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## Growth-compensating phenomena in continuous cultures of *Dunaliella tertiolecta* limited simultaneously by light and nitrate

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### Abstract

Two nitrogen-limited continuous cultures of *Dunaliella tertiolecta* were grown on light/dark cycles. One was submitted to limiting photon flux density (PFD) and the other to nonlimiting PFD. The growth rate was identical in the two cultures despite the difference in the PFD conditions. Once equilibria were reached in both cultures, the PFD were reversed to simulate a decrease and an increase in light conditions. A large suite of variables was measured to characterize the response of the cells, mainly through the interactions of carbon and nitrogen assimilation pathways. A decrease in irradiance led to a rapid decrease in algal biovolume; the biovolume-based growth rate ( $\mu$ ) descended to levels lower than those estimated for cells of the light-limited culture. An increase in irradiance rapidly led to an increase in  $\mu$ , which attained values greater than those observed in high-light cells of the other culture before the shift. For the two cultures before and after the shift, cell carbon and cell volume were strongly correlated, showing the same pattern of diel variations.

The specific C fixation rates ( $\mu\text{C}$ ) of the two cultures before and after the light shift declined significantly during the light periods. Before the light shift,  $\mu\text{C}$  was paradoxically higher in the low light culture and could not be predicted only from the light levels. This suggests that some compensatory phenomena may occur during light and nitrogen limitations. Time variations of cell Chl *a* due to photoacclimation in both cultures were correlated with their N status. Similarly, the regulation of carboxylase activities (Rubisco) by the light levels was sensitive to the degree of N limitation. We found that nitrogen limitation has an overriding effect compared to light for the regulation of cell volume, C fixation and respiration rates, Chl *a* synthesis, electron transport system, and Rubisco activities. In cultures subjected to low irradiance,  $\text{NO}_3^-$  uptake rate decreased several hours into the dark phase, suggesting a time lag between the end of photosynthate production and the exhaustion of compounds necessary for dissolved inorganic nitrogen assimilation. The implications of these results concerning phytoplankton growth modeling in a variable environment are important because there are no existing models that correctly integrate the simultaneous effects of light and nitrogen on primary production. It is shown that the effects of these limitations were not additive in the range of light and N limitations tested in this experiment.

It is now evident that the understanding of physiological processes involved in the acclimation of phytoplanktonic organisms subjected to a variable light or nutrient environment

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requires simultaneous studies of C and N assimilation pathways (Turpin 1991). However, phytoplankton growth models have generally been separately elaborated to represent either the effects of light on C fixation or the effect of N limitation on uptake and assimilation rates of cells. These models carry the implicit assumption that if light (or N) is considered as the limiting growth factor, only the C (or N) pathways need to be considered, and that it is not necessary to take into account the effect of N (or light).

Apart from the fact that the dynamic nature of the marine environment can force phytoplanktonic organisms to adapt simultaneously to light and nutrient variations, the requirement to integrate both N and C metabolisms into a single

study stems from the intrinsic coupling of their metabolism on at least three levels. First, from a structural point of view, the coupling is obvious, because the protein synthesis regulating cell growth requires significant amounts of C and N in a rather strict ratio. Second, from a functional point of view, the effects of N limitation occur at the level of the light and dark reactions of photosynthesis. For example, the efficiency of light capture through pigment complexes depends on the availability of certain apoproteins involved in light-harvesting systems. N deficiency can significantly decrease the Chl *a* content of unicellular algae (Caperon and Meyer 1972; Harrison et al. 1977). Thus, the ecological relevance of studies carried out on N-sufficient biological material is not clear in view of the fact that large oceanic areas are thought to be N limited (Dugdale 1967; Ryther and Dunstan 1971; Thomas 1969, 1970). Similarly, C fixation is carried out by Rubisco, a high-molecular-weight protein that constitutes a major N reservoir for plants (Glover 1989; Osborne and Geider 1986). In the case of N starvation, Cleveland and Perry (1987) have shown that those N pools can be mobilized for other functions, for example amino acid (InAA) production for structural protein synthesis. Finally, from an energetic point of view, primary substrates, which are either reduced (NAD(P)H<sub>2</sub>) or energy-rich (ATP) compounds and are initially produced by light reactions of photosynthesis, are used in a competitive fashion in enzymatic reactions for reduction of CO<sub>2</sub> and oxides of other cellular inorganic elements such as N or S. This energetic competition between assimilation pathways of inorganic C and N may depend on the type of limitation and on the oxidation state of N compounds (Turpin 1991).

The multiplicity of interactions between metabolic pathways allows the organisms to maintain a high growth rate ( $\mu$ ) in suboptimal light or nutrient conditions. The work of Rhee and Gotham (1981) has shown how cells of *Scenedesmus* sp. in continuous culture exposed to a light gradient are able to maintain a stable  $\mu$  by increasing their N quota. These authors suggested that a deficiency in light can be compensated, within certain limits, by an increased availability of dissolved inorganic N. In other words, the increasing levels of internal and subsistence N quota measured along decreasing light levels indicate an increased demand in nutrients at low-light levels.

These compensatory phenomena, also observed by Healey (1985), can be related to the fact that the relationships between certain cell quota and  $\mu$  can be reversed depending on the limiting factor. For example, in the case of N limitation, the relationship between the Chl *a*:C ratio and  $\mu$  is usually positive, whereas it is negative under light limitation (Eppley and Dyer 1965; Laws and Bannister 1980). The same can be said for the relationships between  $\mu$  and cell C: Under N limitation (nitrate or ammonium), the cell C content does not vary with  $\mu$  in a significant manner, whereas it increases in a linear fashion with  $\mu$  under light limitation (Laws and Bannister 1980).

In view of the internal couplings mentioned above, the effects due to a double or multiple limitation on algal growth can be complex, and they have inspired a number of theoretical as well as experimental studies. However, this complexity is not only reflected in cases of multiple limitations.

In fact, the external variability of a limiting resource does not only induce an adaptive readjustment of the mechanisms of its own metabolic pathway but it also modifies assimilation pathways of other nonlimiting resources. For example, a nitrate pulse imposed on N-limited cells can lead to a transient decrease of C fixation under nonlimiting conditions for photosynthesis (Turpin et al. 1988; Collos and Slawyk 1979). In a similar way, nitrite excretion, often attributed to a lack of cofactors or reducing power coming from photosynthesis, can be due to a nitrate pulse in N-limited cells of *Prorocentrum minimum* but not to light changes (Sciandra and Amara 1994). Most production models, be they light- or nutrient-oriented, are generally based on the limiting factor concept. The above remarks suggest that, in a variable environment, it is preferable to use the concept of a limiting process rather than a limiting resource, because an assimilation pathway can be blocked transiently and limit growth, whereas the resource passing through this pathway is not limiting.

To improve our understanding of the overall metabolic adaptations used by phytoplankton, we have used a multi-parameter approach, allowing us to obtain simultaneous information on the main cell quota (N, C, Chl *a*, InAA), processes (C fixation, respiration, N assimilation, growth rate, C and N excretion), and enzymatic activities (carboxylases, respiratory dehydrogenases) under conditions of light and nutrients as close as possible to natural situations for cell growth. *Dunaliella tertiolecta* was chosen because of the massive literature available. Measurements have been carried out using N-limited chemostats submitted to photosynthetically saturating and limiting irradiance levels. It was thus possible to study the changes of physiological indices of adaptation during the period of approach to steady state for two sets of different N/irradiance limiting conditions and also the transient short-term reaction of those indices due to a change in irradiance.

## Materials and methods

In Exp. 1, two NO<sub>3</sub><sup>-</sup>-limited continuous cultures were respectively submitted to saturating and limiting photon flux densities (PFD) with a 12:12 L/D cycle. Once equilibria were reached, the growth irradiance (I<sub>g</sub>) was shifted between the two cultures, and populations were followed until new equilibria were obtained. As only cell number, cell volume, cell quota (N, C, Chl *a*), NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup> were measured in this experiment, it was possible to sample continuous cultures at high frequency without disturbing their steady-state equilibria.

Exp. 2 was conducted under the same conditions as Exp. 1, but with a lower sampling frequency because of the larger number of parameters measured (*see below*). Data for the parameters cited above were very similar in Exp. 1 and 2 and will therefore only be considered in relation to Exp. 1.

In Exp. 3, the parameters examined in Exp. 1 were again measured in several batch and continuous cultures for different combinations of I<sub>g</sub> and light regime (L/D or continuous), resulting in different integrated irradiance over 24 h ( $\Sigma$  PFD) and conditions of N limitation.

The experimental design is given in detail in Bernard et al. (1996). *D. tertiolecta* Butcher (UTEX collection; University of Texas at Austin) was grown in cylindrical borosilicate glass water-jacketed vessels of 6 liters. Source strains were axenic, but the chemostat cultures were not, despite the sterilization of material and handling precautions. DAPI preparations for estimating bacterial abundance made at the beginning and at the end of the experiments revealed that bacterial contamination was negligible ( $<1/1,000$  of the phytoplankton biovolume). The enrichment medium (f/2) had a final concentration of  $46 \pm 1 \mu\text{M NO}_3^-$  and a N:P (atom) of 14. Control measurements showed that  $\text{NH}_4^+$  was produced in the renewal medium during autoclaving, but the final concentration remained always  $<0.5 \mu\text{M}$ . Temperature was maintained at  $18 \pm 0.1^\circ\text{C}$ . The desired PFD (430 and  $72 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , respectively, for the saturating and limiting Ig in Exp. 1 and 2) were obtained by adjusting the distance between lamps and chemostats and by interposing neutral density screens on one of the two cultures. Light permutation was achieved by transferring the neutral screen from one chemostat to the other and by readjusting the lamp-chemostat distance. For Exp. 1 and 2, cultures were named HL (high-light level) and LL (low-light level) before the light shift, and HL(LL) and LL(HL) after. In Exp. 2, no sampling was done 48 h prior to the light shift in order to avoid perturbations of the dynamic equilibrium in cultures HL and LL.

