



Deep Into Oceanic N₂ Fixation

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The biological fixation of dinitrogen (N₂) by marine prokaryotes called diazotrophs is the major source of nitrogen to the ocean, estimated at ~106–120 Tg N y⁻¹ (Gruber, 2004; Gruber and Galloway, 2008). This process contributes importantly to sustain primary production and maintain the global nitrogen inventory. The nitrogen reservoir is further controlled by fixed nitrogen loss processes including sediment burial, denitrification, and anammox (Falkowski, 1997), which exceed fixed nitrogen gains through N₂ fixation, leading to an imbalanced global nitrogen budget (Codispoti et al., 2001; Codispoti, 2007; Eugster and Gruber, 2012). Since the early 1970s, diazotrophic activity has been attributed to autotrophic cyanobacteria constrained to the sunlit and oligotrophic layer of the tropical and subtropical oceans (Zehr, 2011). Yet substantial evidence indicates a high diversity and wide distribution of non-cyanobacterial diazotrophs (bacteria and archaea) in the oceans (Zehr et al., 1998, 2000; Farnelid et al., 2011; Bombar et al., 2016; Moisander et al., 2017). These diazotrophs are potentially not constrained by light as are their cyanobacterial counterparts, and have been detected in wide-ranging environments such as nutrient-rich, cold, and/or dark ecosystems including coastal upwelling regions (Sohm et al., 2011), temperate coastal zones (Bentzon-Tilia et al., 2015), and the deep ocean (Hewson et al., 2007; Hamersley et al., 2011).

Stretching the environmental boundaries, beyond those traditionally thought to constrain N₂ fixation, will likely impact current estimates of nitrogen input to the global ocean. Extending the latitudinal limits from the tropics and subtropics to temperate waters would already represent a considerable increase in the potentially active N₂ fixation area, but spreading this area vertically to the mesopelagic (200–1,000 m) and bathypelagic (1,000–4,000 m) ocean would be immense. Aphotic N₂ fixation rates are usually low when compared to surface activity (<1 nmol N L⁻¹ d⁻¹; see **Table 1** in Moisander et al., 2017) but the volume of the deep ocean is enormous. Consequently, studies comprising both photic and aphotic N₂ fixation measurements report depth-integrated aphotic rates representing 40–95% of the whole water column activity (Bonnet et al., 2013; Rahav et al., 2013; Benavides et al., 2015). Hence, aphotic fixation can account for a significant or even predominant fraction of water column N₂ fixation.

With the mere purpose of illustrating the potential budgetary relevance of the aphotic N₂ fixation to the global fixed nitrogen input, a back-of-the-envelope calculation can be carried out. If we consider a scenario for the mesopelagic zone (where the great majority of published aphotic N₂ fixation measurements were obtained from): taking the lower-end range of aphotic N₂ fixation rates available in the literature (0.01–0.1 nmol N L⁻¹ d⁻¹; **Table 1** in Moisander et al., 2017), and the estimated volume of the mesopelagic zone (2.63 × 10¹⁷ m³; Arístegui et al., 2005), mesopelagic N₂ fixation would range between 13 and 134 Tg N y⁻¹. Fixed nitrogen inputs to the ocean include fluvial inputs, atmospheric deposition and biological N₂ fixation, which add up to 187–279 Tg N y⁻¹ (**Table 1**). Combining denitrification (including sediment burial) and anammox, fixed nitrogen losses add up to 260–475 Tg N y⁻¹ (**Table 1**). Adding mesopelagic N₂ fixation to fixed nitrogen inputs and subtracting losses from gains, we obtain differences ranging from a loss of 183 to a surplus of 114 Tg N y⁻¹ (**Table 1**). Despite this extrapolation may be questionable given that data on aphotic N₂ fixation are so sparse that the spatial distribution of mesopelagic N₂ fixation is

TABLE 1 | Global nitrogen budgets and their variability when considering aphotic N₂ fixation.

