OCEAN ACIDIFICATION

The complex effects of ocean acidification on the prominent N_2-fixing cyanobacterium *Trichodesmium*

Haizheng Hong,[1] Rong Shen,[1] Futing Zhang,[1] Zuozhu Wen,[1] Siwei Chang,[1] Wenfang Lin,[1] Sven A. Kranz,[2] Ya-Wei Luo,[1] Shuh-Ji Kao,[1] François M. M. Morel,[3] Dalin Shi[1]*

Acidification of seawater caused by anthropogenic carbon dioxide (CO_2) is anticipated to influence the growth of dinitrogen (N_2)–fixing phytoplankton, which contribute a large fraction of primary production in the tropical and subtropical ocean. We found that growth and N_2-fixation of the ubiquitous cyanobacterium *Trichodesmium* decreased under acidified conditions, notwithstanding a beneficial effect of high CO_2. Acidification resulted in low cytosolic pH and reduced N_2-fixation rates despite elevated nitrogenase concentrations. Low cytosolic pH required increased proton pumping across the thylakoid membrane and elevated adenosine triphosphate production. These requirements were not satisfied under field or experimental iron-limiting conditions, which greatly amplified the negative effect of acidification.

The ongoing increase in dissolved carbon dioxide (CO_2) in the surface ocean caused by anthropogenic emissions is expected to affect the growth of marine phytoplankton because CO_2 is the substrate for photosynthetic carbon fixation (*1*). However, the concomitant decrease in seawater pH can also affect phytoplankton intracellular pH homeostasis (*2*), as well as the bioavailability of major and trace nutrients (*3*, *4*). Many recent laboratory and field experiments have examined this question and evinced generally modest effects of "ocean acidification" on phytoplankton (*5*). Organisms that have received particular attention are the ubiquitous cyanobacteria *Trichodesmium* spp., prominent dinitrogen (N_2) fixers in oligotrophic oceans (*6*), whose growth is often limited by iron (Fe) (*7*). Paradoxically, some studies have reported considerable increases in the rates of N_2-fixation, photosynthesis, and/or growth under acidified conditions (*8*–*11*), whereas others have documented significant decreases in the same rates under similar conditions in the same isolate of *Trichodesmium erythraeum* IMS101 (*T. erythraeum*) (*12*). In view of the key ecological role played by diazotrophs in the large regions of the oceans that are N-limited, it is important to resolve this discrepancy. In this study, we revisited the question of the effect of acidification on *T. erythraeum*: duplicating the methods used in previous experiments that yielded contrary results, examining the individual effects of high CO_2 and low pH, probing the biochemical basis for the observed effects under Fe-sufficient and Fe-limited conditions, and assessing the response to acidification of naturally occurring *Trichodesmium* populations in the South China Sea.

The growth rates previously observed in cultures of *T. erythraeum* under ambient partial pressure of CO_2 (Pco_2) vary widely, from <0.2 day^{-1} to >0.5 day^{-1}, without a clear understanding of the underlying reasons (*8*–*13*). Like previous investigators, we observed slow growth and N_2-fixation rates in YBCII medium prepared according to the published method (*14*), and these rates increased under acidified conditions (high Pco_2/low pH) as reported (Fig. 1A). Experiments with systematic modifications of the medium revealed contamination by a toxic metal (likely copper) (supplementary text) and by ammonium (NH_4^+), which we found at a concentration of 20 μM (supplementary text). Ammonia (NH_3) crosses biological membranes and is thought to inhibit the oxygen-evolving complex of photosystem II (*15*). Increasing the EDTA concentration to control metal toxicity and using an ultrapure source for $MgCl_2$, which was identified as the source of NH_4^+ contamination, we observed markedly increased growth rates (Fig. 1, A and B). Metal and NH_4^+ toxicity thus likely explains the low and highly variable growth rates of *T. erythraeum* observed by previous investigators in YBCII or similar media (0.16 to 0.41 day^{-1}) (table S1).