**Biomass, growth rate, and inorganic nitrogen**—Cell counts, mean cell volumes ( $V_{\text{cell}}$ ),  $\text{NO}_3^-$ , and  $\text{NO}_2^-$  concentrations were estimated every 1.5 h by an automated sampling system (Malara and Sciandra 1991). The growth rate  $\mu$  was calculated from the logarithm variations in biovolume ( $B_v$ , product of mean cell volume by cell concentration) and dilution rate. To obtain continuous values for  $B_v$ , data were smoothed with a spline interpolation method (Bernard 1995). As the  $B_v$  could be calculated by a continuous function, the derivative was used to calculate the growth rate for each interval, and consequently the mean growth rate was integrated over each light and dark phase ( $\mu_{12}$ ) as well as over each light cycle ( $\mu_{24}$ ). In batch cultures of Exp. 3,  $\mu$  was simply measured from the exponential variation of the  $B_v$ . Control measurements revealed that no  $\text{NH}_4^+$  was present.  $\text{NO}_3^-$  uptake rate  $\rho$  was calculated as follows:

$$\rho = \frac{1}{B_v} \left[ (d \times S_0) - (d \times S) - \frac{\Delta S}{\Delta t} \right]$$

with  $B_v$  being the cell biovolume ( $\text{mm}^3 \text{ liter}^{-1}$ ),  $d$  the dilution rate ( $\text{d}^{-1}$ ),  $S_0$  and  $S$ , respectively, the  $\text{NO}_3^-$  concentrations in the inflowing medium and in the cultures, and  $\Delta S/\Delta t$  the time variation of  $\text{NO}_3^-$  between two measurements.

**Internal quotas**—Samples for Chl *a* were filtered onto GF/C filters under low vacuum and extracted in 90% acetone for 1 h. Calculations were made according to spectrophotometric equations of Strickland and Parsons (1968). A comparison with the spectrofluorometric method of Neveux and Lantoiné (1993) revealed that the above method underestimated the Chl *a* values by  $\sim 20\%$ . Uncorrected values are

presented here. Samples for particulate organic carbon (POC) and nitrogen (PON) were filtered onto precombusted (24 h at  $450^\circ\text{C}$ ) GF/C filters and dried at  $60^\circ\text{C}$  for 24 h. They were stored in a desiccator at  $20^\circ\text{C}$  and analyzed on a Perkin-Elmer 2400 analyzer. Samples for internal free amino acids (InAA) were filtered onto precombusted (12 h at  $500^\circ\text{C}$ ) GF/F filters and stored in liquid nitrogen. For extraction of InAA, the filter was put in 2 ml of methanol and sonicated for 2 h. The sample was then put directly into methanol. Amino acid analysis was carried out according to a method modified from Lindroth and Mopper (1979). The amino acids analyzed were aspartic acid (ASP), glutamic acid (GLU), asparagine (ASN), glutamine (GLN), glycine (GLY), and serine (SER).

**Dissolved carbon**—Alkalinity was measured on discrete samples using the potentiometric analytical method of Perez and Fraga (1987). Continuous pH measurements were carried out with combined glass electrodes (Ross electrode, Orion 8102) and Radiometer 84 pH meters. The electrodes were standardized with a phosphate buffer (National Bureau of Standards 7.413) and a phthalate buffer (NBS 4.008) to determine the electrode slopes. Standardization with Tris buffer (Almgren et al. 1975) was also performed to determine the pH at moles per kg seawater scale. The pH values were acquired every 5 min. The dissolved  $\text{CO}_2$  ( $\text{H}_2\text{CO}_3 + \text{CO}_2$ ) was computed from the pH measurements and interpolated values of alkalinity using the dissociation constants of Dickson and Millero (1987) for carbonic acid, that of Dickson (1990) for boric acid, and the ionic product of water determined by Dickson and Riley (1979).

For dissolved inorganic carbon (DIC) analyses, 50 ml of culture was put into 100-ml glass vials and fixed with 0.1 ml of a saturated  $\text{HgCl}_2$  solution. Samples were kept in the dark at  $4^\circ\text{C}$  until analysis. Daily DIC analyses were carried out with Shimadzu model TOC-5000. DIC was estimated by measuring the gaseous  $\text{CO}_2$  produced by displacing the pH equilibrium of the carbonate system. A sample (20–30  $\mu\text{l}$ ) was directly injected into a reaction chamber where acidification with  $\text{H}_3\text{PO}_4$  (resulting pH 2–3) and bubbling with a pure gas mixture ( $\text{N}_2/\text{O}_2 = 80/20$ , 99.995% quality) yielded DIC in the form of gaseous  $\text{CO}_2$ . It was then analyzed by nondispersive infrared spectrometry and calibrated using carbonate and bicarbonate salt solutions at 20 and 25 mg C liter $^{-1}$ , with a precision of  $\sim 0.1$  mg liter $^{-1}$ .

**Dissolved glycolic acid**—The extraction of dissolved glycolic acid in the culture was performed using liquid-liquid separation with ethyl acetate, and concentration was assessed by HPLC. The glycolic acid dissolved in seawater is extracted in a reproducible manner with a constant yield and measured with a precision superior to  $\pm 7.5\%$  (for details, see Le Boulanger et al. 1994).

**C assimilation and P vs. I curves**—The *P* vs. *I* curves were obtained according to the method of Lewis and Smith (1983). Briefly, the light passed through neutral density filters into the “photosynthetron,” yielding different PFD (controlled at least once a day). The light was provided by lamps of the same type as those used in chemostat cultures.

Table 1. List of abbreviations and parameters.

Symbols	Significance	Unit
HL	Culture grown under high PFD (430)	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
LL	Culture grown under low PFD (72)	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
HL(LL)	HL culture transferred under low PFD (75)	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
LL(HL)	LL culture transferred under high PFD (420)	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
$\Sigma$ PFD	Photon flux density integrated over one light-dark cycle	$\mu\text{mol quanta m}^{-2} \text{ d}^{-1}$
<i>I</i>	Photosynthetically active radiation (PAR) used in the photosynthesis to obtain <i>P</i> vs. <i>I</i> curves	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
<i>I</i> <sub>g</sub>	Growth irradiance: PAR measured in chemostats	$\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
<i>P</i>	Photosynthetic rate or gross production rate	$\mu\text{g C ml}^{-1} \text{ h}^{-1}$
<i>P</i> <sub>m</sub>	Maximum photosynthetic rate	$\mu\text{g C ml}^{-1} \text{ h}^{-1}$
<i>a</i>	Initial slope of <i>P</i> function	<i>P</i> unit/ <i>I</i> unit
<i>b</i>	Scaling constant of <i>P</i> function	$\mu\text{g C ml}^{-1} \text{ h}^{-1}$
$\mu$	Growth rate calculated from the biovolume time variation	$\text{d}^{-1}$
$\mu_{12}$ , $\mu_{24}$	Mean value of the growth rate integrated over 12 and 24 h	$\text{d}^{-1}$
$\mu\text{C}$	Specific production rate (P/POC)	$\text{d}^{-1}$
$\rho$	$\text{NO}_3^-$ uptake rate	$\mu\text{mol mm}^{-3} \text{ h}^{-1}$
RSP	Respiration rate	$\mu\text{mol O}_2 \text{ mm}^{-3} \text{ h}^{-1}$
ETS	Electron transport activity	$\mu\text{mol O}_2 \text{ mm}^{-3} \text{ h}^{-1}$
<i>V</i> <sub>cell</sub> , <i>B</i> <sub>v</sub>	Respectively, mean cell volume and biovolume	$\text{mm}^3 \text{ cell}^{-1}$ , $\text{mm}^3 \text{ liter}^{-1}$
PON, POC, Chl <i>a</i>	Respectively, particulate nitrogen, carbon, and Chl <i>a</i> concentrations	$\mu\text{g liter}^{-1}$
<i>N</i> <sub>v</sub> , <i>C</i> <sub>v</sub> , ( <i>Chl a</i> ) <sub>v</sub>	Respectively, nitrogen, carbon, and Chl <i>a</i> per unit of cell volume	$\mu\text{g mm}^{-3}$
InAA	Internal free amino acids (ASP + GLU + ASN + GLN + SER + GLY)	$\mu\text{g mm}^{-3}$
DIC, DOC	Respectively, dissolved inorganic and organic carbon	$\text{mg liter}^{-1}$

Culture aliquots (1 ml) were incubated for 1 h and DIC was purged following the protocol of Lean and Burnison (1979). The initial activity of the  $\text{NaH}^{14}\text{CO}_3$  added ( $\sim 1 \mu\text{Ci ml}^{-1}$  of culture) was measured from triplicates of 50  $\mu\text{l}$  transferred to scintillation vials containing 100  $\mu\text{l}$  ethanolamine, and subsequently 10 ml of cocktail (Instagel with 10% methanol) was added. The samples were assayed by liquid scintillation (Tricarb 4000, Hewlett Packard). Disintegrations per minute were corrected for blank activity but not for dark activity, because there is no general consensus on this process (Morris et al. 1971). To estimate the photosynthetic rate *P*, DIC data and one isotopic discrimination coefficient (6%) were used (Stemann Nielsen 1952). The gross or net nature of *P* has not yet been clearly established (Jespersen et al. 1992; Peterson 1980; Smith and Platt 1984). We have assumed that the sum of  $^{14}\text{C}$  retained by the cells during a relatively short incubation time (DIC assimilation) and  $^{14}\text{C}$  present in the medium [dissolved organic carbon (DOC) excretion] was more representative of gross production. The parameters (see Table 1) of the empirical function used by Webb et al. (1974),

$$P = P_m [1 - \exp(-aI/P_m)] + b,$$

were estimated using SYSTAT software. Production rates were calculated in the chemostats from their respective *I*<sub>g</sub> values and the *P* vs. *I* curves. This method is not very different from those involving spiked  $^{14}\text{C}$  into the chemostat used by several authors (Laws and Wong 1978) but, requiring smaller sampling volumes, was easier to carry out in this multiparameter study. The specific production rate  $\mu\text{C}$  was calculated after normalization of *P* by POC.

