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Photosynthesis, Growth and Cell Composition of *Spirulina platensis* (*Arthrospira*) Under Elevated Atmospheric CO_2 and Nitrogen Supplement

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Abstract

The consequences of the addition of CO_2 (1%) in cultures of *S. platensis* are examined in terms of biomass yield, cell composition and external medium composition. CO_2 enrichment was tested under nitrogen saturating and nitrogen limiting conditions. Increasing CO_2 levels did not cause any change in maximum growth rate while it decreased maximum biomass yield. Protein and pigments were decreased and carbohydrate increased by high CO_2, but the capability to store carbohydrates was saturated. C:N ratio remained unchanged while organic carbon released to the external medium was enhanced, suggesting that organic carbon release in *S. platensis* is an efficient mechanism for the maintenance of the metabolic integrity, balancing the cell C:N ratio in response to environmental CO_2 changes. CO_2 affected the pigment content: Phycocyanin, chlorophyll and carotenoids were reduced in around 50%, but the photosynthetic parameters were slightly changed. We propose that in *S. platensis* CO_2 could act promoting degradation of pigments synthesized in excess in normal CO_2 conditions, which are not necessary for light harvesting. Nitrogen assimilation was significantly not affected by CO_2, and it is proposed that the inability to stimulate N assimilation by CO_2 enrichment determined the lack of response in maximum growth rate.

Introduction

Since the last two decades, there is a growing need for investigation of basic aspects of photosynthesis and carbon metabolism in the blue-green alga *Spirulina platensis* to ensure optimal biomass

production because of its importance in rural biotechnology and commercial production. One of the most common practices in the aquaculture of *S. platensis* is to provide extra CO_2, either to maintain the pH constant (Qiang et al., 1996) or as an additional inorganic C supply (Vonshak et al., 1996a, b). Stimulation of growth by adding CO_2 is based on the low solubility of gases in the aqueous medium when *S. platensis* is cultured at high temperature (34–40°C), thus additional CO_2 is usually recommended to avoid C limitation under such conditions (Fox, 1996). Nevertheless the consequences of excess CO_2 on biomass quality and photosynthesis have not yet been checked. Because atmospheric CO_2 often limits photosynthetic capacity, many O_2 evolvers show increased photosynthetic rate and biomass in response to elevated CO_2 (Raven, 1991). For this, reason, when CO_2 enrichment on O_2-evolving organisms is examined, CO_2 is usually considered as a mere substrate for photosynthesis and, through this, for growth, although in higher plants, it is well known that CO_2 influences the metabolism in several ways unrelated to being a photosynthetic substrate (Bowes, 1993).Many studies have shown a wide range of effects of CO_2 concentration on metabolic pathways other than C fixation. For instance, C metabolism is linked to N metabolism in at least 50% (Vanleberghe et al., 1990), and in algae CO_2 influences a number of key enzymes both of the C metabolism, i.e. carbonic anhydrase (Fujiwara et al., 1990; Mercado et al., 1996) and Rubisco (Winder et al., 1992; García-Sanchez et al., 1994; Mercado et al., 1996), and N metabolism (Larsson et al., 1985; Fonseca et al., 1997). Furthermore, CO_2 has been though to exert some control on pigment content (García- Sánchez et al., 1994). Nevertheless reports about CO_2 effects on freshwater algae (Jaworski et al., 1981) and more specifically on cyanobacteria (Yunes, 1995) are scarce.

Studies on the influence of additional CO_2 on organic C release by living algal cells are rare, although it is considered a lost of primary production. In percentage it can account from zero (Wood et al., 1992) to >95% of primary production (Fogg et al., 1965). In the green unicellular alga *Dunaliella salina*, organic C release was enhanced in high CO_2 grown cells (Giordano et al., 1994). The amount of organic C released highly depends on nutritional conditions and phase of growth (Chen & Wangersky, 1996) and is considered as the mechanism maintaining

the metabolic integrity in response to environmental conditions (Fogg, 1983; Ormerod, 1983). In *Spirulina platensis*, exo polysaccharides can account for about 10% of the primary production, even during optimal growth conditions (Cornet, 1992), but how a CO_2 surplus can affect C release remains still to be evaluated. In this study, the consequences of CO_2 enrichment on biomass yield, cell composition, photosynthesis and external medium composition, are examined on batch cultured *Spirulina platensis*, carbon losses by respiration and release of organic compounds to the medium are also considered. Cultures are tested under both N sufficiency and N limitation and the effects of CO_2 on N assimilation are studied as well. The role of photosynthesis as the main process mediating the effects of elevated CO_2 levels on growth is discussed.

