We have taken all the comments of the reviewers into account in the revision; replies to each of the comments are provided below in blue fonts.

In response to one of the reviewers' suggestions, we have carefully considered how to appropriately acknowledge the contributions of the data contributors in our revised manuscript, particularly in relation to this database paper. Furthermore, we have strived to ensure the high quality and completeness of both the database and the accompanying paper. To achieve these goals, we extended an invitation to all lead authors of the published data, inviting them to join us as coauthors of the manuscript.

We are delighted to share that nearly 90 lead authors have accepted our invitation and have become coauthors of the revised manuscript. Their involvement has been invaluable, as they have not only revised the manuscript, but also contributed to data analyses, providing unpublished data, and identifying missing historical datasets. The addition of these co-authors has significantly elevated the overall quality of the database and the manuscript, while also exemplifying the collaborative efforts of the entire diazotroph survey community.

Reviewer #1:

Shao et al. present an update of the diazotroph database published in 2012 https://essd.copernicus.org/articles/4/47/2012/

The new version adds up data published between 2012 and 2023, including volumetric and depth-integrated N_2 fixation rates, diazotroph microscope counts and nifH gene counts. This new version also discusses microscope-nifH count comparisons. While this update is valuable for the community as a tool for comparison and contextualization of diazotrophy studies, it fails to account for many diazotrophy studies published between 2012 and 2023. The text has several misinterpretations that need correction. The new version also includes N2 fixation rates proxied with other methods (ARA). I think this is a major problem, since these rates are not currently solidly comparable and downplay the robustness of the database. The manuscript also eliminates nifH gene counts from non-cyanobacterial diazotrophs (NCDs), which is another major issue since NCDs are considered to be outnumber cyanobacterial diazotrophs in the ocean. Finally, the diazotroph microscopy count versus nifH gene count conversion discussion does not seem appropriate here, since very few of the papers listed have compared these approaches on a same given sample, and the issue has been discussed thoroughly in other publications by specialists.
In all, while I acknowledge the effort and usefulness of this manuscript, I advise major revisions as detailed in the comments below.

Response: We thank the reviewer for very constructive and thorough comments. We particularly appreciate the reviewer's suggestion to include PIs of the data sources as coauthors, and we have adopted this suggestion. We are pleased to report that nearly 90 lead authors have agreed to join this collaborative effort. By doing so, we believe that this paper will not only showcase the significant impact of collective research efforts in N_2 fixation, but also the completeness of the database and the quality of the paper can be improved substantially. The new coauthors have identified missing datasets and provided additional comments that will further enhance the quality of the paper. Other general comments have also been addressed:

(1) We have decided not to include the ARA-based data in estimating the global N_2 fixation

rate, but we have included them in the database for those who are interested in using them.

(2) The NCD data have been added to the database as an additional datasheet.

(3) We have decided to keep the comparison of *nifH* gene copies and diazotrophic cell counts in the paper, and we invite you to review our response to the related comments.

Once again, we thank the reviewer for the valuable feedback, which has helped us to improve the quality of our manuscript considerably.

L28: N_2 gas is not inert to diazotrophs.

Response: We have changed the text to "Dinitrogen (N_2) fixation is a process carried out by select prokaryotes (diazotrophs) capable of converting N_2 gas, which is not usable by most organisms, into bioavailable nitrogen (N)."

L31: The balance between N loss/gains in the ETSP has been widely demonstrated to be false in several publications after that of Deutsch et al., see for example (Knapp et al. 2016; Bonnet et al. 2017).

Response: Thanks for the comment. Here, we tried to introduce the general function of nitrogen fixation on the global scale. To avoid misleading, we have revised the text to " Globally, N_2 fixation serves to compensate, at least partially, for fixed N removed via denitrification and anammox (Deutsch et al., 2007; Gruber, 2019)".

L35: Only cyanobacterial diazotrophs can be confidently counted by microscopy.

Response: The text has been revised as "Diazotroph abundance can be estimated from *nifH* gene copies using qPCR assays (Church et al., 2005) or droplet digital PCR (ddPCR) (Gradoville et al., 2017). The abundance of some cyanobacterial diazotrophs can also be obtained by counting them directly using microscopy-based techniques and in some cases flow cytometry."

L36: "NifH gene copies"

Response: Corrected.

L40: This issue has been thoroughly discussed in (Gradoville et al. 2022), validating the use of nifH gene counts as a means to quantify diazotrophs.

Response: Gradoville et al. (2022a) is a regional study in which all the diazotrophs were sampled in two cruises (June 2017 and April 2018) near the Hawaii Islands or along a transect of several hundred kilometers at fixed depths (5 m and 15 m, respectively). Gradoville et al.

(2022a) described their study as: "... expeditions which each spanned >200 km (Fig. 1). While limited, this reflects the most geographically extensive field comparison of *nif*H:cell among taxa to date."

Hence, although Gradoville et al. (2022a) has shown a strong relationship between *nifH* gene counts and diazotrophic (*Crocosphaera*, *Richelia* and *Calothrix*) abundances, it has not sufficiently indicated that this finding is applicable to diazotrophs sampled in other regions or time. Gradoville et al. (2022a) partly attributed the large varieties of *nifH*:cell found in Sargent et al., (2016) and White et al. (2018) to potential methodological issues; but they also concluded that "*nif*H is a useful yet imperfect abundance proxy" and urged "future studies report *nif*H:cell and explore the mechanisms controlling this ratio". Both Drs. Gradoville and White are now coauthors of this paper; they have agreed this interpretion of their paper.

We have therefore decided to keep this sentence in our revised manuscript, followed by an introduction of Gradoville et al. (2022a): " However, a recent regional study spanning over 200 km in the North Pacific Subtropical Gyre has found a statistically significant linear correlation between the abundances of the *nifH* gene and cell counts in the UCYN-B (i.e., *Crocosphaera*) (linear slope = 1.82) and heterocystous cyanobacteria (*Richelia* and *Calothrix*; linear slope from 1.51-2.58) but not in *Trichodesmium* (Gradoville et al., 2022b). A recent discussion highlighted the influence of the uncertainty in gene copy conversion to biomass and the need for further investigations on how to best take advantage of gene copy data for global diazotroph biogeography modelling purposes (Meiler et al., 2022; Zehr and Riemann, 2023); however, there is an agreement that quantifying gene counts is a powerful tool for studying marine diazotroph distributions (Meiler et al., 2023; Zehr and Riemann, 2023). Meiler et al., (2023) proposed a number of topics of study for this field moving forward; Gradoville et al. (2022) concluded that "we hope that future studies report *nifH*:cell and explore the mechanisms controlling this ratio." Both gene based and microscopy cell counts have innate biases, which should be elucidated in future studies. "

L42-47: Other sources of unbalance should be briefly mentioned here.

Response: Thanks for the suggestion. The text has been revised as:

"While the overestimation of the N losses cannot be ruled out, one of possible reasons for this imbalance is the inaccurate estimation of global marine N_2 fixation due to limited spatio-temporal coverage of rate measurements and the different methods employed in N_2 fixation assays (White et al., 2020). Another possible reason is the limited knowledge of ecological niches of N_2 fixing organisms. Over the last decade, the realm of marine N_2 fixation has been expanded to include numerous non-paradigmatic habitats. Coastal (Mulholland et al., 2012; Bentzon-Tilia et al., 2015; Mulholland et al., 2019; Tang et al., 2020; Turk-Kubo et al., 2021), subpolar (Sato et al., 2021; Shiozaki et al., 2018), and even polar ocean regions (Blais et al.,

2012; Sipler et al., 2017; Harding et al., 2018; Shiozaki et al., 2020) have demonstrated N_2 fixation. Notably, N_2 fixation in aphotic waters remains debated (Bonnet et al., 2013; Farnelid et al., 2013; Selden et al., 2021; Rahav et al., 2013; Hamersley et al., 2011; Benavides et al., 2018). Other studies have also suggested that NCDs may be significant contributors to marine N_2 fixation (Shiozaki et al., 2014; Turk-Kubo et al., 2022; Geisler et al., 2020; Delmont et al., 2021; Karlusich et al., 2021; Bombar et al., 2016; Moisander et al., 2017) and may occupy different niches from cyanobacterial diazotrophs (Shao and Luo, 2022)."

