Marseille, 17/05/2018

Dr Douglas G. Capone
Associated Editor – *Biogeosciences*

Dear Associate Editor,

We are very grateful for the opportunity to revise our manuscript entitled *'Transfer of diazotroph-derived nitrogen to the planktonic food web across gradients of N_2 fixation activity and diversity in the Western Tropical South Pacific'*, for publication in *Biogeosciences*. We followed the suggestions from the reviewers, who we thank for the time and effort devoted to the review of the manuscript. Here we send you responses to the reviewers and the revised version of the manuscript in which we have used the 'track changes' mode with different colour for each reviewer to better assess the progress of the manuscript.
Also, we have added Aude Barani as co-author of the manuscript as she performed the cell sorting by flow cytometry.
We thank you for your attention to our manuscript and we hope that you will find this new version ready for publication in *Biogeosciences*.
Sincerely, and on behalf of all co-authors,

Mathieu Caffin

Response to Anonymous Referee #2

We thank Anonymous Referee #2 for the time and effort devoted to the review of the manuscript. Below, we reproduce the reviewer's comments and address their concerns point by point. The reviewer's comments are copied below in regular font with our responses in red. Manuscript changes are shown with additions in bold, deletions in strikethrough.

In this manuscript Caffin et al. examine transfer of diazotroph-derived (DDN) through the foodweb using 15N stable isotope probing, comparing sites dominated by Trichodesmium with a site dominated by UCYN-B as the dominant diazotroph. They find that over 48h in the UCYN-B dominated station, no DDN was detectable in the dissolved pool, whereas a significant fraction was detectable in the Trichodesmium stations. They further characterize DDN to different microbial and zooplankton groups, and find differences between the stations. These results have major ecological implications for our understanding of DDN fate. Overall, I thoroughly enjoyed the manuscript, and highly recommend it for publication. I do have a few general questions and suggestions regarding the interpretation of the results and the context those results are put in. I recognize that putting these results in context of the other research done on the same cruise is difficult to carve out one piece to focus on, but I think the manuscript could use some focusing.

- Regarding whether Tricho releases recalcitrant N and UCYN-B releases labile N, I'm not sure the data really tells us this. It might mostly be a matter of semantics, and how you define labile and recalcitrant. But for me those terms imply different molecules released by the diazotrophs. From the data I don't think we can rule out that Tricho and UCYN-B release the exact same molecules of N, but because of the difference in both the amount of N released and the composition and metabolic state of the resident community, you see different DDN transfer and efficiency. In fact, I think it's interesting, although maybe expected, that you see higher efficiency in the ultra-oligotrophic location, implying that that maybe that community have higher affinity responses and uptake relative to the resident community in the Tricho stations. Prochlorococcus, for example, is likely to be better at high affinity uptake than Synechococcus because of its smaller surface area to volume ratio and adaptation to oligotrophic environments. Maybe this knowledge could help us predict, by knowing community composition and amount of N fixed, how efficient DDN transfer will be?

We agree with the proposition of reviewer #2, that the differences that we observed in DDN release and transfer between the stations is probably the result of contrasted planktonic communities having different affinities due to the trophic state of the station. We have thus taken account to this proposition and discussed this point in the section '4.1 DDN release to the dissolved pool' which is now presented as one of the hypotheses explaining the discrepancy between the two diazotrophs:

"The quantity and quality of N released by diazotrophs to the dissolved pool during N_2 fixation potentially plays a key role in shaping the planktonic and microbial food webs. In this study, Trichodesmium released 14 ± 4 % to 40 ± 57 % of the newly fixed N into the dissolved pool, which is in agreement with values reported in the literature for field studies (Mulholland, 2007; Bonnet et al., 2016a). DON accounted for ~95 % of the DDN released by Trichodesmium (Fig. 2), ~~*which is in agreement*~~ ***accordingly*** *with contributions measured in culture (80 - 90 %; Berthelot et al., 2015) and in the field (Berthelot et al., 2016). The low contribution of NH_4^+ to the DDN release does not mean that it was not released, but is likely the results of immediate consumption by surrounding plankton,* ***which shows a great affinity for NH_4^+.*** ~~*as NH_4^+ is known to the preferred N source for marine plankton. On the opposite*~~ ***Similarly,*** *part of the DON released by Trichodesmium was probably*

uptaken by heterotrophic and mixotrophic plankton (Bronk et al., 2007) ***but a significant fraction was*** ~~*, and part was*~~ *likely refractory (not easily available for organisms)* ***leading to***~~*, explaining*~~ *the observed accumulation in the dissolved pool. If not refractory, the DON would likely have been immediately assimilated as the region where these experiments were performed are strongly limited by N availability (Van Wambeke et al.,* ***2008;*** *this issue****; Bonnet et al., 2008****).*

In the E1 experiment, we noticed a large variability of N_2 fixation and DDN release rates among the three replicates, which explains the high standard deviations (Fig. 2): two replicates exhibited net N_2 fixation rates ~25-30 nmol N L^{-1} 48 h^{-1} and DDN release rates ~7-10 nmol N L^{-1} 48 h^{-1}, whereas in the third replicate, the DDN release (~24 nmol N L^{-1} 48 h^{-1}) exceeded net N_2 fixation rates (5 nmol N L^{-1} 48 h^{-1}). This can be attributed to the decline of Trichodesmium in this replicate as we counted much more degraded trichomes in the third replicate. This suggests that decaying Trichodesmium release DDN more efficiently than healthy Trichodesmium, which has already been observed by Bonnet et al. (2016a). This may also explain why the DDN transfer to non-diazotrophic plankton was slightly higher in E1 (10 ± 2 %) than in E2 (7 ± 1 %), despite both stations were dominated by Trichodesmium.

Conversely to E1 and E2, *the DDN released by UCYN-B (E3), was not quantifiable in our study. However, significant DDN transfer into non-diazotrophic plankton was detected (15 ± 3 % of the total fixed N, Fig. 4), suggesting that the DDN released to the dissolved pool is likely immediately transferred to surrounding communities.* ~~*Contrary to E1 and E2, DON did not accumulate in the dissolved pool, suggesting either DON is released by UCYN but is more labile than DON released by Trichodesmium, or suggesting that UCYN only release NH_4^+ (which is immediately uptaken and thus does not accumulate as in Trichodesmium experiments).*~~ *To our knowledge, this is the first report of DDN release in the field in the presence of a diazotroph community dominated by UCYN-B. Bonnet et al., (2016b) report low release from UCYN-C in coastal waters of the WTSP (16 ± 6 % of total N_2 fixation) compared to Trichodesmium (13 ± 2 % to 48 ± 5 %; Bonnet et al., 2016b). This seems to indicate that the DDN from UCYN is generally lower than the DDN from Trichodesmium. Several hypotheses may explain the differences observed between Trichodesmium and UCYN.* ~~*i) as stated above, the*~~ *DDN* ***compounds released by*** ~~*from*~~ *UCYN may be more* ***bio****-available than the DDN* ~~*from*~~ ***released by*** *Trichodesmium,* ***limiting its accumulation.*** ~~*therefore it does not accumulate in the dissolved pool*~~ ***The lack of accumulation in E3 could also be due to the more severe N limitation of planktonic communities in the ultra-oligotrophic waters as compared to MA waters (Van Wambeke, this issue), and to the nature of the resident community. Prochlorococcus was dominating the planktonic community at LD C (E3) and is known to have a high affinity to to its small surface to volume ratio (Partensky et al., 1999).*** ~~*ii) the*~~ *PCD causing Trichodesmium bloom demise can* ***also be involved in the relatively high*** ~~*enhance the*~~ *DDN* ***release and accumulation during Trichodesmium dominated experiments*** *(Bar-Zeev et al., 2013).* ~~*iii)*~~ ***Exogenous factors, such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) are also suspected to enhance the DDN release. These factors were found to excert a higher pressure in the*** ~~*the E3 experiment (dominance of UCYN-B) was performed in the ultra-oligotrophic waters of the GY where exogenous factors such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) (which ususally enhance N release) are minimal compared to*~~ *MA waters where* ~~*the*~~ *Trichodesmium* ***dominated compared to ultra-oligotrophic waters*** ~~*experiments were performed*~~ *(Bock et al., this issue)****, where UCYN-B dominated. Finally, part of the discrepancy might be due to a methodological artefact: different sampling procedures between E1 and E2 (pump) and E3 (Niskin bottles) as the pump is suspected to induce mechanical stress to the cells which may have potentially affected the DDN release.*** ~~*Lastly, the DDN release measured here*~~

~~*for UCYN-B is close to the one measured in cultures (1.0 ± 0.3 % to 1.3 ± 0.2 % , Benavides et al., 2013; Berthelot et al., 2015), where the exogenous factors are reduced, which would plead for hypothesis ii)*~~*…"*

> As stated by reviewer #2, this knowledge of the affinity responses of surrounding organisms, by knowing community composition and amount of N fixed, could help us to predict how efficient DDN transfer will be. We agree with this comment and we encourage the scientific community to perform further studies on the point to help understanding and prediction the DDN transfer efficiency.

- One of the points that the authors emphasize is novel is that this is the first open ocean study. But I am not getting the full context for moving to the open ocean-what do the authors expect will be different, other than diazotroph identity? If this is the focus, it would be nice to include an expectation in the introduction–do they expect the open ocean DDN transfer to be different from the other studies of coastal or mesocosms performed before by this group? Or the same? For example, P.4 line 15-what was expected, different or similar to what found for coastal? Also P.4 lines 25-27.

> Our group performed similar studies to understand this DDN transfer in coastal water of the WTSP and measured specific transfer rates to the surrounding planktonic communities, but this study provides the first observation of these processes in the open ocean. Previous studies had shown differences of N release between culture and coastal field experiment suggesting a strong influence of the environment on the DDN release. We thus expected to see differences between coastal and open ocean waters in term of release and subsequently in term of transfer. We understand that reviewer #2 would like to see these expectations in the introduction section, thus we have modified the text in the following way: "***The differences of DDN release and transfer rates observed between the different field experiments and the different diazotrophs suggest that these processes strongly depend on the physiological state of diazotrophs and the environment. Yet,*** ~~*To date*~~ *the transfer of DDN to different groups of plankton from different diazotroph (Trichodesmium vs. UCYN) in the open ocean****, where most of global marine N_2 fixation takes place,*** *has never been investigated*."

Then, I think these experiments help give us a context to predict DDN transfer through the food web, so I would like some more discussion in that context at the end: i.e. Will we need to know both diazotroph identity and nutrient conditions to predict DDN transfer? Or other factors? In some ways focusing on "first time in the open ocean" might actually even sell the results a little bit short-is this maybe the first full food web study in this manner as well?

> We agree with the fact that this study gives us a first estimate of the magnitude of the DDN transfer through the food web in the open ocean. Our long-term goal would be to be able to give solid parametrization for models to predict DDN transfer through the food web. This work has been initiated using a 1-D vertical biogeochemical mechanistic model (Gimenez et al., 2016, https://www.biogeosciences.net/special_issue193.html). However, we think that it might be an overkill to say that we could be able to predict the DDN transfer thanks to our measurements in coupled physical-biogeochemical models. More studies should be performed to have a wider understanding of the processes, in particular the effect of physical processes (not taken into account here), the effect of the physiological state of diazotrophs, the trophic status of the water mass, the plankton community composition, etc…

I also have some specific questions and suggestions:

P.8 lines 1-8-Flow sorting before analysis–I would like more information on this method included, when I looked up the referenced Bonnet et al, 2016b, it didn't include flow sorting-is there another paper with these details? If not, more information should be provided in this manuscript in order to verify that you had what was expected on the filter, and the NanoSIMS analysis was on the expected cells.

> The good reference is Bonnet et al., 2016a. This is a mistake that has been corrected in the new version of the manuscript
>
> Information and protocol about flow sorting are available in the supporting information file of Bonnet et al. (2016a) in the section 'Auto- and heterotrophic picoplankton analysis and sorting by flow cytometry' which is available on
>
> https://aslopubs.onlinelibrary.wiley.com/doi/abs/10.1002/lno.10300 .
>
> We understand that it was not clear in the text, thus we have changed the reference 'Bonnet et al. (2016b)' by 'Bonnet et al. (2016a**, Supp. Info.**).

For example, was there any correlated imaging of the filters (i.e. with fluorescence or SEM) to verify and map the cells other than the CCD camera on the NanoSIMS? It would be good to include some more raw data in supplemental with some examples of the NanoSIMS ion and secondary electron images for each group with examples of how ROIs were drawn. Particularly, it seems like the bacteria may have come through in the other sorts, was that a problem and were those identifiable in the NanoSIMS? Prochlorococcus and bacteria for example, might would look similar in the CCD camera?

> We agree with the reviewer that *Prochlorococcus* and heterotrophic bacteria look very similar in the CCD camera of the nanoSIMS but also on a SEM. This is why we use cell sorting to discriminate the different groups, using in autofluoresecence for photosynthtetic cells and SYBR green staining for heterotrophic bacteria. In the new version of the manuscript, we added the figure below in the Supp. Info. showing representative cytograms where populations appeared clearly and were well clustered. This argues for a potentially low level of cross contamination in out samples, even though it cannot be excluded.

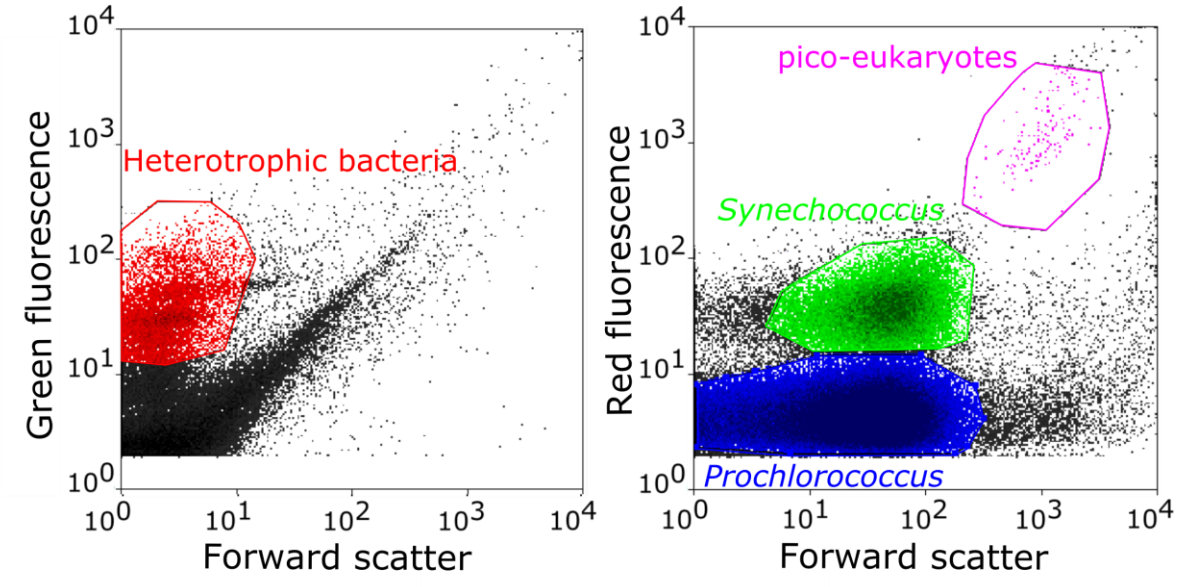

Figure 3: Clustering of planktonic communities by flow cytometry on green fluorescence vs. forward scatter cytograms (left) and red fluorescence vs. forward scatter (right): heterotrophic bacteria (red), *Prochlorococcus* (blue), *Synechococcus* (green), and the pico-eukaryotes (pink)

We agree that it is not clear that how the ROIs were drawn. To clarify this point we have modified the Figure 1 and have added some ROIs on the corresponding images, in the following way.

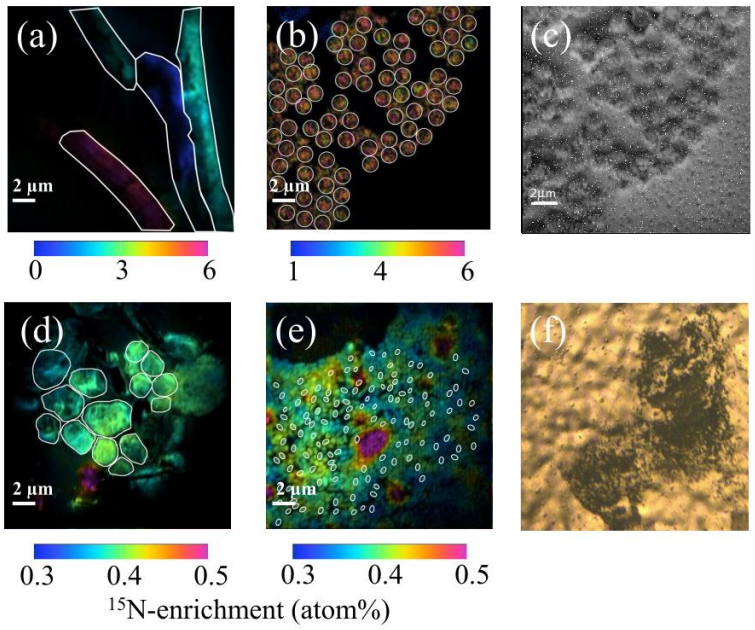

Figure 1: NanoSIMS images showing the ^{15}N-enrichment (a,b,d,e) after 48 h of incubation in the presence of $^{15}N_2$ for *Trichodesmium* (a), UCYN-B (b), Nano-Eukaryotes (d) and *Synechococcus* (e). The ROIs are represented in white line.

NanoSIMS images showing the secondary electrons channel of UCYN ~~(e)~~ (c) and optical camera image of *Prochlorococcus* spotted on the filter before NanoSIMS analyses (f).

