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

David A. Hutchins[1*], Feixue Fu[1], Nathan G. Walworth[1], Michael D. Lee[1], Mak A. Saito[2] and Eric A. Webb[1]

[1] Marine and Environmental Biology, Department of Biological Sciences, University of Southern California, 3616 Trousdale Parkway, Los Angeles, California 90089, USA.

[2] Marine Chemistry and Geochemistry Department, Woods Hole Oceanographic Institution, 266 Woods Hole Road, Woods Hole, Massachusetts 02543, USA.

*Corresponding author: dahutch@usc.edu

Hong et al. (Reports, 5 May 2017, p.527) suggested that previous studies of the biogeochemically-significant marine cyanobacterium *Trichodesmium* showing increased growth and nitrogen-fixation at projected future high CO_2 levels suffered from ammonia or copper toxicity. They reported rates instead decrease at high CO_2 when contamination is alleviated. We present and discuss results of multiple published studies refuting this toxicity hypothesis.

Marine nitrogen-fixing cyanobacteria are important to the global carbon cycle and climate, as they provide vital new nitrogen supplies that allow phytoplankton to draw down atmospheric carbon dioxide (CO_2). Many experiments over the last decade have predicted that the globally-distributed tropical cyanobacterium *Trichodesmium* spp. will grow faster and fix 30-

60% more nitrogen under projected future doubled seawater CO_2 concentrations [*1-6*]. Such CO_2 fertilization of marine nitrogen-fixation could potentially provide a negative feedback on anthropogenic CO_2 emissions [*2,5,6*].

Hong et al. [*7*] argue that these often-reproduced results actually stem from chemical contamination of the widely used *Trichodesmium* artificial seawater culture-medium YBCII. A bad batch of $MgCl_2$ reagent used in their medium preparation led to contamination of their YBCII with ~20 µmol/L of growth-inhibiting ammonia. They speculate that accidental contamination with toxic copper is also likely, although no copper measurements are presented to support this contention. As evidence, they present experiments showing that growth and nitrogen-fixation rates increase when ammonia-free $MgCl_2$ is used to prepare YBCII, or when *Trichodesmium* are grown with higher levels of the trace metal chelator EDTA to bind and detoxify putative copper contamination. Crucially, they also found that the commonly observed CO_2 stimulation of *Trichodesmium* nitrogen-fixation and growth appears to be reversed in their 'uncontaminated' media. They therefore attribute the opposing results seen in nearly all prior studies to ubiquitous, previously unrecognized contamination artifacts [*7*].

This contamination-artifact hypothesis can however be conclusively refuted by examining published studies. Although Hong et al. state that "All previous laboratory studies that have reported positive… effects of acidification… have been carried out with… the growth medium YBCII..." [*S.I.,7*], in fact several *Trichodesmium* studies found large positive effects of high CO_2 in the same ammonia-free, trace-metal clean 'Aquil-tricho' medium they advocate [*5,6,8*]. Additionally, previous CO_2 experiments in both putatively 'contaminated' YBCII and in

Aquil-tricho measured *Trichodesmium* nitrogen-fixation rates that were as high as (or even higher than) the rates measured by Hong et al. in their 'uncontaminated' medium preparations (Fig. 1). We employ our own published results as examples, but most of the multiple previous studies cited by Hong et al. as being likely contaminated [*7*] also documented similarly high nitrogen-fixation rates. Moreover, if all these previous experiments were truly contaminated with 20 µmol/L ammonia as suggested, little or no nitrogen-fixation would have been observed, as *Trichodesmium* nitrogen-fixation is strongly inhibited (~50-100%) by ammonia concentrations of 10-20 µmol/L [*9-11*]. We have studied ammonia inhibition in nitrogen-fixing cyanobacteria [*11,12*], and our measurements show that ammonia concentrations in both YBCII and Aquil-tricho are typically below detection limits (< ~0.5 µmol/L).

