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The relationship between the dissolved inorganic carbon concentration and growth rate in marine phytoplankton

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A range of marine phytoplankton was grown in closed systems in order to investigate the kinetics of dissolved inorganic carbon (DIC) use and the influence of the nitrogen source under conditions of constant pH. The kinetics of DIC use could be described by a rectangular hyperbolic curve, yielding estimations of $K_{G(DIC)}$ (the half saturation constant for carbon-specific growth, i.e. Cμ) and μ_{max} (the theoretical maximum Cμ). All species attained a $K_{G(DIC)}$ within the range of 30–750 μM DIC. For most species, NH_4^+ use enabled growth with a lower $K_{G(DIC)}$ and/or, for two species, an increase in μ_{max}. At DIC concentrations of >1.6 mM, Cμ was $>90\%$ saturated for all species relative to the rate at the natural seawater DIC concentration of 2.0 mM. The results suggest that neither the rate nor the extent of primary productivity will be significantly limited by the DIC in the quasi-steady-state conditions associated with oligotrophic oceans. The method needs to be applied in the conditions associated with dynamic coastal (eutrophic) systems for clarification of a potential DIC rate limitation where cells may grow to higher densities and under variable pH and nitrogen supply.

Keywords: dissolved inorganic carbon; phytoplankton; productivity; kinetics; nitrogen

1. INTRODUCTION

The interspeciation of dissolved inorganic carbon (DIC) (as $CO_{2(aq)}$, HCO_3^- and CO_3^{2-}) in seawater is influenced by pH, alkalinity and temperature (Butler 1982; Takahashi *et al*. 1993). In freshwater environments the $CO_{2(aq)}$ availability has been shown to vary on diel, episodic and seasonal scales, with significant depletions of DIC (Maberly 1996). Despite the greater pH buffering capacity of the carbonate system in the marine environment in comparison with freshwater systems, the supply of DIC to marine phytoplankton changes in response to pH, turbulence (mixing rates) and cell density (Pelgler & Kempe 1988; Rau *et al*. 1989).

At natural $CO_{2(aq)}$ concentrations, $CO_{2(aq)}$ diffusion has been suggested to limit the rate of carbon fixation in marine microalgae, particularly at elevated pH (Riebesell *et al*. 1993; Chen & Durbin 1994). When grown at natural concentrations of $CO_{2(aq)}$, many marine and freshwater microalgae exhibit gas-exchange characteristics which closely resemble those of C_4 higher plants (Badger *et al*. 1980; Miller & Colman 1980; Burns & Beardall 1987). This has been attributed, at least in part, to the derepression of inducible carbon-concentrating mechanisms (CCMs) (Suzuki & Spalding 1989) which may arise as a result of the slow diffusion of CO_2 in the aquatic environment or the slow uncatalysed interconversion of CO_2 and HCO_3^- (Aizawa & Miyachi 1986; Colman 1991; Badger & Price 1992).

The acclimation of many species of algae and cyanobacteria to changes in the availability of DIC has been well documented, including the biochemical, molecular and genetic basis of CCMs (Colman 1991; Price *et al*. 1998). Phytoplankton may possess the ability to use HCO_3^- (Dixon & Merrett 1988; Tortell *et al*. 1997) which comprises 90% of the total DIC in marine systems at an ambient pH of 8.2. Intracellular and, for some species, extracellular forms of carbonic anhydrase appear to be critical in the acclimation process (Badger & Price 1994; Mitchell & Beardall 1996; Iglesias-Rodríguez & Merrett 1997). Intracellular carbonic anhydrase maintains the CO_2 concentration at the active site of ribulose 1,5-bisphosphate carboxylase–oxygenase (Rubisco), which would otherwise only be half saturated at air-equilibrated values of $CO_{2(aq)}$ (Raven & Johnston 1991).