In addition we have added complementary nanoSIMS images with ROIs in the Figure 2 of the Supp Info, in the following way:

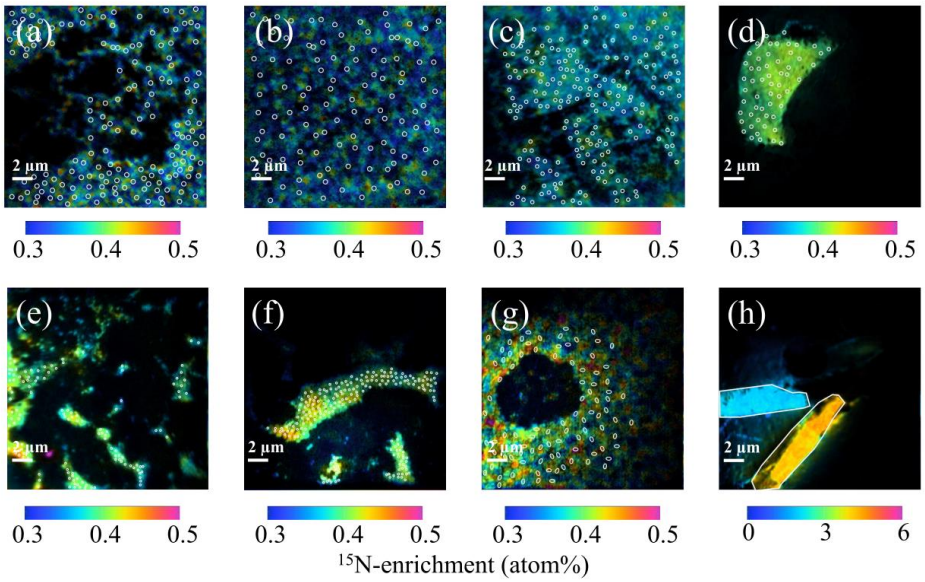

Figure 2: NanoSIMS images showing the ^{15}N-enrichment after 48 h of incubation in the presence of $^{15}N_2$ for *Prochlorococcus* (a,b), pico-eukaryotes (c,d), heterotrophic bacteria (e,f), *Synechococcus* (g), and *Trichodesmium* (h). The ROIs are represented in white line.

P.8 line 24-25, a table of ROIs per sample in supp would help, i.e. n for each analysis

We have added the following table in the Supp. Info. file.

Table 1: Number of ROIs analyzed for diazotrophs (*Trichodesmium* in E1 and E2, UCYN-B in E3), *Synechococcus*, *Prochlorococcus*, bacteria, diatoms, pico-eukaryotes and nano-eukaryotes, for E1, E2 and E3.

Experiment	diazotrophs	*Synechococcus*	*Prochlorococcus*	bacteria	diatoms	pico-euk.	nano-euk.
E1	32	87	32	200	8	111	60
E2	25	156	213	85	33	200	29
E3	192	50	115	70	0	70	0

p.8 line 32-UCYN-B cell diameters from NS images-interesting and not typical-an example in supp would help, was it correlated with other imaging? (i.e. fluor or SEM?).

The UCYN-B cell diameters from nanoSIMS images were 2.3 ± 0.3 µm. Also, UCYN-B cell diameters, measured using a Zeiss Axio Observer epifluorescence microscope were 2.7 ± 0.3 µm. This has been specified in the '2.7 Cell-specific N content and DDN transfer calculations' section in the following way: *"For UCYN-B, cells diameters were directly measured on the nanoSIMS images* ***and further confirmed on microscopic images****."*

P.11 line 5-20 3.3-I couldn't find the information on the T0 values, how many and how analyzed? Everything is relative to the T0 but unclear what the n is.

The T0 values were measured on 5 cells and analyzed on the nanoSIMS, with the same protocol as for all the measurements. T0 here are within the range of T0 values reported by Bonnet et al. (2016a; 2016b) et Berthelot et al. (2016). As the sentence is unclear, we have changed the text in the following way: *"…compared to T0 samples (0.371 ± 0.005 atom%,* ***n=5****), …"*

P.11 Line 15-Sentence "For the three experiments.." -I don't get what this statement means and not sure how it relates to Figure 3

This statement relates to Figure 4 and not Figure 3. We apologize for this mistake which has been corrected in the new version of the manuscript.

P.11 line 26-Again, like the T0, how was the prelabelled plankton measured? NanoSIMS or IRMS? what is the n?

The T0 of the prelabelled plankton were measured by EA-IRMS for the zooplankton experiments. We performed triplicates for each experiment. We have added a new sentence in the Method section to clarify: *"…stop to increase by fixing* $^{15}N_2$. ***The initial*** ^{15}***N enrichment of the*** ^{15}***N pre-labelled plankton was analyzed in triplicates by EA-IRMS.*** *Meanwhile, zooplankton was collected…*". In addition, we have added the number of measurements corresponding to the T0 in the Results section in the following way: "*Before incubation with zooplankton, the isotopic enrichment of the* ^{15}*N pre-labelled plankton averaged 1.035 ± 0.091 atom%* ***(n=9)*** *in the experiments Zoo-1, Zoo-2 and Zoo-3 (dominated by Trichodesmium) and 0.385 ± 0.005 atom%* ***(n=3)*** *in the experiment Zoo-4 (dominated by UCYN-B)."*

P.12 lines 3-4-when the error is bigger than the reported number, I worry this becomes meaningless to report-how else can the data be described?

We acknowledge that the reported error is high, potentially due to the *Trichodesmium* decay in one of the replicate bottle, as mentioned in the discussion section. However, we are confident that the results are still meaningful in regard of the transfer efficiency between diazotroph and non-diazotroph plankton.

P12-Because averaging to T0, lose some information about total N-fixation. Maybe Zoo4 is only different because lower total enrichment?

We averaged T0 of Zoo-1, Zoo-2 and Zoo-3 because there were not significantly different. T0 of Zoo-4 was not averaged with the others experiments because the ^{15}N enrichment was lower as stated by Reviewer #2. As it is unclear, we have modified the text in the following way: *"Before incubation with zooplankton, the isotopic enrichment of the* ^{15}*N pre-labelled plankton* ***was not significantly different in the experiments Zoo-1, Zoo-2 and Zoo-3 (dominated by Trichodesmium)*** *averag****ing*** ~~*ed*~~ *1.035 ± 0.091 atom%* ***(n=9).*** ~~*in the experiments Zoo-1, Zoo-2 and Zoo-3 (dominated by Trichodesmium) and*~~ ***The isotopic enrichment was lower in the experiment Zoo-4 (dominated by UCYN-B) averaging*** *0.385 ± 0.005 atom%* ***(n=3).*** ~~*in the experiment Zoo-4 (dominated by UCYN-B).*~~ *After 24 h of incubation with zooplankton, the* ^{15}*N enrichment of the* ^{15}*N pre-labelled plankton* ***decreased down to 0.431 ± 0.014 atom% on average in Zoo-1, Zoo-2 and Zoo-3, and down to 0.372 ± 0.010 atom% in Zoo-4****."*

P13 line 4-5: but the DDN in the dissolved pool doesn't show release by UCYN-B, the results do imply release because you see DDN transfer but then shouldn't this statement be in the next section?

The DDN measured in the dissolved pool can only come from UCYN-B, and thus show that DDN was released by UCYN-B. However, Reviewer #2 is true when mentioning that seeing DDN transfer implies that DDN was previously released. Thus, we agree that this sentence is suitable in both sections but we think that it makes more sense in this section.

P15 Line 29-Not clear what that 50-95

Here, we mean that diazotrophs contributed from 50 to 95 % to zooplankton biomass in the MA waters. As it is unclear, we have modified the text in the following way: "*This result is in agreement with ~~the ones of Carlotti et al., (this issue) base on natural N isotopic measurements, who revealed~~* ***Carlotti et al. (this issue) results based on ^{15}N isotopic data showing*** *that ~50-95 %* ***and ~10-40 % of the zooplankton N content*** *~~of the zooplankton~~ originates from N_2 fixation in ~~the~~ MA ~~waters~~ and ~~~10-40 % in the~~ GY waters****, respectively****.*"

P16 line 8 "The DDN transfer efficiency was more important..." not sure what is meant by "more important" more important how?

By 'important' we mean 'higher'. The sentence has been clarified in the following way "*The DDN transfer efficiency ~~was~~* ***to non-diazotrophic plankton was higher*** *~~more importantto non-diazotrophic phytoplankton and bacteria (~15 ± 3 %) and zooplankton (~28 ± 8 %)~~ when UCYN-B dominated the diazotroph community than when Trichodesmium dominated ~~(~8 ± 2 % and 7 ± 6 % of transfer to phytoplankton and bacteria, and zooplankton, respectively).~~*"

P.16-last paragraph is a bit confusing and tangential to me. This is just a suggestion, but I would prefer more of a wrap-up on what this data presented means in the context of DDN transfer prediction, e.g. does this help to reconcile the differences between the culture and field studies, or coastal vs. open ocean? What are the implications from the results for predicting transfer through the food web in other areas?

To clarify the conclusion section, we have modified the text in the following way:

"*5. Conclusion and ecological impact* ***of N_2 fixation*** *in the WTSP*

N_2 fixation acts as a natural N fertilizer in the ocean, releasing DDN in the dissolved pool, which is available for surrounding marine organisms. To our knowledge, this study provides the first quantification of DDN transfer to phytoplankton, bacteria and zooplankton communities in open ocean waters. The main interest of this study was to compare DDN transfer and release under contrasting N_2 fixation activity and diversity.

Here, we reveal that Trichodesmium released more DDN than UCYN-B, but a significant part of the DDN released by Trichodesmium ***accumulated in the dissolved pool*** *~~was refractory~~, while the DDN released by UCYN-B was ~~more bio-available (NH_4^+ and labile DON) and likely~~ immediately assimilated by the surrounding plankton communities. The DDN transfer efficiency ~~was~~* ***to non-diazotrophic plankton was higher*** *~~more important to non-diazotrophic phytoplankton and bacteria (~15 ± 3 %) and zooplankton (~28 ± 8 %)~~ when UCYN-B dominated the diazotroph community than when Trichodesmium dominated ~~(~8 ± 2 % and 7 ± 6 % of transfer to phytoplankton and bacteria, and zooplankton, respectively).~~ In the open ocean, most ~~of the~~ N_2 fixation is performed by Trichodesmium (Capone et al., 1997* ***Luo et al., 2012****), thus on a global scale most of the DDN transfer can be attributed to Trichodesmium~~, moreover in the MA waters where Trichodesmium dominated diazotroph community~~. The regions where UCYN are the dominant diazotrophs* ***generally*** *present lower*

N_2 fixation rates than the ones ***where*** ~~*of*~~ *Trichodesmium* ***dominates****, but* ***UCYN provide a continuous source of DDN to surrounding plankton communities*** ~~*it is not negligible and may provide a continuous background of DDN to surrounding plankton communities*~~*. The DDN was* ~~*preferentially*~~ *transferred to pico-plankton, which* ***dominated*** ~~*is the most abundant plankton community in*~~ *the WTSP, suggesting that N_2 fixation fueled the growth of biomass in the N-depleted environment. This is consistent with Caffin et al., (2018), who revealed that N_2 fixation provides >* ~~*more than*~~ *90 % of the new N* ***to*** *the photic layer* ***of the WTSP*** ~~*subsequently transformed into bio-available through DDN release, and indicated that N_2 fixation contributed to 15-21 % of the PP in the MA waters and ~4 % in the GY waters*~~*. On a larger scale view, the simulation performed by Dutheil et al. (this issue) predicts that diazotrophs support a large part of PP (~15 %) in LNLC regions of the Pacific Ocean, comprising the WTSP.*

Overall, this ***study*** ~~*clearly*~~ *indicates that* ~~*in the WTSP the*~~ *N_2 fixation plays a key role on the marine biomass production,* ***the structure of*** ~~*subsequently on*~~ *the planktonic food web* ~~*associated*~~*, and finally on the export* ***of organic matter*** *towards the deep ocean. The DDN can be exported to the deep ocean by different* ***path****ways: i)* ***the*** *direct of export of diazotrophs, ii)* ***the*** *export of non-diazotrophs which benefit****ed*** *from the DDN* ~~*transfer*~~*, and iii)* ***the*** *export of zooplankton which benefit****ed*** *from the DDN* ~~*transfer*~~*. The direct export* ***of diazotrophs accounted for*** ~~*quantification in the WTSP, indicates a direct carbon export associated to diazotrophs of*~~ *~ 30 %* ***of total C export*** *at LD A (E1), 5 % at LD B (E2) and < 0.1 % at LD C (E3) (Caffin et al., 2018).* ***Using a $\delta^{15}N$ budget, Knapp et al., (This issue) found that 50-80 % of exported material was sustained by N_2 fixation (this includes both direct and indirect export of DDN).*** ~~*The low ^{15}N-enrichment of the particulate matter recovered in sediment trap deployed at LD A, LD B and LD C indicates that N_2 fixation significantly contributed to particulate export (Knapp et al., this issue), either by direct or indirect export, in the WTSP.*~~ *Thus, N_2 fixation has ineluctably a key role on the biological carbon pump, as mentioned in Moutin et al. (this issue) who reveal a significant biological "soft tissue" carbon pump in the MA waters* ***almost exclusively*** *sustained* ~~*almost exclusively*~~ *by N_2 fixation, and acting as a net sink* ~~*for*~~ ***of*** *atmospheric CO_2* ***in the WTSP****."*

Figure 1: I think in the figure legend "secondary electrons channel of UCYN (e)" should be (c)? Also, does f correlate with anything? Is there a NanoSIMS image of Prochlorococcus cells?

In the figure legend "secondary electrons channel of UCYN (e)" has been corrected by "secondary electrons channel of UCYN (c)". The image (f) does not correlate with any of the other images. NanoSIMS image of *Prochlorococcus* cells were added in the Supp Info as explained above.

Figure 4: The left pie charts numbers I think should correspond to P.11 lines 19-20 numbers-but they don't-how much N stays with the diazotrophs? Is it 50, 79 and 85

Reviewer #2 is right pie charts numbers did not corresponded to P.11 lines 19-20 numbers. This is a mistake that has been corrected in the new version of the manuscript. The right numbers are 50 ± 40 %, 79 ± 4 % and 85 ± 9 %.

Technical corrections:

P3 Line 15-16-this sentence is confusing to me, lower than what? In the field?

We agree that this sentence is confusing. The DDN release measured in culture studies is much lower than in field studies. We have clarified this sentence in the following way: "***The DDN release is generally much lower in (<5%) in monospecific cultures (Berthelot et al. 2015, Benavides et al. 2013) than in field experiment*** ~~*The DDN released to the dissolved pool measured by this direct approach is generally much lower in culture studies (<5 %) (Benavides et al., 2013a; Berthelot et al., 2016),*~~ *suggesting that external factors such as sloppy feeding and viral lysis have a strong influence on the DDN release by diazotrophs in field.*"

P9 line 21-22 after "Plus an additional..." add "Zoo-2", if that is what that experiment is, confusing.

To avoid confusion we have had "Zoo-2" in the sentence, as recommended by Reviewer #2, in the following way: *"…plus an additional station* ***(Zoo-2)*** *located between LDA and LDB…*"

Response to Carolin Löscher - Referee #1

We thank Carolin Löscher for the time and effort devoted to the review of the manuscript. Below, we address her concerns point by point. The reviewer's comments are copied below in regular font with our responses in blue. Manuscript changes are shown with additions in bold, deletions in strikethrough.

The manuscript by Caffin et al. addresses the important question on how much fixed N is transferred to the dissolved versus the particulate planktonic pool. Caffin et al come up with a nanoSIMS based study to not only make this distinction, but to also show that the composition of the diazotrophic community has an impact on the subsequent channeling of N in the Ocean, and they could identify that Trichodesmium promotes a transfer to the dissolved phase, while UCYN-B would promote transfer to non-diazotrophic plankton (mostly picocyanobacteria, followed by heterotrophs). Intriguingly, a higher share of the N pool was transferred to higher trophic levels when Trichodesmium dominated, however, an overall high transfer efficiency was observed in UCYN-B dominated environments. The manuscript is, to my knowledge, one of the first to address the channeling of N through the food web, with that it critically advances the understanding of N2 fixation in the Ocean. I thus highly recommend publication after addressing the following general and specific recommendations.

General comments:

Overall, the manuscript seems to need a bit of streamlining. I see, this is not an easy job to do and I appreciate the thorough introduction and methodological explanations, as well as the detailed description of the results. However, it seems a bit of an overkill given the obvious key results of the two modes of DDN channeling and its subsequent transfer to higher trophic levels. I recommend to reduce the length of the text in order not to dilute your findings.

We understand that the length of the text can dilute the findings and have thus followed the recommendation of C. Löscher to streamline our manuscript.

Concerning the introduction, as far as we are aware, there are few studies that address the release and fate of DDN in the ocean and we believe it is important to give a detailed state of the art on this topic to introduce the main goals of the present study.

Regarding the Methods section, we reduced the text as much as possible by referring to articles already published (mainly in the same special issue). We changed the text as follows:.

