Nutrient co-limited *Trichodesmium* as nitrogen source or sink in a future ocean.

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Abstract

Nitrogen-fixing (N_2) cyanobacteria provide bioavailable nitrogen to vast ocean regions but are in turn limited by iron (Fe) and/or phosphorus (P), which may force them to employ alternative nitrogen acquisition strategies. The adaptive responses of nitrogen-fixers to global-change drivers under nutrient-limited conditions could profoundly alter the current ocean nitrogen and carbon cycles. Here, we show that the globally-important N_2-fixer *Trichodesmium* fundamentally shifts nitrogen metabolism towards organic-nitrogen scavenging following long-term high-CO_2 adaptation under iron and/or phosphorus (co)-limitation. Global shifts in transcripts and proteins under high CO_2/Fe-limited and/or P-limited conditions include decreases in the N_2-fixing nitrogenase enzyme, coupled with major increases in enzymes that oxidize trimethylamine (TMA). TMA is an abundant, biogeochemically-important organic nitrogen compound that supports rapid *Trichodesmium* growth while inhibiting N_2 fixation. In a future high-CO_2 ocean, this whole-cell energetic reallocation towards organic nitrogen scavenging and away from N_2-fixation may reduce new-nitrogen inputs by *Trichodesmium*, while simultaneously depleting the scarce fixed-nitrogen supplies of nitrogen-limited open ocean ecosystems.

Importance

Trichodesmium is among the most biogeochemically-significant microorganisms in the ocean, since it supplies up to 50% of the new nitrogen supporting open ocean food webs. We used *Trichodesmium* cultures adapted to high CO_2 for 7 years followed by additional exposure to iron and/or phosphorus (co)-limitation. We show that 'future ocean' conditions of high CO_2 and concurrent nutrient limitation(s) fundamentally shift nitrogen metabolism away from nitrogen fixation, and instead towards upregulation of organic-nitrogen scavenging pathways. We show that *Trichodesmium's* responses to projected future ocean conditions include decreases in the nitrogen-fixing nitrogenase enzymes, coupled with major increases in enzymes that oxidize the abundant organic nitrogen source trimethylamine (TMA). Such a shift towards organic nitrogen uptake and away from nitrogen fixation may substantially reduce new-nitrogen inputs by *Trichodesmium* to the rest of the microbial community in the future high-CO_2 ocean, with potential global implications for ocean carbon and nitrogen cycling.

Keywords: nutrient co-limitation, nitrogen fixation, *Trichodesmium*, global change, ocean acidification, microbial ecology, cyanobacteria, evolution, TMA, trimethylamine

68 **Introduction**

69 Oceanic food webs and climate feedbacks are significantly influenced by carbon dioxide (CO_2)
70 and nitrogen (N_2) fixing microbes, thereby contributing to both global productivity and
71 biogeochemistry (1-3) . However, only a handful of studies have investigated the physiological
72 and evolutionary responses of the globally important, photoautotrophic, N_2-fixing,
73 cyanobacterium *Trichodesmium erythraeum* strain IMS101 to high CO_2 under multiple limiting
74 nutrient regimes (4-6). Simultaneous iron (Fe) and phosphorus (P) co-limitation of IMS101 under
75 both low and high CO_2 has been found to yield higher growth rates, reduced cell sizes, and a
76 unique Fe/P protein complement, as compared to single limitation by either Fe or P alone (5, 7).
77 This fitness advantage conferred by Fe/P 'balanced limitation' contrasts with the long-standing
78 Liebig limitation model (8) and has implications for global biogeochemical cycles in both the
79 current and future ocean (3).

80 Oligotrophic populations are predicted to potentially experience longer periods of
81 enhanced nutrient (co)-limitation due to intensifying Fe-stress under high CO_2 (5, 9) and reduced
82 vertical P supplies from increased density-driven stratification (3, 10). However, a major
83 unknown is the potential change to future new nitrogen inputs by globally distributed N_2-fixing
84 microbes (diazotrophs). Indeed, past research has demonstrated significant decreases in growth
85 and N_2 fixation of marine diazotrophs under Fe-limitation, with molecular evidence indicating
86 iron reallocation via reduction in the photosystem I to photosystem II (PSI:PSII) ratio, decreases
87 in metalloenzyme inventories, and increases in Fe-stress PS antennae abundances (5, 11-13).
88 Additionally, *Trichodesmium* has been demonstrated to take up both inorganic (e.g. nitrate and
89 ammonia) and organic (e.g. amino acids) nitrogen species, thereby inhibiting N_2 fixation(14, 15).
90 However, because much of the low-latitude surface ocean is largely nitrogen limited (16), fixed

N sources can be severely depleted, resulting in a marked dependence on Fe and P bioavailability to fuel N_2 fixation (1, 3).

To date, nearly all diazotrophic nitrogen assimilation research has focused on the relationship between N_2 fixation and nitrate, ammonia, and amino acid uptake, resulting in the view that ammonia is the preferred microbial nitrogen source for both diazotrophic and non-diazotrophic microbes (17). Identifying and measuring preferentially scavenged organic nitrogen species by marine microbes has been largely precluded by rapid biochemical turnover *in situ*. Furthermore, microbial consortia aggregating around microbial species of interest can also inhibit discernment between microbial uptake of specific substrates (18).

