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Effect of aeration rates on growth rates and natural abundance $^{13}C/^{12}C$ ratio of *Phaeodactylum tricornutum*

Andrew M. Johnston, John A. Raven

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, United Kingdom

ABSTRACT: Studies of the photosynthetic assimilation of inorganic carbon by the marine diatom *Phaeodactylum tricornutum* showed that the aeration system has a major influence on the dissolved inorganic carbon (DIC) concentration and hence growth rates. The stable isotope ratio of $^{13}C/^{12}C$ was used as an indicator of carbon limitation. Three aeration systems were used. Algae from a well-aerated culture (2 dm^3 min^{-1}) had a specific growth rate of 1.58 d^{-1}, reached a cell density of 12×10^6 cells cm^{-3}, displayed a constant $\delta^{13}C$ value of −23.6 ‰ and the inorganic carbon concentration of the growth media remained above 1.5 mol m^{-3}. Algae from non-aerated cultures had the lowest growth rate (0.42 d^{-1} after 4.5 d), were able to consume DIC faster than the resupply of atmospheric CO_2, and the DIC concentration reached 0.68 mol m^{-3} after 6.5 d of culture. The $\delta^{13}C$ value of these algae rose to −17.83 ‰. Algae that were poorly aerated (0.1 dm^3 min^{-1}) had intermediate growth rates (0.72 d^{-1} at 4.5 d) and reduced the DIC concentration to 0.17 mol m^{-3} after 6.5 d. Algae grown under these conditions displayed the highest $\delta^{13}C$ values, −12.3 ‰. Aeration (2 dm^3 min^{-1}) of a previously unaerated culture restored the DIC concentration of the culture medium to 2.0 mol m^{-3} and reduced the $\delta^{13}C$ from −14.0 ‰ to −23.4 ‰.

INTRODUCTION

Many aquatic photosynthetic organisms have the ability to concentrate dissolved inorganic carbon (DIC) at the site of the primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). This achieves the suppression of photorespiration and photosynthetic gas exchange characterised by high affinities for DIC, low CO_2 compensation points and rates of apparent photosynthesis which are insensitive to changes in the partial pressure of O_2. The mechanism of DIC accumulation is a biophysical one based on the active transport of either CO_2 or HCO_3 or both (Raven 1991). In a small proportion of largely terrestrial higher plants, i.e. C_4 and CAM plants, the repression of photorespiration is achieved by a biochemical concentrating mechanism based on the spatial (C_4) or temporal (CAM) separation of 2 carboxylating enzymes, phosphoenolpyruvate carboxylase (PEPC) and RUBISCO operating in series. The first identifiable product of photosynthetic ^{14}C fixation (in the absence of a 'hydrazine trap') in these plants is a C_4 acid, malate (or less commonly aspartate). The majority of higher plants, C_3 plants, are dependent solely on RUBISCO for their photosynthetic CO_2 fixation and are not able to repress photorespiration. When considering the DIC acquisition mechanisms of marine microalgae the gas exchange data available is consistent with the operation of a DIC concentrating mechanism (Raven 1985). The situation is complicated in some marine microalgae as they have relatively high activities of ß-carboxylation enzymes, PEPC and phosphoenolpyruvate carboxykinase (PEPCK) (Beardall et al. 1976, Holdsworth & Colbeck 1976, Holdsworth & Bruck 1978, Appleby et al. 1980).

An increasingly utilized tool in the study of photosynthesis are stable isotopes. There are 2 stable isotopes of carbon: ^{12}C makes up 98.89 % of the global total and ^{13}C the remaining 1.11 %. RUBISCO discriminates against the heavier ^{13}C and plants which are dependent on RUBISCO have slightly less ^{13}C than

