

Interactive comment on “Programmed cell death in diazotrophs and the fate of organic matter in the Western Tropical South Pacific Ocean during the OUTPACE cruise” by Dina Spungin et al.

Anonymous Referee #2

We would like to thank referee #2 for his insightful comments for clarification and improvement of the manuscript. We have addressed all comments and questions and have revised the manuscript accordingly. Our answers follow the comments in brown.

GENERAL COMMENTS: The manuscript by Spungin et al. Reports on the induction of cell death by nutrient limitation during blooms of the diazotroph *Trichodesmium* (and also diazotrophs associated to diatoms) during a cruise in the South Pacific Ocean. The study analyses whether cell death is a relevant mechanism driving *Trichodesmium* mortality, and whether this contributes to vertical export of organic matter. The aim of this work is to provide evidence of correlation between bloom terminations, cell death and vertical export, with the mediation of specific proteases such as caspase-like and metacaspases. *Trichodesmium* is responsible for roughly half of the nitrogen fixed in the ocean. The study hereby presented focuses on a relevant topic for marine biologists which helps elucidate the impact of cell death of a globally relevant species on the ecosystem, warranting important consequences for the C and N cycles. The paper clearly deserves publication since most of the conclusion are strong.

However, I have several concerns that (in my opinion) need to be addressed by the authors before it can be published.

SPECIFIC COMMENTS:

I have two major concerns. The first, relates to gene expression and activity of metacaspases and caspase like-proteins and their role in a death cascade (initiation and execution of PCD).

As a general comment important to many of the comments below, we would like to note that we have a submitted manuscript in review currently in Environmental Microbiology examining in detail the expression and activity of metacaspases and caspase like proteins and their involvement in PCD in *Trichodesmium* (This manuscript can be sent if requested).

1. The mechanisms by which cell death (CD) (programmed or not programmed) occurs, considering that cell death in phytoplankton leads to the complete demise of the organism/colonies, are always intriguing and there still are many unanswered questions. Among them, which is the proteolytic machinery involved and how it works. Metacaspases, belong to the CD clan of cysteine proteases, were thought to perform similar functions than caspases. It has been shown by multiple labs working with several organisms from yeast, plants and protists that metacaspases are quite distinct in terms of target site specificity from caspases. They target substrate sites are either arginine (R) or lysine (V) at the P1 position. The authors consider this approach right and use a substrate typically hydrolyzed by MCs. I was wondering why this specific (VRPR) substrate was used and no other? and, why in the concentration described, 50mM? Did not the authors test for the optimal substrate concentrations for *Trichodesmium* before the analyses? The reference they give is based on Arabidopsis thaliana assays and that certainly is very different to cyanobacteria. Clarification is needed. Same applies with the caspase like substrate IETD, but in this case, I assume that this has been previously tested according to

Berman-Frank and Bar-Zev former studies.

We chose this specific substrate as recommended in Tsiatsiani et al., 2011 as a fluoregenic substrate with a Arg residues at the P1 position to specifically detect metacaspase activities in cellular extracts. This substrate was experimentally tested with our *Trichodesmium* cultures and was found to suit our purpose. All results and method discussion are currently in review in a paper we have recently submitted to Environmental Microbiology (Spungin et al., in review EM). We used this specific concentration (50 mM) as an equivalent concentration to the IETD used for the determination of caspase-like activity which has been previously shown to be the optimal concentrations on cell extracts (Spungin et al., in review EM). This specific substrate was also checked and calibrated pre-experiments (Spungin et al., in review EM). After calibration, we first applied this method in laboratory experiments under controlled conditions and then in natural samples collected from a bloom in the New Caledonian lagoon. The use of this method during the OUTPACE cruise is after calibration and work on other experiments. To our knowledge we are the first to use specific metacaspase substrates to test direct metacaspase activity in phytoplankton.

