Sustained net CO_2 evolution during photosynthesis by marine microorganisms

Dan Tchernov*, Miriam Hassidim*, Boaz Luz†, Assaf Sukenik‡, Leonora Reinhold* and Aaron Kaplan*

Background: Many aquatic photosynthetic microorganisms possess an inorganic-carbon-concentrating mechanism that raises the CO_2 concentration at the intracellular carboxylation sites, thus compensating for the relatively low affinity of the carboxylating enzyme for its substrate. In cyanobacteria, the concentrating mechanism involves the energy-dependent influx of inorganic carbon, the accumulation of this carbon – largely in the form of HCO_3^- – in the cytoplasm, and the generation of CO_2 at carbonic anhydrase sites in close proximity to the carboxylation sites.

Results: During measurements of inorganic carbon fluxes associated with the inorganic-carbon-concentrating mechanism, we observed the surprising fact that several marine photosynthetic microorganisms, including significant contributors to oceanic primary productivity, can serve as a source of CO_2 rather than a sink during CO_2 fixation. The phycoerythrin-possessing cyanobacterium *Synechococcus* sp. WH7803 evolved CO_2 at a rate that increased with light intensity and attained a value approximately five-fold that for photosynthesis. The external CO_2 concentration reached was significantly higher than that predicted for chemical equilibrium between HCO_3^- and CO_2, as confirmed by the rapid decline in the CO_2 concentration upon the addition of carbonic anhydrase. Measurements of oxygen exchange between water and CO_2, by means of stable isotopes, demonstrated that the evolved CO_2 originated from HCO_3^- taken up and converted intracellularly to CO_2 in a light-dependent process.

Conclusions: We report net, sustained CO_2 evolution during photosynthesis. The results have implications for energy balance and pH regulation of the cells, for carbon cycling between the cells and the marine environment, and for the observed fractionation of stable carbon isotopes.

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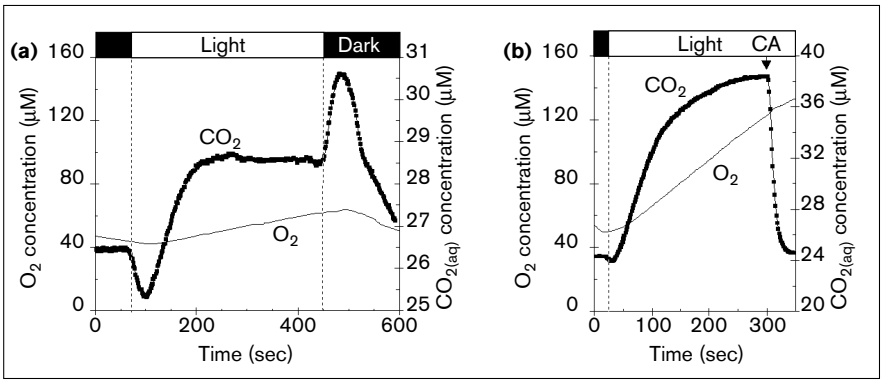
Background

The presence of an inorganic-carbon-concentrating mechanism (CCM [1–6]) has been documented in a number of freshwater and marine photosynthesizing microorganisms, and is most probably widely spread ([7–9] and references therein). By raising the intracellular concentration of CO_2, the CCM compensates for the low affinity for CO_2 of the carboxylating enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) [2–4]. According to a currently accepted model for the CCM in cyanobacteria [4,10–12], influx of inorganic carbon (Ci) into the cells involves active transport, which constitutes the first step in the CCM, resulting in a high internal Ci level. The central premise of the model is that the dominant Ci species in the cytoplasmic pool is HCO_3^-, and that CO_2 and HCO_3^- do not reach chemical equilibrium in this compartment. Light energy is involved in HCO_3^- transport and in the conversion of cytoplasmic CO_2 to HCO_3^-.