In this study, functional genomics and molecular physiology provide strong evidence that following adaptation to high CO_2, *Trichodesmium* allocates the greatest biosynthetic investment into the acquisition of the organic nitrogen substrate trimethylamine (TMA), and potentially other organic nitrogen- and sulfur-containing compounds. This shift in nitrogen acquisition pathways under high-CO_2 conditions is predicted to be mediated by large increases in bacterial flavin-containing monooxygenase (FMO) coupled to global shifts in transcription and translation. This indicates both a fundamental change in nitrogen and global cellular strategy, whereby iron-rich biosynthetic pathways including N_2 fixation and photosynthesis are significantly reduced under Fe-limited, high-CO_2 conditions, in parallel with increased biosynthesis of the predicted TMA-oxidizing FMO enzyme. Additionally, N_2 fixation is inhibited in the presence of exogenous TMA in a manner similar to that seen with nitrate and ammonia, with TMA simultaneously supporting growth rates equivalent to those seen while fixing N_2. Methylated amine compounds like TMA are products of protein putrefaction and degradation of quaternary amine osmoregulators (e.g. glycine betaine)(19) and are thus

ubiquitous in the marine environment, representing a considerable pool of C and N with reported standing concentrations in the nano- to micromolar range (20, 21). Hence, future CO_2 levels and limiting Fe may exacerbate cellular Fe-stress, resulting in a fundamental metabolic shift whereby *Trichodesmium* reallocates resources away from N_2 fixation and towards FMO-mediated organic nitrogen scavenging. The resulting metabolism change observed in *Trichodesmium* necessitates a reassessment of the relationship between new nitrogen inputs and simultaneous removal by this globally important marine N_2-fixer, as well as a broader characterization of the utilization of methylated amines like TMA by biogeochemically-critical marine microbes.

Results and Discussion

Nutrient-limited physiology under increased CO_2

A comprehensive depiction of the experimental design can be found in Fig. S1, and proteomic results and their implications for nutrient limitation theory are presented in Walworth et al. 2016 (5). Here, we focus on steady-state, transcriptional/translational changes to nitrogen source-sink metabolism as they relate to other biogeochemically-important pathways. Briefly summarizing Fig. S1, a single IMS101 population, originally isolated from an individual *Trichodesmium* colony (22), was split into low (380-selected) and high (750-selected) CO_2 treatments of six biological replicates each, and grown for ~7 years (~1,000 and ~1,500 generations for 380- and 750-selected, respectively). Semi-continuous culturing methods were used in a modified trace metal-clean Aquil medium (devoid of ammonia or other fixed nitrogen) containing 25 µM EDTA, standard vitamins and trace metals, with 250 nM Fe and 10 µM phosphate (PO_4^{3-}). Next, Fe/P co-limited treatments were generated by sub-culturing three biological replicates from each CO_2 treatment and incubating them in the aforementioned media except with co-limiting levels of Fe (10 nM) and PO_4^{3-} (0.25 µM) for >1 year. To then mimic

episodic pulses of Fe or P typically experienced by Fe/P co-limited microbes in the oligotrophic ocean (3, 23, 24), either Fe or P was added at replete concentrations to subcultures of the Fe/P co-limited cell lines to generate P and Fe limited lines at both CO_2 levels, respectively. Following a two-month acclimation period to create steady-state conditions, the lines were sampled for molecular analyses.

Growth and N_2 fixation rates were significantly increased under high CO_2 in both nutrient replete (r750) and P-limited (750-P) conditions relative to low CO_2/nutrient replete (r380) and low CO_2/P-limited (380-P) treatments, respectively (Fig. 1). Conversely, no growth rate differences were observed in high CO_2/Fe-limited (750-Fe) and high CO_2/Fe/P-co-limited (750-Fe/P) cell lines relative to their corresponding low CO_2/Fe-limited (380-Fe) and low CO_2/Fe/P-co-limited (380-Fe/P) conditions, respectively. In a recent study, Hong et al. (2017)(9) observed a significant decrease in growth and N_2-fixation rates in both replete (r750) and Fe-limited conditions in high CO_2 (750-Fe) relative to their controls, unlike this study and a number of other previous replete (3-6, 10, 25-28) and high CO_2/Fe-limited studies (5). Hong et al. (2017) (9) attributed these discrepancies to ammonia contamination and metal toxicity in all other past studies, although ammonia was below detection limit in our prepared Aquil media and EDTA concentrations were at 25 µM to control for metal toxicity (29). One central difference in this study and nearly all others relative to Hong et al. (2017) (9) is that *Trichodesmium* cell lines were adapted (4-6) or acclimated (10, 26-28) to low pH as a product of increasing CO_2 partial pressure on seawater carbonate chemistry similar to pH reductions *in situ*. This contrasts with artificially controlling pH with short-term acid/base additions as in Hong et al. (2017), which may also result in other unknown and uncontrolled effects to cell physiology. A second fundamental difference is that our Fe-single limited treatments were generated from pulsing phosphorus into

7-year, high CO_2-adapted cell lines co-limited for iron and phosphorus for ~1 year, similar to persistent co-limitation conditions observed in the oligotrophic ocean (23, 24).