**Carboxylase assays**—Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoenolpyruvate car-

boxylase (PEPc) measurements entail the incorporation of radioactive bicarbonate into stable products. Culture subsamples (20 ml) were filtered onto GF/F filters and kept in liquid nitrogen. All carboxylase assays were made on the same extract. The protocol used has previously been described (Descolas-Gros and De Billy 1987). Each carboxylase assay was carried out in triplicate, and blanks were obtained without the addition of the various substrates (phosphoenolpyruvate, ribulose-1,5-bisphosphate).

**Cell respiration activities**—Rates of cell respiration in 3 ml of cell culture were measured by using an oxygraph (Hansatech) with a thermoregulated chamber at  $18 \pm 0.1^\circ\text{C}$  (Braun, Frigomix-thermomix). Oxygen uptake in darkness was monitored on a graph recorder (Sefram PED), and data were simultaneously stored on a microcomputer for processing. To estimate the contribution of the contaminating bacteria to the overall dark respiration, controls were run with the filtrate passed through GF/F filters. It is assumed that most of the bacteria pass through the filter; considering that the bacterial contamination is rather low, the filtered volumes are too small to lead to the clogging of the filters, that may occur when filtering large volumes of seawater (Savenkoff et al. 1992).

**Electron transport system (ETS) activities**—In the ETS assay from Packard (1971), the response is directly proportional to the dehydrogenase (NADH, NADPH, and succinate dehydrogenase) content of the cell respiratory system. Sample volumes of 10 ml were filtered for measurements of ETS activity on 47-mm GF/F glass-fiber filters at a pressure close to 0.3 atm. To preserve enzymatic activity, GF/F filters were immediately stored in liquid nitrogen. The GF/F glass-fiber filters were processed according to Packard and Williams

(1981). Because the cell culture was aerobic, data were expressed in terms of molecular oxygen uptake ( $\text{ml O}_2 \text{ liter}^{-1} \text{ h}^{-1}$ ) as detailed by Savenkoff et al. (1993). The overall reproducibility of the measurements was  $\sim 20\%$ , ensuring that a significant change of  $0.01 \mu\text{l O}_2 \text{ liter}^{-1} \text{ h}^{-1}$  was detectable.

## Results

**Total biovolume (Bv) of cell population and growth rate ( $\mu$ )**—Diel variations in  $V_{\text{cell}}$  were regular and, as observed by Eppley and Coatsworth (1966), coincided perfectly with the light cycle (Fig. 1b). On a daily time scale,  $V_{\text{cell}}$  varied by  $\sim 40\%$ . During the stabilization phase, oscillations in  $V_{\text{cell}}$  tended to be similar in HL and LL. Bv was lower in LL than in HL (Fig. 1a), reflecting a lower cell density in LL. The 12-h integrated growth rates ( $\mu_{12}$ ) oscillated strongly between the day and the night in both cultures (Fig. 1c). Despite the different Ig imposed on HL and LL,  $\mu_{24}$  were very similar in both cultures and approached the dilution rate of  $0.5 \text{ d}^{-1}$ , indicating that cultures reached steady states. Finally, given the saturating Ig value in HL, the relatively low  $\mu_{24}$  indicated that growth in this culture was effectively N limited before the light shift.

Twenty hours after the light shift, Bv became higher in LL(HL) than in HL(LL) (Fig. 1a). The new steady states reached in both cultures were similar to those observed before the light permutation.  $V_{\text{cell}}$  increased transiently in LL(HL) and decreased for a longer time in HL(LL) (Fig. 1b). The increase in Ig led to an increase in  $\mu_{24}$  for LL(HL), which reached transiently a value greater than that observed in HL (Fig. 1c). Similarly, the decrease in Ig induced a transient decrease in  $\mu_{24}$  for HL(LL) at a lower value than that observed in LL. Three days after the light permutation, the amplitude of diurnal oscillations of  $\mu_{12}$  were systematically greater in HL(LL) than in LL(HL).

The response of  $\mu$  based on exponential variation in non-N-limited cultures exposed to a range of  $\Sigma \text{ PFD}$  showed an expected saturation (Fig. 2), reflecting that the only parameter controlling growth in these unlimited cultures was the amount of quanta received by cells over 24 h. In the range  $0\text{--}7 \text{ mol quanta m}^{-2} \text{ d}^{-1}$ , the increase of  $\mu$  is quasilinear. Such a linearity was also found by Eppley and Coatsworth (1966) with the same species, but in the range  $0\text{--}15 \text{ mol quanta m}^{-2} \text{ d}^{-1}$ . This discrepancy may be due to the different apparatus used to measure irradiance. Inversely,  $\mu$  was insensitive to the augmentation of the  $\Sigma \text{ PFD}$  in chemostat experiments, suggesting that growth was effectively N limited (dashed line). The intersection of the curve with the dashed line corresponds to a situation where light and N are simultaneously limiting, and it can be seen that the cultures submitted to a double limitation have the same growth rate as the culture limited only by light or N. Even if it is clear that there is a positive gradient of N limitation when the  $\Sigma \text{ PFD}$  increases in continuous cultures at steady state (*see below*), and that a compensation phenomenon occurs in N quota when  $\Sigma \text{ PFD}$  changes, the measurements of N quota revealed that for the lowest  $\Sigma \text{ PFD}$  tested in this experiment, the continuous cultures were still more N limited than were the batch cultures. This means that above a given growth

rate (in this study,  $\mu = 0.5 \text{ d}^{-1}$ ), the effects of light and N limitations were not additive.

**Cell quota**—To avoid variability associated with cell division synchrony in a light/dark regime, the different cell quotas were expressed per unit of cell mass rather than per cell. Given the significant correlation between Bv and POC obtained in both cultures (Fig. 3), Bv was chosen to represent the phytoplankton biomass as it could be estimated continuously. Before the light shift, PON concentrations were similar in both HL and LL cultures and, on average, remained close to the input nitrate concentration, in accordance with mass balance predicted from continuous culture theory (Fig. 4a). Because there was a quasi-complete transformation of inorganic N into cell N, this suggests that bacterial activity or dissolved organic nitrogen (DON) excretion–assimilation by phytoplankton could be neglected in a first approximation. Consequently, the higher Bv values in HL resulted in a lower nitrogen quota (Nv) than in LL (Fig. 4b). POC, higher in HL than in LL, showed strong diurnal variations in both cultures (Fig. 4a). C per unit of biovolume (Cv) was very similar in both cultures (Fig. 4c). The difference in mean values of N:C ratios between the two cultures (Fig. 5a) was essentially due to the difference in their N quota, whereas the daily variations of N:C in both cultures reflected changes in cell carbon. Before the light shift, Chl *a* per unit of biovolume (Chl *a*)<sub>v</sub> (Fig. 5b) oscillated around stable values in both cultures. During the stabilization phase, the Chl *a* values per cell (data not shown) as well as the amplitude of variations over 24 h in HL were similar to those observed by Laws and Wong (1978) for the same species at an Ig of  $270 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ , a 12:12 L/D regime and a  $\text{NO}_3^-$ -limited growth rate of  $0.51 \text{ d}^{-1}$ . The Chl *a* contents in HL were, however, much lower than those reported by Falkowski and Owens (1980) for nonlimited cells ( $1.05 \text{ pg Chl } a \text{ cell}^{-1}$ ) at a similar Ig ( $400 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ). Although the general trends are similar, the values for Chl *a* contents measured by Sukenik et al. (1990) are higher than in our study (30% for the lower light levels to 50% for the higher light levels).

After the light shift, POC decreased in HL(LL) and increased in LL(HL) with strong oscillations remaining (Fig. 4a), whereas Cv remained similar in both cultures (Fig. 4c). One day after the light shift, Nv became higher in HL(LL) (Fig. 4b), in the same manner as the N:C ratio (Fig. 5a). (Chl *a*)<sub>v</sub> values increased in HL(LL) and decreased in LL(HL) (Fig. 5b), in agreement with photoacclimation theory in general and results of Falkowski (1984) and Sukenik et al. (1990) in particular concerning the same species. The diurnal oscillations were more marked in HL(LL), with a significant decrease occurring during the light phase. A new steady state was reached only 4 d after the light shift.

The pool of InAA was significantly (Table 2) greater in LL (Fig. 6). All the measured InAA were always higher in LL, except for GLU, which was very close in both chemostats before the light shift (data not shown). In all cases, InAA increased at night and decreased during the day in LL. Such oscillations were not visible in HL.