Catalyzed generation of CO_2 from accumulated HCO_3^- occurs at carbonic anhydrase (CA) sites within the carboxysomes — polyhedral bodies that contain most of the cellular rubisco and CA. CO_2 is thus generated in the close vicinity of carboxylating sites and much of it is immediately fixed. Such CO_2 as leaks from the carboxysomes to the cytoplasm is, in part, scavenged by the light-dependent, CA-like activity that converts cytoplasmic CO_2 to HCO_3^-. The presence of the CCM raises the apparent photosynthetic affinity of the organism for external Ci [3]. The large CO_2 and HCO_3^- fluxes associated with the CCM are likely to affect the stable carbon isotope composition, $\delta^{13}C$, of the organic matter produced [7,13,14].

We have followed CO_2 and HCO_3^- fluxes associated with the CCM in several photosynthetic marine organisms, including the cyanobacterium *Synechococcus* sp. WH7803,

Figure 1



The effect of illumination on $CO_{2(aq)}$ and O_2 concentrations in suspensions of *Synechococcus* WH7803. Cells were grown in artificial sea water containing 1 mM Ci and 20 mM Hepes pH 8.0, 26°C, at a light intensity of 10 μmol quanta m^{-2} sec^{-1}. During measurements (using a membrane inlet quadrupole mass spectrometer) 3 ml cell suspension, corresponding to 5–8 mg chlorophyll*mL-1, were exposed to light/dark cycles. Ci concentration was 2.5 mM, light intensities were (a) 50 μmol quanta m^{-2} sec^{-1} and (b) 420 μmol quanta m^{-2} sec^{-1}. Bovine carbonic anhydrase (Sigma) was provided where indicated (Figure 1b).

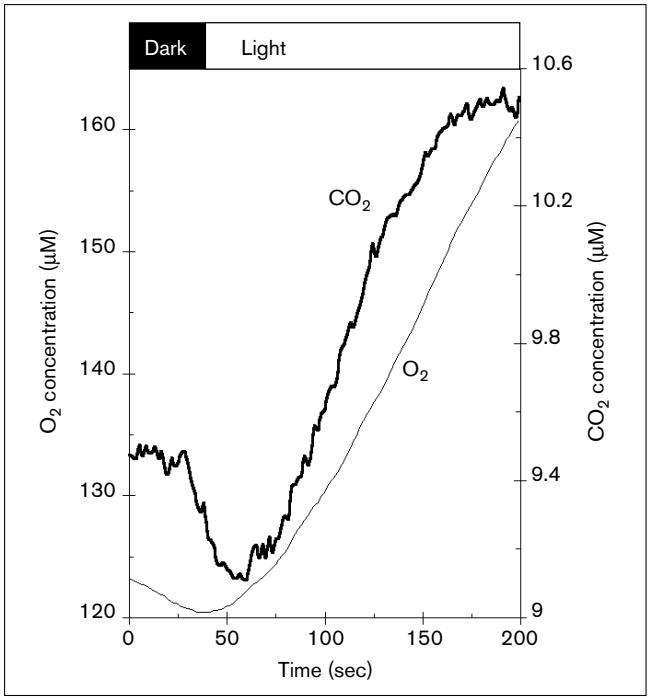
Nannochloropsis sp. (Eustigmatophyceae) and the diatom *Thalassiosira weissflogii*. These organisms are abundant in the ocean and are believed to contribute significantly to its primary productivity. Most of the studies reported here were carried out on *Synechococcus* sp. WH7803, which is capable of growing under a wide range of light intensities by modulating the amount of phycoerythrin and hence the light-harvesting cross-section [15–19]. Further, when exposed for about an hour to light intensities supra-optimal for photosynthesis, it has the ability to lower the efficiency of energy transfer from the harvesting complex to the photosynthetic reaction center. It is also capable of adapting to varying ambient CO_2 concentration by raising the activity of the CO_2-concentrating mechanism when exposed to low ambient CO_2 [20]. We report the unexpected finding that net, sustained CO_2 efflux accompanies CO_2 fixation in these organisms.

