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Effect of ocean acidification on cyanobacteria in the subtropical North Atlantic

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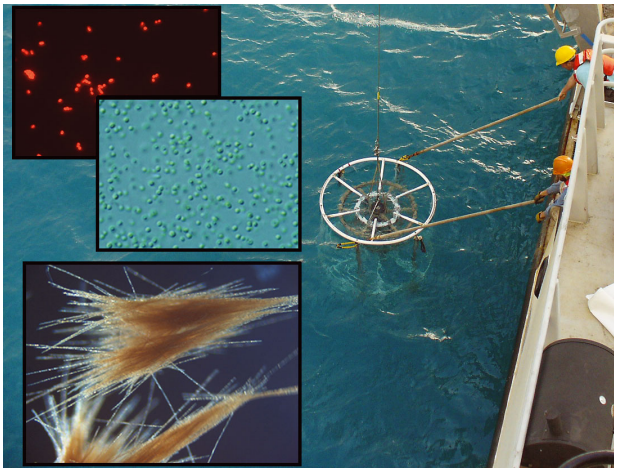
ABSTRACT: Cyanobacteria make significant contributions to global carbon and nitrogen cycling, particularly in the oligotrophic subtropical and tropical gyres. The present study examined short-term (days) physiological and acclimation responses of natural cyanobacterial populations to changes in pH/pCO_2 spanning the last glacial minimum, ~8.4/~150 ppm, to projected year 2100 values of ~7.8/~800 ppm. Fe- and P-replete colonies of *Trichodesmium* increased N_2-fixation rates (nmol N colony^{-1} h^{-1}) at pH 7.8 by 54% (range 6 to 156%) over ambient pH/pCO_2 conditions, while N_2-fixation at pH/pCO_2 8.4 was 21% (range 6 to 65%) lower than at ambient pH/pCO_2; a similar pattern was observed when the rates were normalized to colony C. C-fixation rates were on average 13% (range −72 to 112%) greater at low pH than at ambient pH and 37% (−53 to 23%) greater than at high pH. Whole community assemblages dominated by *Prochlorococcus* and *Synechococcus* (47 to 95% of autotrophic biomass), whether nutrient-replete or P-limited, did not show a clear response of C-fixation rates to changes in pH/pCO_2. Comparison of initial and final C-fixation responses across pH/pCO_2 treatments suggests rapid acclimation of cellular physiology to new pH/pCO_2 conditions. Changes in cell size and pigment content for *Prochlorococcus* and *Synechococcus* were minor and did not vary in a consistent manner with changes in pH/pCO_2. These results for natural populations of all 3 cyanobacteria concur with previous research and suggest that one important response to changes in ocean pH and pCO_2 might be an increase in N_2 and C fixation by *Trichodesmium* under nutrient-replete conditions. The response of single-cell cyanobacteria to changes in pH/pCO_2 will likely be indirect and controlled by the response to other variables, such as nutrients.



At sea ocean acidification experiments show contrasting responses between dominant unicellular and colonial natural cyanobacteria populations. Insets (top to bottom): *Synechococcus* (epifluorescence microscopy), *Prochlorococcus* (bright-field microscopy) and *Trichodesmium* (bright-field microscopy)

Photos: S. Jaeger and M. Lomas

KEY WORDS: Cyanobacteria · North Atlantic · Sargasso Sea · Acidification · Nitrogen fixation · Photosynthesis

INTRODUCTION

Marine cyanobacteria, both unicellular *Prochlorococcus* and *Synechococcus* and colonial *Trichodesmium* spp., play important roles in the ocean carbon cycle and the biological carbon pump, particularly in the subtropical and tropical gyres (e.g. Partensky et

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al. 1999, Capone et al. 2005). The ongoing acidification of the surface ocean by dissolution of anthropogenic carbon dioxide from the atmosphere will likely affect the growth of these and other important phytoplankton and therefore their roles in the ocean carbon cycle. While a large number of studies have examined the effect of high pCO_2/low pH on various cultured phytoplankton (Hutchins et al. 2009 and references therein), only a few of those studies have focused on the growth and physiology of marine cyanobacteria. Furthermore, mesocosm experiments and incubations with natural autotrophic populations have primarily been done in nutrient-rich systems where cyanobacterial contributions to total autotrophic biomass are low enough to be considered negligible (e.g. Tortell et al. 2002, Engel et al. 2005, Kim et al. 2006, Hare et al. 2007, Riebesell et al. 2007, Sommer et al. 2007, Tortell et al. 2008, Feng et al. 2009).

Most of the research on cyanobacterial responses to changes in pH/pCO_2 has focused on the diazotroph *Trichodesmium,* which is thought to account for about half of the total N_2-fixation in the oceans (Barcelos e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007, Kranz et al. 2009, 2010). Only 2 studies have examined the response of other diazotrophs: *Crocosphaera* (Fu et al. 2008) and *Nodularia spumigena* (Czerny et al. 2009). In nutrient-replete culture studies, increasing pCO_2 (decreasing pH) consistently resulted in a substantial increase in both N_2 fixation (35 to 120%) and C fixation (15 to 100%) by *Trichodesmium erythreaum* IMS101 due to the alleviation of inorganic C limitation at low and ambient pCO_2 levels. Growth of *T. erythreaum* at high pCO_2 resulted in elevated cellular C:P and N:P but not C:N ratios. The relative increase in cellular C and N quotas was less than the increase in C- and N_2-fixation rates, suggesting the net release of P-depleted DOM at high pCO_2 (Hutchins et al. 2007). Increased dissolved organic matter production, relative to particulate organic matter production, at high pCO_2 may also be common in natural systems (Yoshimura et al. 2010, Kim et al. 2011) with minimal cyanobacterial populations. To our knowledge, only one study has published similar experiments with natural *Trichodesmium* populations. Hutchins et al. (2009) report that *Trichodesmium* colonies collected from a bloom in the Gulf of Mexico increased N_2-fixation rates by 6 to 41% with a doubling of pCO_2 from 380 ppm to 750 ppm, which is consistent but on the low end of predictions from culture studies. The single cell diazotroph *Croccosphaera* also demonstrated an increase in C-fixation (20 to 100%) and N_2-fixation (40 to 80%) rates at high pCO_2 but only under nutrient-replete conditions; iron-limited cultures did not show any response (Fu et al. 2008). A study of the Black Sea diazotroph *Nodularia spumigena* showed the exact opposite effect of high pCO_2, with a ~25% reduction in N_2-fixation and a ~40% reduction in growth rate (Czerny et al. 2009); this contrasting response may reflect differences between non-heterocystous and heterocystous cyanobacteria, but this is unknown. Based on studies with cultured *Trichodesmium*, ocean acidification would likely result in a positive feedback on the growth and physiology of natural populations, resulting in a positive change in their role in ocean carbon and nitrogen cycles.