Materials and Methods:

Cyanobacterial Culture and Experimental Design:

The Cyanobacterial culture was isolated from Lonar Crater Lake and identified as Spirulina platensis by 16S-rRNA sequencing. Temperature was maintained at 25±0.5°C and aeration at 1 L min^{-1}. Cells were grown in a strictly inorganic medium (Zarrouk, 1966) containing 29.4 or 1 mmol L^{-1} $NaNO_3$ for N sufficient (N+) and N limited (N-) cultures respectively.

Cells were grown under two differentCO2 levels in the bubbling aeration system, atmospheric CO_2 level (0.035%, namely normal CO2) and 1% CO_2 (namely high CO_2). The usual conditions for maintenance (normal CO_2 and N sufficiency) were considered as the control treatment.

Growth:

Culture density was estimated by measuring the optical density (OD) at 750 nm in a spectrophotometer (Beckman DU-7). A lineal regression between OD750 and dry weight was obtained:

gDWL $^{-1}$= D 0.89 OD_{750}−0:02 .(n = 10; p < 0:01)

Consequently OD_{750} has been used to estimate biomass in terms of dry weight.

All cultures started with an OD_{750} of about 0.14. The OD_{750} of the cultures was measured every 24 h until the stationary phase was reached. Maximum growth rate of cultures (r) was calculated by fitting the experimental data of culture density for the

first three days of culture to a exponential function:

$$r = (ln\ DWt - lnDW_0)/t$$

where DW_0 is the initial dry weight. DW*t* is the dry weight for day t and t is the time between both measurements.

Cell Composition:

All samples for biochemical analyses were taken at the fourth day of culture. Total internal C, N and the atomic ratio C/N were measured by means of a C: H: N elemental analyser (Perkin-Elmer 2400 CHN). For the extraction of soluble carbohydrates, soluble proteins and phycocyanin, three samples from each culture were centrifuged (5000 rpm 15 min) and resuspended in extraction buffer (0.1 M phosphate, 4 mM EDTA and 2 mM PMSF; pH 6.5, 4 _C). Then samples of 1 mL were disrupted by sonication (3_30 sec., Vibra- Cell*TM*). After sonication samples were centrifuged (15000 rpm, 15 min) and soluble carbohydrates, soluble proteins and phycocyanin were estimated from the supernatant. Soluble carbohydrates were measured as glucose equivalents according to Kochert (1978). Soluble protein concentration was obtained according to Bradford (1976), and phycocyanin content estimated using the equation of Beer & Esherl (1985). For chlorophyll *a* and total carotenoids, culture samples were filtered through Whatmann GF/F and the filter submersed in N,N-dimethyl formamide for 24 h; Chl *a* and total carotenoids concentrations were determined spectrophotometrically according to Wellburn (1994).

Photosynthesis:

Photosynthesis rate was estimated by means of the oxygen evolution method using a Clark-type oxygen electrode (Yellow Spring, 5331) in 9 mL custom-made Plexiglas chambers at 25° C. The photosynthetic parameters were obtained according to Edwards & Walker (1983). Additionally, optimal quantum yield (F*v*/F*m*) was measured by means of a pulse amplitude modulated fluorometer (PAM-2000), being F*v* the maximal variable fluorescence of a dark adapted sample, and F*m* the fluoresence intensity with all PSII reaction centres closed (Büchel & Wilhelm, 1993).

