Influence of elevated CO2 and nitrogen supply on the carbon assimilation performance and cell composition of the unicellular alga Dunaliella viridis

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The carbon assimilation efficiency and the internal composition of the chlorophyte Dunaliella viridis have been studied under conditions of current (0.035%) and enriched (1%) levels of CO2, with and without N limitation (supplied as nitrate). Results show that both photosynthesis and growth rates are enhanced by high CO2, but the strategy of acclimation also involves the light harvesting machinery and the nutritional metabolism in an N supply dependent manner. D. viridis carried out a qualitative rather than a quantitative acclimation of the light harvesting system leading to increased PSII quantum yields. Total internal C decreased as a consequence of either active growth or organic carbon release to the external medium. The latter process allowed photosynthetic electron transport to proceed at higher rates than under normal CO2 conditions, and maintained the internal C:N balance in a narrow range (under N sufficiency). N limitation generally prevented the effects of high CO2, with some exceptions such as the photosynthetic O2 evolution rate.

Introduction

In the last 200 years atmospheric CO2 concentration has increased as a result of human activity (Vitusek 1994). Such an increase in atmospheric CO2 is especially relevant for photosynthetic organisms because inorganic carbon is their main nutrient. Among them, aquatic primary producers account for about 50% of the total carbon fixation in the biosphere (Falkowski and Raven 1997).

One of the main queries for depicting future scenarios of evolution of atmospheric composition and temperature is whether an atmospheric CO2 increase stimulates primary production, especially in aquatic plants. To answer this question it is necessary to know not only whether photosynthesis is saturated at the present carbon concentration in the oceans, but also whether there is a corresponding effect on growth (Riebesell et al. 1993). Around 30 years ago, Akita and Tanaka (1973) already found that increased levels of CO2 stimulated photosynthesis and growth in C3 plants. More recently, Raven (1991) proposed that those species in which photosynthesis is already saturated by inorganic carbon would decrease their energy requirements for the maintenance of the carbon concentrating mechanisms, thus using a larger proportion of the energy for growth. However, the majority of the studies on the effects of increasing CO2 in aquatic photosynthetic organisms have focused mainly on the uptake of carbon, while unlike their terrestrial counterparts, little attention has been paid to other processes, such as the basic nitrogen metabolism or changes in the biochemical composition. According to Turpin (1991), about 50% of the carbon metabolism may be linked to nitrogen metabolism in plants. In terrestrial higher plants, it has been proposed that the response of plants to increasing CO2 would be similar to that of nitrogen limitation (Webber et al. 1994). This correspondence is seldom applicable to algae due to complex interactions between the metabolism of carbon and nitrogen. However, only a limited number of reports deal with the combined effects of CO2 and N supply in these organisms (e.g. Giordano and Bowes 1997, Gordillo et al. 1999, Andrià et al. 2001).

The release of photosynthetic products (organic carbon) has been claimed to play a role in the regulation of the internal balance between carbon sources and sinks in algae, maintaining the metabolic integrity of the cell under changing environmental conditions, protecting the photosynthetic machinery from an overload of products that cannot be accumulated intracellularly (Fogg 1983, Ormerod 1983). This process would allow electron flow...
to proceed and can account for up to 95–100% of the C fixed by photosynthesis in phytoplankton (Fogg et al. 1965). Changes in irradiance or nutrient concentration can alter the percentage of C released with respect to the total C fixed by photosynthesis (Sharpe 1977, Zlotnik and Dubinsky 1989). The amount of organic carbon released to the external medium is of particular relevance in the ecosystems where D. viridis is found. Microalgae of the genus Dunaliella is among the most ubiquitous eukaryotic organisms in hypersaline environments, and often the major primary producer in salt lakes and in the evaporation ponds of salt works (Nissenbaum 1975, Post 1977, Borowitzka 1981). The evaporation ponds of salt works can show strong photosynthetic activity, leading in many cases to a drop in the formation of commercial grade salt crystals due to the presence of dissolved organic carbon (DOC) in the brine (Baha Al-Deen and Baha Al-Deen 1972).

In this study we analyse the combined effects of increasing CO₂ concentration at different nitrogen status on cell composition and carbon assimilation performance in the chlorophyte D. viridis.