The response of non-diazotrophic cyanobacteria to pCO_2 changes is less well understood. Fu et al. (2007) reported that doubling pCO_2 alone had no significant effect on the growth of either *Synechococcus* or *Prochlorococcus* in culture, whereas 'greenhouse' conditions (doubling pCO_2 and increasing temperature by 4°C) significantly increased growth rates, photosynthetic capacity, and cellular pigment levels in *Synechococcus* but not *Prochlorococcus*. These different responses between *Synechococcus* and *Prochlorococcus* have been attributed to differences in inorganic carbon acquisition systems associated with carbon limitation at low pCO_2 in *Synechococcus* (Fu et al. 2007). In contrast, an enclosure experiment that compared the growth of the natural phytoplankton assemblage (stimulated by addition of nutrients, 10 µmol l^{-1} NO_3^- and 0.5 µmol l^{-1} PO_4^{-3}) at 3 levels of pH/pCO_2 found the only significant difference to be a lower abundance of *Synechococcus* and a higher abundance of picoeukaryotes at high pCO_2 (Paulino et al. 2008). The response of natural cyanobacterial populations to elevated pCO_2 in the nutrient-deplete subtropical and tropical seas remains an important gap in our knowledge of the changing ocean (Rost et al. 2008, Hutchins et al. 2009), particularly given the potential for increased areal extent of the oligotrophic gyres due to ocean warming and increased stratification (e.g. Boyd & Doney 2002).

Here, we report the results of several experiments carried out between July 2009 and April 2010 at the Bermuda Atlantic Time-series Study (BATS) station where we examined the C-fixation responses of natural assemblages dominated by *Synechococcus* and *Prochlorococcus* and the N_2- and C-fixation responses of isolated *Trichodesmium* colonies to pH manipulations. Seasonally, the phytoplankton community at BATS shifts from a spring bloom community co-dominated by nanoeukaryotes, in particular members of the haptophyte group, and *Synechococ-*

cus (e.g. Lomas et al. 2010) to a community dominated by cyanobacteria, of all 3 genera, in late summer (e.g. Orcutt et al. 2001, Steinberg et al. 2001). Of particular note with respect to the goals of the present study is the well documented slow acidification of the surface ocean at BATS (Bates 2007) and the increasing contribution of *Synechococcus* biomass (and the decreasing importance of larger eukaryotes) during the winter/spring bloom (Lomas et al. 2010) over the past 2 decades.

MATERIALS AND METHODS

Field site and experimental design

Experiments were carried out on board the RV 'Atlantic Explorer' at the BATS site (31° 40' N, 64° 10' W), located in the subtropical North Atlantic Ocean ~86 km south-east of Bermuda. *Trichodesmium thiebautii* colonies were collected with a 330 µm net that was hand-towed through the surface mixed-layer (~20 m). Colonies were picked and rinsed in 0.22 µm filtered seawater immediately after the tow and distributed into duplicate experimental bottles filled with filtered (0.2 µm) surface seawater where the pH had been adjusted to represent past (pH ~8.4) and future (pH ~7.8) conditions (see section 'pH/pCO_2 manipulation') as well as a control bottle with no pH adjustment. Both pH treatments and the controls were supplemented with 50 nmol l^{-1} phosphate and 5 nmol l^{-1} $FeCl_3$ ('incubation media'). In Expts A–D, the colonies were allowed to acclimate for 24 h in a shaded (30% surface irradiance) flow-through incubator, while in Expts E to I, rate measurements were begun immediately (Table 1). Colonies in Expts A to D were visually examined after acclimation and showed no obvious detrimental effects of the acclimation period, and all colonies initially added were accounted for after the acclimation period.

For experiments with the bulk phytoplankton community, water was collected using either a rosette sampler (March and April) or a trace-metal clean diaphragm pump (September), gently mixed in a shaded polycarbonate carboy, and dispensed into triplicate acid-washed polycarbonate bottles. Samples were collected from the approximate mid-point of the mixed layer, which in September was ~10 m, in March ~50 m, and in April ~35 m. After pH/pCO_2 adjustment, the following nutrient combinations were added: P-limited treatment: 5 µmol l^{-1} NO_3, 5 µmol l^{-1} Si, 2 nmol l^{-1} Fe; N-limited treatment: 0.5 µmol l^{-1} PO_4, 5 µmol l^{-1} Si, 2 nmol l^{-1} Fe; Replete: 5 µmol l^{-1} NO_3, 0.5 µmol l^{-1} PO_4, 5 µmol l^{-1} Si, 2 nmol l^{-1} Fe. Bottles were placed in a flow-through incuba-

Table 1. Ambient environmental data associated with *Trichodesmium* and natural population experiments. SST: sea surface temperature

Experiment designation	Date (mo/d/yr)	Collection depth (m)	SST (°C)	Duration (d)[a]	Ambient pH (ppm)	Chl *a* (µg l^{-1})	$NO_{3/2}$ (µmol l^{-1})	PO_4 (µmol l^{-1})	Autotrophic carbon (%)
***Trichodesmium* experiments**									
A	7/14/2009	0–30	26.8	1	8.10		<0.03	<0.01	–
B	7/16/2009	0–30	26.8	1	8.12	0.07			~13[b]
C	7/17/2009	0–30		1	8.11				–
D	7/19/2009	0–30	27.3	1	8.08				–
E	7/20/2009	0–30		0	8.14				–
F	8/14/2009	0–30	28.6	0	8.11				–
G	8/15/2009	0–30	28.6	0	8.08	0.06	<0.03	<0.01	~15
H	8/16/2009	0–30	28.8	0	8.10				–
I	8/17/2009	0–30	29.1	0	8.11				–
Whole community experiments									
09-09_Replete[c]	9/10/2009	0–30	28.1	2.8	8.08	0.06			~95[d]
03-10_Replete	3/24/2010	50	18.9	3.2	8.07	0.62			~47
04-10_Replete	4/7/2010	35	18.9	1.5	8.07	0.41			~52