While it has often been assumed that light, phosphorus or nitrogen availability are more likely to limit phytoplankton growth than the availability of DIC (Walsh 1991; Falkowski & Wilson 1992), the possession of inducible CCMs suggests a potential for DIC stress and, hence, carbon rate limitation of growth. However, while there is evidence to suggest that $CO_{2(aq)}$ may rate limit short-term photosynthesis, the ecologically relevant question is whether the carbon availability rate limits growth. Evidence suggests that the growth rate of macrophytes (Madsen 1993; Rivers & Peckol 1995), corals (Marubini & Thake 1999) and terrestrial plants (Bowes 1991, 1993) may be limited by the availability of inorganic carbon. To date, relatively few studies of marine phytoplankton have related the growth rate to DIC (Goldman *et al*. 1974; Goldman & Graham 1981; Riebesell *et al*. 1993; Clark *et al*. 1999). pH has often been used to change the concentration of $CO_{2(aq)}$ by shifting the DIC equilibrium. However, pH also affects the growth rate independently, confounding interpretation of the results. In the present investigation we directly relate the carbon-specific growth rate of a range of marine phytoplankton species from a number of taxa to the availability of DIC at constant pH. Given the link between carbon and nitrogen assimilation in marine phytoplankton and the reported physiological differences as a result of the nitrogen source (Turpin 1991; Levasseur *et al*. 1993), an additional aspect to the study

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Received 18 January 2000 *Accepted* 15 February 2000

Table 1. *Estimations for the values of $K_{G(DIC)}$ and μ_{max}*

(The values are reported ± s.e.s. For an individual species, an asterisk indicates a significant difference at the 95% confidence level between nitrogen sources for the values of $K_{G(DIC)}$ and/or μ_{max}.)

	species	nitrogen source	$K_{G(DIC)}$ (μM)	μ_{max} (day^{-1})
Bacillariophyceae	*Phaeodactylum tricornutum*	NO_2^- [a]	30 ± 3	0.80 ± 0.07
	Thalassiosira pseudonana	NO_3^-	273 ± 7*	1.33 ± 0.11*
		NH_4^+	233 ± 6*	1.75 ± 0.14*
	Thalassiosira weissflogii	NO_3^-	258 ± 4*	1.55 ± 0.08
		NH_4^+	135 ± 2*	1.52 ± 0.05
Chlorophyta	*Stichoccococus bacillaris*	NO_3^-	720 ± 2*	0.77 ± 0.08
		NH_4^+	568 ± 1*	0.83 ± 0.09
Dinophyta	*Alexandrium fundyense*	NO_3^- [b]	468 ± 8	0.36 ± 0.02
	Scrippsiella trochoidea	NO_3^- [b]	280 ± 6	0.40 ± 0.03
Prymnesiophyceae	*Emiliania huxleyi*	NO_3^-	150 ± 5*	1.19 ± 0.10
		NH_4^+	114 ± 6*	1.14 ± 0.15
	Isochrysis galbana	NO_2^- [a]	81 ± 1	0.48 ± 0.01
Raphidophycaee	*Heterosigma carterae*	NO_3^-	673 ± 1	1.21 ± 0.10*
		NH_4^+	663 ± 2	1.62 ± 0.17*

[a] Data from Clark *et al.* (1999) where nitrite was the nitrogen source.
[b] The dinoflagellates did not grow satisfactorily using ammonium; only results from nitrate grown cultures are given.

was to assess the influence of the nitrogen source on the kinetics of DIC use.