"

2.2 Net N_2 fixation rates ***and DDN released to the dissolved pool***

N_2 fixation rates were measured using the $^{15}N_2$ isotopic tracer technique (adapted from Montoya et al. (1996)) ***, as described in Bonnet et al. (this issue)***. ~~*The $^{15}N_2$ bubble technique was intentionally chosen to avoid any potential overestimation due trace metal and dissolved organic matter (DOM) contaminations often associated with the preparation of the $^{15}N_2$-enriched seawater (Klawonn et al., 2015; Wilson et al., 2015) in our incubation bottles as Fe and DOM have been seen to control N_2 fixation or nifH gene expression in this region (Benavides et al., 2017; Moisander et al., 2012). However, the ^{15}N enrichment of the N_2 pool available for N_2 fixation was measured in all incubation bottles to ensure accurate rate calculations. Briefly, 12 mL were subsampled after incubation into Exetainers® fixed with $HgCl_2$ (final concentration 20 mg L^{-1}) that were preserved upside down in the dark at 4°C*~~

~~*until analyzed using a membrane inlet mass spectrometer (MIMS) according to Kana et al. (1994).*~~

~~*At the end of incubations, 2.3 L of the triplicates 4.5 L amended bottles were gently filtered onto pre-combusted (450 °C, 4 h) Whatman GF/F filters (25 mm diameter, 0.7 µm nominal porosity). Filters were stored in pre-combusted glass vials at -20 °C during the cruise, and then dried at 60 °C for 24 h before analysis onshore.* ^{15}*N-enrichments of particulate N collected on filters were determined using an Elemental Analyzer coupled to an Isotope Ratio Mass Spectrometer (EA-IRMS, Integra2 Sercon Ltd).* *The accuracy of the EA-IRMS system was systematically controlled using International Atomic Energy Agency (IAEA) reference materials, AIEA-N-1 and IAEA-310A. In addition, the* ^{15}*N-enrichement of the ambient (unlabeled) particulate N was measured at each station at T0 and was used as the "initial"* ^{15}*N-enrichment as termed in Montoya et al. (1996).*~~

~~*2.3 DDN released to the dissolved pool*~~

The DDN released to the dissolved pool under the form of NH_4^+ *and DON during the* N_2 *fixation process was quantified using the three step diffusion method extensively described in Berthelot et al. (2015) and derived from Slawyk and Raimbault (1995).* ~~*This method enables the differentiation of the nitrate (*~~NO_3^-~~*), *~~NH_4^+~~* and DON pools, and measured their respective* ^{15}~~*N-enrichment.*~~ *As the* NO_3^- *pool was negligible, the total dissolved N (TDN) pool was defined as the sum of DON and* NH_4^+ *pools (TDN = DON +* NH_4^+*). After incubation with the* $^{15}N_2$ *tracer, 300 mL of the filtrate passed through pre-combusted Whatman GF/F filters were collected in 500 mL Duran Schott borosilicate flasks, poisoned with* $HgCl_2$ *(300 µL, final concentration 20 mg* L^{-1}*) and stored at 4°C in the dark until analysis. At the end of each step,* NH_4^+ *and DON fraction were recovered on acidified pre-combusted Whatman GF/F filters, dried 24 h at 60 °C and stored in pre-combusted glass tubes until analysis on* ~~*EA-IRMS*~~ ***an Elemental Analyzer coupled to Isotope Ratio Mass Spectrometer (EA-IRMS, Integra2 Sercon Ltd)*** *as described in Berthelot et al., (2015).* ~~*The DDN release was the sum of all forms of N released in each fraction.*~~

~~*2.4*~~***2.3*** *Inorganic and organic nutrient analyses*

NH_4^+ *concentrations were measured fluorimetrically according to Holmes et al. (1999) on a FP-2020 fluorimeter (Jasco, detection limit = 3 nM).* NO_3^- *and nitrite (*NO_2^-*) concentrations were measured using standard colorimetric procedures (Aminot and Kéroul, 2007) on a AA3 AutoAnalyzer (Seal-Analytical). DON concentrations were measured by the wet oxidation method according to Pujo-Pay and Raimbault (1994).* ~~*After the wet oxidation, the concentration of TDN was measured on a AA3 AutoAnalyzer (Seal-Analytical). DON concentrations were obtained by difference between TDN and Dissolved Inorganic N (*~~NH_4^+~~ *+* ~~NO_3^-~~ *+* ~~NO_2^-~~*) measured in parallel.*~~

~~*2.5*~~***2.4*** *Plankton abundance determination*

The abundance of Trichodesmium and UCYN-B was determined microscopically: 2.2 L of each triplicates $^{15}N_2$*-amended 4.5 L bottles were gently filtered onto 2 µm pore size, 25 mm diameter Millipore polycarbonate filter, fixed with paraformaldehyde (2 % final concentration) for 1 h. Trichodesmium were enumerated on the entire surface of the filter at x100 magnification with a Zeiss Axio Observer epifluorescence microscope fitted with a green (510-560 nm) excitation filter. The number of cells per trichomes was counted on 20 trichomes*

for each experiment; we counted an average of 85 and 115 cells trichome^{-1} for E1 and E2, respectively. UCYN-B were counted on 40 fields (1.3 mm^2 fields; 0-2800 UCYN-B per field) scanned and analyzed with the ImageJ1 software.

Samples for micro-phytoplankton identification and enumeration were collected in each of the triplicate 4.5 L incubated bottles (except for E1 were only one replicate was available) in five 50 mL sterile ***polypropylene*** ~~*PP*~~ *tubes and preserved in acidic Lugol's solution (0.5 % final concentration). Diatoms were enumerated from a 250 mL subsample following the Utermohl method (Hasle, 1978), using a Nikon TE2000 inverted microscope equipped with phase-contrast and a long distance condenser. Diatoms were identified to the lowest possible taxonomic level in one of the three replicates.*

Pico, nano-phytoplankton and heterotrophic bacteria abundances were determined by flow cytometry. After incubation, 1.8 mL were subsample from triplicate 4.5 L bottles into cryotubes, fixed with paraformaldehyde (200 µL, 4 % final concentration) ***for 5 min at room temperature****, flash-frozen in liquid N_2, and stored at -80 °C until analysis on a FACSCalibur (BD Biosciences, San Jose, CA) according to Marie et al. (1999), at the PRECYM flow cytometry platform (https://precym.mio.univ-amu.fr/).* ~~*Before analysis, samples were thawed at room temperature in the dark. For bacterial abundance, 300 µL of each sample was incubated with SYBR Green II (Molecular Probes, 0.5 % final concentration) for 15 min in the dark. For phytoplankton and bacterial abundances measurements, before analysis, 2 µm beads (Fluoresbryte, Polysciences) and Trucount beads (BD Biosciences) were added to the samples. Analyses were run during 1.5 and 3 min at high and medium flow for phytoplankton and bacteria, respectively. The 2 µm beads were used as an internal control and to discriminate picophytoplankton (< 2 µm) from nanophytoplankton (> 2 µm) populations.*~~ *Phytoplankton communities were clustered as Prochlorococcus spp. cell like, Synechococcus spp. cell like, nano-eukaryotes cell like, pico-eukaryotes cell like, and UCYN-B cell like.* ~~*The*~~ *Truecount beads were used to determine the volume analyzed.* ~~*All data were acquired using the CellQuest software (BD Biosciences), and data analysis was performed using the SUMMIT v4.3 software (Dako).*~~

~~*2.7*~~***2.6*** *NanoSIMS analyses*

Just before nanoSIMS analyses, filters were thawed at ambient temperature and sputtered with gold and palladium to ensure conductivity. Analyses were performed on a NanoSIMS N50 (Cameca, Gennevilliers, France) at the French National Ion MicroProbe Facility as previsouly described in Bonnet et al. (2016a, 2016b) and Berthelot et al. (2016). Briefly, high density cells area were retrieved using the nanoSIMS optical camera (Fig.1 f.). Samples were pre-sputtered ~~*with*~~ *prior to analyses for at least 2 min to remove surface contaminants and increase conductivity with a ~22 pA Cesium primary beam. For the analysis, a ~1.2 pA Cesium (16 KeV) primary beam focused onto ~100 nm spot diameter was scanned across a 256×256 or 512×512 pixel raster (depending on the image size, which ranged from 20 µm × 20 µm to 40 µm × 40 µm) with a counting time of 1 ms per pixel. Negative secondary ions (*$^{12}C^-$***,** $^{13}C^-$***,*** $^{12}C^{14}N^-$***,*** $^{12}C^{15}N^-$ ***and*** $^{28}Si^-$*) were collected by electron multiplier detectors, and secondary electrons were imaged simultaneously. A total of 20 serial quantitative secondary ion images were generated to create the final image.* ~~*Mass resolving power was ~8000 in order to resolve isobaric interferences. Data were processed using the LIMAGE software. Briefly, all scans were corrected for any drift of the beam and sample stage during acquisition. Isotope ratio images were created by adding the secondary ion counts for each*~~

*~~recorded secondary ion for each pixel over all recorded planes and dividing the total counts by the total counts of a selected reference mass.~~ Individual cells were easily identified in nanoSIMS secondary electron, $^{12}C^-$, $^{12}C^{14}N^-$ **and** $^{28}Si^-$ images that were used to define regions of interest (ROIs) around individual cells. A total of ~1500 ROI**s** was analyzed. For each ROI, the ^{15}N-enrichment was calculated. ~~50 to 200 cells~~ **~80 cells on average** were analyzed for each plankton group and for each experiment **(see Table 1 SI)**.*

*~~2.9~~ **2.8** Experimental setup for DDN transfer experiments in zooplankton*

*The incubation was stopped by filtering the bottles on 0.2 µm pore size 47 mm membrane filters **in such a way that the ^{15}N enrichment of the food source provided to zooplankton (hereafter referred to as ^{15}N pre-labelled plankton) does not increase during the course of the experiment. For each experiment, the initial ^{15}N enrichment of the ^{15}N pre-labelled plankton was analyzed in triplicates by EA-IRMS. Plankton was then*** ~~and~~ *re-suspend**~~ed ing the particulate matter~~** in 6 1 L bottles filled with 0.2 µm filtered surface seawater collected at the same station~~, in such a way that the ^{15}N enrichment of the food source provided to zooplankton (hereafter referred to as ^{15}N pre-labelled plankton) stop to increase by fixing $^{15}N_2$~~. Meanwhile,…'*

In the context of the discussion of DDN transferred to zooplankton, either directly or indirectly, I would like to see a link to export production, which may be extremely important in the context of enhanced CO2 uptake through certain ecosystem compositions.

We agree with this comment, and thus we have added this paragraph at the end of the section *"**Zooplankton can contribute to organic matter export by production of sinking fecal pellets, active transport to depth and carcasses export. These processes are increasingly recognized as important vectors of organic matter export, and the magnitude of their contributions to organic matter export are highly dependent on regionally variable plankton community structure (Steinberg and Landry, 2017). In the WTSP, where N_2 fixation sustains most of the new primary production (Caffin et al., 2018) and an important fraction of the DDN is transferred to zooplankton, it might play a key role on the export production and hence the CO_2 sink which is the WTSP**."*

I am a bit worried about two things: first, some share of what you measured may be an artefact due to Trichodesmium's sensitivity to mechanical stress, second, samples were taken using two different methods, i.e. from Niskin bottles and from a pump system, the latter of which is suspected to disrupt cells. Please address those concerns.

The underway surface pump is large volume pumping system with large tubing and we paid careful attention to set the final spigot to a gentle flow rate. We previously checked through microscopic observations that fragile cells like diatoms or *Trichodesmium* colonies were not destroyed by this sampling strategy. However, we cannot explain why one of the replicate of the E1 experiment was so different from the others and cannot exclude a potential bias during the sampling with the pump, although from our experience such discrepancies between replicates have already been observed after sampling with Niskin bottles in *Trichodesmium* blooms. We have acknowledged that in the discussion section (see below). Another

explanation could be related to the spatial distribution of *Trichodesmium*, which is very patchy in the ocean during blooms. We may have sampled different populations in different physiological states in different bottles, which may explain the discrepancy between replicates in the E1 experiment.

We have addressed these concerns in the discussion sections, subsection '4.1 DDN release to the dissolved pool' and modified the text in the following way:

" ***Conversely to E1 and E2,*** *the DDN released by UCYN-B (E3), was not quantifiable in our study. However, significant DDN transfer into non-diazotrophic plankton was detected (15 ± 3 % of the total fixed N, Fig. 4), suggesting that the DDN released to the dissolved pool is likely immediately transferred to surrounding communities.* ~~*Contrary to E1 and E2, DON did not accumulate in the dissolved pool, suggesting either DON is released by UCYN but is more labile than DON released by Trichodesmium, or suggesting that UCYN only release* NH_4^+ *(which is immediately uptaken and thus does not accumulate as in Trichodesmium experiments).*~~ *To our knowledge, this is the first report of DDN release in the field in the presence of a diazotroph community dominated by UCYN-B. Bonnet et al., (2016b) report low release from UCYN-C in coastal waters of the WTSP (16 ± 6 % of total* N_2 *fixation) compared to Trichodesmium (13 ± 2 % to 48 ± 5 %; Bonnet et al., 2016b). This seems to indicate that the DDN from UCYN is generally lower than the DDN from Trichodesmium. Several hypotheses may explain the differences observed between Trichodesmium and UCYN.* ~~*i) as stated above, the*~~ *DDN* ***compounds released by*** ~~*from*~~ *UCYN may be more* ***bio****-available than the DDN* ~~*from*~~ ***released by*** *Trichodesmium,* ***limiting its accumulation.*** ~~*therefore it does not accumulate in the dissolved pool*~~ ***The lack of accumulation in E3 could also be due to the more severe N limitation of planktonic communities in the ultra-oligotrophic waters as compared to MA waters (Van Wambeke, this issue), and to the nature of the resident community. Prochlorococcus was dominating the planktonic community at LD C (E3) and is known to have a high affinity to to its small surface to volume ratio (Partenski et al., 1999).*** ~~*ii) the*~~ *PCD causing Trichodesmium bloom demise can* ***also be involved in the relatively high*** ~~*enhance the*~~ *DDN* ***release and accumulation during Trichodesmium dominated experiments*** *(Bar-Zeev et al., 2013).* ~~***iii)***~~ ***Exogenous factors, such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) are also suspected to enhance the DDN release. These factors were found to excert a higher pressure in the*** ~~*the E3 experiment (dominance of UCYN-B) was performed in the ultra-oligotrophic waters of the GY where exogenous factors such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) (which ususally enhance N release) are minimal compared to*~~ *MA waters where* ~~*the*~~ *Trichodesmium* ***dominated compared to ultra-oligotrophic waters*** ~~*experiments were performed*~~ *(Bock et al., this issue)****, where UCYN-B dominated. Finally, part of the discrepancy might be due to a methodological artefact: different sampling procedures between E1 and E2 (pump) and E3 (Niskin bottles) as the pump is suspected to induce mechanical stress to the cells which may have potentially affected the DDN release.*** ~~*Lastly, the DDN release measured here for UCYN-B is close to the one measured in cultures (1.0 ± 0.3 % to 1.3 ± 0.2 % , Benavides et al., 2013; Berthelot et al., 2015), where the exogenous factors are reduced, which would plead for hypothesis iii.*~~

The DDN release plays a key role …"

Specific comments:

p.1

l. 15: What do you mean with atmospheric- I assume dust input? In a way N2 fixation is atmospheric.

> In this sentence 'atmospheric' refers to atmospheric deposition. To avoid any confusion we have clarified the sentence in the following way: *"Biological dinitrogen (N_2) fixation provides the major source of new nitrogen (N) to the open ocean, contributing more than atmospheric* ***deposition*** *and riverine inputs to the N supply."*

l.16: Which technical limitations- such as tracing the isotope fractionation? That's possible at least to a certain degree

> Here, by 'technical limitation' we meant isotope tracking in the different planktonic groups. This technical limitation has been unlocked by the use of nanoSIMS method coupled to cell identification (in situ hybridization or flow cytometry). Nevertheless, the fate of DDN in the planktonic food web is still poorly understood and motivated this study. In order to clarify and keep the abstract concise, we have removed "due to technical limitations".

l. 25: this is somewhat difficult to understand as it seems contradictory to the previous sentences. Please clarify that you are referring to the pool that is transferred to plankton

> We acknowledge that is unclear and seems contradictory to the previous sentences, thus we have removed the sentence.

l. 30: Please add an explanation, here, otherwise it seems contradictory to the previous statements

> We understand that this sentence seems contradictory to the previous statements, in fact we made a mistake in the sentence as we wrote 'more' instead of 'less'. We apologize for this mistake and thus we have corrected the sentence in the following way: *" Regarding higher trophic level, the DDN transfer to the dominant zooplankton species was* ~~*more*~~ ***less*** *efficient when the diazotroph community was dominated by Trichodesmium (~5-9 % of the DDN transfer) than when it was dominated by UCYN-B (~28 ± 13 % of the DDN transfer)."*
>
> In addition, to be clearer, we have modified the previous sentence in the following way:

p.2

l.9: Add the study by Duce et al, 2008.

> We have added this reference in the new version of the manuscript. In addition, we have specified that 'atmospheric input' in this sentence refers to 'atmospheric deposition' as mentioned in a comment above. Thus, the sentence has been modified in the following way: *"At the global scale, N_2 fixation is the major source of new N to the ocean, before atmospheric* ***deposition*** *and riverine inputs (100-150 Tg N yr^{-1},* ***Duce et al., 2008;*** *Gruber, 2008)."*

l. 14: I identified some archaea being important in the Pacific, feel free to add the reference (or even not, Löscher et al, 2014 in ISMEj)

> We have mentioned the archaea in the new version of the manuscript and added the reference Löscher et al (2014) in the following way: *"N_2 fixation is performed by prokaryotic organisms termed diazotrophs, which include the non-heterocystous filamentous cyanobacterium*

Trichodesmium […], unicellular cyanobacteria termed UCYN […], ~~and~~ diverse non-cyanobacterial bacteria […], ***and archaea (Löscher et al., 2014)****."*

l. 31 N2, 2 has to be in subscript

This has been corrected in the new version.

p.4

l.16, l.21: 15N, 15 in upper case

This has been corrected in the new version.

l. 20: Why would Trichodesmium be toxic?

The studies that we referred to in the manuscript mentioned intracellular toxins and toxic compounds into these cyanobacteria that can affect zooplankton.

p. 5

l. 15 onwards is largely the exact same text as in 'In depth characterization of diazotroph activity across the Western Tropical South Pacific hot spot of N2 fixation' by Bonnet et al. As there is no point to repeat that, I would recommend to refer to this manuscript instead of having such a strong overlap.