Determination of NO_3, NO_2 and DOC:

Samples for the determination of nitrate, nitrite and dissolved organic carbon (DOC) in the external medium were taken every 24 h by filtration (Whatman GF/F) and

analysed in an automated system (Bran & Luebbe Traacs 800). Nitrate and nitrite estimations were based on Wood et al. (1967) and Snell & Snell (1949), respectively, and DOC was estimated by means of the persulfate oxidation method using UV radiation, CO_2 dialysis and colorimetric determination (Koprivnjak et al., 1995). Since the initial nitrate concentration in N-sufficient cultures was too high (29.4 mmol L^{-1}) to detect the decrease by cell nutrition in short periods, nitrate consumption was only estimated in N-limited cultures (initial concentration of 1 mmol L^{-1}).

Statistics:

Data presented are the mean of three independent experiments, each consisting of two cultures running in parallel for each treatment. Treatments were compared by one factor analyses of variance followed by a multi range test using Fisher's protected least significant differences (LSD). The confidence level was set at 5%.

Results:

Growth

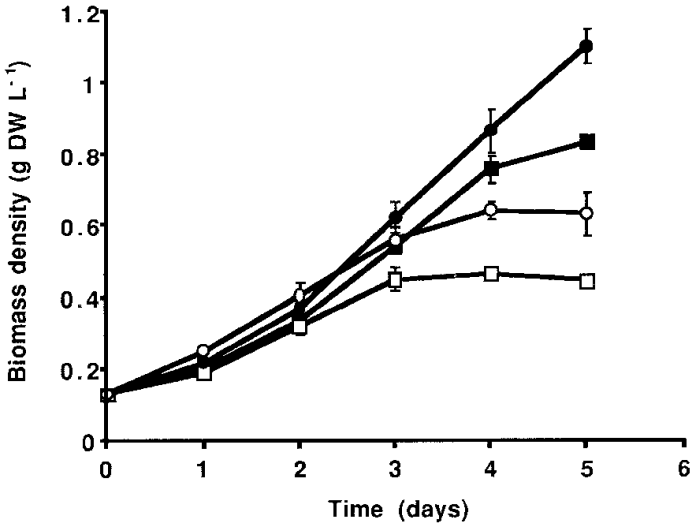

Figure 1. Biomass density of batch cultured *S. platensis* at (■) 1% CO2 and NO_3 saturation, (•) 0.035% CO_2 and NO_3 saturation, (□) 1% CO_2 and NO_3 limitation and (○) 0.035% CO2 and NO− 3 limitation. Standard deviation as a bar when greater than symbol size.

Results of biomass content in the cultures under the different CO_2 and N conditions are shown in Figure 1. Maximum growth rate calculated for the first three days

of exponential growth of the cultures was not significantly different among the treatments, reaching values of 0.45–0.50 d^{-1}. Nevertheless, great differences in biomass yield of the cultures were obtained. Under normal CO_2 and N sufficiency the biomass yield was the highest, while cultures under high CO_2 and N limitation showed the lowest yield. For high CO_2 grown cultures, the low values for biomass yield without variation in maximum growth rate during the exponential phase resulted in that the stationary phase was reached earlier than in normal CO_2-grown cultures. A significant increase in cell carbon content was detected only under nitrogen deficiency at normal CO_2 (697 mg g^{-1} DW), while values were quite constant for the rest of the treatments (513–583 mg g^{-1} DW) (Table 1).

Table 1. Cell composition of S. platensis grown at high (1%) and normal (0.035%) concentration of CO_2, both under N saturation (N+, 29.4 mM NO^-_3) and N limitation (N-, 1 mM NO_3^-). Standard deviation in brackets.

	Normal CO_2		High CO_2	
	N+	N-	N+	N-
Total internal C (mg g^{-1} DW)	574 (83)^a	697 (44)^b	513 (57)^a	583 (48)^a
Total internal N (mg g^{-1} DW)	131 (21)^a	45 (2)^b	108 (12)^c	42 (8)^b
C:N (atomic ratio)	5.1 (0.1)^a	18.1 (0.4)^b	5.5 (0.0)^a	16.6 (3.1)^b
Soluble proteins (mg g^{-1} DW)	285 (9)^a	83 (9)^b	197 (26)^c	70 (9)^b
Soluble carbohydrates (mg eq. Glc g^{-1} DW)	136 (13)^a	881 (114)^b	210 (18)^c	614 (53)^d
Chlorophyll *a* (mg g^{-1} DW)	21.5 (1.3)^a	5.3 (0.9)^b	15.3 (0.4)^c	3.5 (0.9)^d
Total carotenoids (mg g^{-1} DW)	20.2 (3.1)^a	24.1 (3.5)^a	10.5 (0.9)^b	7.9 (1.3)^b
Phycocyanin (PC) (mg g^{-1} DW)	41.6 (1.8)^a	1.1 (0.4)^b	27.8 (4.9)^c	0.6 (0.1)^b