2. MATERIAL AND METHODS

Cultures (table 1) were maintained in artificial seawater medium (Harrison *et al.* 1980) except for the dinoflagellates which were maintained in modified (decreased salinity of 30) K-type medium (Keller *et al.* 1987). Organic buffers were omitted from the media and the culture pH was maintained manually. For species grown in modified K-type media, seawater was first acidified and autoclaved (20 min at 121 °C) to remove the DIC (verified using the Gran titration technique) (Butler 1982). DIC was added in the form of bicarbonate to give initial concentrations of 0.25, 0.50, 1.00 or 2.00 mM. Phosphate (32 μM) plus nitrate or ammonium (50 μM) were added. Data from studies of *Phaeodactylum tricornutum* and *Isochrysis galbana*, in which 50μM nitrite (NO_2^-) was used as the nitrogen source (Clark *et al.* 1999), are also reproduced here. These nutrient regimes resulted in phosphorus-replete cultures limited by either DIC and/or nitrogen. All media were filter sterilized through 47 mm diameter, 0.2 μm pore Durapore filters (Millipore) into 3 l conical flasks, which were then sealed gas tight (i.e. closed systems) with a silicon bung through which three polytetrafluoroethylene (Teflon) tubes were passed. The first tube extended into the culture media for sampling. CO_2-free air replaced the volume of medium removed for sampling, entering the culture vessel via the second tube. This air had first passed through a Dreschel bottle containing 1 M NaOH (thus removing the CO_2) and then through a second Dreschel bottle containing unbuffered high-performance liquid chromatography grade water. The third tube allowed for the maintenance of culture pH at 8.3 ± 0.05 by the addition of small volumes of freshly made acid or alkali (0.025 M HCl or NaOH) via an Acrodisc filter (Gelman, Ann Arbor, MI, USA) (see Clark *et al.* (1999) for further details). Cultures were grown in a constant temperature room (16 °C) at a photon flux density of 200 μmol m^{-2} s^{-1} (measured within water-filled 3-l conical flasks using a Biospherical Instruments QSL 100 meter with a 4π detector; Biospherical Instruments, San Diego, CA, USA) under a 12 L:12 D cycle supplied by cool-white fluorescent tubes.

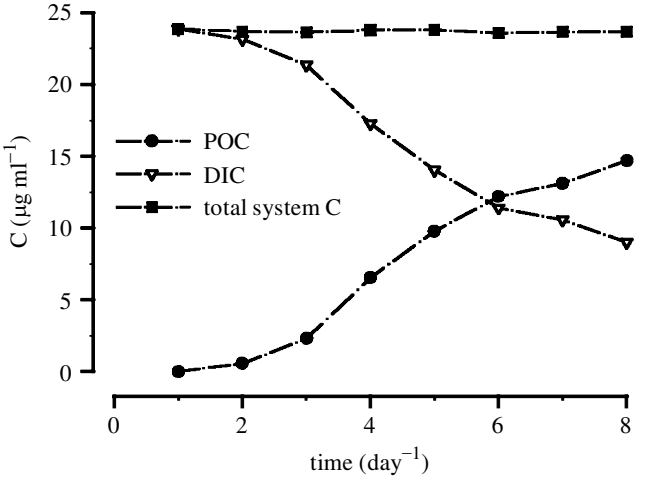

Figure 1. The results for *Thalassiosira pseudonana* using 2.0 mM DIC and 50 μM NO_3^-, demonstrating the depletion of extracellular [DIC] and the increase in particulate organic carbon (POC) with the accounting of total system carbon ± 2%.

Prior to experimentation, stock cultures of all species were maintained in media with an initial DIC concentration of 2.0 mM. The experimental cultures underwent two consecutive batch growth cycles which were started with a < 5% (v/v) inoculation from the previous batch once this had attained the stationary phase (as judged by cell counts). An analysis of variation of the means of the cell counts and biovolume measurements between consecutive growth cycles demonstrated that they were not significantly different ($p \leqslant 0.05$). The data from two growth cycles starting from the four initial DIC concentrations were used here. The cell numbers and biovolume were measured in triplicate (Elzone 282PC particle analyser, Particle Data, Luxembourg City, Luxembourg) and nitrate or ammonium in duplicate at 0, 6 and 12 h into the light phase on each day. The cells were collected by filtration under low pressure (< 75 mm Hg) onto