**Specific carbon assimilation ( $\mu\text{C}$ ) and carboxylating activities**—Before the light shift,  $\mu\text{C}$  decreased systematically in both cultures during the light periods (Fig. 7a). Assimilation

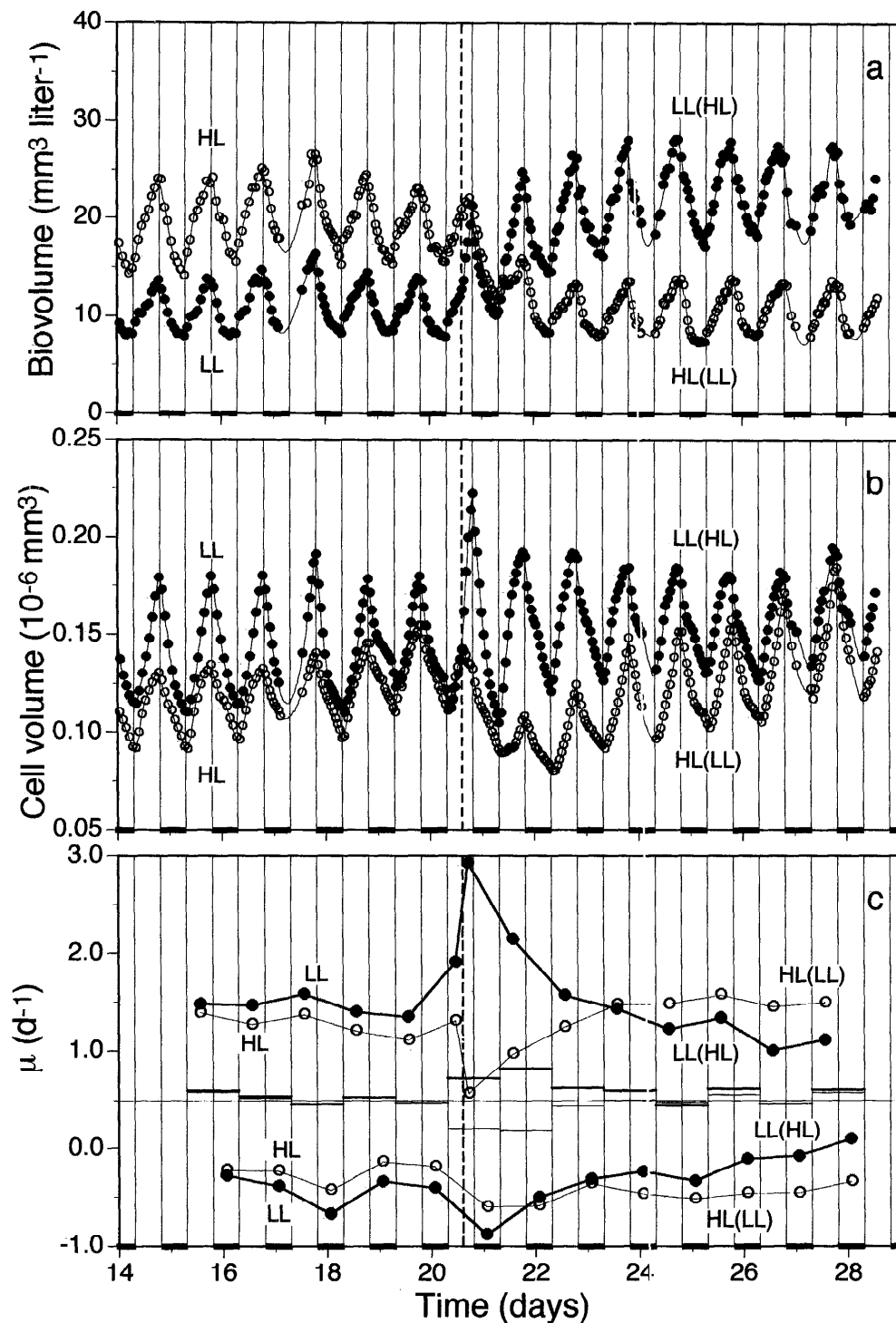


Fig. 1. Experiment 1. Total cell biovolume (a) and mean cell volume (b) of N-limited cultures of *Dunaliella tertiolecta* exposed respectively, to a PFD decrease (open circles) and increase (full circles), with a 12:12 L/D regime. The thin lines are polynomial fits to the light-dark variations of variables. (c) Circles: mean growth rates calculated over 12 h ( $\mu_{12}$ ). The values for the light and the dark phases are connected together. The horizontal segments represent the mean growth rate integrated over 24 h ( $\mu_{24}$ ) for the two cultures exposed, respectively, to a decrease (thin segments) and to an increase (thick segments) of PFD. Axis scale gives the number of days since the beginning of the experiment (horizontal thick bars indicate the dark phases). The vertical broken bar indicates the time of PFD change in the two cultures: HL ( $430 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) to HL(LL) ( $72 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ), and LL ( $75 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) to LL(HL) ( $420 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). The horizontal thin line gives the dilution rate.

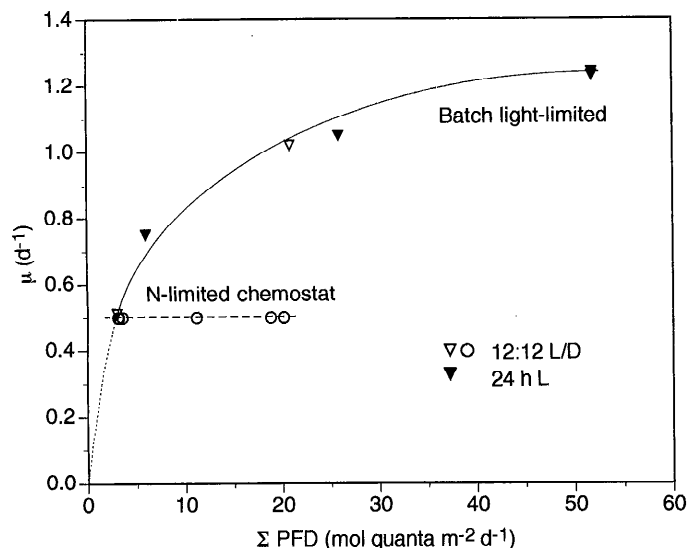


Fig. 2. Experiment 3. Growth rate calculated in batch (triangles) and N-limited cultures ( $d = 0.5 \text{ d}^{-1}$ ; circles) at different  $\Sigma$  PFD obtained in 24 h L (full symbols) and 12:12 L/D (open symbols) light regimes (the curve was hand-fitted).

lations were significantly lower in HL than in LL (Table 2). Two hours after the shift,  $\mu\text{C}$  reached an absolute minimum in HL(LL), much lower than the values measured in LL. In contrast,  $\mu\text{C}$  in LL(HL) achieved a maximum 2 h after the shift, much higher than the values measured in HL.

The systematic decrease in  $\mu\text{C}$  observed in both cultures during light phases (Fig. 7a), as observed by Nelson and Brand (1979) for the same species, could be due to a  $\text{CO}_2$  deficiency in the medium (Fig. 9). During the same time, pH oscillated regularly between 8.5 (at 0800 h) and 8.2 (at 2000 h) in HL and 8.4 and 8.1 in LL. Although the mechanisms of  $\text{CO}_2$  vs. DIC assimilation are not yet completely understood, Burns and Beardall (1987) have shown that, in the  $\text{CO}_2$  range of variations mentioned above, *D. tertiolecta* can increase its cell DIC concentration by a factor of 6.9. This  $\text{CO}_2$ -concentrating mechanism [CCM, see review by Beardall and Raven (1990)] can also be induced in N-deficient medium as shown by Beardall et al. (1982, 1991) for *Chlorella emersonii*. To test the hypothesis of a possible growth limitation by dissolved carbon, two identical cultures were grown under batch conditions, either with unregulated pH (increasing from 8.2 until 9.4 between the beginning and the end of the exponential growth phase) or with pH regulated at a fixed value of 8.4 (data not shown). These cultures could not be distinguished on the basis of their exponential growth rate. In consequence it can be argued that  $\mu\text{C}$  was not limited by  $\text{CO}_2$  in our experiment. More likely, the diurnal variations in  $\mu\text{C}$  were set by the photoperiod, which acted on the cell cycle and the variations in pigment and photosynthetic characteristics of cells (Fig. 5b).