We agree with this comment and the first general comment above that our manuscript needed a bit of streamlining. Thus we have reduced the length of the Methods section and, here in this 'Net N_2 fixation rates' section, we have referred to Bonnet et al. (this issue).

p.8

l.17, l.23, p.9, l.11: please mind the upper and lower cases

This has been corrected in the new version.

p.10

l.28: I would like to see the rates as per day

Our experiments were performed on a 48 h time scale and we performed a N budget to determine where does the fixed N goes after 48 h. This explains why we present N_2 fixation rates over 48 h. At all stations, we also measured them after 24 h, and could present them as well, but we should probably discuss why rates at 48 h are not the double of those after 24 h (mortality or growth of diazotrophes, equilibration of the $^{15}N_2$ bubble, etc), which is not the scope of this study. Therefore, to be consistent with the other measurements (nanoSIMS data, release data, etc…) that were presented for 48 h of incubation and keep focus on the main scientific question of this study, we believe that it is more appropriate to present N_2 fixation rates per 48 h.

p.11

l.1 under the form of DON- sounds awkward, please rephrase

We rephrased the sentence as *"**DON accounted for the major part of the ^{15}N released and accounted for ~93 and ~96 % of the total N release in E1 and E2, respectively."***

l.17 Sentence sounds awkward, please rephrase

> We agree with this comment, thus we have splitted the sentence for more clarity in the following way: *"~~For the three experiments,~~ DDN was mainly transferred to ~~Synechococcus, Prochlorococcus and bacteria in the three experiments and contributed approximately to 98, 92 and 99 % of the transfer in E1, E2 and E3, respectively (Fig. 3)~~ **~~(Fig. 4)~~**. ~~The major part of the transfer took place in~~ pico-**cyanobacteria** ~~phytoplankton~~ (Synechococcus and Prochlorococcus), accounting for 73 ± 15 %, 68 ± 14 % and 65 ± 13 % of the total transfer into non-diazotrophs in E1, E2 and E3, respectively **(Fig. 4).** ~~followed by~~ **The transfer into heterotrophic** bacteria **accounted for** ~~(~~25 ± 5 %, 23 ± 5 % and 34 ± 7 % **of the total transfer**, **in E1, E2 and E3,** respectively ~~(Fig. 4)~~."*

l. 19 What bacteria? I assume, non-phototrophic ones. . .please clarify.

> This is heterotrophic bacteria. This has been clarified in this sentence (see response above).

l. 29 down to what?

> We have specified the final ^{15}N enrichment in the new version. Thus we have modified the sentence in the following way: *"After 24 h of incubation with zooplankton, the ^{15}N enrichment of the ^{15}N pre-labelled plankton **decreased down to 0.431 ± 0.014 atom% on average in Zoo-1, Zoo-2 and Zoo-3, and down to 0.372 ± 0.010 atom% in Zoo-4**."*

p.11

l.7: I don't quite get this conclusion.

> Here we provide the averaged cell-specific N_2 fixation rates for *Trichodesmium* in E1 and E2, and UCYN-B in E3. These rates were calculated as:
>
> Cell-specific N_2 fixation rates = ($^{15}N_{ex}$ x N_{cont}) / N_{sr}
>
> Where $^{15}N_{ex}$ is the cell-specific ^{15}N enrichment, N_{cont} is the cell-specific N content and N_{sr} is the ^{15}N enrichment of the source pool (N_2) in the bottles.

p.12

l.5 + in upper case

> This has been corrected in the new version.

l.27 This is actually worrying, thus all of it may be an effect of how Trichodesmium is treated during the experiments

> We are aware that *Trichodesmium* can be affected by the conditions of the experiments which are discussed in the manuscript. The discrepancy can results from PCD which appears to be highly stochastic and is hard to foresee.

Transfer of diazotroph-derived nitrogen to the planktonic food web across gradients of N_2 fixation activity and diversity in the Western Tropical South Pacific

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Abstract.

Biological dinitrogen (N_2) fixation provides the major source of new nitrogen (N) to the open ocean, contributing more than atmospheric **deposition** and riverine inputs to the N supply. Yet the fate of the diazotroph-derived N (DDN) in the planktonic food web is poorly understood ~~due to technical limitations~~. The main goals of this study were to i) quantify how much of DDN is released to the dissolved pool during N_2 fixation and how much is transferred to bacteria, phytoplankton and zooplankton, ii) to compare the DDN release and transfer efficiencies under contrasting N_2 fixation activity and diversity in the oligotrophic waters of the Western Tropical South Pacific (WTSP) Ocean. We used nanometer scale secondary ion mass spectrometry (nanoSIMS) coupled with $^{15}N_2$ isotopic labelling and flow cytometry cell sorting to track the DDN transfer to plankton, in regions were the diazotroph community was either dominated by *Trichodesmium* or by UCYN-B. After 48 h, ~20-40 % of the N_2 fixed during the experiment was released to the dissolved pool when *Trichodesmium* dominated, while the DDN release was not quantifiable when UCYN-B dominated. ~7-15 % of the total fixed N (net N_2 fixation + release) was transferred to non-diazotrophic plankton within 48 h, with higher transfer efficiencies (15 ± 3 %) when UCYN-B dominated as compared to when *Trichodesmium* dominated (9 ± 3 %). ~~Most of the DDN (>90 %) was transferred to picoplankton (*Synechococcus*, *Prochlorococcus* and bacteria in all experiments.~~ The **pico**-cyanobacteria *Synechococcus* and *Prochlorococcus* were the primary beneficiaries ~~(~65-70 %~~ of the DDN ~~transfer~~ **transferred (~65-70%)**, followed by heterotrophic bacteria (~23-34 %~~ of the DDN transfer~~). The DDN transfer in bacteria was ~~the highest~~ **higher** (34 ± 7 %) **in ~~when~~ the** UCYN-B~~ were~~ dominating ~~**the diazotroph community**~~ **experiment compared to the *Trichodesmium*-dominating experiments (24 ± 5 %)**. Regarding higher trophic level**s**, the DDN transfer to the dominant zooplankton species was ~~more~~ **less** efficient when the diazotroph community was dominated by *Trichodesmium* (~5-9 % of the DDN transfer) than when it was dominated by UCYN-B (~28 ± 13 % of the DDN transfer). To our knowledge, this study provides the first quantification of DDN release and transfer to phytoplankton, bacteria and zooplankton communities in

open ocean waters. It reveals that despite UCYN-B fix N_2 at lower rates compared to *Trichodesmium* in the WTSP, the DDN from UCYN-B is much more available and efficiently transferred to the planktonic food web than the DDN ~~coming~~ **originating** from *Trichodesmium*.

1 Introduction

Nitrogen (N) is one of the basic building blocks of life, though much of the global Ocean surface (~70 %) is oligotrophic and characterized by low N availability, which limits primary productivity and phytoplankton growth (Falkowski, 1997; Moore et al., 2013). In these N-depleted areas of the tropical and subtropical ocean, biological dinitrogen (N_2) fixation (the reduction of atmospheric N_2 into bioavailable ammonia) sustains the major part of new production and organic matter export (Bonnet et al., 2009; Caffin et al., 2018; Capone et al., 2005; Karl et al., 2012). At the global scale, N_2 fixation is the major source of new N to the ocean, before atmospheric **deposition** and riverine inputs (100-150 Tg N yr^{-1}, **Duce et al., 2008;** Gruber, 2008). N_2 fixation is performed by prokaryotic organisms termed diazotrophs, which include the non-heterocystous filamentous cyanobacterium *Trichodesmium* (Capone et al., 1997; Carpenter, 1983), heterocystous cyanobacteria living in symbiosis with diatoms (or diatom-diazotroph associations, termed DDAs; Villareal, 1994), unicellular cyanobacteria termed UCYN (subdivided in Group A, B and C based on the *nifH* gene sequence, Zehr et al., 1998, 2008; Zehr and Turner, 2001) , ~~and~~ diverse non-cyanobacterial bacteria (Bombar et al., 2015; Moisander et al., 2014; Riemann et al., 2010)**, and archaea (Löscher et al., 2014)**. Although considerable efforts have been deployed over the past decades to quantify N_2 fixation, identify the major players, and assess their biogeographical distribution in relation with environmental drivers, the fate of new N provided by N_2 fixation in the ocean, its role on the planktonic food web structure and large-scale biogeochemical fluxes is still poorly understood.

Early studies have reported high dissolved organic N (DON) and ammonia (NH_4^+) concentrations during and following *Trichodesmium* blooms in the Indian Ocean (Devassy et al., 1978, 1979; Glibert and O'Neil, 1999), suggesting that *Trichodesmium* release part of the recently fixed N_2 (hereafter referred to as diazotroph-derived N, DDN) to the dissolved pool, which could subsequently be consumed by the surrounding plankton communities. The first direct release measurements were performed in the early 1990s and showed that *Trichodesmium* colonies isolated from the tropical Atlantic Ocean release ~50 % of the recently fixed N_2 (Glibert and Bronk, 1994). Accumulations of DON and NH_4^+ have subsequently been confirmed near *Trichodesmium* blooms in the Pacific (Karl et al., 1992, 1997) and Atlantic Oceans (Lenes et al., 2001), although not systematic (Bonnet et al., 2016a; Hansell and Carlson, 2001), and in senescent *Trichodesmium* cultures (Mulholland and Capone, 2000), possibly related to the *Trichodesmium* Programmed Cell Death (PCD, Berman-Frank et al., 2004). This DDN release has been attributed i) to endogenous processes such as the dissipation of excess electrons linked to an excess of light (Wannicke et al., 2009) or to a means for the filamentous diazotrophs to transfer fixed N from **N_2**-fixing cells to vegetative cells (Mulholland and Capone, 2000) and ii) to exogenous processes such as viral lysis (Hewson et al., 2004; Ohki, 1999) or 'sloppy feeding' by copepods (O'Neil, 1999).

Numerous studies performed in culture (Hutchins et al., 2007; Karl et al., 1992, 1997) and in the field (Benavides et al., 2013b; Konno et al., 2010; Mulholland and Bernhardt, 2005) focused on quantifying this release, and most of them were performed on *Trichodesmium* and were based on the difference between the measurement of gross N_2 fixation (through the acetylene reduction method, Capone (1993)) and net N_2 fixation rates (through the $^{15}N_2$ isotope labelling method, Montoya et al. (1996)) (Mulholland et al., 2004). It was thus shown that the DDN released to the dissolved pool averages ~50 % (10 to 80 %) of total N_2 fixation. The estimates based on this approach have then be questioned since the discovery of the methodological underestimation of net $^{15}N_2$ rates when the $^{15}N_2$ tracer is injected as a bubble in the incubation bottles (Mohr et al., 2010), leading to a potential overestimation of the DDN release. ~~The application of the new method in which the $^{15}N_2$ is added as dissolved in a subset of seawater previously N_2 degassed (Mohr et al., 2010) has recently shown in *Trichodesmium* cultures that the DDN release represents less than 1 % of total N_2 fixation (Berthelot et al., 2015).~~ An alternative approach based on the direct measurement of the ^{15}N enrichment of both the particulate and dissolved pools (Glibert and Bronk, 1994; Slawyk and Raimbault, 1995) after incubation with $^{15}N_2$ present the advantage of providing ratio of particulate N_2 fixation versus DDN release, without being affected by potential underestimations issues. The studies based on this approach reveal that the proportion of DDN released to the dissolved pool ranges from 10 % to >80 % of total N_2 fixation measured in field (Benavides et al., 2013b; Berthelot et al., 2017; Glibert and Bronk, 1994; Konno et al., 2010). The release appears to be higher when *Trichodesmium* dominates the diazotroph community (Berthelot et al., 2017; Bonnet et al., 2016a; Glibert and Bronk, 1994) than when UCYN dominate (Benavides et al., 2013a; Bonnet et al., 2016b). **The DDN release is generally much lower in (<5 %) in monospecific cultures (Berthelot et al. 2015, Benavides et al. 2013) than in field experiment**~~The DDN released to the dissolved pool measured by this direct approach is generally much lower in culture studies (<5 %) (Benavides et al., 2013a; Berthelot et al., 2016),~~ suggesting that external factors such as sloppy feeding and viral lysis have a strong influence on the DDN release by diazotrophs in field.

The DDN released in the surface ocean is potentially available for surrounding planktonic communities, but its fate in the planktonic food web has poorly been quantified mainly due to methodological locks. Devassy et al. (1979) who reported high DON and NH_4^+ concentrations near *Trichodesmium* blooms were also the first to observe that during the decline of the *Trichodesmium* blooms, diatom abundances increased, followed by a succession of cladocerans, dinoflagellates, green algae and finally copepods. In the tropical Atlantic Ocean, high abundances of non-diazotrophic phytoplankton have also been observed following *Trichodesmium* blooms (Mulholland et al., 2004), and more recently, Chen et al. (2011) showed a positive correlation between abundances of *Trichodesmium* and diatoms in the Kuroshio Current. These studies suggest a link between diazotroph blooms and non-diazotrophic organisms. Studies based on size-fractionation carried out during a *Trichodesmium* bloom incubated in the presence of $^{15}N_2$ report that ~10 % of the fixed N_2 by *Trichodesmium* (recovered in the size fraction >30 µm) was rapidly transferred to non-diazotrophic organisms (recovered in the <30 µm fraction; Bryceson and Fay (1981)). Using similar methods, other studies suggested that during *Trichodesmium* blooms (Garcia et al., 2007; Mulholland et al., 2004) and *Nodularia* and *Aphanizomenon* blooms (Ohlendieck et al., 2000), 5 to 10 % of the DDN is transferred to the picoplankton compartment. However, the methods

based on size fractionation do not discriminate the DDN transfer towards the **non-diazotroph** picoplankton to the **potentially active** N_2 fixation ~~carried out by this same~~ **within** picoplankton (in particular the UCYN-A, one of the most abundant diazotroph in our ocean (Luo et al., 2012)). This method therefore potentially overestimates the DDN transfer and is not applicable to study the DDN transfer associated with UCYN. Moreover, it is not possible with size fractionation methods to determine which populations (e.g. autotrophic vs. heterotrophic plankton, small vs. large plankton) have benefited the most from this source of new N. The recent use of high-resolution nanometer scale secondary ion mass spectrometry (nanoSIMS) coupled with $^{15}N_2$ isotopic labeling and flow cytometry cell sorting (Bonnet et al., 2016a, 2016b; Berthelot et al., 2016;) has proved its efficiency in the quantification of the DDN transfer to specific groups of phytoplankton and bacteria. . Aplying this method during *Trichodesmium* blooms in the coastal Western Tropical South Pacific (WTSP), Bonnet et al. (2016a) revealed that after 48 h 13 ± 2 % to 48 ± 5% of the fixed N_2 was released to the dissolved pool and 6 ± 1 % to 8 ± 2 % of this DDN was transferred to non-diazotrophic plankton, mainly diatoms (45 ± 4 % to 61 ± 38 %) and bacteria (22 ± 27 % to 38 ± 12 %). A mesocosm experiment performed in the New Caledonian lagoon during a UCYN-C bloom (Bonnet et al., 2016b) revealed after 48 h 16 ± 6 % of the fixed N_2 was released to the dissolved pool and 21 ± 4 % of this DDN was transferred to non-diazotrophic plankton, mainly picoplankton (18 ± 4 %) and diatoms (3 ± 2 %). Finally, a comparative study between *Trichodesmium* vs. UCYN blooms simulated thanks to culture isolates (Berthelot et al., 2016), revealed that the DDN transfer to non-diazotrophic plankton is twice as high with *Trichodesmium* as with UCYN. **The differences of DDN release and transfer rates observed between the different field experiments and the different diazotrophs suggest that these processes strongly depend on the physiological state of diazotrophs and the environment. Yet,** ~~To date~~ the transfer of DDN to different groups of plankton from different diazotroph (*Trichodesmium* vs. UCYN) in the open ocean**, where most of global marine N_2 fixation takes place,** has never been investigated.

Regarding higher trophic levels, the low ^{15}N isotopic signature (**$\delta^{15}N$)** of zooplankton reveals that N_2 fixation can significantly contribute to zooplankton N requirements in high N_2 fixation areas (Aberle et al., 2010; Landrum et al., 2011; Loick-Wilde et al., 2012; Mompeán et al., 2013; Montoya et al., 2002; Sommer et al., 2006; Wannicke et al., 2013; Hunt et al., 2016). Few studies reports active grazing of *Trichodesmium* by some specific copepods (Micro- and Macrosetella sp., O'Neil et al. (1996); O'Neil and Roman (1992)). However, *Trichodesmium* has been shown to be toxic for most of the grazers (Guo and Tester, 1994; Hawser et al., 1992; Hawser and Codd, 1992) and the low **$\delta^{15}N$** signature found in zooplankton (indicative of DDN consumption) where *Trichodesmium* thrive most likely originates from indirect transfer mediated by recycling processes (Capone et al., 1994; Capone and Montoya, 2001; Letelier and Karl, 1996) rather than from direct grazing. A recent study based on $^{15}N_2$ labelling in the coastal WTSP (Hunt et al., 2016) reveals that the DDN is less efficiently transferred to zooplankton when *Trichodesmium* and DDA dominate the diazotroph community than when UCYN-C dominate, suggesting that the DDN transfer efficiency to zooplankton strongly depends on the diazotroph involved in the N_2 fixation. To our knowledge, this has never been investigated so far in the open ocean.

The WTSP Ocean has recently been identified as a hot spot of N_2 fixation (Bonnet et al., 2017) and is characterized by trophic and N_2 fixation gradients (Moutin et al., 2017), with oligotrophic waters characterized by high N_2 fixation rates

(631 ± 286 µmol N m^{-2} d^{-1}) mainly associated with *Trichodesmium* in the western part, and ultra-oligotrophic waters characterized by low N_2 fixation rates (85 ± 79 µmol N m^{-2} d^{-1}) mainly associated with UCYN in the eastern part (Bonnet et al., this issue; Stenegren et al., this issue). We performed a series of experiments under contrasting situations (either when *Trichodesmium* or UCYN was dominating the diazotroph community) to study the fate of DDN in the planktonic food web, with the following specific goals: (1) quantify the proportion of DDN released to the dissolved pool relative to total N_2 fixation, (2) quantify the DDN transfer to the non-diazotrophic phytoplankton and bacteria, and (3) quantify the DDN transfer to zooplankton.

2 Material and Methods

2.1 Experimental setup for DDN transfer experiments in phytoplankton and heterotrophic bacteria

This study was carried out during the OUTPACE (Oligotrophic to UlTra oligotrophic PACific Experiment) cruise (DOI: http://dx.doi.org/10.17600/15000900), **which took place** ~~on board the R/V *L'Atalante*~~ in February-March 2015 (austral summer) **on-board the R/V *L'Atalante***. Samples were collected along a ~4000 km west to east zonal transect along ~19°S starting in New Caledonia and ending in French Polynesia, crossing Melanesian archipelago waters (hereafter referred to as MA waters) around New Caledonia, Vanuatu, Fiji up to Tonga and South Pacific Gyre waters located at the western boundary of the South Pacific Gyre (hereafter referred to as GY waters) (see Moutin et al. (2017) for details). Three experiments are reported here (hereafter named E1, E2 and E3). Two were performed at stations located in MA waters: station LD A : 19°12.8'S - 164°41.3'E, and station LD B: 18°14.4'S - 170°51.5'W, where *Trichodesmium* accounted for 95 and 100 % of the total diazotroph community quantified by quantitative PCR (Stenegren et al., 2017). The third experiment was performed in GY waters: station LD C: 18°25.2'S - 165°56.4'W, where UCYN accounted for 82 % of the total diazotroph community (Stenegren et al., 2017).

