

Interactive comment on “Co-occurrence of Fe and P stress in natural populations of the marine diazotroph *Trichodesmium*” by Noelle A. Held et al.

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We thank the reviewer for their thoughtful comments on our manuscript and discuss changes below. We also provide a supplemental file with new tables/figures to be included in the manuscript, as well as the following responses formatted for ease of reading.

Major Remarks

1. The whole conclusion of co-occurring phosphate and iron stress relies on the assumption that protein abundances of IdiA and SphX are good proxies for iron or phos-

C1

phate limitation, respectively. The authors do cite the relevant literature that showed upregulation of the respective markers under the corresponding nutrient stress. What I am missing is information on which fold-changes in protein abundance were measured in the cited studies under the respective nutrient limiting conditions. For example, what are the base levels of IdiA and SphX protein in the cell? If there is three times more SphX than IdiA, such as in Fig 3 for some of the Tricolim samples, does that really indicate co-limitation, or does that just reflect the base level of IdiA? For example, Snow et al., 2015 (Fig. 4) only report a two-fold change for IdiA from ~ 100 fmol/ug to ~ 200 fmol/ug under iron stress. I suggest presenting the evidence for IdiA and SphX being markers for the respective stresses clearly in a table, including the type of experiment (culture or field), absolute quantifications if stated, and fold-changes measured. I also suggest then being a little more careful in wording throughout the paper, and differentiating better how the results from different stations could be interpreted.

The reviewer asks us to address how our results compare with previous reports of IdiA and SphX abundance during nutrient limitation. Per the reviewer, we provide new supplementary tables (Table S8 and S9) containing fold-change values from the literature for IdiA and SphX as during nutrient limitation. The biomarkers increased in abundance during nutrient depletion, however the magnitude of the response varied. This is likely due to experimental differences such as the analytical methodology (Western blots versus LC-MS), nutrient concentrations or growth rate of the culture being examined. For instance, IdiA responded less strongly to iron stress in the Walworth et al., 2016 experiments compared to the Webb et al., 2001 and Snow et al., 2015 experiments, and we hypothesize this is because the Fe-depleted condition had 10 nM added iron as opposed to 0 nM iron in Webb et al. 2001 and Snow et al. 2015. We agree with the reviewer that applying a quantitative framework to this data would be valuable once the necessary data becomes available and now note this in the text.

Based on the consistent responsiveness of IdiA and SphX to nutrient limitation in the laboratory, we concluded that the high relative abundance of these biomarkers was in-

C2

dicative of nutrient stress. The reasoning is that the cells were clearly devoting a large fraction of their proteome to Fe and P uptake, likely at the expense of other nutrient uptake systems such as for organic nitrogen as is discussed later in the text. The reviewer's point that there may be basal expression of IdiA and SphX in replete cells is well taken. Because we report relative abundance data, we cannot directly compare our results to those of other researchers who took different quantitative approaches. However, the possibility of basal expression should be clarified in the text. Carefully calibrated datasets relating IdiA and SphX protein abundance to nutrient limited growth rates, while outside the scope of this paper, would be valuable in facilitating interpretation of this data. Based on the reviewer's comments we have updated Section 3.2 below to clarify the assumptions and caveats in our interpretation, and to highlight the need for calibrated biomarker studies.

3.2 Trichodesmium is simultaneously iron and phosphate stressed throughout its habitat

A surprising emergent observation from the Trichodesmium metaproteomes was the co-occurrence of the iron (IdiA) and phosphate (SphX) stress biomarkers across the samples. The ubiquitous and highly abundant presence of these proteins relative to total protein implied that co-stress may be the norm rather than the exception for Trichodesmium colonies in the field, particularly in the North Atlantic. Even though low-level basal expression of IdiA and SphX has been observed, it was clear that the colonies were devoting a large fraction of their cellular resources to Fe and P uptake, respectively (see Tables S8 and S9) (Webb et al., 2001, Webb et al., 2007, Chappell et al., 2010, Orchard et al., 2010, Snow et al., 2015, Walworth et al., 2016, Frischkorn et al., 2019). This, combined with the responsiveness of IdiA and SphX to nutrient availability in Trichodesmium filaments in the laboratory, indicated that co-stress was occurring.

