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Nitrate Reductase Activity in an Eutrophic Reservoir during the Stratification Cycle

key words: eutrophic, nitrate reductase, nitrogen, reservoir, stratification

Abstract

Nitrate reductase activity was measured during the stratification cycle of the eutrophic reservoir La Concepción. Nitrate was consumed in the epilimnion mainly through assimilatory reduction, and in the hypolimnion through dissimilatory reduction triggered by anoxic conditions. Nitrate reductase activity (NR) scaled to particulate nitrogen (PN) was positively correlated with the external nitrate concentration and the $\text{NO}_3^-/\text{NH}_4^+$ ratio. Using NR as a tool for the assessment of the N cycle dynamics, it can be suggested that at the beginning of the stratification cycle, the phytoplanktonic community was mainly using NO_3^- as N source; and since N was not likely the limiting nutrient at that time of the year, NR was close to its maximum possible.

1. Introduction

La Concepción is an eutrophic reservoir that provides drinking water for a large and very densely populated area in the southern coast of Spain. It has been described as a calcareous reservoir (MARGALEF *et al.*, 1976). The annual cycle is characterized by a single mixing period in winter and the large duration of its stratification period (LUCENA and RODRIGUEZ, 1984). The hypolimnion of La Concepción remains anoxic during a substantial part of the annual cycle due to this hydrological regime and to its nutrient charge (FERNÁNDEZ-ROSADO *et al.*, 1994). Phytoplankton growth in this reservoir has been considered limited by phosphorus (LUCENA and RODRÍGUEZ, 1984). This initial observation has been later supported by different studies on the phosphorus cycle and the C:N:P ratio of settling seston (FERNÁNDEZ, 1986; CLAVERO, 1987; GÁLVEZ *et al.*, 1991). However, this reservoir is an interesting system from the point of view of the nitrogen cycle because several of its processes are known to be greatly affected by O_2 , i.e. the seasonal pattern in O_2 concentration in the water column has a strong influence on nitrogen cycle.

Biologically mediated nitrate reduction occurs in nature with two main purposes, 1) to provide N for growth (assimilatory NO_3^- reduction) and 2) to serve as a final electron acceptor in a number of bacterial respiratory chains in sub-oxygenic conditions. This process is

termed dissimilatory nitrate reduction because nitrate is not used as N-source for growth. The product, nitrite, is released to the medium. After nitrite, dissimilatory nitrate reduction (wide sense) may follow two separate pathways producing NH_4^+ or N_2 as final products (KOIKE and SORENSEN, 1988). The ecological conditions that determine which pathway is used remain poorly understood. During this process, progressively more reduced inorganic N-species are formed and released to the medium. In each reduction step the oxidised form may be used as an electron acceptor. Together with the availability of substrate, this produces a successive dominance of different inorganic N-species in the water column during the annual cycle (WETZEL, 1975).

The activity of the enzyme nitrate reductase from higher plants and several green microalgae used as model organisms (*Chlorella*, *Chlamydomonas*, etc.), has been studied for a long time and there is a great number of uncertainties about its regulation by environmental factors (SOLOMONSON and BARBER, 1990; CAMPBELL, 1996). However, the information on nitrate reductase activity from other algal groups is surprisingly scarce (BERGES, 1997). Since it is well established that NR is induced by nitrate and repressed by ammonium (SYRETT, 1981), it has been suggested by a number of researchers that NR activity might be used as an indicator of nitrate-dependent growth in contrast to ammonium-based growth for algae and higher plants (HERNÁNDEZ *et al.*, 1993). This distinction is particularly relevant for phytoplanktonic communities and, additionally, it is at the base of the current technique to differentiate between new and regenerated production (^{15}N incubations). However, the rationale for using NR to differentiate between nitrate and ammonium use presents a number of difficulties in practice as it has been reported since the pioneering work of EPPLEY *et al.* (1969).

Most studies dealing with NR activity by phytoplankton have been carried out using the *in vitro* method of EPPLEY *et al.* (1969) or subsequent modifications (see BERGES and HARRISON, 1995a for an updated version of the *in vitro* method). The *in vitro* method involves the isolation of the enzyme from its cellular site. The approach adopted in this study is different, NR activity was measured using the so-called *in situ* method (CORZO and NIELL, 1991) because the enzyme is assayed without being extracted from its cellular site, therefore avoiding the possible inactivation of the enzyme by metallic ions, phenolic compounds and proteases released to the extraction medium during cell disruption (BEEVERS and HAGEMAN, 1980). The *in situ* method has been successfully applied to a number of algae, being able to predict the rate of nitrate use under steady state conditions (HERNÁNDEZ *et al.*, 1993; GORDILLO *et al.*, 1997).

Nitrate reductase activity by phytoplankton has been related in the marine environment to a number of factors, such as external nitrate concentration (EPPLEY *et al.*, 1969; EPPLEY *et al.*, 1970; BLASCO and PACKARD, 1974), intracellular nitrate concentration (COLLOS and SLAWYK, 1976), inversely related to external ammonium concentration (EPPLEY *et al.*, 1969; PACKARD and BLASCO, 1974; BERGES *et al.*, 1995) and positively related to the nitrate:ammonium ratio of the external medium (PACKARD and BLASCO, 1974). However, the literature on NR activity in freshwater environments is very scarce. HOCHMAN *et al.* (1986) measured NR activity by the *in situ* method in natural phytoplankton populations from the lake Kinneret. WYNNE and BERMAN (1990) reported NR activity in natural samples from Lake Kinneret in presence of high ambient ammonium concentration suggesting that it could be of dissimilatory origin. HADAS *et al.* (1992), using the same method detected dissimilatory NR activity in protozoa isolated from lake Kinneret. BLOMQUIST *et al.* (1994) reported the induction of NR activity of eukaryotic origin after nitrate enrichment in enclosure experiment in Lake Erken. The activity of NR has been proposed as a bioindicator of ammonia-nitrogen contamination in rivers (ROLLAND and TREMOLIERES, 1995; ROLLAND *et al.*, 1996).

The stratification cycle of La Concepción is a good testing ground for the use of NR activity in freshwater systems because it includes a wide variety of ecological situations from oxygen saturated to anoxic waters, and from nitrate rich to ammonium-dominated waters. In

this paper we study the changes in the NR activity measured *in situ* during the stratification cycle in an eutrophic reservoir. The main objective of our work was to investigate the potential use of the *in situ* method developed in our lab (CORZO and NIELL, 1991; HERNÁNDEZ *et al.*, 1993; GORDILLO *et al.*, 1997) as a tool to improve our knowledge of the nitrogen cycle in La Concepción reservoir.

