6 May 2016

Dear Editor and Reviewers

Attached please find our revised manuscript "Mechanisms of *Trichodesmium* demise within the New Caledonian lagoon during the VAHINE mesocosm experiment". We thank the reviewers for their time and effort in commenting on our manuscript and have taken into account many of their general and specific comments (see point-by-point response). Accordingly, we have made major revisions to the manuscript that we believe will now emphasize and clarify the results and conclusions. Specifically, our major changes include:

1) **Restructuring of manuscript so the reader can easily follow the distinction between the data and results obtained in-situ from the lagoon itself and the results from the experimental incubations of *Trichodesmium* collected from the bloom which developed in the lagoon. Manuscript "results and discussion" now flow in the following order:**

   3. RESULTS

   3.1. Setting the scene -*Trichodesmium* development and bloom within the lagoon.

   3.2. Investigating *Trichodesmium* mortality in experimental microcosms

   3.2.1 Changes in *Trichodesmium* biomass and associated microbial communities.

   3.2.2 Genetic responses of stressed *Trichodesmium*

   3.2.3 PCD-induced demise.

   4. DISCUSSION

   4.1. Mortality processes of *Trichodesmium* – incubation results.

   4.1.1 Grazer and virus influence

   4.1.2 Stressors impacting mortality.

   4.1.3 Programmed cell death (PCD) and markers for increased export flux.

   4.1.4 Changes in microbial community with *Trichodesmium* decline.

   4.2. **Implications for the lagoon system and export flux**.

   5.**CONCLUSIONS AND IMPLICATIONS**

2) **INTRODUCTION** has been revised to set the stage and emphasize the gaps in knowledge. (i.e. We know much more about development, growth and the controls on blooms then on what happens to the large scale blooms and the mortality processes that could impact them.)
3) **METHODS**. We have clearly separated information related to the experimental incubations and have added a supplemental figure (S1) schematic illustrating our experimental flow.
4) **FIGURES**: We have revised the figures so that they are now clearly distinguished as to whether the data displayed in them is from the lagoon or experimental incubations (i.e. experiments 1 and 2).
5) **REMOVAL OF NON-ESSENTIAL DATA**. We have shortened the text considerably and have trimmed non-essential data such as the discussion about arsenate related genes. We have also gone over the text to eliminate redundant discussion and other data throughout the different sections.
6) **CHANGE OF TITLE:** We have removed the word "bloom" attached to the previous title so that from the beginning it is clear we are looking at mortality processes but not specifically in-situ.

We believe these changes have greatly improved the manuscript and hope this will be sufficient for publication in BG.

Please let me know if any further information is needed.

Sincerely,

Ilana Berman-Frank

<u>SPECIFIC ANSWERS TO COMMENTS AND QUESTIONS BY REVIEWERS.</u>

Reviewers comments in black –Our responses in Red

<u>Reviewer 1</u>

Results are interesting and appear technically sound, for the most part.
Overall, this manuscript needs some revision– specifically in 3 sections: 1. Introduction
The Introduction is weak because it lacks discussion of the rationale– e.g., for the
INTRODUCTION lines 84-88– very confusing, and long, sentence; something seems to be missing? Revise this section, as first authors say no blooms developed so bloom in lagoon was "exploited" (is this the best word?), but then authors say they used mesocosms. But methods section indicates bottles/carboys? This needs clarification, or delete, as it is explained again in lines 110-113.

The introduction has now been fully revised and all these specific issues clarified. See below. We have also added figure S1 to fully detail the experimental set up and approach.

METHODS: 115-179: Were 6 net tows done, or 1 tow split among 6 bottles?

For experiment 1, a net tow was taken from several patches of the bloom and the biomass was combined, resuspended in filtered seawater, then split evenly between six 4.6 L bottles. We have added these details in the text. Section 2.2.

121: How many replicate carboys were filled/ sampled?

For experiment 2, we used a Teflon® PFA pump and PVC tubing directly filling nine 20 L polyethylene carboys gently to avoid destroying At each sampling time point, 1 carboy was sampled for the different parameters. Section 2.2.

126-7: Experiment 2 was sampled only on days 23 -25? How frequently? Replication?

Experiment 2 started on day 23 at 17:00, we filtered every 4 hours.
Sampling was done at 17:00 (day 23); 21:00 (day 23); 1:00 (day 24); 5:00 (day 24); 9:00 (day 24); 13:00 (day 24); 17:00 (day 24): 7:00 00 (day 25). This is now illustrated in a schematic of the sampling procedures in supplemental figure S1.