Looking ahead, it is predicted that increasing CO_2 will impact ecosystems more rapidly than increasing sea surface temperature (30), which in turn will affect the adaptive evolution of marine microbes and their contribution to elemental cycling. Hence, following long-term (~7-year) adaptation of IMS101 to high CO_2 (3, 5, 6), we investigated changes to IMS101 molecular physiology and nitrogen acquisition after ~1-year-long exposure to Fe/P-co-limitation, followed by additions of either Fe or P to create steady-state, Fe- and P-single limitation treatments.

Variable nitrogen assimilation strategies under nutrient co-limitation in a future ocean

Below we focus on the dynamics of global transcription and translation as they relate to changing nitrogen metabolic strategies under intensifying nutrient (co)-limitation as a function of high-CO_2 adaptation. A more in-depth discussion on other molecular changes in broad biochemical pathways can be found in supplementary information. Genes in 380-Fe that were differentially expressed (DE) when compared to r380, but not relative to 380-Fe/P, showed significantly different transcript levels in both 380-Fe and 380-Fe/P, respectively, relative to r380 (Fig 2a, Dataset S1). This pool was enriched in Gene Ontogeny (GO) pathways involving nitrogen fixation (downregulated), cytochrome-c oxidase activity (downregulated), copper ion binding (downregulated), nitrate/nitrite transport (upregulated), respiration (downregulated), glutamate synthase (upregulated), and amino acid transport (upregulated). These pathway enrichments indicate a down-regulation of Fe-heavy N_2-fixing enzymes similar to other findings(5, 31) in conjunction with the up-regulation of external nitrogen acquisition genes targeting inorganic (e.g. nitrate; Fig. 3a) and/or possibly organic (e.g. amino acid transport) sources (Dataset S1).

While the 750-Fe pool of DE transcripts relative to r380 was enriched in GO pathways (Fig. S2) involved in nitrogen fixation (downregulated) and cytochrome-c oxidase activity (downregulated), similar to 380-Fe relative to r380, genes involved in nitrate/nitrite transport drastically reduced expression levels in 750-Fe (grey bar) and 750-Fe/P (blue bar) relative to 380-Fe (orange bar) and 380-Fe/P (green bar), respectively (Fig. 3). This massive transcriptional reduction in inorganic nitrogen transport going from low to high CO_2 in Fe-limitation regardless of P concentration also co-occurred with ~50% fewer genes being differentially expressed overall in high CO_2/low Fe, including a considerable reduction of protein synthesis transcripts (Fig. 2b, S2b; Dataset S1).

Interestingly, both transcript and protein abundances of a predicted TMA-oxidizing, flavin-containing monooxygenase (FMO, Tery_3826) harboring the FMO-specific motif(32) and a predicted transmembrane helix (per IMG annotation) sharply increased in 750-Fe (Figs. 2b, 3a), along with the flavin biosynthesis gene, *ribD* (Tery_1661; Dataset S1), relative to all other treatments. In fact, the FMO gene was one of the most highly expressed transcripts detected in the high CO_2, Fe-limited treatment. Furthermore, its general expression level is orders of magnitude greater than other nitrogen acquisition transcripts across all treatments (Fig. 2b, 3b). Transcripts of an annotated ammonia transporter (*amT1;* Tery_4477) remained unchanged across treatments but was consistently expressed in the top ~6% of detected transcripts. Notably, although 380-Fe and 750-Fe growth rates were similar, *ALDO* (Tery_1687; fructose-bisphosphate aldolase) transcript levels also drastically increased in 750-Fe along with the ferric uptake regulator (Tery_3404, *fur*) and Fe-stress/photosynthesis antennae genes (Fig. 2b, Fig. S2b).