Results

A membrane inlet mass spectrometer was used to monitor O_2 and CO_2 exchange in suspensions of the marine photosynthetic organisms investigated. Figure 1a shows that, as expected, net O_2 evolution or uptake was observed following illumination or darkening, respectively, of a dense suspension of *Synechococcus* sp. WH7803. On illumination, the concentration of dissolved CO_2 ($CO_{2(aq)}$) declined briefly, presumably while the intracellular Ci pool was being filled [21], and then rose, indicating sustained net CO_2 evolution during photosynthesis. The steady-state concentration of dissolved CO_2 attained in the light was substantially higher than that in the dark period and significantly exceeded that predicted for equilibrium with the HCO_3^- concentration (Figure 1b). Addition of CA resulted in a sharp decrease in $CO_{2(aq)}$ concentration (Figure 1b), confirming that CO_2 and HCO_3^- were not at chemical equilibrium in the medium during steady-state photosynthesis. The rate of O_2 evolution declined following the addition of CA (Figure 1b), probably because the ambient concentration of $CO_{2(aq)}$, and hence that at the carboxylating site, had been reduced. The brief burst of CO_2 observed following darkening (Figure 1a), was likely to be due to depletion of the internal Ci pool [21].

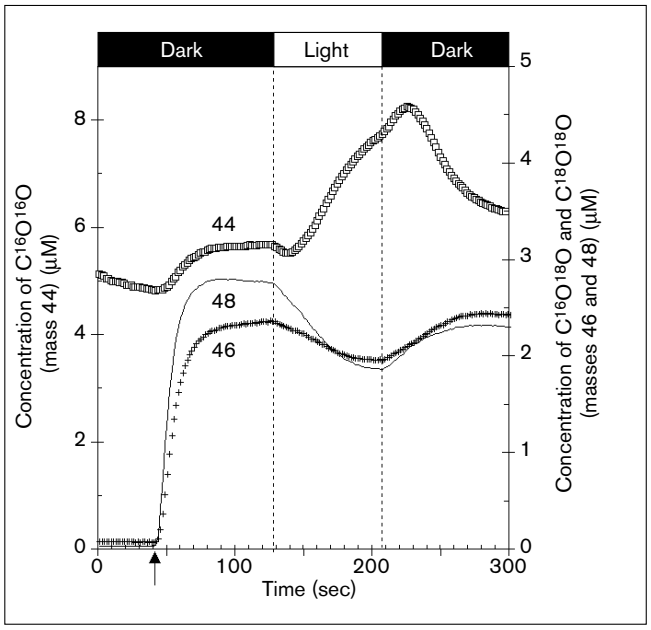
Net CO_2 evolution during photosynthesis was also observed in the marine *Nannochloropsis* sp., though the extent was smaller than in *Synechococcus sp.* WH7803 (Figure 2; see [22]). As could have been expected, however, net CO_2 evolution was not observed with organisms that

Figure 2



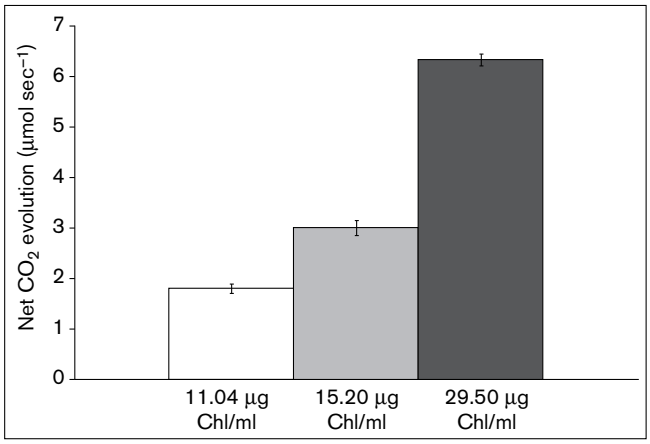
The effect of illumination on $CO_{2(aq)}$ and O_2 concentrations in suspensions of *Nannochloropsis* sp. The experimental conditions were similar to those in Figure 1b.