L50: Diazotroph activity was there before, it is our notion of them that increases, the data available.

Response: Thanks for the comment. The text has been revised and combined into the above paragraph (see response immediately above).

L56-57: I don't think that the dataset assembled here covers enough studies comparing microscopy and nifH based comparisons, and I strongly recommend removing this sentence and section 4.2 from the manuscript.

Response: We thank the reviewer for the comment. We believe it is necessary to include the comparisons of cell counts and *nifH* gene copies in the manuscript for two reasons. First, as discussed in our response to the above comment, the relationship between cell counts and *nifH* gene copies is still an issue undergoing discussions. Second, a large number of measurements have been conducted on diazotrophic cell counts and *nifH* gene copies, particularly those of *Trichodesmium* and *Richelia* (Fig 16, n = 3572 vs. 3098 for *Trichodesmium*; 1309 vs. 1914 for *Richelia*). These comparisons can reveal the overall distributions of cell counts and *nifH* gene copies in specific diazotrophic groups, providing another angle as a meta-analysis that complements previous studies that have directly compared *nifH* and cell counts using a limited number of samples.

We have also slightly revised the texts to more accurately describe our analyses. In the end of Introduction:

"In light of the aforementioned concerns of *nifH*:cell and various N_2 fixation methods (*see* **Section 2.3**), we also discuss the significance of employing different methodological approaches to estimate N_2 fixation rates and abundance metrics. We use the data available in the database to analyze the discrepancies between N_2 fixation rates using $^{15}N_2$ bubble and dissolution methods, and compare the observed ranges of *nifH* gene copies and diazotrophic cell abundance."

At end of the section for analyzing nifH abundance and cell counts, we have also added a full paragraphy and a summary table for discussing this issue:

The application of qPCR assays for *nifH* based abundance (DNA) and expression (RNA) emerged as a critical step forward in our understanding of the distribution, abundance, and physiology (e.g., expression of *nifH*) of diazotrophs (Short and Zehr, 2005; Zehr and Riemann, 2023). Until then, estimating the abundances of diazotrophs were limited to those that could be identified by microscopy, e.g., *Trichodesmium*, heterocystous cyanobacteria (e.g., *Richelia*, *Calothrix, Anabaena, Nodularia, Aphanizomenon*), and some unicellulars (e.g., *Cyanothece*, later *Crocosphaera*). Thus, qPCR enabled the study of diazotrophic targets (and their activity) without the need to microscopy identify them, which came later as some diazotrophs would (and still) require application of FISH techniques for identification (Biegala and Raimbault, 2008). Additionally, qPCR allowed the study of *in situ* activity (gene expression) by diazotrophs without the need for cultivation. Although beyond the scope of the work presented here, important considerations should be taken into account when using microscopy and qPCR datasets (Table S3), for example, in application to biogeochemical models (Meiler et al., 2023).

Table S3. Summary of a few considerations for application and interpretation of qPCR and microscopy counting for enumeration and activity (RNA) of diazotrophs.

Consideration	**Comment**
Cell identity	*Microscopy:* Cross-comparison of cell counts can be difficult as training and experience varies.
Patchy distribution, low abundance	*Both methods:* collection of samples (volumes, depths) are dependent on logistics; collection strategies can vary: size fractionation, gravity filtration (microscopy), etc. *Microscopy*: potential to underestimate/overestimate if the minimum number of cells is not enumerated. *qPCR:* potential to underestimate if targets are below detection of assay (1-10 copies).
Dead or moribund cells	*Both methods* do not distinguish vitality, thus potential to overestimate.
Primer design	*qPCR*: potential to overestimate if primers cross-react with non-targets; potential to underestimate if primers are too specific to a limited/unknown micro- diversity.
Polyploidy	Some bacteria, including data on *Trichodesmium*, *Richelia*, generate multiple genome copies during their life cycle (Sargent et al., 2016; White et al., 2018; Karlush et al., 2021).
Gene copy number	Filamentous cyanobacteria (includes heterocystous cyanobacteria) possess a genome copy in each cell; it is not known for all diazotrophs the number of *nifH* copies/cell, often assumed to be one.
DNA/RNA Extraction efficiency	Not all targets extract uniformly; RNA is prone to degrade

L61: The N_2 fixation rates from Tang et al. 2019 are based on an ARA-$^{15}N_2$ fixation comparison including only 8 data points. This is not robust enough to provide a reliable comparison and downplays the robustness of the $^{15}N_2$-based rates dataset collected here. I strongly recommend removing these from the database and derived basin-scale and global calculations. These may be mentioned as discussion and the Tang paper cited, but not included for quantitative purposes.

Response: Thanks for the comment. Here, we referred to a diazotroph dataset compiled by Tang et al. (2019) and Tang and Cassar (2019) with historical measurements reported by other studies in 2012-2018. There were other in-situ N_2 fixation rates (15 $^{15}N_2$-based and 85 ARA-based measurements) measured by Cassar/Tang's own group (Tang et al., 2019; Tang et al., 2020); these data were also collected into our database. The derived N_2 fixation rates in Tang et al. (2019) were not collected into our database.

We reconsidered ARA-based measurements of N_2 fixation rates and agreed with the reviewer. We have decided not to include the ARA-based data in estimating the global N_2 fixation rate, while keep them in the database for those who are interested in using them.

L72: Removing NCDs is an error in my opinion. NCDs have recurrently been shown to be dominant in the ocean (Farnelid et al. 2011; Delmont et al. 2018, 2021; Riemann, Farnelid, and Steward 2010) and may impact N cycling decisively (Riemann et al. 2022; Turk-Kubo et al. 2022). I strongly recommend that any nifH gene counts of NCDs are added. The previous database included Gamma A and Cluster III. I don't see a solid reason to remove NCDs from the database at this stage, as evidence of their importance increases.

Response: Thanks for the comment. One of the reasons why we did not include NCD data was the existence of a comprehensive NCD dataset compiled by Turk-Kubo et al. (2022). We have now obtained the agreement from Dr. Turk-Kubo to include her NCD dataset in the database (she has agreed to be a coauthor of the revised manuscript). Additional NCD data published in several recent studies have also been added to the revised database. We then accordingly changed the sentence to:

"Additionally we included a compiled NCD dataset (Turk-Kubo et al., 2022) in the database, which contained 7,919 *nifH* gene copy abundances of primarily the most studied phylotype NCD Gamma A (Shao and Luo, 2022; Langlois et al., 2015), also referred to as 24774A11 (Moisander et al., 2012) and UMB (Bird et al., 2005), as well as other phylotypes, and updated that compilation with 469 additional *nifH* gene copy abundances of NCDs published more recently (Turk-Kubo et al., 2021; Sato et al., 2022; Moore et al., 2018; Reeder et al., 2022; Wen et al., 2022; Bonnet et al., 2023). We also collected 468 cell-specific *in situ* N_2 fixation rates and added them to version 2."