Different superscript for significant differences at 5% confidence level.

Total internal N was decreased by CO_2 enrichment under N sufficiency, but the highest differences were caused by N limitation. The C: N atomic ratio was not affected by CO_2. It was low (5.5–5.1) in high nitrogen, increasing to 16.6–18.1 in low nitrogen (Table 1).

Similar results were obtained for soluble protein and phycocyanin, i.e. CO_2

caused a significant decrease, although highest differences were promoted by N limitation (Table 1). Phycocyanin accounted for about 14% of the soluble proteins in *S. platensis* under N sufficiency, independently of the CO_2 concentration, while it only represented 0.8–1.4% under N limitation, indicating a rapid mobilisation of phycocyanin when nitrogen became deficient.

Soluble carbohydrate content was very sensitive to culture conditions and its variation was greater than that found for total internal C. CO_2 enrichment promoted carbohydrate synthesis under N sufficiency but not under N limitation where values were 30% lower than under normal CO_2. N limitation greatly enhanced soluble carbohydrate synthesis, mainly under normal CO_2, where values were six fold higher than in N sufficiency. Chlorophyll *a* was clearly diminished by 20–25% at high CO_2; in this case, the influence of CO_2 was very similar under N sufficiency and N limitation. As expected, nitrogen limitation strongly decreased the chlorophyll *a* content as well, leading to values up to 75% lower than in N-replete cells. Nitrogen effect was similar under both CO_2 conditions. Maximum value was 21 mg g^{-1} DW, found under control conditions, while N limited cultures at high CO_2 showed chlorophyll *a* values as low as 3.5 mg g−1 DW. High CO_2 also induced a significant decrease of total caretenoids concentration to less than 50% of values under normal CO_2. In the other hand, total caretenoids were not affected by N limitation (Table 1).

Crater Lake

Respiration and photosynthesis rates:

Table 2. Dark respiration rates, photosynthetic parameters from Edwards-Walker fitting of the P–I curves, and optimal quantum yield for PSII charge separation (F*v*/F*m*) of *S. plantesis*. Different superscript for significant differences at 5% confidence level.

	Normal CO_2		High CO_2	
	N+	N-	N+	N-
Respiration [1]	31(8)[a]	46(17)[a]	33(3)[a]	116(19)[b]
Pmax[1]	312(53)[a]	209(43)[b]	13(2)[a]	139 (47)[b]
Ic[2]	11(1)[a]	43(16)[b]	13(2)[a]	297(109)[c]
$I_{0.5}$[2]	146(42)[ab]	273(111)[bc]	192(28)[a]	363(132)[c]
α[3]	2.1(0.3)[a]	1.2(0.3)[b]	1.9(0.2)[a]	1.1(0.4)[b]
F*v*/F*m*	0.48(0.02)[a]	0.21(0.3)[b]	0.59(0.01)[c]	0.19(0.03)[b]

