EFFECTS OF NITROGEN AVAILABILITY ON INORGANIC CARBON CONCENTRATING MECHANISMS IN MARINE PHYTOPLANKTON

by

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ABSTRACT

Carbon and nitrogen metabolism are tightly coupled through the enzyme RubisCO, but the effects of nitrogen limitation on cellular carbon acquisition are not well understood. This thesis reports measurements of RubisCO and phosphoenolpyruvate carboxylase (PEPC - an enzyme involved in the C_4 photosynthetic pathway) activity in *Thalassiosira weissflogii* grown under steady-state nitrogen limitation as well as data on the utilization of dissolved inorganic carbon species by this microalgae. These biochemical and physiological parameters were also examined across a natural nitrogen gradient in a field study of phytoplankton assemblages in the Queen Charlotte Sound coastal region.

In nitrogen-limited *T.weissflogii* cultures, RubisCO activity decreased with decreasing growth rate ($r=0.821$; $p<0.001$), while PEPC activity remained unchanged over the range of growth rates tested (1.4 to $0.3 \cdot d^{-1}$). $f HCO_3^-$ values, representing the fraction of dissolved inorganic carbon taken up in the form of HCO_3^-, obtained from ^{14}C uptake experiments decreased from ~0.85 to 0.70 as cells became more severely nitrogen-limited (0.682; $p<0.001$). Carbon uptake kinetics indicated that the decrease in $f HCO_3^-$ was attributed to a decrease in direct HCO_3^- transport, as extracellular carbonic anhydrase activity appeared unaffected by changes in growth rate. A down-regulation of the activity of the carbon concentrating mechanism (CCM) may serve to control elemental ratios within the cell and maintain metabolic balance under nitrogen-limited conditions.

At the seven coastal field stations sampled, there was no relationship between RubisCO activity and nitrate concentration, but a negative trend was observed between PEPC activity and nitrate concentration. The diatom-dominated phytoplankton assemblages studied all utilized HCO_3^- as an exogenous inorganic carbon source and HCO_3^- uptake occurred mainly via a direct transport mechanism. There was no apparent relationship between $f HCO_3^-$ values and nitrate concentration at these stations.

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a	external carbonic anhydrase activity
CA	carbonic anhydrase
CCM	carbon concentrating mechanism
DIC	dissolved inorganic carbon
$fHCO_3^-$	fraction of DIC taken up in the form of HCO_3^-
GF/F	glass fiber filter (nominal pore size 0.7μm)
GF/A	glass fiber filter (nominal pore size 1.6μm)
OAA	oxaloacetic acid
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PEPCK	phosphoenolpyruvate carboxykinase
PAR	photosynthetically active radiation (400 to 900nm)
POC	particulate organic carbon
PON	particulate organic nitrogen
RubisCO	ribulose-1,5-bisphosphate carboxylase / oxygenase

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Chapter 1

General Introduction

Though it is now firmly established that marine phytoplankton play a critical role in the global carbon cycle and climate regulation, very few studies have examined the effects of carbon dioxide (CO_2) concentrations on oceanic primary production. Research has traditionally focused on other macronutrients such as nitrogen, phosphorous and silica that are typically found in low concentrations in ocean surface waters. In contrast, dissolved inorganic carbon (DIC) is not traditionally considered to be a limiting resource in seawater because of high (~2000μM; Morel *et al.* 2002) surface concentrations. However, at pH 8.2 in seawater more than 90% of the total DIC pool in the ocean is present in the form of bicarbonate (HCO_3^-), with less than 1% as free CO_2 (Morel & Herring 1993). Laboratory studies have provided evidence that the growth rate of large marine phytoplankton (particularly diatoms) can potentially be limited by the low CO_2 concentrations in seawater (Riebesell *et al.* 1993).

1.1 RubisCO:

Potential carbon limitation of phytoplankton growth has been attributed to the catalytic inefficiency of Ribulose-1,5-bisphosphate carboxylase / oxygenase (RubisCO), the central carbon fixing and rate-limiting enzyme in photosynthesis. This enzyme is only able to fix inorganic carbon in the form of CO_2 (Cooper *et al.* 1969), and it has a very slow maximum specific turnover rate and a poor affinity for CO_2 attributed, in part, to competition between CO_2 and O_2 at the active site

(Hartman *et al.* 1994). The oxygenase function of RubisCO catalyzes the breakdown of organic carbon in the presence of oxygen, utilizing energy without producing useful biosynthetic compounds. The low concentrations of aqueous CO_2 found in surface seawater (~10 μM) are well below the half-saturation constant of algal RubisCO (K_m range ~20–200 μM; Badger *et al.* 1998). Thus, from a purely biochemical perspective, marine photosynthesis is inherently under saturated with respect to CO_2.

The catalytic inefficiency of RubisCO observed under modern atmospheric conditions of high O_2, low CO_2 stems from the fact that this highly conserved protein evolved ~3 billion years ago in a high CO_2, anaerobic atmosphere. Recently it has been proposed that variations in the kinetic properties of RubisCO between major algal and cyanobacterial taxa of different geological ages may be attributed to the evolution of this enzyme toward higher CO_2 affinity in response to changes in atmospheric CO_2 / O_2 ratios over geological time (Badger *et al.* 1998;Tortell 2000).

1.2 Carbon Concentrating Mechanisms:

Despite the potential for carbon limitation of marine phytoplankton, these organisms are able to maintain rapid growth rates even at low oceanic CO_2 concentrations. In order to compensate for the catalytic inefficiency of RubisCO in low CO_2 environments, phytoplankton and cyanobacteria have evolved various strategies to increase carbon-fixation efficiency. These strategies are collectively known as carbon concentrating mechanisms (CCMs) (*see* Raven 1997; Badger

*et al.*1998; Kaplan & Reinhold 1999) since they act to increase the concentration of CO_2 at the active site of RubisCO, thereby increasing the rate of photosynthetic carbon fixation and suppressing photorespiratory oxygenase activity (Canvin 1990). The CCM is regulated by CO_2 concentration, and can be induced under conditions of low CO_2 and repressed under conditions of high CO_2 (Badger & Price 1992). Not only is there a great deal of discrepancy among phytoplankton in the kinetic properties of RubisCO, there are also differences in the relative efficiencies of their CCMs. As the CO_2 affinity of RubisCO decreases, phytoplankton appear to rely more heavily on CCMs in an effort to accumulate CO_2 at the active site of RubisCO (Tortell 2000).

1.3 Carbonic Anhydrase:

Despite significant differences among phytoplankton in the nature of the CCM, one common feature of many species is their ability to utilize the abundant HCO_3^- pool in seawater. Unlike CO_2, the diffusion of the charged HCO_3^- molecule into the cell is restricted by membrane lipids and by the inside-negative electric potential difference across the plasmalemma (Raven1997). However, species may actively transport HCO_3^- (and/or CO_2) directly into the cell or employ the enzyme carbonic anhydrase (CA), a zinc metalloenzyme that may also contain cobalt or cadmium in the active site (Coleman 1998; Lane & Morel 2000a, 2000b), to catalyze the conversion of HCO_3^- to CO_2, with CO_2 entering the cell (Sültemeyer 1998). (Although the $HCO_3^- \leftrightarrow CO_2$ interconversion occurs spontaneously, it happens very slowly relative to other photosynthetic reactions.)

1.4 CO_2 permeability of algal membranes:

The implications of algal membrane CO_2 permeability for CCM operation and for the diffusive flux of CO_2 from the bulk medium to RubisCO are not fully understood (Raven & Beardall 2003). While a membrane with a high CO_2 permeability may seem advantageous in allowing CO_2 to diffuse easily into the cell, the issue of CO_2 efflux must also be considered. It has been suggested that the energetic costs of CCM operation may be minimized if the membranes between RubisCO and the medium have a low CO_2 permeability and therefore decreased rates of CO_2 leakage from cells relative to C fixation (Raven & Johnston 1991).

1.5 C_4 photosynthesis:

Among those phytoplankton capable of using HCO_3^-, evidence for a functional unicellular C_4 photosynthetic pathway has recently emerged from studies of a marine diatom (*Thalassiosira weissflogii*) (Reinfelder *et al.* 2000; 2004; Morel *et al.* 2002). It has been proposed that HCO_3^- transported into the cell combines with phosphoenolpyruvate (PEP) in the cytoplasm, in a reaction catalyzed by PEP carboxylase (PEPC), to form the C_4 compound oxaloacetic acid (OAA). The OAA is reduced to malate, and malate is then transported into the chloroplast and decarboxylated (via PEP carboxykinase) to regenerate pyruvate and release CO_2 that can then be fixed by RubisCO. Thus, inorganic carbon is concentrated as a C_4 organic acid that is less likely to diffuse back out of the cell than CO_2. Labeling studies have demonstrated that the C_4 pathway is

important in carbon accumulation and photosynthetic carbon fixation in diatoms at low levels of CO_2 (Reinfelder *et al.* 2004).

1.6 Review of Carbon Concentrating Mechanisms across taxa:

To date, laboratory studies of carbon acquisition in phytoplankton have focused mainly on a few model species of cyanobacteria and green algae (*see* review by Kaplan & Reinhold 1999). Comparatively, much less is known about other ecologically significant groups of marine phytoplankton, such as diatoms. Evidence for direct active uptake of both CO_2 and HCO_3^- exists for marine and freshwater cyanobacteria. These organisms may use at least four modes of active DIC uptake: two HCO_3^- transporters and two CO_2 uptake systems, although some cyanobaceria ie: *Prochlorococcus marinus* appear to lack CO_2 uptake systems entirely (*reviewed by* Espie *et al.* 1991; Miller *et al.* 1991; Badger & Price 2003). While no external CA activity has been detected in cyanobacteria, evidence suggests that the HCO_3^- transporter itself may contain a transmembrane CA-like subunit (Price & Badger 1989).

Both direct and indirect mechanisms of HCO_3^- uptake have been documented in green algae (reviewed in Sültemeyer *et al.* 1993; Raven 1997; Amoroso *et al.* 1998; Heurtas *et al.* 2000a; Young *et al.* 2001; Pollock & Colman 2001), in several species of Prymnesiophytes (Nimer *et al.* 1997; Elzenga *et al.* 2000; Bhatti *et al.* 2002; Heurtas *et al.* 2003) and in a few species of dinoflagellates (Nimer *et al.* 1997; *but see* Dason *et al.* 2004).