2. Method

2.1. Sampling Site

La Concepción is an eutrophic reservoir located in the south of Spain (36°32'N, 45°63'W), with a capacity of 61 hm³ and a surface area of 2.14 km². During this study its maximum depth was 40 m. This mass of water has been defined as warm monomictic (LUCENA and RODRIGUEZ, 1984), the stratification period usually lasting from March to October. A single sampling station, located at the deepest area, was chosen to monitor a number of ecological variables during the stratification cycle in 1993. Water samples were collected about noon by means of 5 L Van Dorn bottles triggered at surface, Secchi disc depth (SD), 2 × SD, around thermocline, at the middle depth of the hypolimnion and near the bottom. Temperature and dissolved oxygen concentration were measured in real time at intervals of aprox. 0.5 m throughout the entire water column by means of a YSI-GRANT 3800 water quality-logger.

2.2. Chlorophyll, Bacteria and Nutrients

Water samples were taken as quickly as possible to the laboratory for processing (1–2 h after collection). For chlorophyll *a* determinations, water subsamples of 500 ml for every depth were filtered on Whatman GF/F glass fibre filters at low pressure. Pigment extraction was performed in darkness, in MgCO₃ – neutralized acetone (90%) for 18–24 h at 4 °C. Chlorophyll *a* concentration was measured spectrophotometrically (Beckman DU-7) according to TALLING and DRIVER (1963). Bacteria were stained with DAPI and counted by epifluorescence microscopy according to HOBBIÉ *et al.* (1977). Inorganic nutrients (NO₃⁻, NO₂⁻ and NH₄⁺) were measured by an autoanalyser Bran-Luebbe TRAACS 800 in water samples filtered by Whatman GF/F and stored frozen (–20 °C) until their analysis. The protocols used were based on WOOD *et al.* (1967) for nitrate, SNELL and SNELL (1949) for nitrite and SLAWYK and McISAAC (1972) for ammonium according to the manufacturers.

2.3. Carbon and Nitrogen

For total carbon and nitrogen concentrations in the seston, 500 mL of water was filtered onto 0.7 µm glass fibre filter (GF/F) which were precombusted (500 °C for 30 min). Filters were dried at 105 °C for 48 h and the content of carbon and nitrogen determined with a CNH Elemental Analyzer (Perkin-Elmer Mod. 2400-C).

2.4. Nitrate Reductase Activity

NR activity from the planktonic community was measured as follows: Immediately upon arrival to the laboratory 2 volumes of 1 L of every water sample were filtered through a 47 mm Whatman GF/F glass fibre filter. Every filter was placed into a cryogenic plastic tube and immersed in liquid nitrogen. Later the tubes containing the filters were transferred to a freezer and stored at –20 °C until analysis.

The enzymatic activity is determined as production rate of nitrite, the product of the reaction under saturating product concentration (30 mM KNO₃). The resulting activity is an estimate of the potential activity of the natural sample. For the enzymatic assay, filters were thawed at 30 °C during 4 minutes in their cryogenic tubes, and placed into assay tubes containing 6 ml of the assay medium: 0.1 M

NaH₂PO₄ (pH 8), 0.5 mM EDTA, 1-propanol 5%, 3 mM NADH and 30 mM KNO₃. Previous to the introduction of the filter the reaction mixture was bubbled with N₂ for 3 minutes to remove O₂ and closed hermetically. The anoxic conditions during the assay avoid the competition for NADH between O₂ (as the final electron acceptor in the respiratory chains) and nitrate reduction (CANVIN and WOO, 1979; BEEWERS and HAGEMAN, 1980; REED and CANVIN, 1982). Immediately after the introduction of the filter the test tube was closed, wrapped in aluminium foil and incubated for 30 minutes at 30 °C in a shaking water bath. The incubation must be performed in dark to prevent NO₂⁻ reduction by nitrite reductase. Since NADH from the reaction medium interferes with the colourimetric method used to determine nitrite by inhibiting the formation of the azo dye, it has to be removed with activated charcoal (HERNÁNDEZ *et al.*, 1993; GORDILLO *et al.*, 1997) or oxidised to NAD⁺ with phenazine methosulphate (SCHOLL *et al.*, 1974; SCHEIDELER and NINNEMANN, 1986). In the present work NADH was removed from 1 mL reaction medium by the addition of 0.5 ml of a charcoal suspension (83% w/v). After vigorous shaking the charcoal was removed by centrifugation (2000 × g, 15 min). Then, nitrite was determined in the NADH free solution according to SNELL and SNELL, 1949.

2.5. Nitrate Enrichment Experiments

Nitrate has been reported to induce *de novo* synthesis of nitrate reductase in plant cells. Nitrate enrichment experiment were performed from April to the beginning of August to check whether the NR activity from the planktonic community could be induced by nitrate. Nitrate (50 µM, final concentration) was added to 1L of every water sample previously filtered through a 50 µm mesh (to avoid the presence of large zooplankton during incubation). After NO₃⁻ addition all the samples were incubated for 2 h at 15 °C in white light (Osram Day-Light L 20 W/10 S) at 60 µmol m⁻² s⁻¹. Photon flux density was measured inside the incubation bottles with a Li-Cor Quantum Radiometer (Li-1000 Data Logger) with a Li-189 sensor. The incubation finished when the whole volume (1 L) was filtered through a Whatman GF/F filter. The filter was immediately frozen in liquid N₂ and stored at -20 °C until analysis for NR activity.

3. Results

3.1. Temperature, Oxygen and Chlorophyll *a*

In late April epilimnion (mean temperature = 17.4 °C) and hypolimnion (mean temperature = 11.8 °C) started to differentiate (Fig. 1a). Temperature increased in both compartments as the annual cycle progressed up to reach a maximum at the end of August. However the magnitude of these increases was quite different, in the epilimnion the mean temperature increased in 8.7 °C to 26.1 °C, while in the hypolimnion mean temperature increased only 0.6 °C to 12.4 °C (Fig. 1a). The buoyancy frequency (*N*) is frequently used as a measure of the thermocline strength. Calculation of both *N* and the vertical turbulent diffusion coefficient across the thermocline (*K_v*), from the temperature profiles confirm that maximum stratification was reached at the end of August (*N* = 0.065 rads⁻¹, *K_v* = 3.4 × 10⁻⁷ m² s⁻¹). During a substantial part of the stratification cycle there was only a slight mixing between epilimnion and hypolimnion.