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1.11 % (O'Leary 1981). The differences in the ratio of ^{13}C to ^{12}C between plants are very small so a differential notation is used to compare sample ratios. The term used is $\delta^{13}C$, defined as:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1 \quad (1)$$

where $\delta^{13}C$ is the ratio value (‰); $(^{13}C/^{12}C)_{sample}$ the stable isotope ratio of the sample; and $(^{13}C/^{12}C)_{standard}$ the stable isotope ratio of the standard, PeeDee Belemnite (PDB), a carbonate which is 1.1237 % ^{13}C. A $\delta^{13}C$ value of 0 ‰ represents a $^{13}C/^{12}C$ ratio the same as the PDB standard. A $\delta^{13}C$ value of less than 0, e.g. −27 ‰ (a typical value for C_3 land plants), represents a lower $^{13}C/^{12}C$ value, 1.094 % ^{13}C in this case. Of 2 $\delta^{13}C$ values the more negative value indicates greater discrimination against ^{13}C. $\delta^{13}C$ values have been used in higher plants to distinguish the biochemical pathway. C_3 plants have typical $\delta^{13}C$ values of −20 to −35 ‰ and C_4 plants $\delta^{13}C$ values of −9 to −14 ‰. Algal species exhibit a wide range of $\delta^{13}C$ values; for macroalgae reported values range from −8.8 to −34.5 ‰, (Kerby & Raven 1985, Maberly et al. 1993). In marine microalgae $\delta^{13}C$ ratios have been used as an indicator of ß-carboxylating activity (Descolas-Gros & Fontugne 1985). They suggested that the observed increase in $\delta^{13}C$ ratios, from −22 to −16 ‰, at the end of a culture of *Skeletonema costatum* could be due to the increase in ß-carboxylation activity, caused by decreasing light levels. In this paper we have investigated how the conditions of aeration can influence the growth rates of the diatom *Phaeodactylum tricornutum* and see if the $\delta^{13}C$ value is influenced by controlling the supply of CO_2.

MATERIALS AND METHODS

Algal culture. *Phaeodactylum tricornutum*, strain CCAP 1052/1A, was obtained on agar slopes from Culture Collection of Algae and Protozoa (Windermere, England). The alga was grown in 1 dm^3 flasks containing 200 to 300 cm^3 f/2 media. Three aeration systems were used, fast flow (2 dm^3 min^{-1}), slow flow (0.1 dm^3 min^{-1}) and no aeration but agitated twice a day. Air was obtained from the roof of our department and had a partial pressure of 35 Pa CO_2. Cultures were grown in a photon flux density incident on the flasks of 100 µmol m^{-2} s^{-1} (400 to 700 nm) in continuous light with cool fluorescent tubes and at 20 °C (Geider et al. 1985). Growth rates were calculated from cell counts of at least 200 cells per count made with an Improved Neubauer haemocytometer (Weber, England).

$\delta^{13}C$ analysis. The $\delta^{13}C$ of organic material was measured on a VG SIRA Series II isotope ratio mass spectrometer with a Carlo-Erba CHN analyzer as the combustion unit. Samples were prepared by washing at least 3×10^6 cells, in fresh media, pelleting the cells with a bench centrifuge (4000 × *g* for 10 min), resuspending the cells in 1 cm^3 distilled water, pelleting the cells with an Eppendorf microcentrifuge (10 000 × *g* for 2 min), and finally resuspending the cells in 20 mm^3 distilled water. The cells were transferred to tin boats previously washed in hexane. The samples were acidified with the addition of 2 mm^3 1 kmol m^{-3} HCl, then dried in a 75 °C oven overnight and were then ready for analysis.

For $\delta^{13}C$ determination of source DIC 10 cm^3 of culture was filtered through a Whatman GF/C filter into a 20 cm^3 'Red Top' vacutainer (Becton Dickinson, USA). To measure the $\delta^{13}C$ the samples were acidified with the addition of 1 cm^3 of 6 kmol m^{-3} phosphoric acid. The liberated CO_2 was transferred to the inlet of the mass spectrometer with a helium gas stream and trapped in the liquid nitrogen trap until analyzed. Results are presented as the mean of at least duplicate measurements, with an average standard deviation of ±0.15 ‰ (n = 6) for organic carbon samples and ±0.25 ‰ (n = 5) for inorganic carbon samples.

Determination of DIC concentration. The method used to measure [DIC] was as previously described by Johnston & Raven (1986) using the IRGA acid stripping technique.

RESULTS

Cell growth under different aeration systems

In the well-aerated culture exponential growth lasted 5 d by which time the cell density reached 12×10^6 cells cm^{-3}. The specific growth rate over this period was 1.58 d^{-1} In the poorly-aerated culture exponential growth only lasted 3 d during which the cell density increased to 2.8×10^6 cells cm^{-3}; after this growth slowed (0.72 d^{-1} at Day 4.5) and at 6.5 d the cell density was only 8.1×10^6 cells cm^{-3}. In the non-aerated culture exponential growth lasted less than 3 d; the growth rate was 0.42 d^{-1} at Day 4.5. At 6.5 d the cell density achieved was 5.7×10^6 cells cm^{-3} (Fig. 1).