165: Fluorometer
Changed

Differential expression- Insufficient information on experimental design is given (replication is not described). I'm assuming no replication, which is problematic since results seem so variable. Some discussion of the method used for analysis of DE is needed; sentence in the methods seems to be taken directly from the methods paper
In this manuscript we do not have further biological replicates for each of the metatranscriptomic samples taken from the three time points (T0, T8, T22). However, quantitative real time RT-PCR for representative *Trichodesmium* metacaspase transcripts was conducted for biological replicate samples, and shows similar patterns as provided by the metatranscriptomic analyses (This data is part of a different manuscript Spungin et al. in preparation). Moreover, the tool that we employed for differential expression analysis is specifically designed to infer statistically sound differential expression analysis without biological replicates by modelling biological variation mathematically

(Wu et al., 2010). No sentence was copied from the methods paper. We added a respective explanation to the methods section for the revised paper. Sections 2.9-2.11.

RESULTS: 289-291: Revise this long, confused sentence; why not just state chl a (total biomass) increased from xxx to xxxx
Revised.

292-294– delete this sentence, as this is obvious– or can you estimate what fraction Tricho contributed to the total chl a?
Sentence deleted.

297-8; Should you state that Tricho abundance is measured as # of nif copies?
Inserted where appropriate. In experiment 2, *Trichodesmium* abundance was additionally quantified with counts of trichomes that were estimated from nifH transcript numbers measured by K. Turk-Kubo.

lines 315-317 this repeats the methods
Deleted

Did you look at the samples to verify the absence of other cells (taxa)?
16S analyses were performed; see Fig 3 and results and discussion sections 3.2.1 and 4.1.4.

line 348-9 very wordy ; revise this sentence
Sentence revised

line 363: replace ", which" with "and declined to 0.5 +/- .by day 23
Replaced and changed

lines 372 - 378 appear to be Discussion, not result
This was moved to discussion.

384-5 if increased "significantly", authors should state p-value
As noted in the methods- sections 2.10-2.11 – genes were defined significant if $P>0.98$.

386-91 – Why are arsenate reductase genes results mentioned? Nothing is stated in Introduction to explain why?
Arsenate reductase was primarily mentioned due to its high expression during bloom demise and the danger of higher arsenate influx into the cell through phosphate uptake systems when DIP is low. **However, we agree with the reviewer's comment, and that it is not contributing to the text relating to nutrient stress. We have removed all reference to the arsenate genes from the manuscript.**

Reviewer 2

General comments: I have only few minor comments about this manuscript - overall the manuscript is carefully prepared and well written. The fate of cyanobacterial blooms is relatively little studied and the paper

makes a nice contribution. The results and conclusions are providing new information into this topic, discussing not only mechanistically the drivers of the bloom crash but also discussing the fate of the N and C from the blooms. This paper will be a useful addition to the literature

R121 State how the carboys were cleaned for the experiments
We washed the bottles and carboys with HCl (overnight). At the time of sampling, both bottles and carboys were washed three times with the sampling water before being filled. This is now added to methods. Section 2.2.

R134 nm, not nM
Changed

R151 state the 15N2 gas lot number and whether you made any effort to test for its contamination by 15NH4 or 15NO3. Given the recent evidence that some 15N2 gases are contaminated, can your rate measurements be trusted?
15N2 (98.9 % Cambridge isotope) serial number:18/061501.
The potential contamination level was assessed by the Dadundo group (Dabundo et al. (2014). For the experiment 15N2 Cambridge Isotopes batches (18/061501) were checked for contamination following the method described in Dabundo et al. (2014); it was 1.4 x 10-8 mol of 15NO3- per mol of 15N2 and 1.1 x 10-8 mol NH4+ per mol of 15N2. The application of this contamination level to our samples indicates that our rates may only be overestimated by ~0.05 %, confirming that our present results were unaffected by possible 15N2 stock contamination'. This has been addressed and published in Berthelot et al. 2015. **Added info in section 2.5.**

R155 was filtered
Changed

R155 describe what time of the day 15N2 fixation incubations were initiated and ended, and what time of the day were samples collected for metatranscriptomics
N2 fixation incubations in Experiment 2 lasted for 24h, 40h and 72 hours, 15N2 spike was within an hour of biomass collection from the lagoon at 17:00.

Experiment 1. Samples which were collected and analyzed for metatranscriptomic analyses were collected at- 12:00 (T0), 20:00 (T8), 10:00 (T22).

R200 describe how total protein was determined
Cell extracts from each sampling were divided into a) caspase activity assays, and b) total protein. Total protein was determined using a Pierce BSA protein assay kit (Thermo Scientific product #23225). Added and clarified in the text Section 2.8.