Metal and NH_4^+ toxicity also explains the positive effect of acidification on *T. erythraeum* in YBCII (Fig. 1A). Low pH shifts the NH_3/NH_4^+ equilibrium (pK_a = ~9.5 in seawater, where K_a is the acid dissociation constant) toward a lower concentration of NH_3 and changes the bioavailable concentration of metals (supplementary text) (*16*). When NH_4^+ was minimized and high EDTA used, the positive effect of acidification seen in YBCII medium was reversed (Fig. 1, A and B). *T. erythraeum* then reached a growth rate of >0.5 day^{-1} (Fig. 1B), which is nearly the same as in a natural seawater medium with free trace metal concentrations buffered with 20 μM EDTA ("Aquil-tricho") (Fig. 1C and table S2) (*17*). In Aquil-tricho, the growth and N_2-fixation rates of *T. erythraeum* also decreased at high Pco_2/low pH (Fig. 1C). As previously observed (*12*), these adverse effects were enhanced under Fe-limiting conditions despite increasing the total Fe concentration at low pH to maintain constant the biologically available free iron, Fe′ (table S3) (*4*).

One complication in the response of phytoplankton to ocean acidification is that the increase in Pco_2 and the decrease in pH may have opposite effects on physiology and growth. Hence, we varied Pco_2 and pH independently by adjusting the alkalinity in Aquil-tricho medium prepared with synthetic ocean water (table S4) (*17*). Both the growth and the N_2-fixation rates of *T. erythraeum* at high Fe dropped at low pH (pH 7.8 versus 8.1) but increased slightly at high Pco_2 (800 versus 400 μatm) (Fig. 2, A to C, and supplementary text). Overall, the inhibitory effect of low pH overwhelmed the stimulatory effect of high Pco_2, reducing growth and N_2-fixation under high CO_2/low pH conditions (Fig. 2, A and C). The positive effects of increasing Pco_2 (at constant pH) can be attributed to the down-regulation of the carbon-concentrating mechanisms (CCMs) that saturate the carboxylating enzyme, Rubisco (*18*). This is evidenced by the lower apparent affinities [higher half saturation concentrations ($K_{1/2}$)] for dissolved inorganic carbon at high Pco_2 (Fig. 2D) (*19*). This down-regulation allows energy and resources to be reallocated to other cellular processes, including N_2-fixation (Fig. 2, A to C) (*18*).

A key question regarding the effect of ocean acidification on *Trichodesmium* is whether the pH in the cytosol, where nitrogenase is located, decreases along with seawater pH. Using a fluorescent membrane-permeable probe (*20*), we measured a substantially lower cytosolic pH at a seawater pH of 7.8 compared with 8.1 (Fig. 2E). Symmetrically, the nitrogenase concentration increased at low pH (Fig. 2F), in opposite direction to the daily N_2-fixation (contrast Fig. 2, C and F). This indicates a lower efficiency of the nitrogenase enzyme of *T. erythraeum* at low pH, which may be due to a greater allocation of electrons to protons (H^+) instead of N_2, as evidenced by an enhanced production of H_2 (*12*, *21*).

To probe at the molecular level how acidification affects the biochemistry of *T. erythraeum*, we quantified a number of key proteins under ambient and acidified conditions with either sufficient or limiting Fe supply (Fig. 3). At high Fe, acidification resulted in an up-regulation of several proteins involved in energy generation and pH homeostasis, in addition to nitrogenase (Fig. 3, A and B). This includes the proteins involved in translocation of H^+ across the thylakoid membrane, reflecting the need to maintain cytosolic pH homeostasis and the H^+ gradient necessary for the function of adenosine triphosphate (ATP)

[1]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, Fujian, P.R. China. [2]Department of Earth, Ocean and Atmospheric Science, Florida State University, Tallahassee, FL 32306, USA. [3]Department of Geosciences, Princeton University, Princeton, NJ 08544, USA.
*Corresponding author. Email: dshi@xmu.edu.cn