Dark respiration rates in *S. platensis* ranged from 31 to 46 µmol O_2 mg^{-1} chl *a* h^{-1}, except for N-limited cultures at high CO_2, where respiration increased drastically to 116 *µ*mol O_2 mg^{-1} chl *a* h^{-1} (Table 2). The response curve of net photosynthesis measured as O_2 evolution *versus* irradiance (P–I curves) were affected by CO_2 and N supply (Figure 2). The photosynthetic parameters obtained from Edwards-Walker's fitting are shown in Table 2. The effect of CO_2 enrichment on P*max* was dependent on N status. Increasing CO_2 enhanced P*max* in N-sufficient cultures reaching the highest value of 418 *µ*mol O_2 mg^{-1} chl *a* h^{-1}. The light compensation point for oxygen evolution (I*c*) mainly reflected differences in respiration rate (Table 2). N limitation induced a significant increase in I*c*, especially in high CO_2 grown cultures, reaching values of 300 µmol m^{-2} s^{-1}. The initial slope of the P–I curves (α), namely the photosynthetic efficiency, was not affected by CO_2 enrichment in spite of the enhancement in optimal quantum yield for PSII (F*v*/F*m*, Table 2). The highest values of *α* were found in N saturation, reaching values around 2 µmol O_2 mg^{-1} Chl *a* [_mol photons m^{-2} s^{-1}] h^{-1}, but decreased to 50% under N limitation. This drastic decrease of the efficiency of the photosynthesis at low irradiance under N limitation also affected I*c*.

Irradiance for half-saturation of photosynthesis (I0.5, Table 2) combines both α and P*max*; photosynthesis was half-saturated at 146 and 192 µmol photon m^{-2} s^{-1} at normal and high CO_2, respectively, when nitrogen was supplied in excess, increasing to 273 and 363 µmol photons m^{-2} s^{-1} at normal and high CO_2, respectively, under nitrogen limitation.

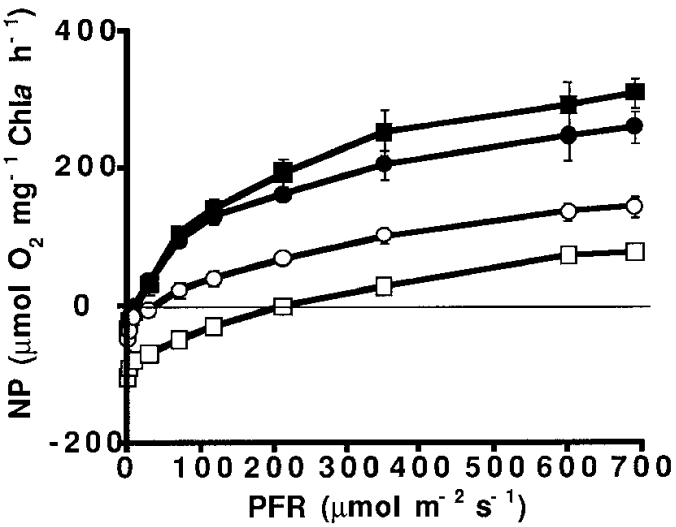

Figure 2.

Net photosynthetic O2 evolution rates (NP) *versus* irradiance in *S. platensis*. Symbols as in Figure 1.

Organic C release:

In addition to respiration, C fixed by photosynthesis can be lost by release of organic compounds. The amount of dissolved organic carbon present in the external medium was daily monitored. This C supposes a loss in primary production efficiency of *S. platensis*. Rates of primary production lost by release of organic C to the medium were dependent on the phase of growth (Figure 3). During the first 24 h, the percentage of primary production lost accounted for 30–50%, regardless of the experimental conditions. The percentage was the lowest during the exponential phase of growth, with values below 5%for all treatments. Great differences between treatments appeared during the stationary phase of growth, in which low CO_2 grown cells released less than 5% while high CO_2 caused a strong reduction in primary production efficiency, being lost more than 30% under N sufficiency and more than 60% under N limitation.