The quick isolation of the hypolimnion from the epilimnion was confirmed by the O₂ evolution (Fig. 1b). The mean O₂ concentration in the epilimnion ranged between 10.8 mg O₂ L⁻¹ and 5.4 mg O₂ L⁻¹ (reached when the stratification was maximum). Seasonal changes in the mean O₂ concentration below the thermocline were higher and by the middle of June, O₂ concentration was below 1 mg O₂ L⁻¹, and it remained very low or undetectable until the middle of October. Therefore, anoxic conditions in the hypolimnion prevailed during a substantial part of the annual cycle (Fig. 1b).

Chlorophyll *a* concentration in the epilimnion ranged from 0.77 to 8.69 µg l⁻¹, with less than 3% of the observed values being higher than 6 µg Chl *a* L⁻¹ (Table 1). Two maxima

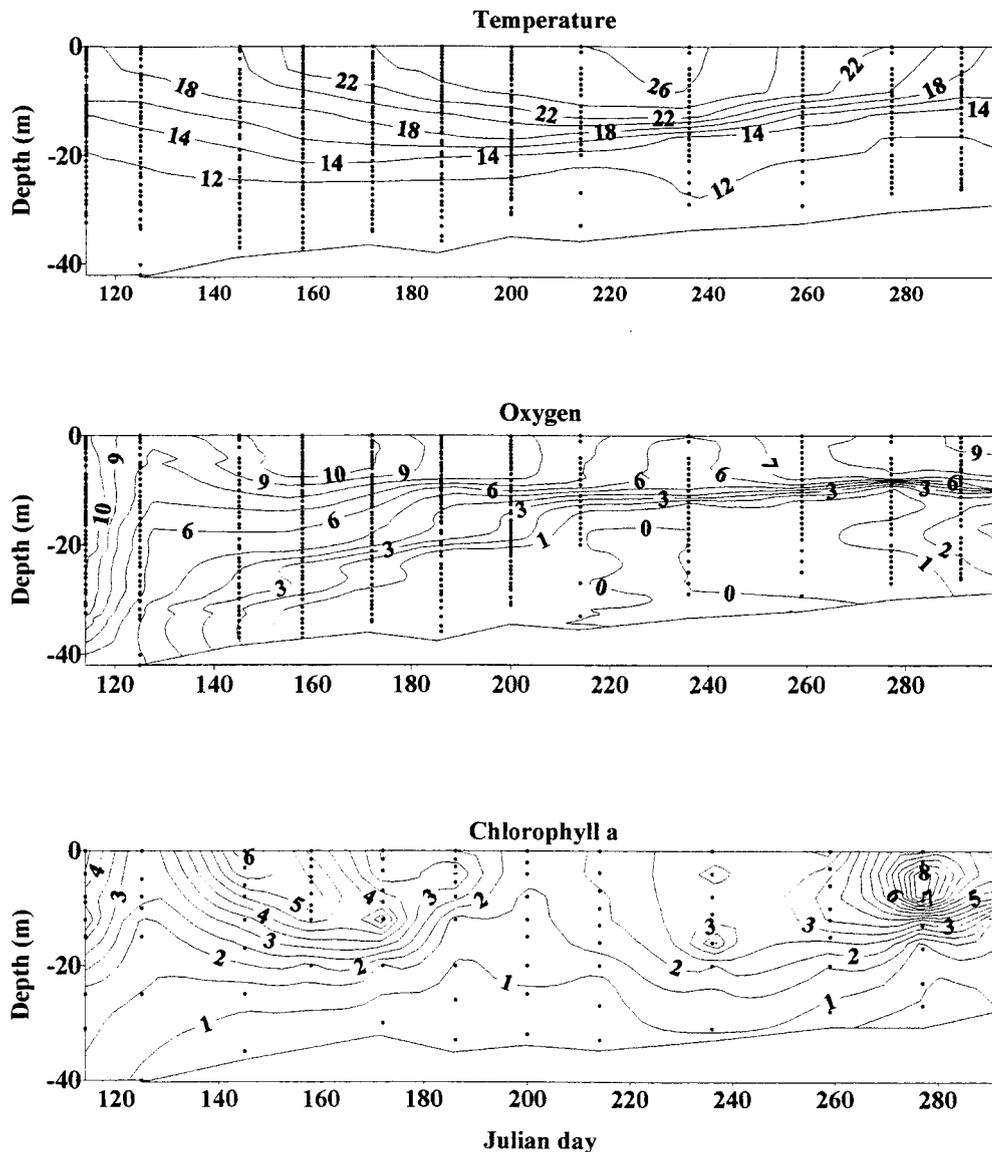


Figure 1. Depth-time distribution of Temperature ($^{\circ}\text{C}$), Oxygen ($\text{mg O}_2 \text{ L}^{-1}$) and chlorophyll *a* ($\mu\text{g L}^{-1}$) during the stratification cycle in La Concepción reservoir in 1993. A single sampling station located in deepest area of the reservoir was selected for this study.

were observed during the study (Fig. 1c). In the first one, in May, the maximum chlorophyll *a* concentration measured was $5.9 \mu\text{g L}^{-1}$. At the beginning of October, the second peak in chlorophyll *a* concentration was more intense, and the highest values were measured just above the thermocline ($8.69 \mu\text{g L}^{-1}$). The species composition of phytoplankton community varied considerably during the study, different species being dominant at distinct times (*Fra-*

Table 1. Mean values of several relevant variables during the stratification cycle in the epilimnion, hypolimnion and whole water column. All data were collected at a single sampling station located at the deepest area of La Concepción reservoir. PC, total particulate carbon; PN, total particulate nitrogen. Nitrate reductase activity appears referred to water volume (NR/L; nmol NO₂⁻ L⁻¹ h⁻¹), Chlorophyll *a* (NR/Chl *a*; nmol NO₂⁻ µg⁻¹ Chl *a* h⁻¹), total particulate N (NR/PN; nmol NO₂⁻ µg⁻¹ N h⁻¹), and bacterial density (NR/Bact; nmol NO₂⁻ 10⁻⁸ Bact h⁻¹).