Taken together, this global shift in transcript profiles in conjunction with the

206 downregulation of nitrate transport and the concurrent upregulation of FMO and *ribD* suggest
207 that high CO_2 intensifies iron limitation (5, 9) resulting in fundamental resource reallocation (e.g.
208 riboflavin) towards increased biosynthetic investment in FMO-mediated, iron-lean organic
209 nitrogen assimilation strategies. The large increase in FMO transcripts and proteins observed in
210 750-Fe suggests *Trichodesmium* preferentially scavenges TMA and possibly other organic
211 nitrogen and sulfur compounds under severe iron stress. Furthermore, FMO transcript and
212 protein abundances are in the top ~0.5% and ~10% of all detected gene transcripts and protein
213 products, respectively, irrespective of treatment, which indicates significant energetic investment
214 into persistent FMO biosynthesis. Conversely, nitrate transport transcripts are only in the top
215 ~50-70% of total transcripts, with no corresponding protein products detected in the proteome.
216 Upon calculating total mean expression of nitrogen acquisition transcripts across all treatments,
217 FMO was consistently expressed at significantly higher levels ($p < 0.0001$) than other nitrogen
218 acquisition genes (Fig. 3a), providing further evidence of persistent and enriched biosynthesis of
219 FMO. Taken together with the ammonia transporter expressed in the top 6% of overall
220 transcripts, these data support the notion that FMO-acquired compounds and ammonia are
221 preferred relative to nitrate acquisition as previously noted for ammonia(17). The TMA protein
222 was significantly more abundant in high-CO_2/low-Fe, low P, and Fe/P co-limitation relative to
223 r380, r750, and 380-Fe conditions, suggesting enhanced energetic investment into TMA enzyme
224 biosynthesis across various CO_2/nutrient-limited global change scenarios (Fig. 3c). It is
225 interesting that FMO transcript and protein levels considerably increased in 750-Fe but not 380-
226 Fe, although Fe-stress genes signaled iron limitation in both conditions. Strain IMS101 N_2-fixing
227 metabolism may be better adapted to low CO_2/low Fe due to prevalent Fe-limitation *in situ* (24)
228 thereby reducing the need to scavenge for nitrogen while molecular metabolic restructuring

induced by high CO_2/low Fe may require increased exogenous nitrogen for growth in an high-CO_2 ocean. Since heterotrophs have been shown to grow on TMA as a sole C and N source (32), *Trichodesmium* may utilize TMA for both C and N (33, 34). It was also recently shown that an associated heterotrophic *Alteromonas macleodii* (*A. macleodii*) strain widely conserved in *Trichodesmium* metagenomic consortia has the genetic potential to cleave DMSP to produce DMS(35), which may be further oxidized by the *Trichodesmium* FMO, as DMS has been shown to have nearly as high an affinity for FMO as that of TMA(32). Hence, TMA- and other FMO-mediated assimilation may be a more general compensatory strategy utilized by *Trichodesmium* for physiological maintenance in the face of fluctuating nutrients, which is evidenced by the significant increase in FMO protein abundance across nutrient-limited treatments (Fig. 3a, bottom panel).

N_2 fixation is strongly inhibited in the presence of 20 µM TMA, just as it is with 20 µM nitrate (Fig. 3d). However, *Trichodesmium* growth rates are virtually unchanged in the presence of 20 µM TMA relative to growth rates supported by N_2 fixation, comparable to results in the presence of 20 µM nitrate, indicating that both fixed nitrogen sources can support growth as efficiently as N_2. In contrast, the presence of 20 µM ammonia was toxic, and the cells were unable to grow or fix N_2 at all (Fig. 3d). One recent study observed enigmatically high growth and N_2 fixation rates in the presence of 20 µM ammonia (9), contrasting with all other previous studies demonstrating drastic repression of N_2 fixation in exogenous ammonia concentrations exceeding >10 µM (15, 17, 36, 37).

Although Hong et al. (2017) (9) observed decreases in the PSI:PSII ratio in 750-Fe conditions similar to this study (Fig. 4a) and others(5, 31, 38), they also observed increases in the iron-rich NifH protein in high CO_2/Fe-limiting conditions (750-Fe), contrasting with our

observed decreases in multiple nitrogenase transcripts and protein subunits in this study (Fig. 4b) and another *Trichodesmium* iron-limited proteome (31). Although Hong et al. (9) suggest more NifH is necessary in 750-Fe due to decreased enzyme efficiency, we observe a contrasting trend via a fundamental shift in nitrogen metabolic strategy, whereby iron-rich gene abundances (e.g. PSI and *nif* subunits) along with nitrate transport transcripts are significantly reduced under severe iron stress concomitantly with increases in TMA-scavenging FMO and riboflavin biosynthesis gene expression (750-Fe; Fig. 4a; Fig. S2).

Among the various forms of available exogenous nitrogen (e.g. amino acids, nitrate, TMA, ammonia), one possible reason FMO may be preferred is that it can oxidize both nitrogen- and sulfur-containing compounds including TMA, which may also serve as potential organic carbon sources, thereby enabling *Trichodesmium* to assimilate several macronutrients by minimizing energetic investment. Additionally, since *Trichodesmium* is physically colonized by a diverse microbial consortium (35, 39) and methylated amine compounds like TMA are ubiquitous as end products of, for example, protein putrefaction (32), *Trichodesmium* may have consistent access to organic nitrogen/carbon sources as a product of biochemical decomposition of cellular components by members of its consortium. This contrasts with the lifestyles of other planktonic, unicellular marine bacteria, which typically harbor much less abundant and diverse consortia. When searching *Trichodesmium* consortia metatranscriptome sequences (35) for FMO via BLASTP (40), no significant hits were observed containing the FMO-specific motif, suggesting it may not be widespread or expressed highly in consortia.