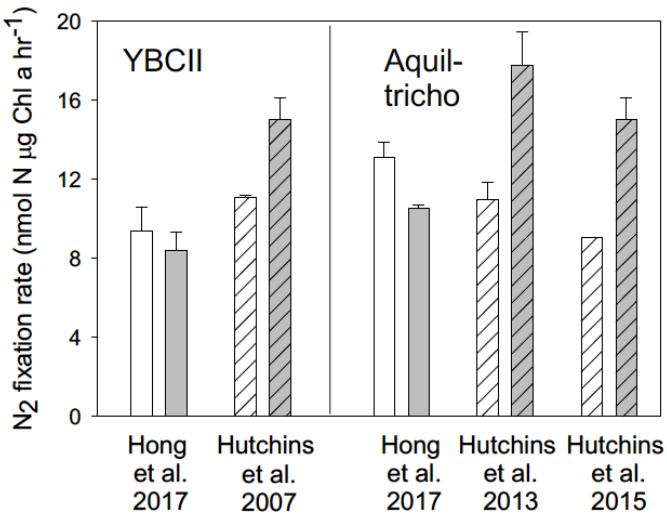

Fig. 1. Reported *Trichodesmium* nitrogen fixation rates in YBCII medium (left) and Aquil-tricho medium (right) in purportedly 'uncontaminated', iron-replete medium formulations from Hong et al. 2017 (open bars) and in previously published studies (hatched bars) in low CO_2 (white bars) and high CO_2 (grey bars) treatments[2,5-7]. Previous studies show no evidence for toxic inhibition of nitrogen fixation, and in fact rates are often higher than in Hong et al. (2017)[7]. Nitrogen-fixation was measured and normalized the same way in all experiments shown; previously published data[2,5,6] was recalculated using the same 4:1 ethylene:N_2 conversion ratio used in Hong et al. (2017)[7].

Also contradicting the toxic-contamination hypothesis is a study that examined seven different nitrogen-fixing cyanobacteria isolates grown across a range of CO_2 concentrations in Aquil-tricho medium [*5*]. In every case, *Trichodesmium* nitrogen-fixation rates closely fit a classic saturation-curve model relative to CO_2 (r^2 = 0.95-1.00); one of these data sets is shown in Fig 2, along with the corresponding Michaelis-Menten enzyme kinetics equation. This strikingly nutrient-like response to a CO_2 concentration gradient cannot be explained by invoking an inhibitory effect of contaminated growth medium.

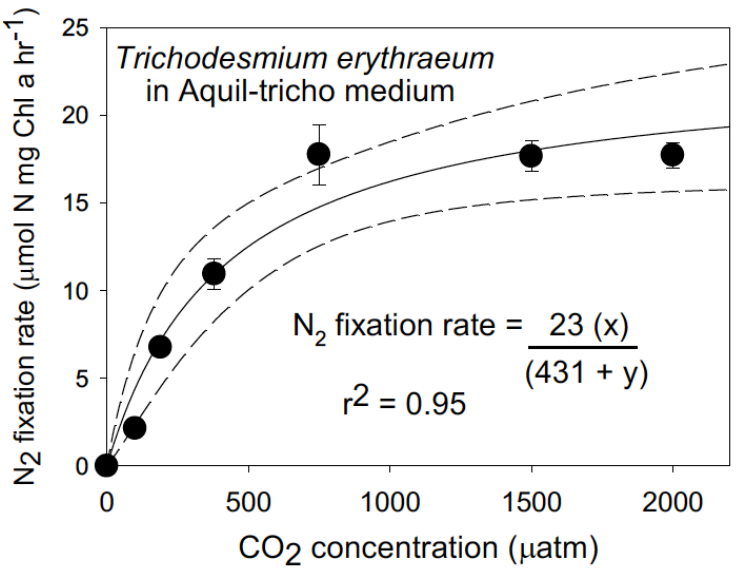

Fig. 2. Results of a prior *Trichodesmium* study that measured nitrogen-fixation rates across a range of CO_2 concentrations and found a highly significant fit to a nutrient-like saturation curve model[5]. The corresponding Michaelis-Menten equation is shown; solid line is the regression and dashed lines are 95% confidence intervals. This classic, well-defined positive response of nitrogen-fixation rates as CO_2 limitation is relieved is fundamentally inconsistent with a toxic effect at either high or low CO_2 concentrations. Nitrogen-fixation was measured and normalized in the same way[5], and recalculated using the same 4:1 ethylene:N_2 conversion ratio as in Hong et al. (2017)[7].