Figure 3



Changes in the concentration of $C^{16}O^{16}O$ (mass 44), $C^{18}O^{16}O$ (mass 46) and $C^{18}O^{18}O$ (mass 48) during dark/light cycles after the addition (indicated by arrow) of ^{18}O-enriched Ci to a suspension of *Synechococcus sp.* WH7803. Light intensity was 50 μmol quanta m^{-2} sec^{-1}, pH 8.15, and the total Ci concentration was 1.0 mM.

Figure 4



The rate of net CO_2 evolution as affected by the density of *Synechococcus sp.* WH7803 cell suspensions. Experimental conditions as in Figure 1b.

possess periplasmic CA, such as certain diatoms (data not shown); in these cells, extracellular CA activity did not allow the $CO_{2(aq)}$ concentration to exceed the CO_2–HCO_3^- equilibrium value and net CO_2 efflux during photosynthesis was consequently not detectable.

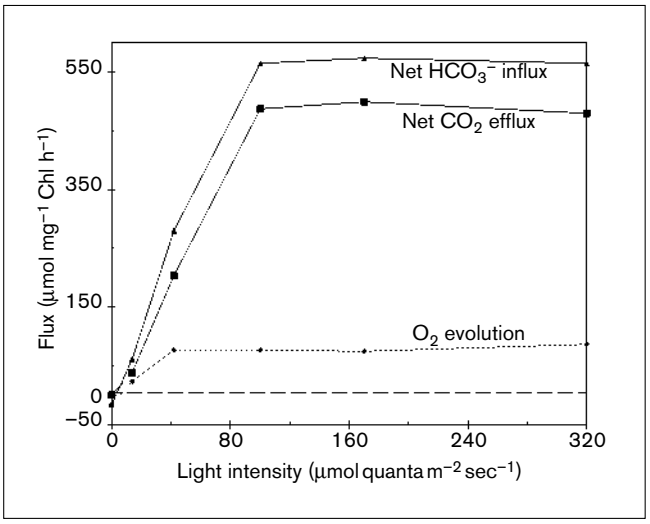
The experiments described above led us to conclude that *Synechococcus* sp. WH7803 does not possess appreciable external CA activity. We made use of this fact to examine whether the CO_2 evolved in the light originated in the intracellular HCO_3^- pool, or alternatively in the unstirred layer surrounding the cells as a result of local acidification. CO_2 containing stable oxygen isotopes, masses 48 and 46 ($C^{18}O^{18}O$ and $C^{16}O^{18}O$, respectively), was supplied to the cell suspension. Since uncatalyzed exchange of oxygen between CO_2 and water is slow, a rapid decline in the abundance of masses 48 and 46 and a rise in mass 44 ($C^{16}O^{16}O$) would indicate that exchange of oxygen between CO_2 and water had been significantly accelerated at intracellular catalysis sites [23]. Figure 3 shows that, upon the addition of ^{18}O-enriched Ci in the dark, the abundance of masses 48, 46 and 44 rose quickly, then declined exceedingly slowly, as expected in the absence of catalysis. Following subsequent illumination the abundance of both 48 and 46 decreased sharply, while that of mass 44 rose, suggesting catalyzed conversion of the former masses to the latter. The simultaneous decline in both masses 48 and 46 also indicates catalysis by CA in the light at an intracellular site and points to an internal source for the effluxing CO_2.

Corroborative evidence that the CO_2 flux originated in the intracellular HCO_3^- pool, and was not generated in the extracellular unstirred layer due to light-dependent acidification, came from experiments where the concentration of the buffer was varied between 5 and 100 mM so as to modify any acidified region within the unstirred layer. This treatment had little effect on the rate of CO_2 evolution (data not shown).