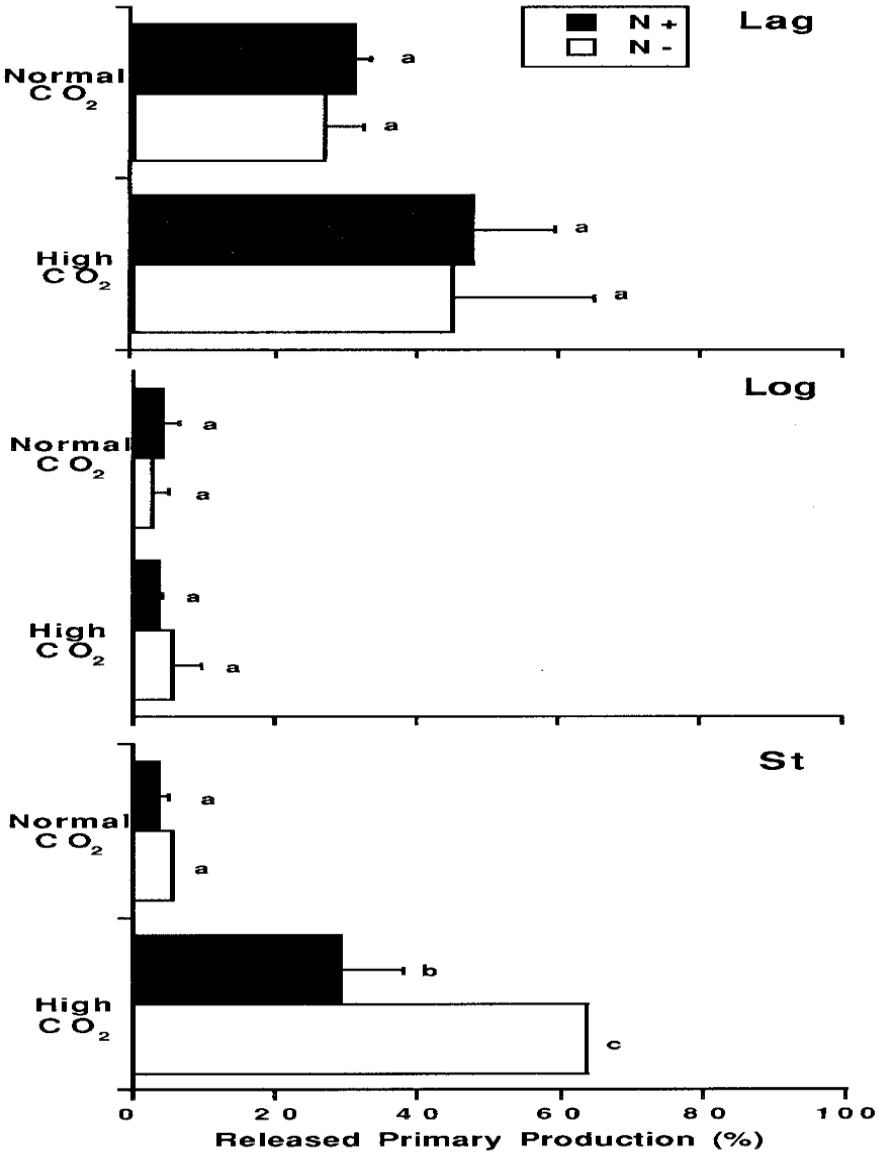

Figure 3.

Percentage of primary production lost by organic carbon release for lag (days 0–1), logarithmic (days 1–3) and stationary (days 3–5) phase of growth. Different letters for significant differences at 5% confidence level.

NO_3 assimilation:

Nitrate consumption was daily monitored in high and normal CO_2 cultures under N limitation. External nitrate dropped down from 1 mM to values of about 0.1 mM in two days, the rate of nitrate uptake being similar in both high and normal CO_2 (Figure 4a). Cultures of *S. platensis* released NO_2^- to the medium (Figure 4b). Nitrogen limited cultures released NO_2^- to levels around 5–10 µM NO_2^- in the medium, that decreased to non-detectable levels after two days. High nitrogen cultures released higher amounts of NO_2^- , that accumulated to concentrations of

about 20 µM in high CO_2 cultures after 5 d and to values >100 μM NO_2^- in normal CO_2 cultures.

