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Implications of Climate Change for Cyanobacteria Over the Western Florida Shelf in the Gulf of Mexico

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IMPLICATIONS OF CLIMATE CHANGE FOR CYANOBACTERIA OVER THE WESTERN FLORIDA SHELF IN THE GULF OF MEXICO

by

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ABSTRACT

IMPLICATIONS OF CLIMATE CHANGE FOR CYANOBACTERIA OVER THE WESTERN FLORIDA SHELF IN THE GULF OF MEXICO

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Concentrations of atmospheric CO_2 are expected to double by year 2100 as a result of anthropogenic activities. Under elevated CO_2 conditions, cyanobacteria may reallocate energy from active accumulation and transport of dissolved inorganic carbon (C) required for photosynthesis to other growth processes. Stimulation of cyanobacterial production on the Western Florida Shelf in the Gulf of Mexico (GOM) could lead to improved nutritional status for the toxic, mixotrophic dinoflagellate *Karenia brevis* that utilizes newly fixed N_2 from co-occurring diazotrophic cyanobacteria and consumes unicellular cyanobacteria via grazing for growth. Culture studies performed by other researchers on the climate induced physiological changes in the GOM diazotroph *Trichodesmium erythraeum* have revealed that this ecologically important cyanobacteria fixes dinitrogen (N_2) and grows at higher rates under elevated pCO_2 compared to present day conditions. Research performed to determine impacts of elevated pCO_2 on primary production and diazotrophic physiology in other important cyanobacterial groups, including coastal *Synechococcus* cf *elongatus*, *Lyngbya* spp. and unicellular diazotrophs is limited, but all currently contribute to bioavailable N pools fueling *K. brevis* blooms. This thesis explores the connection between elevated CO_2 and stimulation of production and N_2 fixation in cyanobacteria to better estimate the nutritional status of GOM phytoplankton, especially *K. brevis,* under future climate conditions.

Incubation experiments were performed using cultured populations of coastal *S.* cf *elongatus* and *T. erythraeum*, and natural populations of *Trichodesmium* spp., *Lyngbya* spp. and a mixed phytoplankton assemblage from the GOM to estimate the impact of elevated pCO_2 and/or elevated temperature on their N and C physiology. Cultured populations experienced stimulation in C fixation at high pCO_2 and N_2 fixation was stimulated in *T. erythraeum.* Natural populations of *Trichodesmium* spp. also

experienced elevated rates of C and N_2 fixation at high CO_2, but significant pCO_2 stimulation of production and N_2 fixation was not observed in *Lyngbya* spp. and the mixed phytoplankton assemblage. The supply of available N and provision of prey species available to fuel *K. brevis* production is likely to increase under future climate conditions because dominant cyanobacteria increase primary production rates and N_2 fixation under elevated pCO_2 and/or temperature conditions.

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CHAPTER I

INTRODUCTION

Atmospheric carbon dioxide (CO_2) levels are increasing at a faster rate than ever observed in the geological record (IPCC 2013). Concentrations of CO_2 in the atmosphere are expected to increase from current concentrations of just over 400 ppm pCO_2 to between 794 and 1132 ppm pCO_2 by year 2100 (IPCC 2013). While we normally think that nitrogen (N) or some other macro or micronutrient limits phytoplankton productivity in the environment, Riebesell et al. (1993) suggested that phytoplankton might experience CO_2 limitation at ambient concentrations in seawater (Riebesell et al. 1993) because the primary carboxylating enzyme, ribulose-1, 5-bioposphate carboxylase oxygenase (RuBisCO) requires CO_2 and RuBisCO has a low affinity for CO_2 relative to concentrations of CO_2 in marine systems (Badger et al. 1998). Many phytoplankton possess C concentrating mechanisms (CCMs) that can respond to changes in pCO_2 and provide a mechanism to concentrate CO_2 around the active site of RuBisCO to compensate for low environmental concentrations (Price et al. 1998, Kaplan & Reinhold 1999, Badger & Price 2003). However, phytoplankton species that lack efficient CCMs, are unable to use bicarbonate (HCO_3^-), or have low affinities for CO_2 and HCO_3^- may be C limited at present day pCO_2 levels but are likely to increase production with increasing CO_2 availability. Many cyanobacteria that lack efficient CCMs might directly benefit from elevated CO_2 availability because they can down-regulate CCMs and re-allocate energy and nutrients elsewhere to promote growth (Beardall & Giordano 2002, Kranz et al. 2009).

Though work has been done to elucidate the impact of variations in CO_2 availability on CCMs in certain groups of cyanobacteria, studies focusing on climate induced changes to other aspects of phytoplankton physiology and ecology are few. Several CO_2 experiments have shown the potential for dramatic changes in phytoplankton growth, photosynthesis and community composition in response to elevated CO_2 conditions (Hein & Sand-Jensen 1997, Tortell et al. 2002, 2008; Fu et al. 2007, 2008a; Feng et al. 2008, 2009; Hutchins et al. 2007, 2009; Riebesell et al. 2007).

However, community responses to the impact of CO_2 enrichment on keystone species in systems plagued by HABs are poorly understood.

Over the Western Florida shelf (WFS) in the Gulf of Mexico (GOM), changes in the abundance of the red tide dinoflagellate *Karenia brevis* in response to variations in environmental conditions is of major concern since blooms of this organism have devastating effects on the economy of the region. Understanding the ecology of *K. brevis* in the GOM will become increasingly important into the future as many co-occurring phytoplankton species that contribute to the nutritional status of this red tide species may respond positively to elevated temperatures and CO_2 availability. Currently *Trichodesmium* spp. fix N_2 at high rates in the GOM, and studies have documented that 52% of the recently fixed N_2 is rapidly released (Mulholland et al. 2006) and available for use by co-occurring organisms (O'Neil et al. 1996, Mulholland et al. 2004a & b, 2006; Mulholland 2007) including *K. brevis* (Mulholland et al. 2014). *Trichodesmium* spp. abundance has been linked with *K. brevis* blooms (Steidinger et al. 1998, Walsh et al. 2006), and researchers recently determined that *K. brevis* is not only capable of uptake of a variety of inorganic forms of N (Bronk et al. 2004, Sinclair et al. 2006, Killberg-Thoreson et al. 2014a), but also assimilates DON exudates from *T. erythraeum* (Sipler et al. 2013). In addition, a more recent study on nutrient release by *T. erythraeum* suggests that 71% of N and 50% of C fixed is released into the environment as total dissolved nitrogen (TDN) and dissolved organic carbon (DOC) (Wannicke et al. 2009).

In addition to diazotrophic cyanobacteria, coastal *Synechococcus* spp., non-diazotrophic cyanobacteria also commonly bloom in the GOM, and this organism is grazed upon by *K. brevis* (Glibert et al. 2009, Procise 2012). While grazing by this mixotrophic dinoflagellate is not fully understood, changes in production of coastal *S.* cf *elongatus* in response to changes in pCO_2 or temperature could change the nutritional supply through the production of prey in support of *K. brevis* bloom formation and maintenance.

Culture experiments on *T. erythraeum* and *Synechococcus sp.* have shown stimulation of various physiological processes with increases in CO_2 availability (Fu et al. 2007, Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007, 2010). If populations of *Trichodesmium* spp. and coastal *Synechococcus* spp. from the WFS

respond positively to increases in pCO_2 and temperature in the same manner there is potential for greater concentrations of new N inputs and prey abundance to support *K. brevis*' mixotrophic growth.

Other co-occurring phytoplankton also contribute to pools of nutrient sources that fuel *K. brevis* growth over the WFS in the GOM. *K. brevis* blooms have co-occurred with blooms of another diazotrophic cyanobacterial group *Lyngbya* spp. in the tributaries draining to the WFS (Paerl et al. 2008), and if N_2 fixation by this organism are also stimulated under future climate scenarios, *K. brevis* is likely to benefit. Currently, warming temperatures have been suggested as the cause for the recent proliferation of *Lyngbya* spp. globally (O'Neil et al. 2012, Paerl & Paul 2012, Paerl et al. 2014), but stimulation of production and N_2 fixation in response to elevated CO_2 conditions has not been assessed for this group of cyanobacteria.

Unicellular diazotrophic cyanobacteria have also been suggested as a significant contributor to new N released into oligotrophic marine environments (Montoya et al. 2004, Zehr et al. 2007) including the GOM (Mulholland et al. 2006). If populations of unicellular diazotrophs are abundant in the GOM and experience stimulation of N_2 fixation or photosynthetic production in response to elevated CO_2 conditions, the input of this newly fixed N could further increase N availability under future climate conditions.

Along the WFS in the GOM, blooms of *K. brevis* appear to have increased over the last several decades as a result of anthropogenic disturbance (Brand & Compton 2007). Further, enhanced CO_2 availability appears to stimulate growth of diazotrophic cyanobacteria which are known provide nutrient inputs that may fuel *K. brevis* blooms. Current results suggest that *Trichodesmium* spp. can sustain moderately sized blooms of *K. brevis* in the GOM (Mulholland et al. 2006) and grazing experiments suggest that ingestion of *Synechococcus* spp. by *K. brevis* might also be important for this red tide species (Glibert et al. 2009, Procise 2012). Stimulation of co-occurring cyanobacteria populations in the GOM is likely to benefit *K. brevis* during both bloom initiation and maintenance phases. Blooms of *K. brevis* are currently responsible for numerous adverse effects to human health (neurotoxic shellfish poisoning and respiratory irritation from brevetoxin production), fishery industries, and thus tourism (Kirkpatrick et al. 2004), so it is important to determine the potential impacts climate change will have on *K. brevis*

through the supply of nutrient sources to support growth.

Statement of the problem

Studies of climate change impacts on the development and maintenance of harmful algal blooms are in their infancy. It is thought that increases in temperature and stratification might promote the growth of cyanobacteria and dinoflagellates, including harmful ones, but while there is now some information on the possible physiological impacts of increased CO_2 availability and temperature on individual phytoplankton species, particularly calcifying algae and diazotrophs, few studies have focused on direct or indirect climate change impacts on other important taxonomic groups or phytoplankton community dynamics as a whole. This research was aimed at determining how cyanobacteria in the GOM might be affected by increases in temperature and/or CO_2 in an effort to estimate the nutritional status of *K. brevis* under future climate scenarios. As part of this study, the individual and interactive effects of temperature and CO_2 availability on cultured populations of coastal *S.* cf *elongatus* and *T. erythraeum* was assessed. Additionally, the physiological response of natural populations of *Trichodesmium* spp., *Lyngbya* spp. and a mixed phytoplankton community in the GOM were studied under elevated CO_2 conditions to estimate the indirect impact these groups of phytoplankton may have on the nutritional status of *K. brevis* into the future. The data obtained from these experiments will allow us to estimate nutrient conditions available to support *K. brevis* blooms in the future and may allow agencies involved in the monitoring and management of *K. brevis* to plan for changes in the magnitude and duration of red tides, thereby facilitating adaptive management.

CHAPTER II

EFFECT OF CARBON DIOXIDE ENRICHMENT AND INCREASED TEMPERATURE ON THE PHYSIOLOGY OF COASTAL *SYNECHOCOCCUS* CF *ELONGATUS* AND *TRICHODESMIUM ERYTHRAEUM* CULTURES ISOLATED FROM THE GULF OF MEXICO OVER THE WESTERN FLORIDA SHELF

Introduction

Atmospheric CO_2 levels are increasing at unprecedented rates as a result of anthropogenic activities (IPCC 2013). This has resulted in increased flux of CO_2 into the ocean and acidification of surface waters. Atmospheric CO_2 concentrations recently reached 400 ppm and are likely to reach between 794 and 1132 ppm by year 2100 (IPCC 2013). If sufficient light is available, macro or micronutrients limit phytoplankton productivity in the environment. Riebesell et al. (1993) suggested that phytoplankton might experience CO_2 limitation at ambient concentrations in seawater (Riebesell et al. 1993) because most of the dissolved inorganic C (DIC) that enters the ocean is rapidly converted to HCO_3^-. The primary carboxylating enzyme, RuBisCO, requires CO_2, but has a low affinity for CO_2 relative to concentrations of CO_2 in marine systems (Badger et al. 1998). To compensate for low environmental concentrations, many phytoplankton possess CCMs that concentrate CO_2 around the active site of RuBisCO (Price et al. 1998, Kaplan & Reinhold 1999, Badger & Price 2003). However, phytoplankton that have low affinities for CO_2, lack efficient CCMs, or are unable to use HCO_3^- may be C limited at present day pCO_2 levels. This limitation might be alleviated with increasing CO_2 availability. Many cyanobacteria, including *Synechococcus* spp. and the diazotroph, *Trichodesmium* spp., may directly benefit from elevated CO_2 availability because they can down-regulate CCMs and reallocate energy and nutrients elsewhere to promote additional growth or biosynthesis when DIC availability increases (Beardall & Giordano 2002, Kranz et al. 2011).

On the WFS in the GOM, blooms of *K. brevis* exert devastating effects on the economy of the region and blooms have been linked to diazotrophic and non-diazotrophic cyanobacteria which provide sources of N and C to fuel their growth. Blooms of *K. brevis* have been related to a variety of factors (Vargo et al. 2008), including new N inputs via dinitrogen (N_2) fixation by *Trichodesmium* spp. (Walsh & Steidinger 2001, Lenes et al. 2001, Mulholland et al. 2006, 2014), and the abundance of *Trichodesmium* spp. has been related with *K. brevis* abundance (Steidinger et al. 1998, Walsh et al. 2006, Lenes & Heil 2010). *Trichodesmium* spp. fix N_2 at high rates in the GOM, and studies have documented that 52% of the recently fixed N_2 is rapidly released (Mulholland et al. 2006, 2014) and available for use by co-occurring organisms (O'Neil et al. 1996, Mulholland et al. 2004a, b, 2006, Mulholland 2007) including *K. brevis* (Sipler et al. 2013). Another recent study suggests that 71% of N and 50% of C fixed by *T. erythraeum* is released into the environment as total dissolved nitrogen (TDN) and dissolved organic carbon (DOC) (Wannicke et al. 2009). Experimental evidence suggests that this group of diazotrophs will thrive and likely increase their growth and N_2 fixation rates under future climate scenarios (Hutchins et al. 2007, Levitan et al. 2007, Barcelos e Ramos et al. 2007), likely increasing the availability of N to *K. brevis* and other co-occurring organisms.

In addition to N inputs through N_2 fixation, cultured isolates (Glibert et al. 2009, Jeong et al. 2005) and natural populations (Procise 2012) of *K. brevis* have been shown to graze on the non-diazotrophic cyanobacteria, *Synechococcus* spp. that commonly bloom in the GOM (Phlips et al. 1999, Glibert et al. 2004). While patterns in the abundance of *Synechococcus* spp. and *K. brevis* are complicated, *K. brevis* can acquire additional N and C via grazing on this ubiquitous organism (Jeong et al. 2005, Glibert et al. 2009, Procise 2012). It is unclear how climate change will impact the growth and abundance of *Synechococcus* spp. populations on the WFS. Some studies suggest that its growth will be unaffected by elevated CO_2 conditions alone (Lomas et al. 2012) while others suggest that coastal *Synechococcus* isolates may respond positively to the effects of increased temperature and pCO_2 (Fu et al. 2007, Lu et al. 2006).

Along the WFS, the question remains as to whether enhanced CO_2 availability will result in increases in the proliferation of cyanobacteria and harmful *K. brevis*

blooms. Current estimates suggest that N_2 fixation by *Trichodesmium* spp. populations observed in the region can sustain moderately sized blooms of *K. brevis* in the GOM (Mulholland et al. 2006). Further, grazing experiments suggest that ingestion of *Synechococcus* spp. by *K. brevis* might also be an important source of nutrition for this red tide species in the GOM (Glibert et al. 2009, Procise 2012). Changes in production of coastal *S.* cf *elongatus* and *T. erythraeum* in response to increases in pCO_2 and temperature will likely impact the transfer of N and C to *K. brevis* through enhanced N_2 fixation and grazing and this may facilitate the bloom intensification by increasing the concentration, duration and/or magnitude of *K. brevis* blooms.

Here we examine the effects of increased temperature and/or CO_2 on the growth of *S.* cf *elongatus*, a potential prey item for *K. brevis* and diazotrophic N_2 fixation by co-occurring *T. erythraeum* on the WFS.

Methods

Culture methods

S. cf *elongatus cultures*

Cultures of *S.* cf *elongatus* (FWRI isolate CCFWC 502) were obtained from FWRI and maintained in semi-continuous culture on f/2 media (Guillard & Ryther 1962, Guillard 1975). Cultures were grown in walk-in environmental chambers maintained at 24°C and 30°C. Incident light was provided by cool white fluorescent lamps at 200 μmoles quanta m^{-2} s^{-1} on a 12:12 light:dark cycle.

Cultures were grown at constant growth rates using semi-continuous culture techniques. Daily culture dilutions were made after measuring *in vivo* fluorescence and calculating the growth rate over the previous 24 hours (μ) (Equation 1). Each day, a fraction of the culture volume was removed and replaced with fresh culture media such that the dilution rate was equal to the growth rate (Fogg 1987). *In vivo* fluorescence measurements and culture dilutions were done at the same time each day to minimize diurnal variations in Chl *a* concentrations. After acclimating cultures to temperature and CO_2 conditions for at least three generations, HCO_3^- uptake and DOC release were measured as described below (Mulholland & Bernhardt 2005, Hutchins et al. 2007, 2009; Fu et al.2008a,b; Feng et al. 2008, 2009).