The experiments were designed according to **Bonnet et al. (2016a, 2016b) and** Berthelot et al. (2016) ~~and Bonnet et al. (2016a)~~. For experiments E1 and E2, seawater was collected by the **surface** underway pumping system at 6 m-depth. For E3, seawater was collected at 55 m-depth using Niskin bottles mounted on a CTD rosette. For all experiments, seawater was collected into 8 HCl-washed-sample rinsed (three times) 4.5 L polycarbonate bottles equipped with septum caps. 5 mL of $^{15}N_2$ gas (98.9 atom% ^{15}N, Cambridge isotopes) were injected into all bottles using a gas-tight syringe. The purity of the $^{15}N_2$ Cambridge isotopes stocks was previously checked by Dabundo et al. (2014) and more recently by Benavides et al. (2015) and Bonnet et al. (2016b), who concluded that the purity is satisfying (2 x 10^{-8} mol:mol N of $^{15}N_2$) and therefore do not alter the results presented below. The bottles were shaken 30 times to facilitate the $^{15}N_2$ dissolution and, except for the T0 set of bottles (see below), were incubated for 48 h in on-deck incubators covered with blue screening (50 % surface irradiance for E1 and E2 and 15 % surface irradiance for E3) and cooled with circulating surface seawater. At T0 and after incubation, a set of 4 bottles were collected and subsampled for the following measurements (see below for methods): N_2 fixation rates, DDN release, quantification of diazotrophs, heterotrophic bacteria and pico-, nano- and microphytoplankton

enumeration, organic and inorganic nutrient concentrations, and ^{15}N-enrichment on diazotrophic and non diazotrophic plankton. Unless otherwise stated, each parameter reported below was measured in triplicates.

2.2 Net N_2 fixation rates and DDN released to the dissolved pool

N_2 fixation rates were measured using the $^{15}N_2$ isotopic tracer technique (adapted from Montoya et al. (1996)), as described in Bonnet et al. (this issue). ~~The $^{15}N_2$ bubble technique was intentionally chosen to avoid any potential overestimation due trace metal and dissolved organic matter (DOM) contaminations often associated with the preparation of the $^{15}N_2$-enriched seawater (Klawonn et al., 2015; Wilson et al., 2015) in our incubation bottles as Fe and DOM have been seen to control N_2 fixation or *nifH* gene expression in this region (Benavides et al., 2017; Moisander et al., 2012). However, the ^{15}N enrichment of the N_2 pool available for N_2 fixation was measured in all incubation bottles to ensure accurate rate calculations. Briefly, 12 mL were subsampled after incubation into Exetainers® fixed with $HgCl_2$ (final concentration 20 mg L^{-1}) that were preserved upside down in the dark at 4°C until analyzed using a membrane inlet mass spectrometer (MIMS) according to Kana et al. (1994).~~

~~At the end of incubations, 2.3 L of the triplicates 4.5 L amended bottles were gently filtered onto pre-combusted (450 °C, 4 h) Whatman GF/F filters (25 mm diameter, 0.7 µm nominal porosity). Filters were stored in pre-combusted glass vials at -20 °C during the cruise, and then dried at 60 °C for 24 h before analysis onshore. ^{15}N enrichments of particulate N collected on filters were determined using an Elemental Analyzer coupled to an Isotope Ratio Mass Spectrometer (EA-IRMS, Integra2 Sercon Ltd). The accuracy of the EA-IRMS system was systematically controlled using International Atomic Energy Agency (IAEA) reference materials, AIEA-N-1 and IAEA-310A. In addition, the ^{15}N enrichement of the ambient (unlabeled) particulate N was measured at each station at T0 and was used as the "initial" ^{15}N enrichment as termed in Montoya et al. (1996).~~

~~2.3 DDN released to the dissolved pool~~

The DDN released to the dissolved pool under the form of NH_4^+ and DON during the N_2 fixation process was quantified using the three step diffusion method extensively described in Berthelot et al. (2015) and derived from Slawyk and Raimbault (1995). ~~This method enables the differentiation of the nitrate (NO_3^-), NH_4^+ and DON pools, and measured their respective ^{15}N enrichment.~~ As the NO_3^- pool was negligible, the total dissolved N (TDN) pool was defined as the sum of DON and NH_4^+ pools (TDN = DON + NH_4^+). After incubation with the $^{15}N_2$ tracer, 300 mL of the filtrate passed through pre-combusted Whatman GF/F filters were collected in 500 mL Duran Schott borosilicate flasks, poisoned with $HgCl_2$ (300 µL, final concentration 20 mg L^{-1}) and stored at 4°C in the dark until analysis. At the end of each step, NH_4^+ and DON fraction were recovered on acidified pre-combusted Whatman GF/F filters, dried 24 h at 60 °C and stored in pre-combusted glass tubes until analysis on ~~EA-IRMS~~ an Elemental Analyzer coupled to Isotope Ratio Mass Spectrometer (EA-IRMS, Integra2 Sercon Ltd) as described in Berthelot et al., (2015). ~~The DDN release was the sum of all forms of N released in each fraction.~~

2.3 Inorganic and organic nutrient analyses

NH_4^+ concentrations were measured fluorimetrically according to Holmes et al. (1999) on a FP-2020 fluorimeter (Jasco, detection limit = 3 nM). NO_3^- and nitrite (NO_2^-) concentrations were measured using standard colorimetric procedures (Aminot and Kérouel, 2007) on a AA3 AutoAnalyzer (Seal-Analytical). DON concentrations were measured by the wet oxidation method according to Pujo-Pay and Raimbault (1994). ~~After the wet oxidation, the concentration of TDN was measured on a AA3 AutoAnalyzer (Seal Analytical). DON concentrations were obtained by difference between TDN and Dissolved Inorganic N (NH_4^+ + NO_3^- + NO_2^-) measured in parallel.~~

2.4 Plankton abundance determination

The abundance of *Trichodesmium* and UCYN-B was determined microscopically: 2.2 L of each triplicates $^{15}N_2$-amended 4.5 L bottles were gently filtered onto 2 µm pore size, 25 mm diameter Millipore polycarbonate filters and fixed with paraformaldehyde (2 % final concentration) for 1 h. *Trichodesmium* were enumerated on the entire surface of the filter at a x100 magnification with a Zeiss Axio Observer epifluorescence microscope fitted with a green (510-560 nm) excitation filter. The number of cells per trichome was counted on 20 trichomes for each experiment; we counted an average of 85 and 115 cells trichome^{-1} for E1 and E2, respectively. UCYN-B were counted on 40 fields (1.3 mm^2 fields; 0-2800 UCYN-B per field) scanned and analyzed with the ImageJ1 software.

Samples for micro-phytoplankton identification and enumeration were collected in each of the triplicate 4.5 L incubated bottles (except for E1 were only one replicate was available) in five 50 mL sterile **polypropylene** ~~PP~~ tubes and preserved in acidic Lugol's solution (0.5 % final concentration). Diatoms were enumerated from a 250 mL subsample following the Utermohl method (Hasle, 1978), using a Nikon TE2000 inverted microscope equipped with phase-contrast and a long distance condenser. Diatoms were identified to the lowest possible taxonomic level in one of the three replicates.

Pico, nano-phytoplankton and heterotrophic bacteria abundances were determined by flow cytometry. After incubation, 1.8 mL were subsample from triplicate 4.5 L bottles into cryotubes, fixed with paraformaldehyde (200 µL, 4 % final concentration) **for 5 min at room temperature**, flash-frozen in liquid N_2, and stored at -80 °C until analysis on a FACSCalibur (BD Biosciences, San Jose, CA) according to Marie et al. (1999), at the PRECYM flow cytometry platform (https://precym.mio.univ-amu.fr/). ~~Before analysis, samples were thawed at room temperature in the dark. For bacterial abundance, 300 µL of each sample was incubated with SYBR Green II (Molecular Probes, 0.5 % final concentration) for 15 min in the dark. For phytoplankton and bacterial abundances measurements, before analysis, 2 µm beads (Fluoresbryte, Polysciences) and Trucount beads (BD Biosciences) were added to the samples. Analyses were run during 1.5 and 3 min at high and medium flow for phytoplankton and bacteria, respectively. The 2 µm beads were used as an internal control and to discriminate picophytoplankton (< 2 µm) from nanophytoplankton (> 2 µm) populations.~~ Phytoplankton communities were clustered as *Prochlorococcus* spp. cell like, *Synechococcus* spp. cell like, nano-eukaryotes cell like, pico-eukaryotes cell like,

and UCYN-B cell like. ~~The~~ Truecount beads were used to determine the volume analyzed. ~~All data were acquired using the CellQuest software (BD Biosciences), and data analysis was performed using the SUMMIT v4.3 software (Dako).~~

2.5 Cell sorting and sampling for nanoSIMS analyses

For flow cytometry cell sorting and subsequent analysis using nanoSIMS, 1 L of one of the 4.5 L bottles was filtered onto 0.2 µm pore size 47 mm polycarbonate filters to preconcentrate the cells and facilitate cell sorting. Filters were placed in a 5 mL cryotube® filled with 0.2 µm filtered seawater and PFA (2% final concentration), and incubated 1 h at room temperature in the dark. The cryovials were vortexed ~20 s to detach the cells from the filter and were stored at -80 °C until analysis. Cell sorting was performed using a Becton Dickinson Influx™ (BD Biosciences, Franklin Lakes, NJ) high speed cell sorter at the PRECYM platform, as described in Bonnet et al. (**2016a, Supp. Info.**). Planktonic groups (pico, nano-phytoplankton, bacteria and UCYN-B (for E3 only)) were separated using the same clusters as for the phytoplankton abundance determination. At the issue of the cell sorter, the cells were directly dropped onto a 0.2 µm pore size, 13 mm diameter polycarbonate filter (Millipore) connected to a low pressure pump in order to concentrate them on a surface as small as possible. The filters were stored at -80 °C until nanoSIMS analyses.

To recover large phytoplanktonic cells (*Trichodesmium* and diatoms), 1 L of the same 4.5 L bottle was filtered on 10 µm pore size 25 mm polycarbonate filters. The cells were fixed with PFA (2 % final concentration), incubated 1 h at ambient temperature and stored at -20 °C until nanoSIMS analyses.

2.6 NanoSIMS analyses

Just before nanoSIMS analyses, filters were thawed at ambient temperature and sputtered with gold and palladium to ensure conductivity. Analyses were performed on a NanoSIMS N50 (Cameca, Gennevilliers, France) at the French National Ion MicroProbe Facility as previsouly described in Bonnet et al. (2016a, 2016b) and Berthelot et al. (2016). Briefly, high density cells area were retrieved using the nanoSIMS optical camera (Fig.1 f.). Samples were pre-sputtered ~~with~~ prior to analyses for at least 2 min to remove surface contaminants and increase conductivity with a ~22 pA Cesium primary beam. For the analysis, a ~1.2 pA Cesium (16 KeV) primary beam focused onto ~100 nm spot diameter was scanned across a 256×256 or 512×512 pixel raster (depending on the image size, which ranged from 20 µm × 20 µm to 40 µm × 40 µm) with a counting time of 1 ms per pixel. Negative secondary ions (**$^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$ and $^{28}Si^-$**) were collected by electron multiplier detectors, and secondary electrons were imaged simultaneously. A total of 20 serial quantitative secondary ion images were generated to create the final image. ~~Mass resolving power was ~8000 in order to resolve isobaric interferences. Data were processed using the LIMAGE software. Briefly, all scans were corrected for any drift of the beam and sample stage during acquisition. Isotope ratio images were created by adding the secondary ion counts for each recorded secondary ion for each pixel over all recorded planes and dividing the total counts by the total counts of a selected reference mass.~~ Individual cells were easily identified in nanoSIMS secondary electron, **$^{12}C^-$, $^{12}C^{14}N^-$ and $^{28}Si^-$** images that were used to define regions of interest (ROIs) around individual cells. A total of ~1500 ROI**s** was analyzed. For each ROI, the ^{15}N-

enrichment was calculated. ~~50 to 200 cells~~ **~80 cells on average** were analyzed for each plankton group and for each experiment **(see Table 1 SI)**.

2.7 Cell-specific N content and DDN transfer calculations

To determine cell-specific N contents, cell sizes of *Trichodesmium* and dominant diatoms were directly measured on each sample collected for microscopy (see section 2.4). For *Trichodesmium*, cell length and width were measured on 25 to 50 cells per sample at x400 magnification with a Zeiss Axio Observer epifluorescence microscope. For diatoms, the cell cross section and apical and transapical dimensions were measured on at least 20 cells using a Nikon TE2000 inverted microscope equipped with phase contrast and a long-distance condenser. For UCYN-B, cells diameters were directly measured on the nanoSIMS images **and further confirmed on microscopic images**. The biovolumes (BV) of *Trichodesmium*, UCYN-B and diatoms were estimated following the geometric model of each cell type (Sun and Liu, 2003). The cellular carbon (C) contents were determined by using the relation between BV and C content according to Verity et al. (1992) for *Trichodesmium* and UCYN-B, and according to Eppley et al. (1970) and Smayda (1978) for diatoms. The N content was calculated based on a C:N ratios of 6 for *Trichodesmium* (Carpenter et al., 2004), 5 for UCYN-B (Dekaezemacker and Bonnet, 2011; Knapp et al., 2012), and a typical Redfield ratio of 6.6 for diatoms.

For *Synechococcus* and *Prochlorococcus* we used the C content reported in Buitenhuis et al. (2012) (255 and 36 fg C cell^{-1}, respectively). For nano-eukaryotes we used the C content reported in Grégori et al. (2001), and converted into N content according to the C:N Redfield ratio of 6.6 (leading to 3.2, 0.45 and 219 fmol N cell^{-1} for *Synechococcus*, *Prochlorococcus* and nano-eukaryotes). For bacteria, an average N content of 2.1 fg N cell^{-1} (Fukuda et al., 1998) was used. For the pico-eukaryotes, the cellular N content of 9.2 ± 2.9 fmol N cell^{-1} was used as reported in Gregori et al. (2001).

The cell-specific N_2 fixation rates and DDN transfer rates (in nmol N **L^{-1} 48 h^{-1}**) to non-diazotrophic phytoplanktonwas calculated for each plankton group analyzed as follows:

$$DD^{15}N = \frac{^{15}Nex}{Nsr} \times N_{con} \times A$$

where ^{15}Nex (atom %) is the excess ^{15}N enrichment of the individual cells measured by nanoSIMS after 48 h of incubation relative to the time zero value, N_{sr} (atom %) is the excess ^{15}N enrichment of the source pool (N_2) in the experimental bottles determined by MIMS, N_{con} is the cellular N content (in nmol N cell^{-1}) of each cell and A is the abundance of the specific plankton group (in cell L^{-1}). Incertitude was estimated on each variable and the final incertitude was estimated using the propagation error rule.

2.8 Experimental setup for DDN transfer experiments in zooplankton

The DDN transfer to zooplankton was measured in four experiments performed at the same stations where the E1, E2 and E3 experiments were performed (hereafter names Zoo-1, Zoo-3 and Zoo-4) plus an additional station **(Zoo-2)** located between LDA and LDB (SD9, 20°57'S – 178°39'E). *Trichodesmium* was dominating the diazotroph community in the Zoo-1, Zoo-2,

and Zoo-3 experiments, and UCYN-B was dominating the Zoo-4 experiment. The experiments consisted in incubations of freshly collected zooplankton in the presence of the natural planktonic assemblage pre-labelled with $^{15}N_2$ as a food source. In parallel to the experiments describes above, 6 additional HCl-washed-sample rinsed (three times) 1 L polycarbonate bottles equipped with septum caps were collected by the underway pumping system at 6 m-depth for Zoo-1, Zoo-2 and Zoo-3 and with Niskin bottles at 55 m-depth for Zoo-4. All bottles were amended with 1 mL of $^{15}N_2$ (98.9 atom% ^{15}N, Cambridge isotopes). The bottles were shaken 30 times to facilitate the $^{15}N_2$ dissolution and incubated in on-deck incubators for 24 h-36 h as described above.

The incubation was stopped by filtering the bottles on 0.2 µm pore size 47 mm membrane filters **in such a way that the ^{15}N enrichment of the food source provided to zooplankton (hereafter referred to as ^{15}N pre-labelled plankton) does not increase during the course of the experiment. For each experiment, the initial ^{15}N enrichment of the ^{15}N pre-labelled plankton was analyzed in triplicates by EA-IRMS. Plankton was then** ~~and~~ re-suspend**ed** ~~-ing the particulate matter~~ in 6 1 L bottles filled with 0.2 µm filtered surface seawater collected at the same station~~, in such a way that the ^{15}N enrichment of the food source provided to zooplankton (hereafter referred to as ^{15}N pre-labelled plankton) stop to increase by fixing $^{15}N_2$~~. Meanwhile, zooplankton was collected using repeated net tows (120 mesh size) before dawn. Animals were recovered on a 120 µm sieve and placed into 4.5 L polycarbonate bottles filled with 0.2 µm filtered surface seawater in the dark for at least 6 h in order to allow them to empty their guts. Living animals were visually identified and the individuals belonging to the genus *Clausocalanus*, which largely dominated the zooplankton community at all stations (Carlotti et al., this issue), were handpicked and 12 animals were dispatched into each of the three 1 L bottles containing the ^{15}N pre-labelled plankton before being incubated in on-deck incubators for 24 h as described above. The three other bottles were immediately filtered after the introduction of animals, first through a 120 µm mesh in order to recover the animals and secondly through precombusted (4 h, 450 °C) GF/F filters, which were used to quantify the isotopic signature of the ^{15}N pre-labelled plankton at the beginning of the experiment, together with the initial NH_4^+ concentrations in the incubation bottles. After 24 h, the triplicate bottles containing the mixture of ^{15}N pre-labelled plankton and zooplankton were filtered in the same way. In addition, the filtrate was recovered as described ~~above~~ in **section 2.2 in** order the measure the NH_4^+ concentration and the ^{15}N enrichment in the dissolved pool by using the two steps diffusion method ~~as described in section 2.3~~. The recovered animals were placed on GF/F filters, which were analyzed by EA-IRMS as described above in section 2.2.