Clearly, net CO_2 efflux (Figures 1,2) will only be observable when the combined CO_2 efflux from all the cells is higher than the rate of hydration of CO_2 to HCO_3^- in the medium — that is, only when the cell density is high enough to achieve this condition. Figure 4 shows that the level of detectable efflux and the steady state $CO_{2(aq)}$ concentration attained were indeed functions of cell density. The cell densities used here were therefore much higher than usually observed under natural conditions. There are no grounds, however, for postulating that net CO_2 efflux does not occur under natural cell densities, though at present, due to detection limitations, this may not be experimentally verifiable. The CO_2 level around the cells, though possibly above that expected at chemical equilibrium, would of course not be as high in nature as that observed in our experiments owing to lower cell densities in natural populations (Figure 4). It is not known whether, under natural conditions, the extracellular CO_2 concentration ever reaches levels high enough for appreciable down-regulation of the CCM.

In order to test whether the requirement for light was related to activation or energization of HCO_3^- uptake [24],

Figure 5



A comparison of the rates of photosynthetic O_2 evolution, net CO_2 efflux and calculated net HCO_3^- influx in *Synechococcus sp.* WH7803 as a function of light intensity. The cells were exposed to the indicated light intensities until a steady state level of ambient $CO_{2(aq)}$ was reached. At this steady state, net HCO_3^- influx was equal to the photosynthetic rate plus net CO_2 efflux. The latter (see text) was equal to the rate of net CO_2 hydration in the medium, which was assessed from the slope of the progress curve for decline in $CO_{2(aq)}$ following injection of a known quantity of $CO_{2(aq)}$ to a cell-free medium (composition as in Figure 1) in a closed system.

and thus of CO_2 efflux, we examined the relationship between these fluxes and light intensity. Figure 5 shows that net HCO_3^- influx saturated at a light intensity 2.5-fold higher than did photosynthetic O_2 evolution, suggesting that light was serving as an energy source. (See Discussion for an explanation of the manner in which the curve for net HCO_3^- influx in Figure 5 was calculated.) At saturating light intensity, HCO_3^- influx was approximately six times the rate of photosynthetic O_2 evolution.

Discussion

The results presented here record the striking observation that, during net photosynthesis by the organisms investigated, the ambient CO_2 concentration was higher than that expected at chemical equilibrium and that this was due to sustained, net CO_2 efflux from the cells. Similar, though transient, effects are visible in figures presented by Badger and Andrews [25] for *Synechococcus* sp. Nageli. The possibility that the CO_2 was generated outside the cell, due to acidification of an unstirred layer by a plasmalemma-located proton-extruding ATPase [26], can be rejected since experiments with stable oxygen isotopes (Figure 3) indicated that the source of the CO_2 was an intracellular CA, as proposed by a quantitative current model [10–12]. Furthermore, raising the buffer capacity of the medium had no effect on CO_2 efflux.

Uptake of Ci from the medium is known to require light for activation and energization [24]. This probably explains the fact that catalyzed exchange of oxygen isotopes between CO_2 and H_2O was only detectable upon illumination, since an appreciable quantity of the ^{18}O-labelled HCO_3^- supplied in the medium will only have been transported to the internal CA sites in the light. In addition, it is possible that light was required to convert the internal CA into an active form. It might also be suggested that the uptake of masses 46 and 48 in the light reflected photosynthetic CO_2 uptake. This interpretation would have to rest, however, on the unlikely assumption that the simultaneous rise in mass 44 (Figure 3) was due to discrimination against this isotope in photosynthesis.