Likewise, contamination does not explain the findings of an experimental-evolution study wherein growth and nitrogen-fixation in six replicate Aquil-tricho-grown *Trichodesmium* cell lines were constitutively increased following ~850 generations of selection at high CO_2 [*6*]. These cell lines now permanently fix nitrogen at higher rates just as if they were growing at elevated CO_2, even when moved back to lower current

CO_2 concentrations, where Hong et al. purport they should again be inhibited due to contaminants. This unique adaptive response is again wholly inconsistent with toxic inhibition.

Despite this contrary evidence strongly suggesting that culture-medium toxicity is irrelevant to the results of most previous studies, we are still left with the puzzling observation that Hong et al. recorded growth rates that were ~25% higher than other published rates [*7*] – even though the nitrogen-fixation rates supporting this rapid growth were similar to, or less than, those in previous studies (Fig. 1). However, this discrepancy is difficult to evaluate, as pertinent details are missing from their growth rate methods text. Although this is a relatively basic analysis, in the case of *Trichodesmium* the protocol chosen is critical. Nitrogen and carbon fixation and growth in this species follow a pronounced diel rhythm [*3,4,6*], so most cell division occurs in the afternoon. One can thus calculate anomalously elevated growth rates similar to those reported by Hong et al., simply by measuring them solely from early morning until late in that (or a subsequent) afternoon. These high growth rates will however retreat to widely published values if experiments are properly sampled over an exact 24-h diel cycle. Unfortunately, this specific information was not provided, as it may have helped to explain why growth rates and other aspects of their study are inconsistent with previous *Trichodesmium* work, including shifts in diel nitrogen-fixation patterns under elevated CO_2 [*4-6*], and trends in the abundance of many key proteins in iron-limited and high-CO_2-grown cells [*8,13*].

We agree with Hong et al. [*7*] that iron limitation negates the positive effects of high CO_2 on nitrogen fixers. We observe similar iron-limited rates at high and low CO_2, however, rather than preferential inhibition by elevated CO_2 [*8,14*]. Although iron limitation indisputably

constrains nitrogen-fixation in much of the current ocean, increased aerosol iron supplies resulting from climate change and anthropogenic pollution may partially alleviate this limitation in the future ocean [*15*].

In conclusion, we certainly concur with Hong et al. [*7*] that the effects of high CO_2 and attendant ocean acidification on *Trichodesmium* are complex, and we applaud them for alerting researchers to potential reagent contamination. It is clearly unwarranted, however, to project an unfortunate contamination problem in one laboratory onto a large, robust, and consistent body of research with important implications for changing ocean ecosystems. The reason that Hong et al. obtain results diametrically opposed to those of nearly every other similar study remains to be determined, but the evidence does not support the suggestion that this is because all other experiments are universally contaminated.

References

1. J. Barcelos e Ramos, H. Biswas, K. G. Schulz, J. LaRoche, U. Riebesell, Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*, *Global Biogeochem. Cycles* 21, GB2028 (2007). doi:10.1029/2006GB002898
2. D. A. Hutchins, F.-X. Fu, Y. Zhang, M. E. Warner, Y. Feng, K. Portune, P. W. Bernhardt, M. R. Mulholland, CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry, *Limnol. Oceanogr.* 52, 1293–1304 (2007). doi:10.4319/lo.2007.52.4.1293