2. Caspase -like activities have been reported in vascular plants, phytoplankton, yeast and protozoa. However, their nature is controversial. Up to date, is still not clear, who is the responsible for the observed caspase-like activity in phytoplankton. In vascular plants some authors have pointed to the serine protease family proteins to perform this hydrolysis (see Bonneau et al., 2008) and/or the vacuolar processing enzyme (Hara-Nishimura and Hatsugai, 2011). It has also been reported that some caspase-like activities are attributable to the plant subtilisin-like proteases-saspases and phytaspases (see Vartapetian et al 2011). Hence, clarify this in the text please. **To me the question is:** Since we are measuring these enzymatic activities in phytoplankton's cell free extracts and not in purified proteins result of gene overexpression, we shall be very careful when ascribing the activity to a species. What I mean is: in a cell free extract there are many proteins potentially users of the mentioned substrates. For this reason, I find the use the term "caspase" is not correct, but instead use the term "caspase-like" throughout the whole MS. It is appropriate that the activity must be referred to as "IETDase, etc Therefore, substitute "caspase activity" by "Caspase-like" (or CL). The same applies to metacaspases, and so VRPRase must be used. Otherwise it can lead to confusion.

We completely agree with the reviewer that as *Trichodesmium* does not have true caspases, the correct form throughout should be "caspase-like" and have corrected this throughout the text to caspase-like activity. In the figures and legend, we have changed nomenclature to reflect the specific substrate: i.e. IETDase or VRPRase cleavage (Figures 3, 4, and 5).

3. By the way, revise the nomenclature of the substrate: "Av-VRPR", what group linked to the peptide is Av? Could possible be that Av is in reality Ac?

Our mistake, It is Ac-VRPR. We have corrected it in the manuscript (lines 167, 399,853, 867,882).

4. Along the same thought, the gene expression measurement is very important, but I must say, that does not mean that the enzymatic activity you are measuring corresponds to the expressed gene, if, as said before, that specific activity has not been measured in a purified protein. Hence, caution is needed on this respect when interpreting your data.

We certainly agree. We do measure metacaspases gene expression, but we do not know if the enzymatic activity we are measuring corresponds to the expressed gene. In our previous experiment in the New Caledonian lagoon (Spungin et al., 2016) we measured MC gene expression via metatranscriptomics during different stages of bloom demise. Also in our submitted manuscript (Spungin et al., in review EM) we measured MC gene expression by applying qRT-PCR for both field

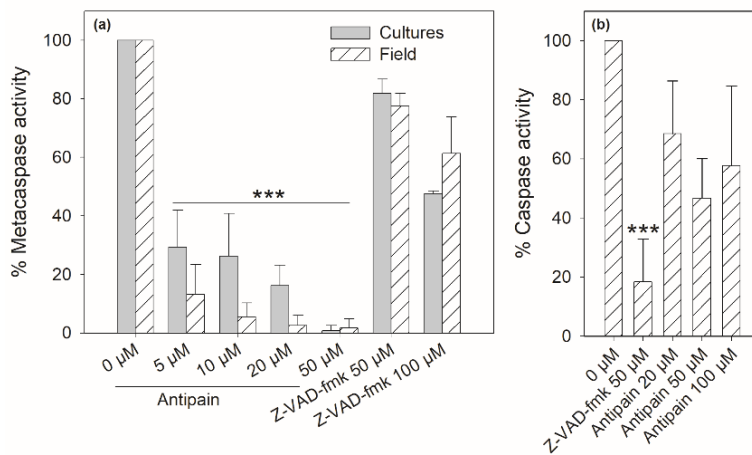
and cultures. We found that MC gene expression is highly elevated during different stages of bloom demise / PCD induction. While we are just beginning to elucidate the roles of the different metacaspases (12 in *Trichodesmium*) we still cannot directly link between expression and activity. Here, in this manuscript we did not specifically examine the MC gene expression as we have previously demonstrated higher expression of metacaspase during bloom demise and PCD induction (Spungin et al. 2016, Bar Zeev et al. 2013). In this study we focused on activity of the metacaspase and caspase-like (as measured by specific substrates) proteins as potential PCD markers. Yet, as the reviewer notes, we do not know what specific protein is responsible for the caspase-like activities and what drivers regulate it, thus it cannot be directly linked to gene expression. We have clarified these points in the text (lines 409-411)

5. Additionally, I think we all must accept that we do not really know if there are initiator or executor CLs or MCs in phytoplankton. Two types of metacaspases (types I and II) are defined based on the presence of a prodomain analogous to the classification of caspases into initiator or executioner caspases. The molecular role of a prodomain in initiator caspases is the recruitment of caspases to multicomponent signaling complexes for caspase activation. However, phytoplankton metacaspases often lack prodomains (Choi and Berges 2013). As I see it, to use this homology can lead into mistake, so I would not describe the enzymes involved as executors of the cell, or initiators of the cascade (although for vascular plants is widely used, it is different, they know exactly which protease which is, and what they do).