Line 82: Group-specific N_2 fixation rates can only be estimated using single-cell approaches. I'm not sure what approach was followed here to derive specific rates, but these can certainly not be estimated with the data collected here. I would rather recommend the authors to collect all *Trichodesmium*, UCYN-B, DDAs and UCYN-A single-cell rates published, which would be

very helpful for the community. See for instance (Foster, Sztejrenszus, and Kuypers 2013; Foster et al. 2011; Benavides et al. 2017; Bonnet et al. 2016; Filella et al. 2022; Krupke et al. 2015; K. Harding et al. 2018; Mills et al. 2020; K. J. Harding et al. 2022; Benavides et al. 2022).

Response: Thanks for the comment and we are sorry for the confusing. The "different groups" here referred to different size groups. In the original database of 2012, N_2 fixation rates in samples with size >10 μm were assigned to *Trichodesmium* and those of smaller sizes were assigned to UCYN. In the revised database, we have corrected and reported them as N_2 fixation rates of size groups > 10 μm and < 10 μm, respectively. In some studies, N_2 fixation rates of *Trichodesmium* and heterocystous cyanobacteria were estimated by multiplying their cell abundance with their cell-specific N_2 fixation rates; we also collected these diazotrophic group-specific data into the new version of the database.

We agree with the reviewer that the cell-specific N_2 fixation rates are important and valuable. The cell-specific N_2 fixation rates suggested by the reviewer, as well as other identified data sources, have been collected into the revised database as a new datasheet.

The paragraph has been revised as:

" N_2 fixation rates were measured for whole seawater samples, for different size fractions (> 10 μm and < 10 μm), or specifically for *Trichodesmium* and heterocystous cyanobacteria. When whole-water N_2 fixation rates were not reported, total N_2 fixation rates were calculated as the sum of the N_2 fixation rates of available groups."

"We also collected 468 cell-specific *in situ* N_2 fixation rates and added them to version 2."

Tables 2 and 4: Many studies are missing in this table, some include (Benavides et al. 2014, 2021; Saulia et al. 2020; Henke et al. 2018; Bonnet et al. 2018; Gradoville et al. 2017; Moreira-Coello et al. 2017; Wilson et al. 2019). Also, in the table some studies are listed as not including counts of some diazotrophs, which needs correction (e.g. Bombar 2011 and Bonnet 2015, 2019 did have qPCR counts). Please revise all these publications thoroughly and correct accordingly.

Response: We thank the reviewer for identifying missing datasets and parameters. We have checked datasets suggested by the reviewer, and have added those parameters collected by this database. With help from other coauthors, much more missing datasets (more than 40 publications) have been identified and added to the revised database. We are confident that nearly all the previous published data have now been included in the database.

L104: The ARA method is rarely used nowadays

Response: The reviewer was correct. We have revised the texts to:
"The commonly used methods for marine N_2 fixation rates include $^{15}N_2$ tracer methods and acetylene reduction assay (Mohr et al., 2010; Montoya et al., 1996; Capone, 1993). However, in the last decade, the community has turned largely to the use of $^{15}N_2$ tracer methods."

L106-107: The ARA to N_2 fixation ratio is highly variable (Mulholland et al. 2006; Benavides et al. 2011; Wilson et al. 2012)

Response: Thanks for this comment. We have added the previously reported range of the conversion factor between acetylene reduction and N_2 fixation:

"Theoretical conversion factors of 3:1 or 4:1 have been used to convert acetylene reduction rates to N_2 fixation rates (Postgate, 1998; Capone, 1993; Wilson et al., 2012), although a wide range of conversion factors from 0.93 to 56 have been reported (e.g., Mague et al., 1974; Graham et al., 1980; Montoya et al., 1996; Capone et al., 2005; Mulholland et al., 2006; Wilson et al., 2012)."

L110: Many other factors affect this difference, including acetylene gas impurity, Bunsen dissolution coefficient, etc.

L112: This is not true. The $^{15}N_2$ method is much more sensitive, does not require biomass preconcentration (biomass is concentrated during filtration, after the incubation), and requires longer incubations for enough tracer to be detectable in biomass. ARA is usually done in 3-4 h incubations and requires biomass pre-concentration to reach detectable signal (Staal et al. 2007; Benavides et al. 2011).

Response: We thank the reviewer for the above two related comments regarding comparing $^{15}N_2$ assimilation and the acetylene reduction assay. We have incorporated the reviewer's comments and corrections and modified the texts as follows:

"When using the $^{15}N_2$ tracer method, samples are incubated in seawater with $^{15}N_2$; the $^{15}N/^{14}N$ ratio of particulate nitrogen is measured at the beginning and at end of the incubation to calculate the N_2 fixation rate (Capone and Montoya, 2001). Most measurements using the $^{15}N_2$ tracer method only counted the fixed N in particulate forms and ignored the N that was fixed but then excreted by diazotrophs in form of dissolved organic N (DON) during incubation, which could theoretically be counted by the acetylene reduction assays (Mulholland, 2007). In some studies using the $^{15}N_2$ tracer method, this missing N was counted by also measuring the ^{15}N enrichment in DON (Berthelot et al., 2017; Benavides et al., 2013a; Berthelot et al., 2015; Benavides et al., 2013b).

Compared to the $^{15}N_2$ tracer method, the acetylene reduction assay needs a shorter incubation time. However, in addition to the uncertainty in converting ethylene production to N_2 fixation, the purity of acetylene gas, trace ethylene contamination, and the Bunsen gas solubility coefficient of produced ethylene can also affect the accuracy of estimated N_2 fixation rates (Hyman and Arp, 1987; Breitbarth et al., 2004; Kitajima et al., 2009). Acetylene used in the assay can even impact the metabolic activities of diazotrophs (Giller, 1987; Hardy et al., 1973; Flett et al., 1976; Staal et al., 2001). Moreover, the acetylene reduction assay needs to pre-concentrate cells for signal detection when diazotrophic biomass is low, which may lead to underestimated N_2 fixation rates by perturbing cells during concentration and filtration (e.g., Capone et al., 2005; Barthel et al., 1989; Staal et al., 2007). In recent years, the acetylene reduction assay has undergone significant advancements. The sensitivity of ethylene detection has been improved by utilizing a reduced gas analyzer (Wilson et al., 2012) and by using highly purified acetylene gas to minimize the ethylene background (Kitajima et al., 2009). However, the preparation of high-purity acetylene with low level of ethylene contamination remains a challenge. More recently, a new method named Flow-through incubation Acetylene Reduction Assays by Cavity ring-down laser Absorption Spectroscopy (FARACAS) has been introduced for high-frequency measurements of aquatic N_2 fixation (Cassar et al., 2018). This method involves continuous flow-through incubations and spectral monitoring of the acetylene reduction to ethylene. By employing short-duration flow-through incubations without cell preconcentration, potential artifacts are minimized. This approach also allows for near real-time

estimates, enabling adaptive sampling strategies."

L120: Wannicke et al. say the opposite of Mohr and Grosskopf.

L123: What White et al. say is that the bubble release method is the most reliable and recommended by the diazotroph research community, with the elimination of rate underestimation benefits overcoming the very unlikely burdens of contamination. This should be corrected in L274-275 as well.