The presence of two carboxylating enzymes, Rubisco and PEPc as  $\beta$ -carboxylase, confirms previous results with the same species (Glover 1989) and other Chlorophyceae (Descolas-Gros and Oriol 1992).  $\beta$ -carboxylase activity remained low in all cases (data not shown) with a  $\beta$ -carboxylase:Rubisco ratio al-

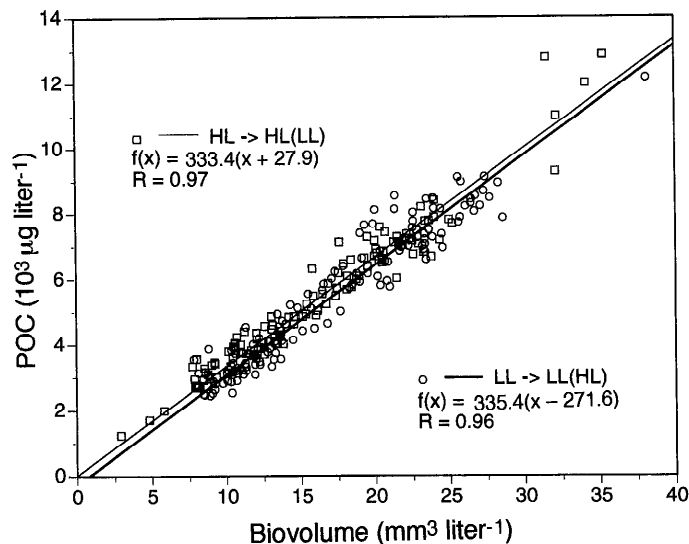


Fig. 3. Experiments 1 and 3. Relationships between total cell biovolume (Bv) and particulate organic C (POC) in N-limited and batch cultures.

ways  $<10\%$ . Before the light shift, activities were significantly (Table 2) higher in LL than in HL (Fig. 7b). The light change did not induce, in the following 24 h, a significant response of the Rubisco activity, neither in HL(LL) or in LL(HL). Higher activities were nevertheless found in HL(LL) 30 h after the light change. Statistical analysis (test of parallelism made on the slopes calculated for each light and dark phase) shows that the slopes of variation were significantly negative during the days and positive at night, (and different from zero), reflecting a diel variation in Rubisco activity.

**Respiration (RSP) and ETS activities**—The most significant difference was observed in ETS activity, which was higher in LL than in HL (Table 2). No difference was observed between HL(LL) and LL(HL). The light decrease stimulated ETS activity in HL(LL), but no difference, due to the light shift up, was induced in LL(HL). RSP values were similar between the two cultures before and after the light shift, and no difference was induced in either culture by the light changes. Diel periodicity did not appear neither in RSP nor in ETS activities.

**Nitrate, nitrite, dissolved glycolic acid, and  $\text{CO}_2$ — $\text{NO}_3^-$  and  $\text{NO}_2^-$**  remained near the detection limit in culture HL before the light shift, so that the calculated values of the N uptake rate  $\rho$  reflect the diel variations of Bv (Fig. 8a). In contrast,  $\text{NO}_3^-$  appeared systematically in the middle of the dark phases in LL, reflecting a decrease in the uptake capacity of cells (Fig. 8b). At the same time,  $\text{NO}_2^-$  was also excreted in the medium in LL and reached a concentration of about  $0.20 \mu\text{M}$  at the end of the dark phases. At the onset of the light phases, surged uptake could be systematically observed, leading to complete disappearance of the  $\text{NO}_3^-$  accumulated in the medium during the night.  $\text{NO}_2^-$  was also reabsorbed when  $\text{NO}_3^-$  became exhausted from the medium. The mean daily uptake rate of  $\text{NO}_3^-$  in LL was about twice



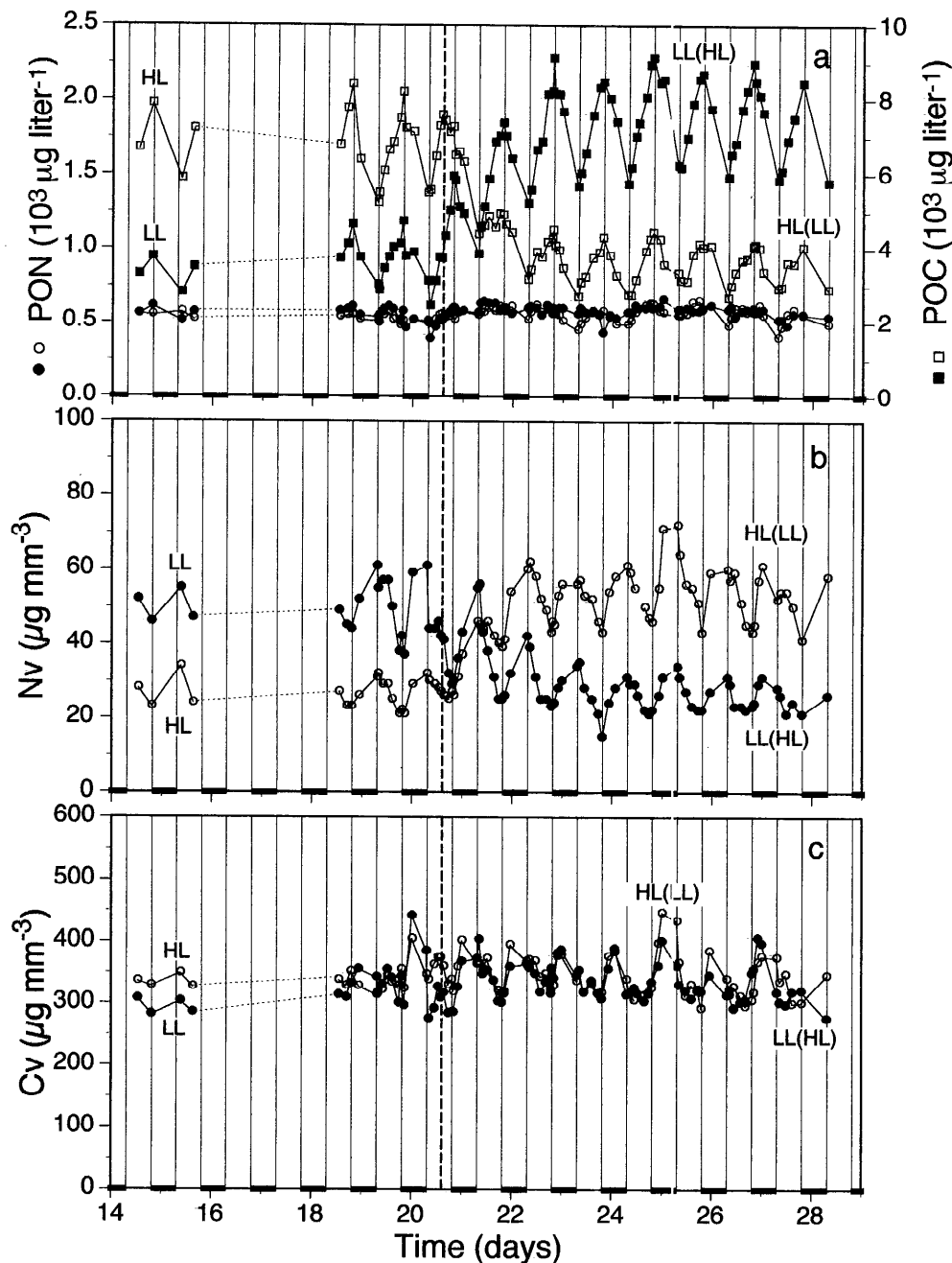


Fig. 4. Experiment 1. Time variations in N-limited continuous cultures of (a) particulate organic nitrogen PON (circles) and particulate organic carbon POC (squares); (b) nitrogen quota  $N_v$ ; and (c) carbon quota  $C_v$ . Others abbreviations and symbols are the same as in Fig. 1.

that in HL, in agreement with the difference in N cell quota. Three days after the light shift, the reverse situation appeared for  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in HL(LL) and LL(HL).

Before the light shift, strong diurnal variations of glycolic acid were evident, with a maximum ( $174 \pm 47 \mu\text{g C liter}^{-1}$  in HL and  $379 \pm 95 \mu\text{g C liter}^{-1}$  in LL) before the end of the light period and undetectable values during the dark phase (Leboulanger et al. unpubl. data). Release took place at the outset of the light phase, at a significantly higher rate

in LL ( $6.1 \pm 0.4 \mu\text{g C liter}^{-1} \text{ h}^{-1}$ ) than in HL ( $2.5 \pm 0.3 \mu\text{g C liter}^{-1} \text{ h}^{-1}$ ). Disappearance of dissolved glycolic acid took place before the end of the light phase, at a rate of  $5.7$  and  $4.2 \mu\text{g C liter}^{-1} \text{ h}^{-1}$ , respectively, in LL and HL cultures. Immediately after the light shift, dissolved glycolic acid exhibited strong variations on a time scale of less than an hour, followed by diurnal variations similar to those of the preshift period. The first event after the light shift was a sharp decrease in dissolved glycolic acid, down to a value close to