Although FMO homologs have been detected across global ocean datasets including within the genomes of the ubiquitous SAR11 clade (32), only a handful of robust cyanobacterial and bacterial homologs to IMS101 were detected with many of the remaining top high-scoring

pairs deriving from metazoans (Fig. S3; high-scoring pair ≥ 70% of original *Trichodesmium* FMO sequence length; e-value ≤ 10^{-5}), yielding in an average percent identity of 27.9% (median = 27.8%) over all pairs. We did detect the FMO genes in another *T. erythraeum* genome (strain 2175) and a *Trichodesmium* metagenome from hand-picked natural colonies (41) demonstrating widespread retention and conservation of FMO. Interestingly, no homologs to other biogeochemically-relevant marine cyanobacteria were detected. Taken together, FMO-mediated scavenging may be ecologically advantageous for *Trichodesmium* in the oligotrophic ocean, given its potentially persistent availability via biochemical degradation, and its relative scarcity in its physically attached consortia as well as other sympatric cyanobacteria.

Fe-single and Fe/P co-limited metabolism in the future ocean

Our data suggest that interaction of limiting Fe with high CO_2 adaptation will intensify iron stress in Fe/P co-limited molecular metabolism. For example, known P-stress genes (e.g., *phoX, sphX, phnG*), but not Fe-stress genes (e.g., *isiA*), were significantly upregulated in 380-Fe/P, as compared to r380, thus suggesting that P-stress may be more prominent than Fe-stress under low-CO_2/co-limiting conditions (Fig. 4; Fig. 5; Fig. S4; Dataset S2, S3). In fact, *isiA* transcripts were significantly downregulated in both P-limited conditions irrespective of CO_2 as well as in 380-Fe/P co-limitation (Fig. 4a). However, transcripts of Fe-stress genes, including *isiA,* significantly increased in 750-Fe/P relative to 380-Fe/P accompanied by a significant decrease in the PSI:PSII ratio, indicating iron stress in high CO_2/co-limitation (Fig. 4, Fig. 5). Finally, this shift toward Fe stress is further evidenced by the high-confidence grouping of 750-Fe with the 380- and 750-Fe/P co-limited treatments (AU-bootstrap p-value > 0.95) resulting from hierarchical clustering of genes exclusively DE (i.e. transcriptional complement; Dataset

297 S3) in both 380- and 750-Fe/P (Fig. 6). Collectively, these data provide strong evidence for the
298 intensification of Fe stress on cellular metabolism upon interacting with high CO_2, regardless of
299 fluctuating P concentrations. Hence, iron-rich metabolic pathways that generate energy and fixed
300 nitrogen will likely be impacted as cells utilize alternative iron- and energy-conserving strategies
301 to acquire nutrients, with consequent reductions in new nitrogen inputs.

302 *P-single and Fe/P co-limited metabolism in the present and future ocean*

303 Upon adaptation to high CO_2, both 750-P growth and N_2 fixation increased relative to 380-P
304 (Fig. 1), while most 750-P-limited transcript levels remained statistically unchanged between
305 380-P and 750-P. This is evidenced by the much smaller number (n = 4) of DE genes between
306 380-P and 750-P as compared to those DE between 380-Fe and 750-Fe (n = 284; Fig. S5; Fig.
307 S6). Translation dominated the GO-enriched pathway pools for downregulated genes relative to
308 both r750 only and 750-Fe/P (Fig. S4; Fig. S6; Dataset S2) as in 380-P. It possible that increased
309 N_2 fixation rates of equivalently sized IMS101 cells under varying CO_2 conditions (e.g. r380 vs
310 r750; 380-P vs 750-P) may be driven by factors contributing to altered enzymatic rates rather
311 than by increases in enzyme abundance, as has been observed for temperature(42). This
312 possibility is supported by either unchanged or decreases in N_2 fixation transcripts and/or protein
313 abundances (e.g., *nifH, nifB, nifK*) in replete, 7-year high-CO_2 adapted IMS101 (r750) relative to
314 replete low CO_2 (r380), despite higher N_2 fixation rates in cells selected under elevated CO_2 (Fig.
315 4)(5). For some N_2 fixation genes, transcript increases were mirrored by increases in protein
316 abundances (e.g., *nifB*, Tery_4133; *nifK*, Tery_4138), though most remained unchanged in the
317 proteomic data (e.g., *nifH*, Tery_4136; *nifD*, Tery_4137). These trends suggest that different
318 nitrogenase genes may be independently regulated at the transcriptional level or have different
319 mRNA half-lives. Taken together, other regulatory mechanisms impacting enzymatic rates, but

not abundances, may be more generally associated with changes in CO_2-impacted physiology under P-limitation.