	Epilimnion (<8 m)			Hypolimnion (>20 m)			Whole water column		
	Mean	Range	n	Mean	Range	n	Mean	Range	n
µg Chl <i>a</i> L ⁻¹	3.2	0.8–8.7	46	0.6	0.1–1.3	27	2.3	0.1–8.7	105
µg PC L ⁻¹	640	158–951	46	375	173–1250	24	527	158–1250	99
µg PN L ⁻¹	103	16–268	46	82.4	13.0–282.0	24	96.7	13–348	99
10 ⁵ Bact mL ⁻¹	9.0	3.9–15.5	39	8.3	2.1–18.4	24	8.4	2.1–18.4	88
NR/L	93.1	29.2–134.1	44	101.3	33.0–242.7	25	98.7	29.2–242.7	94
NR/Chl <i>a</i>	35.6	12.7–126.1	41	361.7	25.4–2022.5	22	133.5	12.7–2022.5	87
NR/PN	1.2	0.2–3.9	41	2.8	0.8–24.2	21	1.7	0.2–24.2	84
NR/Bact	10.9	4.2–18.5	36	14.8	4.0–36.7	21	12.9	4.0–36.7	76
µmol NO ₃ ⁻ L ⁻¹	8.5	0.6–57.1	47	29.5	0–58.9	29	15.9	0–58.9	107
µmol NO ₂ ⁻ L ⁻¹	0.4	0–4.8	47	2.0	0–7.3	29	0.8	0–7.3	107
µmol NH ₄ ⁺ L ⁻¹	0.5	0–3.6	47	10.5	0–52.9	29	3.8	0–52.9	107

gilaria sp., *Ceratium* sp., *Melosira* sp.). *Melosira* sp. was largely responsible for the chlorophyll peak at the beginning of October (GARCÍA, 1995).

3.2. Nitrate, Nitrite, Ammonium and Phosphate

The nitrate concentration (Fig. 2a) in the epilimnion decreased from April (14.7 ± 0.6 µmol NO₃⁻ L⁻¹) to reach a minimum at the beginning of July (0.9 ± 0.1 µmol NO₃⁻ L⁻¹). From this date on, NO₃⁻ concentration above the thermocline remained low (aprox. 1 µmol NO₃⁻ L⁻¹) till early October when the NO₃⁻ concentration started to increase probably due to both a higher flux from hypolimnion due to higher K_v values and to a possible input of NO₃⁻ from the catchment area due to rainfall at the beginning of October. The NO₃⁻ concentration in the hypolimnion changed considerably with depth, being maximal at the bottom (50 µmol NO₃⁻ L⁻¹) and minimal below the thermocline in April. This pattern changed considerably along the year as the O₂ concentration in the hypolimnion decreased.

Nitrite and ammonium concentrations in the epilimnion did not show large seasonal changes (Fig. 2b, c) and remained low during the whole study (<1 µmol L⁻¹). However both nutrients displayed a clear seasonal trend in the hypolimnion. Nitrite concentration in the hypolimnion increased from May reaching maximal values close to the sediment in August (>5 µmol NO₂⁻ L⁻¹). From this date on, NO₂⁻ concentration was below the detection limit for the whole water column until the end of the study. Changes in ammonium concentration in the hypolimnion were considerably larger; near the bottom it increased from 3 µmol NH₄⁺ L⁻¹ at the beginning of May up to more than 50 µmol NH₄⁺ L⁻¹ in September.

The main soluble reactive phosphorus (SRP), available to phytoplankton is that in the epilimnion. Mean SRP in the epilimnion (z < 8 m) increased from April to October from 0.05 to 0.349 µmol L⁻¹ (Fig. 3). Similarly SRP in the hypolimnion increased to reach a maximum (3.5 µmol L⁻¹) close to the sediment in August. The relative availability of NO₃⁻ and SRP as inorganic sources of N and P for the phytoplankton can be investigated through the

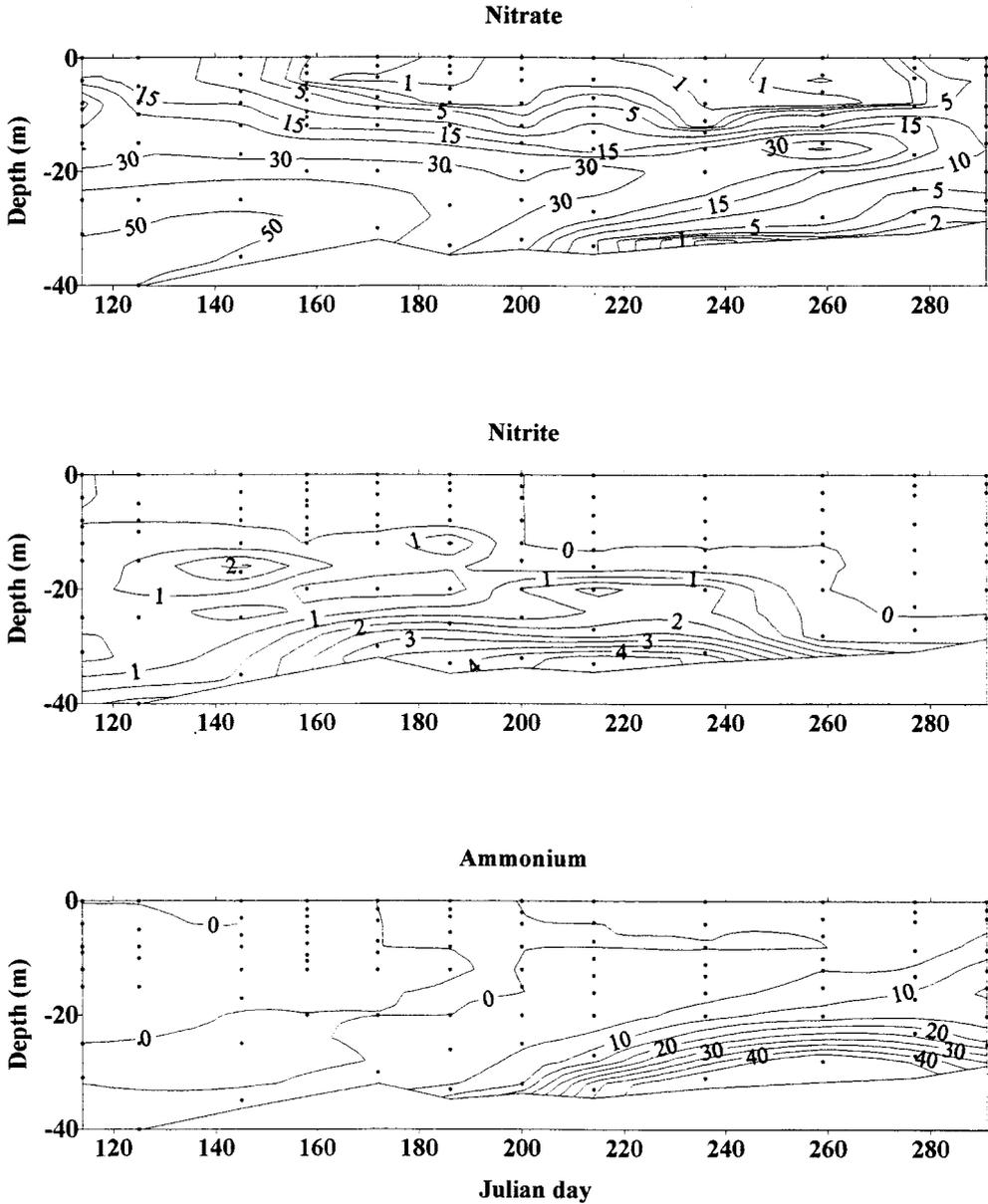


Figure 2. Depth-time distribution of nitrate, nitrite and ammonium concentration ($\mu\text{mol L}^{-1}$) during the stratification cycle in La Concepción reservoir in 1993.