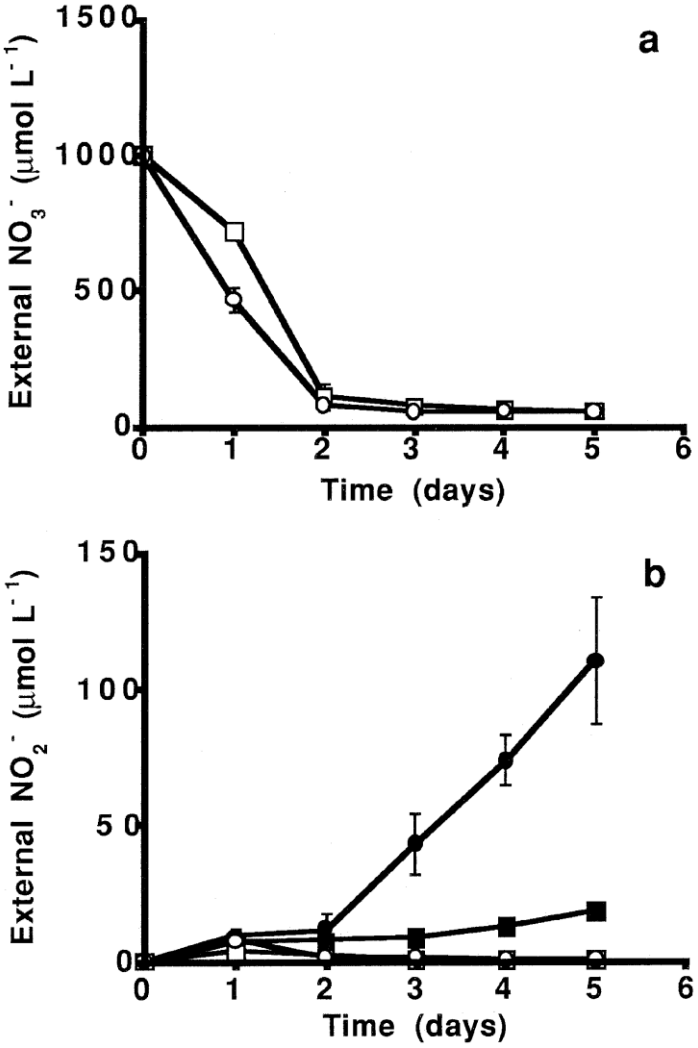

Figure 4.

(a) Nitrate and (b) nitrite in the growth medium of batch cultured *S. platensis*. Symbols as in Figure 1.

Discussion:

Light Harvesting:

Values obtained for respiration rate, light compensation point, α and I0:5 under standard growth conditions (normal CO_2 and N saturation) are on the same range of those obtained by Torzillo & Vonshak (1994), Vonshak et al. (1996a, b) and Vonshak (1997), whose cultures of *S. platensis* were enriched with 1% CO_2. Nevertheless, P*max* was about half of the values reported by those authors for *Spirulina platensis*. This strain of *S. platensis*, i.e. Compère 1968–3786, has been tested in outdoor cultures in raceway ponds, together with other two *S. platensis* strains (e.g. Laporte M132-1 and Laporte 1963–8579). It was found that

biomass yield of strain Compère 1968–3786 was about half of the other two strains tested (Jiménez et al., unpublished data), indicating that low P*max* is characteristic for this strain.

Decrease of pigment content and photosynthesis are a typical response in N limited algae (Turpin, 1991), but in addition, phycocyanin, chlorophyll *a* and total carotenoids were also reduced by CO_2 in *S. platensis*. Nevertheless, in high CO_2 grown cells, P*max* was not decreased but increased (under N sufficiency) or remain unchanged (under N limitation), and α was constant as well (Figure 2, Table 2).We propose that in *S. platensis* CO_2 could act promoting degradation of pigments synthesized in excess that are not necessary for light harvesting. The increase observed in F*v*/F*m* under high CO_2 would provide evidence in that sense (Table 2). García-Sanchez et al. (1994) previously proposed a control role for CO_2 on pigment composition of the red alga *Gracilaria tenuistipitata*.