R219 describe here what sequencing method was used
Sequencing was done at the scientific equipment center in Bar-Ilan University, using an Ion Torrent™ Next-Generation Sequencing Technology (Life Technologies, USA)- clarified in text Section 2.9.

R222 list the number of sequences for each sample after trimming – perhaps include a supplementary table.

The number of reads for each sample is not relevant for OTU clustering, because the pooled reads from all samples are used for this. A sentence clarifying this was added to the respective methods section – Section 2.9-2.11.

R239 describe what time of the day each of these time points were sampled.
T0= 12:00 T8= 20:00 T22= 10:00 Added to text where appropriate.

R251 purified second time with the Zymo kit?
Purified second time with C&C5 (Clean & Concentrator™-5).

R255 state specifics for the N6 primer
It is random. 6 random nucleotides in a row.

R375 What do you mean by T_DIP?
Turn over time of dissolved inorganic phosphorus. This was defined and clarified in Section 3.1 "...Depth-averaged dissolved inorganic phosphorus (DIP) concentrations in the lagoon waters were low at 0.039 ± 0.001 µM, with a relatively stable DIP turnover time (T_{DIP}) of 1.8 ± 0.7 d for the first 15 days, that declined to 0.5 ± 0.7 by day 23 (Berthelot et al., 2015)."

R512 *Trichodesmium*
Changed

R530 add comma after 'Fe' Figures:
Added

Assure that the figures are higher resolution than they were for the review purposes
All figures were reformatted, font and resolution increased.

Reviewer 3

This manuscript is interesting but needs revisions. The initial intent of the study was to follow the demise of a *Trichodesmium* bloom in a mesocosm. While the mesocosms failed, a bloom developed in the wild, so *Trichodesmium* samples were taken in carboys and bottles. The authors follow the collapse and crash of this Trichodesmus bottle/carboy bloom. The authors measure phenotypic and genetic parameters to describe the bloom. The work is interesting but not always clear to the reader.

Introduction lacks sufficient context to understand the relevance of the VAHINE project which is simply referenced, but not described at all. Although the whole issue is about VAHINE it should have a sentence or so to connect the manuscript to the general experimental framework. The Introduction is weak. It lacks a general rationale for the experiment and background about *Trichodesmium* responses to stress.

The introduction has been thoroughly revised and clarified to emphasize the relevance of our experiments to the VAHINE project which is now outlined and referenced to link our work to the general experimental framework.
Our initial objective during the VAHINE project was to study the involvement of PCD in the fate of natural *Trichodesmium* blooms induced in these mesocosms. While *Trichodesmium* was initially present and conditions in the mesocosms appeared favorable, no *Trichodesmium* blooms developed within the mesocosms. Instead, *Trichodesmium* developed and dominated at different phases of the

experimental period outside the mesocosms wasters. During the VAHINE Mesocosm experiment we sampled daily from outside the mesocosms water (where bloom occurred), so this data served us as 'pre bloom' data now clearly defined in Figure 1 and in the first section of the results 3.1..

Furthermore some sentences are difficult to follow and understand, e.g., "While *Trichodesmium* was initially present and conditions in the mesocosms appeared favorable, no *Trichodesmium* blooms developed within the mesocosms with other diazotrophs (such as diatom-diazotroph associations, and unicellular types mainly UCYN-C, as well as UCYN-A and UCYN-B) instead developing and dominating at different phases of the experimental period (Turk-Kubo et al., 2015)."
We have gone over text fully to reduce redundancy and clarify difficult sentences.

Methods section is well written and appropriate, although it wasn't very clear for referee N1, who felt more detail was needed, specifically replication of experiments which many times appear in the results. Additionally, further details about the experimental design would improve the manuscript. A figure showing sampling times, type of assessment (chlorophyll, transcript, nutrients, etc.) will improve the readability of the manuscript
Please see added supplemental scheme (S1) to understand the experimental setup and sampling times.