ratio of their concentration in the water column. Nitrate:Phosphate ratio in the epilimnion changed from very high values (20 times Redfield ratio) in April to values lower than Redfield ratio in the maximum stratification period. The consideration of NO_2^- and NH_4^+ as possible sources of inorganic nitrogen changed little the trend already depicted by the NO_3^- :SRP ratio.

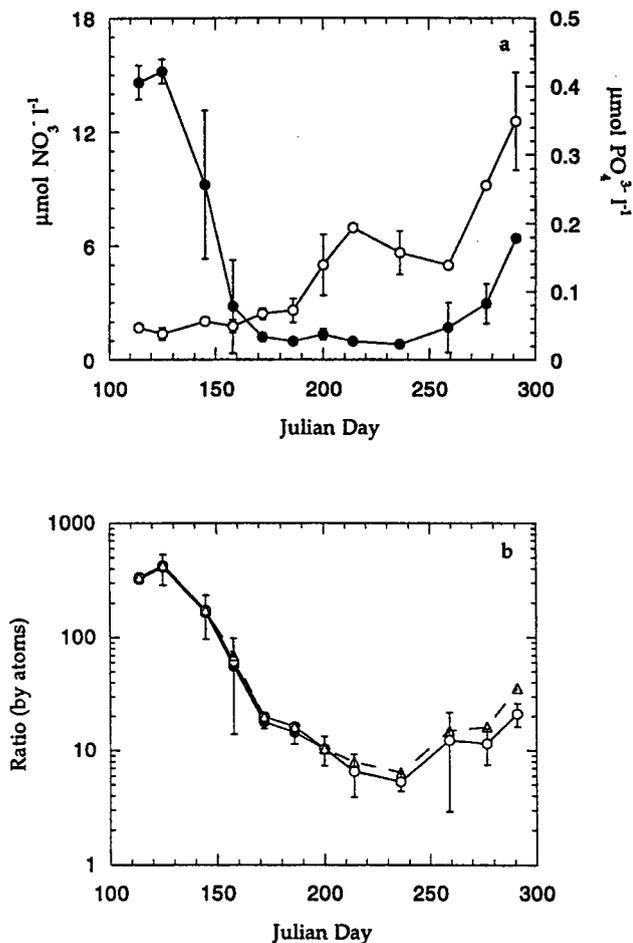
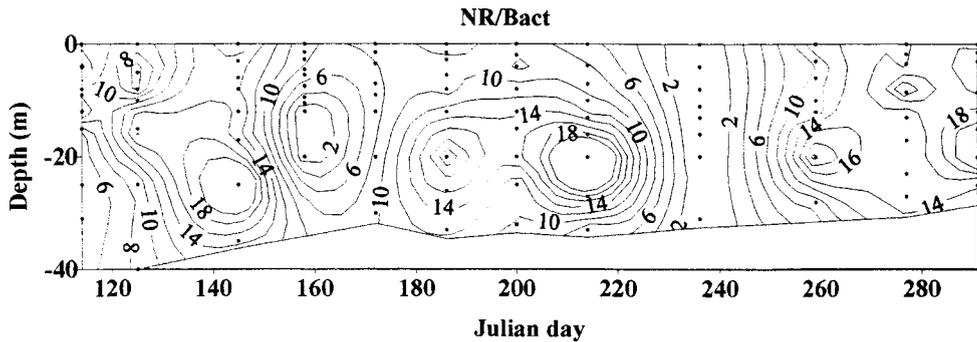
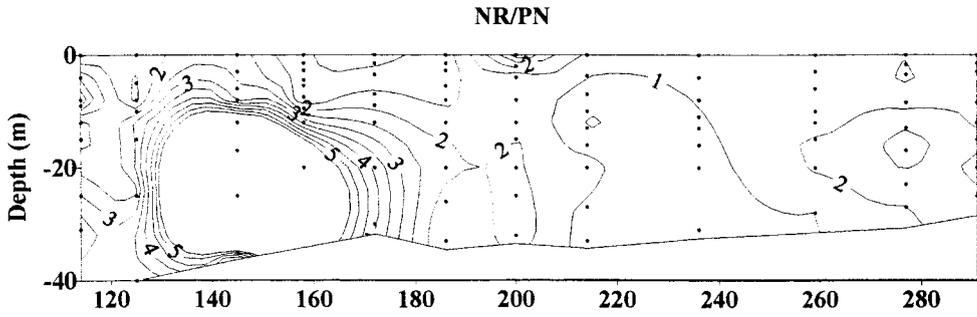
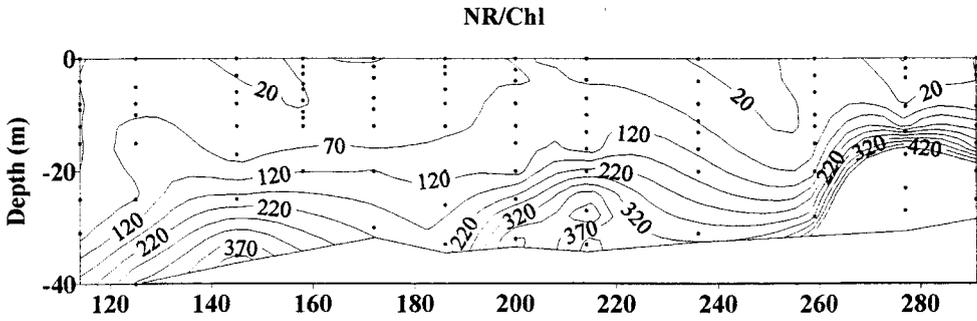
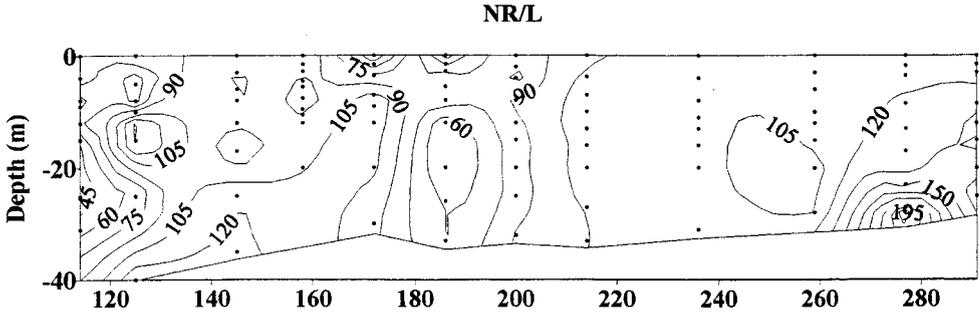