Equation 1. Calculation for daily growth rate based on change in in vivo fluorescence (FL) during initial (T_o) and final (T_f) sampling.

$$\mu = \frac{\ln(FL_F / FL_o)}{T_F - T_o}$$

T. erythraeum cultures

Cultures were grown in walk-in environmental incubators set to 24°C and 30°C. Incident light was provided by cool white fluorescent lamps delivering 200 μmole quanta m^{-2} s^{-1} on a 12:12 light:dark cycle. Once acclimated to ambient or elevated CO_2 conditions for three generations or more, C fixation and N_2 fixation were measured as described below (Mulholland & Bernhardt 2005, Hutchins et al. 2007, Fu et al.2008a,b, 2009; Feng et al. 2008, 2009).

For batch culture experiments, *T. erythraeum* isolate IMS101 was grown in a 30°C walk-in incubator on YBCII media (Chen et al. 1996) for 3 generations before measuring physiological responses to CO_2 enrichment during the lag, exponential and stationary growth phases. Growth rates were calculated from Chl *a* concentrations measured every other day using Equation 1 above, substituting Chl *a* concentration for *in vivo* fluorescence measurements. Carbon and N_2 fixation were measured using tracer techniques (Mulholland et al. 2006) and gross N_2 fixation was estimated using the acetylene reduction technique (Capone 1993) over 12 h incubations during the period when the lights were on as described below.

T. erythraeum IMS101 were also maintained in exponential phase growth under continuous (30°C experiment) or semi-continuous (24°C experiment) culture conditions. In continuous cultures, growth rates approximate dilution rates (Fogg 1987). For these experiments, the dilution rate was set at 400 mL per day (20% of culture volume diluted daily) because this approximated exponential growth rates (0.20 d^{-1}) observed in batch culture experiments. For semi-continuous cultures, daily dilutions were made after estimating growth rates of individual cultures as determined by increases in Chl *a* concentrations or cell number using Equation 1 above. Cultures were then diluted at rates equal to the estimated growth rates. Samples were taken and cultures were diluted at the same time daily to minimize diurnal variations in cell abundance and biomass.

Cells were counted from 0.5mL acid Lugol's preserved samples of well-mixed cultures using an inverted light microscope for the 24°C experiments. Individual filaments and cells were enumerated, but changes in cell abundance were used to determine growth and dilution rates. Ten mL culture samples were sacrificed for Chl *a* determination as a proxy for abundance for the 30°C experiments. The different methods used for determining biomass were selected based on the density of the cultured populations. In dense cultures, it was impossible to enumerate cells along *T. erythraeum* filaments because they overlaid each other.

pH/pCO_2 manipulation

Target pCO_2 treatments were achieved using constant gentle aeration with premixed 750 ppm pCO_2 compressed air or ambient air delivered via an aquarium pump (Hutchins et al. 2007, Rost et al. 2008). Use of standard aquarium air stones maximized diffusion of the gas bubbles into culture media by delivering micro-bubbles with a greater surface area to volume ratio. The diffusive air stone was affixed to the end of tubing, suspended near the base of each individual culture bottle, allowing bubbles to travel through the culture water column before filling the head space of the bottle. Outlet tubing connected to the top of the culture bottle served as a vent for the culture headspace, maintaining a head space CO_2 concentration approximately equal to the culture media CO_2 concentration.

CO_2 concentrations were monitored by making daily pH measurements and using these as a proxy for dissolved inorganic carbon. Cultured populations were maintained at moderate cell densities to avoid drawdown CO_2 concentrations in an amount >5% over the length of the experiments.

Measuring physiological response and culture conditions

Biomass and growth rate measurements

Chl *a* concentrations were determined using the non-acidification fluorometric method (Welschmeyer 1994) and normalized per cell using cell abundance determined via flow cytometry (Olson et al. 1993) for *S.* cf *elongatus*.

Growth rates were calculated with standard equations (Equation 1 above, Parsons et al.1984) using either *in vivo* fluorescence, Chl *a* or cell count data as a biomass metric. For *S.* cf *elongatus*, *in vivo* fluorescence was used as a biomass metric because of the ease and rapidity of this measurement. *T. erythraeum* is too large to be counted via flow cytometry, settles too quickly for *in vivo* fluorescence measurements, and cell number is difficult to enumerate by inverted light microscopy in dense cultures due to the high number of overlapping trichomes. Thus extracted Chl *a* concentrations were measured to estimate biomass in the relatively dense 30°C *T. erythraeum* cultures. Cultures of *T. erythraeum* maintained at 24°C were not as dense so cells were enumerated using cell counts from inverted light microscopy.

Physiological rate measurements

Samples for particulate N (PN) and particulate C (PC) were collected by filtering a known volume of culture onto a combusted Whatman GF/F filter (pore size ~ 0.7 μm) and frozen until analysis. Samples were thawed and desiccated for a minimum of 4 days at 40°C before pelletizing samples in tin disks for elemental analysis. Samples were analyzed on a Europa automated N and C analyzer (ANCA).

Uptake of $^{15}N_2$ and $H^{13}CO_3^-$ was measured in incubations carried out in the daytime and under the same temperature and light conditions as those to which cultures had been acclimated. For $H^{13}CO_3^-$ uptake experiments, subsamples of cultures were collected during the start of the light period and highly enriched (99%) $NaH^{13}CO_3$ was added (200 μM final concentration). Experiments were terminated at the end of the light period by gentle filtration onto pre-combusted (450°C for 2 hours) GF/F filters. For N_2 fixation measurements, aliquots of *T. erythraeum* cultures were enriched with 1 μL $^{15}N_2$ gas (99%) per mL culture at the start of the light period and incubated in gas tight Wheaton bottles for 12 hours. Incubations were terminated by filtering the entire contents of the bottles onto pre-combusted GF/F filters at the end of the light period (Mulholland et al. 2006).

Uptake rates were calculated based on ^{15}N and ^{13}C enrichment of PN and PC above natural abundance levels at the termination of incubation experiments (Montoya et

al. 1996, Mulholland & Capone 2001, Mulholland & Bernhardt 2005, Mulholland et al. 2006).

For *T. erythraeum*, estimates of gross N_2 fixation were performed using the acetylene (C_2H_4) reduction assay in addition to measuring net N_2 fixation using $^{15}N_2$ (Capone 1993, Mulholland et al. 2004). Gross N_2 fixation was calculated using the 4:1 theoretical ratio to calculate N_2 fixation from ethylene (C_2H_2) production (Montoya et al. 1996, Mulholland et al. 2004a).

Particulate C turnover (h^{-1}) was calculated by dividing C specific uptake rates (μmol C L^{-1} h^{-1}) by the measured C within the particulate pool (mass of C in phytoplankton, μmol C L^{-1}). Theoretical C release (h^{-1}) was calculated by dividing cell specific C uptake rates (μmol C $cell^{-1}$ h^{-1}) by cell specific POC (μmol C $cell^{-1}$).

Photosyntheis vs. irradiance (P vs. E) measurements

S. cf *elongatus* photosynthetic production (P) was measured as oxygen evolution using two Rank Brothers polarographic oxygen electrodes, recording changes in voltage signals in mV every second. Electrodes were surrounded by a chamber maintained at 30°C temperature using a flow through water bath system. Illumination was provided by a separate Kodak slide projector, and incident photosynthetically active radiation (PAR) was measured using a radiometer in μmol photons $m^{-2}s^{-1}$. A range of irradiance (E) levels (25, 50, 75, 100, 250, 500 and 1000 μmol photons $m^{-2}s^{-1}$) was achieved using neutral density filters over the projector output.

Spectral output of each projector was measured using an ASD Inc. spectrophotometer, and values of irradiance in terms of photosynthetically utilized (absorbed) radiation (PUR) were calculated after determining *S.* cf *elongatus* culture absorptance as a function of wavelength for each sample via measurements with a UV-spectrophotometer fitted with an integrating sphere to account for light scattered by the sample. Chlorophyll *a* samples were collected in three replicates the day of the P vs. E experiments (Welschmeyer 1994) and an average literature value for the mean Chl *a* absorption coefficient for marine *Synechococcus* (a^*=0.06 mg Chl *a*/ m^{-2}) was used to convert photosynthesis measurements from concentrations of oxygen evolved per mL, to

photosynthetic rates normalized to Chl *a*, to photosynthetic rates in μmol O_2 m^{-2} s^1 (Fu et al. 2007, Morel et al. 1993).

The irradiance used to measure the maximum photosynthetic rate (P_{max}) as a function of pCO_2 was chosen after analysis of the P vs. E data. The irradiance flux of 1000 μmol photons m^{-2} s^{-1} was chosen for determining P_{max} because it was higher than the optimum irradiance for photosynthesis (E_k) for both samples, therefore, the photosynthetic rate being tested was well within the C limited region of the P vs. E curve.

The initial slope (light limited portion) of the P vs. E curve in units of μmol O_2 m^{-2} s^{-1} per μmol photons $m^{-2}s^{-1}$ incident represented photosynthetic efficiency (α) (Greene et al. 1991), P_{max} was the maximum photosynthetic rate observed over the irradiance levels measured (in μmol O_2 $m^{-2}s^{-1}$), compensation irradiance (E_C, the irradiance where photosynthesis balances respiration) was determined by the x-intercept of the least squares linear regression line for the light limited portion of the P vs. E curves for each sample (in μmol photons $m^{-2}s^{-1}$), and the maximum quantum yield of photosynthesis (Φ_p) was determined by the slope of the least squares linear regression line of the light limited portion of the P vs. E curve (based on PUR of the *S.* cf *elongatus* cultures tested).

NH_4^+ and dissolved inorganic C

At the termination of incubations cultures were filtered through a 0.2μm Supor membrane filters and the filtrate was reserved for analysis of NH_4^+ and DOC. Samples were frozen at < -20°C and analyzed as soon as possible. NH_4^+ concentrations were determined using the phenolhypochlorite method (Solorzano 1969) and DOC was analyzed by high temperature combustion (Burdige et al. 1999).

DIC samples were taken from whole culture water at incubation termination by gently transferring a 30mL culture sample to a serum vial with a 60mL syringe attached to small diameter silicone tubing making sure to overflow the sample vial while carefully avoiding sample aeration. Samples were fixed adding mercuric chloride ($HgCl_2$) to reach a final concentration of 0.05% $HgCl_2$, crimp sealed, and refrigerated at <4°C until analysis. Samples were analyzed using standard coulometric methods (DOE 1994).

Results

S. cf elongatus incubations

Cell-normalized Chl *a* concentrations and cell number were substantially higher in *S.* cf *elongatus* cultures grown at 30°C than those grown at 24°C based on results from a 2-factor ANOVA (Table 1). Cell-normalized Chl *a* concentrations were not significantly affected by pCO_2 or the interactive effect of pCO_2 and temperature for either temperature treatment ($F=2.68$, $p=0.117$, $n=24$ and $F=3.28$, $p=0.085$, $n=24$) (Table 1). Temperature affected cell-normalized Chl *a* concentrations such that cultures acclimated to 30°C increased concentrations of Chl *a* per cell by 407% compared to cultures acclimated to 24°C ($F=22.3$, $p<0.001$, $n=24$). Cell number was also significantly elevated with increases in temperature from 24°C to 30°C ($F=46.8$, $p<0.001$, $n=24$), but was not affected by increases in pCO_2 or the interactive effect of pCO_2 and temperature ($F=0.032$, $p=0.860$, $n=24$ and $F=4.24$, $p=0.053$, $n=24$).

Table 1. Mean pH, cell-normalized Chl *a*, cell abundance, and cell-normalized PC for *S.* cf *elongatus* cultures grown at 24°C and 30°C under ambient and high pCO_2 conditions. Standard deviations from replicate cultures are in parentheses (n=6). A 2-factor ANOVA indicated significant differences between temperature treatments for Chl *a* ($F=22.3$, $p<0.001$, $n=24$) and cell abundance ($F=46.8$, $p<0.001$, $n=24$), but pCO_2 effects and combined pCO_2 and temperature effects did not cause significant differences between treatments (Chl *a*, $F=2.68$, $p=0.117$, $n=24$ and $F=3.28$, $p=0.085$, $n=24$, cell abundance, $F=0.03$, $p=0.860$, $n=24$ and $F=4.23$, $p=0.053$, $n=24$).

Treatment		pH	pg Chl *a* $cell^{-1}$	10^3 cells mL^{-1}	pmol C $cell^{-1}$
24°C	Ambient	8.03 (0.04)	0.04 (0.02)	362 (219)	1.2 (0.55)
	High CO_2	7.86 (0.07)	0.05 (0.01)	185 (109)	1.4 (0.61)
30°C	Ambient	8.22 (0.25)	0.23 (0.14)	707 (206)	1.9 (0.77)
	High CO_2	8.10 (0.08)	0.12 (0.03)	991 (202)	1.2 (0.33)

In cultures acclimated at 24°C, mean C fixation rates were 0.005 ± 0.003 pmol C $cell^{-1}$ h^{-1} and 0.014 ± 0.005 pmol C $cell^{-1}$ h^{-1} for cultures acclimated to ambient and 750 ppm pCO_2, respectively (Fig. 1A). Cultures grown at 30°C fixed 0.03 ± 0.01 pmol C $cell^{-1}$ h^{-1} under ambient pCO_2 conditions and 0.04 ± 0.01 pmol C $cell^{-1}$ h^{-1} under elevated

pCO_2. A two-factor ANOVA indicated significant differences in C fixation with regard to both temperature and pCO_2 treatments for the *S.* cf *elongatus* experiments. Carbon fixation rates increased significantly in elevated pCO_2 treatments at both temperatures ($F=13.95$, $p=0.001$, $n=24$). Further, C fixation rates were significantly higher in cultures acclimated at 30°C (0.03 ± 0.01 pmol C $cell^{-1}$ h^{-1}) than in those grown at 24°C (0.01 ± 0.01 pmol C $cell^{-1}$ h^{-1}) ($F=64.9$, $p<0.001$, $n=24$). There was no interactive effect between pCO_2 and temperature ($F= 0.644$, $p=0.432$, $n=24$).

Significantly elevated growth rates (217%) were observed in *S.* cf *elongatus* populations grown at 30°C relative to those grown at 24°C ($F=9.15$, $p=0.011$, $n=16$), however, growth rates were not significantly affected by pCO_2 enrichment ($F=0.159$, $p=0.697$, $n=16$) or combined pCO_2 and temperature treatment effects ($F=0.010$, $p=0.922$, $n=16$) based on results from a 2-factor ANOVA (Fig. 1B, Table 2).

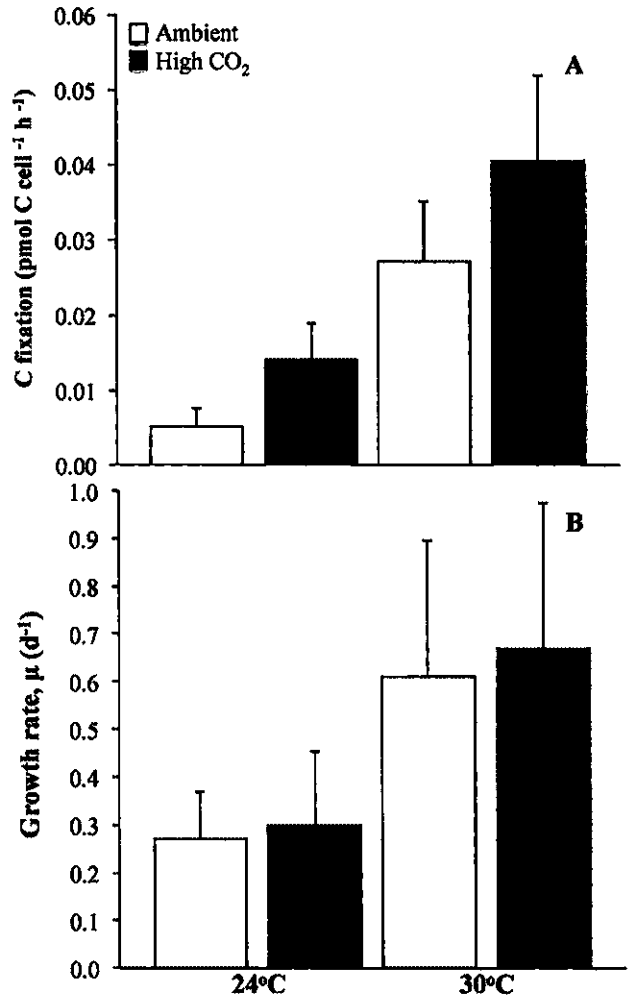

Figure 1. Hourly C uptake rates per cell (A) and specific growth rates (B) in steady state semi-continuous cultures of *S.* cf *elongatus* growing at 24°C or 30°C, and at ambient (white bars) or elevated (750 ppm) pCO_2 (black bars). Error bars represent one standard deviation determined by replicate cultures (n=6 for A, n=4 for B). Data were analyzed using a 2-factor ANOVA. Significant differences were observed in C fixation for both pCO_2 and temperature treatment effects ($F=13.957$, $p=0.001$, $n=24$ and $F=64.9$, $p<0.001$, $n=24$), but C fixation did not respond to the interactive effect of pCO_2 and temperature manipulations ($F=0.644$, $p=0.432$, $n=24$). Growth rate increased significantly with changes in temperature ($F=9.15$, $p=0.011$, $n=16$), but significant differences between treatments were not observed between pCO_2 treatments and the combined pCO_2 and temperature treatments ($F=0.159$, $p=0.697$, $n=16$ and $F=0.010$, $p=0.922$, $n=16$).