While diatoms contribute disproportionately to global carbon and silica biogeochemical cycles (Eppley & Peterson 1979; Sarmiento & LeQuere 1996), carbon acquisition strategies in these microalgae are relatively unknown. Evidence from laboratory studies suggests that HCO_3^- utilization is also predominant in this group, although there appears to be considerable variability in carbon acquisition strategies between species and even for one single species, depending on external conditions. Several studies have presented indirect evidence for HCO_3^- transport in diatoms (Burns & Beardall 1987;Colman & Rotatore 1988, 1995; Dixon & Merrett 1988). More direct evidence exists from measurements of CA-catalyzed HCO_3^- dehydration at the cell surface coupled to a CO_2 transport system (Rotatore & Colman 1992; Rotatore *et al.* 1995; Nimer *et al.* 1997; Sültemeyer *et al.* 1998; Rost *et al.* 2003) and from carbon uptake studies (Korb *et al.* 1997; Elzenga *et al.* 2000; Burkhardt *et al.* 2001; Morel *et al.* 2002).

1.7 Limiting nutrients and dissolved inorganic carbon acquisition:

Phytoplankton must expend considerable resources to acquire inorganic carbon. For example, energy is required for the active transport of carbon into cells, thus low light may impair the ability of phytoplankton to actively concentrate carbon. Cells that produce carbonic anhydrase may have increased trace metal requirements such as zinc, cobalt and cadmium (Coleman 1998; Lane & Morel 2000a, 2000b) because these metals are cofactors in this enzyme. Indeed, laboratory studies have shown an interaction between zinc and CO_2 limitation

mediated through carbonic anhydrase (Morel *et al.* 1994). Since RubisCO represents a large nitrogen sink for the cell, accounting for up to 30% of cellular protein (Ellis 1979), the availability of nitrogen may affect the efficiency of cellular carbon fixation. In summary, the ability of each species to obtain and use DIC may depend on the availability of other potentially limiting resources such as light, trace metals and nitrogen (Raven & Johnston 1991; Beardall *et al.* 1998).

1.8 Atmospheric CO_2 rise – ecological implications:

If resource availability does affect inorganic carbon acquisition, the physiological mechanism of carbon acquisition should influence the growth response of natural phytoplankton assemblages to changing levels of CO_2. It is expected that by the end of the next century, we will observe a doubling of surface ocean CO_2 levels (from current partial pressure values of ~360ppm up to ~750ppm; Houghton *et al.* 1995). The implications of this predicted increase in CO_2 concentration for the functioning and productivity of marine ecosystems are unknown. However, preliminary studies have shown that shifts in phytoplankton community composition may occur with changes in CO_2 concentration (Tortell *et al.* 2002). Field incubation experiments in an Equatorial Pacific phytoplankton assemblage revealed that the relative abundance of diatoms decreased significantly under low [CO_2] relative to high [CO_2] while the abundance of *Phaeocystis*, a colonial prymnesiophyte, increased at low [CO_2] (Tortell *et al.* 2002). While this evidence suggests that certain phytoplankton taxa may be better adapted for survival in low CO_2 conditions than others, the nature of this

competitive edge remains unclear. Factors that influence phytoplankton species distribution and succession are important because the relative contributions of different phytoplankton species or groups (ie: silicifying vs calcifying phytoplankton) to the carbon pump are highly variable (Rost *et al.* 2003).

1.9 Study organism:

Thalassiosira weissflogii is a particularly attractive model diatom as it is easy to grow in the lab, specifically in stressful chemostat culture conditions (Li & Goldman 1981). Previous studies of DIC uptake strategies in this species provide evidence of highly efficient uptake systems for both CO_2 and HCO_3^- (Nimer *et al.* 1997; Lane & Morel 2000a; Burkhardt *et al.* 2001; Morel *et al.* 2002). More controversial evidence of a C_4 photosynthetic pathway (Reinfelder *et al.* 2000; 2004; Morel *et al.* 2002) has been presented for this diatom. However, other than studies of the impact of zinc on carbon uptake in *T. weissflogii* (Morel *et al.* 1994), there is overall little information on the impacts of other resources on microalgal CCMs.

1.10 Field Studies:

In addition to physiological studies of carbon uptake strategies employed by model species in the laboratory, field studies are also important. However, field research is often expensive, time consuming and associated with many logistical challenges. Additionally, it can be difficult to interpret experimental results since natural phytoplankton assemblages are composed of many different

species that experience considerable variation in growth conditions (ie: light levels, CO_2 and nutrient concentrations). As a result of these complexities, there have been relatively few field studies investigating the pathways of inorganic carbon assimilation and fixation in natural plankton communities (Berman-Frank *et al.* 1994, 1996, 1998; Tortell *et al.*1997, 2002; Tortell & Morel 2002; Cassar *et al.* 2004). Although regular fluctuations in nutrient and CO_2 concentrations do occur in natural coastal systems, the specific impacts of resource limitation on carbon uptake mechanisms are not well understood. Despite the difficulties associated with field work, understanding the mechanisms of carbon uptake occurring in the field is important as it is these natural mixes of phytoplankton that are controlling primary productivity in the oceans.

1.11 Thesis Objectives:

Specifically, my research goals were to:

1) Investigate the effect of nitrogen limitation on activities of RubsiCO and PEPC in *Thalassiosira weissflogii.*

2) Investigate the effect of nitrogen limitation on the species of dissolved inorganic carbon taken up by *Thalassiosira weissflogii* (ie: HCO_3^- vs. CO_2 contribution).

3) Observe activities of RubisCO and PEPC across a nitrogen gradient in the Queen Charlotte Sound coastal region.

4) Investigate the source of inorganic carbon utilized by phytoplankton assemblages in the Queen Charlotte Sound coastal region.

Chapter 2

Effects of nitrogen limitation on inorganic carbon concentrating mechanisms in *Thalassiosira weissflogii*

2.1 Introduction

Laboratory studies have provided evidence that the growth rate of large marine phytoplankton, particularly diatoms, can be limited by CO_2 diffusion (Riebesell *et al.* 1993). This finding has been attributed to the catalytic inefficiency of Ribulose-1,5-bisphosphate carboxylase / oxygenase (RubisCO), which is only able to fix inorganic carbon in the form of CO_2 (Cooper *et al.* 1969). Despite the potential for carbon limitation of marine phytoplankton growth, these organisms are able to maintain rapid growth rates under low oceanic CO_2 concentrations with the use of carbon concentrating mechanisms (CCMs) (Canvin 1990; Raven 1997; Badger *et al.*1998; Kaplan & Reinhold 1999). Phytoplankton may actively transport HCO_3^- (and/or CO_2) from the external medium directly into the cell or employ the enzyme carbonic anhydrase (CA) to catalyze the conversion of HCO_3^- to CO_2, with CO_2 entering the cell (Sültemeyer 1998). Additionally, species may use HCO_3^- in a C_4 photosynthetic pathway that functions as an intracellular component of the CCM (Reinfelder *et al.* 2000; 2004; Morel *et al.* 2002).

2.1.1 Limiting resources – implications for DIC acquisition:

Phytoplankton must expend considerable resources in order to operate CCMs. For example, energy is required for the active transport of inorganic carbon into cells (Raven & Lucas 1985), thus low levels of PAR (photosynthetically active radiation: 400 to 700nM) may impair the ability of phytoplankton to actively concentrate carbon. The long-term effects of acclimation to growth-limiting irradience on the function of the CCM have been investigated in a cyanobacterium (Beardall 1991) and in a few species of macroalgae (Kübler & Raven 1994, 1995). More recently, Young and Beardall (2004) demonstrated that CCM investment in the microalgae *Dunaliella tertiolecta* was limited by sub-saturating irradiance during growth.

Even under high photon flux densities, photosynthetic energy harvesting can be compromised by macronutrient and iron deficiency (Martin *et al.*1989; Greene *et al.* 1992; Sunda & Huntsman 1997; Geider *et al.* 1998; Young & Beardall 2003). Cells that produce carbonic anhydrase may have increased trace metal requirements such as zinc, cobalt and cadmium (Coleman 1998; Lane & Morel 2000a, 2000b) because these metals are cofactors in this enzyme. Indeed, laboratory studies have shown an interaction between zinc and CO_2 limitation mediated through carbonic anhydrase (Morel *et al.* 1994). Also, since RubisCO represents a large nitrogen sink for the cell, the availability of nitrogen may affect the efficiency of cellular carbon fixation. Understanding how the availability of other potentially limiting resources affects the ability of each species to obtain

and use DIC is necessary in order to appreciate the ecological relevance of CCMs in natural phytoplankton populations.

2.1.2 Carbon / nitrogen interactions:

Since carbon fixation is expensive in terms of nitrogen due to the high nitrogen requirements associated with RubisCO, there is the potential for carbon-nitrogen interactions. It is firmly established that nitrogen deficiency causes a decrease in photosynthetic capacity through effects on both the enzymatic component of the photosynthetic apparatus and light-harvesting pigment protein complexes (Eppley & Renger 1974; *see* review by Turpin 1991; Geider *et al.* 1998; Keller *et al.* 1999). The negative impact of nitrogen limitation on the cellular content and activity of RubisCO is also well documented (Lapointe & Duke 1984; Falkowski *et al.*1989; Beardall *et al.* 1991; Geider *et al.* 1998). Although it has been suggested that CCM activity may improve nitrogen-use efficiency by increasing the CO_2 concentration at the active site of RubisCO (Raven & Johnston 1991), the effects of nitrogen limitation on regulation of CCM function are not well understood (Beardall *et al.* 1982; 1991; Beardall & Giordiano 2002; Spalding *et al.* 2002; Young & Beardall 2004).

2.1.3 Study Goals and Hypotheses:

The main goal of this research was to investigate the effects of nitrogen limitation on CCM function in the diatom *Thalassiosira weissflogii* grown under ambient CO_2 concentrations. Measurements of RubisCO and PEPC activities

were made using cells cultured under varying degrees of nitrogen limitation. In addition, ^{14}C uptake experiments were used to quantify the CO_2 vs. HCO_3^- contribution to photosynthesis. My main hypothesis was that CCM function would be upregulated under increasing levels of nitrogen limitation. Specifically, I expected to observe a decrease in RubisCO activity, but an increase in PEPC and extracellular carbonic anhydrase activity and direct HCO_3^- transport with decreasing nitrogen-limited growth rate.