Concentration of DIC in culture media

The initial [DIC] is often less than the 2 mol m^{-3} DIC expected in air-equilibrated seawater at pH 8.0. Autoclaving culture media for 15 min can reduce the [DIC] to 0.26 mol m^{-3}. This is followed by a slow re-equilibration

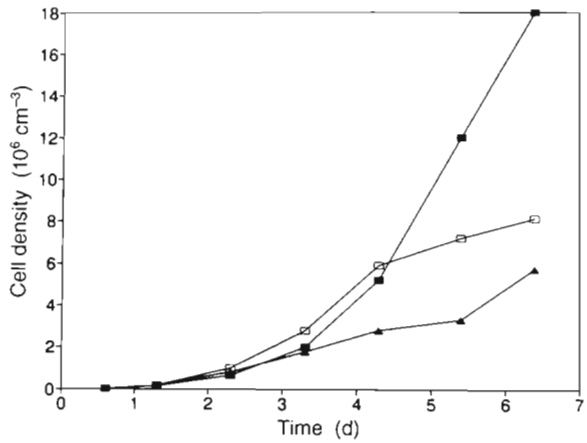

Fig. 1. *Phaeodactylum tricornutum*. Growth under a photon flux density of 100 µmol m^{-2} s^{-1} (400 to 700 nm) at 20 °C with different aeration systems: (■) 2 dm^3 min^{-1}, (⁻) 0.1 dm^3 min^{-1} and (▲) non-aerated batch culture

with atmospheric CO_2 and the [DIC] increases to 1.1 mol m^{-3} after 48 h. Aeration increases the [DIC] to the air-equilibrium value 1.9 mol m^{-3}. In the experimental media there was a slight increase in the well-aerated cultures to 2 mol m^{-3} DIC. In the well-aerated culture the [DIC] remained at or above 2 mol m^{-3} DIC for the length of the experiment. As the cell density increased, the [DIC] of the poorly aerated culture fell to 0.17 mol m^{-3} DIC after 6.5 d. In the non-aerated culture the rate of cell growth was not as great as in the poorly aerated culture and the decrease in the [DIC] was less, the [DIC] being 0.68 mol m^{-3} after 6.5 d (Fig. 2). The re-equilibration of DIC following autoclaving of the media will be with atmospheric CO_2, which in laboratory conditions may have a variable $\delta^{13}C$ value, depending on the time of day and number of people in the laboratory. We now take the precaution of allowing our media to cool down on the roof of our department and for the following 3 d it is kept in an unused room.

Carbon stable isotope ratios

In the well-aerated culture the $\delta^{13}C$ ratio of organic C remained within the range −24.3 to −22.8 ‰ but in the poorly-aerated culture the $\delta^{13}C$ ratio increased from −21.8 to −12.2 ‰ at Day 4.5. In the non-aerated culture, where the rate and amount of growth were not as great as in the poorly-aerated culture, the $\delta^{13}C$ of organic C showed less of an increase, rising from −21.6 to −17.8 ‰ at Day 6.5 (Fig. 3).

There was no systematic change in the $\delta^{13}C$ of the source DIC in the well-aerated culture, the value ranging from −2.2 to −0.2 ‰. The poorly aerated culture showed the greatest change in $\delta^{13}C$ values, falling from −0.6 to −20.2 ‰ at Day 5.5. The decline in $\delta^{13}C$ of source DIC in the unaerated medium was more gradual, from −2.4 to −16.5 ‰ at Day 6.5 (Fig. 3).