3 Results

3.1 Description of the biogeochemical context at the studied stations

Regarding the chlorophyll *a* and nutrient concentrations, the four studied stations were divided into two main sub-regions (Table 1): i) stations of E1/Zoo-1, Zoo-2 and E2/Zoo3 were located in the oligotrophic MA waters characterized by surface chlorophyll *a* concentrations of 0.159-0.377 µg L^{-1} and NO_3^- and PO_4^- concentrations below 50 nmol L^{-1}, ii) station E3/Zoo-4

located in the ultra-oligotrophic GY waters presenting lower chlorophyll *a* concentrations than in MA waters (0.053 µg L^{-1}), NO_3^- concentration below 50 nmol L^{-1}, and PO_4^- concentration ~110 nmol L^{-1}.

Trichodesmium dominated the diazotroph community (95 – 100 % of total *nifH* gene copies detected by qPCR, Stenegren et al., this issue) at 6 m-depth in the MA waters (E1/Zoo-1, Zoo-2 and E2/Zoo3), while UCYN-B dominated (82 % of *nifH* gene copies) at 55 m-depth in the GY waters (E3/Zoo-4 experiment). MA waters were characterized by a higher abundance of pico-phytoplankton (*Synechococcus* and *Prochlorococcus*) and bacteria abundances (125-200 and 271-424 10^{11} cells m^{-2}, respectively) than GY waters (~110 and ~290, 10^{11} cells m^{-2}, respectively; Bock et al., this issue). ~~In both regions, *Prochlorococcus* dominated pico-phytoplankton biomass (Table 1)~~.

3.2 N_2 fixation and DDN release**d to** ~~**in**~~ **the dissolved pool**

Net N_2 fixation rates were 20.1 ± 13.4, 49.9 ± 2.4 and 3.2 ± 0.3 nmol N L^{-1} 48 h^{-1} for the E1, E2 and E3 experiments, respectively (Fig. 2). The DDN released to the dissolved pool (the sum of DON and NH_4^+) was 14 ± 9 and 8 ± 2 nmol N L^{-1} 48 h^{-1} in E1 and E2, and was below quantification limits in E3. Considering gross N_2 fixation as the sum of net N_2 fixation and DDN release rates (Mulholland et al., 2004), the DDN released **to** ~~**in**~~ the dissolved pool accounted for 40 ± 27 and 14 ± 4 % of gross N_2 fixation in E1 and E2, respectively (Fig. 4). ~~Most of the ^{15}N released was under the form of DON, which accounted for ~93 and ~96 % of the total N release.~~ **DON accounted for the major part of the ^{15}N released and accounted for ~93 and ~96 % of the total N release in E1 and E2, respectively.**

3.3 Cell-specific ^{15}N enrichment and DDN transfer to non diazotrophic plankton

NanoSIMS analyses performed on individual trichomes (E1 and E2) and UCYN-B cells (E3) revealed significant ($p<0.05$) ^{15}N-enrichment after 48 h of incubation (Fig. 1 a, b) compared to T0 samples (0.371 ± 0.005 atom%**, n=5**), indicating active N_2 fixation during the experiments: 1.946 ± 0.837 atom% (n=32) in E1, 1.523 ± 0.477 atom% (n=25) in E2 and 4.707 ± 0.210 atom% (n=192) in E3 (Fig. 3). Cell-specific N_2 fixation rates of *Trichodesmium* were 252.7 ± 50.5 fmol N $cell^{-1}$ 48 h^{-1} in E1 and 341.5 ± 68.3 fmol N $cell^{-1}$ 48 h^{-1} in E2, and cell-specific rates of UCYN-B were 18.6 ± 3.8 fmol N $cell^{-1}$ 48 h^{-1} in E3.

NanoSIMS analyses performed on non-diazotrophic plankton (diatoms and cell-sorted *Synechococcus* (Fig. 1 d), *Prochlorococcus*, bacteria, pico- and nano-eukaryotes (Fig. 1 e)) also revealed significant ^{15}N-enrichment as compared to T0 values ($p<0.05$) (Fig. 3). The ^{15}N-enrichment of the non-diazotrophic plankton (all groups pooled together) was not statistically different ($p>0.05$) between E1 and E2. However, it was significantly lower ($p<0.05$) in E3 compared to E1 and E2.

Over the 48 h of the experiment, 10 ± 2 % of the total DDN was transferred to non-diazotrophic plankton in E1, 7 ± 1 % in E2, and 15 ± 3 % in E3 (Fig.4). ~~For the three experiments,~~ DDN was mainly transferred to ~~*Synechococcus, Prochlorococcus* and bacteria in the three experiments and contributed approximately to 98, 92 and 99 % of the transfer in E1, E2 and E3, respectively (Fig. 3) **(Fig. 4)**. The major part of the transfer took place in~~ pico-**cyanobacteria** ~~phytoplankton~~

(*Synechococcus* and *Prochlorococcus*), accounting for 73 ± 15 %, 68 ± 14 % and 65 ± 13 % of the total transfer into non-diazotrophs in E1, E2 and E3, respectively **(Fig. 4).** ~~followed by~~ **The transfer into heterotrophic** bacteria **accounted for** ~~(~~25 ± 5 %, 23 ± 5 % and 34 ± 7 % **of the total transfer**, **in E1, E2 and E3,** respectively ~~(Fig. 4)~~. Lastly, ~~50 ± 40 %, 10 ± 2 % and 40 ± 27 %~~ **50 ± 40 %, 79 ± 4 % and 85 ± 9 %** of the newly fixed $^{15}N_2$ remained in the pool of diazotrophs (corresponding to the major group of diazotrophs detected at each stations and analysed by nanoSIMS (*Trichodesmium* or UCYN-B), other potential diazotrophs that have not been targeted by qPCR (such as ~~diazotrophic heterotrophic bacteria~~ **non-cyanobacterial diazotrophs**), and other groups of non-diazotrophic plankton to which $^{15}N_2$ was transferred but that were not analyzed by nanoSIMS due to their very low abundance) for E1, E2 and E3, respectively.

3.4 DDN transfer to zooplankton

Before incubation with zooplankton, the isotopic enrichment of the ^{15}N pre-labelled plankton **was not significantly different in the experiments Zoo-1, Zoo-2 and Zoo-3 (dominated by *Trichodesmium*)** averag**ing** ~~ed~~ 1.035 ± 0.091 atom% **(n=9).** ~~in the experiments Zoo-1, Zoo-2 and Zoo-3 (dominated by *Trichodesmium*) and~~ **The isotopic enrichment was lower in the experiment Zoo-4 (dominated by UCYN-B) averaging** 0.385 ± 0.005 atom% **(n=3).** ~~in the experiment Zoo-4 (dominated by UCYN-B).~~ After 24 h of incubation with zooplankton, the ^{15}N enrichment of the ^{15}N pre-labelled plankton **decreased down to 0.431 ± 0.014 atom% on average in Zoo-1, Zoo-2 and Zoo-3, and down to 0.372 ± 0.010 atom% in Zoo-4**. Meanwhile, the ^{15}N enrichment of zooplankton increased as compared to T0 values (0.383 atom% on average) and reached 0.482, 0.376, 0.513 and 0.368 atom% on average in Zoo-1, Zoo-2, Zoo-3 and Zoo-4, respectively. As the ^{15}N enrichment of the initial source food (^{15}N pre-labelled plankton) was different between the four stations/experiments, and in order to compare the results obtained among experiments, we normalized the values as the percentage of initial amount of ^{15}N atoms in excess in the ^{15}N pre-labelled plankton transferred to the different compartments (i.e. conserved in the ^{15}N pre-labelled plankton pool, transferred to the zooplankton pool and to the NH_4^+ pool) at the end of the incubation. In the experiments where *Trichodesmium* dominated the diazotroph community (Zoo-1, Zoo-2, and Zoo-3), 19 ± 7 % to 48 ± 21 % of the initial $DD^{15}N$ remained in the phytoplankton pool, 5 ± 5 % to 9 ± 13 % was transferred to the zooplankton and 0.4 ± 0.3 to 7 ± 3 % was transferred to the NH_4^+ pool (Fig. 5). In the Zoo-4 (where UCYN dominated the diazotroph community, Table 1), a greater proportion of $DD^{15}N$ was conserved in the phytoplankton (76 ± 34 %) but a greater transfer to the zooplankton was also observed (28 ± 8 %, Fig. 5). The recovery of the initial $DD^{15}N$ was comprised between 29 % and 100 %, suggesting that the remaining fraction was released to the DON pool. Interestingly, the recovery of the $DD^{15}N$ was in surplus in the Zoo-4 experiment (112.5 ± 8.5%), suggesting that the $DD^{15}N$ transfer in the DON pool is close to zero.

4 Discussion

4.1 DDN release to the dissolved pool

The quantity and quality of N released by diazotrophs to the dissolved pool during N_2 fixation potentially plays a key role in shaping the planktonic and microbial food webs. In this study, *Trichodesmium* released 14 ± 4 % to 40 ± 57 % of the newly fixed N into the dissolved pool, which is in agreement with values reported in the literature for field studies (Mulholland, 2007; Bonnet et al., 2016a). DON accounted for ~95 % of the DDN released by *Trichodesmium* (Fig. 2), ~~which is in agreement~~ **accordingly** with contributions measured in culture (80 - 90 %; Berthelot et al., 2015) and in the field (Berthelot et al., 2016). The low contribution of NH_4^+ to the DDN release does not mean that it was not released, but is likely the results of immediate consumption by surrounding plankton**, which shows a great affinity for NH_4^+.** ~~as NH_4^+ is known to the preferred N source for marine plankton. On the opposite~~ **Similarly,** part of the DON released by *Trichodesmium* was probably uptaken by heterotrophic and mixotrophic plankton (Bronk et al., 2007) **but a significant fraction was** ~~, and part was~~ likely refractory (not easily available for organisms) **leading to**~~, explaining~~ the observed accumulation in the dissolved pool. If not refractory, the DON would likely have been immediately assimilated as the region where these experiments were performed are strongly limited by N availability (Van Wambeke et al., **2008;** this issue**; Bonnet et al., 2008**).

In the E1 experiment, we noticed a large variability of N_2 fixation and DDN release rates among the three replicates, which explains the high standard deviations (Fig. 2): two replicates exhibited net N_2 fixation rates ~25-30 nmol N L^{-1} 48 h^{-1} and DDN release rates ~7-10 nmol N L^{-1} 48 h^{-1}, whereas in the third replicate, the DDN release (~24 nmol N L^{-1} 48 h^{-1}) exceeded net N_2 fixation rates (5 nmol N L^{-1} 48 h^{-1}). This can be attributed to the decline of *Trichodesmium* in this replicate as we counted much more degraded trichomes in the third replicate. This suggests that decaying *Trichodesmium* release DDN more efficiently than healthy *Trichodesmium*, which has already been observed by Bonnet et al. (2016a). This may also explain why the DDN transfer to non-diazotrophic plankton was slightly higher in E1 (10 ± 2 %) than in E2 (7 ± 1 %), despite both stations were dominated by *Trichodesmium*.

Conversely to E1 and E2, the DDN released by UCYN-B (E3), was not quantifiable in our study. However, significant DDN transfer into non-diazotrophic plankton was detected (15 ± 3 % of the total fixed N, Fig. 4), suggesting that the DDN released to the dissolved pool is likely immediately transferred to surrounding communities. ~~Contrary to E1 and E2, DON did not accumulate in the dissolved pool, suggesting either DON is released by UCYN but is more labile than DON released by *Trichodesmium*, or suggesting that UCYN only release NH_4^+ (which is immediately uptaken and thus does not accumulate as in *Trichodesmium* experiments).~~ To our knowledge, this is the first report of DDN release in the field in the presence of a diazotroph community dominated by UCYN-B. Bonnet et al., (2016b) report low release from UCYN-C in coastal waters of the WTSP (16 ± 6 % of total N_2 fixation) compared to *Trichodesmium* (13 ± 2 % to 48 ± 5 %; Bonnet et al., 2016b). This seems to indicate that the DDN from UCYN is generally lower than the DDN from *Trichodesmium*. Several hypotheses may explain the differences observed between *Trichodesmium* and UCYN**.** ~~i) as stated above, the~~ DDN **compounds released by** ~~from~~ UCYN may be more **bio-**available than the DDN ~~from~~ **released by** *Trichodesmium*, **limiting**

its accumulation. ~~therefore it does not accumulate in the dissolved pool~~ **The lack of accumulation in E3 could also be due to the more severe N limitation of planktonic communities in the ultra-oligotrophic waters as compared to MA waters (Van Wambeke, this issue), and to the nature of the resident community. *Prochlorococcus* was dominating the planktonic community at LD C (E3) and is known to have a high affinity to its small surface to volume ratio (Partensky et al., 1999).** ~~ii) the~~ PCD causing *Trichodesmium* bloom demise can **also be involved in the relatively high** ~~enhance the~~ DDN **release and accumulation during *Trichodesmium* dominated experiments** (Bar-Zeev et al., 2013)**.** ~~iii)~~ **Exogenous factors, such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) are also suspected to enhance the DDN release. These factors were found to excert a higher pressure in the** ~~the E3 experiment (dominance of UCYN-B) was performed in the ultra-oligotrophic waters of the GY where exogenous factors such as viral lyses (Fuhrman, 1999) and sloppy feeding (O'Neil and Roman, 1992b; Vincent et al., 2007) (which ususally enhance N release) are minimal compared to~~ MA waters where ~~the~~ *Trichodesmium* **dominated compared to ultra-oligotrophic waters** ~~experiments were performed~~ (Bock et al., this issue)**, where UCYN-B dominated. Finally, part of the discrepancy might be due to a methodological artefact: different sampling procedures between E1 and E2 (pump) and E3 (Niskin bottles) as the pump is suspected to induce mechanical stress to the cells which may have potentially affected the DDN release.** ~~Lastly, the DDN release measured here for UCYN-B is close to the one measured in cultures (1.0 ± 0.3 % to 1.3 ± 0.2 % , Benavides et al., 2013; Berthelot et al., 2015), where the exogenous factors are reduced, which would plead for hypothesis iii.~~

The DDN release plays a key role in the transfer of N from diazotrophs to the surrounding non-diazotrophs, only it is not a good indicator of the DDN transfer efficiency as we observed that DDN transfer to non-diazotrophs was higher when the release was low (E3) than when it was high (E1 and E2). This has already been observed in coastal waters of the WTSP by (Berthelot et al., 2016).

4.2 DDN transfer efficiency and pathways in the WTSP

Here we report for the first time data on the transfer of DDN to the planktonic food web under contrasting diazotroph community composition in the open ocean. We reveal that 7 ± 1 % to 15 ± 3 % of the DDN is transferred to the non-diazotrophic plankton (Fig. 4) at short time scales (48 h), which is in the same order of magnitude than the transfer (~10 %) reported in coastal waters of the WTSP (Bonnet et al., 2016a; Berthelot et al., 2016). In terms of efficiency, despite UCYN-B fix at lower rates compared to *Trichodesmium*, the DDN originating from UCYN-B is more efficiently transferred to non-diazotrophic plankton (15 ± 3 % of total fixed N in the E3 experiment, Fig. 4) compared to the DDN originating from *Trichodesmium* (10 ± 2 % and 7 ± 1 % in the E1 and E2 experiments, respectively). This results is in accordance with the fact that we did not detect any accumulation of ^{15}N-labelled N forms in the dissolved pool (see section above)~~. suggesting a higher availability of the DDN released by UCYN-B.~~

Several studies have proven that a fraction of the DDN release is transferred to surrounding non-diazotrophic plankton, and one of them (Bonnet et al., 2016a) conclude that diatoms are the major beneficiaries of the DDN originating

from *Trichodesmium* and develop extensively during/after *Trichodesmium* blooms in the coastal WTSP ocean. Despite *Trichodesmium* is rarely recovered in sediment traps (Chen et al., 2003; Walsby, 1992), these authors hypothesize a ~~potential~~ tight coupling between *Trichodesmium* blooms and export of organic matter as diatoms are efficient exporters of organic carbon to depth (Nelson et al., 1995). In contrast, in the present study, > 90 % of the DDN was transferred to picoplankton (*Synechococcus*, *Prochlorococcus* and bacteria), whatever the station studied (Fig. 4). The cyanobacteria *Synechococcus* and *Prochlorococcus* were the primary beneficiaries (73 ± 15 %, 68 ± 14 % and 65 ± 13 % of the DDN transfer, Fig. 4), which is consistent with Bonnet et al. (this issue) who observed a positive correlation between N_2 fixation rates and the abundance of *Synechococcus* and *Prochlorococcus* in the WTSP. We attributed this difference between the present study and the Bonnet et al., (2016a) study to the phytoplanktonic populations present in ambient waters at the time of the experiments. In the Bonnet et al., (2016a) study, diatoms were accounting for ~30 % of the non-diazotrophic phytoplankton biomass at T0, whereas, diatoms were **scarce** ~~quasi absent~~ (1 % of the non-diazotrophic phytoplankton biomass) in our offshore experiments, i.e. too low to show a significant increase in 48 h, even if they benefited from the DDN. **However, in E2 (LD B), the diatom abundances were the highest of the three experiments (Leblanc et al., this issue) and the bloom at this station was mainly composed of diatoms and *Trichodesmium*, suggesting that *Trichodesmium* contributed to sustain this bloom.**

~~On the opposite, picoplankton was dominating here with *Prochlorococcus* accounting > 60 % of the picoplankton C biomass (Bock et al., this issue). In the present study, diatom abundances were to low at T0 to show a significant increase in 48 h, even if they benefited from the DDN. However, in E2 the diatom abundances were the highest of the three experiments and de Verneil et al. (2017) mentioned that the bloom observed at LD B was composed of diatoms and *Trichodesmium*, suggesting that *Trichodesmium* contributed to sustain this bloom.~~ In the E1 and E2 experiments, where *Trichodesmium* was the dominant diazotroph (Stenegren et al., this issue), the DDN was preferentially transferred to *Synechococcus*, while it was preferentially transferred to *Prochlorococcus* in E3 where UCYN-B was the dominant diazotroph (Stenegren et al., this issue). This suggests a possible coupling between *Synechococcus* and *Trichodesmium* as ever mentioned by Campbell et al. (2005), who report higher *Synechococcus* abundances inside *Trichodesmium* blooms compared to surrounding waters, while it is not the case for other plankton groups. This difference may also be linked with the communities present at the time of the experiments: *Prochlorococcus* accounted for ~65 % of pico-phytoplankton in E1 and E2, while it accounted for ~80 % in GY in E3. While the transfer of DDN to *Prochlorococcus* and *Synechococcus* together was roughly equivalent for E1 and E2 (~70 %, Fig. 4), *Synechococcus* abundances increased by 150 % in E1, and *Prochlorococcus* increased by 12 % during the time course of the experiment, while none of the populations increased in abundance in E2 (Fig. 1 Supp. Info.). This result is in agreement with Bock et al. (this issue) who report an increase of the grazing pressure with the decrease of the oligotrophic degree as E1 was performed in more oligotrophic waters than E2.