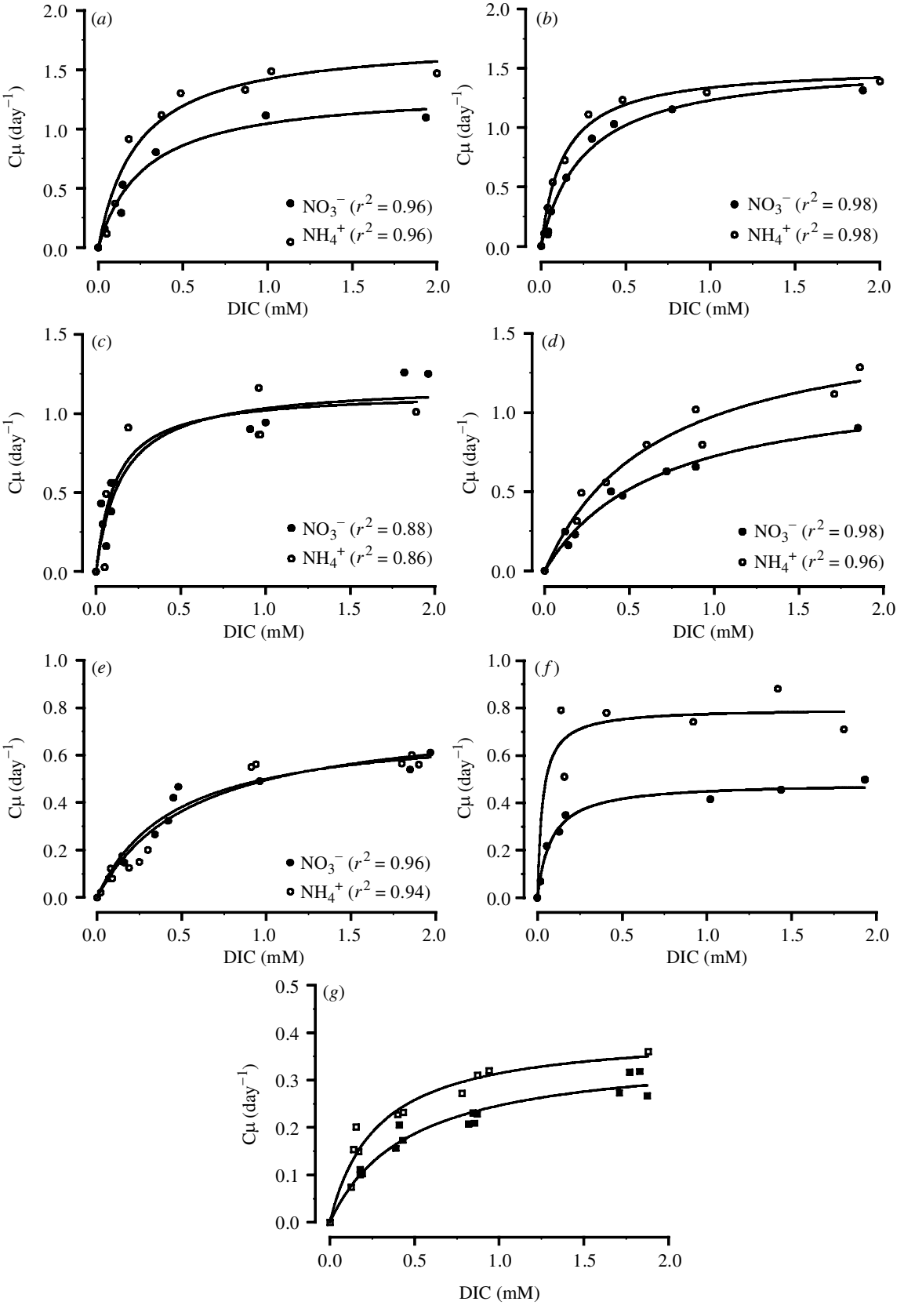

Figure 2. A rectangular hyperbolic (Michaelis–Menten type) curve fitting through the data describing the variation in Cμ with [DIC] for the range of species investigated. See also table 1. (*a*) *Thalassiosira pseudonana*, (*b*) *Thalassiosira weisflogii*, (*c*) *Emiliania huxleyi*, (*d*) *Heterosigma carterae*, (*e*) *Stichoccocus bacillaris*, (*f*) open circles, *Phaeodactylum tricornutum* (NO_2^-, $r^2 = 0.95$) and closed circles, *Isochrysis galbana* (NO_2^-, $r^2 = 0.98$), and (*g*) closed squares, *Alexandrium fundyense* ($r^2 = 0.95$) and open squares, *Scrippsiella trochoidea* ($r^2 = 0.94$).

pre-ashed, 13 mm, Gelman A/E filters at 6 h into the light phase and subsequently used for elemental carbon and nitrogen analysis.