Fe/P co-limited transcription in the present and future ocean

Photosynthesis-related (e.g., Tery_0983, Tery_0998, Tery_5048, Tery_5049, Tery_0728), ferredoxin (e.g., Tery_0916, Tery_4539, Tery_5051), heme-containing (e.g., Tery_1714, Tery_0335), and N_2-fixing genes (Fig. 2; Fig. 4; Fig. 5; Fig. S7) were all downregulated in 380-Fe/P. This indicates a potential reduction and/or reallocation of Fe away from these pathways in the co-limited cells coinciding with reduced cell size (and therefore cellular elemental quotas), growth, and N_2 fixation relative to the replete conditions. Additionally, downregulation of GO-enriched pathways in 380-Fe/P involved in translational machinery and RNA binding (Fig. 5; Fig. S7) are consistent with observations in yeast and bacteria (i.e., stringent response) where proteins involved in cell growth and division (e.g., ribosomal proteins) have been observed to be downregulated in minimal (e.g. 380-Fe/P) vs rich (e.g. r380) medium (43, 44). Moreover, the upregulated 380-Fe/P (relative to r380) transcriptional complement also contained many genes involved in precursor metabolisms, potentially allowing for greater cellular plasticity and metabolic flexibility in low-nutrient regimes (5). Accordingly, we identified a set of upregulated proteins specific to 380-Fe/P co-limitation that included many involved in precursor metabolisms (i.e., isoprenoid biosynthesis); a finding consistent with prior observations in yeast maintained in minimal media(44). For example, 3-dehydroquinate synthase (Tery_2977, *aroB*) is upregulated solely in 380-Fe/P and is part of the shikimate pathway that produces chorismate, a precursor to aromatic amino acids including phenylalanine, tyrosine, and tryptophan (45)(Fig. S8).

Following 7 years of adaptation to high CO_2 and one year of growth at high CO_2 Fe/P co-

limitation conditions (750-Fe/P), transcript levels had shifted across broad metabolic pathways, presumably orchestrating the maintenance of similar growth and N_2 fixation relative to 380-Fe/P conditions (Fig. 5; Fig. S7). Of those genes that were exclusively DE during Fe/P co-limitation, only ~5% (n = 26; Dataset S3) from the 380-Fe/P complement (n = 313) remained in the 750-Fe/P complement (n = 174) (Dataset S3). In other words, these genes were either upregulated (n = 15) or downregulated (n = 11) only in 380-Fe/P and 750-Fe/P co-limitation, relative to their respective replete reference conditions (r380 and r750). Shared upregulated genes include the universal stress global response regulator (Tery_2353, *uspA*), the aforementioned GTP-binding gene (Tery_1904), and a carbohydrate selective porin gene (Tery_0838, *oprB*). The consistent upregulation of these genes suggests that both 380- and 750-Fe/P co-limited cellular physiology respond to environmental flux by consistently transcribing genes involved in general stress, bio-timer regulation, and potential organic carbon uptake, as has been observed in other cyanobacteria(33, 34). Shared downregulated genes include a 5′,5‴-P1,P4-tetraphosphate phosphorylase (Tery_0108, *ap$_4$A*), which has been implicated in bacterial stress responses and gene regulation (46), and the mutator gene, *mutT* (Tery_4056), which has been demonstrated to suppress transversion mutations (A•T to C•G) (47). Accordingly, *E. coli* Δ*mutT* deletion mutants exhibited a 1000-fold increase in unidirectional A•T to C•G transversion frequencies. Other studies have postulated that mutators can accelerate adaptive evolution under certain circumstances via increased mutation rates(48). Hence, the significant reduction in transcription of *mutT* in both long-term 380-Fe/P and 750-Fe/P treatments may help enable adaptive mutation by possibly de-repressing transversion mutations in the face of low-nutrient, stress-inducing environments.

Conclusions

Here, we show a marked shift in nitrogen metabolism going from low CO_2/Fe-limited to high CO_2/Fe-limited regimes whereby iron-heavy pathways are significantly reduced, and cellular investment is reallocated toward a predicted FMO-mediated organic nitrogen scavenging, relative to acquisition pathways for inorganic nitrogen substrates. This fundamental iron-saving strategy may also enable simultaneous assimilation of several other required elements including C and S from methylated amines like TMA. Our data also suggest that intensifying Fe-stress under high CO_2 may shift Fe/P co-limited metabolism into a more Fe-limited metabolic state. This trend further highlights the need for iron-saving metabolic strategies for nutrient limitation in a future high-CO_2 ocean. Additionally, N_2 fixation but not growth was inhibited in the presence of TMA. If interactive global change factors intensify nutrient limitation leading to enhanced organic nitrogen scavenging and reduced N_2 fixation by *Trichodesmium*, future work must consider the relationship between N_2-fixation-mediated new-nitrogen inputs and simultaneous removal from the fixed nitrogen pool. The shifting balance between these two processes may have global implications for the role of *Trichodesmium* to the future ocean biomes. Finally, transcript patterns in Fe/P co-limited metabolism also suggest reductions in iron-heavy pathways in exchange for increases in precursor-related genes that may aid cellular plasticity in response to varying nutrient concentrations. Although limitations in this long-term experiment and others (49) include a steady-state environment lacking natural variability as well as the use of a single strain of *Trichodesmium*, we believe our results demonstrating molecular reallocations of nitrogen-acquisition and iron-sparing expression provide robust indicators of potential *in situ* strategies some *Trichodesmium* populations may employ while under co-varying nutrient limited regimes. We highlight consistent molecular patterns in genes under Fe- and/or P-limitation while co-varying CO_2, which strengthens broad

388 trends outlined above. Future field work investigating co-limitation should aim to compare *in*
389 *situ* molecular patterns with those that we have identified in culture to try and help elucidate
390 universal indicators of co-limitation in the future ocean.