Thank you for this valid clarification. We have deleted these suggestions from the text, so we do not define these enzymes as executors or initiators.

6. Last but not least, just would like to know your opinion on this actual heated-debate: Do you think that at the time being caspase-like proteins, in phytoplankton, could hydrolyse R or V?

We have extensively worked on metacaspases vs caspases- like proteins trying to elucidate the differences/ common roles of both in *Trichodesmium* in lab and field extracts (Spungin et al., in review EM). As our experiments find a significant positive correlation between both activities, we have done a series of inhibitor experiments. In vitro treatment with antipain efficiently inhibited metacaspase activity, confirming the arginine-based specificity of *Trichodesmium* metacaspases (see Fig. 1). Our biochemical activity and inhibitor observations demonstrate that metacaspases and caspases-like activities are likely distinct and are independently activated under stress and coupled to PCD in our experiments of both laboratory and field populations. However, caspase-like activity was somewhat sensitive to the metacaspase inhibitor, antipain, showing a ~30-40% drop in activity. This hints at some catalytic crossover between these two catalytic activities in *Trichodesmium* that further should be studied. We have also inserted this issue to the discussion in this manuscript (lines 452-471).



(Fig. 1 Spungin et al., in review EM)

7. The second major concern relates to the fact of bloom/ cell dismissal in the water column.

When working in the field, dead cells are rarely seen at later stages (Berges and Choi 2014) or not seen (Segovia et al., 2018), only because they have been cleared away from the system. Any source of energy that cellular debris may provide to the neighborhood will be immediately used by other species within the food web. So, it is very unlikely to see cellular rests consequence of CD on the water column. Yet, POC downward flux is the way to have some estimates. In my opinion and experience, this can be applied to cultures in the lab under controlled conditions, but I find it truly complicated in natural communities / ecosystem level. Please, clarify how this fits within your sampling/sample analyses time framework. Has that to do with the blooming condition excluding other components of the trophic web of the niche?

The assumption that most dying and dead cells are utilized quickly and recycled within the food web and upper surface layer, may be correct especially in the surface layers of the oligotrophic oceanic regions. Yet, when high biomass blooms occur (as with *Trichodesmium* blooms) the fate of the extensive biomass is more complicated (Bonnet et al., 2015). PCD induced cell death, combined with buoyancy loss, can lead to rapid sinking to depth of the biomass at a speed that would prevent large feeding events on this biomass. This may be determined by POC downward fluxes easy to measure in the lab and extremely complex in the open ocean as you mentioned. We previously measured POC export in our lab under controlled conditions (Bar-Zeev et al., 2013). In this specific experiment however, as mentioned in the text, we had also deployed sediment traps (150, 325, and 500 m depth). In these sediment traps we measured POC fluxes, but also have specific indications (*NifH* reads) of *Trichodesmium* and other diazotrophs which were blooming for several days at the surface. This indicates that under bloom conditions when biomass is high some of the cell pellets do sink down out of the food web. This has also been added and discussed in the text (Lines 593-604).

8. Nothing is said about viruses affecting C losses, which is important for C cycling and definitively affects C export. Viruses were not measured the text says. But in my opinion, this shall at least be discussed and do not directly exclude this possibility as a possible cause for bloom demise. Is there any long-term study done on *Trichodesmium* blooms termination affected by viruses that at least allows you to compare with other situations?