2.2 Materials and Methods

2.2.1 Culture Conditions:

The diatom *Thalassiosira weissflogii* [(Grunow) G. Fryxell et Hasle 1977] obtained from the Canadian Center for the Culture of Microorganisms (Isolate ACTIN, CCMP1336 from Gardiner's Island, NY) was cultured in Aquil / synthetic ocean water medium with *f*/2 concentrations of trace elements and vitamins (Price *et al.* 1988/89: Appendix A) at a temperature of ~21±1°C. The cultures were illuminated continuously with ~200μmol photons·m^{-2}·s^{-1}. Only milli-Q water (18.2 Ω) was used in the preparation of the culture medium and all medium carboys, culture vessels and tubing were acid washed and sterilized using both autoclave and microwave methods. In non-aerated, unstirred nutrient-replete batch cultures, cells were harvested during the exponential phase of growth when cell density was ~ 4×10^4 cells·ml^{-1}. Growth rates (μ, day^{-1}) were calculated as the slope of the natural log of fluorescence (*in vivo* chlorophyll) values over time (Brand & Guillard 1981).

Nitrogen-limited cells were obtained using a continuous chemostat culture method. In a common batch culture, it is impossible to separate the effects of an overall deterioration in cell metabolism occurring in an aging culture from the effects of nutrient limitation. In a continuous chemostat culture, however, the effects of nutrient (ie: nitrogen) limitation on photosynthesis can be studied without the compounding variables inherent in batch culture experiments (Li & Goldman 1981). The chemostat is a flow-through system in which individual cells are suspended in a constant volume at (or near) a steady state of growth established by the continuous flow of fresh (nitrogen-containing) media into the culture vessel, and the continual removal of part of the culture. In this way cells are maintained at exponential growth rates under constant and limiting concentrations of nitrogen.

Continuous chemostat cultures were grown in 1-L glass side-arm flasks, stirred with a Teflon coated stir bar and constantly aerated with H_2SO_4 scrubbed, water-saturated air passed through a 0.2μm Acrodisc filter capsule. Initial culture medium contained 50μM of $NaNO_3$, 10μM of $NaH_2PO_4 \cdot H_2O$ and 100μM of $Na_2SiO_3 \cdot 9H_2O$; fresh media was delivered continuously to the culture vessel at pre-set dilution rates controlled by multi-channel Manostat peristaltic pumps. Constant volume within the culture vessel was maintained via an overflow arm as new media was introduced. Dilution rates (D) were calculated using daily estimates of the volume of effluent collected (E) and total culture volume (V) where D = E/V. Cultures were monitored daily (using overflow samples) for fluorescence (Fluorometer: Turner 10-AU), cell abundance and cell size (Coulter

particle count and size analyzer: Model Z2) to ascertain steady-state conditions. Nitrate concentrations were measured regularly using a Nitric Oxide Analyzer (Antek: Model 7020). Chemostats were sacrificed for experiments after cultures had maintained steady-state growth (determined via stable fluorescence and cell density ± 15%) for a minimum of five generation times. Growth rates in steady-state chemostat cultures were assumed to equal the dilution rates (Li & Goldman 1981). Two independent chemostat trials were conducted: the first trial took place over a six month period while the second trial was completed over the span of two months. In trial one, the number of experimental replicates at each growth rate is inconsistent due to chemostat complications such as inconsistent flow rates caused by air bubbles in tubing and a power outage in the culture incubator. There were two experimental replicates completed for each growth rate in trial two.

2.2.2 Physiological Assays:

a) Particulate Organic Carbon and Particulate Organic Nitrogen: A 20ml sample from log phase batch culture or sacrificed chemostat was filtered through a 15mm combusted (450°C for 4.5 h) glass-fiber filter (GF/F nominal pore size 0.7μm). The filters were dried at 55°C for a minimum of 24 h and analyzed using a Carlo Erba NCS elemental analyzer NA1500. Both filter and container blanks were subtracted from the samples (UC Davis Facility; Maureen Soon, UBC).

b) RuBP-carboxylase / PEP-carboxylase activity: 250ml of log-phase batch or chemostat culture was concentrated onto a 25mm glass-fiber filter (GF/A nominal pore size 1.6μm) and immediately resuspended into 4ml of ice-cold extraction buffer (modified from MacIntyre & Geider 1996: Appendix B). Cells were disrupted by homogenization for 3×40sec periods in an ice-bath. Crude cell extracts were clarified using a microcentrifuge at 14 000×g for 2×10s, and the supernatants were retained for enzyme assays.

Enzyme activities were determined by RubisCO / PEP-dependent ^{14}C fixation (assay modified from Smith *et al.* 1983; MacIntyre & Geider 1996; Hatch *et al.* 1978). Seven 200μl aliquots of cell homogenate were sampled into microtubes: 3 each for RuBP and PEP plus 1blank. Following a pre-incubation period of 15 minutes at 25°C in the dark, the reaction was initiated by the addition of either 20μl of RuBP stock (23mM) or 20μl of PEP stock (50mM). RuBP and PEP stock were omitted for the blanks. After samples were incubated for 3 minutes on the bench, 5μl of 50μCi/mL $NaH^{14}CO_3$ solution was added. Reactions were stopped after 30 minutes by the addition of 100μL of 6N HCl, and samples were left shaking in a fume hood overnight to drive off any unfixed ^{14}C. Degassed samples were transferred to 7ml scintillation vials and 5ml scintillation cocktail (Fisher Scintisafe 50%) was added. After mixing, the ^{14}C activity in samples was measured by standard liquid scintillation procedures (Beckman Coulter LS 6500 Scintillation Counter). ^{14}C counts (disintegrations per minute) were normalized to total protein in samples. Protein was measured using a Bio-Rad® Protein Assay.

c) HCO_3^- VS CO_2 Uptake - Isotope disequilibrium technique: To examine inorganic carbon uptake by nitrogen-limited *T.weissflogii* cells during steady state photosynthesis, the isotope disequilibrium technique described by Elzenga *et al.* (2000) was employed with several minor modifications (*see* Tortell & Morel 2002):

500ml of steady-state nitrogen-limited chemostat culture was concentrated onto a 5μm polycarbonate filter and re-suspended into 14ml Bicine seawater buffer (20mM Bicine, pH 8.5 ± 0.05). The cell suspension was stored in a 15°C water bath until used. 2.5ml of concentrated cell suspension (~ 2×10^6 cells·ml^{-1}) was acclimated at 15°C for 4 minutes in a rapidly stirred, thermostated O_2 electrode chamber. Cell suspensions were continually mixed with a Teflon-coated magnetic stir bar and illuminated by a Kodak Carousel 4200 slide-projector bulb with a photon flux of ~400μE·m^{-2}·s^{-1}. Radiolabeled carbon uptake was initiated by injecting 10μCi ^{14}C solution into the electrode chamber. Immediately prior to experiments the ^{14}C spike solution was prepared by diluting 10μCi primary isotope stock (*NEN Dupont:*1μCi·μl^{-1} ; ~50mCi·$mmol^{-1}$) into 90μl of pH 7.0 (± 0.05) 50mM HEPES buffer. 200μl samples were removed from the electrode chamber at intervals between 5 and 180 seconds and rapidly dispensed into scintillation vials containing ml 50% HCl (~6N) in order to track the time course of cellular ^{14}C incorporation into organic matter. The acid solution immediately stopped photosynthesis, converting all inorganic carbon to CO_2. Samples were left to degas on a shaker table overnight in a fume hood in order to remove all unfixed carbon. After degassing was complete, 5ml scintillation

cocktail (Fisher Scintisafe 50%) was added to each vial, and the ^{14}C activity in samples was measured using liquid scintillation procedures. Blank samples, consisting of ^{14}C added to bicine buffer without cells, were also run in order to correct for small amounts of residual inorganic carbon present in samples. All experiments were run in duplicate.

To analyze the results of isotope disequilibrium experiments, a model fit was used to extract HCO_3^- / CO_2 uptake rates and external carbonic anhydrase activity from the ^{14}C time course data. Equations given by Elzenga *et al.* (2000) were fit to data by non-linear regression using Sigma Plot:

$$\mathbf{DPM_t} = \underbrace{Vt*(1-f)/a*(a*t+49*(1-\exp(a*t)))}_{\mathbf{CO_2\ contribution}} + \underbrace{Vt*f/(a*1.16)*((a*1.16)*t-.24*(1-\exp(-(a*1.16)*t)))}_{\mathbf{HCO_3^-\ contribution}}$$

Where:

DPM_t = fixed radioactivity at time (t)
Vt = total rate of fixation of carbon in acid stable compounds
f = fraction of HCO_3^- taken up by the cells
1- *f* = fraction of CO_2 taken up by the cells
a = rate constant of equilibration (~carbonic anhydrase activity)

2.3 Results

2.3.1 Growth Rate and Biochemical Parameters:

Table 2.1: Comparison of biochemical parameters between different nitrogen-limited chemostat culture growth rates and chemostat vs. batch culture cells ± standard error.