391 **Materials and Methods**

392 *Culturing Methods*

393 Details of nutrient-limited culturing methods can be found at Walworth et al. (2016)(5)
394 and in the supplementary materials and methods. For the growth experiment comparing TMA
395 with other N sources, IMS101 was grown in four N treatments: 1) N_2 2) 20µM $NaNO_3$ 3) 20µM
396 TMA (Trimethylamine, C_3H_9N) 4) 20µM NH_4Cl. For all treatments, triplicate cultures were
397 maintained semi-continuously at 26^{o}C under a light intensity of 150µmol photons $m^{-2}s^{-1}$
398 irradiance (12L/12D cycle), cultures were diluted every other day, and 20µM N sources were
399 added after every dilution. Growth and nitrogen fixation rates were measured after 10
400 generations as described above and below.

401 Aquil medium was bubbled with 0.2µm-filtered prepared air/CO_2 mixtures (Praxair) and
402 in-line high-efficiency particulate air (HEPA) filters were employed to avoid Fe contamination
403 from particles in the gas tanks or lines. The pH was monitored daily with DIC being measured at
404 the final sampling. The acetylene reduction method was used to measure N_2 fixation rates of
405 *Trichodesmium* (4, 14, 25). Samples from each experimental triplicate (20 mL) were incubated
406 in gas-tight vials for 1 h after being injected with 2 mL acetylene in 23 mL of headspace. The
407 amount of ethylene produced was then analyzed by injecting a 200 µl aliquot of headspace gas
408 into a gas chromatograph (model: GC-8A, Shimadzu Scientific Instruments, Columbia, MD,
409 USA). An ethylene accumulation to N_2 fixation conversion ratio of 3 was used to calculate N_2
410 fixation rates. Upon steady state growth, *Trichodesmium* filament abundance and lengths were

411 measured in a 1mL-phytoplankton counting chamber using epifluorescence microscopy and
412 significant differences between nutrient conditions were calculated using two-way ANOVA
413 along with the Tukey test. For RNA sampling, cultures were swiftly and gently filtered onto 5
414 µm polycarbonate filters (Whatman) during the middle of the photoperiod, immediately flash
415 frozen, and stored in liquid nitrogen until RNA extraction.

416 *RNA isolation and extraction for Illumina Sequencing*

417 Samples for RNA were taken concomitantly with proteome samples in Walworth et al.
418 2016 (5) during the middle of the photoperiod for each replicate. Briefly, cells were swiftly and
419 gently filtered at 11 a.m. onto 5 µm polycarbonate filters (Whatman), immediately flash frozen,
420 and stored in liquid nitrogen until RNA extraction. RNA was extracted from 2 randomly chosen
421 biological replicates per treatment using the Ambion MirVana miRNA Isolation Kit (Thermo
422 Fisher Scientific) in an RNase free environment according to the manufacturer's instructions
423 followed by two incubations with Ambion's Turbo DNA-free kit to degrade trace amounts of
424 DNA. RNA was then submitted to the UC San Diego Institute for Genomic Medicine (IGM)
425 core for library preparation and sequencing (http://igm.ucsd.edu/genomics/services.shtml).
426 Briefly, rRNA removal and library construction was done with the Ribo-Zero rRNA Removal
427 Kit (Illumina) and TruSeq Stranded RNA Library Prep Kit (Illumina), respectively, and
428 multiplexed libraries were sequenced using the Illumina Hi-Seq yielding single-end 50 base pair
429 read libraries. Raw fastq files have been deposited in NCBI's Gene Expression Omnibus (50) and
430 are accessible through GEO Series accession number GSE94951. All protein spectral data used
431 in the above analyses can be found in Supplementary Data 4.

432 *Expression analysis*

Differential expression was done as previously described (6) and detailed methods can be found in the supplementary materials and methods.

Gene Ontology (GO) enrichment analysis

As in Walworth et al. 2016 (6), Gene Ontology (GO) annotations for *Trichodesmium* were downloaded from the Genome2D web server (http://pepper.molgenrug.nl/index.php/bacterial-genomes). Next, the "phyper" function in "R" (R Core Team 2014) was used to test for significant enrichment of GO categories among the treatments and p-values were corrected with the Benjamin and Hochberg method(51) using the "p.adjust" function ($p \leq 0.1$). Finally, genes in enriched GO categories were manually checked.

Phylogenetic analysis

FMO sequences for *T. erythraeum* strains IMS101 and 2175 were taken from the Integrated Microbial Genomes (IMG) website (https://img.jgi.doe.gov/), and *Trichodesmium* environmental metagenomic sequence data was downloaded from Walworth et al. 2015 (41). The BLASTP algorithm was used to search sequences against the RefSeq protein database (52) in which all high-scoring pairs were retained if the aligned portion spanned >70% of the original query length with an evalue < 10^{-5}. Sequences were then aligned with MUSCLE v3.8.31 with default settings(53), and spurious sequences and poorly aligned regions were removed with trimAl 1.2rev59 (54). RAxML (55) was used for all maximum likelihood phylogenetic analyses with the following settings: -f a -p 12345 -m PROTCATLG -N 100 -x 12345.