S. cf *elongatus* C turnover increased significantly in populations acclimated to high pCO_2 relative to those grown under ambient pCO_2 ($F=37.8$, $p<0.001$, $n=24$), but did

not vary with temperature (F=1.0, p=0.333, n=24) or combined pCO_2 and temperature treatment effects (F=0.64, p=0.432, n=24). Data were analyzed using a 2-factor ANOVA. Mean C turnover times were higher in elevated pCO_2 treatments in both 24°C and 30°C acclimated cultures (Fig. 2, Table 2).

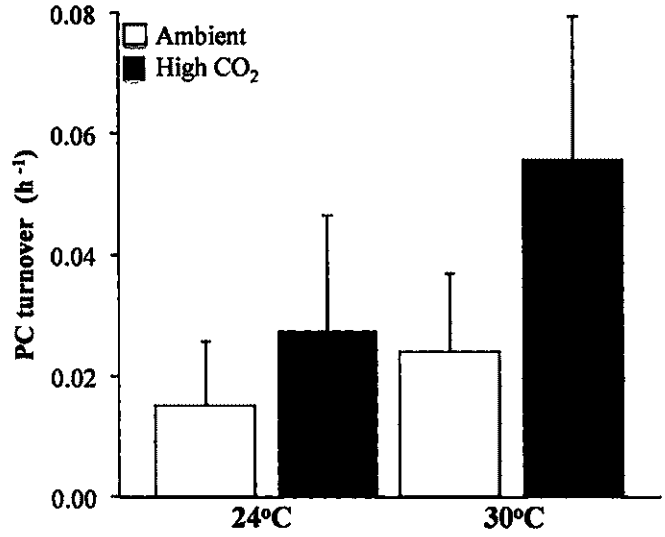

Figure 2. Mean rates of C turnover measured in steady state semi-continuous cultures of *S.* cf *elongatus* growing at 24°C or 30°C, and ambient (white bars) or elevated pCO_2 (black bars). Error bars represent one standard deviation determined from replicate cultures (n=6). A 2-factor ANOVA indicated significant differences in C turnover between pCO_2 treatments (F=37.8, p<0.001, n=24), but significant differences were not observed for temperature or combined pCO_2 and temperature effects (F=0.985, p=0.333, n=24 and F=0.644, p=0.432, n=24).

Steady state DOC concentrations were used to estimate DOC production in cultures. Biomass normalized DOC production rates for *S.* cf *elongatus* were 2.9± 1.3 pg C (pg PC)$^{-1}$ and 4.0± 0.27 pg C (pg PC)$^{-1}$ in ambient and elevated pCO_2 treatments, respectively, grown at 24°C. A 2-factor ANOVA indicated that temperature affects DOC production. DOC production was much lower in experiments conducted at 30°C, and did not vary with pCO_2 or combined temperature and pCO_2 effects (F=1.61, p=0.240, n=12 and F=1.614, p=0.240, n=12). DOC production measured 0.76± 0.20 pg C (pg PC)$^{-1}$ under ambient pCO_2 and 0.76 ± 0.22 pg C (pg PC)$^{-1}$ at elevated pCO_2 (Fig. 3, Table 2).

Temperature effects significantly decreased *S.* cf *elongatus* DOC production at 30°C relative to 24°C from a mean of 3.4 ± 1.0 pg C (pg PC)$^{-1}$ to 0.76 ± 0.22 pg C (pg PC)$^{-1}$ ($F=44.4$, $p<0.001$, $n=12$).

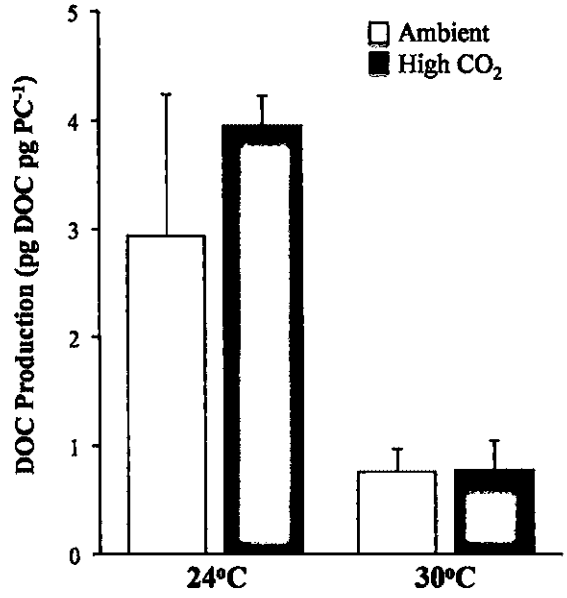

Figure 3. Mean C-normalized DOC production in steady state semi-continuous cultures of *S.* cf *elongatus* acclimated at 24°C or 30°C, and ambient (white bars) or elevated pCO_2 (black bars). Error bars represent one standard deviation determined from replicate cultures ($n=3$). A 2-factor ANOVA indicated significant differences between treatments caused by temperature ($F=44.4$, $p<0.001$, $n=12$), but DOC production did not vary significantly based on pCO_2 and combined temperature and pCO_2 effects ($F=1.61$, $p=0.240$, $n=12$ and $F=1.614$, $p=0.240$, $n=12$).

Table 2. Mean daily growth rate, hourly C turnover rate, theoretical hourly cellular C release and DOC production normalized to *S.* cf *elongatus* POC for semi-continuous cultures of *S.* cf *elongatus* acclimated to temperatures of 24°C or 30°C, and ambient or high pCO_2. Standard deviations from replicate cultures in parentheses (n=6). A 2-factor ANOVA indicated a significant increase in growth rate at elevated temperature conditions ($F=9.152$, $p=0.011$, $n=16$), a significant increase in C turnover at elevated pCO_2 ($F=37.8$, $p<0.001$, $n=24$) and a significant increase in DOC production at 24°C relative to 30°C ($F=44.4$, $p<0.001$, $n=12$). Significant differences were not found for the following parameters and independent treatment variables: growth rate across pCO_2 or combined pCO_2 and temperature combinations ($F=0.16$, $p=0.697$, $n=16$ and $F=0.010$, $p=0.922$, $n=16$), C turnover across temperature and combined temperature and pCO_2 combinations ($F=1.00$, $p=0.333$, $n=24$ and $F=2.82$, $p=0.109$, $n=24$) and DOC production across pCO_2 and temperature and pCO_2 combinations ($F=1.61$, $p=0.240$, $n=12$ and $F=1.61$, $p=0.240$, $n=12$).

Treatment		μ d^{-1}	PC turnover h^{-1}	Cellular C release $(x10^{-6})\ h^{-1}$	DOC pg DOC (pg PC)$^{-1}$
24°C	Ambient	0.27 (0.10)	0.02 (0.01)	5.1 (1.8)	2.9 (1.3)
	High CO_2	0.31 (0.15)	0.03 (0.02)	12 (3.6)	4.0 (0.27)
30°C	Ambient	0.61 (0.29)	0.02 (0.01)	24 (13)	0.76 (0.20)
	High CO_2	0.67 (0.31)	0.06 (0.02)	56 (24)	0.77 (0.29)

Photosynthesis versus irradiance (P vs. E) data obtained from samples of pooled replicate cultures from the 30°C *S.* cf *elongatus* culture experiment indicated that photosynthetic efficiency (α) and quantum photosynthetic efficiency (ϕ_p) was not significantly different between pCO_2 treatments determined by results from an ANCOVA ($F=0.671$, $p=0.5500$, $n=5$) (Fig. 4, Table 3). Photosynthetic parameters alpha (α) and the maximum quantum yield of photosynthesis (ϕ_p) were not statistically different across pCO_2 treatments for *S.* cf *elongatus*, however, quantitative differences in respiration rates were measured in high pCO_2 treatments relative to controls incubated at present day pCO_2. The saturating irradiance for photosynthesis (E_k) for *S.* cf *elongatus* acclimated to high pCO_2 was half that for *S.* cf *elongatus* acclimated to ambient pCO_2 (Table 3).

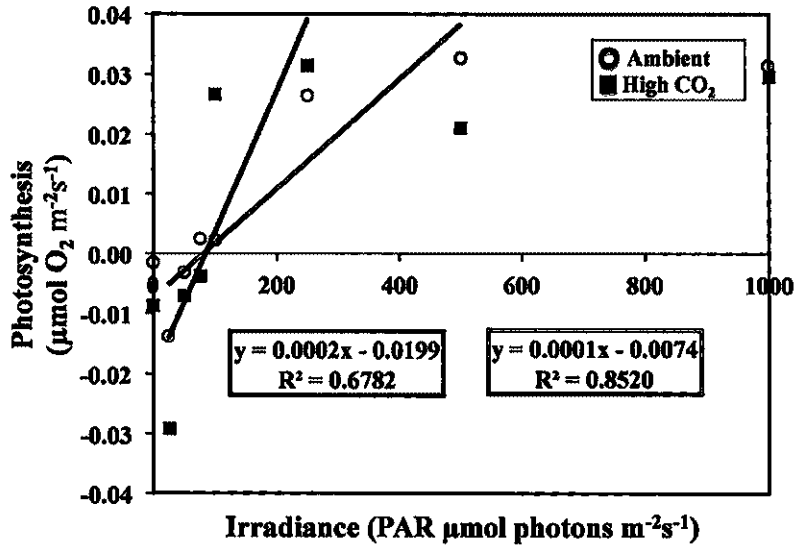

Figure 4. Photosynthesis (P) vs. irradiance (E) results for *S.* cf *elongatus* acclimated to 30°C under ambient pCO_2 (open circles) or high pCO_2 (black squares). Photosynthesis was measured over incident irradiance levels of 0 -1000 µmol photons $m^{-2}s^{-1}$, and the x-intercept of the least squares linear regression lines (double line, ambient pCO_2 and single line, high pCO_2) plotted through the light limited portion of the P vs. E curve determined the compensation irradiance (E_c), and the slope of the line determined photosynthetic efficiency (α). An ANCOVA indicated no significant difference in photosynthetic efficiency between pCO_2 treatments (F=0.671, p=0.5500, n=5).

Table 3. Photosynthesis (P) vs. irradiance (E) experimental results for coastal *S.* cf *elongatus* acclimated to 30°C. Results included the following photosynthetic parameters: maximum photosynthetic rate, P_{max} (µmoles O_2 m^{-2} s^{-1}), compensation irradiance, E_c (µmoles photons $m^{-2}s^{-1}$), maximum phosyntheic efficiency, α (µmoles O_2 µmoles photons incident), saturation irradiance, E_k (µmoles photons $m^{-2}s^{-1}$), maximum quantum yield of photosynthesis, ϕ_p (µmoles O_2 µmoles photons absorbed), respiration (µmoles O_2 $m^{-2}s^{-1}$) and the photosynthetic quotient, P_g:R (determined by the quotient of gross photosynthesis to respiration). Standard deviations could not be calculated because aliquots from replicate cultures were pooled to create a single sample for each pCO_2 treatment (n=1).

pCO_2	P_{max}	E_k	α	E_c	ϕ_p	R	P_g:R
380	0.03	500	0.0002	67.5	0.003	-0.001	24.4
750	0.03	250	0.0007	66.0	0.010	-0.009	4.58

T. erythraeum Incubations

Batch culture results

Chl *a* concentrations in *T. erythraeum* batch cultures acclimated at 30°C were not significantly affected by pCO_2 based on results from a repeated measures ANOVA (F=2.31, p=0.2040, n=18). Data were not distributed normally, so a custom F-table was created for the ANOVA based on 10,000 randomized iterations of the dataset (Manly 1997). Mean concentrations of Chl *a* were 4.9 ± 0.62, 11 ± 0.85, 72 ± 11 μg L^{-1} for cultures under ambient pCO_2 and 5.4 ± 0.66, 12 ± 1.4 and 84 ± 12 μg L^{-1} for cultures growing under elevated (750 ppm) pCO_2 conditions during lag, exponential and stationary phase growth, respectively.

T. erythraeum growth rates (μ, d^{-1}) followed a typical pattern wherein rates increase as cultures transition from lag to exponential growth and then decrease substantially during stationary phase (Fig. 5A). A repeated measures ANOVA indicated that growth rates did not vary with pCO_2 (F=0.087, p=0.772, n=18).

Carbon fixation by *T. erythraeum* was significantly stimulated by elevated pCO_2 based on results from a repeated measures ANOVA (F=19.6, p=0.0155, n=18). Data for C fixation was not distributed normally so a custom F-table was created for the ANOVA based on 10,000 randomized iterations of the dataset (Manly 1997). Mean rates of C fixation were 0.79 ± 0.09, 0.72 ± 0.69 and 1.4 ± 0.22 μmol C μg Chl a^{-1} d^{-1} for cultures acclimated to ambient pCO_2 conditions, and 1.4 ± 0.18, 2.0 ± 1.0 and 3.5 ± 1.1 μmol C μg Chl a^{-1} d^{-1} for cultures growing at elevated pCO_2 during lag, exponential and stationary growth phases, respectively (Fig. 5B).

Gross N_2 fixation determined using the acetylene reduction technique (Capone 1993, Mulholland et al. 2004) was negligible during the lag growth phase for both pCO_2 treatments, and a repeated measures ANOVA indicated that gross N_2 fixation did not vary with pCO_2 (F=6.51, p=0.0549, n=18). Gross N_2 fixation data were not distributed normally so a custom F-table was created for the ANOVA based on 10,000 randomized iterations of the dataset (Manly 1997). Mean gross N_2 fixation rates were 31 ± 8.9 and 20 ± 15 μmol N μg Chl a^{-1} d^{-1} for cultures under ambient CO_2 and 32 ± 1.2 and 76 ± 34 μmol N μg Chl a^{-1} d^{-1} for cultures acclimated to high CO_2 during exponential and stationary phase growth, respectively (Fig. 5C).

Net N_2 fixation rates measured using stable isotope tracer methods did not vary with pCO_2 according to statistical results from a repeated measures ANOVA ($F=2.67$, $p=0.1734$, $n=18$). The data for net N_2 fixation was not normally distributed so a customized F-table was prepared for the ANOVA analysis using 10,000 randomized iterations of the dataset (Manly 1997). Mean rates of net N_2 fixation for cultures growing under ambient CO_2 conditions were 0.28 ± 0.30, 26 ± 28, 20 ± 5.1 nmol N μg Chl a^{-1} d^{-1} for lag, exponential and stationary growth phases, respectively. Mean rates of N_2 fixation for cultures acclimated to elevated pCO_2 conditions were 0.95 ± 0.61, 58 ± 25 and 20 ± 4.4 nmol N μg Chl a-1 d^{-1} during lag, exponential and stationary growth phases, respectively (Fig. 5D).

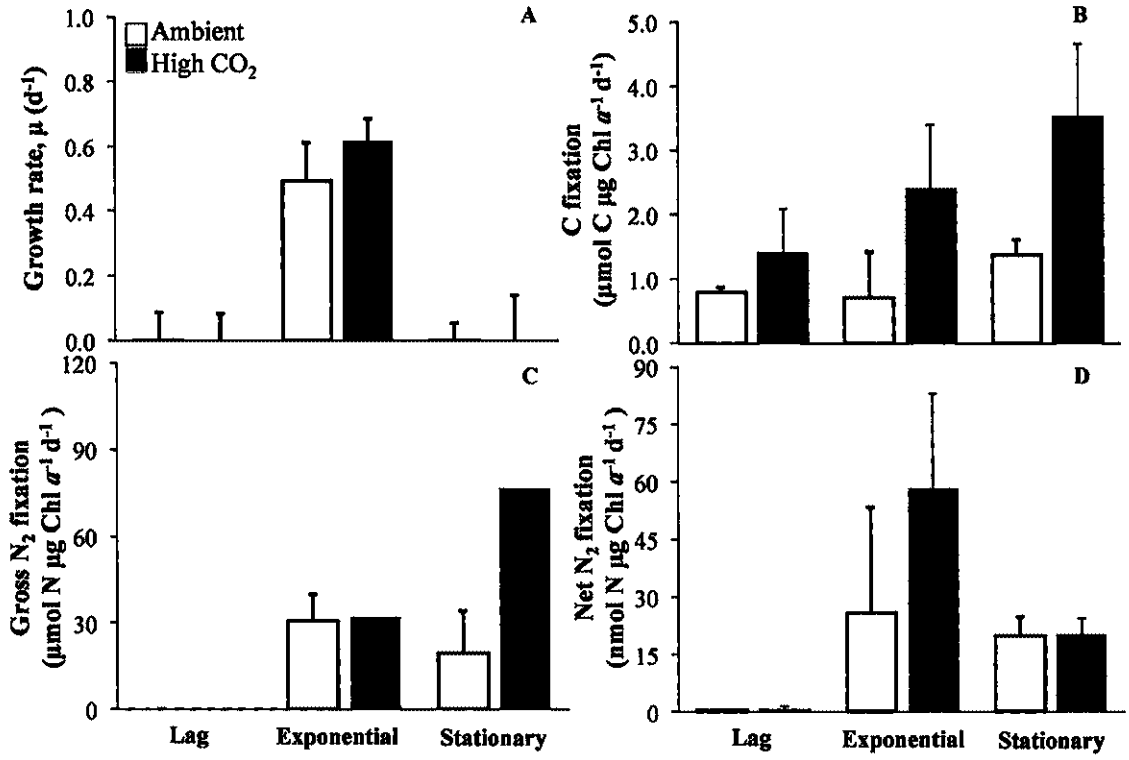

Figure 5. Mean daily growth rates (A), daily C fixation rates determined using $H^{13}CO_3^-$ (B), daily gross N_2 fixation rates determined using the acetylene reduction method (C), and daily net N_2 fixation rates measured using $^{15}N_2$ (D) in batch cultures of *T. erythraeum* during lag, exponential and stationary phase growth, and growing under ambient (white bars) or high (750 ppm) pCO_2 (black bars) conditions. Rates are normalized to Chl *a* concentrations. Error bars represent the standard deviation determined from replicate cultures (n=3). Measurements were made only after cultures were acclimated to pCO_2 conditions for 3 generations. A repeated measures ANOVA indicated significant differences for pCO_2 effects for C fixation (F=19.6, p=0.0155, n=18), but did differences between pCO_2 treatments were not significant for growth rate, gross N_2 fixation or net N_2 fixation (F=0.087, p=0.772, n=18, F=6.51, p=0.0549, n=18 and F=2.67, p=0.1734, n=18

Continuous and semi-continuous culture results

T. erythraeum Chl *a* concentrations were not affected by pCO_2, temperature or combined treatment effects (F=0.226, p=0.8778, n=12, F=1.21, p=0.3467, n=12 and F=0.106, p=0.9423, n=12) (Table 4). A 2-factor ANOVA was performed for Chl *a* concentration data after preparing a customized F-table based on 10,000 randomized iterations of the dataset because non-normality of Chl *a* concentration distributions (Manly 1997).