[a]Duration of acclimation period to manipulated C system conditions prior to tracer incubation
[b]Estimated contribution of *Trichodesmium* colonies to autotrophic C using colony abundances for these months from Orcutt et al. (2001) and C per colony from the present study (Fig. 2). Total algal C estimated from chl *a* and C:chl *a* ratio for the Bermuda Atlantic Time-series Study (BATS) as in natural population experiments
[c]Experimental notation, MM-YY_nutrient condition
[d]Combined contribution of *Synechococcus* and *Prochlorococcus* carbon to total algal carbon (see 'Materials and methods'). Total algal C estimated from chl *a* and C:chl *a* ratio at BATS. *Synechococcus* and *Prochlorococcus* cell C estimated from flow cytometer forward scatter signal as in J. R. Casey et al. (unpubl.)

tor shaded to ~30% of surface irradiance. In many cases, rate measurements were made immediately after pH/pCO_2 adjustment/nutrient amendment, but in all experiments, the community was allowed to grow for 1 to 3 d under pH-adjusted conditions prior to final measurement of C-fixation and other parameters. Final nutrient, but not Fe, concentrations were measured. With the exception of the N-limited treatments, dissolved inorganic nitrogen concentrations were >1 µmol l^{-1}.

pH/pCO_2 manipulation

In all the low pH experiments, the pH was reduced to ~7.8, the approximate predicted year 2100 value. We used only high-purity HCl addition for the *Trichodesmium* incubations to minimize the chance of contamination. For the longer lasting incubations with bulk phytoplankton in which the DIC drawdown might have become substantial, we added equimolar additions of high-purity HCl and $NaHCO_3$, effectively mimicking the ongoing rise in CO_2, which also increases DIC. As shown by Shi et al. (2009), the method makes no difference as long as the DIC drawdown is small. In the high pH experiments, the pH was increased to 8.4, similar to last glacial minimum values, using high-purity NaOH. The pH was measured using an electrode calibrated on the USA National Bureau of Standards scale. These measurements were converted to the total hydrogen ion scale by intercalibration with spectrophotometric measurements of pH (on the total hydrogen ion scale) using thymol blue (Zhang & Byrne 1996). All pH values are reported on the total hydrogen ion scale. Based on pH and average DIC concentrations at BATS (N. Bates pers. comm.), the estimated pCO_2 values for each treatment were as follows: low pH: 800 to 820 ppm CO_2, ambient pH: 350 to 405 ppm CO_2, high pH: 150 to 165 ppm CO_2 (Lueker et al. 2000). pH measurements are a very good indicator of change in CO_2 concentrations due to consumption or gas exchange (Gattuso et al. 2010). Values changed little between the beginning and the end of the incubation, suggesting that the carbon system was stable (data not shown).

Biomass measurements

Chlorophyll *a* (chl *a*) samples were collected by gentle vacuum filtration onto glass fiber filters (GF/F) and stored in liquid nitrogen. Onshore, the filters were extracted overnight in a 90% acetone and 10% water solution at −20°C, and chl *a* was quantified fluorometrically (Parsons et al. 1984). Particulate organic material was collected by gentle filtration on precombusted GF/F filters and stored at −20°C. Prior to analysis, the filters were exposed to fuming HCl to remove inorganic carbon and then dried in an oven at 60°C. Particulate organic carbon (POC) and nitrogen (PON) masses and isotopic composition were measured using an elemental analyzer coupled to an isotope ratio mass spectrometer (e.g. Orcutt et al. 2001). Particulate organic phosphorus (POP) was analyzed on a magnetic sector field HR-ICP-MS (Element 2, ThermoFinnigan) following the method of Tang & Morel (2006).

Rate measurements

Trichodesmium thiebautii colonies (in incubation media) were sealed in 30 ml glass serum vials to which $^{15}N_2$ gas (Cambridge Isotope Laboratory, 98% enriched) and ^{13}C-$NaHCO_3$ (Cambridge Isotope Laboratory, 98% enriched, 100 µmol l^{-1} final concentration for an atom% enrichment of 4.5%; the addition alters pH by ≤0.02 units) were added and then incubated for 6 to 8 h around local noon at ~30% incident irradiance in a flow-through incubator (Orcutt et al. 2001). N_2-fixation rates were quantified based on the incorporation of $^{15}N_2$ into biomass and calculated following the methods of Montoya et al. (1996). C-fixation rates were measured by quantifying the incorporation of ^{13}C-$NaHCO_3$ into particulate biomass and calculated using equations analogous to those for N_2-fixation (e.g. Slawyk et al. 1977). DIC concentrations for these calculations were estimated from the BATS climatology (e.g. Bates et al. 1996). Relative changes in N_2- and C-fixation rates were calculated as follows:

$$\text{fractional change} = \frac{(R_{trt} - R_{amb})}{R_{amb}} \quad (1)$$

where R_{trt} is the rate for a treatment (either high or low pH), and R_{amb} is the rate at ambient pH.

For the bulk phytoplankton community experiments, C-fixation rates were measured using ^{14}C-bicarbonate. To a 100 ml seawater sample, 5 µCi ^{14}C-bicarbonate was added, incubated for 2 to 3 h, centered on local noon, at ~30% incident irradiance in a flow-through incubator, and then gently filtered through a GF/F filter. The filter was saturated with 2% HCl and allowed to degas overnight to remove inorganic carbon before addition of scin-

tillation fluid and counting on a Perkin Elmer Tri-Carb 2900 TR low activity liquid scintillation analyser. Samples for total ^{14}C activity added were taken at the beginning of the incubation and preserved with an equal volume of β-phenethylamine. C-fixation rates were calculated based upon the calculated ^{14}C specific activity for each experiment and activity retained in the particulate fraction (Hopkinson et al. 2010).