Interestingly, biomarker abundance was not necessarily associated with nutrient concentrations in the surface ocean, suggesting that the colonies were experiencing stress

C3

despite variation in nutrient availability (Figure 3 C-D). SphX abundance varied up to 7.5 fold and were negatively associated with dissolved phosphate concentrations, though analytical differences across the field expeditions may have forced this relationship (Figure S2). Oceanographically, SphX was most abundant in the P-deplete, summer-stratified North Atlantic gyre (JC150 expedition) compared with winter waters near the Amazon river plume (Tricolim expedition) or at station ALOHA, where phosphate concentrations were greater (Hynes et al., 2009; Sañudo-Wilhelmy et al., 2001; Wu et al., 2000). IdiA varied up to 8 fold but there was no observable relationship with dFe concentrations at the surface. Instead, IdiA may be responsive to other factors such as the varying iron requirements of the populations/species examined. It should be highlighted that in this study only Trichodesmium colonies were considered, so factors such as colony size may affect iron availability and biomarker expression. Additionally, because the surface ocean iron inventory is low, transient inputs such as from the Sahara desert can dramatically impact iron availability on short time scales, and the time scale of these inputs relative to changes in biomarker abundance is not well understood (Kunde et al., 2019). Carefully calibrated datasets relating IdiA and SphX abundance to nutrient-limited growth rates of Trichodesmium in both the filamentous and colonial forms would facilitate further interpretation of this data.

2. Figure 3, and the corresponding Figure S2 are nice and the basis for some important claims being made in part 3.2. of this manuscript. However, these claims should be supported with the necessary statistics, and it would help if Fig S2 was not in the Supplement, but presented together. For example, in line 210 ff, the authors claim that a) "Biomarkers for iron (IdiA) and phosphate (SphX) stress were highly abundant and positively associated with surface Fe or P concentrations" and b) "IdiA varied up to 8 fold, and increased moving West to East across the JC150 transect, consistent with an observed decrease in dFe concentrations". For a) I think the authors mean "negatively", not "positively", correlated. And while I believe this correlation for SphX, it is not obvious for IdiA. For b) I cannot see increasing protein abundance from west Please prove this statistically before claiming it.

C4

First, we thank the reviewer for correcting our mistake in line 210 – we did indeed mean to write “negatively.” For clarity, we have moved the panels in Figure S2 alongside Figure 3 in the main text. Figure S2 now provides scatter plots of IdiA and SphX versus dFe and phosphate concentrations in the surface ocean (see updated figures section at the end of this document). There was a statistically observable relationship between SphX and dissolved phosphate, however the relationship may be forced by the different analytical approaches used on the JC150 versus Tricolim expeditions as is noted in the text below. By contrast there was no statistically observable relationship between IdiA and dFe, even though laboratory experiments clearly indicated that IdiA was a good biomarker of Fe stress in *Trichodesmium*. There are many factors that could influence this association, particularly iron speciation, changes in iron quotas, and factors such as colony size, which are not controlled in the field. We thank the reviewer for calling this point to our attention as it provides an opportunity to discuss these points in the updated Section 3.2 (see Major Remarks #1).

3. Given that only 1 sampling station is NOT in the Atlantic, please remove all claims that generalize the findings, e.g. “co-stress is the norm rather than the exception” (l. 18) → add “in the Atlantic”, if wanting to keep this. Or in line 60f: “simultaneously Fe and P stressed throughout the worlds oceans” – this statement cannot be made with just one station outside the Atlantic.

Change accepted.

Specific Comments

Abstract

The abstract is missing some specificity.

line 19: nitrogenase was most abundant – compared to what? Please rephrase: more abundant than under . . line 22: is confronted by the biophysical limits – when? Under which conditions is it confronted by this? line 24f: be more specific. The last sentence

C5

is true for any microbe.

We agree with these points and have updated the text accordingly. On line 24f, we wished to highlight the importance of considering multiple nutrients for *Trichodesmium* specifically, given the historical emphasis on either Fe or P stress.

Abstract. *Trichodesmium* is a globally important marine microbe that provides fixed nitrogen (N) to otherwise N limited ecosystems. In nature, nitrogen fixation is likely regulated by iron or phosphate availability, but the extent and interaction of these controls are unclear. From metaproteomics analyses using established protein biomarkers for iron and phosphate stress, we found that co-stress is the norm rather than the exception for field *Trichodesmium* colonies. Counter-intuitively, the nitrogenase enzyme was more abundant under co-stress than under single nutrient stress, consistent with the idea that *Trichodesmium* has a specific physiological state under nutrient co-stress, as opposed to single nutrient stress. Organic nitrogen uptake was observed to occur simultaneously with nitrogen fixation. Quantification of the phosphate ABC transporter PstA combined with a cellular model of nutrient uptake suggested that *Trichodesmium* is generally confronted by the biophysical limits of membrane space and diffusion rates for iron and phosphate acquisition in the field. Colony formation may benefit nutrient acquisition from particulate and organic nutrient sources, alleviating these pressures. The results highlight that to predict the behavior of *Trichodesmium*, both Fe and P stress must be evaluated and understood simultaneously.

Introduction

Line 36: colloquialism

Change accepted.