The DIC was determined in a cell-free medium by the Gran titration technique described by Butler (1982). From the titration, the amount of weak acid or alkali required to correct each culture pH to 8.3 ± 0.05 was calculated and added. Extracellular nitrate or ammonium were determined using an Alpkem RFA/2 (Alpkem Corp., Clackamas, OR, USA), microsegmented, flow nutrient analyser. The cellular carbon and nitrogen were measured using Europa Scientific Roboprep and Tracer-mass instruments (Europa, Crewe, UK) using isoleucine as the standard.

The culture biovolume was linearly related to the particulate organic carbon (POC) during nitrogen-replete growth for all species ($r^2 = 0.97$). The coefficient of variation for the triplicate biovolume determinations was lower ($< 5\%$) than for the duplicate POC measurements; the carbon-specific growth ($C\mu$) was therefore determined from the rate of biovolume specific growth. $C\mu$ was determined using the equation

$$C\mu = \frac{\ln(BV_2) - \ln(BV_1)}{(t_2 - t_1)}, \quad (1)$$

where BV_1 and BV_2 were the biovolume values at times t_1 and t_2, respectively. Only those values for $C\mu$ corresponding to nitrogen-replete growth (extracellular inorganic nitrogen $> 20\,\mu M$) were used in the construction of the relationship between $C\mu$ and the extracellular concentration of DIC ([DIC]). The $C\mu$:[DIC] relationship was constructed by plotting the 24 h averaged $C\mu$ against the corresponding [DIC]. The half saturation constant for $C\mu$ ($K_{G(DIC)}$) and the theoretical maximum rate of growth (μ_{max}) were determined using an iterative fit procedure (Biosoft's FigP) from the equation

$$C\mu = \mu_{max} \frac{[DIC]}{[DIC] + K_{G(DIC)}}. \quad (2)$$

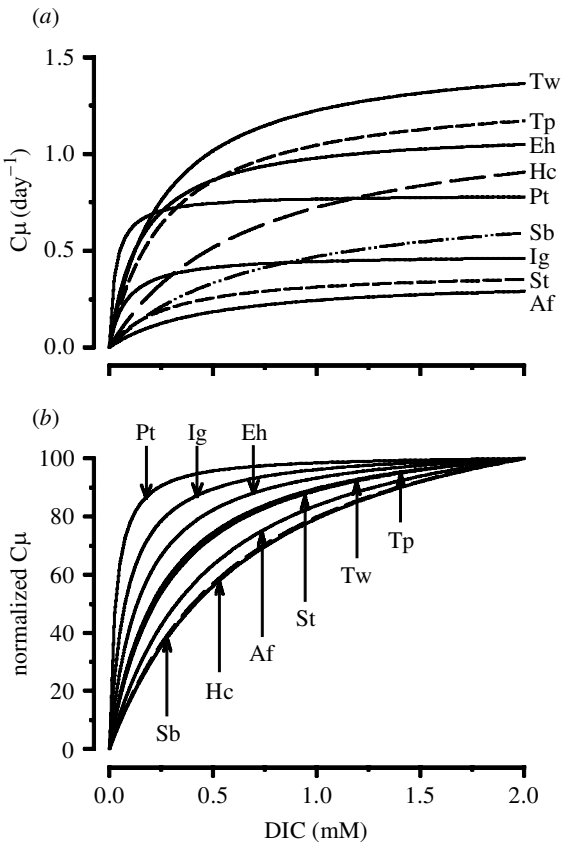

Figure 3. (*a*) Co-plots of the curve fits for all species compiled from the nitrate and nitrite data sets in figure 2. (*b*) These have been normalized to set $C\mu$ at 100% with an extracellular [DIC] of 2.0 mM. Bacillariophyceae: Pt, *Phaeodactylum tricornutum*; Tp, *Thalassiosira pseudonana*; Tw, *Thalassiosira weisflogii*. Chlorophyta: Sb, *Stichoccocus bacillaris*. Dinophyta: Af, *Alexandrium fundyense*; St, *Scrippsiella trochoidea*. Prymnesiophyceae: Eh, *Emiliania huxleyi*; Ig, *Isochrysis galbana*. Raphidophyceae: Hc, *Heterosigma carterae*.