Materials and methods
Plant material and experimental design
D. viridis Teodoresco was isolated from the athalassic lake Fuente de Piedra in Málaga (Spain) and maintained in culture in the laboratory in the medium described by Johnson et al. (1968) at a salinity of 1 M NaCl at 25 ± 1°C. For experimentation, D. viridis was batch cultured in a duplicate series of 250 ml in Perspex cylinders at a constant photon fluence rate (PFR) of 100 μmol m⁻² s⁻¹, provided by cool-white fluorescent lamps (OSRAM (Munich, Germany) daylight L20W/10S) measured by a spherical sensor (LiCor 193 SB) connected to a LiCor-1000 radiometer (LiCor, Lincoln, NB, USA). All cultures started at a cell density of 10⁶ cells ml⁻¹ and were aerated at 1 l min⁻¹ according to Jiménez et al. (1996).

Treatments corresponded to two different CO₂ levels in the bubbling aeration system, non-manipulated air (atmospheric CO₂ level of 0.035%) and 1% CO₂ (namely high CO₂), and nitrate provided to the cultures at two different concentrations, i.e. high nitrate (5 mM) and low nitrate (0.5 mM which was almost completely consumed after 48 h). The usual conditions for maintenance (non-manipulated air and high nitrate) were considered as the control treatment.

Cell counting and growth rate
Cell density was measured daily using a Coulter Counter Multisizer-II (Luton, UK), with a 0.07-mm orifice tube. Maximum specific growth rate (r, day⁻¹) was calculated by fitting the experimental data of cell density for the first 3 days of cultivation to an exponential function.

Cell composition
Samples for cell composition analysis were taken the fourth day of culture. Total internal carbon and nitrogen were measured with a C:H:N elemental analyser (Perkin-Elmer (Wellesley, MA, USA) 2400CHN) after filtration of 1 ml of culture (Whatman GF/F). Three replicates from each culture were dried overnight at 60°C before the analysis. Soluble protein concentration was measured according to Bradford (1976) in pelleted cells (5 min at 1000 g). Pigment analysis was performed after extraction with N,N-dimethyl formamide also from pelleted cells during 24 h at 4°C; pigment concentrations were calculated according to Wellburn (1994).

Photosynthesis and chlorophyll fluorescence
Photosynthesis and dark respiration rates were estimated as O₂ evolution rates by means of a Clark-type electrode (YSI 5331) installed in a 9-ml Perspex chamber at a constant temperature of 25°C. Samples were taken the fourth day of culture. Photosynthesis was measured at culture irradiance (100 μmol m⁻² s⁻¹) and at saturating irradiance (600 μmol m⁻² s⁻¹). Optimal quantum yield (Fv/Fm) and effective quantum yield at growth irradiance (ΔF/Fm') of photosystem II (PSII) charge separation were measured by means of a pulse amplitude modulated fluorometer (PAM-2000, Waltz, Effeltrich, Germany). F₀ is the basal fluorescence, Fv is the maximal variable fluorescence of a dark adapted sample, Fm the fluorescence intensity with all PSII reaction centres closed, F the variable fluorescence at any time during induction and Fm' the light saturated fluorescence according to Genty et al. (1989). Additionally, for D. viridis, the photochemical quenching (qP) and non-photochemical quenching (NPQ), as well as the relative electron transport rate (rETR) were calculated as:

\[ qP = \frac{(F_m' - F_i) - (F_m' - F_0)}{F_m' - F_0} \]
\[ NPQ = \frac{(F_m - F_m')}{F_m'} \]
\[ rETR = \frac{\Delta F}{F_m'} \times \text{PFR} \]

For clarity in the discussion of processes, we preferred to use the PSII excitation pressure (1–qP), rather than qP. All measurements were performed on the fourth day of cultivation.

Determination of DOC
Samples for the determination of dissolved organic carbon (DOC) in the medium were taken daily and analysed in an automated system (Traacs 800, Bran & Luebbe (Norderstedt, Germany). After removing the pre-existing inorganic carbon in the sample by acidification, organic carbon was oxidized to CO₂ by means of persulphate oxidation and UV radiation. After CO₂ dialysis, proportional changes in pH were determined colourimetrically using phenolphthalein (Koprivnjak et al. 1995). The percentage of assimilated carbon released as DOC was calculated as the increase in DOC divided by the sum of increase in DOC plus increase in total cell carbon in the culture during a specific period of time, and multiplied by 100.
Statistics

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

Results

Total biomass production of *D. viridis* was enhanced at high CO2 (Fig. 1). In N sufficiency, maximal cell density was 60 x 10⁶ cell ml⁻¹ at high CO2 and only 20 10⁶ cell ml⁻¹ in non-CO2 enriched cultures. Cell doubling rates (r) were also enhanced at high CO2 in N sufficiency. Nitrogen limitation prevented the enhancing effects of CO2, and maximal biomass and r were greatly reduced (Table 1).