Flow cytometry

Samples for analytical flow cytometric analysis were collected from the whole community experiments and fixed with paraformaldehyde (0.5% final concentration, ~4°C for 1 to 2 h) before storage in liquid nitrogen until analysis. Samples were analyzed on a Becton Dickinson (formerly Cytopeia) influx cytometer as described by Lomas et al. (2010). Cyanobacteria were identified as either *Synechococcus* or *Prochlorococcus* based on cell size and the presence or absence of phycoerythrin, respectively. Based upon these gating criteria, the number of cells in each identified population was enumerated and converted to cell abundances using the volume analyzed method (Sieracki et al. 1993). Geometric mean values of red fluorescence (relative chlorophyll content), orange fluorescence (relative phycoerythrin content), and forward laser scatter (FSC, relative size) for gated *Synechococcus* and *Prochlorococcus* populations were calculated using FCS Express v3.0. The geometric mean FSC signals for *Synechococcus* and *Prochlorococcus* were used to calculate mean cellular POC using a direct POC_{cell} vs. FSC calibration curve specific to this instrument (J. R. Casey et al. unpubl.). In brief, the geometric mean FSC and POC_{cell} (via elemental analyzer) were determined on a wide range of cultures and flow cytometrically sorted natural populations up to ~20 µm in size. A robust direct correlation was found between mean FSC and POC_{cell} regardless of sample origin or size. In the present study, the mean FSC *Synechococcus* and *Prochlorococcus* was used to calculate POC_{cell}, and this POC_{cell} value was converted to POC per taxonomic population by multiplying by abundance, summed across *Synechococcus* and *Prochlorococcus*, and expressed as a percentage of the total autotrophic C estimated from chl *a* and the C:chl *a* ratio for each cruise (data not shown).

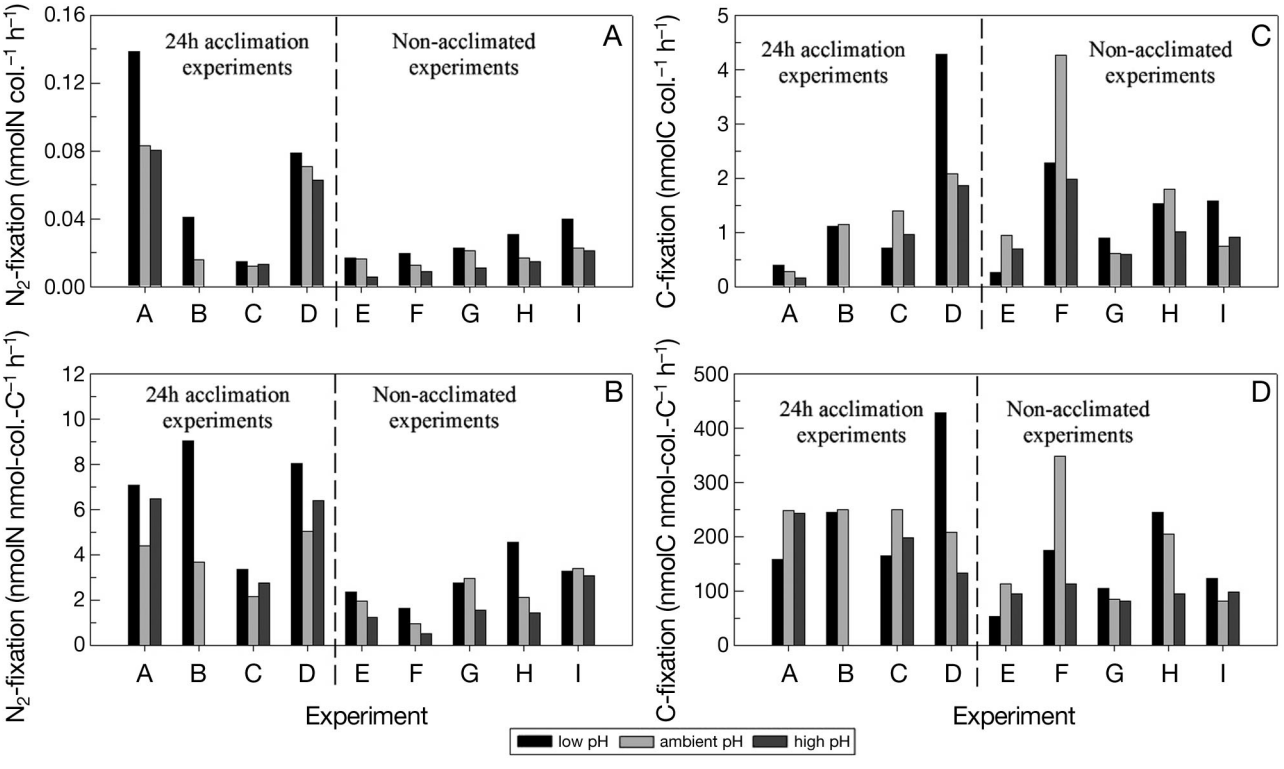

Fig. 1. *Trichodesmium thiebautii*. (A,B) N_2- and (C,D) C-fixation rates in response to low (black), ambient (light grey) and high (dark grey) pH for each of the 9 independent experiments, in terms of N or C fixed (A,C) per colony (col.) or (B,D) normalized to colony C content. See Table 1 for experimental descriptions

RESULTS AND DISCUSSION

Trichodesmium incubations

Colony normalized N_2-fixation rates at ambient pH/pCO_2 averaged (±SD) 0.031 ± 0.026 (range 0.014 to 0.082; n = 9; Fig. 1A) nmol N colony^{-1} h^{-1}, which is at the low end of rates previously measured at BATS using $^{15}N_2$ incubations (0.05 to 0.5 nmol N colony^{-1} h^{-1}; Orcutt et al. 2001). A possible reason for this is the size of the colonies sampled. Orcutt et al. (2001) measured an average colony N quota of ~100 to 200 nmol N colony^{-1} which is slightly higher than the values of ~50 to 125 nmol N colony^{-1} observed in the present study (Fig. 2A). Due to the possibility of changes in colony size or elemental composition between sampling points, N_2-fixation rates were also normalized to colony POC (Fig. 1C,D). In all 9 *Trichodesmium theibautii* experiments, nutrient-replete (see 'Methods') N_2-fixation rates increased at low pH relative to rates at ambient pH with no significant difference (Student's *t*-test, p = 0.372) between experiments with (0.032 ± 0.026 N colony^{-1} h^{-1}) and without (0.030 ± 0.029 N colony^{-1} h^{-1}) the 24 h acclimation period (Fig. 1A). Further, normalization to colony C did not change the general pattern of data, and N_2-fixation rates were significantly higher (Student's *t*-test; p < 0.01) at low pH (Table 2). On average, the N_2-fixation rate at low pH was 54% (range 6 to 156%) greater (HSD and *t*-tests, p < 0.05) than at ambient pH conditions (Table 2). The results of our field experiments with *T. thiebautii* are very similar, qualitatively and quantitatively, with those obtained in cultures of *T. erythraeum* (Barcelos e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007) and *Trichodesmium* spp. from the Gulf of Mexico (Hutchins et al. 2009). N_2-fixation rates decreased in all but one incubation at high pH relative to ambient pH (Fig. 1A, Table 2). N_2-fixation rates at high pH were on average 21% (range 4 to 63%) lower than at ambient *p*H (HSD and *t*-tests, p < 0.05, Table 2) but still measurable at 0.027 ± 0.028 nmol N colony^{-1} h^{-1}. This is in contrast to the findings of Hutchins et al. (2007), who found that at low pCO_2 (~180 ppm, similar to our low pH experiment), cultured *T. erythraeum* would not grow. This finding suggests that there may be species-specific thresholds for growth at low pCO_2 or that natural populations of *Trichodesmium* have a greater resilience to low pCO_2, and perhaps other sub-optimal environmental conditions, than cultured representatives; this highlights the need to study the effects of ocean acidification with natural planktonic assemblages as well as cultured representatives.