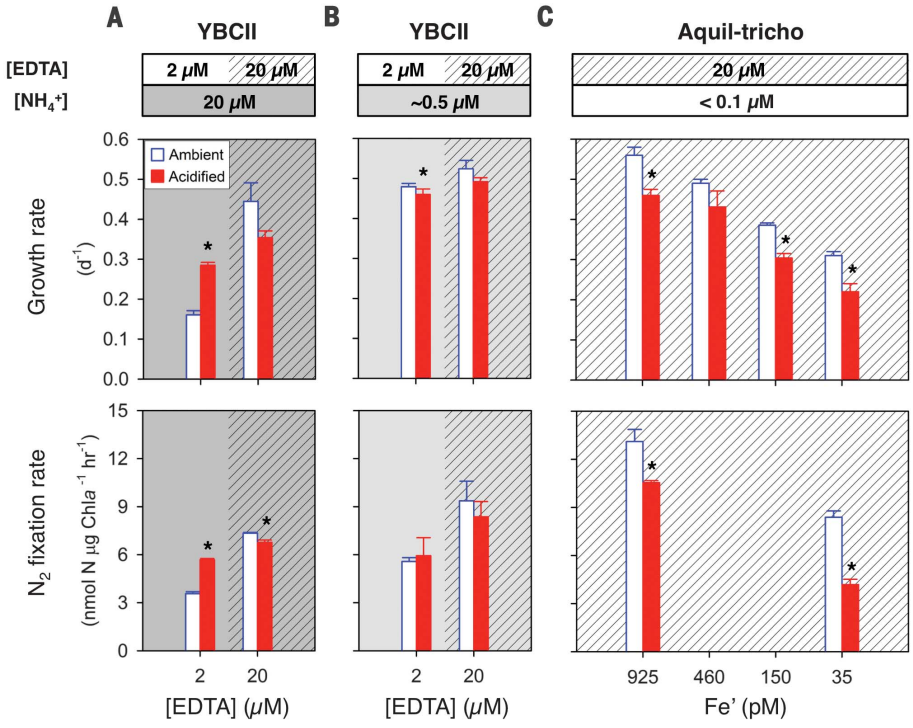

Fig. 1. The effect of ocean acidification on growth and short-term N_2-fixation of *T. erythraeum*. (**A** to **C**) Specific growth rates and short-term N_2-fixation rates of *T. erythraeum* in [(A) and (B)] the artificial medium YBCII, with varying concentrations of EDTA (2 or 20 μM illustrated without or with slashes, respectively) or background NH_4^+ (~0.5 or 20 μM illustrated by light gray or gray backgrounds, respectively), and (C) 20 μM EDTA-buffered natural seawater Aquil-tricho medium (background $[NH_4^+]$ < 0.1 μM) at varying inorganic Fe concentrations (Fe′) under ambient and acidified conditions (table S4). N_2-fixation rates were measured with the acetylene reduction method and converted by using a 4:1 ratio of ethylene production to N_2-fixation, which may underestimate the actual rates at high pH relative to those at low pH (*12*, *21*). Error bars represent the SD of biological replicates (n = 2 to 4). Asterisks denote significant changes under acidified conditions compared with ambient conditions (P < 0.05, Student's t test).