Figure 3. (a) Mean epilimnetic concentration of soluble reactive phosphorus (open circles) and nitrate (closed circles) during the stratification cycle in La Concepción reservoir in 1993. Standard deviation represented as bars ($n = 3 - 6$). (b) NO_3^- :SRP (closed circles), $[\text{NO}_3^- + \text{NO}_2^-]$:SRP (open circles), $[\text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+]$:SRP (triangles) mean ratios in the epilimnion. For clarity, standard deviations have only been plotted for NO_3^- :SRP data ($n = 3 - 6$).

3.3. Nitrate Reductase Activity

Mean NR activity per litre (NR/L) during the studied period was $98.7 \pm 30.9 \text{ nmol NO}_2^- \text{ L}^{-1} \text{ h}^{-1}$ and it ranged from 29 to $243 \text{ nmol NO}_2^- \text{ L}^{-1} \text{ h}^{-1}$ (Fig. 4a, Table 1). When the NR activity was scaled to the chlorophyll *a* concentration (NR/Chla, $\text{nmol NO}_2^- \mu\text{g Chla}^{-1} \text{ h}^{-1}$) the larger

Figure 4. Seasonal changes of nitrate reductase activity. NR activity was scaled to volume of sampled water (NR/L; $\text{nmol NO}_2^- \text{ L}^{-1} \text{ h}^{-1}$), Chlorophyll *a* concentration (NR/CHL; $\text{nmol NO}_2^- \mu\text{g Chla}^{-1} \text{ h}^{-1}$), total particulate nitrogen (NR/PN; $\text{nmol NO}_2^- \mu\text{g N}^{-1} \text{ h}^{-1}$) and bacterial density (NR/BACT, $\text{nmol NO}_2^- \times 10^{-8} \text{ Bact h}^{-1}$).



values appeared close to bottom due to the low chlorophyll concentration at this depth (Fig. 4b). This is somehow misleading because high values of Chl-specific NR activity point out the existence of other sources than primary producers of this activity in the hypolimnion. Three maxima were observed along the stratification cycle, the first and third ones were coincident in time with peaks in the chlorophyll concentration in the epilimnion (Figs. 1c, 4b) and they were also coincident with relatively large values of NR/L mainly in mid October. The second peak in NR/Chl a appeared in the period of maximum stratification, when NR/L showed little variation with depth but chlorophyll a concentration below the thermocline was very low.

Total particulate nitrogen has been proposed as a suitable variable to scale the nitrate reductase activity in the aquatic environment (DUGDALE and WILKERSON, 1991; BERGES and HARRISON, 1995b). Nitrate reductase activity per unit of particulate nitrogen (NR/PN, nmol NO $_2^-$ μ g N $^{-1}$ h $^{-1}$) reached the maximum values in the hypolimnion between mid May and mid June (Fig. 4c). These high values in NR/PN were due both to high values in nitrate reductase activity per litre and low values of particulate nitrogen. The epilimnetic values were also relatively high at that date compared with the rest of the cycle. At this time of the year a phytoplankton bloom was taking place in the epilimnion. Once the bloom finished, the NR/PN remained generally low the rest of the cycle until the second annual peak in chlorophyll a concentration in October. At this date the NR/PN was maximum just below the thermocline coinciding with the minimum in the vertical profile of particulate nitrogen.

Nitrate reductase activity was also scaled to the abundance of bacteria (NR/Bact, nmol NO $_2^-$ 10^{-8} Bact. h $^{-1}$) since the bacterial community is a likely source of this activity both in the assimilative and in the dissimilative pathways. Maximum values of NR/Bact mainly appeared just below the thermocline (Fig. 4d). They were measured during the spring and the autumn blooms, but also at the beginning of August when chlorophyll a concentration in the euphotic layer was low. In this last case the high values of NR/Bact were mainly due to the very low bacterial abundance measured in this period.

Changes in NR activity did not present a clear pattern either in depth or through the year, probably, due to the various sources of this activity in aquatic environments (Fig. 4a). However, we observed a weak but significant correlation of NR activity in the epilimnion scaled to volume of water (NR/L) with chlorophyll a ($r = 0.312$, $n = 41$, $P < 0.05$) and NR activity in the whole water column scaled to bacterial density (NR/Bact) with chlorophyll a ($r = 0.600$, $n = 36$, $P < 0.01$). When NR activity was scaled to particulate nitrogen (NR/PN), it was positively correlated with NO $_3^-$ concentration ($r = 0.427$, $n = 41$, $P < 0.01$) and with the ratio NO $_3^-$:NH $_4^+$ ($r = 0.483$, $n = 22$, $P < 0.05$). However, NR/L was not correlated with NO $_3^-$ and negatively correlated with NO $_3^-$:NH $_4^+$ ratio ($r = -0.577$, $n = 23$, $P < 0.01$), which was unexpected.

3.4. Nitrate Enrichment Experiments

Nitrate is known to be one of the major environmental variables inducing nitrate reductase activity in plant cells (SOLOMONSON and BARBER, 1990). However, the addition of 50 μ mol NO $_3^-$ to 1 L confined samples taken at different depth and at different date along the stratification process did not increase the NR activity significantly. The comparison of the NR activity values before and after the incubation by paired t-test revealed that there were no significant differences ($P < 0.05$). Similar results were obtained when the data corresponding to the epilimnion and hypolimnion were analysed separately to rule out the possibility of a difference between the epilimnion, where the abundance of phytoplankton was obviously higher, and the hypolimnion data. These results were unexpected because the NO $_3^-$ concentration in the epilimnion, although relatively high ($> 10 \mu$ mol NO $_3^-$ L $^{-1}$) at the beginning of the stratification, it was low ($< 1 \mu$ mol NO $_3^-$ L $^{-1}$) during most part of the period in which the enrichment experiment was performed (April to August).