A central assumption from Spungin et al. is that the microcosms "carboys" experiments is representative of the natural environment and therefore the phenomena observed during the experiment, i.e., the collapse and its causes, are equivalent to the natural sea bloom. While this might be true, the reviewer disagrees with this interpretation. Indeed Spungin et al. mention in line L312 that "Based on previous experience (Berman-Frank et al., 2004), resuspension of *Trichodesmium* cells in the extremely high densities of the surface blooms (Fig. 2a-c) would cause an almost immediate crash of the biomass." If this is true why would these blooms be equivalent? Furthermore, no data is presented nor mentioned about the timescale, cell physiology and causes of the eventual collapse of the bloom at its natural environment. No control about the behavior of *Trichodesmium* in the bottle environment is provided (Did the natural bloom also collapse in 22 hours? How? Levels of nutrients?).
One of the principal problems of tracking the natural bloom decline in the lagoon water (rather than in the VAHINE mesocosms as originally planned) is the spatial and temporal patchiness and quick disappearance of the biomass. We could track an increase in the percentage of *Trichodesmium* in the lagoon water in the days preceding the surface bloom as described in result section 3.1. On the day the large surface accumulations appeared, the variability in densities was high with our 0900 am sampling showing 0.39 µg chl a^{-1} L^{-1} by 12:00 the extensive biomass appeared on surface with values of 1-5 mg chl a^{-1} L^{-1}, and remained visible although at lower densities still at 1700. By the following morning this biomass had disappeared. Disappearance could be due to physical transport of the biomass either horizontally or vertically. Unfortunately, we could not continue to track this biomass within the lagoon water in the framework of the experiment. Thus we cannot describe or quantify the demise of the accumulated biomass in the lagoon any further as the reviewer requests.
We have now included the explanations for this in the text . Sections introduction and 3.1, 4,2.

We have now completely revised the results and discussion to separate between the lagoon data and the experimental incubation data. See beginning of our letter.

We agree with the reviewer that processes occurring within carboys, bottles, or even mesocosms do not fully reflect the reality occurring in the lagoon which may be either more or less intense and will be further complicated by additional parameters such as turbulence/grazing etc. However, as we

were interested in examining the subcellular responses to different stressors and focusing especially on mortality processes we could track these within the enclosed systems that contained collected biomass from various patches and from two different time points (12:00 and 17:00) and were incubated under ambient temperature and light.

A deep and thorough discussion about the differences between the microcosms environment with respect to the natural environment is expected, however it was not provided. For example, PCD phenotype cells were quantified in the bottle setup, but not necessarily this mechanism would be at the natural environment.

To clarify the differences between what occurs in the natural environment and what has been illustrated here we modified the text completely so that the differences between the systems are clearer. The results and discussion have been restructured so as to clearly discuss first the lagoon system and then the experimental microcosms (see explanation above).

Another main weak point of the manuscript is the large amount of the results described, without biological interpretation. Some examples are: L361-L368, interpretation of DIP turnover. L376-402. Gene expression changes for several pathways are described, but no physiological interpretation is provided.

This has now been changed. We have removed non-essential results that contributed little information to the main message (i.e. information on expression of arsenate related genes) and have tried to distill and explicate the results we describe with an ecological/physiological perspective.

Another result than needs further insight is the low number of reads mapping to *Trichodesmium* genome for T8 and T22 samples (line L277). While T0 has a percentage of 52% mapped reads (low rate but within reasonable range), only 5% and 3% mapped for T8 and T22 samples. An explanation for this result is expected. If they did not match to *Trichodesmium* genome, to what other organism do they map (use BLAST)? This result needs to be explained, interpreted and discussed.

This mapping percentage to the *Trichodesmium* genome reflects the dying *Trichodesmium* biomass and was expected. The sequencing data is effectively a metatranscriptome and a large fraction of reads in T8 and T22 mapped to Alteromonas, which grew when *Trichodesmium* declined (see Fig. 3 in the revised MS), but many other bacteria are expected to be present at lower numbers in these environmental samples, too. For this MS however, we were only interested in the transcriptomic response of *Trichodesmium* , which is why we did not map (or BLAST) all the data. A BLAST against NCBI of 50,000,000 reads is computationally intense and thus only done if necessary.

The transcript expression figures should not display RPM, but log2 changes from FPKM with respect to initial conditions. RPM is not a good measure to compare different transcripts because different transcripts have different transcript lengths and counts are biased by transcript length. I suggest to use cufflinks package to quantify transcript abundance (FPKM, not RPM) and statistical tests for differential expression. Final figures should present log2 fold changes to illustrate expression dynamics or log10 FPKM if absolute value needs to be illustrated.

We agree that RPM is not a good measure to compare different transcripts. We did not aim at comparing transcripts amongst each other, but at comparing the expression of one given transcript at three time points. For this purpose, RPM is valid. However, because we did present multiple

transcripts in one figure, it might indeed be better if the reader can directly compare these transcripts' expression amongst each other.

