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 x $10^6$ 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 5.5 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 β-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
measured on a VG SIRA Series I1 isotope ratio mass
spectrometer with a Carlo-Erba CHN analyzer as the
combustion unit. Samples were prepared by washing
at least 3 \times 10^6 cells, in fresh media, pelleting the
cells with a bench centrifuge (4000 \times g for 10 min), resus-
pending the cells in 1 cm^3 distilled water, pelleting the
cells with an Eppendorf microcentrifuge (10 000 \times 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 acid-
ified 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 Dickin-
son, 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 \pm 0.15 \% \text{O} (n = 6) for organic
carbon samples and \pm 0.25 \% \text{O} (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 \times 10^9 cells cm^{-3}. The specific growth rate over this
period was 1.58 d^{-1}. In the poorly-aerated culture ex-
ponential growth only lasted 3 d during which the
cell density increased to 2.8 \times 10^9 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 \times 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 \times 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. Auto-
claving culture media for 15 min can reduce the [DIC] to
0.26 mol m^{-3}. This is followed by a slow re-equilibration

MATERIALS AND METHODS

Algal culture. Phaeodactylum tricornutum, strain
CCAP 1052/1A, was obtained on agar slopes from
Culture Collection of Algae and Protozoa (Winder-
mere, England). The alga was grown in 1 dm^3 flasks
containing 200 to 300 cm^3 /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 \mu mol 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
stable isotope ratio of the sample, PeeDee Belemnite
(PDB), which is 1.1237 \% 13C. A \delta^{13}C value of 0 \% represents a 13C/12C 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 13C/12C value. 1.094 \% 13C in this case. Of 2 \delta^{13}C values the more negative value indicates
greater discrimination against 13C. \delta^{13}C values have
been used in higher plants to distinguish the bio-
chemical 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 val-
ues, 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 \beta-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 costa-
tum could be due to the increase in \beta-carboxylation activity, caused by decreasing light levels. In this
paper we have investigated how the conditions of aer-
ation 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.

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

where \delta^{13}C is the ratio value (%); \delta^{13}C_{\text{sample}} the
stable isotope ratio of the sample; and \delta^{13}C_{\text{standard}}
the stable isotope ratio of the standard, PeeDee Belemnite
(PDB), which is 1.1237 \% 13C. A \delta^{13}C value of 0 \% represents a 13C/12C 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 13C/12C value. 1.094 \% 13C in this case. Of 2 \delta^{13}C values the more negative value indicates
greater discrimination against 13C. \delta^{13}C values have
been used in higher plants to distinguish the bio-
chemical 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 val-
ues, 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 \beta-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 costa-
tum could be due to the increase in \beta-carboxylation activity, caused by decreasing light levels. In this
paper we have investigated how the conditions of aer-
ation 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.
with atmospheric CO₂ 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 and amount of growth were not as great as in the poorly-aerated culture, where the rate and amount of growth were shown less of an increase, rising from -21.6 to -17.8 % at Day 6.5 (Fig. 3).

There was no systematic change in the δ¹³C 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 δ¹³C values, falling from -0.6 to -20.2 % at Day 5.5. The decline in δ¹³C 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 × 10⁶ cells cm⁻³, a [DIC] of 0.34 mol m⁻³ and a δ¹³C ratio for organic C of -14.0 %. The growth rate was 0.28 d⁻¹. Aeration at 2 dm³ 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
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 CO₂ then DIC may be a possible exception to the above assumption. If the carbon content of *Phaeodactylum tricornutum* is around $12.4 \times 10^{-12}$ g cell$^{-1}$ (Geider et al. 1985) a cell density of $1 \times 10^6$ 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 CO₂ 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$_{org}$, 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$_{org}$, 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$_{org}$ ratio values caused by increased $\beta$-carboxylation activity in response to self-shading (see Descolas-Gros & Fontugne 1985) is doubtful. A review of the role of $\beta$-carboxylation and its effect on $\delta^{13}$C in algae is in preparation (Johnston unpubl.). The fact that in a well-aerated culture the cell density reached either $18.7 \times 10^6$ cells cm$^{-3}$ (Fig. 1) and the organic carbon $\delta^{13}$C value remained constant (Fig. 3) or $32.3 \times 10^6$ cells cm$^{-3}$ (Fig. 4) and the organic carbon $\delta^{13}$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 $\delta^{13}$C values. When aeration was poor or non-existent the organic C $\delta^{13}$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 CO₂, the maximum photosynthetic rate ($P_{max}$) of *Phaeodactylum tricornutum* was 22.43 mmol C (10$^6$ cells)$^{-1}$ h$^{-1}$ and the half-saturation concentration (K$_C$) 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$_2_{eq}$ 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 HCO₃⁻ 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 δ¹³C value under such conditions is a reflection of this limitation as the algae discriminate less against the heavier ¹³C when the DIC pool becomes depleted. The inorganic C ending up with a more negative δ¹³C 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 (1976) enriched the DIC concentration to 20 mol m⁻³ DIC and used an ascorbate absorption tube to prevent exchange with atmospheric CO₂. Even with these precautions the δ¹³C 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 effect on the δ¹³C 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. (1986) reported that the degree of aeration influenced the δ¹³C of Skeletonema costatum. At 18 °C the values were −13.4 % (source CO₂ −5 %) in slow aeration and −19.4 % (source CO₂ −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 δ¹³C 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 δ¹³C values produced by the decreased solubility of CO₂ at higher temperatures and hence an increased limitation of photosynthesis by CO₂ 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 δ¹³C has a specific δ¹³C value. There are a number of environmental factors which can influence the δ¹³C 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 δ¹³C value had an average of −23.3 % (± 0.8, n = 6). A δ¹³C source value of −21.1 % at pH 8.0 and 20 °C represents a δ¹³C of −1.9 % for HCO₃⁻ and −11.51 % for CO₂ using the equilibrium isotopic fraction between dissolved CO₂ and HCO₃⁻ of −9.55 % (Mook et al. 1974). Comparisons of fractionation among cultures use the equation (Farquhar et al. 1989):

\[ \Delta = \frac{\delta^{13}C_{\text{source}} - \delta^{13}C_{\text{organic}}}{1 + \delta^{13}C_{\text{organic}}} \]  

\( \Delta \) (%o) 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 % (CO₂) and 21.5 % (HCO₃⁻). 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 CO₂ and increase the amount of discrimination. P. tricornutum grown in 5 kPa CO₂ has a δ¹³C of −54.0 % and the δ¹³C 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 δ¹³C 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.

Acknowledgements. This work is supported by NERC Grant GR3/6197. The VG SIRA Series II isotope ratio mass spectrometer was supplied under NERC Grant GR3/7967. We thank Dr Stephen Maberly for his helpful discussion during this work.

LITERATURE CITED


This article was submitted to the editor


Manuscript first received: July 10, 1992
Revised version accepted: September 21, 1992