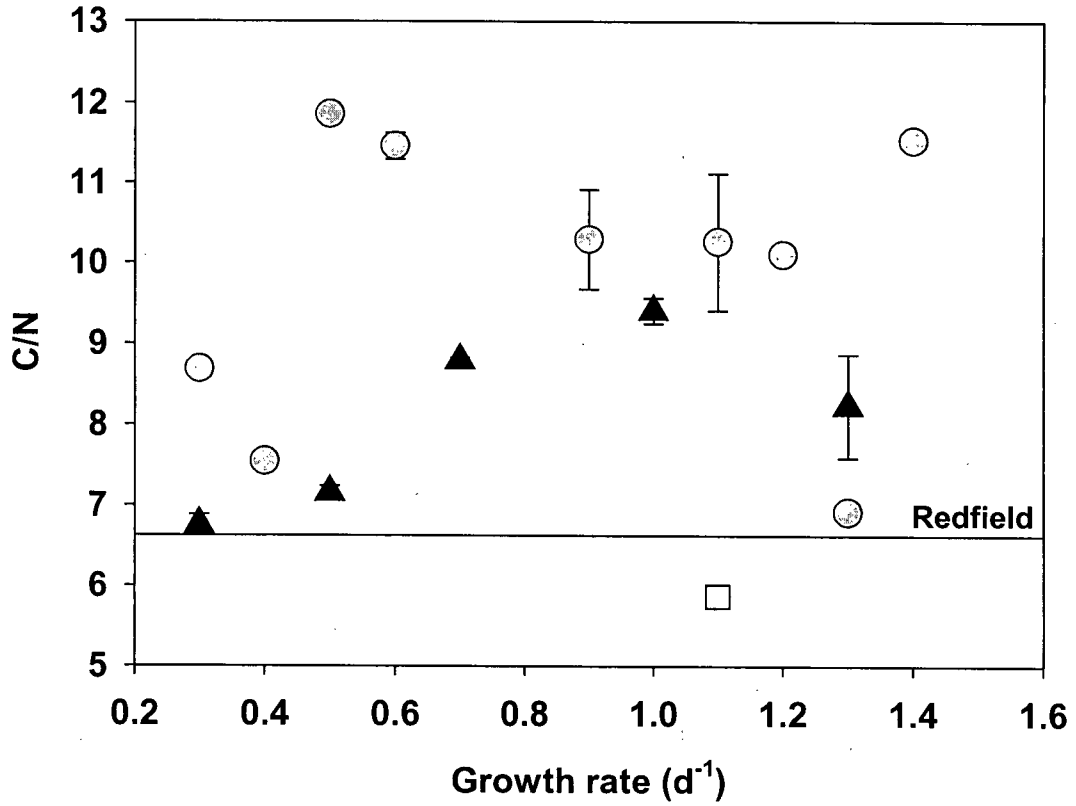
Parameter	Growth-rate dependence ($p<0.05$)	N-limited chemostat cells	Batch-cultures	P value chemostat vs. batch cells
Residual NO_3^- (μM)	No	0.53±0.21	N/A	N/A
Fluor / Cell Density ratio ($\times 10^{-5}$)	Yes ($p<0.001$)	8.71±2.55	62.2±13.0	$p<0.001$
Cell Volume ($fl \cdot cell^{-1}$)	No	82.51±5.58	132.47±1.18	$p<0.001$
C/N ratio	No	7.81±0.745	5.87±0.047	$p<0.05$

Several biochemical parameters provided evidence that the steady-state growth of chemostat-cultured cells was in fact limited by nitrogen supply (Table 2.1). Measurements made using the Nitric Oxide Analyzer confirmed that less than 1μM residual nitrate remained in all steady-state chemostat cultures, irrespective of growth rate. By comparison, autoanalyzer measurements (performed by Chris Payne, UBC) confirmed that other potentially limiting nutrients (ie: phosphorous and silica) were present in excess quantities ([P] = 2.6μM ± 0.29; [Si] = 86.2μM ± 3.3). There was no significant change in cell volume or C/N ratio (Fig 2.1) between growth rates. However the steady-state ratio of fluorescence to cell density decreased more than two-fold between the highest ($1.4 \cdot d^{-1}$) and lowest ($0.3 \cdot d^{-1}$) growth rates.

Unstirred, non-aerated batch cultures had significantly higher fluorescence/cell density ratios and larger cell volumes than nitrogen-limited

chemostat cells. The C/N ratio in batch-cultured cells was significantly lower than that observed in chemostat-grown cells over the range of growth rates tested (Fig 2.1). C/N ratios of chemostat cells were also elevated with respect to the Redfield ratio, a representative of the average elemental composition of marine phytoplankton (Redfield 1934).

Figure 2.1: Effect of N-limited growth rate on C/N ratios in *T. weissflogii*. Results presented for two separate experimental chemostat trials (circles = trial 1; triangles = trial 2) and for independent batch culture experiments (square) ± standard error.

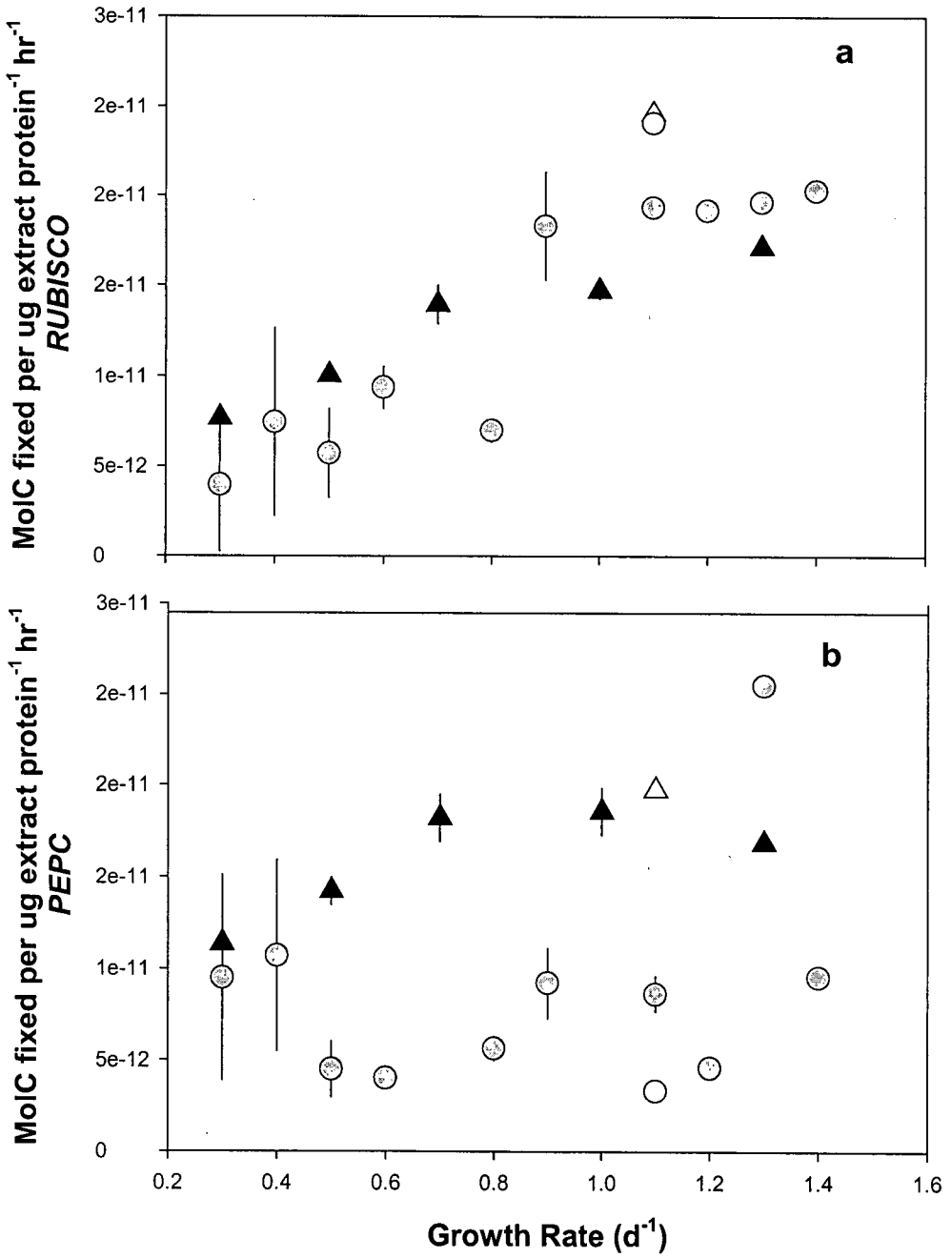


2.3.2 RuBP-carboxylase / PEP-carboxylase activity:

There was a significant correlation between RubisCO activity and growth rate ($r=0.821$; $p<0.001$) in the chemostat cultures (Fig 2.2a). Results demonstrate a 5-fold decrease in activity from highest to lowest nitrogen-supply rate ($1.4 \cdot d^{-1}$ to $0.3 \cdot d^{-1}$), and activities appeared to plateau at growth rates above ~$1.1 \cdot d^{-1}$. Non-aerated, unstirred batch cultured cells (growth rate ~$1.1 \cdot d^{-1}$) had higher RubisCO activities than chemostat-cultured cells at an equivalent growth rate.

While RubisCO activity was strongly correlated with growth rate, there appeared to be no such trend in PEPC activity (Fig 2.2b). PEPC activity remained relatively unchanged throughout the range of growth rates tested. Although PEPC activity did not appear to be regulated by nitrogen-supply, activities across all flow rates were maintained at rates comparable to those of RubisCO.