In the revised version of this MS, we thus present expression data as the log2 of the fold change with respect to T0, as suggested by the reviewer. We now use the fold-change calculated by the Bayesian tool ASC (Analysis of Sequence Counts), which we have used in this work to infer statistically sound differential expression (see Methods of the original MS). The advantage of this tool is that it accounts for biases introduced by different library sizes (in this case, the "library" is the sum of all reads mapped to *Trichodesmium*), like the "zero-count" problem in small libraries: In small libraries, rare transcripts will more often have zero reads than in larger libraries, leading to a smaller set of expressed transcripts. As a consequence, if simple library size normalization is performed, transcripts in the smaller library will get too many counts (artificial upscaling). ASC accounts for this as well as for missing replication and is rather conservative compared to other tools in calling differentially expressed transcripts (Wu et al, 2010).

The use of important terms like "bloom" or "demise" should be use much more strictly.
At the beginning of the manuscript, we define the term "bloom" as dense surface accumulation. This is a common term for this phenomenon (see Behrenfeld, 2014. Climate mediated dance of the Plankton. Nature Climate Change : "...seasonal blooms – period of high biomass concentration"). The term "demise" denotes the mortality/death of the cells and crash of biomass. However, to clarify this further, these terms have been reviewed throughout and changed where appropriate.

T0 (Fig. 8b)." may appear to refer to the actual bloom in the lagoon, while it actually may refer to the description of the phenomena in the carboys. It is unclear to the reader. Clear differentiation about the description of the phenomena in the lagoon versus the carboys should be expressed more clearly. Maybe a table would help?

All measurements and results described from experiment 1 and 2 were obtained from sampled water incubated in the bottles/carboys. We have clarified this in the method text and also provided a scheme in supplemental figure 1.

Discussion: generally the discussion is too long and many irrelevant hypotheses that were not tested are discussed. I recommend to shorten the section and specifically address issues directly related to the work, not tangential hypotheses that were not addressed by the current work, e.g., L576-L596.

The discussion was revised considerably to reflect the new structure differentiating clearly between lagoon and microcosm experiments. We have also shortened it substantially to eliminate non-essential hypotheses and results.

Finally, figures resolution is low, and font size of text within figures should be increased.
All figures were reformatted, font and resolution increased.

Also acronyms should be fully spelled first time they appear in the main text, e.g, DIP (Dissolved Inorganic Phosphorus) is never defined at the manuscript.

Checked and defined in text.

L435-L438, when comparing pre- to bloom values, a coherent measure should be used, e.g., average to average or max to max. Instead several ranges are used and it is not clear to the reader how the fold increase was calculated.

Yes the reviewer is right, we now clarified it in text, and calculated the fold changes between maximum concentration from pre bloom days and maximum concentration during the bloom.

L439, cellular internal components do not "collapse", they rather "structurally degrade".

Changed

L476. It is unclear to the reader to what experiment the authors refer to when they mention a "for the three weeks of the experiment". In their work, two experiments were conducted of 22 and 44 hours respectively. Maybe authors refer to the monitoring period of the lagoon?
This has been revised and clarified. See Fig S1 and methods 2.1.

L493. The use of term "fastidious" is inappropriate

Changed

L506/L516. While L506 states that no specific *Trichodesmium* phage has been isolated, L516 mentions that authors could not identify *Trichodesmium* specific phages on their experiments. Probably the use of term "found" or "characterized" is more appropriate than "identify".
Changed

L514-L516. The arguable hypothesis about "Nonetheless, virus infection itself may be a stimulant for community N2 fixation perhaps by releasing key nutrients (i.e., P or Fe) upon lysis of surrounding microbes" should be further elaborated or at least referenced.

Referenced. See Weitz, J. S., and Wilhelm, S. W.: Ocean viruses and their effects on microbial communities and biogeochemical cycles, F1000 Biol Rep, 4, 17, 2012.

L526-535. Authors conclude that increase of phoA transcripts can be interpreted as an acclimation to low P availability, however, P availability was not measured and other causes are equally plausible as, e.g., general stress, which were not tested.

We have reworded the discussion around nutrient stress specifically P stress – see Section 4.1.2.

L680-L685. Authors mention hypotheses, not conclusions.
Revised

L688-L691. Conclusion should be rewritten to be more accurate. What happens in bottles can it be transferred to what happens in situ? Could a shallow lagoon be comparable to the ocean, are these systems comparable?
Conclusions (section 5) have been rewritten to reflect what our results specifically demonstrate. However, the concluding paragraph also discusses the implications of our research in natural bloom settings. We left this as we feel it is essential for providing the larger picture.

Other typos include: L94 "bloom to crash" instead of "bloom crash" L151 extra "%" symbol L152 "at" should be removed L170 "then" instead of "than" Figure 4, Y-axis label is wrong. It reads "% of 16S tags", but scale is from 0-1. L323 "High respective rates" should be changed to "Relatively high rates". L351 "2 d" should be "2 day" L432 ug is missing between 700 and GX.
Changed.