Response: Thanks for pointing out these two mistakes. We have carefully revised whole section:

"The original $^{15}N_2$ tracer method involved addition of a known volume of $^{15}N_2$-labelled bubbles to the incubation bottle (named *original $^{15}N_2$ bubble method* hereafter). However, this method was later found to underestimate rates because N_2 gas solubility is low and tracer additions take a long time to equilibrate (Mohr et al., 2010; Großkopf et al., 2012; Jayakumar et al., 2017). To address this issue, the *$^{15}N_2$ dissolution method* has been employed, which involves pre-preparing $^{15}N_2$-enriched seawater to maintain a constant $^{15}N_2$ atom% enrichment throughout the incubation (Mohr et al., 2010), similar to the method described in Glibert and Bronk (1994). However, the $^{15}N_2$ dissolution method does not always yield higher N_2 fixation rates than the original $^{15}N_2$ bubble method (Table S4 in Großkopf et al., 2012; Saulia et al., 2020); it is still not conclusive what control the magnitude of the underestimation (if it exists) by the original $^{15}N_2$ bubble method. Compared to the original $^{15}N_2$ bubble method, the $^{15}N_2$ dissolution method is more susceptible to the introduction of contaminants (e.g., metals) during the preparation of the $^{15}N_2$ inoculum due to its more complex process, which can alter the diazotrophic activities and abundance, thereby impacting the accuracy of N_2 fixation measurements. (Dabundo et al., 2014; Klawonn et al., 2015). For example, Needoba et al. (2007) reported that a low but detectable amount of Fe^{3+} contamination can be measured when protecting the needle of the gas-tight syringe with a commercially available tubing. Additionally, pH and other chemical properties of the inoculum may be altered during its preparation, further affecting the measurements of N_2 fixation. Despite these limitations, the $^{15}N_2$ dissolution method remains the predominant assay for measuring N_2 fixation rate due to its ability to satisfy the fundamental assumption of constant $^{15}N_2$ atom% enrichment over the incubation period.

More recently, a modified $^{15}N_2$ bubble method, known as the *$^{15}N_2$ bubble release method*, has been proposed as an alternative to the $^{15}N_2$ dissolution method (Klawonn et al., 2015; Chang et al., 2019; Selden et al., 2019). This method involves adding $^{15}N_2$ gas to the incubation bottles and mixing for a brief period (~15 min) to facilitate $^{15}N_2$ equilibration, then removing the gas bubble. Compared to the original $^{15}N_2$ bubble method, the $^{15}N_2$ bubble release method ensures a uniform $^{15}N_2$ atom% enrichment throughout the incubation. Moreover, it causes less interference with the incubation matrix than the $^{15}N_2$ dissolution method. However, the slow and gentle rocking of incubation bottles required to stimulate gas dissolution has been suggested

to negatively affect diazotrophs, although no robust studies have yet been performed to assess this criticism (Wannicke et al., 2018; White et al., 2020). Moreover, the $^{15}N_2$ bubble release method requires a handling step and additional costs for preparing tracers may not be allowed (White et al., 2020). Ultimately White et al. (2020) "advise employing either the dissolution or bubble release method, whichever is best suited to the specific research objectives and logistical constraints" with additional recommendations on the need for determination of detection limits for all rate measurements."

The first sentence of 4.1has also been revised as:

"To date, the discrepancy in N_2 fixation rates estimated using different $^{15}N_2$ tracer methods remains unclear."

L150: There are 4 UCYN-A sublineages (Farnelid et al. 2016).

Response: We have corrected the text as follows:

"Four sublineages of UCYN-A, including UCYN-A1, UCYN-A2, UCYN-A3, and UCYN-A4, have been identified (Thompson et al., 2014; Farnelid et al., 2016)."

L328: UCYN-A has been found in symbiosis with other eukaryotic algae (Zehr et al. 2016)

Response: The text has been revised as:

"The conversion factor for UCYN-A is also updated because it has been found to live symbiotically with haptophyte *Braarudosphaera bigelowii* and relatives (Thompson et al., 2012; Hagino et al., 2013). "

L370: The first version of the database included all the authors that had contributed to its construction with their seagoing expeditions, laboratory analyses and publications. I humbly find it sad and somewhat unfair that this is not the case in this update.

Response: We highly value the reviewer's comment and agree proper coauthorship credit is important for all contributors. Initially, our plan to publish an updated global marine diazotrophic database was too simplistic and lacked careful thinking. Since the first global marine diazotrophic database was published in 2012, our group has continuously updated the database with newly published data. In recent years, we have received numerous requests for an updated version of the database, which prompted us to consider publishing it for wider usage.

We have extended an invitation to all lead authors to join us as coauthors of the manuscript. Please refer the texts at the beginning of this file for our detailed response to this suggestion.

Reviewer #2:

This manuscript by Shao and Xu et al. describes an updated version 2 of the global oceanic diazotroph database. It build upon the previous version by adding additional measurements of

marine diazotrophic abundance, N_2 fixation rates, microscopic and qPCR-based diazotropic abundance. The spatial coverage significantly improved most notably in the Indian Ocean. The newly revised estimate for global N_2 fixation rate is significantly higher (+123 Tg N yr^{-1}, almost doubled) when calculating using a standard arithmetic mean, although surprisingly the geometric mean did not significantly change. A brief analysis and discussion of the $^{15}N_2$ bubble vs. dissolution indicated a potential general underestimation from the bubble method particularly at high rates, however noting the comparison of samples were from different times so it is not a formal error analysis (which the authors acknowledge). The database is available to download from the provided link in the abstract.

Overall, I find this to be an important update to the database mainly due to the significant increase in included measurements and spatial coverage. The database is transparent and mostly well described. The analysis and first preliminary quantification of the $^{15}N_2$ bubble vs. dissolution is also an important contribution. Perhaps some additional details/analysis could be provided (see comments below), but additional analyses can also be performed independently by users who download the data for their specific interest. There is one important aspect that needs additional clarification in my view before I would endorse this manuscript for publication (global N_2 fixation rate calculation, see below).

-Christopher Somes
GEOMAR Helmholtz Centre for Ocean Research Kiel

Response: We thank Dr. Somes for his positive and constructive comments, which have helped us improve the quality of this paper substantially. Please see our responses below.

Major Comment: Global N2 fixation calculation description

Since this paper will likely often be cited for revising the global N_2 fixation rate significantly upwards, the description of this calculation should be more transparent and comprehensive:

line 266 (Table 5 caption): "Data are first binned to 3x3 grids…"

This needs to be better described. For example, was there any type of interpolation method used or simple averaging of all measurements in each bin? It would be interesting to know what percentage of bins in each ocean basin has data coverage. How do you define the Southern Ocean region and is that area removed from the other southern regions?

How was the vertical coordinate handled? Is it evenly spaced or according to the depths ranges in Figure 7?

It is not clear to me how the "Areal sum" calculation was made based on the "Mean N_2 fixation rate" (Table 5). Does the "Mean N_2 fixation" rate include all measurements or only the "Depth-integrated N_2 rates", which requires 3 measurements in the vertical? If the vertical coordinate is uneven, do measurements that get binned into a larger volume in larger deeper layers have more weight on the depth-integrated rate than shallower layers?

When calculating the "Areal Sum", do you assume that the "Mean N2 fixation rate" extrapolates across the entire region or do you only consider the area of the bins that have data coverage? For example, the Indian Ocean has about 36% of the bins compared to the South Pacific. Therefore I was expecting a much larger decrease when calculating the Areal Sum

relative to the Mean N_2 fixation rate for the Indian Ocean compared to the South Pacific. However this relative decrease is quite subtle in Table 5 between these regions. I acknowledge there is no truly perfect way to estimate a global ocean N_2 fixation rate with the current coverage, but all of the assumptions and details that go into the calculation should be specifically stated and described.

Response: Here we respond Dr. Somes's general comments regarding the description of calculating the global marine N_2 fixation rate.

We followed the procedure used in the previous database paper (Luo et al., 2012) to estimate the global marine N_2 fixation rate. However, as reminded by the reviewer, we should describe the method in this paper, which has been added in section 2.4 in the revised manuscript.