	Codispoti et al., 2001	Galloway et al., 2004	Gruber, 2008	Jickells et al., 2017
SOURCES				
Pelagic photic N ₂ fixation	117	106	120	164
River inputs (DON+PON)	76	48	80	34
Atmospheric deposition	86	33	50	39
Mesopelagic N ₂ fixation	13 to 134	13 to 134	13 to 134	13 to 134
Total sources without considering aphotic N ₂ fixation	279	187	250	237
Total sources considering aphotic N ₂ fixation*	292 to 413	200 to 321	263 to 384	250 to 371
SINKS				
Benthic denitrification	300	206	180	
Water column denitrification	150	116	65	
Sediment burial	25	16	25	
Total sinks	475	338	270	260
Balance without considering aphotic N ₂ fixation	-196	-151	-20	-23
Balance considering aphotic N ₂ fixation	-183 to -62	-138 to -17	-7 to 114	-10 to 111

All fluxes are expressed in Tg N y⁻¹. *The range of aphotic N₂ fixation rates considered is 13.45–134.45 Tg N y⁻¹, see the main text.

unknown, it does illustrate that aphotic N₂ fixation could be important to global nitrogen budget considerations, and thus deep N₂ fixation should be further explored. Considering the stock of fixed nitrogen in the mesopelagic zone (Gruber, 2008) and the range of mesopelagic N₂ fixation rates estimated here (i.e., 13–134 Tg N y⁻¹), N₂ fixed and eventually remineralized to nitrate in the mesopelagic zone would turn over in 4 to 43 y.

The currently available dataset (Table 1 from Moisander et al., 2017, this issue) lacks robustness because (i) the number of measurements is limited and geographically sparse, and (ii) methodological difficulties are entailed in the detection of low N₂ fixation rates. While aphotic N₂ fixation has been consistently reported in several tropical and temperate waters (Table 1; Moisander et al., 2017, this issue), it is unknown whether it occurs homogeneously throughout the dark water column or only in micro-niches where suitable conditions are found. Such hospitable niches may comprise aggregates, or organic matter accumulation zones like ecotones, fronts or water mass boundaries (Benavides et al., 2015; Bombar et al., 2016). Only a few studies have documented *nifH* gene expression in aphotic waters (e.g., Jayakumar et al., 2012), and it is debated whether reported abundances of non-cyanobacterial diazotrophs can account for measured rates of N₂ fixation when considering published cell specific rates of cultivated strains (Turk-Kubo et al., 2014; Bentzon-Tilia et al., 2015). This introduces uncertainty to the reliability of measuring especially low N₂ fixation rates (Gradoville et al., 2017), and emphasizes the need for continued refinement of the ¹⁵N₂ incorporation method (Moisander et al., 2017).

In this context, it is pertinent to consider the methodological difficulties encompassed in the detection of low N₂ fixation rates using ¹⁵N₂ as a tracer. The precision of N₂ fixation rates may be affected by (i) a slower than theoretically assumed dissolution of the ¹⁵N₂ bubble in seawater (Mohr et al., 2010; Großkopf et al., 2012), (ii) the contamination of ¹⁵N₂ gas

stocks with nitrogenous species other than N₂ (Dabundo et al., 2014), and (iii) failure to measure time zero δ¹⁵N values of the particulate nitrogen pool. As any other tracer method, ¹⁵N₂-based N₂ fixation rates are subject to a number of other sources of error, including variability in incubation and/or filtration time among replicates, sample particle size and its retention in filters varying with filter pore size (Bombar et al., 2018), as well as heterogeneous distribution of particles in Niskin bottles (Suter et al., 2017). Moreover, the vast majority of ¹⁵N₂-based published N₂ fixation measurements report net rates, whereas the leakage of ¹⁵N-labeled dissolved organic nitrogen and/or ammonium can be significant in certain cases (e.g., Berthelot et al., 2017).

Most of the compiled aphotic rates (Moisander et al., 2017, this issue) were measured using the bubble method (Montoya et al., 1996), and should be considered as minimum estimates, despite the fact that they were performed in cold waters (typically ~10°C), which enhances gas dissolution and hence optimizes isotopic equilibrium in seawater samples enriched with ¹⁵N₂ gas. Moreover, the majority of the studies i) used an isotope brand that provides high purity ¹⁵N₂ gas, affecting aphotic N₂ fixation rates by <1% when ¹⁵N-labeled nitrogen molecules other than N₂ are taken up (Benavides et al., 2015) and/or ii) provided time zero δ¹⁵N values of the particulate nitrogen pool at each sampling depth, making their results robust (Bonnet et al., 2013; Rahav et al., 2013; Benavides et al., 2015, 2016). Finally, the variability between replicates in all terms included in the N₂ fixation calculation equation (as outlined in Montoya et al., 1996) may throw back minimum quantifiable rates values below estimated aphotic N₂ fixation rates (Gradoville et al., 2017). Propagating errors of the data (Birge, 1940), in five out of the nine aphotic N₂ fixation studies currently available, results in minimum quantifiable rates ranging from 0.01 to 2.7 nmol N L⁻¹ d⁻¹ (Table S1), suggesting

that most of the aphotic N₂ fixation rates published are significant.