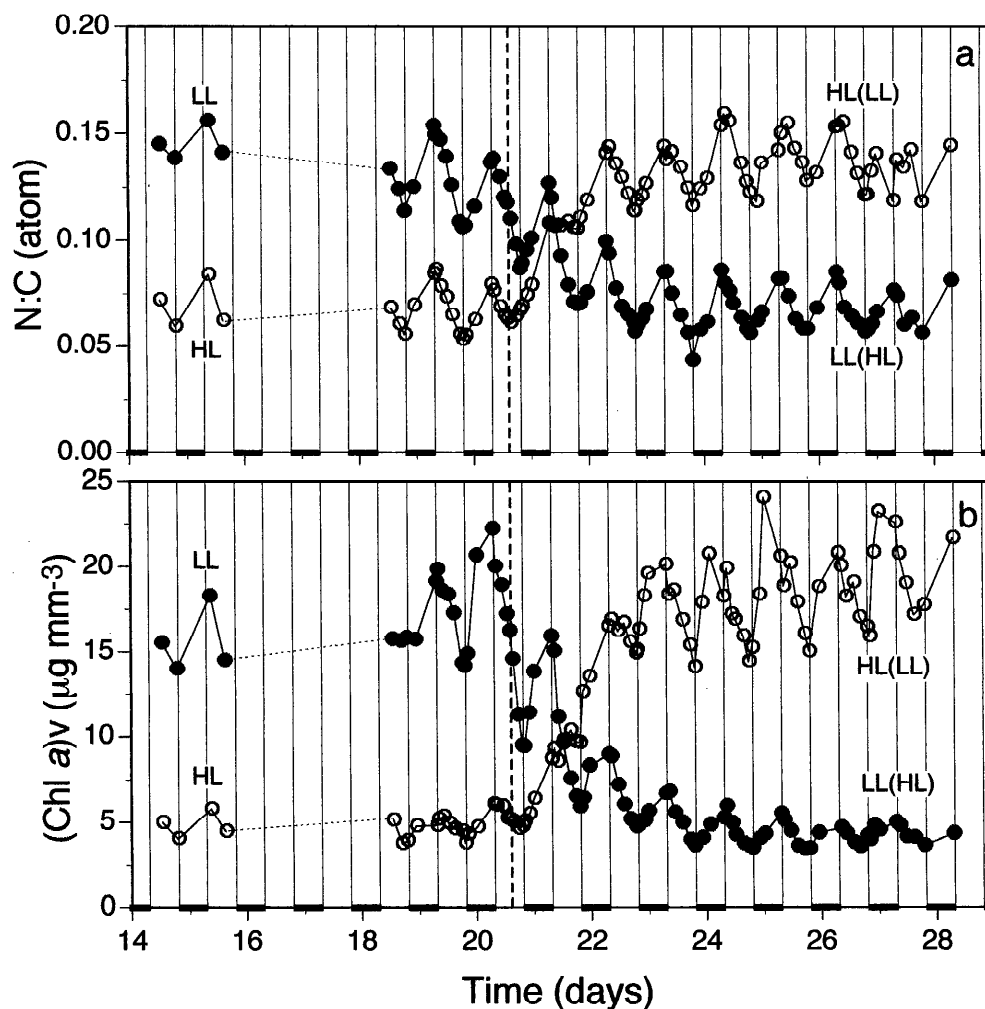


Fig. 5. Experiment 1. Time variations of N:C ratio (a) and Chl *a* quota (b) in N-limited continuous cultures. Other abbreviations and symbols are the same as in Fig. 1.

the detection limit in both cultures during a period of less than 20 min. However, mean release rates were higher in HL(LL) than in LL(HL) on day 21.

Despite bubbling and stirring, the CO<sub>2</sub> concentration ranged from 12.3 to 5.1 µmol CO<sub>2</sub> liter<sup>-1</sup> between the beginning and the end of the light period (Fig. 9), whereas DIC levels ranged from ~2.0 to 2.3 mmol liter<sup>-1</sup>. Note that

the light shift induced an inversion of mean equilibria between the two cultures.

#### Discussion

*Combined effects of PFD and N limitations on growth rate, V<sub>cell</sub>, and cell quota*—The protocol used in this study

Table 2. Experiment 2. Mean value of the different parameters measured before and after the light change in two N-limited cultures. The Student's *t*-test was used to test the difference either between HL and LL cultures before and after the light shift (paired samples) or between the two light conditions experienced by each culture (unpaired samples). (\*Test not significant at *P* = 0.05).

	HL	LL	HL(LL)	LL(HL)	<i>t</i> -test HL/LL	<i>t</i> -test HL(LL)/ LL(HL)	<i>t</i> -test HL/HL(LL)	<i>t</i> -test LL/LL(HL)
InAA (µg mm <sup>-3</sup> )	3.02	4.54	3.07	4.06	<i>P</i> <0.001	<i>P</i> <0.05	<i>P</i> <0.05	*
µC (d <sup>-1</sup> )	0.87	1.10	0.82	1.29	<i>P</i> <0.01	<i>P</i> <0.05	<i>P</i> <0.05	*
Rubisco (µmol CO <sub>2</sub> mm <sup>-3</sup> h <sup>-1</sup> )	0.18	0.26	0.18	0.20	<i>P</i> <<0.0001	*	*	*
RSP (10 <sup>-3</sup> µmol O <sub>2</sub> mm <sup>-3</sup> h <sup>-1</sup> )	0.79	1.16	0.68	0.70	*	*	*	<i>P</i> <0.02
ETS (10 <sup>-3</sup> µmol O <sub>2</sub> mm <sup>-3</sup> h <sup>-1</sup> )	0.19	0.32	0.30	0.23	<i>P</i> <0.0001	*	<i>P</i> <0.03	<i>P</i> <0.05

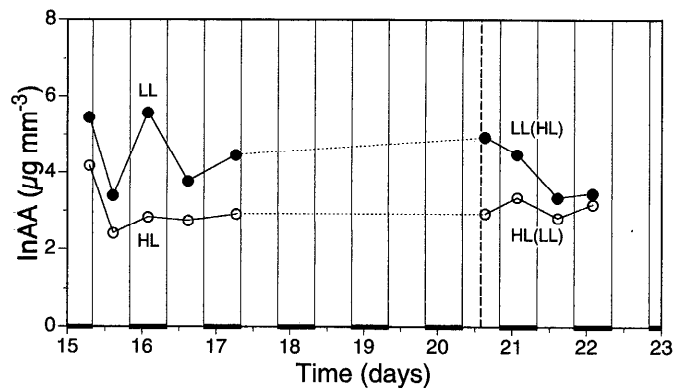


Fig. 6. Experiment 2. Time variations of InAA in N-limited continuous cultures. Data presented are the mean values calculated for each dark and clear phases. Others abbreviations and symbols are the same as in Fig. 1.

allowed us to grow two N-limited continuous cultures of *D. tertiolecta* at the same rate, with a less severe degree of N limitation for the cells submitted to lower PFD. The possibility of obtaining similar steady-state growth rates for different combinations of nutrient and light limitation has been reported previously by Rhee and Gotham (1981) and Healey (1985) in  $\text{NO}_3^-$ -limited cultures. In our study, we show how light stress performed on different N/PFD-limited steady-state cultures generate transient phases in which the differing sources of growth limitation can be identified. During these phases, the growth rate may differ within a large range from its steady-state value (Fig. 1c).

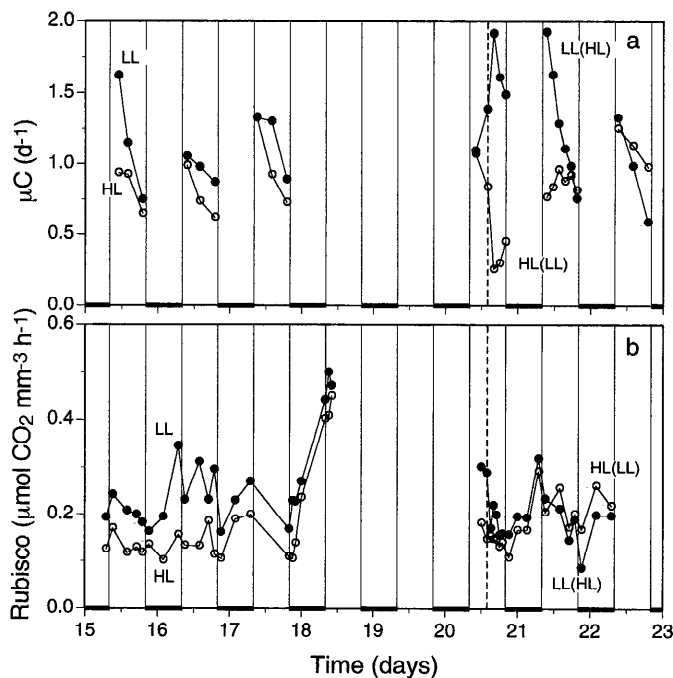


Fig. 7. Experiment 2. Time variations of the specific carbon assimilation rates  $\mu_C$  (a) and Rubisco activities (b) in N-limited continuous cultures. Other abbreviations and symbols are the same as in Fig. 1.

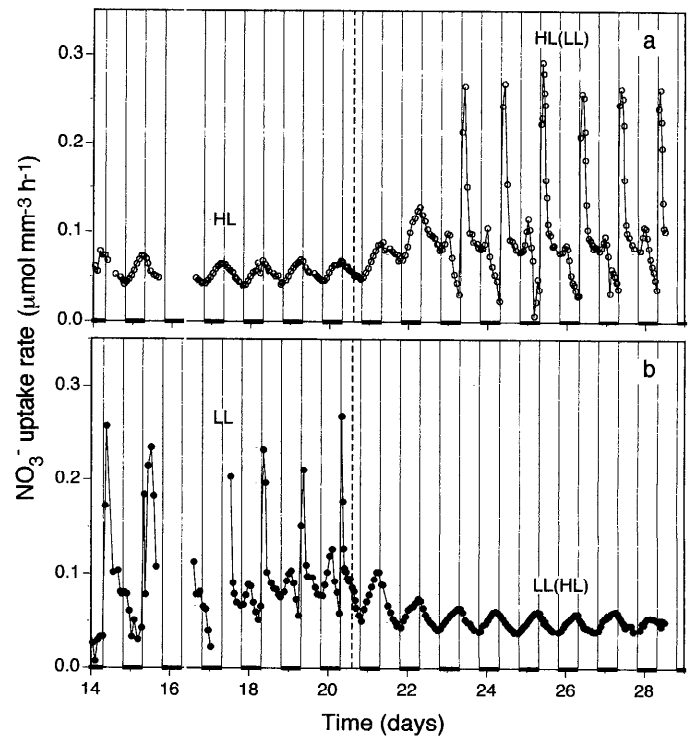


Fig. 8. Experiment 1. Time variations of  $\text{NO}_3^-$  uptake rates in N-limited continuous cultures. Other abbreviations and symbols are the same as in Fig. 1.