Here are some quick answers to the reviewer's questions:

The data used in the estimation is the depth-integrated N_2 fixation rates integrated from surface to the depth of the deepest data (up to 200 m; see section 2.1). The measurements in each vertical profile were linearly interpolated, which was not clearly described in the original manuscript. We have revised the sentence (in Section 2.1) to: " The measurements within a profile were first interpolated linearly with depth, with the shallowest datum representing the level between the sea surface and the depth of that datum. The profile was then integrated from the sea surface to the deepest recorded measurement. Most vertical profiles of N_2 fixation rates were measured within the euphotic zone, with a few studies extending measurements to several hundred meters or deeper. In these cases, we only integrated to the deepest data point above 200 m, taking into account the scarcity of aphotic N_2 fixation measurements in the global ocean and their controversial contribution to the global budget (Benavides et al., 2018). As a result, it was possible that certain measurements below the euphotic zone but above 200 m were included in the integration. However, these measurements would typically have minimal impact on the depth-integrated N_2 fixation rates due to their low rates and limited vertical extent in this range."

The arithmetic and geome mean of the data in each bin was calculated first, and then these means in each basin were averaged further.

The Southern Ocean was defined as the area south of 45°S and was excluded from other basins when calculating the global rates. Additionally, due to very limited data coverage, the N_2 fixation rates of the Southern and Arctic Oceans have been excluded from the estimation of global marine N_2 fixation.

The percentage of bins with data coverage in each ocean basin have been added in the revised table.

When calculating the areal sum, we extrapolated the mean N_2 fixation rate of each basin across the entire basin, i.e., the mean N_2 fixation rate was multiplied by the area of each basin. We have listed the areas of every ocean basin in the table.

The description of the methods in calculating global marine N_2 fixation rate was added to Section 2.4:

“The estimation of the global marine N_2 fixation rate involved four steps. First, we calculated

the arithmetic or geometric means of depth-integrated N_2 fixation rates within each 3° latitude × 3° longitude bin. Second, these mean values were further averaged using either arithmetic or geometric methods to determine the mean N_2 fixation rates for different ocean basins, which included the North Atlantic, South Atlantic, North Pacific, South Pacific, Indian, Arctic, Southern Oceans, and the Mediterranean Sea. Third, we multiplied the arithmetic or geometric mean of each basin by its respective area to estimate the total N_2 fixation rate for that specific basin, except when there was insufficient spatial coverage available. Finally, we obtained the global marine N_2 fixation rate by summing up the individual rates calculated for each basin, with the errors associated with basin rates propagated properly (Glover et al., 2011).

In the first two steps, the geometric means were derived from positive N_2 fixation rates (NF_+): if μ and SE represented the mean and standard error of $\ln(NF_+)$, respectively, the geometric mean was e^{μ}. The confidence interval for the geometric mean, based on the standard error, ranged between e^{μ}/e^{SE} and $e^{\mu} \cdot e^{SE}$ (Thomas, 1979). To address the issue of not including zero-value N_2 fixation rates, we adjusted the geometric means by multiplying them with the percentage of zero-value data within each 3° × 3° bin (in the first step) or within each basin (in the second step)."

The authors do not give much context on interpreting the geometric vs. arithmetic mean despite that it is mentioned multiple times throughout the manuscript and gives a significantly different result. From what I understand, geometric mean is less sensitive to the high-end rates compared to arithmetic mean. Does this mean that most of the increase in the arithmetic mean is driven by newly included high-end rates? It would be valuable to know how much of the large increase in the arithmetic areal sum is driven by additional spatial coverage versus generally higher rate values. I would suggest to include a histogram of the previous version in one of the supplementary figures for comparison. If newly included rate values tend to be significantly higher, it would be interesting to know how much of that may be attributable to growing numbers of the dissolution method compared to bubble method (i.e. based on Figure 10).

Response: Dr. Somes was correct in interpreting geometric versus arithmetic means. As our N_2 fixation data were approximately log-normally distributed, their geometric mean is near the most frequently observed rate (i.e., the peaks of the distribution of the log-transformed N_2 fixation rates). Meanwhile, high N_2 fixation rates do occur and should be included in estimating global N_2 fixation. Hence, the arithmetic means should be used in estimating global N_2 fixation if sufficient data have been sampled. However, if the number of samples is small, some occasionally observed high N_2 fixation rates can greatly elevate the estimated global rate while we cannot know if these high N_2 fixation rates are typical. This was the reason that we presented both the geometric and arithmetic means of N_2 fixation rate.

In the revised manuscript, the increase in the arithmetic-mean-based estimation of global marine N_2 fixation, compared to that in Luo et al. (2012), was caused mostly by (1) the much higher estimation for the South Pacific Ocean and North Atlantic Ocean and (2) the estimation for the Indian Ocean for which the estimation of N_2 fixation was not made in Luo et al. (2012).

We also conducted additional analyses of data distributions to explore reasons why the

arithmetic means can be much higher using version 2 compared to using version 1 of the database, and found that version 2 considerably extends both the left and right tails of the data distribution. We concluded the previous assessments of the global marine N_2 fixation rate were likely underestimated due to the absence of these new measurements.

The text has been revised and new figures have been produced:

"The substantial increase was mostly driven by notable changes in the South Pacific, North Atlantic, and Indian Oceans. In the South Pacific Ocean, numerous high N_2 fixation rates were observed in the western subtropical region over the past decade (**Fig. 12**), resulting in a substantial increase of 68±23 Tg N yr^{-1} in the estimated N_2 fixation rate for this basin (**Table 8**). It is worth noting that these newly recorded measurements in the western subtropics of the South Pacific Ocean might even be underestimated since most of them were obtained using the original $^{15}N_2$ bubble method. In the North Atlantic Ocean, the estimated N_2 fixation rate also experienced an increase of 30±9 Tg N yr^{-1} for (**Table 8**), without any discernible pattern regarding the locations of the new high N_2 fixation measurements (**Fig. 13**). Furthermore, in the Indian Ocean, the improved data coverage in version 2 (**Fig. 8a**) supported the estimation of an N_2 fixation rate of 35±14 Tg N yr^{-1} for this basin (**Table 8**), which was not possible to calculate using version 1 due to insufficient data availability.

However, when estimating the global marine N_2 fixation rate using geometric means, both version 1 and version 2 yielded similar rates of approximately 50 Tg N yr^{-1} (**Table 9**). The N_2 fixation rates in each basin tended to follow a log-normal distribution (**Fig. 14; Table S2**), with the geometric mean aligning near the peak of the distribution. In the South Pacific Ocean, as discussed earlier, version 2 included a substantial number of newly observed high N_2 fixation rates, but it also incorporated a significant number of rates that were much lower than those in version 1 (**Fig. 14c**). This could be partially attributed to enhanced detection limits in measurements. Consequently, while version 2 yielded a much higher arithmetic mean N_2 fixation rate compared to version 1 for the South Pacific Ocean (**Table 8**), their geometric means remained quite similar (**Table 9**). In the North Pacific Ocean, for the same reasons, the arithmetic mean N_2 fixation rates obtained from both versions were very close, while the geometric mean from version 1 could be even higher than that from version 2 (**Tables 8 & 9; Fig. 14a**). These analyses reveal that, despite the similarity in geometric means of N_2 fixation rates obtained from both versions of the database, the higher arithmetic means in version 2 were not coincidental. Instead, they were a direct outcome of the improved measurement methods and the expanded spatial and temporal coverage of marine N_2 fixation over the past decade. Consequently, previous assessments of the global marine N_2 fixation rate were likely

underestimated due to the absence of these new measurements."