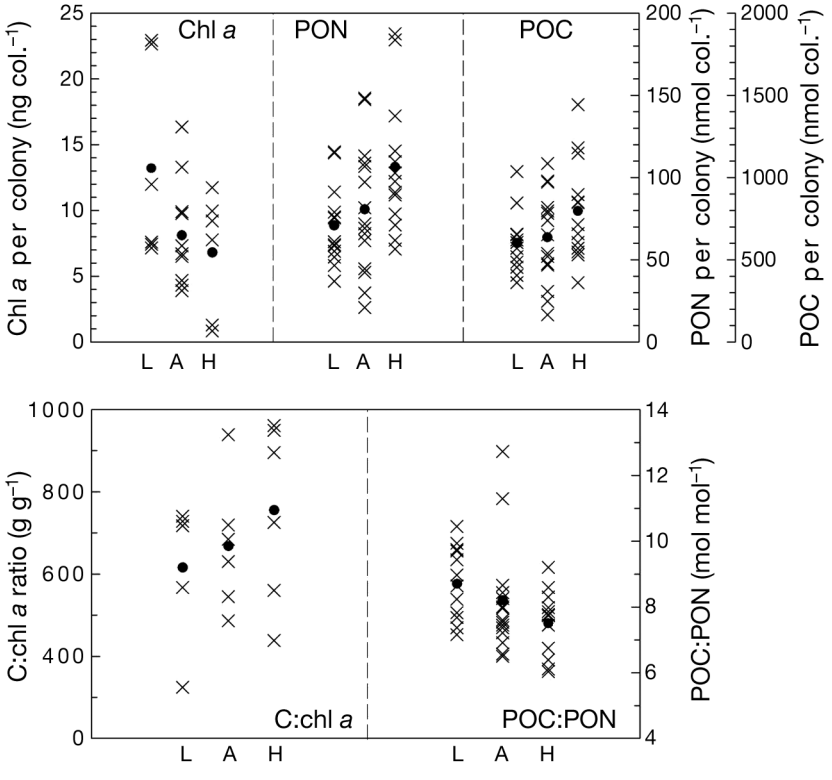

Fig. 2. *Trichodesmium thiebautii*. Biochemical characterization in response to changes in pH. (A) Chlorophyll *a* (chl *a*) per colony (col.) (left partition), particulate organic nitrogen (PON) per colony (middle partition), and particulate organic carbon (POC) per colony (right partition). (B) Colony C:chl *a* ratio (left partition) and colony C:N ratio (right partition). ×: values for all experiments grouped by pH treatment; filled circle: mean. L, A, H represent low, ambient and high pH treatments, respectively

The C-fixation rates were more variable but nonetheless show that in 6 out of 9 experiments, C-fixation rates decreased with increasing pH (Fig. 1C). On average, C-fixation rates were 22 to 24% (range –53 to 23%) lower at high pH than at ambient pH and 37% (–72 to 112%) lower than at low pH, but the differences were not significant regardless of normalization (Table 2). These relative changes are in good agreement with previous work in cultures of

Table 2. Fractional changes in N_2- and C-fixation rates at high and low pH relative to rates measured at ambient pH. Data are presented for colony normalized and colony (col.) C normalized rates. Negative values represent decreases in rates relative to rates at ambient pH. Statistical tests report values for low pH vs. high pH comparisons for the same normalized rate measurements. nd: no data

Experiment	N_2-fixation (nmol N col.$^{-1}$ h^{-1})		N_2-fixation (nmol N nmol-col.-C^{-1} h^{-1})		C-fixation (nmol C col.$^{-1}$ h^{-1})		C-fixation (nmol C nmol-col.-C^{-1} h^{-1})	
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH
A	0.66	−0.04	0.62	0.47	0.40	−0.44	−0.36	−0.02
B	1.56	nd	1.44	nd	−0.03	nd	−0.02	nd
C	0.22	0.11	0.57	0.28	−0.49	−0.31	−0.34	−0.20
D	0.11	−0.11	0.60	0.27	1.06	−0.10	1.06	−0.36
E	0.06	−0.63	0.21	−0.37	−0.72	−0.27	−0.53	−0.16
F	0.57	−0.29	0.678	−0.46	−0.47	−0.53	−0.50	−0.67
G	0.10	−0.48	−0.07	−0.48	0.45	−0.04	0.24	−0.03
H	0.82	−0.12	1.17	−0.32	−0.14	−0.44	0.20	−0.54
I	0.74	−0.09	−0.04	−0.09	1.12	0.23	0.52	0.21
Average	0.54	−0.21	0.58	−0.09	0.13	−0.24	0.03	−0.22
SD	0.49	0.24	0.51	0.38	0.67	0.25	0.53	0.29
Statistic	Student's *t*-test p = 0.001		Student's *t*-test p = 0.009		Mann-Whitney rank sum p = 0.36		Mann-Whitney rank sum p = 0.31	

Trichodesmium erythraeum, which showed a 15 to 128% increase in C-fixation from present conditions to future high pCO_2 conditions (750 ppm; Hutchins et al. 2007). The two experiments where C-fixation rates decreased at low pH (C and G; Fig. 1B) were also 2 of the lowest in terms of N_2-fixation response, suggesting that some other constraint in those experiments may have mitigated the response to changing pCO_2.

