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.