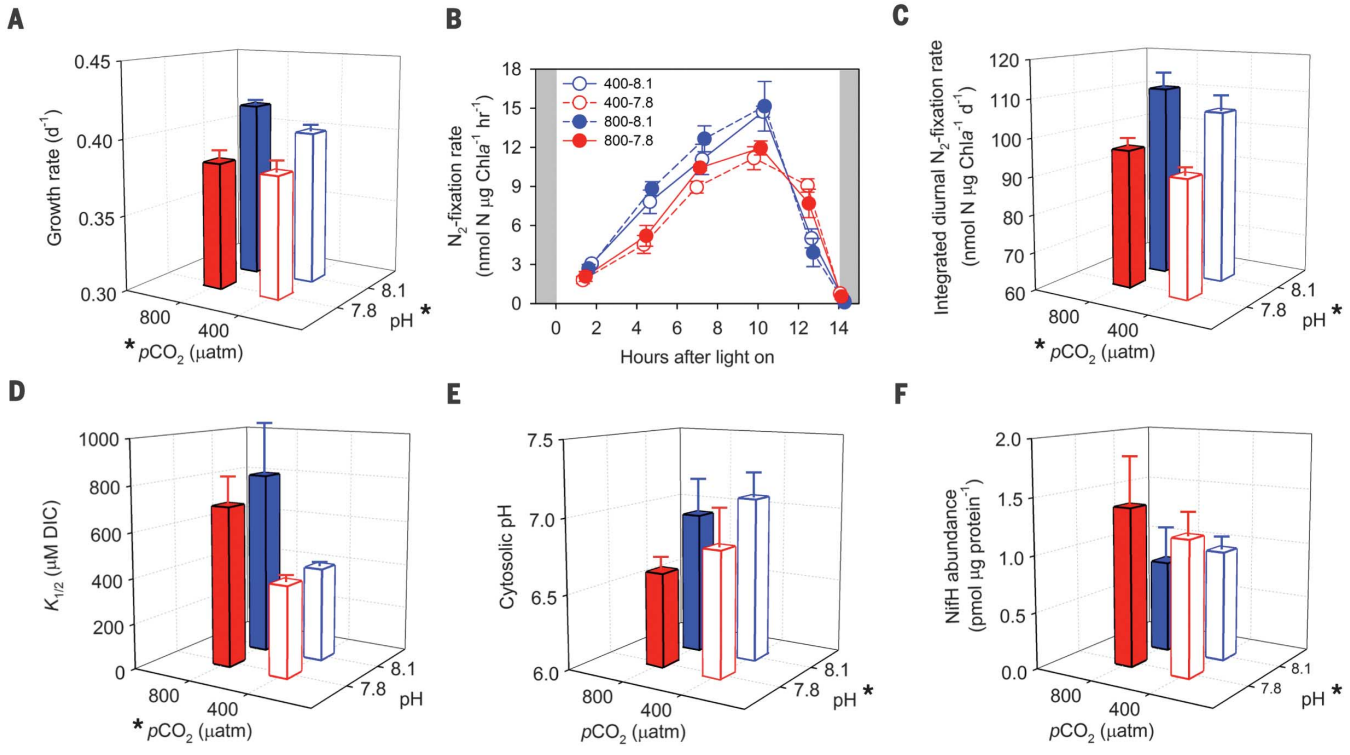

Fig. 2. Separate effects of increasing P_{CO_2} and decreasing pH on *T. erythraeum*. (**A**) Specific growth rates, (**B**) endogenous rhythm of N_2-fixation rate over a diurnal cycle (the gray areas indicate the dark phase), (**C**) integrated diurnal N_2-fixation rates, (**D**) half saturation concentrations ($K_{1/2}$) of photosynthesis, (**E**) cytosolic pH, and (**F**) NifH concentration of steady-state growing *T. erythraeum* at high Fe (Fe_T = 1 μM) in Aquil-tricho medium prepared with synthetic ocean water, where P_{CO_2} and pH were varied independently (table S4) (*17*). $K_{1/2}$, cytosolic pH, and NifH concentration were determined at the middle of photoperiod (7 to 9.5 hours). Blue and red denote pH 8.1 and 7.8, respectively; solid bars and circles denote 800 μatm CO_2, and open bars and circles denote 400 μatm CO_2. Error bars represent the SD of biological replicates (n = 4). Significant differences between CO_2 treatments or between pH treatments are denoted by asterisks beside the axis titles of P_{CO_2} or pH (P < 0.05, two-way analysis of variance).