Line 58: add . . .Pho box, a regulatory DNA sequence, which is necessary. . .

Change accepted

Line 65f: Fe and P stress were positively associated – only as co-stress? If yes, say

C6

so.

They were associated both individually and under co-stressed.

Also say how Fe, P, and N statuses are closely linked.

We suggest these are linked via a currently unknown regulatory network; change accepted.

Methods

Line 119: what does that mean? Which precursors, of what? Was every protein normalized to the top 3 precursor intensities? Make this clear also to a reader who is not familiar with the specifics of proteomics analysis.

Relative abundance was measured by averaging the peptide precursor/MS1 intensities for the 3 most abundant peptides in the protein, then normalizing this value to the total precursor intensity. Text has been updated:

Raw spectra were searched with the Sequest algorithm using a custom-built genomic database (Eng, Fischer, Grossmann, and MacCoss, 2008). The genomic database consisted of a publically available *Trichodesmium* community metagenome available on the JGI IMG platform (IMG ID 2821474806), as well as the entire contents of the CyanoGEBA project genomes (Shih et al., 2013). Protein annotations were derived from the original metagenomes. SequestHT mass tolerances were set at +/- 10ppm (parent) and +/- 0.8 Dalton (fragment). Cysteine modification of +57.022 and methionine modification of +16 were included. Protein identifications were made with Peptide Prophet in Scaffold (Proteome Software) at the 95% protein and peptide identification levels. Relative abundance was measured by averaging the precursor intensity (area under the MS1 peak) of the top 3 most abundant peptides in each protein, then normalizing this value to total precursor ion intensity. Normalization and global false discovery rate (FDR) calculations, which were 0.1% at the peptide level and 1.2% at the protein level, were performed in Scaffold (Proteome Software). FDR

C7

was calculated by Scaffold using the probabilistic method by summing the assigned protein or peptide probabilities and dividing by the maximum probability (100%) for each. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016225 and 10.6019/PXD016225 (Perez-Riverol et al., 2019). Statistical tests of relationships between proteins were conducted with the scipy stats package (<https://docs.scipy.org/doc/scipy/reference/stats.html>) using linear Pearson tests when the relationship appeared to be linear and a Spearman rank order test when this was not the case.

Line 120: How is the FDR defined? What does “0.1% peptide” and “1.2% protein” mean?

Text updated (see above paragraph):

FDR was calculated by Scaffold using the probabilistic method by summing the assigned protein probabilities and dividing by the maximum probability (100%) for each. Different FDRs can be assigned for peptides versus proteins depending on which probabilities are used for the calculation.

Line 128: Which peptides were selected? We have added to the supplemental Tables S6 and S7 which describe the peptides selected for quantitation

Results and Discussion

Line 178: change “most” to “all but one”

Change accepted

Line 232: please rephrase. What exactly is common in marine bacteria. For sure, all bacteria have regulatory networks.

Change accepted. A recent review of regulatory genes found that regulatory networks may be particularly more abundant in marine organisms (Held et al., 2019).

C8

This indicates that the cell's N, P and Fe statuses are linked, perhaps involving a regulatory network which are particularly common in marine bacteria (Figure 5) (Held et al., 2019).

Line 255ff: skip this justification sentence

With respect we prefer to leave this sentence in because exploratory metaproteomics is not yet widely used as an analytical tool in oceanography. We hope this study and others like it will encourage its adoption. We do welcome editorial advice or further discussion on this point.

Section 3.4. Throughout this section, I think the use of the term ligand is not the norm. For ABC transporters, the word "ligand" is typically used for whatever binds to and is transported by the transporter. The part of the transporter binding the substrate is usually called "ligand-binding protein".

We agree with the reviewer that "ligand" is often used to describe a chemical compound, for instance a siderophore, which can be transported by an ABC transporter. However specifically in the uptake kinetics literature "ligand" is used to describe the ABC transporter itself (i.e. the protein that binds the nutrient). To avoid confusion we have updated the text to use the word "transporter" or "protein" instead.

Line 260: change to ". . .required for both iron and phosphate uptake"

Change accepted

Line 305ff: rewrite sentence. Hard to understand.

Updated, and hopefully clearer now!

For a given surface area: volume quotient, we define nutrient limitation to be caused by either membrane crowding or diffusion limitation depending on which model calculated a higher minimum nutrient concentration.

Line 316ff on cylinders: Shouldn't the Trichodesmium filament, instead of a single Tri-

C9

chodesmium cell, be considered for these models? The effective cell surface of a Trichodesmium cell is reduced by its contact to the neighboring cells.