The increases in N_2-fixation and C-fixation under nutrient-replete, low pH conditions are consistent with the upregulation of cellular machinery leading to enhancement of growth rates (growth rates themselves were not likely to have increased over this short acclimation and measurement period) as previously shown (Barcelos e Ramos et al. 2007, Hutchins et al. 2007, Levitan et al. 2007). There are at least 2 possible physiological explanations for this observation. First, it is possible that the specific activity of the enzymes mediating these rate processes (i.e. nitrogenase and RUBISCO) changes in response to changes in pH (Levitan et al. 2010). Second, upregulation may be attributed to reallocation of cellular energy demand from the C concentrating mechanism allowing for enhancement of overall cellular growth rates (Kranz et al. 2010). Genetic evidence suggests *Trichodesmium* has a C concentrating mechanism similar to other cyanobacteria (Badger et al. 2006); however, the observation that C-fixation at high pH is depressed at short (Fig. 1) and acclimated timescales (Hutchins et al. 2007) suggests that the ability to acquire inorganic carbon is limited or energetically expensive at ambient and lower pCO_2 levels. Consistent with the hypothesized upregulation of cellular growth at low pH is an average increase in mean colony chl *a* content at high pCO_2 relative to chl *a* per colony at low pCO_2 (Fig. 2A; 1-way ANOVA, p = 0.108), which would provide the additional cellular energy needed for the energy intensive pathways of N_2- and C-fixation. However, we do not have data on variable fluorescence that would explain whether the hypothesized change in energy allocation is due to increased energy transfer efficiency or due to changes in chl *a* and therefore total light harvested. The average increase in the colony C:chl *a* ratio with increasing pH (Fig. 2B) might suggest the latter, but this remains to be determined. Interestingly, there was a slight, but not significant (1-way ANOVA, p = 0.099), decrease in the particulate C:N ratio during incubation at high pH (C:N = 7.5 ± 0.83) and low pH (C:N = 6.5 ± 0.85; Fig. 2B), consistent with the slightly higher stimulation of colony-normalized N_2-fixation relative to C-fixation (Fig. 1). The ratio of C and N-specific uptake rates (i.e. N_2 fixation normalized to colony N and C fixation normalized to colony C) at high pH (ratio = 6.6 ± 2.9) and low pH (6.3 ± 2.3) also suggests little change in the cellular demand for C:N as a function of inorganic carbon cycle changes (data not shown). Further research is required to determine whether this stability of the cellular C:N ratio is actively controlled by physiological mechanisms (e.g. changes in enzyme specific activity) and whether there are conditions under which they become uncoupled.

Whole community incubations

During September 2009, March 2010, and April 2010, similar incubation experiments were performed at varying pH/pCO_2 with the natural phytoplankton assemblage, of which 47 to 95% of the autotrophic carbon biomass was attributed to *Prochlorococcus* and *Synechococcus* (Table 1). The remainder of the autotrophic biomass was dominated by pico- and nanoeukaryotes (data not shown). When ^{14}C experiments were performed immediately after pH adjustment and nutrient addition (September and March only), a significant ($p = 0.002$, *t*-test, 2-tailed distribution) increase in chl *a* normalized C-fixation (i.e. assimilation number) with decreasing pH was observed (Table 3). The relative change in assimilation number over the full experimental pH range was 25 to 33% (Table 3). This differential effect was short-lived as no significant difference in assimilation number between pCO_2 conditions was observed after 1 to 3 d of incubation in any treatment or month (Table 3). There were differences between the initial and final assimilation number values, likely due to variable nutrient status in the various treatments at the end of the incubation. For example, in all P-limited and replete incubations during all of the cruises, the chl *a* concentration (as a proxy for biomass) and *Synechococcus* cell number consistently increased. In the March and April experiments, chl *a* increased to >2 µg l^{-1}, which likely resulted in substantial consumption of added nutrients, whereas in September, chl *a* also increased 3-fold but remained <0.2 µg l^{-1}, and nutrients were not substantially reduced. Regardless of the treatment or month, the relative change in assimilation number over the full range of pH treatments did not exceed 18% and averaged 11 ± 7%. These results suggest that natural phytoplankton assemblages dominated by cyanobacteria have the ability to immediately respond, via changes in assimilation number, to either increases or decreases in the ambient pCO_2 condition. On the acclimation time scale (days), however, the assimilation numbers equalized, likely through adjustment of the C concentrating mechanism. Therefore, the pCO_2 response in single cell cyanobacteria, unlike that observed above for *Trichodesmium*, appears to be transient. On the timescale of cell division, photosynthesis adjusts to optimize growth at the new conditions, and therefore, cell growth is unlikely to be controlled by changes in the inorganic carbon cycle but rather by macronutrient or micronutrient availability (Beardall et al. 2009).

As expected, net cell growth was low and did not vary with pH treatment in either the P-limited 'control' or P-replete treatments. The trends in net growth across pH treatments were not consistent between nutrient treatments (Table 3). *Synechococcus* cell numbers increased with decreasing pH in the absence of added phosphate but decreased with decreasing pH when phosphate was added. The same general pattern as a function of pH held for *Prochlorococcus.* However, overall, there was a substantial drop in cell abundance between initial values and those at the end of the incubation. These data support prior research that tight grazer control on picoplankton under increasing pCO_2 might be maintained as long as the picoplankton community composition does not change significantly, as was the case in these experiments (Rose et al. 2009). In addition, they suggest that there may be an interaction between nutrient status and pH manipulation that should be considered further in additional experiments, but treatment responses are likely to be small.

