layer, formed primarily of picocyanobacteria, is relatively close to the nutrient-rich anoxic zone, and nutrient diffusion may partly explain the higher development of deep phototrophic populations. In summer, when nutrient exhaustion in upper layers reduces phytoplankton density, light can penetrate deep into the lake, particularly in wavelengths of 540–600 nm, which are efficiently harvested by these phycoerythrin-containing picocyanobacteria. Our results show that at the end of stratification the deep populations were N limited, because internal N supply, mainly from settled

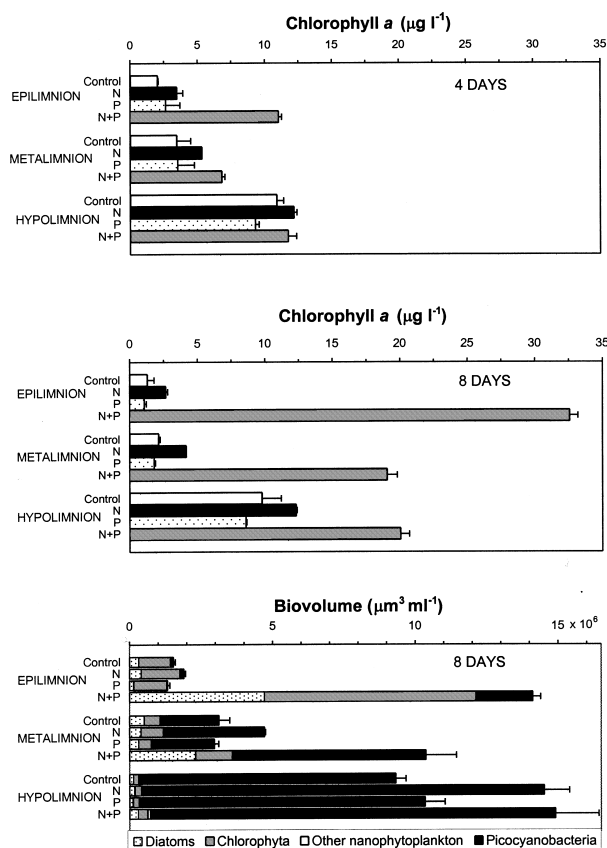


Fig. 4. Top: Chl *a* concentrations in microcosms with different nutrient treatments for each depth strata on days 4 and 8. Bottom: mean total biovolumes of autotrophic plankton in the different nutrient and depth treatments at day 8 (proportions of the different algal groups are indicated). All graphs show the means \pm SD of the three replicates.

spring primary production, became insufficient at this time. Furthermore, the low groundwater flux in summer (Figure 3) reduces N supply from the water itself and by its associated turbulence, as summer advances. Nitrogen limitation of picoplankton growth has been reported from enrichment bioassays in other lakes during certain periods (Stockner and Shortreed, 1988), although this limitation may also change seasonally [e.g. (Beatty and Parker, 1996)]. On the other hand, our results also show that P is less limiting at this depth, because N and N+P additions did not yield significantly different responses, except for slightly higher Chl contents.

In summer, when groundwater flow is limited, atmospheric deposition of nutrients may be particularly important in lakes that have negligible anthropogenic impacts from terrestrial run-off. In Lake El Tejo, where the catchment area is small (a perched aquifer), much of the input falls directly into it; therefore, it is most affected by the composition of precipitation. In Europe, atmos-

pheric deposition of N is very high over much of the continent, but in central-eastern Spain deposition is much lower in relation to northern Europe [Figure 1; (European Environmental Agency, 1998)]. The Iberian Peninsula is mainly downwind from the Atlantic Ocean and from the Sahara desert. These areas lack N emissions (Galloway, 1998), resulting in almost negligible contribution of atmospheric N from transboundary inputs. Additionally, nitrogen oxide and ammonia emissions are low in the area surrounding Lake El Tejo, thus probably further reducing atmospheric deposition as compared with most of Europe. Commensurate with the low atmospheric deposition, dissolved inorganic N concentrations in Lake El Tejo were much lower than in mountain lakes in central and northern Europe, where concentrations are frequently $>20 \mu\text{M N-NO}_3^-$ [e.g. (Mosello *et al.*, 1991; Kopáček *et al.*, 2000)].

On the other hand, the deposition of P-rich dust associated with thunderstorms transporting P-rich Saharan dust (muddy rain) is relatively common in eastern Spain during spring and summer (Carrillo *et al.*, 1990; Avila, 1996). Dust deposition stimulates terrestrial and aquatic systems in the Mediterranean (Loÿe-Pilot *et al.*, 1986; Psenner, 1999), and may be an important P source for a closed-basin lake such as Lake El Tejo, enhancing primary productivity. Reactive N is also present in Saharan dust, but the relative supply of N and P is lower than the Redfield ratio (Morales-Baquero *et al.*, 1999). These authors found that the N deficiency relative to P in pristine mountain lakes of Spain was associated with deposition of P-rich Saharan dust. Similarly, Psenner reported that atmospheric dust supplied 39% of the P, but only 18% of the N, for an Austrian lake (Psenner, 1984). Among European Union countries, the average N surplus from all sources is lowest on the Iberian Peninsula and in part of Austria (Crouzet *et al.*, 1999), where Psenner's evaluations were made.

The balance between the much reduced N supply by sublacustrine inflows during summer, the higher atmospheric inputs of P relative to N deposition and the reduction of internal nutrient loads due to water column stability may explain the N limitation found in Lake El Tejo at the end of stratification. This limitation would probably change in other periods of the year, as a consequence of mixis, increased N-rich groundwater flow into the lake, and the lower aeolian P supply relative to that of N when atmospheric deposition becomes associated with oceanic fronts lacking Saharan dust.

Regardless of the atmospheric or geological factors influencing nutrient inputs to Lake El Tejo, it is clear that the phytoplankton in all three lake strata was N limited in the early fall, as N addition nearly doubled Chl levels and significantly increased phytoplankton abundance.

Schindler argued that lakes should seldom be N limited, because cyanobacteria might be able to make up any N deficit in the system (Schindler, 1977). However, at least at the end of the stratification, the phytoplankton in Lake El Tejo remain N limited; but N-fixing cyanobacterial taxa were very rare throughout the year in this lake, even though they could have been competitively dominant under the N-limited conditions. As we do not know the extent in time of N limitation prior to the bioassay, one possibility may be that this limitation would not remain long enough to allow these cyanobacteria to develop dense populations. However, N-fixing cyanobacteria may also have been limited by other nutrients such as iron or molybdenum, or even by the low P levels in the lake (Wurtsbaugh, 1988). Although Schindler's argument is enticing, the large proportion of N-limited lakes (Elser *et al.*, 1990) suggests that we should be trying to understand the reason(s) why cyanobacteria fail to overcome the deficit, rather than assuming that they are overcoming it.

We suggest that in lakes relatively free of surface run-off, nutrient supply by atmospheric deposition might be a key factor in controlling nutrient deficiency for phytoplankton growth, at least during periods in which other sources are less important. Consequently, in many north-temperate lakes, which receive large external loads of N due to atmospheric deposition, P limitation is more likely to appear than in lakes where this N deposition is much lower.

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