3. RESULTS

During the batch growth of all species in closed systems, the total system carbon (DIC + algal-C) varied by $< 2\%$ (e.g. figure 1), validating the use of the Gran titration technique and experimental procedure in this study. $C\mu$ could be related to the [DIC] by a rectangular hyperbolic (Michaelis–Menten type) function for all species (equation (2) and figure 2). Estimates of the theoretical maximum rate of $C\mu$ (μ_{max}) and of the [DIC] which would support the half μ_{max} (i.e. $K_{G(DIC)}$) were determined (table 1). Statistically significant differences between nitrogen sources for the values of $K_{G(DIC)}$ and μ_{max} are also presented in table 1.

Co-plotting the data enabled a direct interspecies comparison of the variation in $C\mu$ with [DIC] (figure 3*a*). Generally, diatoms such as *Thalassiosira pseudonana* and *Thalassiosira weissflogii* attained higher rates of $C\mu$ at any given [DIC], while the dinoflagellates attained the lowest. Normalizing the data to a [DIC] of 2.0 mM (the typical seawater concentration) removed the variation in the comparisons due to μ_{max} and enabled a visual comparison of the relative 'performance' of each species (figure 3*b*). While there was considerable variation between species, even the poorest competitor (*Stichoccococus bacillaris*) still attained a growth rate of *ca.* 80% of the normalized growth rate at 1 mM DIC (figure 3*b*). There was no relationship between μ_{max} and $K_{G(DIC)}$. For example, although the dinoflagellate *Scrippsiella trochoidea* attained one of the lowest absolute $C\mu$ at any given [DIC], $C\mu$ approached saturation at a similar rate to that of the diatoms *T. pseudonana* and *T. weissflogii*.

4. DISCUSSION

CO_2 limitation of phytoplankton growth rates has been suggested in a number of studies in which growth rate measurements were made at elevated pH. However, as pointed out by Goldman (1999), growth at an elevated pH may not necessarily be a consequence of CO_2 limitation as inferred by Riebesell *et al.* (1993) and Chen & Durbin (1994). In seawater, the availability of essential but sparingly soluble nutrients for marine algal growth, such as phosphorus and trace metals, is highly dependent on pH. In addition, growth at an elevated pH results in gross alterations in the membrane transport processes and metabolic functions involved in intracellular pH regulation (Smith & Raven 1979; Raven 1980, 1993). As such, it is virtually impossible to infer any effects on μ due simply to changes in the CO_2 availability when the pH and CO_2

covary. In the present study, the pH of the closed system cultures was constant (± 0.05). As a result, the growth rate response of each species was directly related to changes in the DIC (and, hence, CO_2) availability alone. This current study is the most comprehensive to date using this technique.

A number of studies have applied Michaelis–Menten functions in describing the kinetics of DIC acquisition in marine phytoplankton, cyanobacteria and macrophytes (Caperon & Smith 1978; Turpin *et al*. 1985; Madsen 1993; Riebesell *et al*. 1993; Gimmler & Slovik 1995). In the present investigation, the relationship between the rate of carbon-specific growth ($C\mu$) and the extracellular DIC concentration for a taxonomically diverse range of marine phytoplankton could be described by a Michaelis–Menten function. The kinetics results enabled an estimation of the [DIC] which would support half μ_{max} (i.e. an estimation of affinity—$K_{G(DIC)}$). The diatom *P. tricornutum* attained the highest affinity for DIC, which was over an order of magnitude greater than that attained for the toxic dinoflagellate *Alexandrium fundyense*. However, the diatom *T. pseudonana* had a similar $K_{G(DIC)}$ to that of the dinoflagellate *S. trochoidea*, while both were higher (i.e. lower affinity) than the high calcifying strain of the coccolithophorid *Emiliania huxleyi*. These results indicate that a low growth rate (low μ_{max}) does not necessarily equate to a poor capacity for using DIC (high $K_{G(DIC)}$).