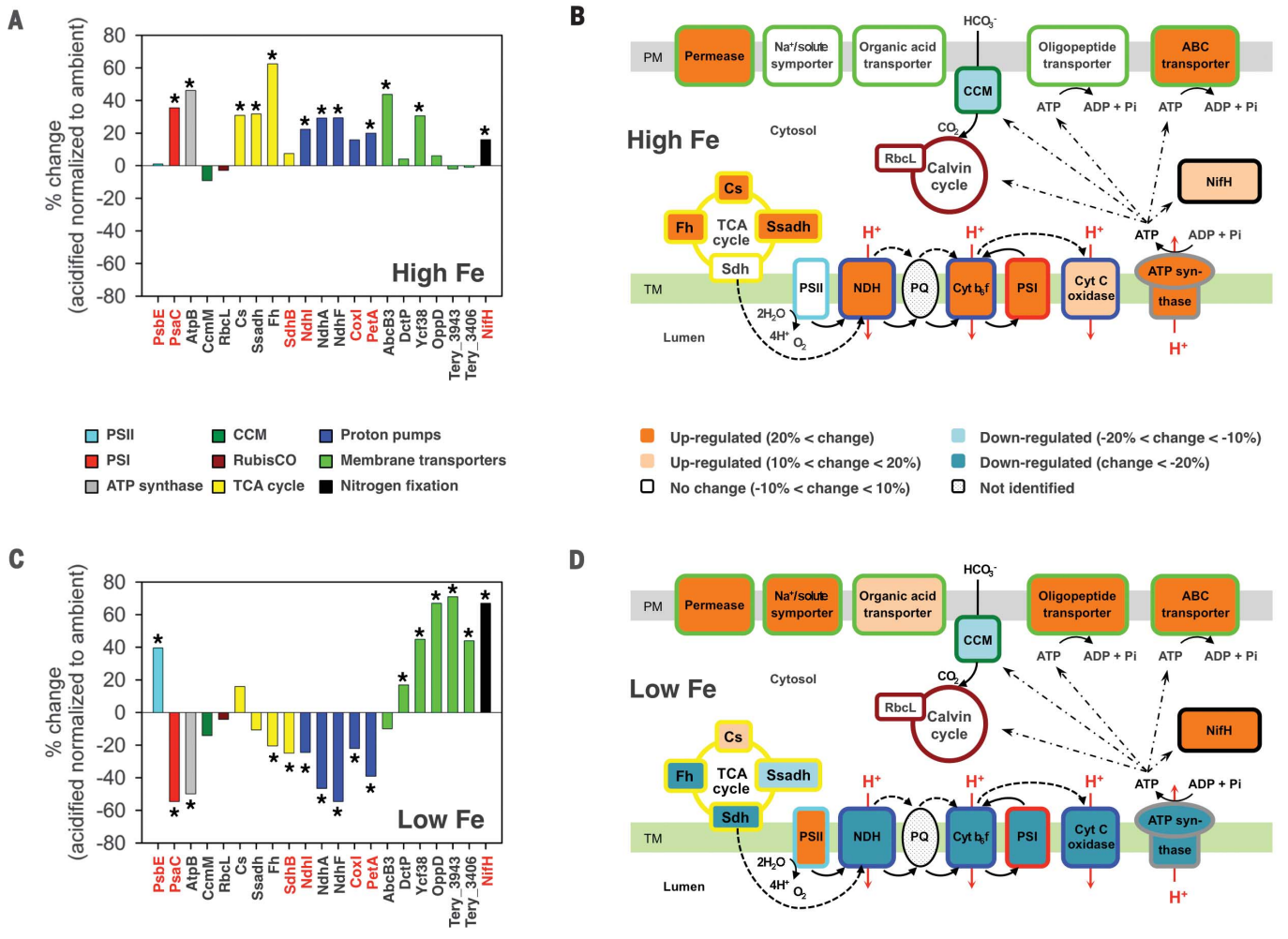

Fig. 3. Changes of protein abundance in response to ocean acidification in *T. erythraeum*. (**A** and **C**) Percentage change (acidified normalized to ambient condition) (table S4) of abundance of key proteins involved in photosynthesis, energy generation, carbon fixation, cytosolic pH homeostasis, and N_2-fixation in *T. erythraeum* at high (~925 pM Fe′) and low (~35 pM Fe′) Fe concentrations in 20 μM EDTA-buffered natural seawater Aquil-tricho medium. Data are mean of three biological replicates (n = 3; error bars not shown). Asterisks denote significant changes in protein abundance in response to acidification (fold changes of ≥1.2 or ≤0.83 with 5% false discovery rate cutoff, Student's t test followed by Benjamini-Hochberg correction). Fe-containing proteins are denoted by names in red. (**B** and **D**) Schematic representation of the functions of the proteins quantified in (A) and (C), respectively, and of their change in abundance in response to acidified condition at high and low Fe concentrations. Colors at the periphery of the protein pictograms correspond to bar colors in (A) and (C), and those in the center indicate the extent of up- or down-regulation of these proteins. PM, plasma membrane; TM, thylakoid membrane.

synthase. Evidence for an increased production of ATP at low pH is seen in the up-regulation of ATP synthase and tricarboxylic acid (TCA) cycle enzymes, as well as an increase in the PSI/PSII ratio, which likely indicates a more active cyclic electron flow (*18*). The increase in ATP production partly mitigates the effects of acidification on N_2-fixation by improving electron transfer to the substrate (*22*), and on cellular homeostasis as shown by the up-regulation of transporters and permeases in the plasma membrane (Fig. 3, A and B).