Effects of aeration on a previously non-aerated culture

A culture which had previously been non-aerated for 7.5 d had a cell density of 2.43×10^6 cells cm^{-3}, a [DIC] of 0.34 mol m^{-3} and a $\delta^{13}C$ ratio for organic C of −14.0 ‰. The growth rate was 0.28 d^{-1}. Aeration at 2 dm^3 min^{-1} had the effect, after a short lag, of increasing the growth rate to 2.05 d^{-1} for 3 d after which it fell back to 1.05 d^{-1}. By the end of the experiment the cell density reached

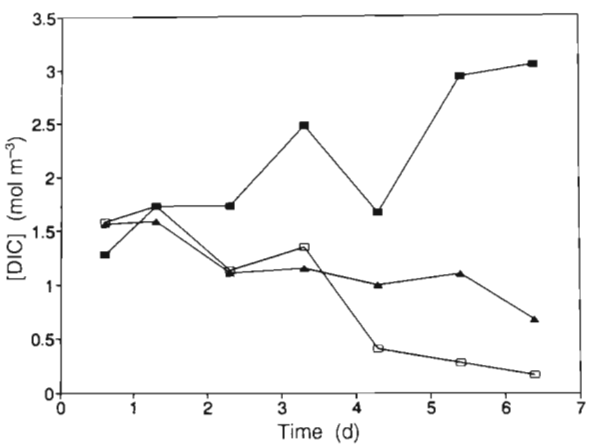

Fig. 2. Change in DIC concentration during culture of *Phaeodactylum tricornutum* grown in different aeration systems: (■) 2 dm^3 min^{-1}, ( ) 0.1 dm^3 min^{-1} and (▲) non-aerated batch culture. Each point is the mean of 2 measurements

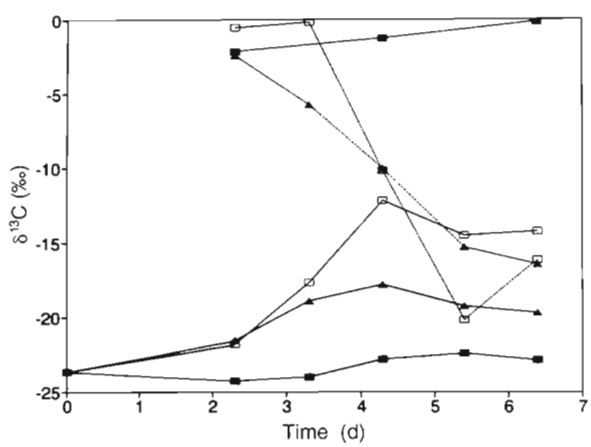

Fig. 3. $\delta^{13}C$ of the organic C of *Phaeodactylum tricornutum* grown (solid lines), and of source C (dotted lines), in 3 aeration systems: (■) 2 dm^3 min^{-1}, ( ) 0.1 dm^3 min^{-1} and (▲) non-aerated batch culture. Each point is the mean of 2 measurements

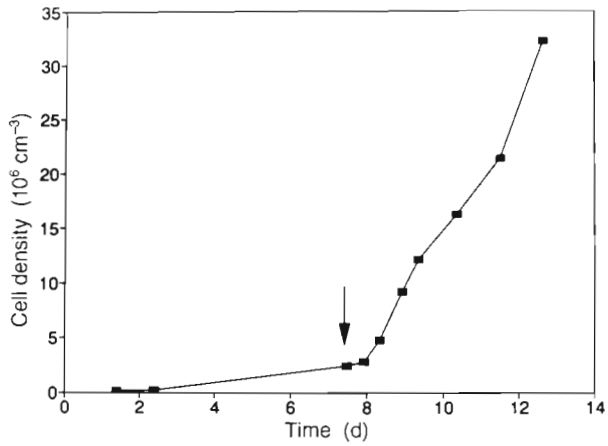

Fig. 4. *Phaeodactylum tricornutum*. Effect on cell density of aeration of a previously non-aerated culture grown in a photon flux density of 100 µmol m^{-2} s^{-1} at 20 °C. The arrow indicates the commencement of aeration at a rate of 2 dm^3 min^{-1} Each point is the mean of 2 measurements