Over the periods where ambient $CO_{2(aq)}$ concentration is constant (Figure 1), the sum of the rates of the processes generating CO_2 in the external medium — dehydration of HCO_3^- plus efflux of CO_2 from the cells — will be equal to that of the processes consuming CO_2 in the medium (hydration of CO_2 plus CO_2 influx into the cells). Net CO_2 efflux can thus be calculated from this relationship using rate constants for interconversion of CO_2 and HCO_3^- in sea water [27] and the observed concentrations of CO_2 and HCO_3^-. With the aid of this value for net CO_2 efflux, net HCO_3^- influx can be estimated since at the steady state:

$$CO_{2\,(influx)} + HCO_3^-{}_{(influx)} = CO_{2\,(efflux)} + HCO_3^-{}_{(efflux)} + \text{photosynthetic rate} \quad (1)$$

Alternatively, net HCO_3^- influx can be assessed from the sum of net CO_2 efflux plus photosynthesis, deduced from O_2 evolution, assuming an O_2:CO_2 exchange ratio of 1. Figure 5 shows that the curve for net HCO_3^- influx lies well above that for O_2 evolution (and hence CO_2 fixation), and continues to rise at photon flux densities beyond those which saturate photosynthetic O_2 evolution. Note that the photosynthetic rate reached a maximum value at a relatively low light intensity (40 μmol quanta m^{-2} sec^{-1}) reflecting the fact that the cells had been grown at, and were thus adapted to, a low fluent flux of 10 mol quanta m^{-2} sec^{-1}. When the cells were grown at a higher light intensity, the same qualitative effects were observed but the curves shown in Figure 5 were shifted toward higher light intensity.

The significance of this energy-dependent Ci circulation, apparently futile, and its possible role is yet to be established, but it could confer some protection against damage to the photosynthetic reaction center occasioned by excess light energy. The large cross-section of the light-energy-absorbing pigments, following acclimation to a low light intensity [16,18], renders the photosynthetic machinery susceptible to photodamage at higher light intensities [17,19,20]. The observation that, at a saturating light intensity, net HCO_3^- uptake was six-fold larger than CO_2 fixation raises the possibility that Ci cycling may serve as a

means for dissipating excess light energy by rapid consumption of ATP or ATP equivalents (such as proton gradients). The amount of energy that can be dissipated, in ATP equivalents, is not known. However, assuming that 4 moles of HCO_3^- are transported per mole of ATP hydrolyzed (in analogy to the approximately 4 H^+ ions transported per ATP consumed, [28]), HCO_3^- transport would use additional energy equivalent to about 1.5 ATP per CO_2 fixed in photosynthesis — that is, it would raise the theoretical ATP consumption from 3 to 4.5 moles for each mole of CO_2 fixed (see [12]). At sub-saturating light intensities, the dissipation of ATP would be less, since the ratio of CO_2 effluxing to that fixed is lower (Figure 5), reducing futile energy consumption. A further pathway of energy dissipation necessitated by massive HCO_3^- uptake will be the neutralization of the OH^- ions remaining in the cytoplasm following net CO_2 efflux.

On the basis of the above argument, maintenance of the internal pH would require up to 1.5 ATP equivalents per CO_2 fixed, at saturating light intensity. Clearly, at lower fluent flux the energy demand would be reduced concomitantly with the reduction in the ratio CO_2 leaked:CO_2 fixed. Intracellular circulation between CO_2 and HCO_3^- (due to formation of CO_2 from HCO_3^- in the carboxysome, some leak of CO_2 to the cytoplasm and its energy-dependent conversion to HCO_3^- in the cytoplasm, [3,4,6]) is yet another process where low-CO_2-adapted cells, capable of significant HCO_3^- uptake, would demand a larger amount of energy for each CO_2 fixed. Indeed, it is well established that the maximal quantum yield of photosynthetic O_2 evolution (measured at the lower range of light intensities) is considerably higher in cyanobacteria grown high-CO_2 [25].