Despite the diversity of culture conditions experienced by the cells during the steady and transient phases, the relationship between cell volume and cell carbon remains surprisingly stable and similar between HL and LL cultures (Fig. 3). Three points support the hypothesis that  $V_{\text{cell}}$  variation was principally driven by processes of C exchange (i.e. fixation, respiration, and excretion). First, the volume variation was principally driven by the light-dark regime and thus by photosynthesis. Second, diurnal variations of dissolved  $\text{CO}_2$  (Fig. 9), DIC (not shown), and glycolate in the medium are in phase with the light-dark cycle. Third, the light value of  $\mu_{12}$  (calculated from  $B_v$  variation, Fig. 1c) and  $\mu_C$  (calcu-

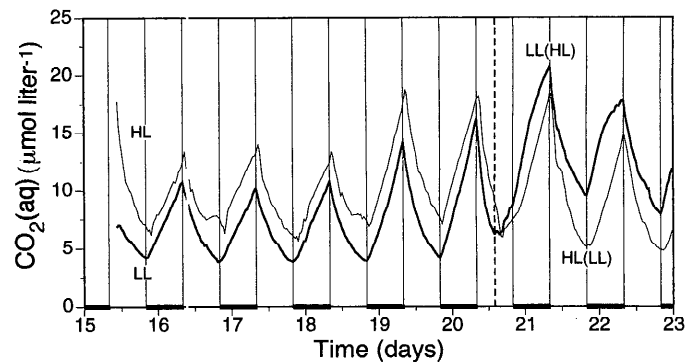


Fig. 9. Experiment 2. Time variation of dissolved  $\text{CO}_2$  in N-limited continuous cultures. Other abbreviations and symbols are the same as in Fig. 1.

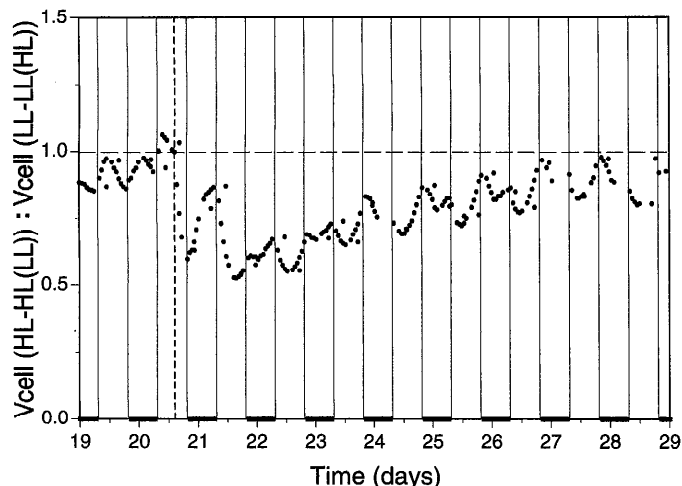


Fig. 10. Experiment 1. Time variations of the mean cell volume ratio between cultures HL-HL(LL) and LL-LL(HL). Others abbreviations and symbols are the same as in Fig. 1.

lated from C fixation, Fig. 7a) present the same qualitative differences between HL and LL cultures. Although Avron (1992) has shown that ionic and glycerol exchanges regulate the cell volume of *D. tertiolecta* in the short term, Riisgård (1981) has shown that photosynthetic activity is essential, especially through the synthesis of low molecular metabolites (Ahmad and Hellebust 1985; Dor 1985; Reed and Stewart 1985). Claustre and Gostan (1987) have also observed close relationships between Vcell and C metabolism in Haptophyceae during light shifts, in particular through carbohydrate synthesis. In addition, they showed that the changes in photosynthetic production (calculated by a theoretical model for non-N-limited cells) and Vcell are similar.

Our results are also consistent with those of Thompson et al. (1991), according to which the volume of *D. tertiolecta* is correlated positively with  $I_g$ , but the relationship disappears under N limitation. Falkowski and Owens (1980) have also observed a positive relationship between  $I_g$  and the volume of *D. tertiolecta* under nonlimiting conditions, but the relationship was negative between  $I_g$  and the C quota, with the latter contrary to what is generally observed in other species. In our experiment, Vcell was unexpectedly higher in LL than in HL, or at least equal (Figs. 1b, 7a), reflecting the interference between the light and N limitations. Also, note that the expected difference in Vcell due to the different  $I_g$  occurred at around day 21 (Fig. 10), i.e. when the N quota became similar in HL(LL) and LL(HL) (Fig. 4b).

The interfering effects of N limitation with PFD conditions are also visible on pigment adaptation. The differences mentioned between our results and those of other authors are probably due to the effect of N limitation in our study. Fig. 11 shows the relationships between N:C and Chl *a*:C during the transient period following the light shift. The greatest difference in Chl *a*:C is observed between HL and LL cultures, i.e. when cultures also show the greatest difference in N:C. The irradiance decrease led, in HL(LL), to an expected increase in Chl *a*:C, which took several days before reaching the level in LL. Inversely, the higher initial N:C ratio

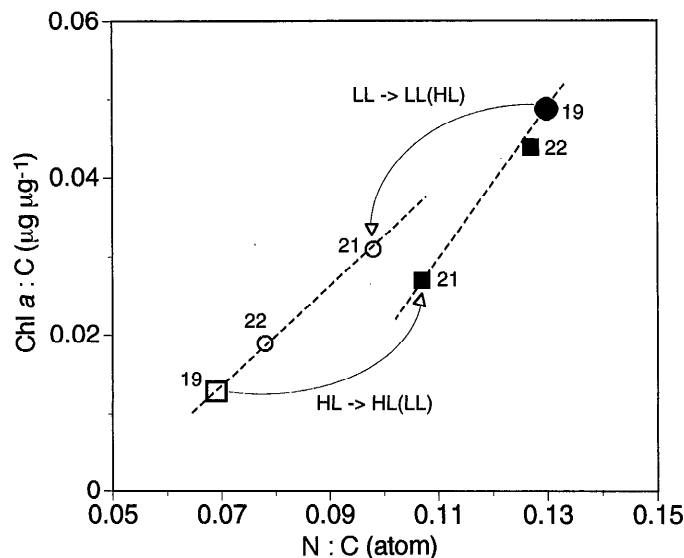


Fig. 11. Experiment 1. Relations between N:C and Chl *a*:C ratios measured during the transient period following the light decrease in chemostat HL→HL(LL) (squares) and chemostat LL→LL(HL) (circles). Numbers indicate days of experiment. Large symbols represent values obtained at steady state just before the light perturbation. Low and high PFD conditions are, respectively, represented by full and open symbols. Arrows indicate the change of light conditions.

in LL allows the cells to retain a high Chl *a* content after the light increase.

Obviously, the N quota is involved in the relationship between irradiance and Chl *a*. In Fig. 11, the hidden variable is the irradiance. By joining points corresponding to the same  $I_g$ , the positive relationship between N and Chl *a* quota, reported by many authors for continuous cultures, is apparent. It can also be noted that the slope of the relationship is lower for cultures exposed to high PFD, indicating a weaker influence of the N quota on Chl *a* content at high irradiance.

Our results also differ from those of other studies in the time scale of acclimation and emphasize the importance of the light regime in the dynamic adaptation of phytoplankton. The older studies on *D. tertiolecta* were carried out under constant illumination and our results indicate a difference in the time scale necessary to reach a new steady state. For example, in the case of Sukenik et al. (1990), the acclimation was reached in 48 h, whereas our results indicate a significantly longer time to acclimate. This is probably due to our use of the more realistic light-dark cycle, which supplies less energy to the cells on a daily basis.

*Combined effects of PFD and N limitations on Rubisco activity and photorespiration*—Several studies have shown that both Rubisco carboxylase activity and photosynthetic capacity decline with decreasing PFD in different algae species. More specifically in *D. tertiolecta*, Beardall (1989) and Beardall et al. (1976) found that, in batch cultures, Rubisco activity was correlated with photosynthetic activity. This has been interpreted as a strategy to decrease photosynthetic

costs at low irradiance (Richardson et al. 1983). With N limitation, we found that Rubisco activity, as  $\mu\text{C}$ , was not correlated with the irradiance level (Fig. 7), but with the N status. This observation and the lack of immediate response of Rubisco activity to the light change are consistent with reports about the secondary role of Ig on Rubisco regulation (Campbell and Ogren 1992; Portis 1992). The effect of light seems to vary with the degree of N limitation (Duke et al. 1986). For example, Paasche (1971) reported that *D. tertiolecta* cells growing on ammonium as a N source have a greater cell volume and higher photosynthetic rate and Rubisco carboxylase activity than cells growing on nitrate. This has been interpreted as the consequence of a globally greater protein synthesis and therefore larger photosynthetic enzyme pools. Glover (1989) reported that in nitrate-limited continuous cultures of *Phaeodactylum tricornutum*, the photosynthetic rate, the carboxylase activity and protein levels decrease with N limitation.