Table 4. Mean pH, Chl *a* concentrations, Chl *a* normalized PN and PC concentrations, and dissolved NH_4^+ concentrations in experimental *T. erythraeum* cultures grown at 24°C and 30°C under ambient and high pCO_2 conditions. Standard deviations from replicate cultures are in parentheses (n=3). A 2-factor ANOVA found no significant difference in Chl *a* concentrations based on pCO_2, temperature, or combined pCO_2 and temperature effects (F=0.226, p=0.8778, n=12, F=1.21, p=0.3467, n=12 and F=0.106, p=0.9423, n=12).

Treatment		pH	µg Chl *a* L^{-1}	µg PN µg Chl a^{-1}	µg PC µg Chl a^{-1}	NH_4^+ µM
24°C	Ambient	7.96 (0.15)	11 (16)	91 (71)	790 (609)	1.3 (0.1)
	High CO_2	7.79 (0.13)	7.2 (4.0)	80 (30)	463 (441)	2.1 (0.7)
30°C	Ambient	8.32 (0.02)	4.2 (2.8)	16 (7.1)	1240(1421)	2.5 (0.1)
	High CO_2	8.18 (0.02)	3.5 (1.0)	14 (3.3)	566 (238)	3.3 (0.7)

Growth rates were not significantly affected by pCO_2 (F=0.131, p=0.0726, n=12), temperature (F=1.785, p=0.218, n=12) or combined temperature and treatment effects (F=0.002, p=0.966, n=12) as determined by results from a 2-factor ANOVA.

Carbon fixation rates by *T. erythraeum* varied significantly with pCO_2 (F=14.3, p=0.0070, n=12), but did not change significantly in response to either temperature or combined temperature and pCO_2 treatment effects (F=0.188, p=0.6934, n=12 and F=0.034, p=0.8240, n=12). A 2-factor ANOVA was used for analysis of C fixation data after preparing a customized F-table based on 10,000 randomized iterations of the C fixation dataset because C fixation values were not normally distributed (Manly 1997). At 24°C mean C fixation rates under ambient conditions were 13 ± 0.6 µmol C µg Chl^{-1} h^{-1} and 23 ± 1.8 µmol C µg Chl^{-1} h^{-1} for cultures acclimated to 750 ppm pCO_2 (Fig. 6). Cultures grown at 30°C fixed 11 ± 8.1 µmol C µg Chl^{-1} h^{-1} under ambient conditions and 23 ± 6.1 µmol C µg Chl^{-1} h^{-1} under elevated CO_2 (Fig. 7).

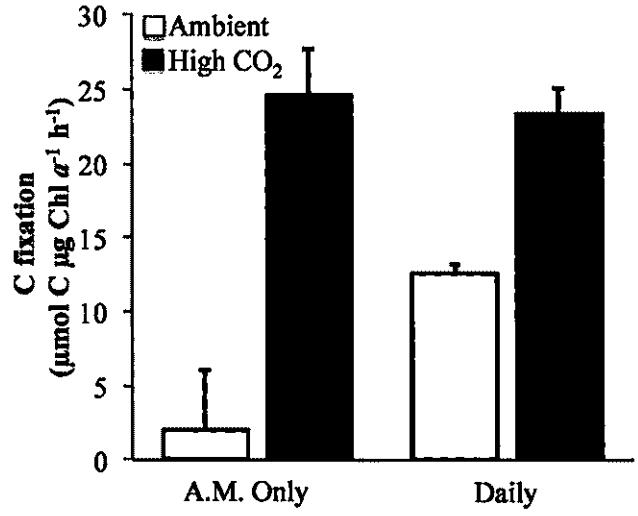

Figure 6. Mean hourly C fixation rates in steady state semi-continuous cultures of *T. erythraeum* growing at 24°C under ambient (white bars) or elevated pCO_2 (black bars) during the morning or over the entire light period. C fixation rates are normalized to Chl *a*. Error bars represent one standard deviation determined from replicate cultures (n=3). A 2-factor ANOVA indicated significant differences between treatments with respect to pCO_2 (F=14.3, p=0.0070, n=12), but C fixation did not vary significantly with temperature or temperature and pCO_2 in combination (F=0.188, p=0.6934, n=12 and F=0.034, p=0.8240, n=12).

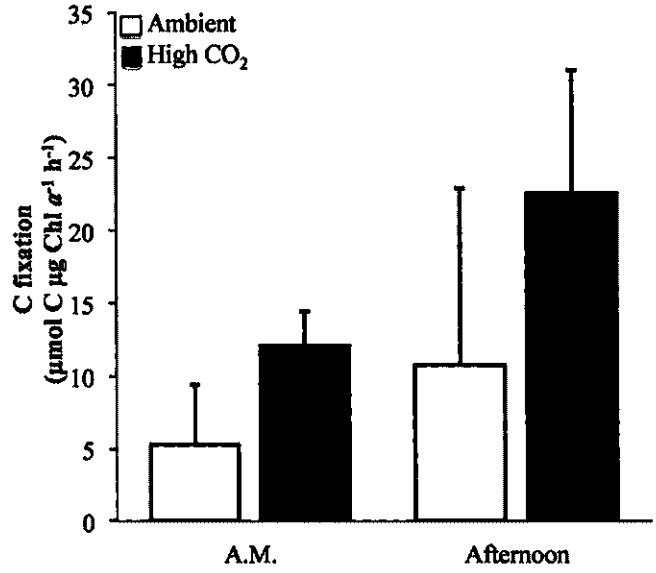

Figure 7. Mean hourly C fixation rates in steady state continuous cultures of *T. erythraeum* growing at 30°C under ambient (white bars) or elevated pCO_2 (black bars) measured during incubations spanning the period when the lights came on through noon, or a period starting at noon and ending at sunset. Chl *a* concentrations were used to normalize C fixation rates. Error bars represent one standard deviation determined from replicate cultures (n=3). A 2-factor ANOVA indicated significant differences between treatments with respect to pCO_2 ($F=14.3$, $p=0.0070$, $n=12$), but C fixation did not vary significantly with temperature or temperature and pCO_2 in combination ($F=0.188$, $p=0.6934$, $n=12$ and $F=0.034$, $p=0.8240$, $n=12$).

T. erythraeum C turnover did not vary significantly with pCO_2, temperature or combined temperature and pCO_2 treatments as indicated by a 2-factor ANOVA ($F=0.833$, $p=0.4069$, $n=12$, $F=1.13$, $p=0.330$, $n=12$ and $F=0.022$, $p=0.8570$, $n=12$). Data for C turnover were not normally distributed, so a customized F-table was prepared based on 10,000 randomized iterations of the dataset to perform the ANOVA (Manly 1997). Mean C turnover for cultures maintained at 24°C were 0.7 ± 0.9 and 1.0 ± 0.7 h^{-1} for populations grown under 24°C, and 0.5 ± 0.2 and 0.6 ± 0.4 for populations grown under 30°C at ambient and high CO_2, respectively (Fig. 8).

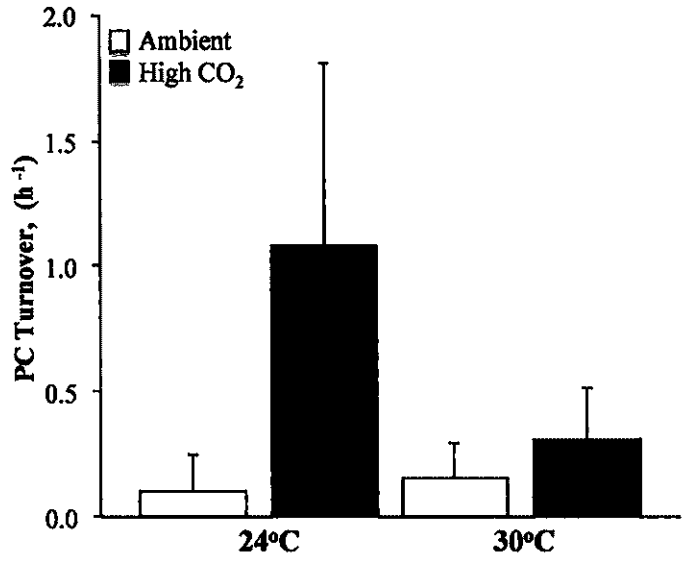

Figure 8. Mean hourly POC turnover rates due to C fixation in steady state cultures of *T. erythraeum* growing at 24°C or 30°C, and acclimated to ambient (white bars) or high pCO_2 (black bars) conditions. Error bars represent one standard deviation determined from replicate cultures (n=3). A 2-factor ANOVA indicated that C turnover did not vary significantly with pCO_2, temperature or combined temperature and pCO_2 treatment effects (F=0.833, p=0.4069, n=12, F=1.13, p=0.330, n=12 and F=0.022, p=0.8570, n=12).

Gross N_2 fixation rates differed significantly for experiments at 24°C and 30°C determined by a 2-factor ANOVA (F=104.6, p<0.001, n=12). Cultures growing at the higher temperature fixed N_2 at lower rates than those grown at the lower temperature (Fig. 9 A, C). Mean gross N_2 fixation rates were 260 ± 10.1 and 266 ± 85.5 μmol N μg Chl^{-1} d^{-1} in cultures acclimated to 24°C, and 3.5 ± 1.7 and 11.4 ± 7.4 μmol N μg Chl^{-1} d^{-1} in cultures acclimated to 30°C for populations acclimated to ambient and high pCO_2, respectively. Gross N_2 fixation did not vary significantly with pCO_2 or combined temperature and pCO_2 effects (F=0.078, p=0.787, n=12 and F=2.241, p=0.973, n=12).

Net N_2 fixation for cultured populations of *T. erythraeum* varied significantly with pCO_2, temperature and combined temperature and pCO_2 effects as determined with a 2-factor ANOVA (F=7.83, p=0.0273, n=12, F=28.1, p=0.0009, n=12 and F=21.3, p=0.0034, n=12). Net N_2 fixation data were not normally distributed, so a customized F-table was prepared based on 10,000 randomized iterations of the dataset to perform the ANOVA (Manly 1997). In the 30°C continuous culture experiment measurements of net

N_2 fixation rates were higher under ambient pCO_2 relative to high pCO_2 conditions, but N fixation was significantly stimulated under high pCO_2 for the semi-continuous culture experiment performed at 24°C ($F=28.1$, $p=0.0009$, $n=12$). The interaction of pCO_2 and temperature manipulations significantly elevated N_2 fixation rates measured for populations incubated at 24°C under elevated pCO_2 conditions relative to all other pCO_2 and temperature combinations. At 24°C mean N_2 fixation rates of 22 ± 7.0 nmol N μg Chl^{-1} d^{-1} and 56 ± 14 nmol N μg Chl^{-1} d^{-1} were determined for ambient cultures and high pCO_2 cultures, respectively, where cultures grown under elevated pCO_2 in 30°C temperature fixed N at mean rates of 11 ± 2.4 nmol N μg Chl^{-1} d^{-1} compared to ambient cultures that fixed at rates of 19 ± 3.5 nmol N μg Chl^{-1} d^{-1} (Fig. 9 B, D).

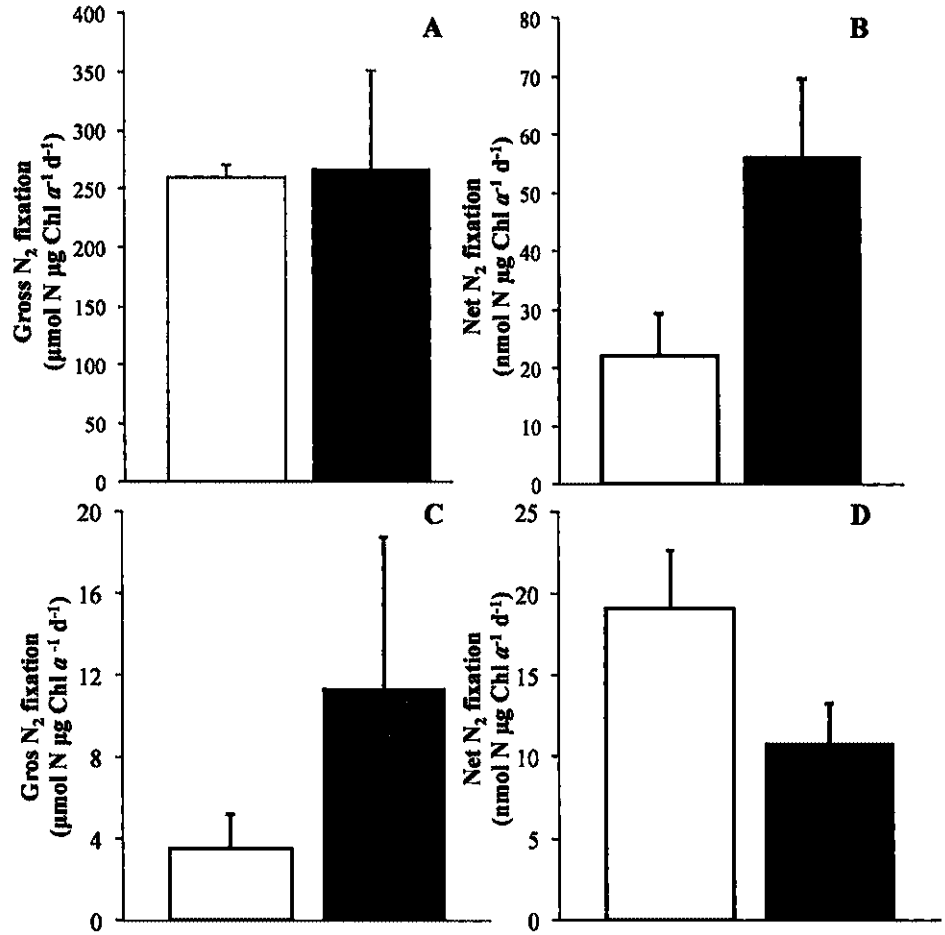

Figure 9. Mean daily N_2 fixation rates measured in steady state semi-continuous cultures of *T. erythraeum* growing at 24°C (A, B) and in continuous cultures of *T. erythraeum* growing at 30°C (C, D) under ambient (white bars) or high pCO_2 (black bars) conditions. Gross N_2 fixation rates (A, C) were estimated using the acetylene reduction method and net N_2 fixation rates (B, D) were determined with $^{15}N_2$. Error bars represent one standard deviation determined from replicate cultures (n=3). A 2-factor ANOVA indicated significant differences in both gross and net N_2 fixation between temperature treatments ($F=104.6$, $p<0.001$, $n=12$ and $F=28.1$, $p=0.0009$, $n=12$). Net N_2 fixation was stimulated by pCO_2 and combined temperature and pCO_2 effects as well ($F=7.83$, $p=0.0273$, $n=12$ and $F=21.3$, $p=0.0034$, $n=12$). Differences between pCO_2 treatments and combined temperature and pCO_2 treatments for gross N_2 fixation were not significant ($F=0.078$, $p=0.787$, $n=12$ and $F=2.241$, $p=0.973$, $n=12$).

Discussion

S. cf elongatus Incubations

Cell-normalized C fixation rates (Fig. 1A) increased significantly in populations of *S.* cf *elongatus* grown at elevated temperature or pCO_2, and C turnover (Fig. 2) increased significantly at high pCO_2, but the interactive effect between temperature and pCO_2 treatments was not significant. Results from experiments using the coastal GOM *S.* cf *elongatus* isolate were consistent with those observed in another study investigating the effects of increased temperature and pCO_2 on the photosynthetic physiology of an oceanic *Synechococcus* sp. isolate. In that study, growth rates increased by a factor of 2.3 and the maximum quantum yield of photosynthesis responded positively to both increases in pCO_2 and temperature independently, and in combination although changes in temperature alone explained the observed significant differences between experimental treatments (Fu et al. 2007). In another study, Lu et al. (2006) showed that growth rates and abundance of a coastal *Synechococcus* sp. isolate increased more in response to increases in temperature and pCO_2 than an oceanic isolate suggesting a high degree of variability that may be due to differences at the genomic level of between ecotypes.