Declarations

Availability of data and materials

All RNA-Seq data used in this study have been deposited as raw fastq files in NCBI's Gene Expression Omnibus (50) and are accessible through GEO Series accession number GSE94951.

All protein spectral data used in the above analyses can be found in Walworth et al. 2016 (5) in Supplementary Data 4 (DOI: 10.1038/ncomms12081). Physiological and proteome data are archived through the U.S. National Science Foundation Biological and Chemical Oceanography Data Management Office (http://www.bco-dmo.org/dataset/649904).

Conflict of Interest

The authors declare that they have no competing interests.

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Author contributions

F.-X.F., D.A.H., and E.A.W. designed research; N.G.W., M.D.L., X.C., and F.-X.F. performed research; N.G.W., M.D.L., X.C., F.-X.F., D.A.H., M.A.S, and E.A.W. analyzed data; and N.G.W., M.D.L., F.-X.F., D.A.H., M.A.S, and E.A.W. wrote the paper.

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646 **Figures**

647 **Figure 1 Cell physiology** Cell-specific growth rates in day^{-1} (top panel), N_2-fixation rates
648 (middle panel), and cell sizes (assessed using the proxy carbon content per filament length (pg
649 C/μm)) (bottom panel) are shown. Experimental conditions are labeled on the left side of all
650 plots and color coded by nutrients. Data are shown as means and standard errors (n = 6).
651 Different letters denote statistical significance.
652
653 **Figure 2 Fe-limited vs Fe/P co-limited transcripts under 380 and 750 μatm CO_2** (a)
654 Scatterplot of 380-Fe differentially expressed (DE) genes relative to 380-Fe/P (blue) and 380-Fe
655 DE genes relative to both r380 and 380-Fe/P (magenta). Center diagonal shows a 1:1 line while
656 the other lines show 2:1 and 1:2, respectively. Insets are the same plot zoomed out to display
657 highly expressed genes. (b) Similarly, scatterplot of 750-Fe DE genes relative to either 750-Fe/P
658 (blue) or both r750 and 750-Fe/P (magenta). Select genes are labeled. The different colored
659 circles indicate genes within significantly enriched Gene Ontology (GO) pathways between the
660 two conditions.
661
662 **Fig. 3 (a) Distributions of nitrogen acquisition transcripts across treatments, log_2 fold**
663 **changes of transcript and protein abundances per treatment, and (b) physiological data of**
664 **IMS101 grown on several nitrogen species.** (a) Top panel displays the distribution of
665 transcripts per gene across all treatments. Different letters signify significantly different mean
666 values. Middle panel shows log_2 fold changes of gene transcripts relative to the r380 condition
667 across experimental treatments. Bottom panel shows log_2 fold changes of normalized spectral
668 counts of the FMO gene relative to r380. Stars indicate statistical significance relative to r380.
669 (b) Growth and N_2 fixation rates are shown when IMS101 is grown without fixed nitrogen (N_2)
670 and in the presence of either NO_3, TMA, or NH_4 at 20 μm. Different letters signify significantly
671 different mean values.
672
673 **Fig. 4 (a) Log_2 fold changes of normalized transcript and protein abundances of**
674 **photosystem genes and (b) genes from various pathways.** (a) Top panels shows log_2 fold
675 changes of gene transcripts relative to the r380 condition across experimental treatments. Bottom
676 panels shows log_2 fold changes of normalized spectral counts of the FMO gene relative to r380.
677
678 **Figure 5 Replete vs Fe/P co-limited transcripts under 380 and 750 μatm CO_2** (a) Scatterplot
679 of 380-Fe/P differentially expressed (DE) genes relative to r380 (tan) and 380-Fe/P DE genes
680 relative only r380 and no other 380-nutrient limitations (magenta). Center diagonal shows a 1:1
681 line while the other lines show 2:1 and 1:2, respectively. Insets are the same plot zoomed out to
682 display highly expressed genes. (b) Similarly, scatterplot of 750-Fe/P DE genes relative to r750
683 (tan) and 750-Fe/P relative only r750 and no other 750-nutrient limitations (magenta). Select
684 genes are labeled. The different colored circles indicate genes within significantly enriched Gene
685 Ontology (GO) pathways between the two conditions.
686
687 **Figure 6 Hierarchical clustering of 380- and 750-Fe/P gene transcript complements**
688 Hierarchical clustering of Bray-Curtis dissimilarities with multiscale bootstrap resampling
689 calculated from normalized transcripts of all DE genes in both 380-Fe/P and 750-Fe/P
690 complements. Values at nodes are approximately unbiased (AU) p-values. Boxes highlight high-
691 confidence clusters with AU p-values > 0.95.