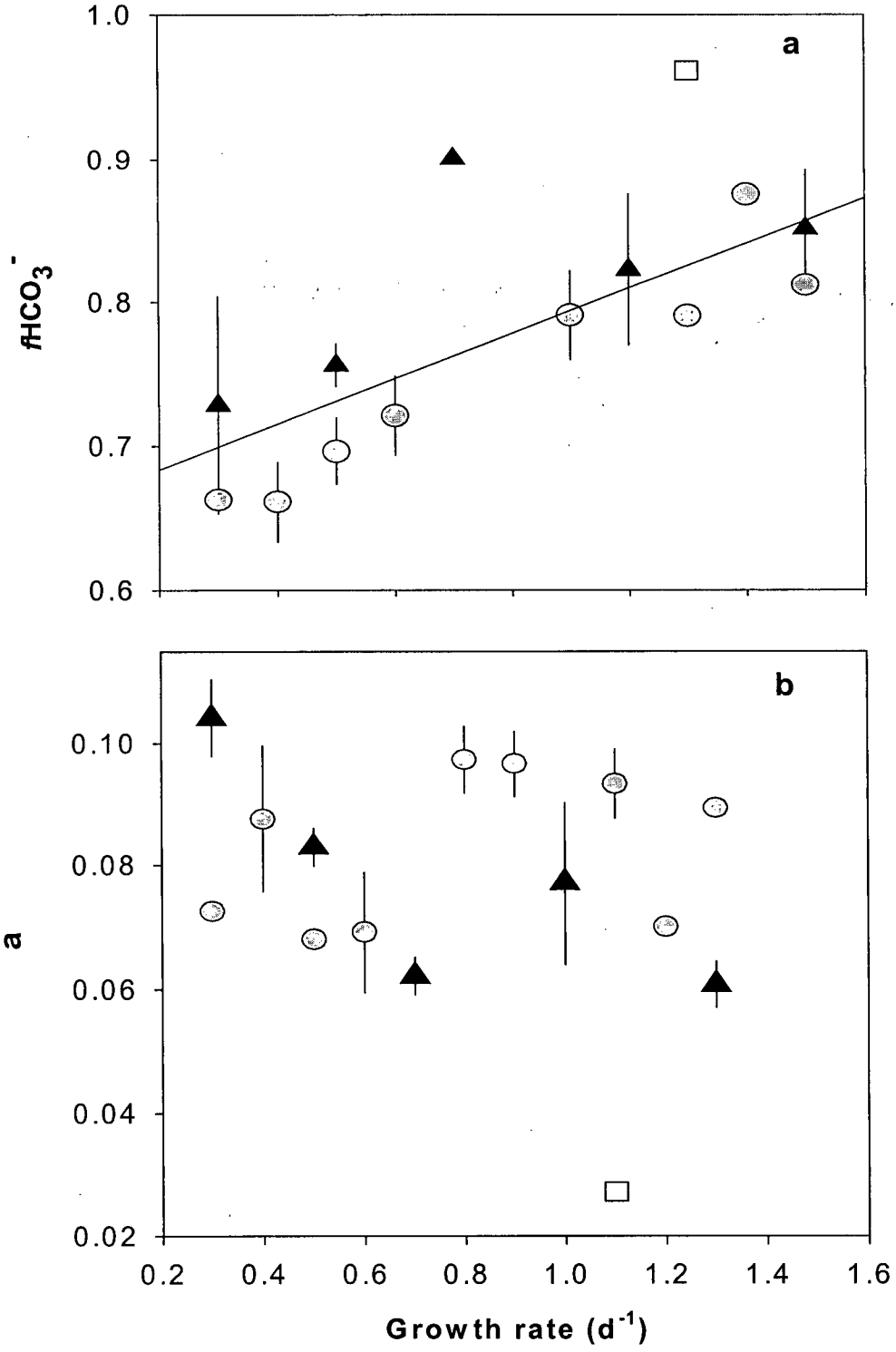
Figure 2.2: Effect of N-limited growth rate on a) RubisCO (r=0.821; p<0.001) and b) PEPC activities in *T. weissflogii.* Results presented for two separate experimental trials (closed circles = trial 1; closed triangles = trial 2) and for independent batch-culture experiments (trial 1 = open circles; trial 2 = open triangles) ± standard error.



2.3.3 HCO_3^- vs. CO_2 Uptake:

There was a significant correlation between $fHCO_3^-$ (the fraction of HCO_3^- taken up by the cells) and growth rate ($r = 0.682$; $p<0.001$), with $fHCO_3^-$ values decreasing from ~0.85 – 0.65 as growth rate decreased from 1.4 to 0.3·d^{-1} (Fig 2.3a). Non-stirred, unaerated batch cultures had $fHCO_3^-$ values ~10% greater than chemostat cultured cells at equivalent growth rates (~1.1·d^{-1}). Values of ***a*** (analogous to carbonic anhydrase activity) were not correlated with growth rate, and carbonic anhydrase activities at all growth rates were elevated relative to batch cultured cells (Fig 2.3b).

Figure 2.3: Effect of N-limited growth rate on a) $f HCO_3^-$ (r=0.682; p<0.001) and b) **a** (~CA activity) in *T. weissflogii.* Results presented for two separate experimental trials (circles = trial 1; triangles = trial 2) and for independent batch-culture experiments (open squares) ± standard error.



2.4 Discussion

2.4.1 Biochemical parameters:

When algal growth is limited by nitrogen availability, cells typically exhibit elevated C/N ratios, decreased levels of chlorophyll, nitrogen and protein per cell and decreased cellular rates of photosynthesis and fluorescence yield (Eppley & Renger 1974; *see* review by Turpin 1991; Geider *et al.* 1998; Keller *et al.* 1999). While C/N ratios in my chemostat-cultured cells were elevated relative both to the Redfield ratio and to the batch culture samples, I did not observe the linear increase in C/N with decreasing nitrogen supply as I would have expected. Neither the amount of $C \cdot cell^{-1}$ nor $N \cdot cell^{-1}$ was significantly affected by growth rate in my chemostat cultures. While stable values of $C \cdot cell^{-1}$ with decreasing growth rate are consistent with the literature, the fact that I did not see a decrease in $N \cdot cell^{-1}$ is puzzling. These C/N ratios should be interpreted with caution as the measured values of total POC and PON on my filters were at or near the threshold detection limit of the instruments. A more rigorous sampling protocol, including the collection of more cell biomass on filters, may help to clarify this result.

2.4.2 Regulation of light-saturated photosynthesis – RubisCO:

RubisCO accounts for a major portion of total cell protein, and my results suggest that RubisCO activity in *T.weissflogii* is strongly regulated by nitrogen supply or, alternatively, by growth rate. This finding is consistent with previous studies that have shown a decrease in both cellular RubisCO content and/or

activity under nitrogen-limitation (Beardall *et al.*1982; Lapointe & Duke 84; Osborne & Geider 1986; Falkowski *et al.* 1989; Plumley & Schmidt 1989; Beardall *et al.* 1991; Geider *et al.* 1998). I did not quantify the amount of RubisCO present in my nitrogen-limited chemostat cells, so the assumption that observed changes in RubisCO activity represent changes in the amount of the enzyme present presumes that activation and deactivation of the enzyme is not regulating the rate of photosynthesis at saturating irradiences (MacIntyre *et al.*1997; Geider *et al.* 1998). The relationship between enzyme activity and protein content is complicated by the fact that large pools of RubisCO are catalytically inactive (LaCoste-Royale & Gibbs 1987; McKay & Gibbs 1991; Badger et al 1998), possibly due to the role of this enzyme as a nitrogen store (Ekman *et al.* 1989; Falkowski *et al.* 1989; Plumley & Schmidt 1989; Geider *et al.* 1993; Garcia-Ferris & Moreno 1994). Determination of the quantity of RubisCO present using the Western blot technique in addition to enzyme activity would help to clarify this result for *T. weissflogii.*

2.4.3 CCMs and nitrogen-use efficiency:

Although carbon and nitrogen metabolism are tightly coupled through RubsiCO, the relationship between activity of the CCM and nitrogen-limited growth rate is not well understood. It has been proposed that CCMs may increase cellular nitrogen-use efficiency (rate of CO_2 fixation per unit of nitrogen in RubisCO) by increasing the concentration of CO_2 next to the active site of RubisCO, thereby decreasing the enzyme's wasteful photorespiratory reactions

(Raven & Johnston 1991; Beardall *et al.* 1982, 1991). Spalding *et al.* (2002) report a transiently decreased expression of both small and large subunits of RubisCO when the CCM is induced under low CO_2 conditions, and an enhanced affinity for DIC with increasing nitrogen-stress has recently been demonstrated in *Dunaliella tertiolecta* (Young & Beardall 2004). However, earlier studies on the response of the CCM to nitrogen-limitation have produced mixed results, including an apparent decrease in affinity for CO_2 in two species of green microalgae (Beardall *et al.*1982, 1991; Beardall & Giordano 2002).

My results do not provide evidence of an upregulation of CCM activity under increasing nitrogen-stress in *T. weissflogii*. While PEPC activity was maintained at high levels (comparable to those of RubisCO) throughout chemostat cultures at all growth rates, there was no apparent regulation of PEPC activity with growth rate. This suggests that the C_4 photosynthetic pathway is not directly influenced by nitrogen supply. In contrast, data obtained from ^{14}C uptake experiments illustrated that while HCO_3^- uptake accounted for a substantial proportion (more than 65%) of the total inorganic carbon taken up even at the lowest growth rates, the HCO_3^- contribution in fact decreased as cells became more severely nitrogen-limited or, alternatively, as their growth rates decreased. The absence of a corresponding decline in carbonic anhydrase activity (implied from values of ***a***) established that the observed decrease in $fHCO_3^-$ was attributed to a decrease in direct HCO_3^- transport. It is possible that this reduction in direct HCO_3^- uptake may be due to the decreased carbon demand of cells that are growing more slowly.

2.4.4 Summary:

In summary, these results suggest that as nitrogen supply and RubisCO activity decreases, CCM activity in *T. weissflogii* remains unchanged with respect to the function of the internal C_4 photosynthetic pathway, but is actually down-regulated with respect to direct HCO_3^- transport from the outside medium. Since carbon-fixation rates associated with slower growth will create a lower demand for net DIC-uptake, it is possible that nutrient acquisition mechanisms, including CCMs, may be co-regulated in order to control elemental ratios and maintain metabolic balance during periods of nutrient stress. Investigating the effects of phosphate limitation on the function of the CCM may help to distinguish the impacts of decreased nitrogen-supply from growth rate effects.

Chapter 3

Inorganic carbon acquisition in Queen Charlotte Sound phytoplankton assemblages

3.1 Introduction

Despite the established importance of marine phytoplankton in the global carbon cycle, the physiological mechanisms of dissolved inorganic carbon (DIC) acquisition in phytoplankton remain poorly understood. In particular, the extent to which phytoplankton utilize CO_2 vs. HCO_3^- as sources of inorganic carbon for photosynthesis, and active vs. diffusion-based mechanisms of DIC uptake is currently debated. Virtually all studies of carbon acquisition in phytoplankton to date have focused on a handful of model organisms grown in monospecific, nutrient-replete laboratory batch cultures (ie: Korb *et al.* 1997; Sültemeyer *et al.* 1998; Elzenga *et al.* 2000; Burkhardt *et al.* 2001; Morel *et al.* 2002; Rost *et al.* 2003). While these controlled studies help us to understand the complex physiological processes involved in carbon acquisition at a cellular level, the results do not necessarily explain processes occurring in complex natural phytoplankton assemblages.

3.1.1 Challenges associated with field studies:

As a result of the complex and logistically challenging nature of field work, the pathways of inorganic carbon assimilation and fixation in natural phytoplankton assemblages remain poorly studied (Berman-Frank *et al.* 1994, 1996, 1998; Tortell *et al.*1997, 2000; Tortell & Morel 2002; Cassar *et al.* 2004).