In contrast to culture studies, field experiments performed on natural populations dominated by *Synechococcus* spp. exhibit higher rates of C fixation under experimentally elevated pCO_2 (Lomas et al. 2012). The authors of this study suggest that factors such as nutrient limitation could have limited the ability of *Synechococcus* spp. to respond to increased DIC availability (Lomas et al. 2012). Unlike many oligotrophic natural systems, in culture studies, macro- and micro-nutrients were all supplied at saturating concentrations. A more thorough investigation on the combined and interactive effects between temperature, CO_2 availability, nutrient availability and other environmental stressors on *Synechococcus* spp. is necessary to better estimate this group's response to climate change variables in the environment.

In addition to C fixation rate measurements, I calculated DOC production normalized to *S.* cf *elongatus* POC and found that DOC production was significantly higher in cultures growing at the lower temperature (24°C) and that DOC production did not vary significantly among pCO_2 treatments (Fig 3).

Potential exists for *S.* cf *elongatus* to reallocate energy involved in photosynthetic production under elevated pCO_2 conditions because the E_k for photosynthesis under ambient conditions is approximately double that for *S.* cf *elongatus* acclimated to high CO_2 (Table 3). Since populations of *S.* cf *elongatus* grown under high pCO_2 appear to require half of the incident irradiance to achieve maximum photosynthesis compared to populations acclimated to ambient pCO_2 conditions, quantum efficiencies for photosynthesis may improve for *S.* cf *elongatus* grown under high pCO_2 relative to populations acclimated to ambient conditions. Thus two cultures growing under the same light source will have different photosynthetic yields, growth and C metabolism when grown at different pCO_2 (Table 3).

Under steady state conditions at high pCO_2 *S.* cf *elongatus* decreased mean cell-normalized Chl *a* concentration by 48% and particulate C by 36% (Table 1), whereas respiration rates increased 900% (Table 3). This suggests that C release increased by 133% (Table 2). At the same time, mean specific growth rates of *S.* cf *elongatus* and overall biomass were greater in cultures acclimated at elevated pCO_2 conditions, but differences between treatments were not found to be significant. Therefore *S.* cf *elongatus* isolated from the WFS grew at similar rates regardless of pCO_2 treatment, even though photosynthetic and C fixation rates were higher in *S.* cf *elongatus* cultures acclimated at high pCO_2 relative to those acclimated to ambient pCO_2. I speculate that cultures of *S.* cf *elongatus* increase their respiration rate to accommodate the accelerated release of recently fixed C in cells acclimated to high pCO_2 (Table 3). The reason for the disagreement between DOC production measurements and the calculated theoretical cellular C release remain unclear. However, cyanobacteria have been shown capable of DOC uptake (Paerl et al. 1993, Kang et al. 2004, Andrade & Costa 2007, Yu et al. 2009) and the uptake of recently respired C could have masked its production based on measurements of DOC concentrations.

The variability in growth and C metabolism exhibited in response to elevated pCO_2 and temperature complicate my ability to assign causative factors to responses of *S.* cf *elongatus* cultures unequivocally. By pooling data and calculating overall means for temperature and pCO_2 treatments I was able to identify temperature as the key variable affecting changes in *S.* cf *elongatus* growth rates (Fig. 10). Pooling data for C fixation

rates, I observed that both temperature and pCO_2 effects significantly affected C fixation (Fig.11).

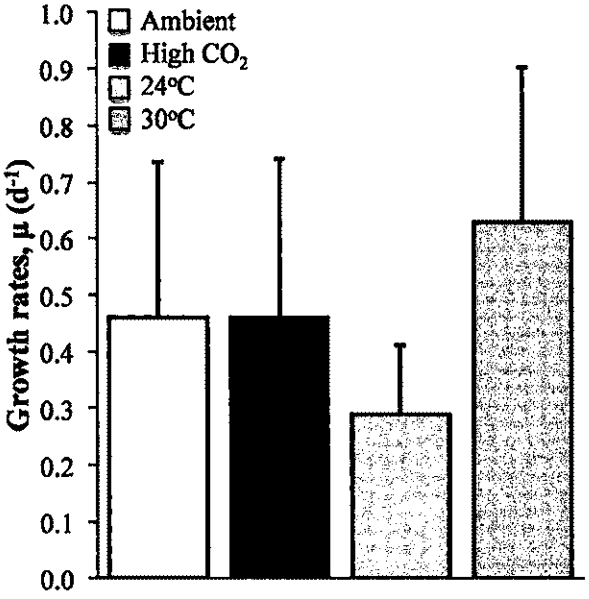

Figure 10. Mean daily growth rates determined by *in vivo* fluorescence pooled from the results of steady state semi-continuous culture experiments on *S.* cf *elongatus* growing at temperatures of 24°C and 30°C, under ambient or high pCO_2 conditions. Error bars represent standard deviations from replicate cultures (n=12). A 2-factor ANOVA indicated that growth rates are significantly increased under elevated temperature conditions (F=9.15, p=0.011, n=24), but are unaffected by pCO_2 and combined pCO_2 and temperature treatments (F=0.16, p=0.697, n=24 and F=0.01, p=0.922, n=24).

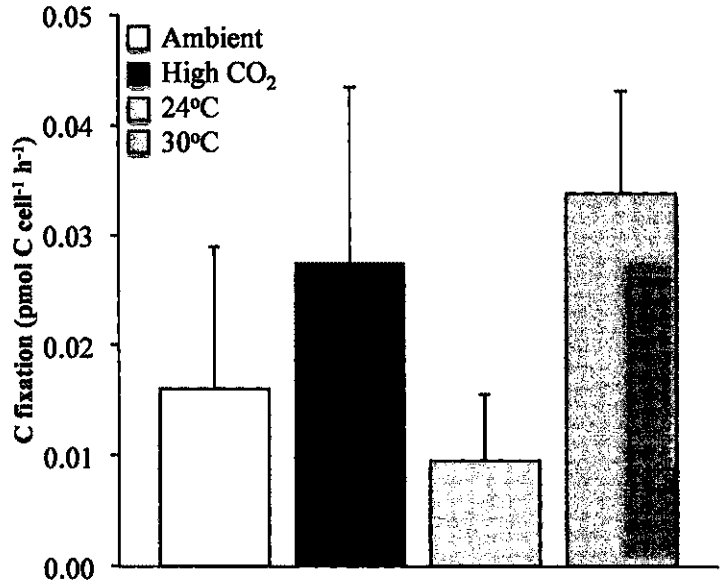

Figure 11. Mean cell-normalized C fixation rates pooled from the results of steady state semi-continuous culture experiments on *S.* cf *elongatus* growing at temperatures of 24°C and 30°C, under ambient or high pCO_2 conditions. Error bars represent standard deviations from replicate cultures (n=12). A 2-factor ANOVA indicated that growth rates are significantly increased under elevated temperature and pCO_2 conditions independently ($F=64.9$, $p<0.001$, $n=24$ and $F=14.0$, $p=0.001$, $n=24$), but are unaffected by combined pCO_2 and temperature treatments ($F=0.64$, $p=0.432$, $n=24$).

Future investigations on the combined effects of pCO_2 and temperature on growth and C fixation by *Synechococcus* spp. are necessary to better understand the natural variability within populations of *Synechococcus* spp. Published studies on coastal and oceanic *Synechococcus* isolates have documented large differences in C fixation in response to changes in temperature and pCO_2 between ecotypes (Lu et al. 2006). Research on the magnitude of difference in physiological rates between species ecotypes has found similar magnitudes of difference between physiological comparisons of different phytoplankton genera (Schaum et al. 2012). The variability between ecotypes of the same species needs to be fully explored before results from this study can be extrapolated to basin or global scales.

While provocative, the suggestion that the enhanced rates of C fixation observed in cultures of *S.* cf *elongatus* grown at high pCO_2 are translated into increased respiration

rates rather than increased growth, a replicated P vs. E experiment is warranted to verify this result statistically. Further, the effect of temperature was not assessed since the data presented here were limited to populations grown at 30°C where growth rates were already higher than those grown at 24°C. In addition, *S.* cf *elongatus* has been documented to exhibit differences in photoacclimation periods based on C status (Mackenzie et al. 2004) and so the impact of light acclimation on P vs. E results should be considered in future experiments.

T. erythraeum incubations

Carbon fixation increased significantly at high pCO_2 for all culture experiments conducted on *T. erythraeum.* Chl *a* concentrations, growth rates, and N fixation rates (both gross and net) showed no significant differences between pCO_2 treatments for the batch culture experiments conducted at 30°C, and statistically significant differences in Chl *a*, growth rate and C turnover were not determined for *T. erythraeum* growing in steady state under 24°C or 30°C. Gross and net N_2 fixation were significantly affected by temperature, but only net N_2 fixation varied significantly over pCO_2 and combined temperature and pCO_2 treatments, prohibiting generalized conclusions on the effect of increased pCO_2 on N_2 fixation of *T. erythraeum* in this study. However, many of the observations made as part of this study agree with published results from CO_2 enrichment experiments done previously with cultured *T. erythraeum* populations (Levitan et al. 2007, 2010; Kranz et al. 2011, Hutchins et al. 2007, Barcelos e Ramos et al. 2007) and *Trichodesmium thiebautii* populations (Lomas et al. 2012).

Growth rates in *T. erythraeum* batch cultures grown at 30°C did not show a stimulation in response to elevated pCO_2 as has been reported in many other studies of *T. erythraeum* growing under 25°C (Levitan et al. 2007, 2010; Kranz et al. 2011). Though not statistically different from one another, exponential phase growth rates measured for *T. erythraeum* in this study were 25% higher in elevated pCO_2 treatments grown at 30°C. This agrees with the observed range of pCO_2 enhanced growth rates (~25-29%) observed in semi-continuous cultures grown at 25°C reported by Hutchins et al. (2007) and Barcelos e Ramos et al. (2007).

Like *S.* cf *elongatus*, C fixation by *T. erythraeum* was enhanced in cultures growing at elevated pCO_2 (Figs. 6 & 7). In batch cultures acclimated to elevated pCO_2, C fixation rates by *T. erythraeum* increased by 77%, 233% and 156% during lag, exponential and stationary growth phases, respectively in cultures acclimated to 30°C at elevated versus present day pCO_2. Additionally, C fixation rates measured in steady-state cultures acclimated to elevated pCO_2 were always greater than those measured under ambient conditions, regardless of temperature treatment. Under steady state continuous or semi-continuous culture conditions acclimated to ambient and elevated pCO_2, C fixation increased by 185% in cultures grown at 24°C, and increased 111% for populations grown at 30°C. Increases in C fixation under elevated pCO_2 have been observed previously for *T. erythraeum*, however the magnitude of this increase varied among studies. For example, in semi-continuous cultures of *T. erythraeum*, increases in C fixation in response to elevated pCO_2 ranged from 28% - 51% in cultures of the Atlantic isolate (IMS101) (Kranz et al. 2011, Hutchins et al. 2007), 15-54% in a Great Barrier Reef isolate from the Pacific Ocean (GBRTRLI101), and just 13% in short term incubations of natural populations of *T. thiebautii* at the Bermuda Atlantic Time-series Study (BATS) station (Lomas et al. 2012). Combined, these data suggest that primary production by this organism will be enhanced with increasing pCO_2 under future climate scenarios. If increases in C fixation translate into increased growth rates in the natural environment, blooms of *T. erythraeum* could grow in size in response to climate change in the GOM and elsewhere. Potentially larger blooms of *T. erythraeum* are likely to release a greater load of new N to the environment especially if these populations also fix N_2 at the elevated rates, as has been observed in previous culture studies (Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007) and this could stimulate the growth of co-occurring phytoplankton (Mulholland et al. 2004, 2014).

In contrast to previous studies, statistically significant changes in N_2 fixation in response to temperature and pCO_2 treatments could not be made based on gross N_2 fixation, though elevated pCO_2 was found to cause significant differences in net N_2 fixation for steady state cultures of *T. erythraeum* in this study. Gross N_2 fixation rates between replicate cultures were not significantly different from one another with regard to pCO_2, but temperature caused significant differences in rates observed at 24°C and

30°C. The combined effects of temperature and pCO_2 resulted in significantly higher net N_2 fixation rates in cultures acclimated at 24°C and elevated pCO_2, but combined pCO_2 and temperature effects did not cause significant differences in gross N_2 fixation rates in this study (Fig. 9). For *T. erythraeum* IMS101, gross N_2 fixation increased 35-400% (Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007, 2010) and for *T. erythraeum* GBRTRLI101 gross N_2 fixation increased by 63% (Hutchins et al. 2007) in response to elevated pCO_2 in semi-continuous cultures grown at 25°C. Increases in gross N_2 fixation of 35% (Hutchins et al. 2007) and 114% (Levitan et al. 2010) were measured in populations of IMS101 acclimated to 25 or 31°C, respectively, and N_2 fixation rates by natural populations of *T. thiebautii* collected at the BATS station increased by 54% at elevated pCO_2 on average under temperature conditions ranging from 27-29°C (Lomas et al. 2012).

Methodological reasons may account for some of the variability in our observed pCO_2 effects on N_2 fixation. The majority of studies examining the effects of pCO_2 on N_2 fixation by *Trichodesmium* spp. employed the acetylene reduction method, which estimates gross N_2 fixation (Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007, 2010). Both acetylene reduction and $^{15}N_2$ tracer methods (which measures net N_2 fixation) (Mulholland et al. 2006) were used to determine N_2 fixation in this study. Net N_2 fixation was stimulated at high pCO_2 only in cultures of *T. erythraeum* acclimated at 24°C. In cultures acclimated at the higher temperature, only gross N_2 fixation was stimulated (Fig. 9). This suggests that the recently fixed N_2 may not have been incorporated into biomass in cultures grown at the higher temperature, rather it could have been released (Mulholland et al. 2006). If enhanced N_2 fixation at higher temperature and pCO_2 results in enhanced rates of release of bioavailable N, this could have a great impact on planktonic communities that are usually limited by the availability of N in environments were *Trichodesmium* spp. occur (Carpenter & Capone 2008).

Rates of net N_2 fixation measured in this study employed the $^{15}N_2$ bubble method and were an order of magnitude lower than those obtained measuring gross N_2 fixation by acetylene reduction (Fig. 9) indicating that methodological differences could have come into play in these experiments. The bubble method is thought to underestimate net N_2 fixation because the $^{15}N_2$ gas addition may not be fully equilibrated over the course of

incubation experiments leading to an underestimate of net N_2 fixation (Mohr et al. 2010, Großkopf et al. 2012). While probably underestimates of N_2 fixation, statistically significant differences in rates of N_2 fixation rates between treatments were observed when using the $^{15}N_2$ bubble method but not when using the acetylene reduction method because of the large variability between replicate cultures. Additional investigation into the effect of pCO_2 on N_2 fixation in cultured populations of *T. erythraeum* is warranted, and should include sample sizes greater than three to improve the power of detection of statistically significant differences between treatment groups.

Release of recently fixed N_2 by *Trichodesmium* spp. can be substantial (Mulholland et al. 2004, 2006, Lenes & Heil 2010) so any increase to the stock of *Trichodesmium* spp. in the GOM or any increase in the release of recently fixed N_2 could provide new N to fuel *K. brevis* production (Sipler et al. 2013) or that of co-occurring microbes (Mulholland & Capone 2001, Sheridan et al. 2002).

Environmental conditions such as nutrient and light availability also play an important role in the diazotrophic capability of *Trichodesmium* spp. (Carpenter & Capone 2008). Decreases in rates of N_2 fixation have been observed in phosphorus (P) limited cultures (Mulholland & Bernhardt 2005). Decreases in growth rates may occur under conditions of depleted P concentrations because *T. erythraeum* must divert energy from growth into increasing alkaline phosphatase activity to satisfy its P demands using the organic P pool (Mulholland et al. 2002). However, increases in rates of N_2 fixation in cultures of *T. erythraeum* at high pCO_2 were independent of available P concentrations (Hutchins et al. 2007). Available iron (Fe) concentrations can also limit diazotrophic growth because this element is required in higher amounts in N_2 fixing organisms (Carpenter & Capone 2008). During one Saharan dust deposition event on the WFS in the GOM, researchers documented a two order of magnitude increase in *Trichodesmium* spp. populations, and dissolved organic N concentrations also increased three to four times, suggesting that the influx of available Fe stimulated growth and N_2 fixation by *Trichodesmium* spp. as well as the release of recently fixed N_2 (Lenes et al. 2001). Future studies assessing the effects of elevated pCO_2 on *Trichodesmium* spp. N_2 fixation should consider Fe and other potentially limiting elements that may have interactive effects with pCO_2 and temperature under future climate conditions. Light levels also

impact rates of N_2 fixation by *T. erythraeum* through the production of energy. CO_2 stimulation of N_2 fixation in cultures of *T. erythraeum* was highest at sub-saturating light levels representative of mid- and bottom-level regions of the euphotic zone in comparison with saturating light levels observed in the surface oceans (Garcia et al. 2011), indicating that *T. erythraeum*'s position in the water column, and incident light levels in the surface waters of the GOM will also play a role in determining the extent to which N_2 fixation increases under future climate scenarios.