Viruses have been increasingly invoked as key agents terminating phytoplankton blooms. Infection

by phages has been invoked as the mechanism of *Trichodesmium* bloom crashes, (Brown et al., 2013; Hewson et al., 2004; Ohki, 1999) but it has yet to be unequivocally demonstrated in long term *Trichodesmium* blooms. We did study this in a natural bloom of *Trichodesmium* in the new Caledonian lagoon. Virus like particles were measured from samples collected from the bloom during different stages of demise. Enumeration of virus-like particle numbers did not indicate that a massive, phage-induced lytic event of *Trichodesmium* occurred there. This issue was discussed and published in Spungin et al., 2016 Biogeosciences.

As *Trichodesmium* spp. are not grazed by predominant copepods of the water column because of toxins, we believe that PCD and particularly viral lysis may be considerable sources of mortality. Virus infection may also induce PCD in *Trichodesmium*: Virus infection has been shown to increase the cellular production of reactive oxygen species (Vardi et al., 2012), which in turn can stimulate PCD in algal cells (Berman-Frank et al., 2004; Bidle, 2015; Thamtracoln et al., 2012). Viral attack can also directly trigger PCD as part of an antiviral defense system activated to limit virus production and prevent massive viral infection (Bidle, 2015).

We have now mentioned and discussed this further in the text (Lines 376-385).

References

Bar-Zeev, E., Avishay, I., Bidle, K. D., and Berman-Frank, I.: Programmed cell death in the marine cyanobacterium *Trichodesmium* mediates carbon and nitrogen export, The ISME journal, 7, 2340-2348, 2013.

Berman-Frank, I., Bidle, K., Haramaty, L., and Falkowski, P.: The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway, Limnol. Oceanogr., 49, 997-1005, 2004.

Bidle, K. D.: The molecular ecophysiology of programmed cell death in marine phytoplankton, Annual review of marine science, 7, 341-375, 2015.

Bonnet, S., Berthelot, H., Turk-Kubo, K., Fawcett, S., Rahav, E., l'Helguen, S., and Berman-Frank, I.: Dynamics of N₂ fixation and fate of diazotroph-derived nitrogen in a low nutrient low chlorophyll ecosystem: results from the VAHINE mesocosm experiment (New Caledonia), Biogeosciences, 12, 19579-19626, doi:10.5194/bgd-12-19579-2015, 2015.

Brown, J. M., LaBarre, B. A., and Hewson, I.: Characterization of *Trichodesmium*-associated viral communities in the eastern Gulf of Mexico, FEMS Microbiol. Ecol., 84, 603-613, 2013
Hewson, I., Govil, S. R., Capone, D. G., Carpenter, E. J., and Fuhrman, J. A.: Evidence of *Trichodesmium* viral lysis and potential significance for biogeochemical cycling in the oligotrophic ocean, Aquatic Microbial Ecology, 36, 1-8, 2004.

Ohki, K.: A possible role of temperate phage in the regulation of *Trichodesmium* biomass, Bulletin de l'institute oceanographique, Monaco, 19, 287-291, 1999.

Spungin, D., Bidle, K.D and Berman-Frank, I. The roles of Metacaspases in programmed cell death of the marine cyanobacterium *Trichodesmium*. Environmental microbiology, In review.

Spungin, D., Pfreundt, U., Berthelot, H., Bonnet, S., AlRoumi, D., Natale, F., Hess, W. R., Bidle, K. D., and Berman-Frank, I.: Mechanisms of *Trichodesmium* demise within the New Caledonian lagoon during the VAHINE mesocosm experiment, Biogeosciences, 13, 4187-4203, 2016.

Thamtracoln, K., Korenovska, O., Niheu, A. K., and Bidle, K. D.: Whole-genome expression analysis reveals a role for death-related genes in stress acclimation of the diatom *Thalassiosira pseudonana*, Environ. Microbiol., 14, 67-81, 2012.

Tsiatsiani, L., Van Breusegem, F., Gallois, P., Zavialov, A., Lam, E., and Bozhkov, P.: Metacaspases, Cell Death & Differentiation, 18, 1279-1288, 2011.

Vardi, A., Haramaty, L., Van Mooy, B. A., Fredricks, H. F., Kimmance, S. A., Larsen, A., and Bidle, K. D.: Host–virus dynamics and subcellular controls of cell fate in a natural coccolithophore population, *P. Natl. Acad. Sci.*, 109, 19327–19332, 2012.