Because the cyanobacteria were analyzed using flow cytometry, information was also gained on relative cell size and pigment content (assessed by fluorescence) for *Synechococcus* and *Prochlorococcus* as a function of pH and nutrient conditions. Cell size (based upon forward light scatter as a proxy) changed little (≤20%) between the initial population and most nutrient treatments and was only related to pH (increasing with increasing pH) in the September experiment for *Synechococcus* (Table 3). In March, the mean cellular chl *a* and phycoerythrin fluorescence for *Synechococcus* and *Prochlorococcus* decreased ~50% in all tests, likely due to photoacclimation, as cells were incubated at irradiances higher than what they were exposed to in the field. Consistent with this explanation is the much smaller reduction in cellular pigment fluorescence during the incubation in April when the mixed layer depth had shoaled to ~60 m within the euphotic zone (~95 m; Siegel et al. 2001) from the >200 m in March (BATS unpubl. data). Cellular pigments, both chl *a* and phycoerythrin, were largely independent of changes in pH treatment, with the exception of *Synechococcus* in September, which showed a consistent decrease in pigment per cell with a decrease in pH (Table 3). *Prochlorococcus* exhibited no change in cellular pigment levels in response to pH or nutrient treatments, suggesting that the 2 important single cell cyanobacteria do not have the same physiological response to the same environmental manipulations. Our data from the fall and spring cruises when

Table 3. Summary of whole community, *Synechococcus* and *Prochlorococcus* response to pCO_2/pH manipulations. Initial values were taken immediately after pH was adjusted; all others (T_f) were at the end of the incubation period. Data are mean (SD). –: no sample collected. Initial samples were only collected from the ambient (A) condition, so low (L) and high (H) columns are left open. Flow cytometer fluorescence values are normalized to values for 0.53 µm beads. Error estimates for flow cytometry data were derived as the coefficient of variation (CV) for each parameter for each of the cyanobacteria populations; n = 503 to 2771 individual cells counted for *Prochlorococcus* and n = 1912 to 15 071 individual cells for *Synechococcus*. CVs were FSC_{syn} = 90.3 %, FSC_{pro} = 58.8 %, RFL_{syn} = 49.5 %, RFL_{pro} = 63.0 %, OFL_{syn} = 48.4 %, and OFL_{pro} = 52.1 % (FSC: forward scatter; RFL: red fluorescence; OFL: orange fluorescence). To all treatments, 5 µmol l^{-1} NO_3, 0.5 µmol l^{-1} PO_4, 5 µmol l^{-1} Si and 2 nmol l^{-1} Fe was added except as follows: P-limited, PO_4 was omitted; N-limited, NO_3 was omitted. L, A, H: low, ambient, and high pH treatments, respectively; S, M, A: September, March, and April sampling efforts, respectively; PE: phycoerythrin; rfu: relative fluorescence units

Parameter	Month	Initial			T_f P-limited			T_f N-limited			T_f replete		
		L	A	H	L	A	H	L	A	H	L	A	H
	S	7.76	8.08	8.30	–	–	–	–	–	–	7.76	8.08	8.30
	M	pH 7.70	8.07	8.37	7.77	8.14	8.45	–	–	–	7.80	8.14	8.42
	A	7.68	8.08	8.37	7.67	8.05	8.32	7.70	8.09	8.37	7.70	8.09	8.36
Whole community													
Assim. no.	S	0.44 (0.09)	0.39 (0.06)	0.35 (0.04)	–	–	–	–	–	–	0.94 (0.20)	0.83 (0.23)	0.88 (0.03)
(mol C g^{-1}	M	0.63 (0.00)	0.50 (0.00)	0.47 (0.00)	0.30 (0.05)	0.29 (0.03)	0.26 (0.04)	–	–	–	0.39 (0.03)	0.35 (0.03)	0.34 (0.00)
chl *a* h^{-1})	A	–	–	–	0.41 (0.05)	0.33 (0.08)	0.35 (0.06)	0.48 (0.08)	0.55 (0.05)	0.43 (0.05)	0.33 (0.05)	0.30 (0.01)	0.03 (0.02)
Chl *a* (µg l^{-1})	S		0.05 (0.01)		–	–	–	–	–	–	0.16 (0.02)	0.18 (0.03)	0.18 (0.03)
	M		0.34 (0.00)		1.38 (0.69)	1.37 (0.72)	1.69 (0.88)	–	–	–	3.15 (1.61)	4.16 (2.09)	4.16 (2.09)
	A		0.69 (0.00)		0.77 (0.15)	0.85 (0.03)	0.81 (0.00)	0.42 (0.03)	0.39 (0.03)	0.44 (0.03)	2.31 (0.25)	2.49 (0.00)	2.20 (0.12)
Particulate	S		–		–	–	–	–	–	–	–	–	–
carbon	M		–		11.83 (2.46)	11.68 (1.94)	12.41 (0.45)	–	–	–	13.40 (1.31)	15.17 (1.10)	14.79 (0.26)
(µmol C l^{-1})	A		8.83 (0.76)		12.66 (1.70)	18.08	14.46 (0.76)	16.18 (1.57)	17.69 (2.41)	16.59 (0.98)	17.88 (1.96)	18.17 (0.73)	16.50 (0.99)
Particulate	S		–		–	–	–	–	–	–	–	–	–
nitrogen	M		–		1.38 (0.28)	1.60 (0.34)	1.54 (0.06)	–	–	–	1.91 (0.25)	2.18 (0.14)	2.24 (0.09)
(µmol N l^{-1})	A		0.99 (0.04)		1.49 (0.12)	2.19	1.56 (0.00)	1.22 (0.04)	1.36 (0.13)	1.27 (0.02)	2.72 (0.27)	2.82 (0.11)	2.47 (0.14)
Particulate	A		–		–	–	–	–	–	–	–	–	–
phosphorus	S		–		–	–	–	–	–	–	–	–	–
(µmol P l^{-1})	M		–		0.066 (0.007)	0.090 (0.022)	0.067 (0.006)	–	–	–	0.176 (0.011)	0.236 (0.051)	0.239 (0.016)
C:N ratio	S		–		–	–	–	–	–	–	–	–	–
(mol mol^{-1})	M		–		8.59 (0.19)	7.35 (0.38)	8.08 (0.59)	–	–	–	7.36 (0.42)	6.85 (0.20)	6.67 (0.18)
	A		8.93 (0.44)		8.47 (0.49)	8.27	9.28 (0.51)	13.20 (0.84)	13.02 (0.84)	13.06 (0.76)	6.57 (0.11)	6.45 (0.10)	6.68 (0.05)
C:P ratio	S		–		–	–	–	–	–	–	–	–	–
(mol:mol^{-1})	M		–		–	–	–	–	–	–	–	–	–
	A		–		183.5 (56.4)	132.8 (21.6)	179.5 (8.6)	–	–	–	77.1 (14.2)	66.0 (11.9)	62.0 (4.4)
Synechococcus													
Cell abundance	S		10.1 (4.5)		–	–	–	–	–	–	51.1 (4.6)	48.0 (2.2)	46.1 (4.3)
($\times 10^3$ cells ml^{-1})	M		49.7		100	63.3	65.1	–	–	–	12.4	76.3	99.3
	A		16.1		49.3	45.6	29.5	–	–	–	30.7	47.9	48.2
Forward scatter	S		1.57 (0.22)		–	–	–	–	–	–	2.36 (0.04)	2.56 (0.07)	2.82 (0.11)
(FSC_{syn}; rfu)	M		2.26		1.74	1.69	1.71	–	–	–	1.66	1.68	1.67
	A		2.25		2.21	2.19	2.18	–	–	–	2.23	2.20	2.20
Chl *a*	S		0.34 (0.15)		–	–	–	–	–	–	1.26 (0.33)	1.09 (0.06)	1.01 (0.36)
fluorescence	M		2.76		1.16	1.15	1.16	–	–	–	1.48	1.38	1.39
(RFL_{syn}; rfu)	A		1.87		1.48	1.39	1.35	–	–	–	1.51	1.52	1.53
PE	S		0.56 (0.28)		–	–	–	–	–	–	2.89 (0.59)	2.18 (0.07)	1.70 (0.47)
fluorescence	M		3.85		1.94	1.98	2.01	–	–	–	1.83	1.66	1.45
(OFL_{syn}; rfu)	A		2.66		2.04	1.73	1.56	–	–	–	2.09	2.06	1.79