Conclusions

I found that coastal *S.* cf *elongatus* and *T. erythraeum* are both affected by increases in temperature and pCO_2. Carbon fixation appears to be C limited for both species of cyanobacteria studied. Reallocation of energy for biosynthesis is likely when CO_2 concentrations are elevated and active inorganic C transport requires less energy to achieve maximal C fixation rates. However, much work remains to be performed to better understand the impacts of climate change on marine cyanobacteria including diazotrophs. Culture studies are inherently variable and larger experimental replication would improve the statistical power to draw conclusions. Because of the labor-intensive nature of these studies, small sample sizes in this and other studies done to date yield a high degree of variability within and between studies. Future studies should also consider multiple stressors that impact rates of cyanobacterial growth and N_2 fixation in the environment. While culture studies are useful, it is paramount that we be able to extrapolate results to natural populations of cyanobacteria in the environment. Currently, there is no practical means to incorporate factors such as variability in the physiological status of natural populations, the impact of grazers, and changes in light and stratification as populations are mixed in the surface waters. Increases in temperature and pCO_2 associated with climate change will likely shift the ecology and biogeochemical function of these quantitatively important cyanobacterial groups in the GOM and elsewhere. Increasing abundances of cyanobacteria and release of organic C and various forms of N in species with diazotrophic capability will alter dissolved nutrient and organic matter concentrations under elevated temperature and pCO_2 in the future. These changes are likely to fundamentally alter foodwebs. In the GOM where *K. brevis* blooms plague the

coastal economy, blooms may become more intense if the growth and activity of these cyanobacterial groups are favored in the future and their production enhances the provision of nutrients to fuel their growth.

CHAPTER III

EFFECT OF CARBON DIOXIDE ENRICHMENT ON NATURAL PHYTOPLANKTON POPULATIONS ISOLATED FROM THE GULF OF MEXICO OVER THE WESTERN FLORIDA SHELF

Introduction

Several experiments have demonstrated dramatic changes in phytoplankton growth, photosynthetic rates and community composition in response to elevated pCO_2 (Hein & Sand-Jensen 1997, Tortell et al. 2002, 2008; Fu et al. 2007, 2008a,b; Feng et al. 2008, 2009; Hutchins et al. 2007, 2009; Riebesell et al. 2007). However, few to date have examined community responses to the impact of CO_2 enrichment in systems experiencing harmful algal blooms (HABs) (Fu et al. 2007). Along the WFS in the GOM, the red tide dinoflagellate *K. brevis* exerts devastating impacts on the economy of the region. *K. brevis* blooms are responsible for numerous adverse effects to human health (neurotoxic shellfish poisoning and respiratory irritation from brevetoxin production), marine fisheries, and tourism (Kirkpatrick et al. 2004) in western coastal communities in Florida. Much work has been done to improve our understanding of the physiology and ecology of *K. brevis* blooms on the WFS (Tester & Steidinger 1997, Walsh & Steidinger 2001, Mulholland et al. 2002,Vargo et al. 2002, 2008; Magaña & Vilareal 2006, Redalje et al. 2008, Paerl et al. 2008, Vargo 2009), but most studies have yet to quantify the nutritional contribution of co-occurring phytoplankton populations other than *Trichodesmium* spp. to the nutrient budget required to sustain reoccurring red tides in the region.

Elevated pCO_2 has been show to stimulate N_2 and C fixation rates. As discussed in the previous chapter, stimulation of N_2 fixation by *T. erythraeum* in response to high pCO_2 could result in additional new N inputs to the WFS that could stimulate *K. brevis* growth. Increases in N_2 fixation rates by *Trichodesmium* spp. acclimated to about twice the present day pCO_2 range from 35 to 400% (Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007, 2010; Barcelos e Ramos et al. 2007, Lomas et al. 2012, Chapter 2). Carbon fixation rates by *T. erythraeum* more than doubled in cultures acclimated under

elevated pCO_2 conditions (Chapter 2), consistent with a 39% to 51% increase documented in another study (Hutchins et al. 2007). For another diazotrophic cyanobacterium, *Crocosphaera*, C and N_2 fixation rates increased by 140-180% and 120-200%, respectively, under elevated pCO_2 (750 ppm) compared to rates measured at ambient and lower pCO_2 (Fu et al. 2008). Together these findings indicate CO_2 enrichment affects both C and N metabolism in cyanobacterial diazotrophs.

It has been estimated that more than half of the recently fixed N_2 is rapidly released by *Trichodesmium* spp. into the environment as organic N (Mulholland et al. 2006, 2014; Wannicke et al. 2009) contributing to available pools of dissolved N and C that can support growth of co-occurring organisms (O'Neil et al. 1996, Mulholland et al. 2004a, b, 2006; Mulholland 2007, Sipler et al. 2013), including *K. brevis* (Mulholland et al. 2004a, 2014; Sipler et al. 2013). Past measurements documented that a bloom of *Trichodesmium* spp. may have supplied up to 40% of the N required by the 2001 WFS *K. brevis* bloom, and that the nutrients released during decay of a co-occurring *Trichodesmium* spp. bloom could have supplied *K. brevis* with nutrients necessary to sustain a large populations for two months (Lenes & Heil 2010, Mulholland et al. 2009). If *Trichodesmium* spp. continue to play a large role in fueling *K. brevis* blooms, future climate conditions will likely increase nutrient flow from *Trichodesmium* spp. to *K. brevis* through the release and bioavailability of dissolved labile compounds.

Lyngbya spp., another co-occurring diazotrophic non-heterocystous group of cyanobacteria common in the coastal regions and tributaries of the GOM, may also contribute new N to support *K. brevis* blooms (Paerl et al. 2008). Over the last decade, *Lyngbya* spp. has proliferated. Blooms are more frequent and persistent and appear to be spreading geographically in coastal zones around the world (O'Neil 2012). Rising temperatures have been suggested as one possible contributor to the geographic expansion and increase in *Lyngbya* spp. abundance worldwide (O'Neil 2012, Watkinson et al. 2005). However, to date no experiments have been conducted to determine the direct impact increasing pCO_2 might be having on the production of this species.

Dinitrogen and C fixation by diazotrophs other than *Trichodesmium* spp. and *Lyngbya* spp. may also contribute bioavailable N on the WFS and these groups may also experience metabolic stimulation under elevated pCO_2, however, little is known about the

response of other diazotrophic cyanobacterial groups to higher temperatures and elevated pCO_2. Unculturable picocyanobacteria are distributed widely and in oceanic realms previously thought to be inhospitable to diazotrophic groups (Moisander et al. 2010, Mulholland et al. 2012). It is unclear whether these groups might also be metabolically stimulated under future climate scenarios and contribute new N and fixed C to these ecosystems. Picocyanobacteria without diazotrophic capabilities can also contribute to the nutritional status of *K. brevis* in the GOM. *Synechococcus* cf *elongatus*, a non-diazotrophic cyanobacterium that commonly blooms in the GOM, is grazed upon by *K. brevis* (Jeong et al. 2005, Glibert et al. 2009, Procise 2012). A study investigating the effects of increased temperature and pCO_2 on the photosynthetic physiology of oceanic *Synechococcus* isolates found that growth rates and photosynthetic yields responded positively to both increases in pCO_2 and temperature independently, and in combination (Lu et al. 2006, Fu et al. 2007). Carbon fixation and turnover was also stimulated in a coastal *S.* cf *elongatus* isolate from the WFS in response to increases in pCO_2 and temperature (Chapter 2). Consequently, potential increases in the abundance and activity of *S.* cf *elongatus* could enhance the availability of prey to *K. brevis* under future climate scenarios.

Along the WFS in the GOM, it is unclear how increases in temperature and pCO_2 will affect community primary production, cyanobacteria-specific production, N_2 and C fixation and extracellular nutrient release. It is also unclear how these potential impacts may affect the proliferation of harmful *K. brevis* blooms under future climate conditions. Here, I examine the effects of increased pCO_2 on the growth, and C and N_2 fixation by natural WFS populations that included *Trichodesmium* spp., *Lyngbya* spp., and a mixed coastal microbial community.

Methods

Phytoplankton collection and incubation methods

During 2007, short-term CO_2 enrichment experiments were conducted on natural populations of *Trichodesmium* spp. to determine whether N_2 fixation was enhanced (Hutchins et al. 2009). In October 2009, samples were collected to measure the short-term effects of elevated pCO_2 on N_2 and C fixation in natural planktonic assemblages and

in populations dominated by *Lyngbya* spp. collected along the WFS in the GOM. Experiments were conducted aboard the *R/V Pelican*.

Trichodesmium spp.

From 23-26 October 2007 *Trichodesmium* spp. filaments and colonies were collected daily along a cruise track on the WFS in the GOM using a 202 μm mesh phytoplankton net. *Trichodesmium* sp. colonies were isolated using inoculating loop and placed into three replicate incubation bottles containing 0.2μm filtered seawater equilibrated to ambient (~386 ppm) or elevated pCO_2 (750 ppm) and acclimated to these conditions over 4 days (Hutchins et al. 2009). Each day, C and N_2 uptake incubations were initiated by adding highly enriched (96-99%) ^{13}C or ^{15}N-labeled HCO_3^- or N_2 gas to three replicate bottles. Incubation bottles were placed in deck-board, flow through incubators and covered with a single layer of neutral density screen for 4 to 6 hours during daytime. Incubation experiments were terminated by gentle filtration through pre-combusted (450°C for 2 h) Whatman GF/F filters (pore size ~ 0.7 μm). Some of the N_2 fixation data for this experiment were published in Hutchins et al. (2009) and are redrawn and presented with C fixation data in greater detail here. Experiments were performed prior to my starting my thesis work at ODU, but I was involved in the sample and data processing for these incubations.

Lyngbya spp.

Populations of the filamentous diazotrophic cyanobacteria *Lyngbya* spp. were collected from floating mangrove propagules in surface waters along the same cruise track during 6-8 October 2009. *Lyngbya* spp. samples were removed from collected propagules and combined in a clean bucket of 0.2μm FSW before being apportioned by wet weight in the Florida Fish and Wildlife Institute laboratories into two sets of three replicate 2L polycarbonate incubation bottles. Replicate bottles were bubbled with either ambient (~386 ppm) or CO_2-enriched (750 ppm) air mixtures and each bottle was inoculated with four *Lyngbya* spp. mat filament fragments weighing 0.1g (wet weight) each. Incubations were placed in deck-board, flow through incubators and covered with a single layer of neutral density screen. Five hundred milliliters water was removed and

replaced with fresh 0.2μm FSW daily to prevent nutrient depletion in incubation bottles. After 2 days, treatment and control bottles were sacrificed and the four mat fragments were used to measure: C and N_2 fixation, and Chl *a*, PN, and PC concentrations. Samples were wrapped in foil, frozen immediately and were transported to Old Dominion University (ODU) where their final wet weights were measured to estimate growth rates. Growth rates were calculated based on the differences in wet weight at the start and termination of the two-day incubation. Weight was used to measure abundance for *Lyngbya* spp. because of the difficulty in accurately counting cells or measuring Chl *a* content in this densely matted cyanobacterium. Filtrate (0.2 μm) was reserved for DOC analyses.

Natural planktonic populations

Natural phytoplankton community assemblages were collected using a plankton net (202 μm) towed at 10m at a station located at 26° 25.84 N', 82°00.51 W' around mid-day on October 4, 2009. Populations were dominated by diatoms. Natural water collected from the same station was filtered through a 0.2 μm Supor filter, placed in three replicate 2L polyethylene bottles, and amended with forty milliliters of concentrated plankton. Replicate bottles were bubbled with either ambient (~386 ppm) or CO_2-enriched (750 ppm) air mixtures as described in detail below. Incubation bottles were placed in a deck-board incubator and covered with two layers of neutral density screen to simulate light conditions at the depth where the plankton community was collected. Nutrient limitation was alleviated by exchanging 300 mL of incubation volume (15% of total volume) with 0.2 μm FSW collected from the same station and pre-equilibrated to the appropriate pCO_2. Incubations were terminated after 2 days and samples were collected to measure concentrations of Chl *a*, PN, PC, and DOC and rates of C and N_2 fixation.

pH/pCO₂ manipulation

Target pCO_2 treatments were achieved using constant gentle aeration with premixed 750 ppm pCO_2 compressed gas or ambient air delivered via an aquarium pump for the high pCO_2 and ambient pCO_2 treatments, respectively (Rost et al. 2008). Use of

standard aquarium air stones maximized diffusion of the gas bubbles into incubation media by delivering micro-bubbles with a greater surface area to volume ratio. The diffusive air stone was affixed to the end of inlet tubing and suspended near the base of each individual incubation bottle, allowing bubbles to travel through the water column before filling the head space of the bottle. Outlet tubing connected through the top of the bottle served as a vent for the incubation headspace, maintaining a headspace CO_2 concentration approximately equal to the seawater CO_2 concentration.

CO_2 concentrations were measured in DIC samples collected at the termination of experimental incubations to ensure that algal populations did not substantially drawdown CO_2 (>5%) over the course of incubations.

Measuring physiological response and incubation conditions

Sample analysis

Biomass and nutrient concentrations. Chl *a* concentrations were determined using a standard fluorometric method and growth rates were calculated using either wet weight or Chl *a* concentration as a metric for biomass using standard equations (Welschmeyer 1994). Samples for particulate N (PN) and particulate C (PC) were collected by filtering a known sample volume onto a combusted (450°C for 2 h) GF/F filter, samples were immediately frozen until analysis. Samples were thawed and desiccated for a minimum of 4 days at 40°C before pelletizing samples for analysis. Samples were analyzed on a Europa automated nitrogen and carbon analyzer (ANCA).

At the termination of experiments, samples were filtered through 0.2μm Supor membrane filters and the filtrate was reserved for analysis of DOC and ammonium. Samples were frozen at < -20°C and analyzed as soon as possible. NH_4^+ was determined using the phenolhypochlorite method (Solorzano 1969). DOC was analyzed by high temperature combustion (Burdige et al. 1999). Whole water DIC samples were also collected at the termination of incubations. Samples were gently transferred to a 30mL serum vial until over flowing using a 60mL syringe and piece of silicone tubing. Care was taken to avoid exposure of samples to air and samples were fixed adding $HgCl_2$ (final concentration of 0.05% $HgCl_2$) to avoid biological activity in collected samples.

Samples were crimp sealed, and refrigerated at <4°C until analysis. Samples were analyzed using standard coulometric methods (DOE 1994).

C and N_2 fixation rate measurements and C turnover times

To measure bicarbonate uptake, samples were enriched by 10% with highly enriched (98%) $NaH^{13}CO_3$. Samples were incubated for 12 h in the light and then terminated by filtration. For N_2 fixation rate measurements by *Trichodesmium* spp. and *Lyngbya* spp. incubation bottles were amended with 1 µL 99.99% $^{15}N_2$ gas per mL seawater and incubated in gas tight Wheaton bottles for 12 hours before terminating experiments via gentle filtration (Mulholland et al. 2006). Uptake rates were calculated based on ^{15}N and ^{13}C enrichment of particles at the end of the incubations relative to the natural abundance of these isotopes in PN and PC prior to isotope enrichment (Montoya et al. 1996, Mulholland & Capone 2001, Mulholland & Bernhardt 2005, Mulholland et al. 2006). Theoretical C turnover due to phytoplankton was calculated by dividing C specific uptake rates (µmol C L^{-1} h^{-1}) by the particulate C pool.

Results

Trichodesmium spp. incubations

At the end of each daily incubation period, rates of C and N_2 fixation were significantly higher in elevated pCO_2 treatments (750 ppm) than in controls maintained at ambient pCO_2 concentrations (Fig. 12). A paired t-test indicated significant differences between pCO_2 treatments for N_2 fixation and C fixation ($t=-3.45$, $p=0.007$, $n=20$ and $t=-1.92$, $p<0.0001$, $n=24$). The data for C fixation was not normally distributed so a customized t-table was created based on 10,000 randomized iterations of the dataset to perform the t-test (Manly 1997).

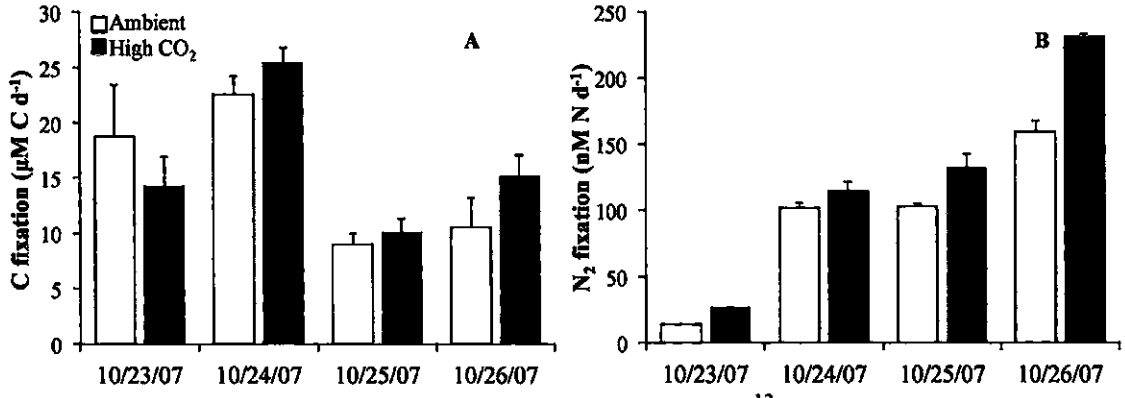

Figure 12. Mean daily C uptake rates (A) measured with $H^{13}CO_3^-$ and daily N_2 Fixation rates (B) measured with $^{15}N_2$ in natural populations of *Trichodesmium* spp. incubated under ambient (white bars) and high (750 ppm) (black bars) pCO_2 during a 2007 research cruise along the WFS in the GOM. Error bars represent one standard deviation determined from replicate incubations (n=3), with exception of N_2 fixation measured on 10/23/07 where only one incubation was performed at each pCO_2 treatment. A paired t-test indicated significant differences between C and N_2 fixation pCO_2 treatments (=-1.92, $p<0.0001$, n=24 and t=-3.45, p=0.007, n=20).