Increasing CO2 resulted in a significant decrease of the total cell C from 12.5 to 8.6 pg C cell⁻¹ in N sufficiency, and from 15.6 to 10.5 pg C cell⁻¹ in N limitation (Table 1). Total cell nitrogen also decreased at high CO2, regardless of the external N supply. As a result, the C:N ratio decreased at high CO2 from 8 to 7 in N sufficiency, but did not change from values around 12 in N limitation.

The soluble protein concentration was significantly reduced by nitrogen limitation, as well as by high CO2. Chlorophyll a was not affected by high CO2 in N sufficiency but in N-limitation the cell content was reduced by 50% (Table 1). The total carotenoids content decreased at high CO2, and also at N-limitation. The ratio chlorophyll b/chlorophyll a is an indirect indication of antenna size. In *D. viridis* this ratio was not significantly affected by N limitation or high CO2.

High CO2 induced an increase in the rate of photosynthesis (as O2 evolution) (Table 2), especially at high N. Dark respiration was increased mainly by nitrogen deficiency, while CO2 had little effect. Optimum quantum yield for PSII (Fv/Fm) slightly increased at high CO2 when N was supplied in excess. N limitation decreased Fv/Fm and prevented the positive effect of CO2. However CO2 induced a significant increase in ΔF/Fm' even in N-limitation (but to a lower extent). The relevance of the effect of CO2 on these light harvesting-related parameters (antenna size and quantum yield) in *D. viridis* was further investigated by examining the relative electron transport rate between the two photosystems (rETR), as well as the photochemical (qP) and non-photochemical quenching (NPQ).

Relative electron transport rate was largely affected by both CO2 and N supply (Fig. 2A). CO2 enhanced rETR in N sufficiency to about double the values reached in non-manipulated air. N limitation decreased rETR and prevented the enhancing effect of CO2. Photochemical quenching is better expressed as 1–qP that corresponds to the PSII excitation pressure. The highest values corresponded to N sufficient cells, especially in nonenriched air, where excitation pressure reached saturation. N-limitation caused a drastic drop in 1–qP for both CO2 conditions. Accordingly, Fig. 2C shows highest NPQ for N-limited cultures. In N sufficient cells, NPQ is higher at high CO2 in low light, but as 1–qP reached saturation in nonenriched air treatment, NPQ surpassed those of CO2-enriched cultures.

*D. viridis* released a variable proportion of the carbon fixed to the culture medium, in the form of organic compounds (Fig. 3). The level of CO2 and N, and also the phase of growth, influenced the extent of this process. At low CO2 (both at low and high NO3⁻) and at high CO2 + low NO3⁻ there was a similar accumulation of dissolved organic carbon in the growth medium, reaching 10 mM C after 7 days of culture. However, high CO2 + high NO3⁻ induced a significant increase of the organic carbon released by the cells, reaching 27 mM C in the same period of time. The percentage that this organic carbon released to the medium represented over the total assimilated carbon varied depending on the growth phase (Fig. 3). During the lag phase (days 0–2), 50–60% of the assimilated carbon was released, independently of the growth conditions; during the phase of logarithmic growth (days 2–5), carbon release represented 25% of the assimilated carbon at high nitrate (independently of the CO2 level), and it increased to 50 and 60% at low and high CO2, respectively, when nitrogen was limiting. During the stationary phase (days 5–7) organic carbon released to the medium accounted for >80% of the carbon assimilation in nitrogen deficiency, and 70% in nitrogen sufficiency at high CO2, while it represented only 35% at high NO3⁻ and low CO2.

Discussion

It has long been debated whether phytoplankton species are growth-limited by current levels of CO2 in the aquatic systems, i.e. whether an increase in atmospheric CO2 could stimulate growth (Riebesell et al. 1993). The species studied here, the chlorophyte *D. viridis*, has previously been used as model species for the study of inorganic carbon uptake. This species possesses carbon
concentrating mechanisms (Goyal et al. 1992, Rotatore et al. 1995) but further analyses on its metabolism are scarce (Giordano and Bowes 1997). Photosynthesis and growth rate stimulation by increased CO2 in N sufficiency were not the only response, but mechanisms of acclimation in the light harvesting system and the nutritional metabolism were also involved. The decrease in chlorophyll at high CO2 is a relatively common response (Gordillo et al. 1999, Andrés et al. 2001), and even a qualitative pigment changes were also inferred from the pigment ratio, Table 1) remained unchanged. Qualitative pigment changes were also observed by Poza-Carrío et al. (2001) in the cyanobacterium Nostoc sp., where high levels of CO2 induced an increase in the number of phycobilisomes, but a decrease in their size. Similarly, for D. viridis, CO2 could promote an increase in the number of PSII reaction centres of smaller size. According to Sukênik et al. (1987), Dunaliella is able to modify PSII excitation by altering the number of chlorophyll molecules bound to each protein in the light-harvesting pigment-protein complexes.