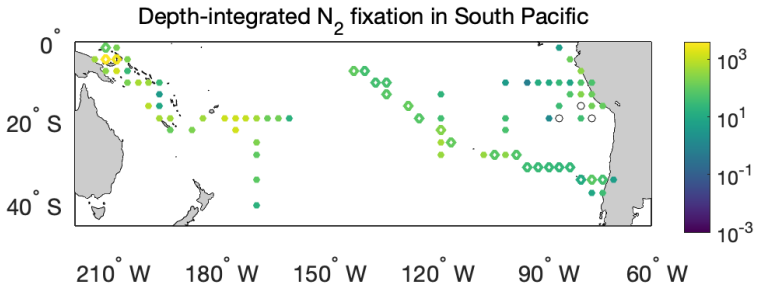

Figure 12. Depth-integrated N_2 fixation rates in the South Pacific Ocean (μmol N m^{-2} d^{-1}). The shown data are arithmetic mean rates in 3° latitude ×3° longitude bins. Empty diamonds and filled circles denote the existing data in the version 1 of the database and the new data added to version 2, respectively.

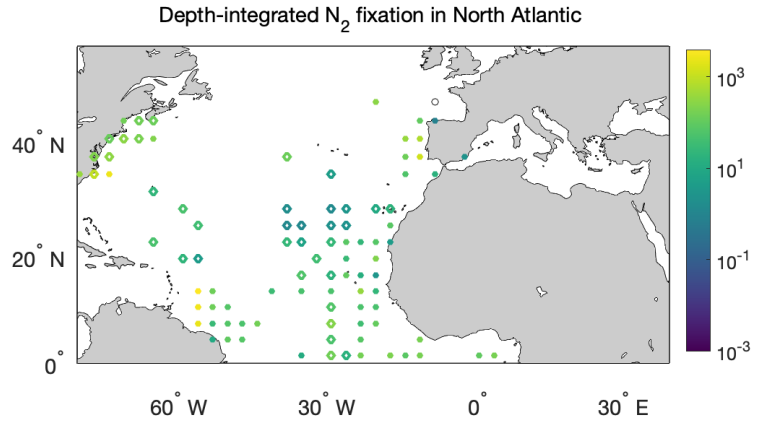

Figure 13. Depth-integrated N_2 fixation rates in the North Atlantic Ocean (μmol N m^{-2} d^{-1}). The shown data are arithmetic mean rates in 3° latitude ×3° longitude bins. Empty diamonds and filled circles denote the existing data in the version 1 of the database and the new data added to version 2, respectively.

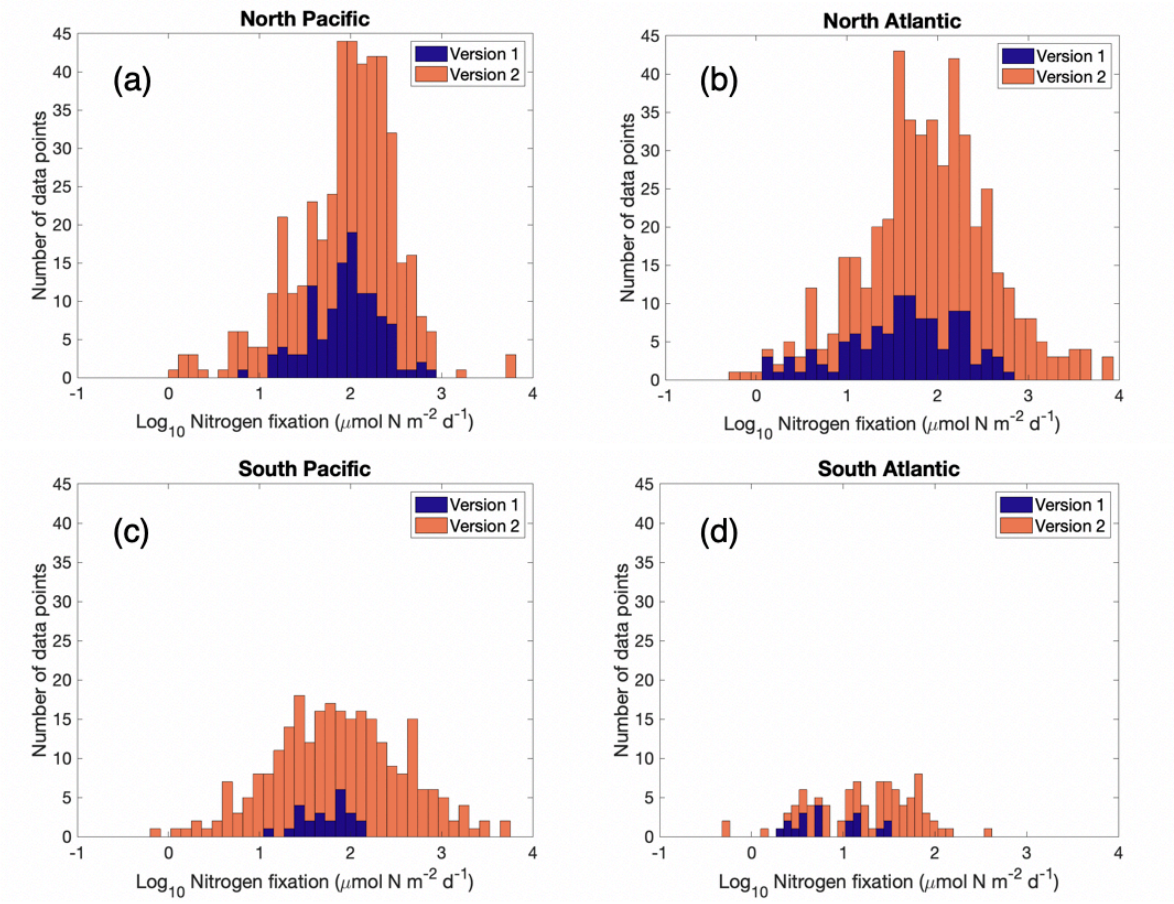

Figure 14. Comparison of the distribution of log-transformed N_2 fixation rates between the two versions of the database. Note that the zero-value data are not included because of the log-transformation. The comparison is performed for data in (**a**) North Pacific, (**b**) North Atlantic, (**c**) South Pacific, and (**d**) South Atlantic Oceans.

Minor Comments:

line 84 and data file: Metadata
In the data file, the meta data are titled "Surface …", yet they are associated with a specific depth, so are they really surface? I am used to seeing chlorophyll expressed by volume not area.

Response: Thank you for pointing out the mistakes. In the volumetric spreadsheets, the meta data were measured at the same depths as the diazotrophic data, and the word "surface" has been deleted from their names. Similarly, the chlorophyll concentration in the volumetric datasheets should be in unit of mg m^{-3}, which has been corrected in the revised database.

In the depth-integrated datasheet, considering the large vertical variations of environmental parameters and chlorophyll, we collected their near-surface values. We have corrected their names to "Near-surface xx".

lines 127-129: daily vs. daytime vs. nighttime normalization
I am still a little confused about the time normalization with this brief description. If the incubation is only performed during the day, you convert hours to day by 12 hr/day which assumes no rates at night? I see that incubation hours vary a lot and in some cases not a multiple of 12 hours or 1 day. Perhaps you can describe how individual studies typically convert to a daily rate depending on the incubation period. Would it make more sense to multiply by the daytime of each location during the time of sampling instead of assuming 12 hours?

Response: We have divided the N_2 fixation rate data into two spreadsheets based on the incubation period (24 hrs or < 24 hrs), only the N_2 fixation rates with incubation period of 24 hours were used in this estimation or method comparision. The method has now been descried with more details:

"The majority of N_2 fixation rates (9,405) were measured with incubation periods of 24 hours and were reported as daily rates. In contrast, 2,416 samples were incubated for less than 24 hours and hourly N_2 fixation rates were reported. Diel cycles of N_2 fixation vary among samples and/or diazotrophic groups, and substantial errors may be introduced when extrapolating N_2 fixation rates incubated for less than 24 hours to daily rates (White et al., 2020). Therefore, the N_2 fixation rates measured with incubation periods of less than 24 hours were collected into separated datasheets in our database and were not used in further analyses within this study. Please note that the incubation periods of whole diurnal cycles (e.g., 24, 48, or 72 hours) were used in Konno et al. (2010). The samples in Yogev et al. (2011) were incubated between 24 to 30 hours. The reported daily N_2 fixation rates by these two studies were also included in the 24-hour datasheets and were used in our estimation of the global marine N_2 fixation rate (see below)."