Table 3 (continued)

Parameter	Month	Initial			T_f P-limited			T_f N-limited			T_f replete		
		L	A	H	L	A	H	L	A	H	L	A	H
	S	7.76	8.08	8.30	–	–	–	–	–	–	7.76	8.08	8.30
	M pH	7.70	8.07	8.37	7.77	8.14	8.45	–	–	–	7.80	8.14	8.42
	A	7.68	8.08	8.37	7.67	8.05	8.32	7.70	8.09	8.37	7.70	8.09	8.36
Prochlorococcus													
Cell abundance	S		25.2 (2.6)		–	–	–	–	–	–	7.8 (0.5)	8.1 (1.2)	11.1 (1.9)
($\times 10^3$ cells ml^{-1})	M		10		15.3	13.1	12.3	–	–	–	7.1	17.5	9.2
	A		9.7		11.5	11.2	7.8	–	–	–	3.6	7.8	7.1
Forward scatter	S		0.38 (0.05)		–	–	–	–	–	–	0.36 (0.00)	0.36 (0.00)	0.36 (0.00)
(FSC_{proi}, rfu)	M		0.44		0.41	0.40	0.41	–	–	–	0.33	0.39	0.31
	A		0.58		0.55	0.55	0.57	–	–	–	0.47	0.48	0.49
Chl *a*	S		0.06 (0.00)		–	–	–	–	–	–	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)
fluorescence	M		0.18		0.08	0.08	0.08	–	–	–	0.08	0.08	0.08
(RFL_{proi}, rfu)	A		0.11		0.08	0.08	0.08	–	–	–	0.08	0.08	0.08
PE	S		–		–	–	–	–	–	–	–	–	–
fluorescence	M		0.03		0.03	0.03	0.03	–	–	–	0.03	0.03	0.04
(OFL_{proi}, rfu)	A		0.03		0.03	0.03	0.03	–	–	–	0.03	0.03	0.03

cyanobacteria are very important contributors to autotrophic carbon biomass show relatively little direct effect of pH on net growth (by changes in cell number), carbon assimilation and cellular pigment levels. This is in accord with the results of the laboratory study of Fu et al. (2007), in which most of the effect observed in *Synechococcus* growth was caused by high temperature rather than increased pCO_2. These observations suggest that, unlike *Trichodesmium*, which is limited by inorganic carbon at low pCO_2, single cell cyanobacteria are not C-limited at low pCO_2.

CONCLUSIONS

According to the results of the present field study, the only consistent and significant effect of decreasing pH on the cyanobacterial phytoplankton at BATS was an increase in N_2- and C-fixation by *Trichodesmium*. This increase, which was seen in isolated *T. thiebautii* colonies in the field, is similar to what has been observed in several studies with pure cultures of *T. erythreum* IMS101 (e.g. Barcelos e Ramos et al. 2007, Hutchins et al. 2007). The implication is that rising pCO_2 in surface seawater could lead to a substantial augmentation of N_2-fixation rates by *Trichodesmium* and thus an increasing input of fixed N into marine ecosystems where this organism thrives. A major caveat is that, like most field measurements, our results were obtained under conditions of excess P and Fe, 2 nutrients that often limit the growth of *Trichodesmium* in the ocean (Paerl et al. 1994, Lenes et al. 2001, Sañudo-Wilhelmy et al. 2001, Achilles et al. 2005). Stratification will likely only increase in a future pCO_2-enriched ocean, thus reducing vertical nutrient inputs in the subtropical and tropical gyres where *Trichodesmium* is most important (Sarmiento et al. 2004). Hutchins et al. (2007) showed that under P limitation, *Trichodesmium* maintained near maximum growth rates due a reduction in cellular P quota, likely through substitution of sulfolipids for phospholipids (Van Mooy et al. 2009), thus countering potential reductions in growth rate associated with increased nutrient limitation associated with increased stratification (e.g. Rost et al. 2008).

Bulk communities dominated by *Synechococcus* and *Prochlorococcus*, while showing a short term pH response (depression of C-fixation at high pH), rapidly acclimate and up-regulate photosynthetic capacity such that pH-dependent responses are no longer evident after 1 to 3 d, the temporal resolution of these

experiments. Our observations of small and non-significant effects of pH on *Prochlorococcus* and *Synechococcus* growth and physiological parameters are also in accord with the results of laboratory experiments with pure cultures (Fu et al. 2007) and further highlight the differences in response of similar phytoplankton to the same change in an environmental parameter. While a number of studies of phytoplankton response to increased pCO_2 have reported a variety of potentially significant responses (reviews by Fabry 2008, Hutchins et al. 2009), it may be important to remember that many experiments in which no measurable effect of increasing pCO_2 were detected have probably not been published. In the field of ocean acidification, all responses are important to understand how future autotrophs will respond to these environmental changes.

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