The strategy proposed for D. viridis can be further developed by examining changes in the quenching characteristics of the excitons reaching the reaction centres. In N sufficiency, lower PSII excitation pressure (1–qP) at high CO2 would correspond to a higher demand for electrons exerted by C fixation that would act as a sink for excitons, as reflected by a higher photosynthetic electron transport rate (Fig. 2A). This is supported by the excitation pressure being alleviated (Fig. 2B), thus avoiding saturation. Under normal CO2 conditions (N sufficient cells), lower rETR could lead to the saturation observed in 1–qP, and consequently, to an increase in NPQ at relatively high irradiance (Fig. 2C). On the other hand the drastically reduced 1–qP values registered in N-limitation would correspond to a lower capacity to harvest light. This is supported by the poor pigmentation, and low Fv/Fm and ΔF/Fm′ values observed in N limitation. As a consequence NPQ is dramatically enhanced. Although xanthophylls, and especially zeaxanthin, could potentially participate in NPQ (Masojidek et al. 1999), no significant amount of these compounds were found in the present study (not shown).

The release of organic carbon to the external medium has been proposed as a mechanism for maintaining the metabolic integrity of the cell (Fogg 1983, Ormerod 1983). According to Wood and Van Valen (1990), organic carbon release would be a sink mechanism protecting the photosynthetic apparatus from an overload of products that cannot be invested in growth or stored. It

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**Table 1. Growth rate and internal components of D. viridis batch cultured with nonenriched (air) and 1% CO2-enriched air (+CO2), in N-sufficient (N+), and N-limited (N–) conditions. Standard deviations in brackets (n=6), different superscripts for significant differences (P<0.05).**

<table>
<thead>
<tr>
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<th>Air</th>
<th>+ CO2</th>
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<td></td>
<td>N+</td>
<td>N–</td>
<td>N+</td>
<td>N–</td>
</tr>
<tr>
<td>r(d⁻¹)</td>
<td>0.55 (0.04)a</td>
<td>0.40 (0.02)b</td>
<td>0.70 (0.02)c</td>
<td>0.60 (0.03)</td>
</tr>
<tr>
<td>Total C (pg cell⁻¹)</td>
<td>12.5 (0.3)a</td>
<td>15.6 (0.6)b</td>
<td>8.6 (0.4)c</td>
<td>10.5 (0.2)b</td>
</tr>
<tr>
<td>Total N (pg cell⁻¹)</td>
<td>1.79 (0.07)a</td>
<td>1.43 (0.05)b</td>
<td>1.40 (0.08)b</td>
<td>0.95 (0.06)c</td>
</tr>
<tr>
<td>C:N (atomic ratio)</td>
<td>8.1 (0.4)a</td>
<td>12.7 (0.2)b</td>
<td>7.2 (0.1)c</td>
<td>12.9 (0.9)b</td>
</tr>
<tr>
<td>Soluble Proteins (pg cell⁻¹)</td>
<td>47.1 (3.3)a</td>
<td>38.8 (3.4)b</td>
<td>33.6 (0.2)bc</td>
<td>28.4 (0.5)c</td>
</tr>
<tr>
<td>Chlorophyll a (fg cell⁻¹)</td>
<td>347 (38)a</td>
<td>300 (4)b</td>
<td>395 (15)c</td>
<td>191 (4)c</td>
</tr>
<tr>
<td>Chlorophyll b (fg cell⁻¹)</td>
<td>127 (12)a</td>
<td>93 (15)b</td>
<td>134 (11)c</td>
<td>70 (22)b</td>
</tr>
<tr>
<td>Carotenoids (fg cell⁻¹)</td>
<td>161 (6)a</td>
<td>152 (5)c</td>
<td>137 (4)b</td>
<td>117 (12)c</td>
</tr>
<tr>
<td>Chl b/Chl a</td>
<td>0.24 (0.01)a</td>
<td>0.31 (0.01)c</td>
<td>0.24 (0.01)c</td>
<td>0.37 (0.11)a</td>
</tr>
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**Table 2. Photosynthetic rates and both optimum and effective quantum yields for PSII charge separation of D. viridis batch cultured with nonenriched (air) and 1% CO2-enriched air (+CO2), in N-sufficient (N+), and N-limited (N–) conditions. Standard deviations in brackets (n=6), different superscripts for significant differences (P<0.05).**