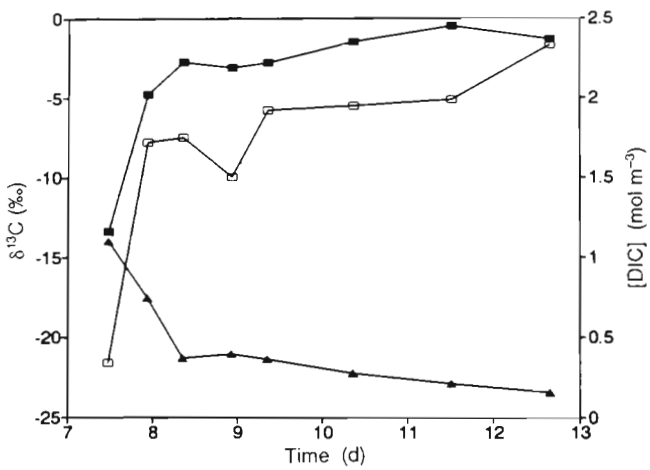

Fig. 5. Effect on inorganic C concentration (■), $\delta^{13}C_{organic}$ (○) and $\delta^{13}C_{source}$ (▲) of aeration at 2 dm^3 min^{-1} of a previously non-aerated culture of *Phaeodactylum tricornutum* grown in a photon flux density of 100 µmol m^{-2} s^{-1} at 20 °C

32.3×10^6 cm^{-3} (Fig. 4). Within half a day the [DIC] had been restored to 2.0 mol m^{-3} DIC. Within 2 d the δ^{13}C of organic C had decreased to –21.3 ‰ and by the end of the experiment had reached –23.4 ‰. The δ^{13}C of the source C had been restored to –0.5 ‰ (Fig. 5).

DISCUSSION

Batch culture has been and still is a common method of growing algae in the laboratory. One of the main problems associated with this method is that the growing algae consume nutrients which are not replaced and so they experience a constantly changing chemical environment. This can be avoided to a certain extent by enriching the media with an excess of nutrients so that no matter how much the algae consume the concentration of nutrients will remain saturating In nearly all cases this practice will overcome nutrient limitation during exponential phase of growth. If a requirement of an experiment is to maintain air-equilibrium concentrations of CO_2 then DIC may be a possible exception to the above assumption. If the carbon content of *Phaeodactylum tricornutum* is around 12.4×10^{-12} g cell^{-1} (Geider et al. 1985) a cell density of 1×10^6 cells cm^{-3} represents 1.03 mol organic C m^{-3}, half the normal [DIC] of seawater. If, during rapid growth based on the DIC available to the alga already in solution, the resupply of DIC from atmospheric CO_2 by aeration (or diffusion through the cotton wool stopper in non-aerated cultures) is not sufficient to maintain air-equilibrium DIC concentrations the [DIC] will decline. *P. tricornutum* is able to reduce the [DIC] to as low as 0.17 mol m^{-3} (Fig. 2). The agitation caused by the slow aeration appears to stimulate cell growth compared to non-aerated cultures with a parallel decrease in [DIC] (Fig. 2) and this is reflected in the greater changes in [DIC], $\delta^{13}C_{organic}$ and $\delta^{13}C_{source}$. When the culture is well aerated all the parameters that have been measured in the study remain constant (growth rates, [DIC], $\delta^{13}C_{organic}$ and $\delta^{13}C_{source}$).

The suggestion that high cell densities at the end of exponential phase of *Skeletonema costatum* are responsible for the less negative $\delta^{13}C_{organic}$ ratio values caused by increased ß-carboxylation activity in response to self-shading (see Descolas-Gros & Fontugne 1985) is doubtful. A review of the role of ß-carboxylation and its effect on δ^{13}C in algae is in preparation (Johnston unpubl.). The fact that in a well-aerated culture the cell density reached either 18.7×10^6 cells cm^{-3} (Fig. 1) and the organic carbon δ^{13}C value remained constant (Fig. 3) or 32.3×10^6 cells cm^{-3} (Fig. 4) and the organic carbon δ^{13}C value re-established a constant more negative value (Fig. 5) suggests that self-shading, and hence low photon flux density, does not cause an increase in δ^{13}C values. When aeration was poor or non-existent the organic C δ^{13}C rose to as high as –12.2 ‰ (Fig 3). In a low pH medium, pH 5.5, where nearly all the DIC is in the form of CO_2, the maximum photosynthetic rate (P_{max}) of *Phaeodactylum tricornutum* was 22.43 nmol C (10^6 cells)$^{-1}$ h^{-1} and the half-saturation concentration ($K_{0.5}$) was 3.51 mmol m^{-3} DIC. The acidic conditions do not appear to have affected the P_{max} (see Johnston & Raven 1986). With a decrease in DIC due to poor aeration and a likely increase in pH the change in $CO_{2\,aq}$ will be significant