Table 5.: "n" is missing in Indian Ocean

Response: Corrected.

Figure 7:
Why do you choose geometric mean over the more commonly used arithmetic mean in this figure? Does it look significantly different if you use arithmetic means?

Response: The general spatial pattern of N_2 fixation was similar when using either geometric or arithmetic means, except for some high arithmetic means. In order to demonstrate these high values in the global ocean, we then have changed to present arithmetic means of N_2 fixation in the revised manuscript (Fig. 7 attached below).

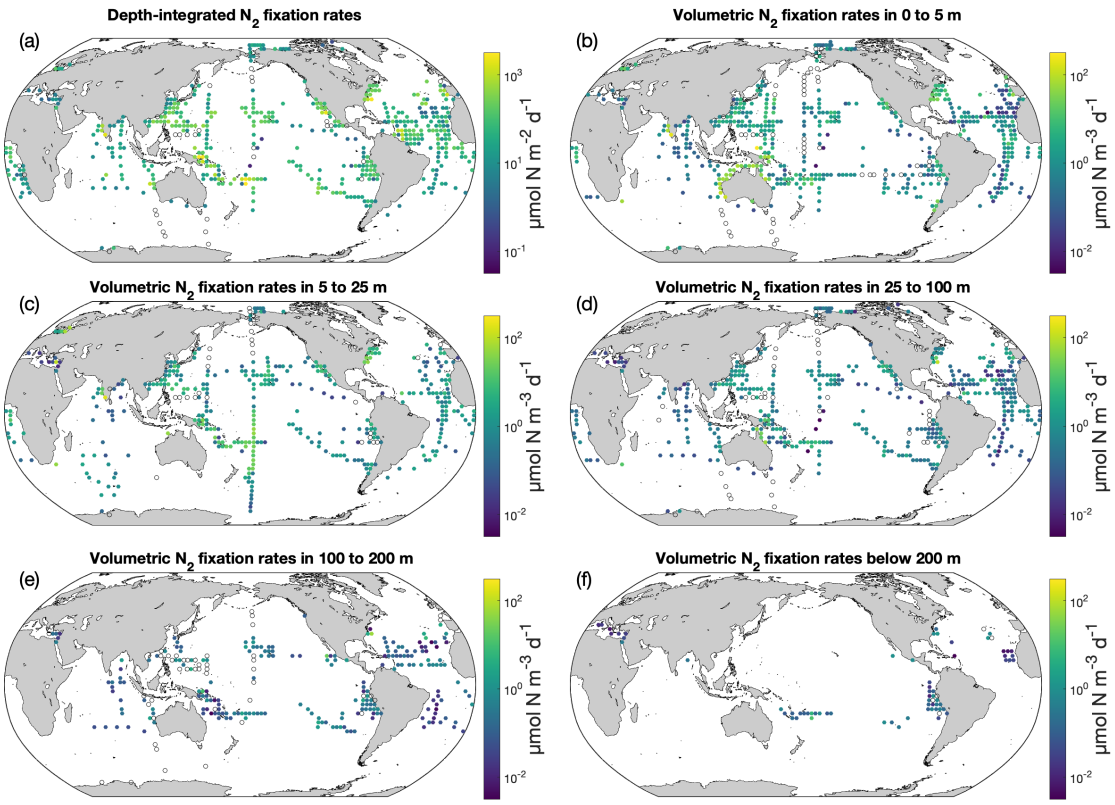

Figure 8. N_2 fixation rates in the version 2 of the database. The panels show (a) depth-integrated data and volumetric data in (b) 0–5 m, (c) 5–25 m, (d) 25–100 m, (e) 100-200 m, and (f) below 200 m. For a clear demonstration, arithmetic mean N_2 fixation rates in 3° latitude × 3° longitude bins are shown. Zero-value data are denoted as black empty circles. Only rates measured with incubation periods of 24 hours are included.

I would be interested to see a euphotic vs. aphotic depth-integrated rate. I am curious how much the generally low to moderate rates occurring below 100 meters contribute to the total depth-integrated rate since they can occupy more volume. Perhaps adding a < 100m and >100m panel would be useful? At what depths are the deepest N2 fixation measurements?

Response: We have generated an averaged vertical profile of N_2 fixation rates from sea surface to the deepest (4000 m; Hallstrøm et al., 2022) N_2 fixation measured (**Figure R1** attached below). Using the average vertical profiles of this figure, the total N_2 fixation below 200 m would be 2.5 times magnitude of that above 200 m.

However, the contribution of aphotic to the global budget has been discussed elsewhere (Benavides et al., 2018). This paper recoganized that the scarce N_2 fixation measurements in the dark ocean prevented a reliable estimate, which was still true after 5 years as shown in the figure below. We then decided not to include the estimate of aphotic N_2 fixation in our paper, considering it could be somehow unreliable and beyond scope of this paper.

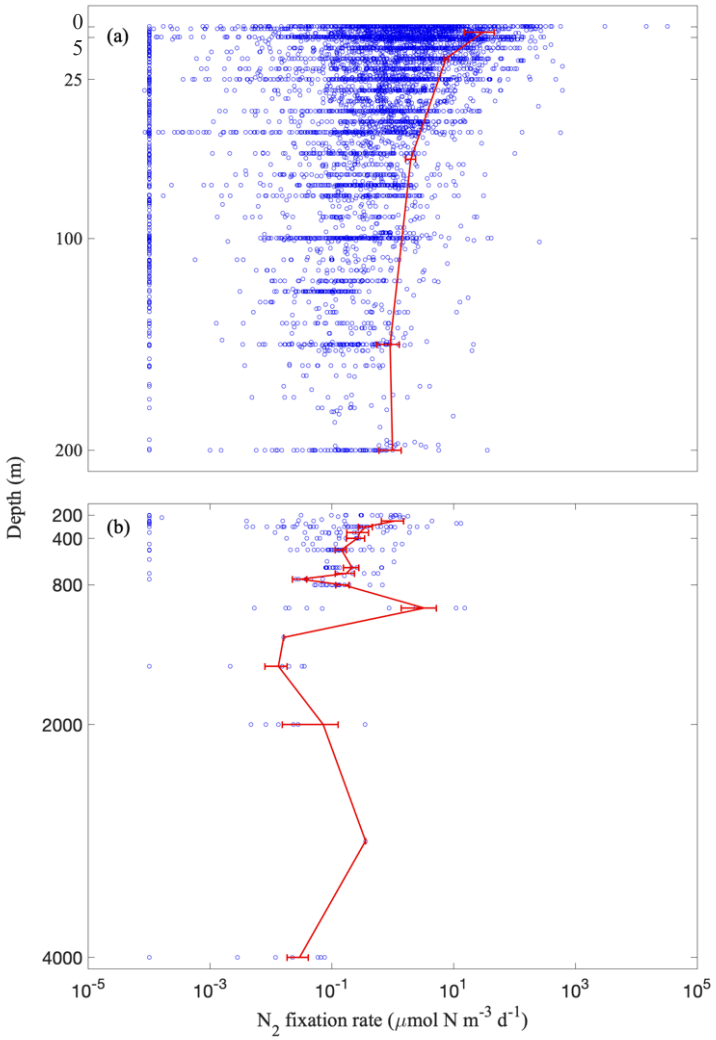

Figure R1. Vertical profile of N_2 fixation rates in the global ocean. Blue circles represent the reported N_2 fixation rates, and the red circles and error bars are the means and standard errors in depth intervals marked in the y-axis. The x-axis is in a log scale to better show the distribution of low N_2 fixation rates.