When Fe is limiting, the large increase in nitrogenase concentration necessitated by its reduced efficiency at low pH is made at the expense of Fe-rich proteins involved in the photosynthetic and respiratory electron transfer chains as well as H^+ translocation into the lumen for ATP production (Fig. 3, C and D). Plasma membrane transporters are significantly increased to compensate for the reduced H^+ pumping and maintain cytosolic pH homeostasis. Besides nitrogenase and membrane transporters, PSII is up-regulated considerably at low pH and Fe, as previously observed (*12*, *23*). Overall, acidification under Fe-limited conditions requires a reallocation of Fe among proteins to compensate for the loss of N_2-fixation efficiency, which affects electron flow (fig. S1), energy production, and pH homeostasis (Fig. 3, C and D).

Our laboratory results predict that ocean acidification may have opposite effects on *Trichodesmium* depending on whether it is limited by inorganic carbon or by iron. When *Trichodesmium* is organized in large colonies, the supply of CO_2 may become limiting, and the positive effects of increasing CO_2 can dominate over the negative effects of low pH (Fig. 2). This is a plausible explanation for the observed increase in N_2-fixation rate at high CO_2 by *Trichodesmium* spp. colonies collected from the Gulf of Mexico and the Sargasso Sea (*24*, *25*). Opposite effects of low pH and high P_{CO_2} may also help explain the variable responses to acidification of *Trichodesmium* spp. that have different growth rates and sizes at station ALOHA (A Long-term Oligotrophic Habitat Assessment) in the North Pacific (*26*, *27*).

Growth and N_2-fixation of *Trichodesmium* are known to be limited by Fe in vast regions of the oceans, such as in the South Atlantic and South Pacific (*7*, *28*). But to our knowledge, there has been no field study of the effect of acidification on N_2-fixation in Fe-limited regions. To test whether our laboratory results can be extended to natural *Trichodesmium* populations, we conducted experiments at three stations in the northern South China Sea (fig. S2), where surface Fe concentrations are very low and likely limit N_2-fixation