Besides *Prochlorococcus* and *Synechococcus*, heterotrophic bacteria were the second beneficiaries of the DDN, especially when the diazotroph community was dominated by UCYN-B (34 ± 7 % of the DDN transfer, Fig. 4). In this experiment, bacteria abundances increased by 70 % on average (Fig. 1 Supp. Info), which is in agreement with Berthelot et al., (2016) and Bonnet et al., (2016a; 2016b), who reported similar bacterial increases in the coastal WTSP. When

Trichodesmium was the dominant diazotroph, 23-25 % was transferred to bacteria, whose abundance increased by 135 % and 15 % in E1 and E2, consistent with Sheridan et al. (2002), who reported higher bacterial abundances in *Trichodesmium* blooms than in surrounding waters. The significant DDN transfer from *Trichodesmium* to bacteria concurs with *Trichodesmium* and bacteria association that has been largely highlighted in the last decades (Hmelo et al., 2012; Paerl et al., 1989; Sheridan et al., 2002). That is in accordance with Van Wambeke et al. (this issue), who report that N_2 fixation fuels 3-35 % of bacterial production in MA waters. Then, we could not discriminate the DDN transfer to pico- and nano-eukaryotes, but for diatoms, the DDN transfer represented a low contribution to the overall transfer into non-diazotrophs in this region of the open ocean.

4.3 Transfer of DDN to zooplankton

Regarding higher trophic levels, the experiments performed here show that the DDN transfer to the major group of zooplankton present in this ecosystem (the copepod *Clausocalanus*) was less efficient (Fig. 5) when the diazotroph community was dominated by *Trichodesmium* (~5-9 %) than when it was dominated by UCYN-B (~28 %). This result is consistent with a previous study based on analogous $^{15}N_2$ labelling method in coastal waters of the WTSP (Hunt et al., 2016), which also report a higher DDN transfer efficiency in the presence of UCYN.

Regarding the DDN transfer from UCYN-B, although the transfer experiments to phytoplankton and bacteria (E3) and zooplankton (Zoo-4) were not performed in the same incubation bottles, they consistently report lower ^{15}N-enrichments in all the studied pools as compared to the experiments performed when *Trichodesmium* dominated, but in fine, the DDN transfer efficiency was more important in the presence of UCYN. We observed that a larger fraction of DDN was conserved in the UCYN-B pool than in the *Trichodesmium* pool, and a larger part of the DDN was missing (likely associated to the DON pool) with *Trichodesmium* than with UCYN-B (Fig. 5). These observations are consistent with the transfer experiments E1, E2, and E3 which show that *Trichodesmium* released more DDN in the dissolve pool (DON + NH_4^+) than UCYN-B. The DDN released in the NH_4^+ pool did not presented significant differences between *Trichodesmium* (Zoo-1, Zoo-2 and Zoo-3) and UCYN-B (Zoo-4), and in all experiments, the DDN contribution was low in the NH_4^+ pool, as it was immediately assimilated by surrounding organisms as explained in section 4.1. We suggest that the DDN transfer was higher with UCYN than with *Trichodesmium* since the UCYN-B can be directly grazed due to their small size (2-3 µm) as mentioned in Hunt et al. (2016), who revealed high UCYN abundances in the copepods guts based on qPCR data, while less *Trichodesmium* were measured. This pleads for a direct transfer of DDN from UCYN-B to zooplankton and an indirect transfer from *Trichodesmium* through non-diazotrophs. At the ecosystem level, even if the DDN transfer efficiency (~15 %) to zooplankton from UCYN-B is higher than the one of *Trichodesmium*, the ultimate quantity of DDN transferred to secondary producers is higher when *Trichodesmium* dominates, as cell-specific N_2 fixation rates of *Trichodesmium* (~250-340 fmol N cell^{-1} 48 h^{-1}) are far higher than those of UCYN-B (~19 fmol N cell^{-1} 48 h^{-1}). This result is in agreement with ~~the ones of Carlotti et al., (this issue) base on natural N isotopic measurements, who revealed~~ **Carlotti et al. (this issue) results based on ^{15}N isotopic data showing** that ~50-95 % **and ~10-40 % of the zooplankton N content** ~~of the zooplankton~~

originates from N_2 fixation in ~~the~~ MA ~~waters~~ and ~~~10-40 % in the~~ GY waters**, respectively.** Finally, the DDN transferred to zooplankton, either directly or indirectly, may be released in the dissolved pool as NH_4^+, providing additional NH_4^+ from DDN in the environment that is likely assimilated by organisms in N-depleted waters. Thus, zooplankton N release appears as another DDN transfer pathway to the microbial communities in the WTSP.

Zooplankton can contribute to organic matter export by production of sinking fecal pellets, active transport to depth and carcasses export. These processes are increasingly recognized as important vectors of organic matter export, and the magnitude of their contributions to organic matter export are highly dependent on regionally variable plankton community structure (Steinberg and Landry, 2017). In the WTSP, where N_2 fixation sustains most of the new primary production (Caffin et al., 2018) and an important fraction of the DDN is transferred to zooplankton, it might play a key role on the export production and hence the CO_2 sink which is the WTSP.

5. Conclusion and ecological impact of N_2 fixation in the WTSP

N_2 fixation acts as a natural N fertilizer in the ocean, releasing DDN in the dissolved pool, which is available for surrounding marine organisms. To our knowledge, this study provides the first quantification of DDN transfer to phytoplankton, bacteria and zooplankton communities in open ocean waters. The main interest of this study was to compare DDN transfer and release under contrasting N_2 fixation activity and diversity.

Here, we reveal that *Trichodesmium* released more DDN than UCYN-B, but a significant part of the DDN released by *Trichodesmium* **accumulated in the dissolved pool** ~~was refractory~~, while the DDN released by UCYN-B was ~~more bio-available (NH_4^+ and labile DON) and likely~~ immediately assimilated by the surrounding plankton communities. The DDN transfer efficiency ~~was~~ **to non-diazotrophic plankton was higher** ~~more important to non-diazotrophic phytoplankton and bacteria (~15 ± 3 %) and zooplankton (~28 ± 8 %)~~ when UCYN-B dominated the diazotroph community than when *Trichodesmium* dominated ~~(~8 ± 2 % and 7 ± 6 % of transfer to phytoplankton and bacteria, and zooplankton, respectively).~~ In the open ocean, most ~~of the~~ N_2 fixation is performed by *Trichodesmium* (Capone et al., 1997 **Luo et al., 2012**), thus on a global scale most of the DDN transfer can be attributed to *Trichodesmium*~~, moreover in the MA waters where *Trichodesmium* dominated diazotroph community~~. The regions where UCYN are the dominant diazotrophs **generally** present lower N_2 fixation rates than the ones **where** ~~of~~ *Trichodesmium* **dominates**, but **UCYN provide a continuous source of DDN to surrounding plankton communities** ~~it is not negligible and may provide a continuous background of DDN to surrounding plankton communities~~. The DDN was ~~preferentially~~ transferred to pico-plankton, which **dominated** ~~is the most abundant plankton community in~~ the WTSP, suggesting that N_2 fixation fueled the growth of biomass in the N-depleted environment. This is consistent with Caffin et al., (2018), who revealed that N_2 fixation provides > ~~more than~~ 90 % of the new N **to** the photic layer **of the WTSP** ~~subsequently transformed into bio-available through DDN release, and indicated that N_2 fixation contributed to 15-21 % of the PP in the MA waters and ~4 % in the GY waters~~. On a larger scale view, the simulation performed by Dutheil et al. (this issue) predicts that diazotrophs support a large part of PP (~15 %) in LNLC regions of the Pacific Ocean, comprising the WTSP.

Overall, this **study** ~~clearly~~ indicates that ~~in the WTSP the~~ N_2 fixation plays a key role on the marine biomass production, **the structure of** ~~subsequently on~~ the planktonic food web ~~associated~~, and finally on the export **of organic matter** towards the deep ocean. The DDN can be exported to the deep ocean by different **path**ways: i) **the** direct of export of diazotrophs, ii) **the** export of non-diazotrophs which benefit**ed** from the DDN ~~transfer~~, and iii) **the** export of zooplankton which benefit**ed** from the DDN ~~transfer~~. The direct export **of diazotrophs accounted for** ~~quantification in the WTSP, indicates a direct carbon export associated to diazotrophs of~~ ~ 30 % **of total C export** at LD A (E1), 5 % at LD B (E2) and < 0.1 % at LD C (E3) (Caffin et al., 2018). **Using a $\delta^{15}N$ budget, Knapp et al., (This issue) found that 50-80 % of exported material was sustained by N_2 fixation (this includes both direct and indirect export of DDN).** ~~The low ^{15}N enrichment of the particulate matter recovered in sediment trap deployed at LD A, LD B and LD C indicates that N_2 fixation significantly contributed to particulate export (Knapp et al., this issue), either by direct or indirect export, in the WTSP.~~ Thus, N_2 fixation has ineluctably a key role on the biological carbon pump, as mentioned in Moutin et al. (this issue) who reveal a significant biological "soft tissue" carbon pump in the MA waters **almost exclusively** sustained ~~almost exclusively~~ by N_2 fixation, and acting as a net sink ~~for~~ **of** atmospheric CO_2 **in the WTSP**.

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Table 1: Environmental conditions at stations where experiments were performed. Station position, depth of sampling, concentrations of Chl a, NO_3^-, NH4+, DON, PO_4^-, DOP, dominant phytoplankton communities and dominant diazotrophs.

Experiment		E1 / Zoo-1	Zoo-2	E2 / Zoo-3	E3 / Zoo-4	References
Position	Lat. - Lon.	19°12.8'S 164°41.3'E	20°57'S 178°39'E	18°14.4'S 170°51.5'W	18°25.2'S 165°56.4'W	
Depth	m	6	6	6	55	
Chl a	µg L^{-1}	0.197	0.159	0.377	0.053	Dupouy et al., this issue
NO_3^-	nmol L^{-1}	30	< 10	20	20	
NH_4^+	nmol L^{-1}	5	2	8	1	
DON	µmol L^{-1}	6.20	5.20	6.00	5.15	
PO_4^-	µmol L^{-1}	10	10	20	110	
DOP	µmol L^{-1}	0.15	0.17	0.17	0.15	
Prochlorococcus	10^{11} cells m^{-2} integrated on the upper photic layer	122 ± 31		183 ± 27	110 ± 9	Bock et al., this issue
Synechococcus		3 ± 2		16 ± 9	0.5 ± 0.2	
Bacteria		271 ± 73		424 ± 108	290 ± 32	
Dominant diazotroph	% nifH gene copies	*Trichodesmium* 95 %	*Trichodesmium* 99 % *	*Trichodesmium* 100 %	UCYN-B 82 %	Stenegren et al., this issue

Figure captions

Figure 1: NanoSIMS images showing the ^{15}N-enrichment (a,b,d,e) after 48 h of incubation in the presence of $^{15}N_2$ for *Trichodesmium* (a), UCYN-B (b), Nano-Eukaryotes (d) and *Synechococcus* (e). The ROIs are represented with white line. NanoSIMS images showing the secondary electrons channel of UCYN ~~(e)~~ (c) and optical camera image of *Prochlorococcus* spotted on the filter before NanoSIMS analyses (f).

Figure 2: N_2 fixation rates (dark grey, nmol N L^{-1} 48 h^{-1}), DDN release nmol N L^{-1} 48 h^{-1}) as NH_4^+ (light grey) and DON (white) for each experiment (E1, E2 and E3). Error bars represent the standard deviation of triplicate incubations.

Figure 3: Left panels: box-plot of the 15N-enrichment measured in diazotrophs (*Trichodesmium* for E1 and E3, and UCYN-B for E3). Right panels: ^{15}N-enrichment measured in non-diazotrophic plankton: *Synechococcus*, *Prochlorococcus*, Bacteria, Diatoms, Pico-Eukaryotes and Nano-Eukaryotes for each experiment. Black dotted line indicates the natural isotopic enrichment.

Figure 4: DDN fate after 48 h for each experiment. Left pie charts: Orange: DDN remained in diazotrophs (orange), yellow: DDN released to the dissolved pool, Dark blue: DDN transferred to non diazotrophic plankton Right pie charts, from dark blue to light blue: Relative DDN transferred to *Synechococcus*, *Prochlorococcus*, bacteria, diatoms, pico-eukaryotes and nano-eukaryotes in E1 (top), E2 (middle) and E3 (bottom pie chart)..

Figure 5: DD^{15}N transferred (%) in the NH_4^+ pool (white), zooplankton (light grey) and remained in the phytoplankton pool (dark grey) after 24 h of incubation. Error bars represent the standard deviation of triplicate incubations and the propagated analytical errors

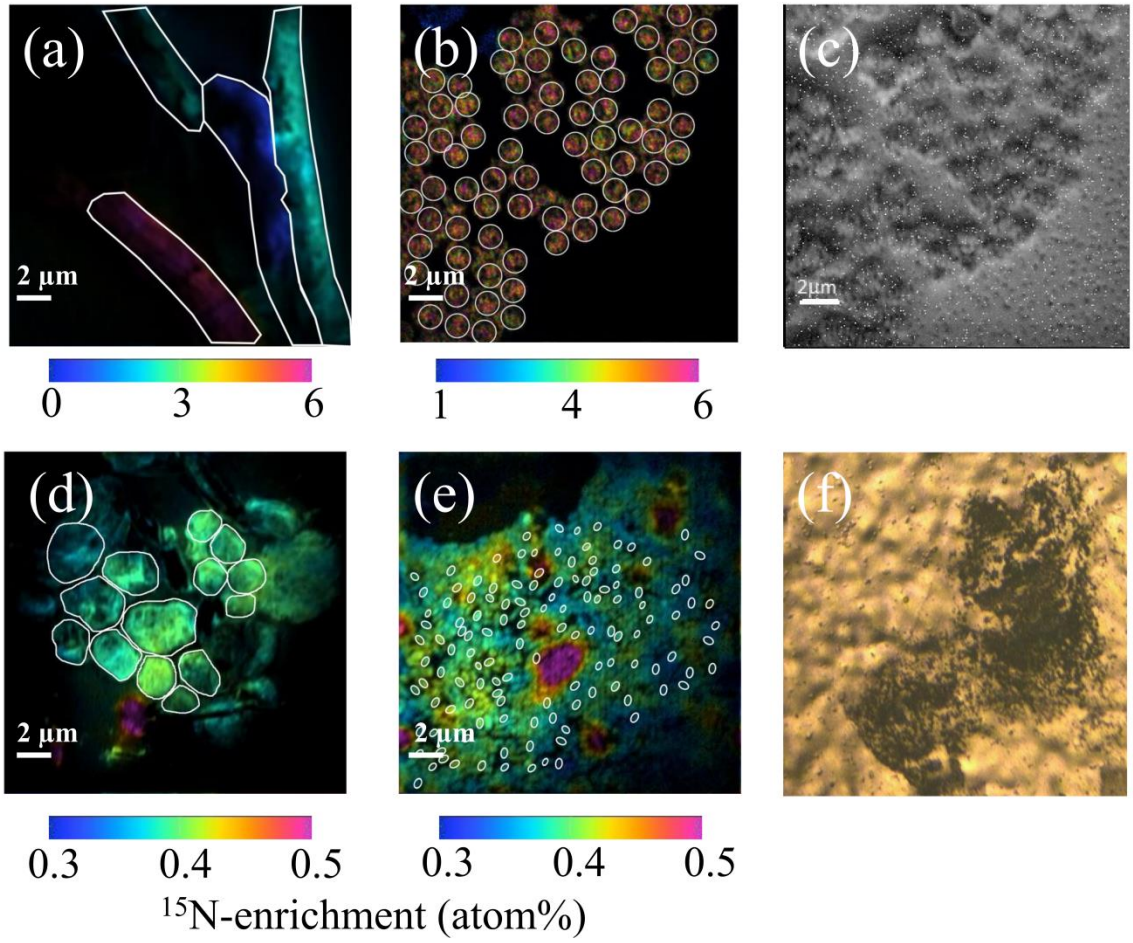
(a)
2 µm
(b)
2 µm
(c)
2µm
0
3
6
1
4
6
(d)
2 µm
(e)
2 µm
(f)
0.3
0.4
0.5
0.3
0.4
0.5
15N-enrichment (atom%)

Figure 1

5

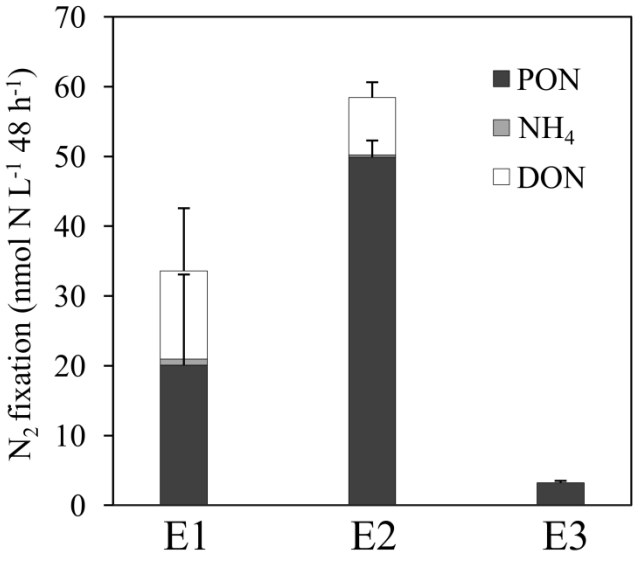
70
60
50
40
30
20
10
0
N_2 fixation (nmol N L^{-1} 48 h^{-1})
PON
NH_4
DON
E1
E2
E3