Using the estimation of $K_{G(DIC)}$ for each species, the calculated average $K_{G(CO2)}$ (at pH 8.3 using the equations of Butler (1982)) was 1.33 μM $CO_{2(aq)}$. The $K_{M(CO2)}$ for Rubisco, which catalyses the primary carboxylation in photosynthetic autotrophs, is 6.6–185 μM for microalgae and 100–125 μM for cyanobacteria (Jordan & Ogren 1981; Read & Tabita 1994; Raven *et al*. 2000). Given the order of magnitude difference between the $K_{G(CO2)}$ and the $K_{M(CO2)}$ for algal Rubisco, there is at least circumstantial evidence for the operation of CCMs in these marine phytoplankton. The fact that all species attained 90% of the normalized $C\mu$ at DIC concentrations of > 1.6 mM (and as low as 0.3 mM for *P. tricornutum*) (figure 3*b*), while the typical seawater DIC concentration is 2.0 mM, presumably reflects the efficiency of algal CCMs. Although diffusive CO_2 entry may satisfy the demands for photosynthesis (Gleitz *et al*. 1996), definitive evidence for purely diffusive CO_2 entry in marine microalgae is scarce. On the contrary and consistant with the results of the present study, the operation of CCMs appears ubiquitous in a wide taxonomic range of aquatic phototrophs (see Raven 1991). Thus, the structure of models of phytoplankton growth based solely on diffusive CO_2 entry (Riebesell *et al*. 1993; Goericke *et al*. 1994, Laws *et al*. 1995, 1997) may need to be reconsidered.

The kinetics of steady-state growth (Goldman & Graham 1981; Miller *et al*. 1984) and photosynthesis (Badger & Andrews 1982; Miller *et al*. 1984) in photoautotrophic microorganisms have been separately related to DIC availability. Measurements of short-term photosynthesis are not necessarily related to the growth rate, since such measurements are to a large degree dependent on the physiological state of the cells at the time of sampling (Goldman & Graham 1981). The results reported for *E. huxleyi*, a coccolithophorid of great biogeochemical significance (Holligan *et al*. 1983), highlight the limitations of predictions based on photosynthetic performance. While photosynthetic oxygen evolution in *E. huxleyi* may only be 50% saturated with CO_2 at ambient DIC concentrations (Paasche 1964), the results of the present study indicated that the coccolith-producing *E. huxleyi* strain we used attained 90% of normalized $C\mu$ at a [DIC] of 0.80 mM. Thus, although photosynthesis may be limited by $CO_{2(aq)}$ at ambient DIC concentrations (Paasche 1964; Sekino & Shiraiwa 1994), this does not result in an ecologically relevant limitation of the growth rate. Further, Takano *et al*. (1995) demonstrated that, in *E. huxleyi*, the calcification rates rather than growth rates were stimulated at DIC concentrations of 13.2 mM. Consistent with this, an extrapolation of our results suggests only a 6% increase in the growth rate of *E. huxleyi* when grown at 13.2 mM rather than 2.0 mM DIC.

The quantitative and qualitative supply of nitrogen to phytoplankton varies over spatial and temporal scales in the marine environment. To our knowledge, the influence of the nitrogen source on carbon acquisition kinetics has not previously been reported in marine phytoplankton. With the exception of *Heterosigma carterae*, the use of NH_4^+ consistently resulted in a lower $K_{G(DIC)}$ (i.e. an increased capability for making use of a low [DIC]) (table 1). For *H. carterae* and *T. pseudonana*, NH_4^+ use resulted in a higher μ_{max} (table 1). The decreased $K_{G(DIC)}$ with NH_4^+ use may be partly a consequence of boundary layer acidification during the assimilation of NH_4^+ (the assimilation of nitrate results in an increase in pH.) A shift in the equilibrium of the carbonate system towards CO_2 due to boundary layer acidification would enhance the supply of CO_2 to the cell surface.

It has been shown for the first time that, under conditions where DIC declines with constant pH, phytoplankton exhibit a Michaelis–Menten relationship between $C\mu$ and [DIC]. In the oligotrophic marine environment, where the bulk of primary productivity takes place, pH variations are of minor significance. Under such conditions, the results of the present study suggest that carbon availability will not limit the extent or rate of biomass accumulation and, hence, primary productivity. In contrast, significant pH variations may take place in coastal ecosystems, a site of importance in the global carbon budget (Smith & Hollibaugh 1993). Under such conditions, the potential for a DIC rate limitation of growth, together with the interaction between the nitrogen source and DIC assimilation, requires clarification.

This work was supported by the Natural Environment Research Council (UK) through a studentship to D.R.C.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.