Oceanographic field studies are particularly difficult as they are often extremely expensive, time consuming and hampered by unfavorable weather conditions that may interfere with or prohibit sampling altogether. Experimental procedures that are easily carried out in a laboratory are often logistically difficult to reproduce at sea. For example, the low biomass of natural phytoplankton assemblages can make it difficult to obtain enough cells to perform experiments or make measurements. Interpretation of experimental results can be challenging since natural phytoplankton assemblages are composed of many different species whose carbon uptake systems have not yet been studied. Furthermore, these communities experience considerable variation in growth conditions (ie: light levels, CO_2 and nutrient concentrations) and the specific impacts of resource limitation on carbon uptake mechanisms are not well understood.

3.1.2 Importance of field studies:

Despite their inherent limitations, field experiments are important because it is natural mixes of phytoplankton that are controlling primary productivity in the oceans. Coastal marine waters experience regular fluctuations in nutrient and CO_2 concentrations that can influence phytoplankton growth. In the lab I examined the function of the carbon concentrating mechanism over a range of nitrogen concentrations in one species of marine diatom. This complementary field study is a preliminary step to examine carbon uptake in natural coastal phytoplankton assemblages over a nitrogen gradient.

3.1.3 Study Goals and Hypotheses:

The goals of this field study of phytoplankton assemblages in the Queen Charlotte Sound coastal region were to:

1) Examine trends in activities of enzymes involved in dissolved inorganic carbon assimilation (RubisCO and PEPC) across a nitrogen gradient.

2) Investigate the source of dissolved inorganic carbon utilized by phytoplankton assemblages.

My main hypothesis was that CCM function would be upregulated in regions where nitrogen levels were low. Specifically, I expected to observe a decrease in RubisCO activity, but an increase in PEPC and extracellular carbonic anhydrase activity and direct HCO_3^- transport in those phytoplankton assemblages found in areas with low levels of nitrate as compared to those assemblages growing in areas with high nitrate concentrations.

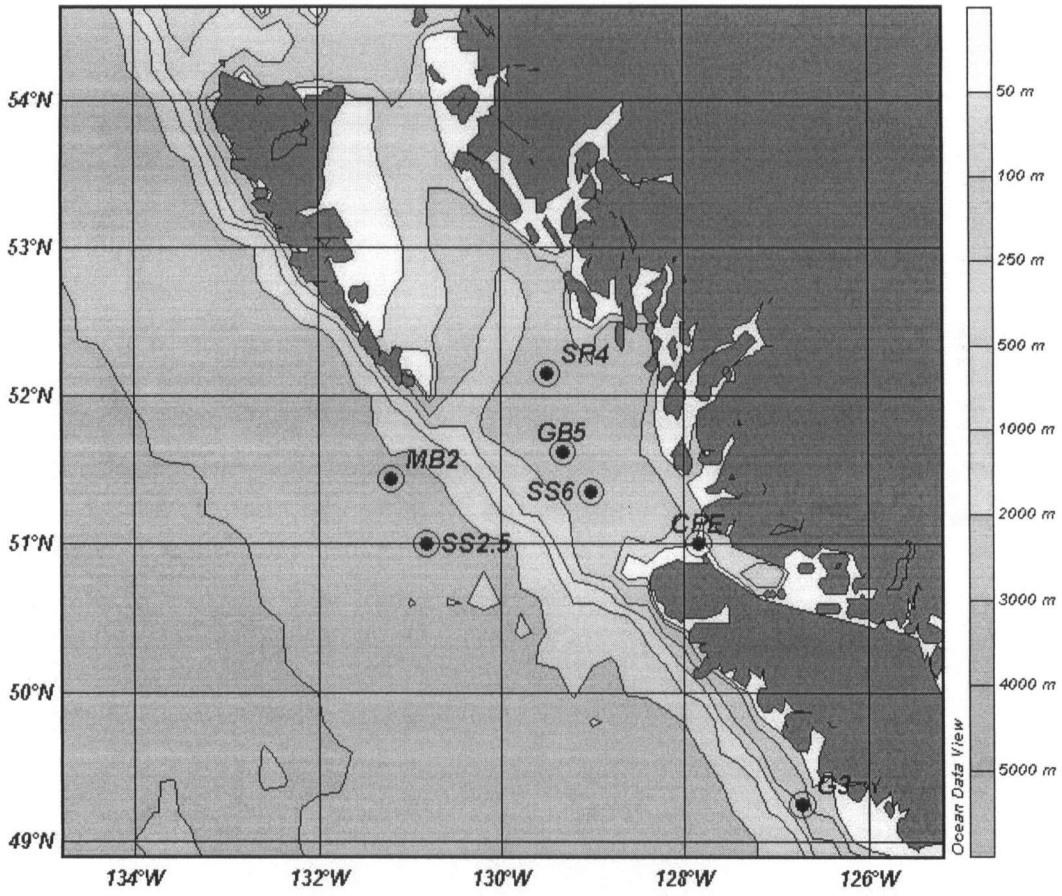
3.2 Material and Methods

3.2.1 Field site:

Field sampling was conducted from August 11–19 of 2004 in coastal waters of the Queen Charlotte Sound aboard the CCGS John P. Tully. Sampling sites were located along two cross-shelf transects extending into offshore waters and along-shore following the shelf break (Fig 3.1). Seasonally intense upwelling events carry nutrient and CO_2-rich deep waters to the surface in this coastal region, supporting high levels of primary productivity dominated mainly by large

(>30μM) solitary and chain-forming diatoms (Lalli & Parsons 1994). A large degree of spatial and temporal variability exists both in nutrient levels and phytoplankton biomass in these waters. At each station, nutrient concentrations were determined with a shipboard autoanalyzer, and total chlorophyll *a* (Chl *a*) was determined by fluorometric analysis later in the lab (Lindsay Richier, Varela lab, UVic: method in Parsons *et al.* 1984).

Figure 3.1: Map showing location of sampling stations in the Queen Charlotte Sound.



3.2.2 In-situ sampling and isotope disequilibrium experiments:

a) Enzyme activity: All samples were collected from the chlorophyll maximum (depth ranging from ~0.2 – 22.6m) using 10L Niskin bottles on a rosette. Several 1L phytoplankton samples taken from Niskin bottles were concentrated onto 25mm glass-fiber filters (GF/A: nominal pore size 1.6μm) and frozen in liquid nitrogen for subsequent laboratory analysis of RubisCO and PEPC expression. Enzyme assays were conducted as described in methods section of Chapter 2 with one minor modifcation: 10μl of 50μCi·ml^{-1} NaH^{14}CO$_3$ solution was added instead of 5μl in order to compensate for lower biomass on filters.

b) Particulate Organic Carbon and Particulate Organic Nitrogen: A 500mL sample was filtered through a 25mm combusted (450°C for 4.5 h) glass-fiber filter (GF/F: nominal pore size 0.7μm) Whatman® filter and rinsed with 10ml of nitrogen-free synthetic ocean water (SOW). The filters were stored in a glass dessicator for the duration of the cruise then dried at 55°C for 24hrs when back in the lab. C/N ratios were determined using an isotope ratio mass spectrometer (U.C. Davis Facility).

c) Isotope disequilibrium experiments: Phytoplankton were collected by passing water directly from 2×10L Niskin bottles through 3µm in-line polycarbonate filters (filtered only by gravity). Microalgae were re-suspended off filters into 14ml of seawater buffer containing 20mM Bicine adjusted to pH 8.5 (±0.05) and isotope disequilibrium experiments were immediately begun. Experiments were conducted as described in methods section of Chapter 2 with one minor modification: 20μl of 50μCi·ml^{-1} NaH^{14}CO$_3$ solution was added instead

of 10μl in order to compensate for lower biomass in cell suspension. Leftover cell-suspension was fixed with formaldehyde to a final concentration of 2% for later determination of phytoplankton community taxonomic composition. Phytoplankton were categorized into two main groups based on size: cells <10µM (mainly nanoflagellates and small diatoms) and cells ≥10µM (mainly larger diatoms and dinoflagellates).

3.3 Results

3.3.1 Biochemical Parameters:

Table 3.1: Chemical and biological properties of sampling stations.

Station ID	Sampling Depth (dbar)	Nitrate (µM)	Silicate (µM)	Phosphate (µM)	Chl *a* ($\mu g \cdot L^{-1}$)
GB5	19.9	5.7	11.5	0.76	1.96
MB2	22.6	5.5	11.8	0.75	1.20
SP4	8.6	0.2	5.5	0.38	1.50
SS2.5	20.4	3.5	10.4	0.65	0.96
CPE	0.2	17.6	33.3	1.68	5.50
SS6	14.9	4.4	9.2	0.66	1.77
G3	12.6	1.1	12.8	0.46	—

Over the seven stations sampled, nitrate concentrations were positively correlated with measurements of Chl *a* ($r = 0.934$; $p<0.01$; Table 3.1). With the exception of the highly productive inshore station CPE, nitrate levels ranged from 0.2–5.7µM and Chl *a* levels were between 0.96–1.96$\mu g \cdot L^{-1}$. There were no clear differences between onshore and offshore sites with respect to phytoplankton biomass or nutrient concentrations. Microscopic examination of phytoplankton samples revealed that diatoms >30µM, including *Coscinodiscus spp.*;

Cylindrotheca spp., and Pseudo-nitzschia spp., dominated at all stations sampled except for station SP4, where nanoflagellates and small diatoms <10µM were more abundant.