The reviewer is correct that membrane limitation would be exacerbated for cells living in filaments, as the surface area exposed to the surrounding environment would be reduced. We considered modeling filamentous cells but decided to consider only single cells for clarity since this is the most conservative scenario (i.e. the one in which Trichodesmium would theoretically have the most exposure to the environment and be the least limited). We have added a sentence to the discussion of the model highlighting the focus of the model (single cells) but mentioning that filamentous cells would have lower surface area.

While this model may be directly applicable to some N₂-fixing cyanobacteria such as Groups B and C, which have roughly spherical cells, Trichodesmium cells are not spheres but rather roughly cylindrical (Hynes et al., 2012). Thus, we repeated the model calculations for cylinders with varying radii (r) and heights ($2r$ or $10r$) based on previous estimates of Trichodesmium cell sizes (Bergman et al., 2013; Hynes et al., 2012). Cylinders have lower surface area: volume quotient than spheres of similar sizes. In addition, the rate constant (kD) for diffusion, which is a function of cell geometry, is greater. This increases the slope of the diffusion limitation line such that membrane crowding is important across a greater range of cell sizes (Figure 7c-d). Trichodesmium cell sizes vary in nature, for instance the cylinder height can be elongated, improving the surface area: volume quotient. However, the impact of cell elongation to radius r and height $10r$ on both diffusion limitation and membrane crowding is subtle (Figure 7e-f). Furthermore, though not explicitly considered here, cylindrical cells living in filaments would have reduced surface area available for nutrient uptake. Thus, we conclude that in certain scenarios, lack of membrane space could hypothetically limit Fe and perhaps P acquisition by Trichodesmium.

Line 361: Reference missing for mucus production being a "hallmark of Trichodesmium colony formation"

C10

Citation added (Eichner et al., 2019)

Line 362f: If mucus acts as a diffusive barrier, it also does the opposite of “protecting them [the cells] from oxygen”, namely preventing O₂ to diffuse out of the cells during photosynthesis, which was also shown in Eichner et al, 2019.

Note added to this effect:

A key hallmark of *Trichodesmium* colony formation is production of mucus, which can capture particulate matter and concentrate it within the colony (Eichner et al., 2019). In addition to particle entrainment, the mucus layer can benefit cells by protecting them from oxygen and/or concentrating oxygen during photosynthesis, facilitating epibiont associations, regulating buoyancy, defending against grazers and helping to “stick” trichomes together (Eichner et al., 2019; Lee et al., 2017; Sheridan, 2002). However, these benefits come at a cost because the mucus layer hinders diffusion to the cell surface (Figure 9), reducing contact with the surrounding seawater. Despite this, the benefits of colony formation seem to outweigh the costs, since *Trichodesmium* forms colonies in the field, particularly under stress (Bergman et al., 2013; Capone et al., 1997; Hynes et al., 2012).

Line 384: Which specific regulatory systems should be characterized? What do you mean by chemical phases?

We don't know yet which regulatory systems should be examined! For a review of marine regulatory systems and their often unknown functions see Held et al. 2019. These results suggest that one or more regulatory networks may control Fe, P, and N status in tandem with one another in *Trichodesmium* cells, and we hope this work will stimulate future research on this topic. By chemical phases we meant dissolved versus particulate nutrient sources, since *Trichodesmium* is known to use both – we've clarified this now:

Future studies should aim to characterize the specific regulatory systems, chemical

C11

species and phases (i.e. dissolved versus particulate nutrient sources), and symbiotic interactions that underlie *Trichodesmium*'s unique behavior and lifestyle.

Figures

Figure 1: Please use the same numbers on the figure and the legend, or at least also add the figure numbering top the legend.

Figure 1 has been updated (see supplemental file).

Figure 2: Please increase the font size on the legend, and add a legend name like “# of times a protein appeared in the same cluster” – consider changing the legend to a percentage. Please also say in the caption what the color legend shows.

Figure 2 and its caption have been updated

Figure 3: Please state in the caption how the protein abundance values were normalized.

Figure 3 caption has been updated (see supplemental file)

Figure 4: Please adjust font sized throughout panels. How were the dashed lines in c and d defined? Based on what do they denote Fe- or P-stress? And why are they different in c and d?

Figure 4 font sizes have been updated. The dashed lines in (C) and (D) were drawn by hand to help the reader to visually understand the intersection of Fe and P stress. However, given the important discussion about *IdiA* and *SphX* abundances raised by the reviewer, we can see how this might be misleading and have removed the dotted lines.

Figure 5: Does not necessarily need to be a figure if wanting to save space.

We welcome editorial advice on this but would advocate for including the figure as we think it illustrates the discussion in section 3.3, particularly for visual learners.

C12

Please also note the supplement to this comment:
<https://www.biogeosciences-discuss.net/bg-2019-493/bg-2019-493-AC2-supplement.pdf>

Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2019-493>, 2020.