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>+ CO2</th>
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<tbody>
<tr>
<td></td>
<td>N+</td>
<td>N–</td>
<td>N+</td>
<td>N–</td>
</tr>
<tr>
<td>Pmax* (fmol O₂ cell⁻¹ h⁻¹)</td>
<td>36 (6)a</td>
<td>18 (2)b</td>
<td>77 (9)c</td>
<td>21 (4)b</td>
</tr>
<tr>
<td>APS** (fmol O₂ cell⁻¹ h⁻¹)</td>
<td>12 (3)a</td>
<td>−1 (1)b</td>
<td>38 (4)c</td>
<td>1 (0)b</td>
</tr>
<tr>
<td>Dark Respiration (fmol O₂ cell⁻¹ h⁻¹)</td>
<td>8 (1)a</td>
<td>17 (2)b</td>
<td>13 (1)ab</td>
<td>24 (5)c</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.68 (0.02)a</td>
<td>0.61 (0.01)b</td>
<td>0.71 (0.00)c</td>
<td>0.61 (0.02)b</td>
</tr>
<tr>
<td>ΔF/Fm/ΔFm/ΔFm′**</td>
<td>0.32 (0.01)a</td>
<td>0.13 (0.02)b</td>
<td>0.43 (0.01)c</td>
<td>0.17 (0.02)d</td>
</tr>
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*Measured at saturating PFR (600 μmol m⁻² s⁻¹).
**Measured at growth PFR (100 μmol m⁻² s⁻¹).
has long been recognized that conditions favouring release seem to be species- and growth phase-dependent (Huntsman 1972, Hellebust 1974). In *D. viridis* organic carbon release seemed to be a highly significant process, influenced by the growth phase and able to respond to treatment conditions. The relatively high release values found in the lag phase would reflect the so-called dilution stress, rather than the effect of the treatments at that stage. Previously, Giordano et al. (1994) and Gordillo et al. (1999) found that short-term incubation under high or low CO2 conditions were not sufficient to produce a significant difference in *D. salina* and the cyanobacterium *Spirulina platensis*, respectively. Differences in organic carbon release due to N limitation appeared in the exponential phase of growth, while CO2 influenced the percentage of carbon being released only in the stationary phase of growth. The latter is in agreement with the pattern found for *S. platensis* (Gordillo et al. 1999). In N sufficiency, the increase in the release of organic carbon observed at high CO2 in the stationary phase of growth coincides with lower internal C levels. These low levels of internal carbon would be in agreement with the global strategy proposed for *D. viridis* (in N sufficiency) according to which the accumulation of photosynthetic products are reduced to avoid overload and produce a high demand for photosynthates, which leads to increased electron flow between photosystems as discussed above. The process would then divert assimilated C to either the production of new biomass, or the release to the external medium once the culture conditions do not allow further exponential growth. The consequence is also the maintenance of the internal C:N balance in a tight

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**Fig. 2.** Relative PSII electron transport rate (rETR) (A), PSII excitation pressure as 1–qP (B), and non-photochemical quenching (C) of *D. viridis* grown with-non-manipulated air (circles) or 1% CO2-enriched air (squares), in N sufficient (N+, black symbols) and N limited conditions (N−, open symbols). Standard deviations as bars when bigger than symbol size (n = 6).

**Fig. 3.** Percentage of assimilated C released lost to the medium as dissolved organic carbon in the lag (0–2 days), logarithmic (2–5 days) and stationary (5–7 days) phase of growth, batch cultured with-non-manipulated air or 1% CO2-enriched air (+ CO2), in N sufficient (N+) and N limited conditions (N−). Standard deviations as bars. Different letters for significant differences (P < 0.05, n = 6).
range (Table 1) as previously observed, not only in *D. viridis* (Gordillo et al. 2001a), but also in the green seaweed *Ulva rigida* (Gordillo et al. 2001b) and the cyanobacterium *Spirulina platensis* (Gordillo et al. 1999).

In conclusion, it is not sufficient to know whether CO₂ stimulates photosynthesis and biomass production. Different mechanisms can also be involved in the acclimation strategy, e.g. internal composition and C assimilation efficiency. Regarding C–N interactions, although N limitation generally prevented the effects of high CO₂, this was not always the case (e.g. CO₂ led to lower internal C in *D. viridis*, regardless of the N supply). Organic C release seemed to be a highly relevant acclimative process allowing high electron flow and the maintenance of the cellular C:N balance.

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