3.3.2 RuBP-carboxylase / PEP-carboxylase activity:

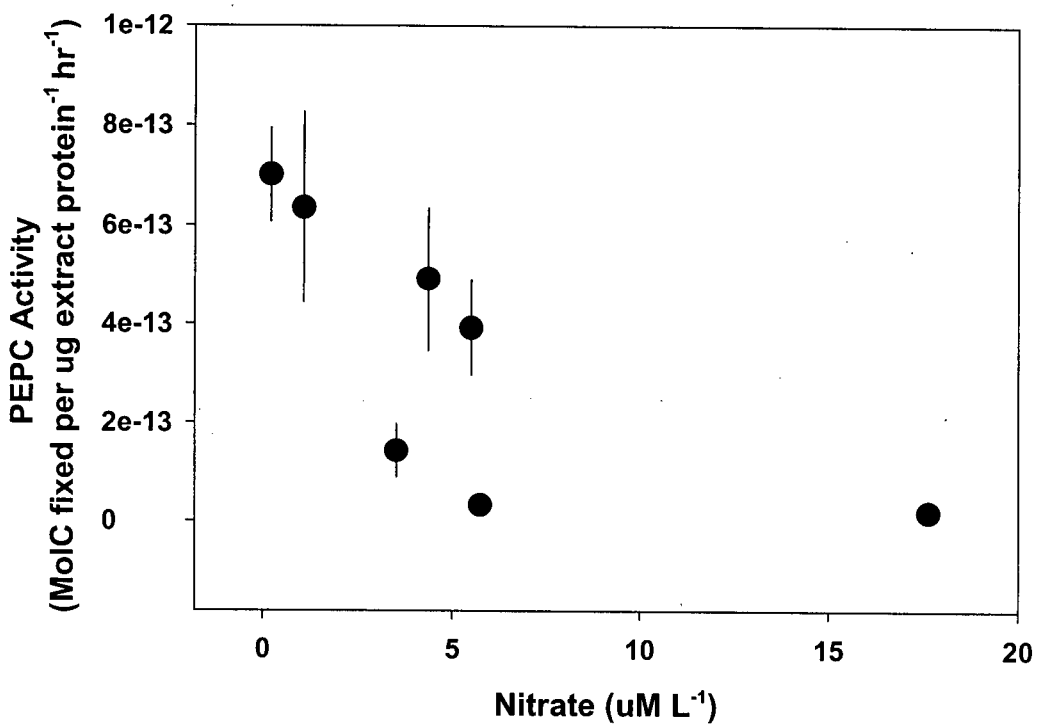
Table 3.2: Data analysis of enzyme assays and C/N ratios ± standard error.

Station ID	C/N	RubisCO Activity ($x10^{-12}$) (Mol C fixed·µg extract protein^{-1} ·hr^{-1})	PEPC Activity ($x10^{-13}$) (Mol C fixed·µg extract protein^{-1} ·hr^{-1})
GB5	6.08	0.78 ± 0.014	0.33 ± 0.022
MB2	5.92	1.36 ± 0.030	3.92 ± 0.951
SP4	5.22	1.09 ± 0.013	7.01 ± 0.933
SS2.5	5.31	0.32 ± 0.032	1.43 ± 0.534
CPE	5.84	1.37 ± 0.012	0.18 ± 0.046
SS6	5.28	2.21 ± 0.014	4.90 ± 1.44
G3	5.84	2.19 ± 0.033	6.35 ± 1.91

C/N ratios varied little between stations, ranging from ~5.2 to ~6.1 (Table 3.2). There was significant variation in RubisCO activity between stations ($p<0.001$), with a seven-fold difference between the highest and lowest measured activities. However, these data do not support any correlation between RubisCO activity and C/N ratio, nitrate concentration or level of Chl *a*. Measurements of RubisCO activity obtained in the field, ranging from 0.32 – 2.21 Mol C fixed·µg extract protein^{-1}·hr^{-1}, were of the same order of magnitude as those observed in lab-grown monocultures of *T. weissflogii* at the lowest growth rates (0.3·d^{-1}; see Results Chapter 2).

The relationship between PEPC activity and nitrate concentration was on the threshold of statistical significance ($p\sim0.05$), with PEPC activity increasing as

Figure 3.2: Relationship between PEPC activity and nitrate concentration (r=0.712; p~0.05) ± standard error.



nitrate concentration decreased (Fig 3.2). Field measurements of PEPC activities were approximately one order of magnitude lower than those of lab-grown *T.weissflogii* growing at $0.3 \cdot d^{-1}$ (see Results Chapter 2).

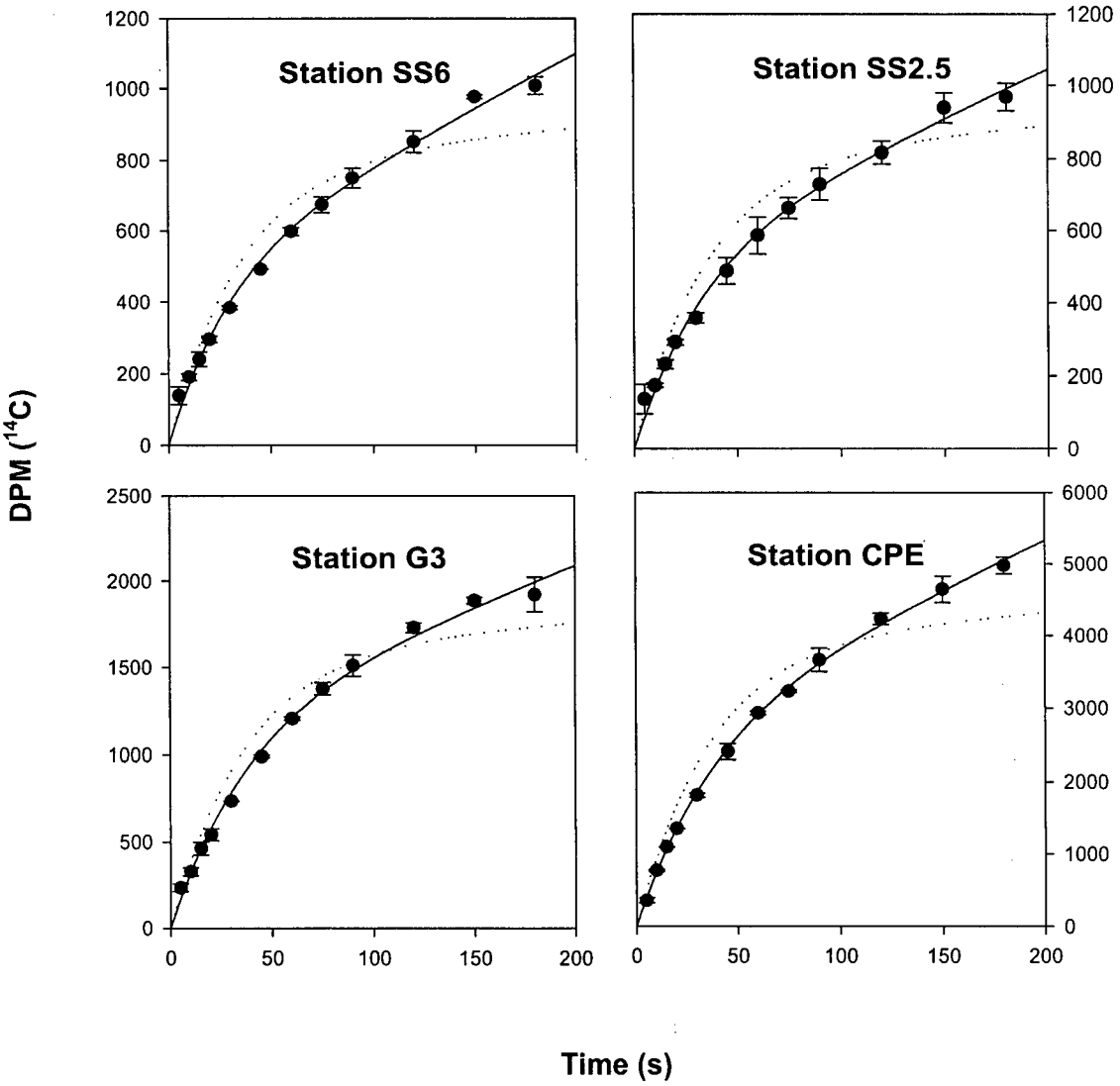
3.3.3 HCO_3^- vs. CO_2 Uptake:

Table 3.3 Data analysis of isotope disequilibrium experiments and C/N ratios.

Station ID	C/N	$fHCO_3^-$	*a*
GB5	6.08	—	—
MB2	5.92	—	—
SP4	5.22	0.9298±0.030	0.0272±0.022
SS2.5	5.31	0.8762±0.024	0.0299±0.007
CPE	5.84	0.8903±0.012	0.0272±0.004
SS6	5.28	0.8885±0.012	0.0332±0.005
G3	5.84	0.8589±0.027	0.0272±0.005

At all five stations where disequilibrium experiments were performed, $fHCO_3^-$ values indicate that more than 86% of the total carbon demand could be accounted for by HCO_3^- uptake (Table 3.3; Fig 3.3). Low values of ***a*** (external CA activity) suggest that at these stations HCO_3^- was taken up mainly via direct active transport. While $fHCO_3^-$ values were not significantly correlated with nitrate concentration or C/N ratios, the highest $fHCO_3^-$ value (obtained at station SP4) was associated with the lowest nitrate concentration and C/N ratio, and the highest PEPC activity. Additionally, this was the only station dominated by small diatoms and nanoflagellates rather than large diatoms.

Figure 3.3: Time course of ^{14}C assimilation during isotope disequilibrium experiments with phytoplankton assemblages at four of the stations sampled (± standard error). The dotted line indicates the best fit of the equation assuming only use of CO_2 and no contribution of HCO_3^-.



3.4 Discussion

3.4.1 RubisCO:

Since RubisCO is only able to fix inorganic carbon in the form of CO_2 (Cooper *et al.*1969) this enzyme is undersaturated with respect to the concentrations of CO_2 found in surface seawater (Badger *et al.* 1998). RubisCO represents a large nitrogen-sink for the cell (Ellis 1979), and while the effect of nitrogen-limitation on the cellular content and activity of RubisCO in lab-grown phytoplankton cultures is well documented (Lapointe & Duke 1984; Falkowski *et al.*1989; Beardall *et al.* 1991; Geider *et al.* 1998; *also see* Results Chapter 2), it is unclear how combined RubisCO activities of complex phytoplankton assemblages vary over nitrogen gradients in the field. Despite the significant correlation between nitrate and Chl *a* levels, RubisCO activity did not vary predictably over the range of nitrogen concentrations reported in this field study. This is not the result I would have expected based on the findings of my lab experiments. However, my nitrogen-limited chemostat cultures of *T. weissflogii* had residual nitrate levels less than 1μM at all experimental growth rates tested (*see* Results Chapter 2), while five of the seven stations sampled had nitrate concentrations >3.5$\mu M \cdot L^{-1}$. Since the half-saturation constant for nitrate uptake in coastal marine phytoplankton ranges from 0.98 to 4.2 μM (Raymont, J.E.G.) it is possible that the nitrate levels at these field stations may not have been low enough to elicit a change in the cellular content of RubisCO. Alternatively, variations in cellular growth stage (ie: exponential vs. stationary growth phase) may have influenced RubisCO activity.