Besides its carboxylating activity, Rubisco may also act as an oxygenase. The observed cell production of glycolic acid is the result of photorespiration: oxidation of ribulose-1,5-bisphosphate into 3-phosphoglycerate and phosphoglycolate (Artus et al. 1986). In certain cases, the glycolic acid resulting from phosphoglycolate dephosphorylation is not immediately reinjected into the cell metabolism but can be excreted into the medium, where it can be eventually recycled (Edenborn and Litchfield 1987; Moroney et al. 1986; Ogren 1984). The carboxylase and oxygenase functions are in competition because they are carried out by the same enzyme. Campbell and Ogren (1990), Mächler et al. (1987), and Portis (1992) showed that N limitation induces an accumulation of glyoxylate, an intermediate of the photorespiratory pathway, and that this compound induces a decrease in Rubisco activation under light. Therefore, there is a joint decrease of the carboxylating and oxygenating functions of Rubisco under N limitation. This point, and the fact that N limitation was more severe in HL culture than in LL culture, explains why the glycolate production is lower in HL than in the LL culture and confirms the results of Osborne and Geider (1986) that Rubisco activity in unicellular algae depends also on N limitation.

The protocol used in this study allowed us to follow the covariation of  $\mu\text{C}$ , the carboxylating activity of Rubisco and photorespiration. The systematic decrease in  $\mu\text{C}$  observed during light periods in both HL and LL (Fig. 7a) has also been observed for  $\mu$  by Nelson and Brand (1979) for the same species. Based on the methodology used in our study,  $\mu\text{C}$  is more representative of gross production, and its diurnal variations express the changes in C fixation rather than those in respiration. Note, too, that glycolate excretion also takes place before the end of the light period (Leboulanger et al. unpubl. data). If we assume that this excretion rate reflects the photorespiratory activity, then the parallel decreases in  $\mu\text{C}$  and glycolic acid production can be attributed to a drop in carboxylating and photorespiratory activities of Rubisco during light periods. Such a decrease is visible in the carboxylating activity of the Rubisco on certain days (Fig. 7b). The regulation of the carboxylase and photorespiratory functions of Rubisco is complex because it depends, among other things, on respective concentrations of  $\text{O}_2$ ,  $\text{CO}_2$ ,

and C substrate (Portis 1992). The simplest hypothesis explaining the parallel variation of the two functions either under N limitation or during light periods is that the size of the Rubisco enzyme pool itself changes. In this case, the light regulation of Rubisco activity may be canceled by the N status if the pool of Rubisco, which represents a large reserve of N in the cells, is degraded in periods of N starvation. The mobilization of the Rubisco pool to the production of InAA used for synthesis of proteins and enzymes necessary for growth has been reported in N-limited cultures of *Chaetoceros gracilis* by Cleveland and Perry (1987).

*Interference between PFD and N limitations on dissolved inorganic nitrogen (DIN) assimilation and respiration*—Laws and Wong (1978) observed that the  $\text{NO}_3^-$  uptake rate of *D. tertiolecta* in continuous cultures decreases significantly at night. In our experiments, this phenomenon was observed only in continuous cultures exposed to low Ig (Fig. 8). Clearly, the loss of ability to take up  $\text{NO}_3^-$  during the dark phases was correlated with the low C reserves in the cell resulting from low Ig experienced during the previous light phase. The fact that  $\text{NO}_3^-$  uptake resumed immediately after the onset of the light phase indicates that  $\text{NO}_3^-$  absorption is coupled with photosynthesis, and that the strength of this coupling depends on the photosynthetic activity during the day and on the level of C reserves. Interestingly, the drop in dark uptake capacity did not occur at the first dark phase after the light shift in HL(LL) culture. Fig. 8a shows that the consequence of the cell Bv decrease observed in HL(LL) was a higher uptake rate (days 21–23). This led to an expected increase in N quotas (Fig. 4b) and to a decrease in C:N ratio (Fig. 5a) because C fixation decreased during the same time (Fig. 7a). The first drop in  $\text{NO}_3^-$  uptake measured at night corresponds to the time where the energy and substrate demand for N assimilation were not balanced by photosynthetic and respiratory pathways. On day 23,  $\text{NO}_3^-$  uptake decreased only after several hours into the dark phase (Fig. 8a), suggesting a time lag between the end of photosynthate production and the exhaustion of compounds necessary for DIN assimilation (Grant 1968; Grant and Turner 1969). The simultaneous accumulation of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  suggests that assimilation is partly blocked at the level of  $\text{NO}_2^-$  reductase, this step requiring reduction factors generally coming directly from photosynthetic activity.

Oxidative metabolism within the cell converges toward the cell respiratory system where electron transfer is coupled to energy conversion. A change in the cell activity induced by a change in an environmental parameter (light, temperature, nutrients) might result in a change in the cell respiratory rate that can occur through two processes. One corresponds to a real variation of the electron transfer rate of the respiratory system, whose activity is regulated without any change in its composition. The other concerns the composition of the respiratory system itself that might vary through synthesis, proteolysis, and mechanisms of activation or deactivation. Therefore, the measurement of oxygen uptake alone cannot distinguish between these two possibilities whereby a cell reacts to an external stimulation or stress. To address this problem, we have combined dark RSP measurements that provides the specific oxygen uptake rate, and

the ETS assay, the response of which is directly proportional to the dehydrogenase content of the cell respiratory system. Depending on experimental conditions, we observed the two situations. In LL and less N-limited cultures,  $\text{NO}_3^-$  uptake rate as well as ETS activity were higher than in more N-limited and HL cultures. Although proteins were not measured directly, one can presume from the difference in InAA quota and  $\text{NO}_3^-$  uptake and C fixation rates that the C demand for protein synthesis was probably higher in LL than in HL culture. This allowed the LL culture to maintain a similar respiration rate as in HL culture by means of a higher dehydrogenase content. It is noticeable that the positive relationships generally found between RSP and  $\mu\text{C}$  in N-limited cultures and between RSP and  $\mu$  in light-limited cultures (Laws and Caperon 1976; Laws and Bannister 1980; Laws and Wong 1978) do not appear in the case of double limitation. These relationships are widely used in mathematical modeling where both light and N control growth.

*Modeling the N and light limitations*—The main value of the approach used here lies in the simultaneous description of the phytoplankton dynamics and multiple metabolic processes involved in cell adaptation. Although  $\mu$  remains ultimately dependent upon processes controlling protein synthesis, our results show that there are no simple relationships between the rate of those processes and  $\mu$ , when growth is limited by both irradiance and N. Our results show that the effect of irradiance on specific C fixation is tightly dependent on the cell N status. The increase of  $\mu\text{C}$  by the Ig conditions is influenced at the level of both light and dark reactions. The mechanisms of photoacclimation are favored in N-rich cells, and those of C assimilation, partly under the influence of Rubisco carboxylating activity, also depend on N availability. The relationships observed by numerous authors between RSP and  $\mu$  for individual N or light limitations are not valid in cases of multiple limitations. While the cellular energy potential, emanating from the photosynthetic capacity, constitutes an important common denominator for energy demands associated with C and N assimilation pathways, the N pool is also a common denominator for enzymatic requirements associated with the same pathways. Our work shows that, due to those interdependencies, the relationships that traditionally appear between processes and quota under conditions of either light or N limitation are no longer valid under conditions of double limitation.

It is instructive to see that the light shift imposed on two cultures having similar growth rates does not lead to symmetrical changes in  $\mu$  and  $\mu\text{C}$ , at least during a transient period. The asymmetry indicates a difference in the nature of the limitation and therefore a difference in potential response to environmental fluctuations. The implications of those results concerning phytoplankton growth modeling in a variable environment are important, because there are no existing models that correctly integrate the simultaneous effects of light and N on primary production. This problem is usually swept aside by the use of empirical hypotheses. One of them is that of the minimum: for a set of light–nitrogen (Ig, N) combinations, one can calculate a set of growth rates  $\mu(\text{N})$  and  $\mu(\text{Ig})$  from classical one-variable models. The variable for which  $\mu$  is the lowest is then considered as more

limiting and as the only one to be taken into account for growth calculations, the other one being assumed to be without effect:  $\mu = \min[\mu(\text{N}), \mu(\text{Ig})]$ . Another frequently used approach is to calculate  $\mu$  from the product of two functions, respectively, of N and I:  $\mu = \mu'(\text{N}) \times \mu'(\text{Ig})$ . This last assumption would be correct if the functions  $\mu(\text{N})$  and  $\mu(\text{Ig})$  were, respectively, independent of Ig and N, which is in contrast with our findings. The results expressed in Fig. 2 are more favorable to the minimum law; when light limitation is added to N limitation, the growth rate remains unchanged, at least under our experimental conditions. But in any case, the minimum law will never be able to reproduce the fact that under light-limiting conditions cells are able to sustain higher  $\mu\text{C}$  if they are less N limited. Our study should be continued in order to see how  $\mu(\text{Ig}, \text{N})$  varies in a wider range of Ig and N, for the compensatory mechanism demonstrated in the present study probably works only within a range of values of Ig and N.

In open systems such as chemostats, the maintenance of the same growth rate  $\mu$  in cultures limited differently by light and N does not lead to the same C production. Indeed, even if  $\mu\text{C}$  is lower in HL than in LL, the mass of C produced per day remains higher in HL, due to the higher amount of C fixed by cells under high light conditions.

One can then presume that the models elaborated separately for the effects of N or Ig on the various metabolic rates and growth rates will not be of great use to attain a level of modeling necessary to integrate their coupled effects, especially in a variable environment. In the same way that under a strict N-limitation regime, the N quota has proved to be a rather good internal index of growth rate, at least under steady-state conditions, it is necessary to find out whether a similar intracellular criterion that would integrate the simultaneous effects of light and N also exists. Obviously, this criterion would be associated with processes whose activity is tightly regulated by light regime and N availability. Our study shows that Rubisco activity and pigment quota are such examples.

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