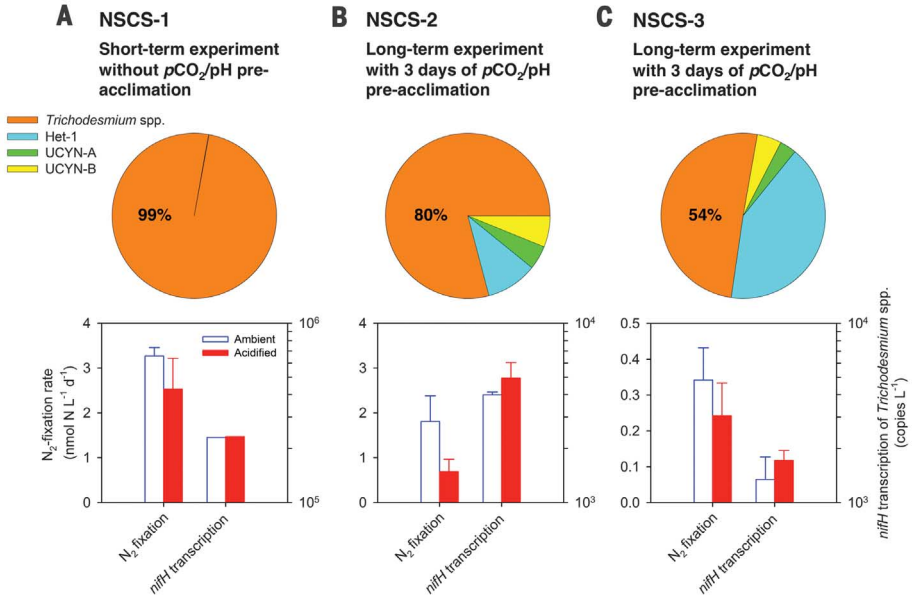

Fig. 4. Diazotroph community composition, and the effect of ocean acidification on N_2-fixation and *Trichodesmium* spp. *nifH* gene transcription in NSCS surface seawater. (**A** to **C**) Relative contribution (percent of total quantified *nifH* genes) of four different *nifH*-containing cyanobacteria, N_2-fixation rate of the diazotroph community, and *nifH* gene transcription of *Trichodesmium* spp. under ambient and acidified conditions (table S5) at the end of the manipulation experiments conducted at (A) NSCS-1, (B) NSCS-2, and (C) NSCS-3. For each experiment, the pie chart of diazotroph community composition shows the average of all treatments because there was no significant difference in *nifH* gene abundance of the phylotypes. Error bars represent the SD of biological replicates (n = 3). Differences between ambient and acidified conditions were significant for the N_2-fixation rates of the three experiments (P = 0.036) and for *nifH* transcription at NSCS-2 and NSCS-3 (P = 0.005) (Student's t test for normalized compiled data).

(fig. S3) (*29*). *Trichodesmium* spp. dominated the diazotroph community at Northern South China Sea 1 (NSCS-1) (99% of *nifH* genes) and accounted for a large fraction of it at NSCS-2 and NSCS-3 (80 and 54% of *nifH*, respectively) (Fig. 4). In all experiments, *Trichodesmium* spp. was present as free trichomes, and as in the laboratory experiments, the N_2-fixation rate decreased under acidified conditions. As expected, transcripts of the nitrogenase gene *nifH* were unchanged by acidification in the short-term experiment (Fig. 4A), whereas they increased significantly when the diazotrophs were acclimated for three days (Fig. 4, B and C, and table S5). This indicates a decrease in N_2-fixation efficiency by nitrogenase at low pH, which is consistent with our laboratory results.

The ongoing acidification of seawater caused by anthropogenic CO_2 will lead to various direct or indirect effects on marine phytoplankton. Our study reconciles previous results that show opposite effects of acidification on *Trichodesmium* and demonstrates a significant decrease in N_2-fixation by this prominent diazotroph at the seawater pH expected for year 2100, particularly under the Fe-limited conditions that prevail in large oceanic regions (*7*). Because *Trichodesmium* is estimated to contribute up to 50% of marine N_2-fixation (*30*), acidification could lead to a decline in the supply of new nitrogen to oceanic ecosystems, and this effect would be magnified if other diazotrophs were similarly affected.