C assimilation

Nitrogen limitation caused photoassimilated C to be redirected towards the synthesis of carbohydrates instead of proteins and chlorophyll. This response has been widely observed in many algal species (Turpin, 1991). As a consequence, cell C and N content was unbalanced as it is reflected by the high C:N values obtained. Protein and chlorophyll decrease and carbohydrate increase by CO_2 enrichment has been previously observed in a number of species (Loehle, 1995); in *S. platensis* these changes did not influence the C:N ratio, being in agreement with those reported by Fox (1996) for this species. The capability to store soluble carbohydrates intracellularly seemed to be saturated under high CO_2 and the excess of photoassimilated C could be released to the growth medium, which is evidenced by the increase in the percentage of primary production released at the end of the exponential phase of growth (Figure 3). This was especially relevant under N limitation, where additional CO_2 caused a loss of 60% of the primary production, released to the medium as organic carbon, while internal stored carbohydrates were lower than in normal CO_2-grown cells. Organic carbon release is considered a mechanism to maintain metabolic integrity in response to environmental conditions (Wood et al., 1992). In S. platensis it could then be considered as an efficient mechanism in response to ambient CO_2 increase, able to maintain the balance

between carbon and nitrogen at high CO_2 levels. The increase in organic C release to the external medium as a consequence of CO_2 enrichment has been previously reported in the green unicellular alga *Dunaliella salina* by Giordano et al. (1994). Although external organic carbon has been considered as a quality problem in outdoors cultures of *S. platensis*, few studies have tested the factors influencing this loss of photosynthetic carbon. Cornet (1992) reported that S. platensis released around 10% of produced polysaccharides even under optimal growth conditions which are in agreement with the low values of C release obtained in this work during the exponential phase of growth (Figure 3).

Nitrogen assimilation and Growth:

CO_2 enrichment slightly affected the total internal nitrogen content, while it was drastically affected by N limitation. Phycobiliproteins have been proposed as the main N pool that is mobilised under N demand in red algae (Vergara & Niell, 1993) and cyanobacteria (Allen & Smith, 1969; Wyman et al., 1985). The ratio phycocyanin/soluble proteins did not vary either at normal or high CO_2 levels, PC accounting for about 14% of the total soluble proteins at N sufficiency, decreasing drastically under nitrogen limitation, indicating that PC is mobilised in case of N demand, being N metabolites redirected towards the synthesis of non-pigmented proteins rather than pigmented ones, as reported by Vergara & Niell (1993) for the red macroalga *Corallina elongata*. On the other hand, the pool of cell nitrogen that forms part of soluble proteins did not vary significantly among the treatments, and it accounted for about 30% of total cell nitrogen, thus indicating that in *S. platensis* PC hardly could act as a major N-storage, thus, the structural proteins accompanying pigments in the membrane complexes should be the main responsible of internal N decrease as proposed by Markager & Sand-Jensen (1994).

It is commonly reported that the enhancement of growth in response to CO_2 enrichment occurs only when nitrogen assimilation is also increased. In higher plants, where the effects of CO_2 enrichment have been extensively studied, the stimulation of growth by CO_2 enrichment is usually a transitory effect caused by enhanced photosynthesis, leading just to an increase in soluble carbohydrates, being proposed that the lack of enhancement of the specific growth rate in the long-term is a

consequence of an absence of parallel stimulation of the nitrogen assimilation (Loehle, 1995). Gordillo et al. (unpublished data) have found a stimulation of growth by CO_2 in the green macroalga *Ulva rigida* related to an increase in nitrate reductase activity. Larsson et al. (1985) reported that CO_2 stimulation of growth rate correlated with high nitrate uptake rates and increased internal N content in the unicellular chlorophyta *Scenedesmus obtusiusculus*. Yunes (1995) described an increase in growth rate in the cyanobacterium *Anabaena variabilis* in parallel with an enhancement of nitrite assimilation. In this work, nitrate assimilation was not stimulated by CO_2 (Figure 4) and the internal N content was only slightly changed by CO_2 enrichment (Table 2). This might be the reasons to explain why the maximum growth rate of this species is not enhanced by CO_2 enrichment. It has been proposed that high CO_2 affects C and N metabolism indirectly, as a consequence of soluble carbohydrates accumulation which could be the responsible of the control of photosynthesis (The so-called down regulation, Webber et al., 1994) and nitrogen assimilation (Larsson et al., 1985, Krapp & Stitt, 1994). The down regulation of photosynthesis cannot be discarded to occur in N limited *S. platensis* whilst an enhancement of nitrogen assimilation by the soluble carbohydrates level is hardly possible on the view of the data presented here.

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