3. S. A. Kranz, S. Dieter, K.-U. Richter, B. Rost, Carbon acquisition by *Trichodesmium*: The effect of pCO_2 and diurnal changes, *Limnol. Oceanogr.* 54, 548–559 (2009). doi:10.4319/lo.2009.54.2.0548
4. M. Eichner, S. A. Kranz, B. Rost, Combined effects of different CO_2 levels and N sources on the diazotrophic cyanobacterium *Trichodesmium*, *Physiol. Plant.* 152, 316–330 (2014). doi:10.1111/ppl.12172 Medline
5. D.A. Hutchins, F.-X. Fu, E.A. Webb, N. Walworth, A. Tagliabue. Taxon-specific responses of marine nitrogen fixers to elevated carbon dioxide concentrations, *Nature Geoscience* 6, 790-795 (2013). doi: 10.1038/ngeo1858
6. D.A. Hutchins, N. Walworth, E.A. Webb, M.A. Saito, D. Moran, M.R. McIlvin, J. Gale, Fu, F.-X., Irreversibly increased N_2 fixation in *Trichodesmium* experimentally adapted to high CO_2, *Nature Communications* 6, 8155 (2015). doi:10.1038/ncomms9155
7. H. Hong, R. Shen, F. Zhang, Z. Wen, S. Chang, W. Lin, S.A. Kranz, Y.-W. Luo, S.-J. Kao, F.M.M. Morel, D. Shi, The complex effects of ocean acidification on the prominent N_2-fixing cyanobacterium *Trichodesmium*, *Science*, 356, 527- 531 (2017). doi:10.1126/science.aal2981
8. N.G. Walworth, F.-X. Fu, E.A. Webb, M.A. Saito, D. Moran, M.R. Mcllvin, M.D. Lee, D.A. Hutchins, Mechanisms of increased *Trichodesmium* fitness under iron and phosphorus co-limitation in the present and future ocean, *Nature Communications* 7, 12081, (2016) doi10.1038/NCOMMS12081

9. K. Ohki, J.P. Zehr, P.G. Falkowski, Y. Fujita. Regulation of nitrogen-fixation by different nitrogen sources in the marine non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB1067, *Archives of Microbiology* 156, 335–337, (1991). doi:10.1007/BF0024870
10. M.R. Mulholland, K. Ohki, D.G. Capone, Nutrient controls on nitrogen uptake and metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria), *Journal of Phycology* 37, 1001-1009, (2001)
11. F.-X. Fu, P.R.F. Bell, Factors affecting N_2 fixation by the cyanobacterium *Trichodesmium* spp. GBRTRLI101, *FEMS Microbiology Ecology* 45, 203-209, (2003).
12. Garcia, N.S., Hutchins, D.A. Light-limited growth rate modulates nitrate inhibition of dinitrogen fixation in the marine unicellular cyanobacterium *Crocosphaera watsonii, PLoS One* 9, e114465, (2014). DOI:10.1371/journal.pone.0114465
13. J.T. Snow, D. Polyviou, P. Skipp, N.A.M. Chrismas, A. Hitchcock, R. Geider, C.M. Moore, T. S. Bibby, Quantifying integrated proteomic responses to iron stress in the globally important marine diazotroph *Trichodesmium*, *PLoS ONE* 10, e0142626, (2015). doi.org/10.1371/journal.pone.0142626
14. F.-X. Fu, M.R. Mulholland, N. Garcia, A. Beck, P.W. Bernhardt, M.E. Warner, S.A. Sañudo-Wilhelmy, D.A. Hutchins, Interactions between changing pCO_2, N_2 fixation, and Fe limitation in the marine unicellular cyanobacterium *Crocosphaera, Limnology and Oceanography* 53, 2472- 2484, (2008).
15. D.A. Hutchins**,** P.W. Boyd, Marine phytoplankton and the changing ocean iron cycle, *Nature Climate Change* 6, 1071-1079, (2016). doi: 10.1038/NCLIMATE3147