3.4.2 C_4 photosynthesis:

Since carbon-fixation is expensive in terms of nitrogen, the availability of nitrogen may affect the efficiency of cellular carbon fixation (Raven & Johnston 1991). Laboratory labeling studies have demonstrated that a unicellular C_4 photosynthetic pathway is important in carbon accumulation and photosynthetic carbon fixation in diatoms at low levels of CO_2 and Zn (Reinfelder *et al.*2000, 2004; Morel *et al.* 2002). However, the relationship between PEPC activity and nitrate concentration remains uncertain. PEPC activities did not appear to be regulated by nitrogen supply in nitrogen-limited chemostat cultures of *T. weissflogii* (see Results Chapter 2). The negative trend between PEPC activity and nitrate concentrations observed at the seven stations sampled in this field study suggests that PEPC activity may be up-regulated when nitrogen levels are low. The induction of PEPC activity at low levels of nitrogen may help to save nitrogen in RubisCO by making the enzyme more efficient.

These RubisCO and PEPC enzyme data should be interpreted with caution as there was only one sample obtained per station and the activities measured, particularly of PEPC, were low compared to lab-grown *T. weissflogii* cultures. It is possible that these low activities may be explained by variations in the phytoplankton growth phase. While lab cultures were sampled for enzyme assays during the exponential phase of growth, phytoplankton sampled in the field may have been in stationary phase. More rigorous sampling, including the collection of more phytoplankton biomass on filters over a range of depths (ie. depth profiles), may help to clarify the results of these enzyme assays.

3.4.3 HCO_3^- VS CO_2 uptake:

All phytoplankton assemblages studied utilized HCO_3^- as an exogenous inorganic carbon source. The observed kinetics of ^{14}C incorporation suggest that carbon uptake occurred mainly via a direct transport mechanism. Laboratory studies on inorganic carbon acquisition in diatoms have shown that HCO_3^- utilization is common in this group (Burns & Beardall 1987; Nimer *et al.* 1997), although there appears to be considerable variability in carbon acquisition strategies between species and even for one single species, depending on external conditions (Elzenga *et al.* 2000; Burkhardt *et al.* 2001; Morel *et al.* 2002). Additionally, the use of HCO_3^- as a carbon source in natural diatom-dominated phytoplankton assemblages has been demonstrated (Tortell *et al.*1997; Tortell *et al.* 2000; Tortell & Morel 2002; Cassar *et al.* 2004). However, reports on the contribution of direct active HCO_3^- uptake vs. indirect, CA-mediated HCO_3^- use are mixed. Evidence of external CA expression in coastal Pacific phytoplankton assemblages was demonstrated by Tortell *et al.* (2000). Subsequently, data from isotope disequilibrium experiments performed in the eastern subtropical and Equatorial Pacific region showed that diatom-dominated assemblages expressed external CA and transported CO_2 derived from the catalyzed dehydration of HCO_3^- (Tortell & Morel 2002), although authors acknowledged that direct HCO_3^- transport may have been present. In contrast, negligible CA activity was detected in diatom-dominated Southern Ocean phytoplankton assemblages (Cassar *et al.* 2004). These authors report data from

isotope disequilibrium experiments confirming that 50% of the DIC was obtained through direct HCO_3^- uptake, the other half being active or passive CO_2 uptake.

It is interesting to note that in three of the five stations sampled, the model rate-constant for HCO_3^- / CO_2 equilibration was not different from uncatalyzed rates, implying that absolutely no CA activity was present at these stations. In support of this finding, we were unable to detect any CA activity in these samples using membrane inlet mass spectrometry (MIMS) (measurements made by P. Tortell). However, a large amount of phytoplankton biomass is required to make reliable measurements of CA activity via MIMS, so it is possible that there simply was not enough phytoplankton biomass on the filters.

3.4.4 Effect of nitrogen on CCM function:

While the function of the CCM has been studied in relation to CO_2 concentrations, the relationship between nitrogen availability and active inorganic carbon uptake mechanisms remains unclear. Results from my lab experiments with chemostat-cultured *T. weissflogii* suggest that while HCO_3^- uptake remains high even under extreme nitrogen-limitation, HCO_3^- transport may in fact be down-regulated with decreasing nitrogen-supply. In this field study, $f HCO_3^-$ values were not correlated with nitrate concentrations or C/N ratios, although station SP4 had the lowest nitrate concentration and C/N ratio and the highest PEPC activity and $f HCO_3^-$ values of all the stations sampled. Interestingly, this was also the only station dominated by small diatoms and nanoflagellates, suggesting that a more detailed investigation of phytoplankton community

structure (ie. mainly small diatoms or mainly nanoflagellates) may be important for the interpretation of these results. It is difficult to draw any conclusions on the relationship between nitrate-supply and HCO_3^- uptake at these stations since, as previously discussed, five of the seven stations sampled had nitrate concentrations that were potentially non-limiting.

3.4.5 Summary:

In summary, while RubisCO activity was not regulated by nitrogen supply at the stations sampled in this field study, the negative trend observed between nitrate concentration and PEPC activity suggests that the function of the C_4 photosynthetic pathway may have been enhanced under low levels of nitrogen. Inorganic carbon acquisition occurred mainly via direct HCO_3^- transport at all stations sampled and did not appear to be influenced by nitrogen levels.

FINAL CONCLUSIONS

1) RubisCO activity was positively correlated with nitrogen-limited growth rate in *T.weissflogii*. The function of the C_4 photosynthetic pathway, as determined by PEPC activity, was not influenced by nitrogen-limited growth rate.

2) HCO_3^- uptake accounted for 65 – 85% of the total dissolved inorganic carbon taken up by *T. weissfloggii* over the range of growth rates tested. The decrease in $fHCO_3^-$ (the fraction of dissolved inorganic carbon taken up in the form of HCO_3^-) observed with decreasing growth rate was attributed to a decrease in direct HCO_3^- transport.

3) There was no relationship between RubisCO activity and nitrate concentration at the seven diatom-dominated field stations sampled, but a negative trend was observed between PEPC activity and nitrate concentration.

4) All phytoplankton assemblages studied utilized HCO_3^- as an exogenous inorganic carbon source and HCO_3^- uptake occurred mainly via a direct active transport mechanism.

FUTURE RESEARCH

This thesis has provided preliminary physiological data on the effects of nitrogen supply on carbon-uptake systems in *T. weissflogii* and in coastal phytoplankton assemblages of the Queen Charlotte Sound. Suggestions for future research include:

1) Assessment of the effects of CO_2 concentration on carbon acquisition in nitrogen-limited phytoplankton.

2) Investigation of silica (Si)-limitation on carbon-uptake mechanisms of cells grown under high and low CO_2.

3) Characterization of N and Si / carbon interactions for different phytoplankton species representing different taxonomic / functional groups (ie: coccolithophores, nanoflagellates).

4) Analysis of the expression of particular genes involved in DIC acquisition under nutrient / carbon limitation (using the recently sequenced genome of *T. pseudonana).*

5) Assessment of effects of CO_2 on competitive interactions in nutrient-limited phytoplankton. Competition experiments could be conducted in the laboratory (using chemostats) as well as in the field (using incubation experiments) and could help to predict potential long-term ecological shifts that may result from future variations in atmospheric CO_2 levels.

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Appendix A

Aquil Culture Medium
(Price *et al.* 1988/89)

Substance	Final Concentration (M)
Aquil Salts	
NaCl	4.20×10^{-1}
Na_2SO_4	2.88×10^{-2}
KCl	9.39×10^{-3}
$NaHCO_3$	2.38×10^{-3}
KBr	8.40×10^{-4}
H_3BO_3	4.85×10^{-4}
NaF	7.14×10^{-5}
$MgCl_2 \cdot 6H_2O$	5.46×10^{-2}
$CaCl_2 \cdot 2H_2O$	1.05×10^{-2}
$SrCl_2 \cdot 6H_2O$	6.38×10^{-5}
Nutrients	
$NaH_2PO_4 \cdot H_2O$	1.00×10^{-5}
$NaNO_3$	3.00×10^{-4}
$Na_2SiO_3 \cdot 9H_2O$	1.00×10^{-4}
Trace metals	
Na_2EDTA	5.00×10^{-6}
$FeCl_3 \cdot 6H_2O$	4.51×10^{-7}
$ZnSO_4 \cdot 7H_2O$	4.00×10^{-9}
$MnCl_2 \cdot 4H_2O$	2.30×10^{-8}
$CoCl_2 \cdot 6H_2O$	2.50×10^{-9}
$CuSO_4 \cdot 5H_2O$	9.97×10^{-10}
$Na_2MoO4 \cdot 2H_2O$	1.00×10^{-7}
Na_2SeO_3	1.00×10^{-8}
Vitamins	
B_{12}	5.50×10^{-7} g·L^{-1}
Biotin	5.00×10^{-7} g·L^{-1}
Thiamine HCl	1.00×10^{-4} g·L^{-1}

Appendix B

Permeabilization Buffer
(Modified from MacIntyre *et al.* 1996)

50mM Bicine (pH 7.8) [163g·mol^{-1}; 815mg·100ml^{-1}]

1mM EDTA [200μl 0.5M EDTA·100ml^{-1}; 0.5M EDTA = 4.653g·25ml^{-1}]

10mM $MgCl_2$ [1ml 1.0M $MgCl_2$·100ml^{-1}; 1.0M $MgCl_2$ = 5.083g·25ml^{-1}]

1.5M Glycerol [=glycerin; 92g·mol^{-1}; 1.26g·ml^{-1}; 11ml·100ml^{-1}]

10 mM $NaHCO_3$ [84·100ml^{-1}]

5mg BSA (bovine serum albumen)

* Adjust pH with NaOH (to pH 7.8)

5mM DTT [154g·mol^{-1}; 77mg·100ml^{-1}]

0.1% Triton-X-100 [250μl·100ml^{-1}]

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*RuBP stock (store frozen): 23mM; 5mg $Na_4RuBP·1.5\ H_2O$ [429g·mol^{-1}] in 0.5ml of QH_2O

*PEP stock (store frozen): 50mM; 19mg Na_3PEP [378g·mol^{-1}] in 1ml of QH_2O