The cycling of Ci reported here has relevance to the fractionation of stable carbon isotopes, $\delta^{13}C$, by organisms that possess a CCM. The universal carboxylating enzyme, rubisco, discriminates against ^{13}C-CO_2 [13,14]; the $\delta^{13}C$ of the organic matter formed thus reflects this discrimination, as does the isotopic composition of the internal Ci pool, which serves as the substrate for the carboxylation reaction. The $\delta^{13}C$ of the internal Ci pool will be strongly affected, firstly by the rubisco fractionation resulting in ^{13}Ci enrichment of the pool, and secondly by the balance of the fluxes of the various Ci species, inward and outward, and of the carboxylation rate. The nature of the Ci species taken up — CO_2 and HCO_3^- — and their relative proportion will also affect the $\delta^{13}C$ of the internal pool. At isotopic equilibrium, HCO_3^- is approximately 9% richer in ^{13}C than is $CO_{2(aq)}$ [29,30], so uptake of HCO_3^- will tend to increase the abundance of ^{13}C in the internal Ci pool. The Ci cycling relative to the photosynthetic rate thus has relevance to the potential use of $\delta^{13}C$ as a paleobarometer for CO_2 concentration [31,32] and for oceanic productivity, and should be taken into account. Finally, while in general marine photosynthesis operates as a sink for atmospheric CO_2, it remains to be seen whether organisms engaged in massive cycling of Ci through their CCMs serve as a CO_2 source for preferential CO_2 users in their immediate vicinity in the phytoplankton consortium.

Conclusions

Massive HCO_3^- uptake by the marine microorganisms investigated here, followed by formation of CO_2 at intracellular CA sites, results in net CO_2 efflux during photosynthesis and an ambient CO_2 concentration above that expected at chemical equilibrium between Ci species. The cycling of Ci is dependent on light energy, and increases with rising light intensity. Furthermore, energy is also required for the maintenance and regulation of internal pH in the face of excess OH^- ions, which tend to accumulate as a result of the substantial HCO_3^- uptake and CO_2 efflux. Since the Ci fluxes continue to rise at light intensities that saturate photosynthesis, they may serve as a means for dissipating excess light energy. This may well be of particular ecological advantage in an organism such as *Synechococcus sp.* WH7803, which is capable of developing a large light-energy-absorbing cross-section, and which may consequently be sensitive to photodamage. The massive Ci fluxes would also be expected to affect the stable carbon isotope composition ($\delta^{13}C$) of the internal Ci pool and hence of the organic matter produced. Correct use of the $\delta^{13}C$ of organic and calcified matter as a paleobarometer will thus depend on better understanding of the interrelations between the Ci fluxes and the $\delta^{13}C$ of the Ci pool.

Materials and methods

Organisms and growth conditions

Synechococcus sp. WH7803 and *Nannochloropsis sp.* were grown in artificial sea water supplemented with f/2 nutrients [33], 1 mM inorganic carbon and 20 mM Hepes-NaOH pH 8.0. *Thalassiosira* cultures were grown in filtered sea water supplemented with f/2 and sodium silicate. Cells of *Synechococcus sp.* WH7803 and *Nannochloropsis* sp. were grown at 24°C, the *Thalassiosira* cultures were maintained at 16°C. Continuous illumination was provided by VHO fluorescence tubes at 10 μmol quanta m^{-2} sec^{-1} (*Synechococcus* and *Thalassiosira*) or 30 μmol quanta m^{-2} sec^{-1} (*Nannochloropsis*). Cultures at mid exponential growth were used in the experiments reported here.

Gas exchange measurements

Gas exchange measurements were performed with the aid of a membrane inlet quadrupole mass spectrometer (Balzers QMG 421) using 3 ml cell suspension. Simultaneous measurements of argon concentration (not shown) were used to assess the stability of the system. Dissolved CO_2 masses 48 and 46 were obtained by dissolving $NaHCO_3$ in ^{18}O-enriched (95%) water.

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