Figure 2

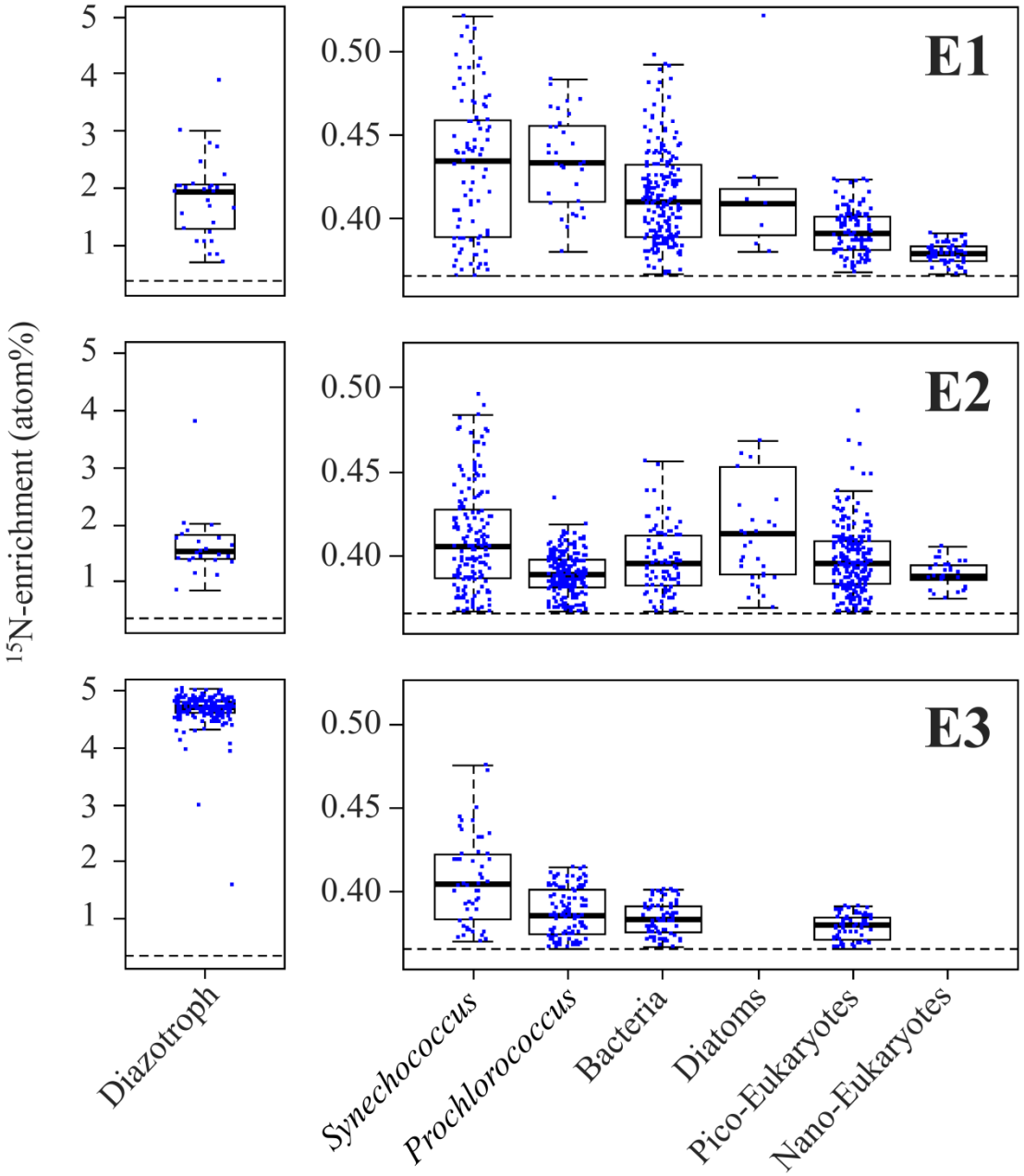
E1
E2
E3
15N-enrichment (atom%)
Diazotroph
Synechococcus
Prochlorococcus
Bacteria
Diatoms
Pico-Eukaryotes
Nano-Eukaryotes

Figure 3

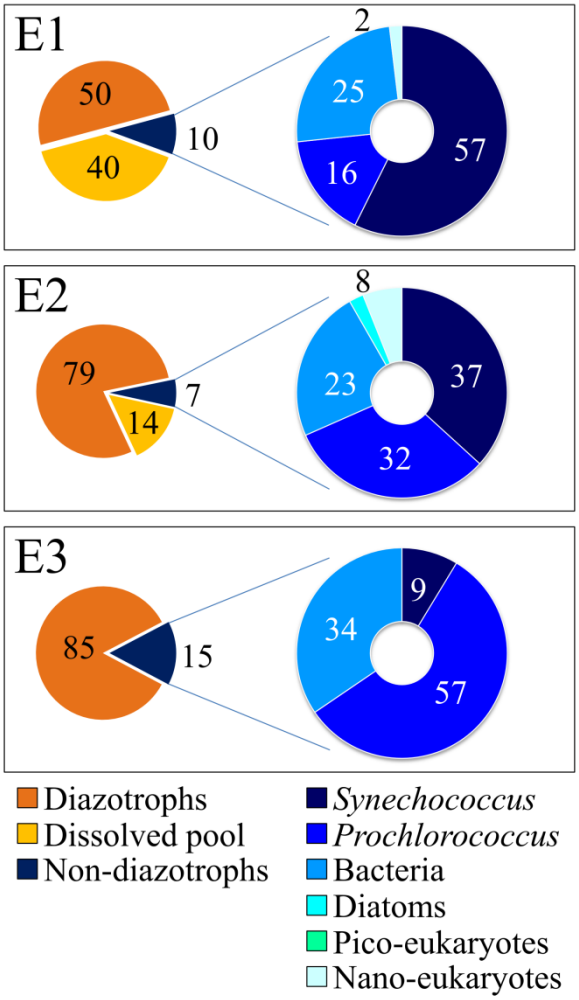
E1
50
40
10
2
25
16
57
E2
79
14
7
8
23
32
37
E3
85
15
34
9
57
Diazotrophs
Dissolved pool
Non-diazotrophs
Synechococcus
Prochlorococcus
Bacteria
Diatoms
Pico-eukaryotes
Nano-eukaryotes

Figure 4

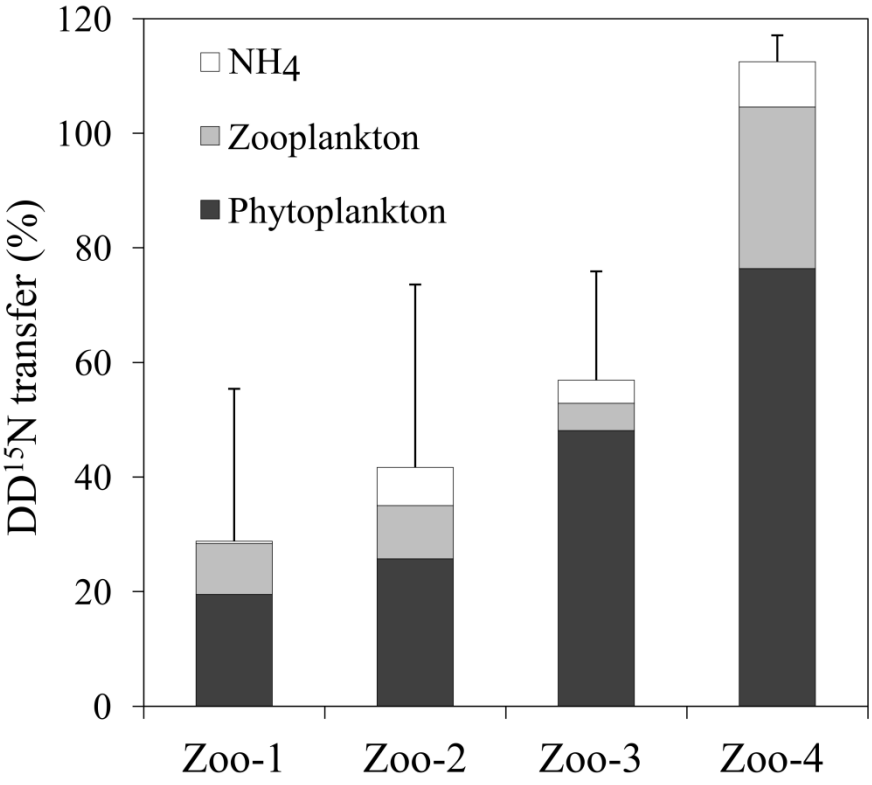
120
100
80
60
40
20
0
DD15N transfer (%)
NH4
Zooplankton
Phytoplankton
Zoo-1
Zoo-2
Zoo-3
Zoo-4

Figure 5

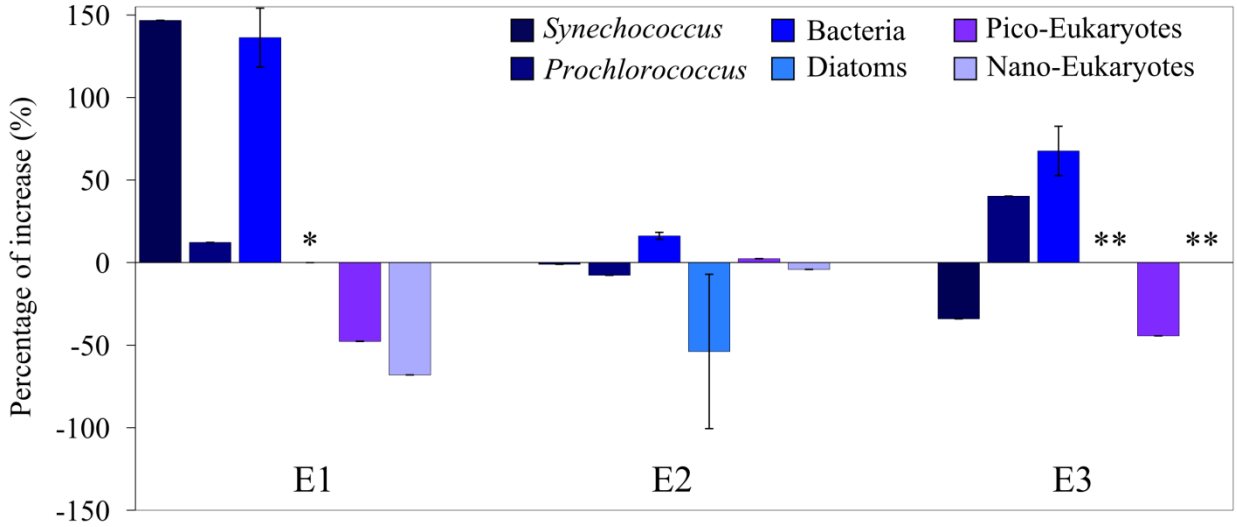

Figure 1: Relative increase of cells abundance associated to *Synechococcus*, *Prochlorococcus*, bacteria, diatoms, pico-eukaryotes and nano-eukaryotes (blue pattern) after 48 h for E1, E2 and E3. Error bars represent the standard deviation of triplicate counts and the propagated analytical errors. * Diatoms were not counted at T0. ** Diatoms and nano-eukaryotes were not analyzed for E3.

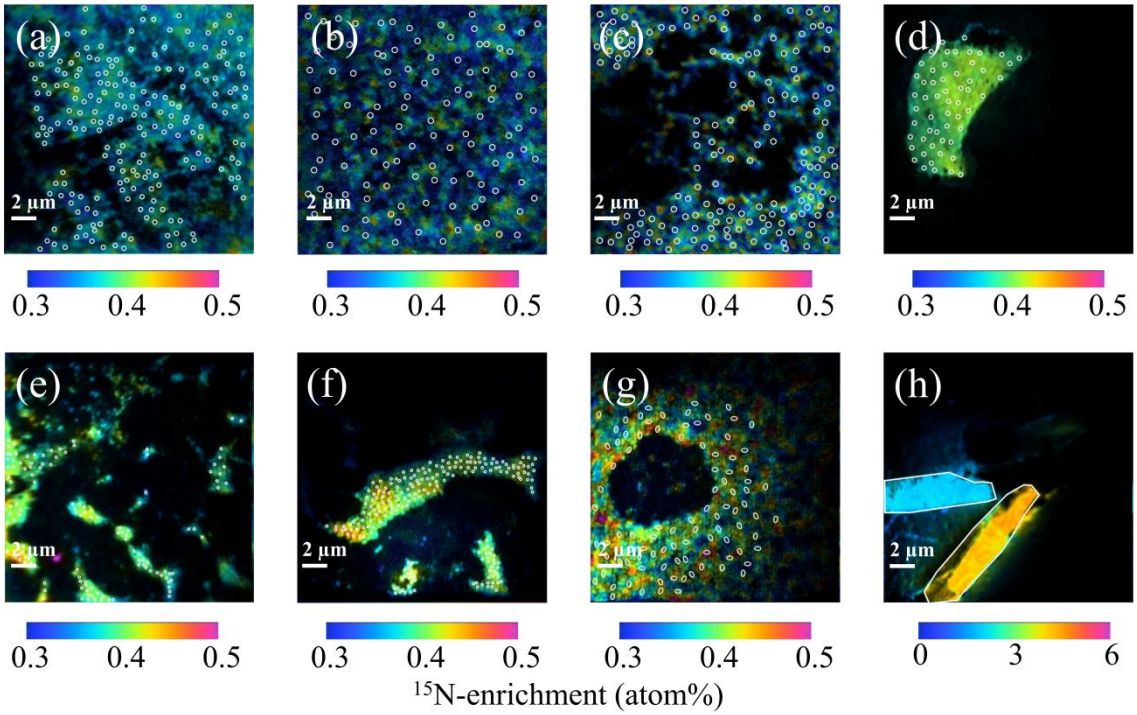

Figure 2: NanoSIMS images showing the ^{15}N-enrichment after 48 h of incubation in the presence of $^{15}N_2$ for *Prochlorococcus* (a,b), pico-eukaryotes (c,d), heterotrophic bacteria (e,f), *Synechococcus* (g), and *Trichodesmium* (h). The ROIs are represented in white line.

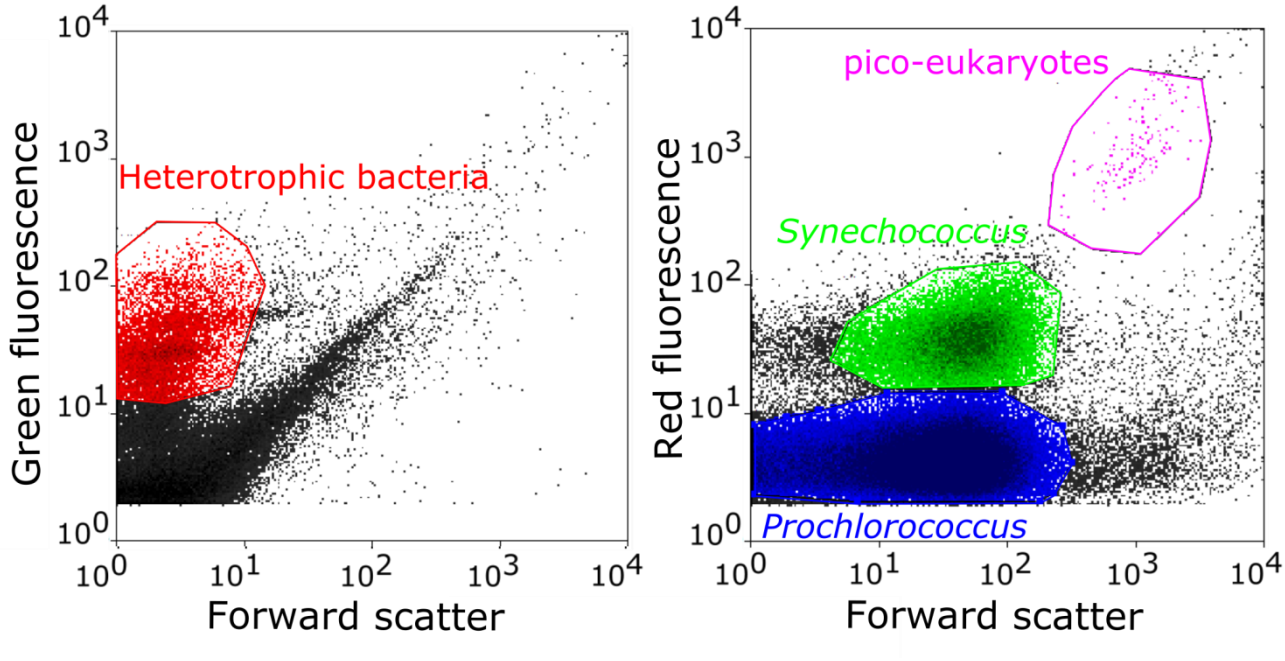

Figure 3: Clustering of planktonic communities by flow cytometry on green fluorescence vs. forward scatter cytograms: heterotrophic bacteria (red), *Prochlorococcus* (blue), *Synechococcus* (green), and the pico-eukaryotes (pink)

5

Table 1: Number of ROIs analyzed for diazotrophs (*Trichodesmium* in E1 and E2, UCYN-B in E3), *Synechococcus*, *Prochlorococcus*, bacteria, diatoms, pico-eukaryotes and nano-eukaryotes, for E1, E2 and E3.

Experiment	diazotrophs	*Synechococcus*	*Prochlorococcus*	bacteria	diatoms	pico-euk.	nano-euk.
E1	30	87	32	200	8	111	60
E2	25	156	213	85	33	200	29
E3	192	50	115	70	0	70	0