TECHNICAL COMMENT

OCEAN ACIDIFICATION

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

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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 that these 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 past decade have predicted that the globally distributed tropical cyanobacterium *Trichodesmium* spp. will grow faster and fix 30 to 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 growth-inhibiting ammonia (~20 μmol/liter). 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" [supplementary text of (*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 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 use our own published results as examples, but most of the multiple previous studies cited by Hong *et al.* as being likely contaminated also documented similarly high nitrogen fixation rates. Moreover, if all these previous experiments were truly contaminated with ammonia (20 μmol/liter) as suggested, little or no nitrogen fixation would have been observed, as *Trichodesmium* nitrogen fixation is strongly inhibited (~50 to 100%) by ammonia concentrations of 10 to 20 μmol/liter (*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/liter).

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 to 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.

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 after ~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 because of 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

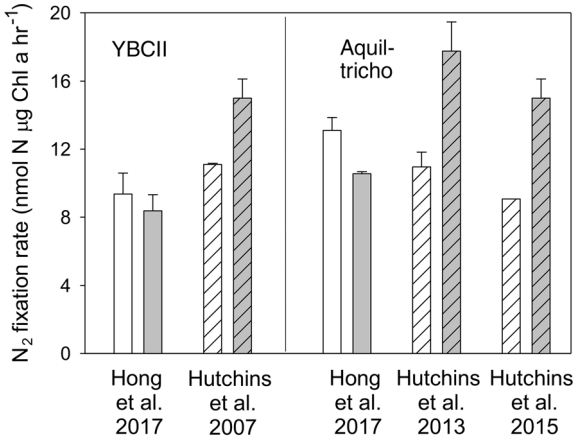

Fig. 1. Prior *Trichodesmium* CO_2 studies refuting the Hong *et al*. culture medium toxicity hypothesis. Reported *Trichodesmium* nitrogen fixation rates in YBCII medium (**left**) and Aquil-tricho medium (**right**) in purportedly "uncontaminated" iron-replete medium formulations from Hong *et al*. (open bars) and from previously published studies (hatched bars) in low CO_2 (white bars) and high CO_2 (gray 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*. Nitrogen fixation was measured and normalized the same way in all experiments shown; previously published data (*2*, *5*, *6*) were recalculated using the same 4:1 ethylene:N_2 conversion ratio used in Hong *et al*.

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left with the puzzling observation that Hong *et al.* recorded growth rates that were ~25% higher than other published rates—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 that afternoon (or a subsequent afternoon). These high growth rates will, however, retreat to widely published values if experiments are properly sampled over an exact 24-hour 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*).

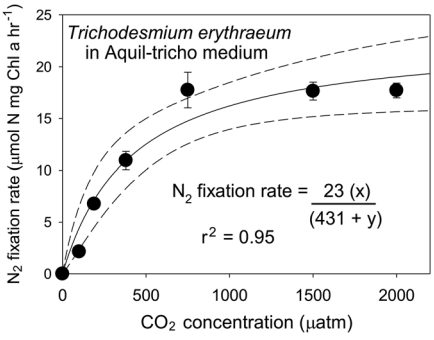

Fig. 2. *Trichodesmium* N_2 fixation saturation curve as a function of CO_2 concentration. Results of a prior *Trichodesmium* study that measured nitrogen fixation rates across a range of CO_2 concentrations and found a highly significant fit to a nutrient-like saturation curve model (*5*). The corresponding Michaelis-Menten equation is shown; the 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*.

We agree with Hong *et al.* 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.* 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.

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