Section 4.1/Figure 10:
As mentioned above, I think is a useful first investigation into methodological uncertainties on N2 fixation rates. Is there enough data coverage to do a similar analysis for acetylene reduction?

Response: We thank Dr. Somes to recognize the value of our analyses. We compared the N_2 fixation rates measured using the $^{15}N_2$ tracer methods and from the acetylene reduction (ARA) method. However, there were too limited pairs of data available (n=16 and 6 for ARA vs. $^{15}N_2$ dissolution method and ARA vs. the original method, respectively) to be included in the manuscript (see **Figure R2** attached blow for your reference).

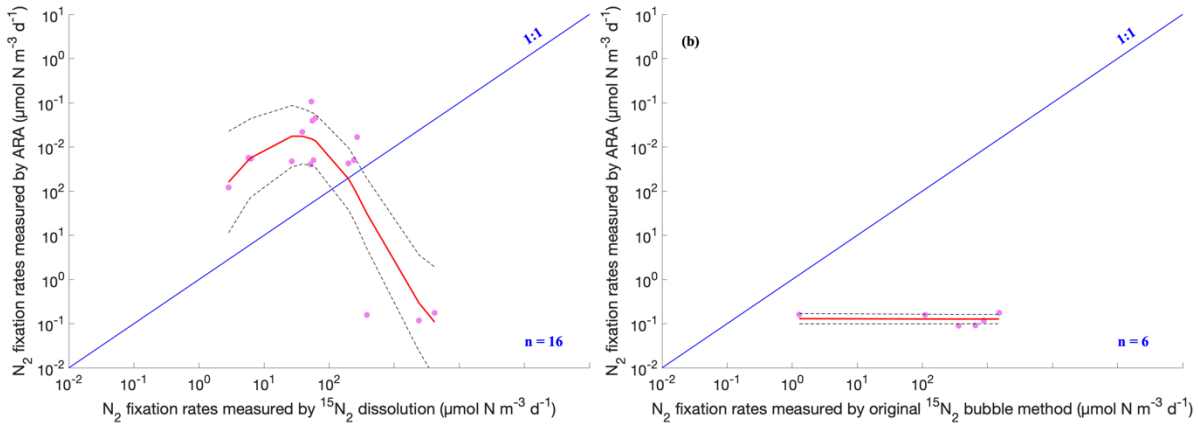

Figure R2. Comparison of measured N_2 fixation rates using the $^{15}N_2$ tracer methods and acetylene reduction (ARA) assays. (a) The ARA versus the $^{15}N_2$ dissolution; and (b) the ARA versus the original $^{15}N_2$ bubble method. The pink dots are measurements. The fitted results using the generalized additive model (GAM) and confidence intervals are represented by the red solid line and the dashed black lines, respectively. The blue lines is the 1:1 ratio of the measurements using the compared methods.

Please be also noted that there were mistakes when pairing $^{15}N_2$ dissolution and $^{15}N_2$ bubbling measurements in the original manuscript, which have been corrected in the revised manuscript (new **Fig. 13** attached below). The texts have been revised as follows:

"To date, the discrepancy in N_2 fixation rates estimated using different $^{15}N_2$ tracer methods remains unclear. As shown above, the volumetric N_2 fixation rates obtained by the original $^{15}N_2$ bubble method and the $^{15}N_2$ dissolution method spanned a similar range (**Fig. 1**), while the average rates using the former method were significantly lower than that measured using the latter method (one-tailed Wilcoxon test, $p<0.001$, n = 2460 and 1128). With substantial data accumulated over the past decade, we further compared N_2 fixation rates measured using the two methods at close locations and sampling time, although the samples were not identical. We first binned data collected from the same months, horizontal locations (3° latitude × 3° longitude) and depth intervals (0–5 m, 5–25 m, 25–100 m, and 100–200 m), and calculated the average rates for each method in each bin. The results showed that the original $^{15}N_2$ bubble method produced lower rates than the $^{15}N_2$ dissolution method in 69% of the cases (**Fig. 13**). Furthermore, our analysis employing the generalized additive model (GAM) revealed that the relationship between the rates measured using the original $^{15}N_2$ bubble method and those obtained through the $^{15}N_2$ dissolution method closely adhered to the 1:1 line, albeit with slightly lower values in the former (**Fig. 15**). It is crucial to reiterate that the rates being compared were derived from different samples, emphasizing the necessity for more future investigations that directly compare the two methods using the same samples with controlled parameters such as temperature, volume of injected $^{15}N_2$ and incubation volume. Despite this limitation, our analysis suggests that the extensive body of historical marine N_2 fixation rate data obtained

through the original $^{15}N_2$ bubble method still holds a value , particularly in the examination of spatial and temporal variations in N_2 fixation.

We also used the same procedure to compare the N_2 fixation rates measured using the acetylene reduction assays and the $^{15}N_2$ tracer methods. However, there were insufficient pairs of data available for reliable comparisons (n = 16 for acetylene reduction versus the $^{15}N_2$ dissolution method; n = 6 for acetylene reduction versus original $^{15}N_2$ bubble method)."

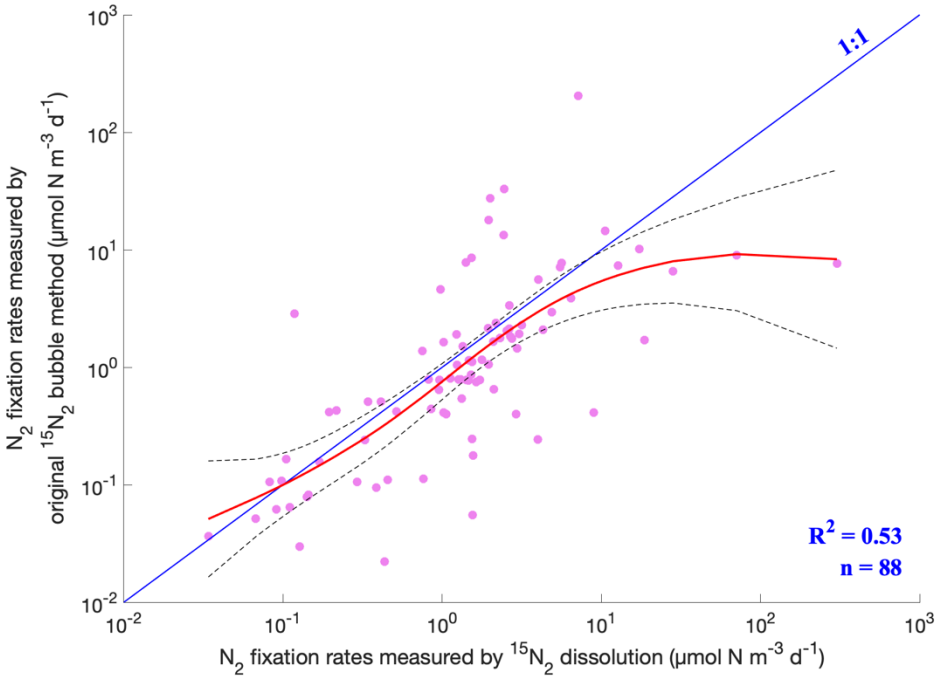

Figure 15. Comparison of measured N_2 fixation rates using the original $^{15}N_2$ bubble method and the $^{15}N_2$ dissolution method. The pink dots are measurements. The fitted results of the two methods by the generalized additive model (GAM) and confidence intervals are represented by the red solid line and the dashed black lines, respectively. Only the N_2 fixation rates measured with incubation periods of 24 hours were included in this analysis.

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