Whole community incubation

Over the two-day incubation period Chl *a* concentrations and picocyanobacteria abundance declined substantially in the incubation bottles (Table 5). Results from a t-test indicated that DOC and NH_4^+ concentrations were unaffected by pCO_2 manipulations (t=-0.792, p=0.487, n=3 and t=0.215, p=0.850, n=3).

Table 5. Mean Chl *a*, PC, PN, DOC, and NH_4^+ concentrations in mixed plankton communities acclimated under present day (ambient) and elevated pCO_2 for 2 days. Initial samples were collected from the GOM on 4 October 2009. N=3, with exception of the initial NH_4^+ measurements where one replicate sample was discarded due to contamination. A t-test indicated no significant differences in DOC and NH_4^+ were caused by pCO_2 manipulations (t=-0.792, p=0.487, n=3 and t=0.215, p=0.850, n=3).

T, CO_2 Treatment	Chl *a* $\mu g\ L^{-1}$	PC μM	PN μM	DOC μM	NH_4^+ μM
T_o, Ambient	0.92 (0.27)	n.d.	n.d.	n.d.	0.59 (NA)
T_f, Ambient	0.42 (0.11)	213 (37.8)	14.6 (6.90)	213 (0.5)	0.82 (0.06)
T_f, High CO_2	0.38 (0.13)	169 (11.3)	8.55 (1.35)	256 (2.4)	0.96 (1.78)

Carbon fixation and C turnover were not stimulated for the community exposed to elevated CO_2 conditions relative to the community maintained under ambient pCO_2 conditions at the end of the two-day incubation period (Figs. 13 & 14). A t-test indicated that C fixation and C turnover were not significantly affected by pCO_2 manipulations (t=-1.70, p=0.330, n=3 and t=1.56, p=0.340, n=3). Carbon fixation measured 0.35 ± 0.15 μmol C L^{-1} h^{-1} in elevated pCO_2 incubations and 17 ± 0.03 μmol C L^{-1} h^{-1} in the ambient pCO_2 incubations on average at the end of the experiment (Fig. 13). Mean C turnover measured 0.0248 ± 0.0124 d^{-1} in the high pCO_2 treatment and 0.0098 ± 0.0036 d^{-1} in the ambient pCO_2 control bottles (Fig. 14).

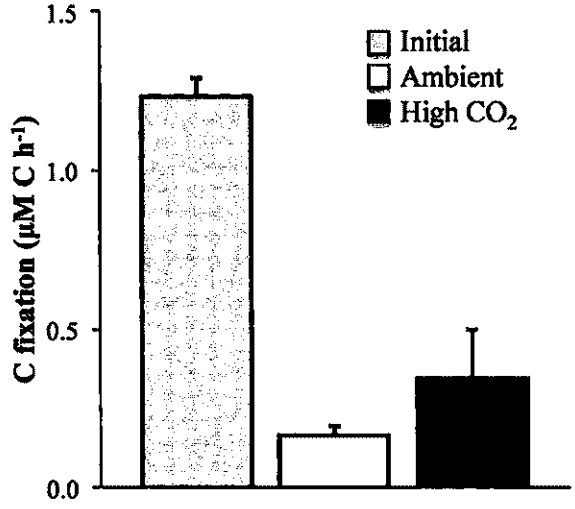

Figure 13. Mean C fixation rates measured in a natural plankton assemblage collected along the WFS in the GOM (grey bar) and after a 2-day incubation at either ambient (white bar) or elevated (black bar) pCO_2. Error bars represent one standard deviation determined from replicate samples and statistical differences between pCO_2 treatments were not observed based on results from a t-test (t=-1.70, p=0.330, n=3).

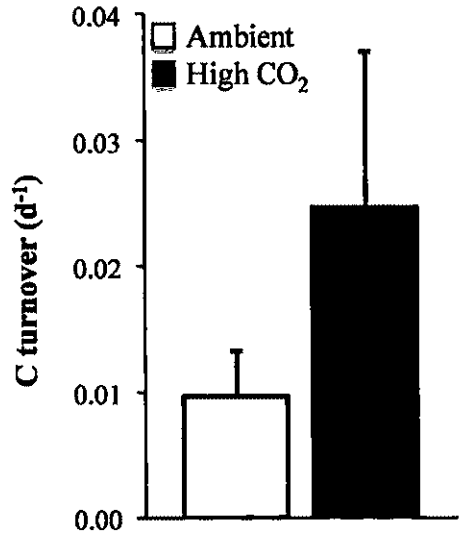

Figure 14. Mean plankton community C turnover due to photosynthesis in natural plankton communities from the WFS in the GOM incubated for 2 days under ambient (white bar) and high (black bar) pCO_2. Error bars represent one standard deviation determined from replicate samples and statistical differences between pCO_2 treatments were not observed based on results from a t-test (t=1.58, p=0.340, n=3).

Dinitrogen fixation rates also decreased in the final measurements after the two-day incubation period relative to initial measurements. Initial measurements of N_2 fixation rates made prior to the two-day incubation period measured 2.05 ± 0.60 nmol N L^{-1} h^{-1}. Two days later, final measurements of N_2 fixation measured 0.59 ± 0.05 nmol N L^{-1} h^{-1}at ambient and 1.10 ± 0.16 nmol N L^{-1} h^{1}elevated pCO_2 (Fig. 15). A t-test indicated no significant difference between N_2 fixation for the two pCO_2 treatments (t=-4.44, p=0.120, n=3).

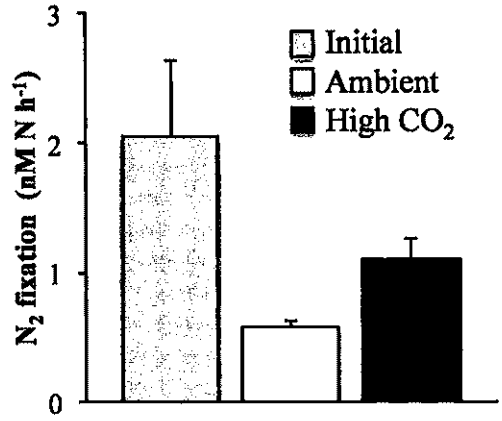

Figure 15. Mean hourly N_2 fixation rates measured in natural plankton assemblages collected from along the WFS in the GOM in October 2009 (grey bar) and after a 2-day incubation under ambient (white bar) and elevated (750 ppm) pCO_2 (black bar). Error bars represent one standard deviation determined from replicate samples. Dinitrogen fixation did not vary significantly between pCO_2 treatments based on results from a t-test (t=-4.44, p=0.120, n=3).

Chl-normalized rates of C and N_2 fixation measured in natural assemblages incubated at elevated pCO_2 were similar to those measured in water samples prior to the two-day incubation experiment (Fig. 16). An independent samples t-test indicated no significant difference between pCO_2 treatments for Chl-normalized rates of C or N_2 fixation (t=-1.25, p=0.428, n=3 and t=-1.63, p=0.345, n=3).

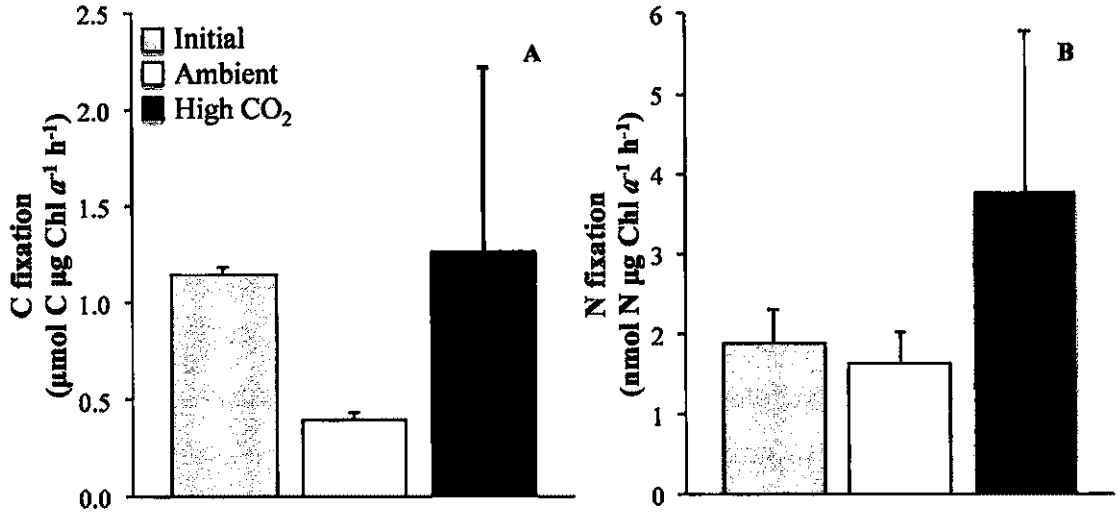

Figure 16. Mean hourly Chl-normalized C (A) and N_2 (B) fixation rates in natural plankton assemblages collected from the fluorescence maximum along the WFS in the GOM immediately after collection (grey bars), and after 2-day incubations under ambient (white bars) and elevated pCO_2 (black bars). Error bars represent one standard deviation determined from replicate samples or incubations and rates of C and N_2 fixation did not vary significantly with pCO_2 ($t=-1.25$, $p=0.428$, $n=3$ and $t=-1.63$, $p=0.345$, $n=3$).

DOC production per unit C biomass (PC) measured 1.51 ± 0.15 pg DOC (pg PC)$^{-1}$ under elevated pCO_2 and 1.04 ± 0.44 pg DOC (pg PC)$^{-1}$ under ambient pCO_2. A t-test indicated that the difference between pCO_2 treatments was not significant ($t=-1.75$, $p=0.198$, $n=3$) (Fig. 17).

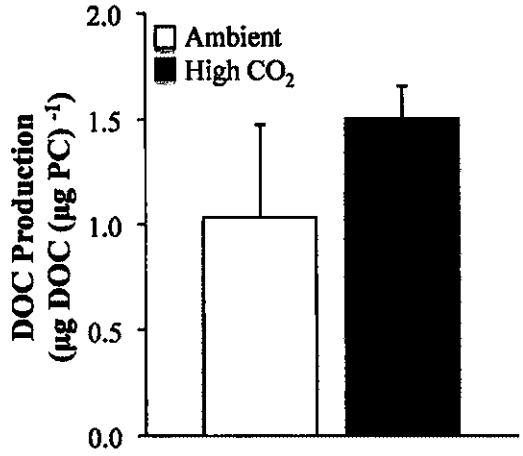

Figure 17. Mean biomass (PC)-normalized DOC concentrations measured in natural plankton communities from the WFS in the GOM at the termination of a 2-day incubation under ambient (white bar) and elevated (black bar) pCO_2. Error bars represent one standard deviation determined from replicate samples. A t-test indicated no significant difference in DOC production measured at high pCO_2 relative to measurements at ambient pCO_2 (t=-1.75, p=0.198, n=3).

Lyngbya spp. incubation

Growth rates calculated based on the change in wet weight mass were not significantly different in ambient and elevated CO_2 treatments determined with a t-test (t=-0.762, p=0.489, n=3) (Table 6). Similarly, biomass-normalized Chl *a* concentrations and NH_4^+ concentrations were unaffected in *Lyngbya* spp. populations incubated under varying levels of pCO_2 according to results from a t-test (t=0.277, p=0.796, n=3 and t=-0.524, p=0.628, n=3) (Table 6).

Table 6. Mean biomass-normalized Chl *a* concentrations, growth rate (based on increase in wet weight of mat fragments) and NH_4^+ concentrations measured after 2-day incubations of *Lyngbya* spp. at ambient and elevated (750 ppm) pCO_2. Statistically significant differences were not observed between pCO_2 treatments for Chl *a*, µ, or NH_4^+ concentrations using a t-test (t=0.277, p=0.796, n=3, t=-0.762, p=0.489, n=3 and t=-0.524, p=0.628, n=3, respectively).

Treatment	Chl *a* mg g_{ww}^{-1}	μ_{ww} d^{-1}	NH_4^+ µM
Ambient	368 (222)	0.16 (0.05)	3.69 (1.67)
High CO_2	417 (208)	0.19 (0.06)	4.77 (3.13)

Absolute C and N_2 fixation rates were not calculated for *Lyngbya* spp. because sample masses exceeded the highest standard used to calibrate the automated C and N analyzer. However, the transport rate of $^{15}N_2$ from the dissolved to the particulate pool was calculated and used to estimate the absolute N_2 fixation rate. Results from a t-test indicated that rates of N_2 fixation did not vary significantly between pCO_2 treatments (t=-1.89, p=0.199, n=3). Dinitrogen fixation rates measured 0.051 ± 0.002 d^{-1} when growing under high pCO_2 and 0.015 ± 0.014 d^{-1} at ambient pCO_2 (Fig. 18A).

DOC concentrations were similar between pCO_2 treatments for *Lyngbya* spp. Mean concentrations of DOC under ambient pCO_2 were 149± 34 µM and DOC concentrations in *Lyngbya* spp. populations exposed to high pCO_2 were 176 ± 65 µM (Fig. 18B). Statistically significant differences in DOC concentration were not observed between pCO_2 treatments based on results from a t-test (t=-0.639, p=0.557, n=3).

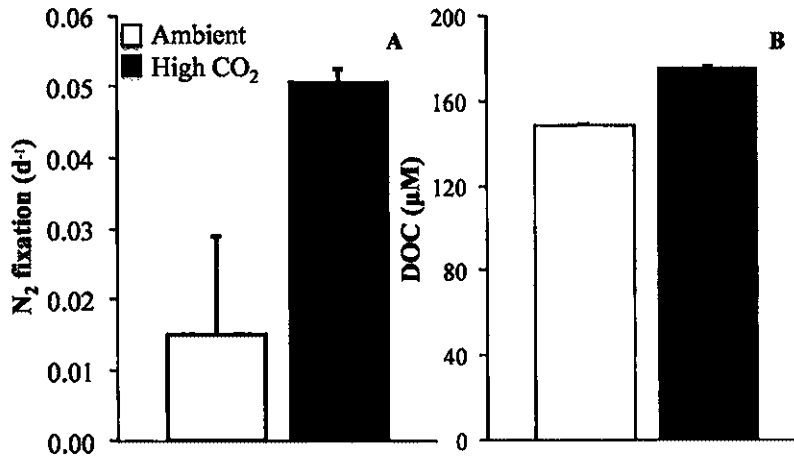

Figure 18. Mean specific N_2 fixation rates (A) and DOC concentrations (B) measured in incubation experiments with *Lyngbya* spp. populations collected along the WFS in the GOM in October 2009. Uptake incubations and DOC concentrations were measured after acclimating populations for two days at ambient (white bars) and elevated (black bars) pCO_2. Error bars represent one standard deviation determined from replicate samples (n=3). Statistically significant differences between pCO_2 treatments were not found for measurements of N_2 fixation or DOC concentration using a t-test (t=-1.89, p=0.199, n=3 and t=-0.639, p=0.557, n=3).

Discussion

CO_2 enrichment experiments conducted using natural plankton assemblages and populations of *Trichodesmium* spp. and *Lyngbya* spp. collected during research cruises on the WFS in the GOM suggest that increases in pCO_2 could substantially alter functioning of natural plankton populations. Uptake of N_2 was greater in populations incubated at elevated pCO_2 and DOC concentrations were greater in populations incubated at elevated pCO_2 conditions for both the *Lyngbya* spp. and whole community phytoplankton populations suggesting changes in the coupling between autotrophic and heterotrophic metabolisms.

Trichodesmium spp. incubations

Carbon fixation increased by an average of 11% in short-term incubations of natural populations of *Trichodesmium* spp. at high pCO_2 compared with populations incubated under ambient pCO_2 conditions. Elevated pCO_2 also stimulated N_2 fixation by an average of 46% in these same incubations (Fig. 12) supporting culture and natural population incubation results demonstrating enhancement of diazotrophy at elevated pCO_2 (Hutchins et al. 2007, Kranz et al. 2011, Levitan et al. 2007, 2010; Barcelos e Ramos et al. 2007, Lomas et al. 2012, Chapter 2).