REFERENCES AND NOTES

1. U. Riebesell *et al.*, *Nature* **450**, 545–548 (2007).
2. A. R. Taylor, A. Chrachri, G. Wheeler, H. Goddard, C. Brownlee, *PLOS Biol.* **9**, e1001085 (2011).
3. T. Tanaka *et al.*, *Biogeosciences* **5**, 669–678 (2008).
4. D. Shi, Y. Xu, B. M. Hopkinson, F. M. M. Morel, *Science* **327**, 676–679 (2010).
5. K. Mackey, J. J. Morris, F. M. M. Morel, S. A. Kranz, *Oceanography* **28**, 74–91 (2015).
6. D. G. Capone, J. P. Zehr, H. W. Paerl, B. Bergman, E. J. Carpenter, *Science* **276**, 1221–1229 (1997).
7. J. A. Sohm, E. A. Webb, D. G. Capone, *Nat. Rev. Microbiol.* **9**, 499–508 (2011).
8. J. Barcelos e Ramos, H. Biswas, K. G. Schulz, J. LaRoche, U. Riebesell, *Glob. Biogeochem. Cycles* **21**, GB2028 (2007).
9. D. A. Hutchins *et al.*, *Limnol. Oceanogr.* **52**, 1293–1304 (2007).
10. S. A. Kranz, S. Dieter, K.-U. Richter, B. Rost, *Limnol. Oceanogr.* **54**, 548–559 (2009).
11. O. Levitan *et al.*, *Glob. Change Biol.* **13**, 531–538 (2007).
12. D. Shi, S. A. Kranz, J. M. Kim, F. M. M. Morel, *Proc. Natl. Acad. Sci. U.S.A.* **109**, E3094–E3100 (2012).
13. M. Eichner, S. A. Kranz, B. Rost, *Physiol. Plant.* **152**, 316–330 (2014).
14. Y. B. Chen, J. P. Zehr, M. Mellon, *J. Phycol.* **32**, 916–923 (1996).
15. M. Drath *et al.*, *Plant Physiol.* **147**, 206–215 (2008).
16. F. M. M. Morel, J. G. Hering, *Principles and Applications of Aquatic Chemistry* (Wiley Inter-Science, 1993).
17. Materials and methods are available as supplementary materials.
18. S. A. Kranz, M. Eichner, B. Rost, *Photosynth. Res.* **109**, 73–84 (2011).
19. M. R. Badger, G. D. Price, B. M. Long, F. J. Woodger, *J. Exp. Bot.* **57**, 249–265 (2006).
20. G. K. Dixon, C. Brownlee, M. J. Merrett, *Planta* **178**, 443–449 (1989).
21. D. N. Pham, B. K. Burgess, *Biochemistry* **32**, 13725–13731 (1993).
22. R. V. Hageman, R. H. Burris, *Biochim. Biophys. Acta* **591**, 63–75 (1980).
23. N. G. Walworth *et al.*, *Nat. Commun.* **7**, 12081 (2016).
24. D. A. Hutchins, M. R. Mulholland, F.-X. Fu, *Oceanography* **22**, 128–145 (2009).
25. M. W. Lomas *et al.*, *Aquat. Microbiol. Ecol.* **66**, 211–222 (2012).
26. D. Böttjer, D. M. Karl, R. M. Letelier, D. A. Viviani, M. J. Church, *Glob. Biogeochem. Cycles* **28**, 601–616 (2014).
27. M. R. Gradoville, A. E. White, D. Böttjer, M. J. Church, R. M. Letelier, *Limnol. Oceanogr.* **59**, 645–659 (2014).
28. C. M. Moore *et al.*, *Nat. Geosci.* **2**, 867–871 (2009).
29. J. Wu *et al.*, *Glob. Biogeochem. Cycles* **17**, 1008 (2003).
30. C. Mahaffey, A. F. Michaels, D. G. Capone, *Am. J. Sci.* **305**, 546–595 (2005).

ACKNOWLEDGMENTS

The authors thank B. Hopkinson for helpful discussions and Y. Zhang for technical assistance with the analysis of diazotroph community structure. The authors gratefully acknowledge the captain and crew of the R/V *Dongfanghong* 2 for their help on the experiments conducted in the northern South China Sea region. This work was supported by the National Key Research and Development Program of China (no. 2016YFA0601203), the National Science Foundation of China (nos. 41222040 and 41376116), the Recruitment Program of Global Youth Experts of China, and a grant from the U.S. NSF to F.M.M.M. The data presented in all figures are available at https://dx.doi.org/10.1594/PANGAEA.874127.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/356/6337/527/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 to S5
References (*31*–*55*)

3 November 2016; accepted 12 April 2017
Published online 27 April 2017
10.1126/science.aal2981

Science

The complex effects of ocean acidification on the prominent N_2-fixing cyanobacterium *Trichodesmium*

Haizheng Hong, Rong Shen, Futing Zhang, Zuozhu Wen, Siwei Chang, Wenfang Lin, Sven A. Kranz, Ya-Wei Luo, Shuh-Ji Kao, François M. M. Morel and Dalin Shi

Science **356** (6337), 527-531.
DOI: 10.1126/science.aal2981originally published online April 27, 2017

Reconciling pH and future productivity

The differential effects of reduced seawater pH and increased carbon dioxide on marine phytoplankton productivity have not been resolved. Hong *et al.* found that previous experimentation did not account for variable metal concentrations or for ammonia contamination. After controlling for these variables, experimentation, protein expression analysis, and field data showed that low pH, coupled with the low ambient iron availability in the open ocean, inhibits nitrogen fixation, whereas elevated CO_2 is fertilizing. Overall, the deleterious effects of decreased pH trump the beneficial effects of increased CO_2. Thus, it seems that in a future, more acidic ocean, phytoplankton productivity is likely to be suppressed.

Science, this issue p. 527