and probably lead to DIC-limitation, hence the reduction in growth rates. Some of this shortfall will be made up by enhanced HCO_3 utilization (Johnston & Raven unpubl.) but in poorly aerated cultures there does appear to be some degree of DIC-limitation evident from reduction in growth rates. The increase in $\delta^{13}C$ value under such conditions is a reflection of this limitation as the algae discriminate less against the heavier ^{13}C when the DIC pool becomes depleted. The inorganic C ending up with a more negative $\delta^{13}C$ than the organic C in the poorly aerated culture was unexpected (Fig. 3). Before an apparent inverse discrimination can be used to account for such an observation a more detailed mass balance analysis of total cell carbon, source carbon and extracellular organic carbon would be required. In an attempt to overcome potential problems of DIC resupply, Wong & Sackett (1978) enriched the DIC concentration to 20 mol m^{-3} DIC and used an ascarite absorption tube to prevent exchange with atmospheric CO_2. Even with these precautions the $\delta^{13}C$ values did not remain constant. Having increased the DIC concentration the status of DIC acquisition must have been altered and given the results of this study is likely to have had some affect on the $\delta^{13}C$ values. Rees (1984) showed that the affinity for DIC decreased to 0.44 mol m^{-3} DIC from 0.12 mol m^{-3} DIC when *P. tricornutum* was grown in 30 mol m^{-3} DIC as compared to air-equilibrated cultures.

Degens et al. (1968) reported that the degree of aeration influenced the $\delta^{13}C_{organic}$ of *Skeletonema costatum*. At 18 °C the values were −13.4 ‰ (source CO_2 −5 ‰) in slow aeration and −19.4 ‰ (source CO_2 −7.6 ‰) in violently aerated cultures. This difference can be related to either the decrease in diffusion pathlength in the well-aerated cultures, a decreased affinity for DIC (Johnston & Raven unpubl.) or a combination of the two. Restoring air-equilibrium concentrations of DIC has the effect of re-establishing a constant $\delta^{13}C$ value, −22.9 ‰ (± 0.6, n = 3; Fig. 5). These observations are consistent with the suggestion that an increase in temperature causes an increase in $\delta^{13}C$ values produced by the decreased solubility of CO_2 at higher temperatures and hence an increased limitation of photosynthesis by CO_2 supply (Rau et al. 1989, Fry & Wainright 1991, Sackett 1991).

We are not in a position to state categorically that a specific alga supplied with a DIC source of a given constant $\delta^{13}C$ has a specific $\delta^{13}C$ value. There are a number of environmental factors which can influence the $\delta^{13}C$ value. The current work has shown that when grown in a well-aerated medium at 20 °C and a photon flux density of 100 µmol m^{-2} s^{-1} the $\delta^{13}C$ value had an average of −23.3 ‰ (± 0.8, n = 6). A $\delta^{13}C$ source value of −2.1 ‰ at pH 8.0 and 20 °C represents a $\delta^{13}C$ of −1.9 ‰ for HCO_3 and −11.51 ‰ for CO_2 using the equilibrium isotopic fraction between dissolved CO_2 and HCO_3 of −9.55 ‰ (Mook et al. 1974). Comparisons of fractionation among cultures use the equation (Farquhar et al. 1989):

$$\Delta = \frac{\delta^{13}C_{source} - \delta^{13}C_{organic}}{1 + \delta^{13}C_{organic}} \quad (2)$$

Δ (‰) can be presented in relation to the total DIC or the individual species, and for *Phaeodactylum tricornutum*, from a well-aerated culture with the source DIC and organic C values quoted above, is 21.4 ‰ (DIC), 11.7 ‰ (CO_2) and 21.5 ‰ (HCO_3). This value indicates the degree of discrimination by *P. tricornutum* is not as large as its potential, 30 ‰, the value of resource saturated RUBISCO. It is possible to grow *P. tricornutum* at higher than air-equilibrium concentrations of CO_2 and increase the amount of discrimination. *P. tricornutum* grown in 5 kPa CO_2 has a $\delta^{13}C$ of −54.0 ‰ and the $\delta^{13}C$ of the source DIC is −30.4 ‰, which corresponds to a Δ of 25.0 ‰. Conversely when the alga is DIC-limited the level of discrimination is less. If laboratory studies are to be used to model natural environments it is imperative the [DIC] and the $\delta^{13}C$ of source inorganic carbon and organic carbon are measured to ensure that the DIC status of the algal culture is constant and well defined, so aiding comparisons with other studies.

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