4. Discussion

Oxygen concentration is determinant in the control of different biological activities relevant for the nitrogen cycle in aquatic environments. In La Concepción reservoir, where anoxic conditions prevail in the hypolimnion during a large part of its annual cycle, the oxygen availability appeared to be a key factor in the regulation of the nitrogen metabolism of its microbial community. Annual changes in oxygen concentration, especially in the hypolimnion, determined the alternation of dominance of different processes during the annual cycle. As the stratification cycle progressed the thermocline strengthened and determined very low values of the vertical turbulent diffusion coefficient, therefore reducing considerably the exchange between both compartments. The O₂ reserve in the hypolimnion began to exhaust at the bottom where the organic matter accumulates. Anoxic conditions appeared first close to the bottom at the beginning of June and by August the whole hypolimnion became anoxic (Fig. 1).

Nitrate reduction mediated by organisms was responsible for NO₃⁻ depletion both in epilimnion and hypolimnion. Above the thermocline, since O₂ concentration were high, it can be assumed the assimilatory NO₃⁻ reduction pathway to be responsible for the NO₃⁻ consumption, probably mainly due to phytoplankton. In the hypolimnion, however, NO₃⁻ depletion may, in principle, be due to both assimilatory NO₃⁻ reduction and/or more likely to dissimilatory NO₃⁻ reduction, both being mainly mediated by bacteria. Anoxic conditions and the relatively high NO₃⁻ concentration favoured the development of microbial communities able of oxidising the organic matter using NO₃⁻ as a final electron acceptor. There was clear evidence of dissimilatory NO₃⁻ reduction in the hypolimnion because the NO₃⁻ depletion was triggered by anoxic conditions. NO₃⁻ concentration 50 cm above the sediment remained close to 50 μM from April to the middle of June. From this date on, being O₂ concentration nearly zero, the NO₃⁻ depletion rate was 1.03 μmol L⁻¹d⁻¹ till the beginning of August when NO₃⁻, close to the bottom, was already undetectable. Hence, NO₃⁻ in the hypolimnion decreased as anaerobic conditions propagated from the bottom to the thermocline. As the result of the depletion of NO₃⁻ in the epilimnion and hypolimnion a dramatic peak of relatively high concentrations appeared just below the thermocline in August (Fig. 2a).

Nitrate, nitrite and ammonium seasonal peaks appeared sequentially (Fig. 2), in an order determined by their redox state, from more oxidised to more reduced. Despite NO₂⁻ is produced in the first reaction during dissimilatory NO₃⁻ reduction, it did not accumulate probably because it followed further reduction. Nitrite can be used as well as a terminal electron acceptor in many anaerobic respiratory chains presents in prokaryotes, although it yields less energy than the utilisation of NO₃⁻. Dissimilatory NO₂⁻ reduction is a rather complex and not well understood process with two possible outcomes. Nitrite may be reduced to NH₄⁺, or to N₂O and/or N₂. Although it is out of the scope of this paper to make a detailed mass balance for inorganic N, we have calculated that approximately 60% of NO₃⁻ in the hypolimnion seemed to end up as NH₄⁺ at the maximum stratification period (Table 2). In addition, total inorganic nitrogen in August decreased to 7.8% in respect to the April value. This could be due to denitrification. If this were the case, the dissimilatory NO₃⁻ reduction to NH₄⁺ would be the most important pathway in La Concepción reservoir.

The values for NR activity (scaled to any of the variable used here) measured in this study are in the high range of the activity measured by others in field studies both in marine and freshwater systems (BLASCO *et al.*, 1984; KRISTIANSEN, 1987). For Lake Kinneret the values given by HOCHMAN *et al.* (1986) were around two times lower than the mean values reported in this study. BLOMQUIST *et al.* (1994), reported NR activities, scaled to chlorophyll *a*, one order of magnitude lower than reported here, for an enclosure experiment in lake Erken. Unfortunately, the difficulty to find highly significant correlations between NR activity and several other variables supposed to be relevant for NO₃⁻ assimilation seems to be characteristic of field studies dealing with NR activity. This is likely due to the diversity of possi-

Table 2. Nitrate, nitrite and ammonium mass balance for the hypolimnion at the beginning of the stratification and at its maximum. Data are expressed as mmol m^{-2} . Concentrations at the bottom of the water column (approximately 50 cm above the sediment) are included as well. Concentrations are expressed as $\mu\text{mol L}^{-1}$.

	24th April		2th August	
	mmol m^{-2}	$\mu\text{mol L}^{-1}$	mmol m^{-2}	$\mu\text{mol L}^{-1}$
NO_3^-	572.8	50	163.9	undetectable
NO_2^-	10.3	0.2	34.9	5.2
NH_4^+	3.2	undetectable	338.7	45.5
	583.3		537.5	

ble sources for NR activity in aquatic environments. NR/L and chlorophyll *a* were weakly correlated (although significant) in La Concepción reservoir. If a substantial portion of the biomass were supported by regenerated production (NH_4^+ as a nitrogen source) it could explain the low correlation between NR/L and chlorophyll *a*. However, we think this is not the case at least in the initial part of the stratification cycle. The variable used to scale the NR activity seems to be critical to obtain good correlations. When NR activity was scaled to bacteria (NR/Bact), chlorophyll *a* variability explained 36% of the NR/Bact variance. Several researcher have suggested that the best variable to scale NR activity or nitrate uptake rates would be total particulate nitrogen (TPN) (DUGDALE and WILKERSON, 1991; BERGES and HARRISON, 1995b; DICKSON and WHEELER, 1995). In our study, NR activity was positively correlated with either external NO_3^- concentration or $\text{NO}_3^-/\text{NH}_4^+$ when it was scaled to TPN. Similar correlations have been found by others (EPPELY *et al.*, 1969; KRISTIANSEN, 1987; BERGES *et al.*, 1995).