If increases in C and N_2 fixation translate into increased growth rates, blooms of *Trichodesmium* spp. in the GOM could grow in magnitude and/or duration under future climate scenarios. This could dramatically increase new N inputs into areas where *Trichodesmium* spp. occur such as the WFS in the GOM. Release of recently fixed N by *Trichodesmium* spp. can be substantial (Mulholland et al. 2004, 2006, 2014) and this N is bioavailable to co-occurring phytoplankton along the WFS in the GOM, including the harmful alga, *K. brevis* (Mulholland et al. 2004, 2006; Lenes & Heil 2010, Sipler et al. 2013). *K. brevis* blooms have been associated with concurrent or preceding *Trichodesmium* spp. blooms (Steidinger et al. 1998, Walsh et al. 2006), so any increase to the stock of *Trichodesmium* spp. and the availability of fixed N in the GOM could fuel *K. brevis* production.

Whole community incubations

Biomass declined substantially by the end of the two-day CO_2 enrichment experiment relative to that measured in the initial water samples collected. The mixed phytoplankton population (dominated by diatoms) used for this experiment was collected from the Chl *a* maximum which was located at about 10m depth during mid-day on the day experiments were initiated. This collapse was unexpected but may have been due to exhaustion of nutrients in incubation bottles, turbulent conditions created by bubbling of incubations to achieve experimental pCO_2 conditions or the physiological state of the phytoplankton community at the time of sampling. Carbon and N_2 fixation declined dramatically under both pCO_2 treatments relative to rates measured just after water samples were collected. However, N_2 fixation rates were measureable in all samples in the absence of *Trichodesmium* spp. suggesting that other diazotrophs were present in the natural microbial assemblage, as has been observed previously along the WFS (Mulholland et al. 2006). While genetic identification of diazotrophs in the natural plankton assemblage was beyond the scope of this study, the presence of diverse nitrogenase genes and measurements of active N_2 fixation has been observed now in a range of oceanic and coastal environments (Montoya et al. 2004, Zehr et al. 2007, Mulholland et al. 2012). Further investigation into the identity of active diazotrophs in the region is warranted since it appeared that at least some portion of the plankton assemblage used for this study experienced stimulation of C and N_2 fixation under high pCO_2.

Despite the overall lower productivity in treatment and control bottles after the two-day incubation period, there were observable differences between the two pCO_2 treatments. Chl-normalized C fixation rates were more than twice as high in plankton assemblages incubated under high CO_2 relative to those supplied with ambient pCO_2 (Fig. 16). Similarly, Chl-normalized N_2 fixation rates were 86% greater in assemblages incubated at high pCO_2 relative to those at ambient pCO_2 conditions. DOC concentrations were 20% greater, and NH_4^+ concentrations were 17% greater in the high pCO_2 treatments compared with ambient pCO_2 incubations and PC normalized DOC production was 45% higher in the elevated pCO_2 treatments than the ambient pCO_2 incubations. Stimulation in physiology and release of organic matter and nutrients under

high CO_2 conditions was not found to be statistically significant, however. Greater sample sizes are recommended for future investigations into enhanced physiology of GOM phytoplankton populations under elevated CO_2 conditions to improve the power of statistical determinations on pCO_2 effects since high variability in physiological rates were measured in the incubations included in this study. While increases in NH_4^+ concentrations were observed between the two pCO_2 treatments after the two day incubation period, it was unclear whether the differences were due to increased rates of N_2 fixation and subsequent release of NH_4^+ under high CO_2, or due to death and decay of organic matter within the bottles over the two-day incubation period.

Lyngbya spp. incubations

These are the first results measuring N_2 fixation by *Lyngbya* spp. under elevated pCO_2. *Lyngbya* spp. growth rates, concentrations of Chl *a* and NH_4^+ were not significantly different in populations incubated for 2 days under high CO_2 relative to those incubated at ambient pCO_2. Measurements of $^{15}N_2$ uptake were more than 200% greater under high CO_2 in comparison with *Lyngbya* spp. incubated under ambient pCO_2, which may have contributed to the higher NH_4^+ concentrations in the high CO_2 incubations. Still, pCO_2 did not cause statistically significant differences in N_2 fixation for *Lyngbya* spp. in this study. DOC concentrations were not statistically different under high pCO_2 compared to measurements in *Lyngbya* spp. incubations under ambient pCO_2, as well. A high degree of variability between physiological rates, DOC and NH_4^+ concentrations measured in ambient versus high CO_2 incubations likely limited the power of detection of statistically significant differences between pCO_2 treatments for the *Lyngbya* spp. incubations. Future studies into pCO_2 effects on the growth and N_2 fixation of *Lyngbya* spp. should include a larger sample size to improve the power of statistical testing.

Lyngbya spp. proliferate in many tropical and subtropical coastal waters and the toxins these species release negatively impact many co-occurring organisms including submerged aquatic vegetation (Watkinson et al. 2005), corals (Kuffner & Paul 2004), benthic organisms (Estrella et al. 2011) and sea turtles (Arthur 2008) in addition to causing irritation of skin, eyes and respiratory systems in humans (Osborne et al. 2001).

Proliferation of *Lyngbya* spp. under future climate scenarios not only has the likelihood to increase the availability of new N in the GOM, but also the likelihood of drastically modifying the ecology of larger organisms in the ecosystem as a result of toxin.

Higher rates of N_2 fixation by *Lyngbya* spp. under future climate scenarios may also stimulate the growth of *K. brevis* populations through the provision of new N and this could exacerbate impacts on the WFS from the production of multiple toxins. Like *Trichodesmium* spp., blooms of *Lyngbya* spp. (Paerl et al. 2008) have been associated with *K. brevis* blooms in the past, and if future conditions stimulate the production of this diazotroph as suggested by many researchers (Paul 2008, O'Neil et al. 2012, Paerl & Paul 2012, Paerl & Otten 2013, Paerl et al. 2014), inputs of bioavailable new N could stimulate growth of *K. brevis*. Paerl et al. (2008) determined that *K. brevis* production was limited by N and that *K. brevis* production was stimulated by adding bioavailable N. The abundance of diazotrophs and their co-occurrence with *K. brevis* may stimulate production by this organism and lead to an increased number, duration or magnitude of blooms in the future.

Lyngbya spp. mats contain a diverse microbial assemblage and growth of non-diazotrophic microbes in these assemblages may take up newly fixed N_2 rapidly. For example, ammonium oxidizing bacteria and archaea were found in *Lyngbya* spp. mat communities in Hawaii, and these organisms were able to denitrify NH_4^+ exudates from *Lyngbya* spp. before this N was able to diffuse away (Sobolev et al. 2013). Viruses are another agent that facilitate the release of bioavailable N from *Lyngbya* spp. populations (Hewson et al. 2001). Positive correlations between phytoplankton host abundance and bacterial abundance have been observed (Danovaro et al. 2011), and if viruses follow the same trend, greater abundances of *Lyngbya* spp. in the future may induce greater abundances of associated viruses as well. Further research into the microbial loop associated with *Lyngbya* spp. is warranted. Quantifying nutrient release from death and decomposition of *Lyngbya* spp. mat communities will be important for assessing their contribution to the nutrient demand of co-occurring attached or planktonic communities. Alternatively, the accumulation of recycled nutrient compounds may inhibit further N_2 fixation by *Lyngbya* spp.

While *Lyngbya* spp. is known to be harmful, little is known about the diazotrophic capability of *Lyngbya* spp. because it has been difficult to measure. While production by *L. majuscula* has been shown to be stimulated with additions of Fe, N and P (Watkinson et al. 2005, Ahern et al. 2007, 2008, Ahern 2008), these are the first studies to examine the response of *Lyngbya* spp. to elevated pCO_2. While there was some debate as to whether *Lyngbya* spp. fix N_2 at all (Jones et al. 2011), the preponderance of evidence suggests that it does (Jones 1990, Dennison et al. 1999, Lundgren et al. 2003, Elmetri & Bell 2004, O'Neil & Dennison 2005) and our results appear to confirm that either *Lyngbya* spp. or some organism living in close association to *Lyngbya* spp. is actively fixing N_2. Measurements of N_2 fixation by *Lyngbya* spp. could have been due to stimulation of the production of microscopic organisms attached to the mat fragments, or perhaps a diazotrophic endosymbiont. Previous studies determined that *L. majuscula* contains nitrogenase only transiently, during the night (Lundgren et al. 2003). The incubations done as part of this study were conducted over 24 hours, so encompassed both light and dark periods precluding the determination of the timing of N_2 fixation for *Lyngbya* spp.

DOC production

DOC measurements were higher in high pCO_2 treatments for whole community and *Lyngbya* spp. incubations. It is unclear whether *K. brevis* will benefit directly from increases in DOM production, but *K. brevis* is a known mixotroph capable of uptake of organic compounds (Sipler et al. 2013). A study examining the bioavailability of DOM fractions on the WFS found a strong positive correlation between *K. brevis* cell density and concentrations of DOM produced in situ in comparison with terrestrial DOM sources (Mendoza et al. 2012). Additionally, other studies support *K. brevis'* utilization of DON released by *T. erythraeum* (Bronk et al. 2004, Sipler et al. 2013). Other studies have determined that some cyanobacteria, including *Synechococcus* spp. and *Lyngbya* spp., are capable of utilizing DOC via photoheterotrophic metabolisms (Paerl et al. 1993, Kang et al. 2004, Andrade & Costa 2007, Yu et al. 2009), so future experiments should focus on the characterization and bioavailability of the DOM released by GOM phytoplankton whose extracellular release of organic matter is elevated under high CO_2.

Observed increases in DOC production at high pCO_2 will not necessarily stimulate *K. brevis* production. In a study performed in Hunter Estuary, Australia, Hitchcock et al. (2010) found that DOC additions induced decreases in Chl *a* in natural phytoplankton communities. However, increases in Chl *a* concentrations were observed when natural populations were amended with both DOC and N. This suggests that the combination of enhanced N_2 fixation and DOC release might enhance production by *K. brevis* and possibly other co-occurring phytoplankton in the GOM.

Phytoplankton DOC exudates are also available for uptake by bacteria (Baines & Pace 1991, Morán et al. 2002, Cherrier et al. 2014). Increases in labile DOC production by phytoplankton associated with ocean warming and increases in pCO_2 (Engel et al. 2011), could stimulate bacterial metabolisms (Fork & Heffernan 2014, Engel et al. 2014). Enhanced bacterial production stimulated by abundant labile DOC induces a greater consumption of dissolved oxygen (Hitchcock et al. 2010) that could shift net community metabolism and enhance the expansion of low oxygen zones (Stramma et al. 2008, Diaz & Rosenberg 2008) and oceanic N losses through denitrification. The GOM already experiences vast zones of hypoxia and these zones could expand if future climate favors heterotrophic processes (Engel et al. 2014). Phylogentic sequencing of the microbial communities in hypoxic zones of the GOM have already revealed community shifts from dominance of grazing dinoflagellates to parasitic dinoflagellates (Rocke et al. 2012) indicating the strong possibility for altered phytoplankton community structure under future climate scenarios when DOC concentrations are elevated.

My preliminary studies suggest that future studies should be aimed at quantifying DOC release by phytoplankton and consequent impacts on heterotrophic and net community metabolism in a warmer, high pCO_2 environment. Previous experiments have examined the production of DOC with respect to light. For example, DOC production by GOM populations incubated under high light conditions released more DOC (Morán et al. 2002). In another study, release of DOC by phytoplankton was greater under higher light conditions, and as a result, bacterial populations consumed DOC at almost three times the rate of bacterial populations incubated with filtrate from the lower light phytoplankton incubations (Cherrier et al. 2014). Enhanced DOC production associated with increases in temperature and pCO_2 could dramatically alter

microbial communities and shift net community metabolism in the ocean, and contribute to the expansion of oxygen minimum zones and the biogeochemical processes they facilitate.

Conclusions

Results from these experiments suggest that natural communities and the biogeochemical processes they mediate could be fundamentally altered by higher pCO_2. This will likely have ramifications for foodwebs and the biogeochemical functioning of aquatic systems. Stimulation of N_2 fixation under high pCO_2 indicates that *Trichodesmium* spp. will release greater loads of new N into the GOM system in the future. Elevated rates of C fixation observed for *Trichodesmium* spp. and the mixed phytoplankton assemblage in this study suggest that primary production of many species of GOM phytoplankton should increase under high CO_2 conditions, but it is unclear whether elevated C metabolisms will translate into a greater abundance of these organisms, or instead result in increased release of DOC exudates.

Elevated DOM concentrations on top of increases in concentrations of recently fixed N_2 will elevate stocks of bioavailable nutrients in the GOM. However, it is unclear who will benefit most, *K. brevis*, a mixotroph who appears to be N limited, other co-occurring phytoplankton, or heterotrophic bacteria? The answer to this question may have important implications with regard to net community metabolism and the expansion of oxygen minimum zones.

CHAPTER IV

CONCLUSIONS

The experiments conducted as part of this thesis examining the impact of elevated temperature and/or pCO_2 on the physiology and biogeochemical functioning of coastal *S.* cf *elongatus* and *T. erythraeum* cultures indicated that climate change could favor growth, C fixation, N_2 fixation (for *T. erythraeum*) and the release of recently fixed C and N by these important cyanobacterial groups in the future. However, research has demonstrated that differences among *Synechococcus* ecotypes (Lu et al. 2006) and different strains of *T. erythraeum* (Gradoville et al. 2014) may complicate predictions of species-specific responses to elevated pCO_2. In fact, intraspecies physiological variability has been shown to approximate physiological variation between phytoplankton genera in some cases (Schaum et al. 2012). Still, the value of culture experiments in making determinations about altered physiology under future climate conditions remains important because of the difficulty in conducting experiments on natural phytoplankton communities that include factors that promote or limit primary production including physical forces, grazing impacts, and interactions with co-occurring phytoplankton, bacteria and viruses in the microbial community.

Culture experiments using coastal isolates of the cyanobacteria *Synechococcus* and *T. erythraeum* and field experiments conducted using natural populations of the diazotrophic cyanobacteria *Trichodesmium* spp. and *Lyngbya* spp. and natural plankton populations collected from the GOM all suggest that C fixation by cyanobacteria and N_2 fixation by cyanobacterial diazotrophs may increase in response to higher temperatures and pCO_2 over the next century. Any increase in the load of new N released into the GOM will improve the nutritional status of *K. brevis* (Mulholland 2014) and other GOM phytoplankton into the future (O'Neil et al. 1996, Mulholland et al. 2004a, b, 2006, Mulholland 2007). It will be important to determine the diversity of diazotrophic cyanobacteria in the GOM since other species not tested in this project may also exhibit stimulation of N_2 fixation under elevated pCO_2 conditions, including *Richelia* spp. found in association with diatoms in the GOM (Knapke 2012). Also, observed increases in DOC production under high CO_2 for some of the phytoplankton studied here will likely

contribute to an elevation in the load of substrate available to GOM bacteria (Sellner 1997, Engel et al. 2014) or photoheterotrophic cyanobacteria (Paerl et al. 1993, Kang et al. 2004, Andrade & Costa 2007, Yu et al. 2009, Zehr 2011). Whether *K. brevis*, other co-occurring phytoplankton, bacteria or viruses result as the group that benefits most from anticipated increases in the N and DOM pool under future climate scenarios remains to be determined.

Elevated nutrient and organic matter release rates could radically alter marine food webs in the future by both changing the food supply available to higher trophic levels and altering the production of bioavailable N and DOC that fuel microbial communities. Shifts in the major trophic pathways can alter net community metabolism thereby affecting the productivity of oceanic systems. Any change that promotes the shift from autotrophic dominance to net heterotrophy will undoubtedly reduce dissolved oxygen concentrations (Hitchcock et al. 2010), expanding hypoxic regions in marine systems into the future (Stramma et al. 2008, Diaz & Rosenberg 2008).

We have only begun to understand the physiological effects of changing climate on aquatic microbial communities. Because these are the base of aquatic food webs, changes in the composition and biogeochemical function of plankton communities could fundamentally alter marine food webs in the future. In the GOM, this could mean more devastating blooms of the red tide dinoflagellate *K. brevis*. However, there are many potentially confounding factors that could contribute to shaping aquatic foodwebs in the future and these include physical factors such as changes in terrestrial runoff volume delivered to the basin (Scavia et al. 2002), water column stratification (Smayda 1970, Paerl & Paul 2012), atmospheric deposition of nutrients (Lenes et al. 2001), and circulation patterns (Tester & Steidinger 1997). Biological and chemical shifts including changes in species specific phytoplankton production in situ (Lomas et al. 2012), bacterial or viral production and the resulting remineralization of dissolved inorganic nutrients, changes to the chemical characterization of allocthonous organic matter delivered in terrestrial runoff to the basin (Walsh et al. 2006) and mortality of larger organisms releasing nutrients into the dissolved pool during decay (Walsh et al. 2006, Killberg-Thoreson et al. 2014b) all need to be considered when making predictions about the expected microbial ecology of the GOM under future climate scenarios.

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Professional History

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Conference Contributions & Publications

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2014	Mulholland MR, Bernhardt PW, Ozmon IM, Procise LA, Garrett M, O'Neil JM, Heil CA, Bronk DA (2014) Contribution of diazotrophy to nitrogen inputs supporting *Karenia brevis* blooms in the Gulf of Mexico. *Harmful Algae*, in press
2009	Ozmon IM, Bernhardt PB, Mulholland MR (2009) Effects of CO_2 and Nutrient Enhancement on Planktonic Estuarine Communities. Poster presented at the Coastal and Estuarine Research Federation Conference, Portland, Oregon, November 2009