Nitrate reductase activity in field studies is difficult to scale for a number of reasons. The first one is related to the fact that the same activity (NO_3^- reduction) may serve for two quite different purposes: assimilatory and dissimilatory NO_3^- reduction. Another problem is the taxonomic distribution of both activities, which preclude for instance the scaling to chlorophyll being entirely satisfactory (Fig. 4b). Assimilatory NO_3^- reduction is present mainly in autotrophs but also in heterotrophic bacteria (PARSONS *et al.*, 1981; HORRIGAN *et al.*, 1988; KIRCHMAN *et al.*, 1992). Dissimilatory NO_3^- reduction is mainly associated with a number of groups of heterotrophic bacteria in anaerobic conditions (HOCHTEIN and TOMLINSON, 1988); although there are also some reports on dissimilatory NO_3^- reduction in several groups of eukaryotes in anoxic environments (FINLAY *et al.*, 1983; HADAS *et al.*, 1992; ZVYAGILSKAYA *et al.*, 1996). The third reason is a methodological one. It is likely that both, the *in vitro* NR assay as used by EPPELY *et al.* (1969) including subsequent modifications, and the *in situ* assay as used by HOCHMAN *et al.* (1986) or CORZO and NIELL (1991), are able to measure both assimilatory and dissimilatory NO_3^- reduction at the same time (PACKARD *et al.*, 1978; HADAS *et al.*, 1992). Moreover, some NR enzymes, particularly prokariotes, cannot use the NADH added in the assays as a source of reducing power, resulting in an underestimation of the activity. This methodological difficulty obviously represents a serious problem when using nitrate reductase activity as a semi-quantitative biochemical index to assess NO_3^- utilisation by primary producers. It is essential to improve our methods to distinguish at least assimilatory from dissimilatory NO_3^- reduction.

The lack of any consistent increase in NR activity in response to the NO_3^- enrichment in the incubations performed with the natural samples suggests that the natural community was

either: 1) in N-sufficient conditions, i.e. its growth being limited by a different resource, for instance phosphate, 2) using NH_4^+ as N source, which is well known to inhibit NR activity (SYRETT, 1981), or 3) the incubation time was too short to detect any induction of NR activity by NO_3^- . This last possibility seems unlikely since in many other experimental systems, 2 h has been a period of time long enough to detect induction of NR activity by NO_3^- (TISCHNER and LORENZEN, 1979; HIPKIN and CANNONS, 1987; CORZO and NIELL, 1992).

The Redfield ratio (REDFIELD *et al.*, 1963) is frequently used as characteristic of natural phytoplankton communities. The deviation from the Redfield ratio in the availability of N and P in the water column for phytoplankton may be used to identify which nutrient is more likely to limit growth. The ratio $[\text{NO}_3^-]:[\text{SRP}]$ in the epilimnion changed considerably during the study; it presented a very large average (171.3 ± 214.4 , $n = 20$) from April to June, being more than 95% of data higher than the Redfield ratio (Fig. 3). Therefore phytoplankton during that period seemed more likely to be P-limited than N-limited. As the annual cycle progressed, the $[\text{NO}_3^-]:[\text{SRP}]$ ratio in the epilimnion decreased to reach a minimum in August (4.4); from this date on $[\text{NO}_3^-]:[\text{SRP}]$ ratio increased slightly until the end of the sampled period. Nitrate was the main form of inorganic nitrogen in the epilimnion, the inclusion of NO_2^- and NH_4^+ , or even dissolved organic nitrogen, as suitable sources of N for phytoplankton stress even further the likely existence of P-limitation with respect to N at least from April to early July as suggested from previous work (LUCENA and RODRIGUEZ, 1984; GALVEZ *et al.*, 1991).

The NR activity of the phytoplankton is not likely to be influenced by an inhibitory effect of NH_4^+ , at least at the beginning of the stratification period. It is well known that NH_4^+ may inhibit both NO_3^- uptake and nitrate reductase (ULLRICH, 1983). However, NH_4^+ concentration in the whole water column was very low (from undetectable to $0.24 \mu\text{mol NH}_4^+ \text{L}^{-1}$) during the initial part of the stratification period, and coincident with NO_3^- concentrations at least one order of magnitude higher. Additionally, vertical transport from hypolimnion was also negligible due to the low NH_4^+/z during that period of the year. In addition, the sole observed rate of NO_3^- consumption seems to be enough to support the observed standing stocks of chlorophyll *a*, therefore NH_4^+ was of little importance as N source for phytoplankton at this time of the year. During the initial phase of the spring bloom (April–May) the NO_3^- consumption rate in the epilimnion was $0.45 \mu\text{mol NO}_3^- \text{L}^{-1} \text{d}^{-1}$ (depletion rate + net vertical flux of NO_3^-), and NR/PN reached the highest values of the studied period. From this figure, 12.8% is the contribution of the net upward vertical flux calculated from the NO_3^- concentration gradient across the thermocline and K_v derived from the temperature profiles. Nitrite consumption in the epilimnion was also detected although at the beginning of the bloom only accounted for 1.3% of NO_3^- consumption rate. Assuming that nutrients were being used at a stoichiometry close to the Redfield ratio, the net inorganic carbon fixation rate should have been close to $35.6 \mu\text{g C L}^{-1} \text{d}^{-1}$. By using a suitable Chl*a*:C ratio, net carbon fixation rate can be transformed to a net increase rate for chlorophyll *a*. However, Chl*a*:C ratios for phytoplankton cultures are highly variable, ranging from 0.003 to >0.1 (CLOERN, 1995). Field measurements of Chl*a*:C and C:N are less straightforward because it is not possible to separate the carbon content of phytoplankton from detrital and other sources of planktonic carbon. The mean Chl*a*:C ratio measured in this study for the epilimnetic community was 0.006 ± 0.002 ($n = 9$), therefore within the range, but close to the minimum for cultures. Using this value as a characteristic of the phytoplankton in this reservoir, albeit its limitation, we found that according to the measured net NO_3^- consumption rate, the increase rate in Chlorophyll *a* could have been as much as $0.2 \mu\text{g L}^{-1} \text{d}^{-1}$, which is larger than the maximal net increase rate for chlorophyll *a* measured in our study ($0.16 \mu\text{g L}^{-1} \text{d}^{-1}$). If the real value for the phytoplankton Chl*a*:C ratio were higher, the amount of chlorophyll *a* produced by mol of NO_3^- used would have been even larger. Therefore we think that NR activity could not be induced in the incubation experiment because the phytoplankton community was using NO_3^- as N-source in a non-limiting way.

The use of NR activity (specially NR/PN) qualify the main conclusion that at the beginning of the stratification process nitrate was the main source of N; and that this was not the limiting nutrient for that period. As the annual cycle progressed primary production was likely to be N-limited, this limitation being maximal when the stratification was the highest.

5. Acknowledgements

This work was supported by the projects AMB99–1088 of the CICYT of the Spanish Ministry of Education and Culture and RNM-214, RNM-0176 from Plan Andaluz de Investigación. FRANCISCO JL GORDILLO was supported by the Spanish Ministry of Education and Culture.

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Manuskript received June 13th, 2000; revised January 20th, 2001; accepted February 28th, 2001