The potentially high budgetary significance of aphotic N₂ fixation to the global nitrogen budget calls for further studies that will establish the geographical and temporal distribution of aphotic N₂ fixation and consolidate the volumetric rates published thus far. In future studies, we encourage researchers in the field of marine nitrogen cycling to place emphasis on documenting N₂ fixation in the aphotic ocean and identifying environmental drivers of aphotic N₂ fixation: including oxygen, dissolved organic matter availability and particle colonization (Riemann et al., 2010; Benavides et al., 2015; Bombar et al., 2016). The availability of more data is essential to facilitate modeling and assessment of the distribution and magnitude of aphotic N₂ fixation in the global ocean (i.e., association with water masses, ecotones or density fronts). Eventually, a more comprehensive understanding of the ecophysiology of aphotic N₂ fixers and their contribution to global nitrogen input, will reveal their ecological importance and may help answer such question as what are the evolutionary advantages of the energetically-expensive process of N₂ fixation in an environment rich in dissolved inorganic nitrogen, and how does it affect oceanic carbon sequestration.

REFERENCES

- Aristegui, J., Agustí, S., Middelburg, J. J., and Duarte, C. M. (2005). "Respiration in the mesopelagic and bathypelagic zones of the oceans," in *Respiration in Aquatic Ecosystems*, eds P. A. Del Giorgio and P. Williams (Oxford: Oxford University Press), 182–206.
- Benavides, M. H., Moisaner, P., Berthelot, H., Dittmar, T., Grosso, O., and Bonnet, S. (2015). Mesopelagic N₂ fixation related to organic matter composition in the Solomon and Bismarck Seas (Southwest Pacific). *PLoS ONE* 10:e0143775. doi: 10.1371/journal.pone.0143775
- Benavides, M., Bonnet, S., Hernández, N., Martínez-Pérez, A. M., Nieto-Cid, M., Álvarez-Salgado, X. A., et al. (2016). Basin-wide N₂ fixation in the deep waters of the Mediterranean Sea. *Glob. Biogeochem. Cycles* 30, 952–961. doi: 10.1002/2015GB005326
- Bentzon-Tilia, M., Traving, S. J., Mantikci, M., Knudsen-Leerbeck, H., Hansen, J. L. S., Markager, S., et al. (2015). Significant N₂ fixation by heterotrophs, photoheterotrophs and heterocystous cyanobacteria in two temperate estuaries. *ISME J.* 9, 273–285. doi: 10.1038/ismej.2014.119
- Berthelot, H., Benavides, M., Moisaner, P. H., Grosso, O., and Bonnet, S. (2017). High-nitrogen fixation rates in the particulate and dissolved pools in the Western Tropical Pacific (Solomon and Bismarck Seas). *Geophys. Res. Lett.* 44, 8414–8423. doi: 10.1002/2017GL073856
- Birge, R. T. (1940). The propagation of errors. *Am. Phys. Teacher* 7, 351–357. doi: 10.1119/1.1991484
- Bombar, D., Paerl, R. W., Anderson, R., and Riemann, L. (2018). Filtration via conventional glass fiber filters in ¹⁵N₂ tracer assays fails to capture all nitrogen-fixing Prokaryotes. *Front. Mar. Sci.* 5:6. doi: 10.3389/fmars.2018.00006
- Bombar, D., Paerl, R. W., and Riemann, L. (2016). Marine non-cyanobacterial diazotrophs: moving beyond molecular detection. *Trends Microbiol.* 24, 916–927. doi: 10.1016/j.tim.2016.07.002
- Bonnet, S., Dekazemacker, J., Turk-Kubo, K. A., Moutin, T., Hamersley, R. M., Grosso, O., et al. (2013). Aphotic N₂ fixation in the eastern tropical South Pacific Ocean. *PLoS ONE* 8:e81265. doi: 10.1371/journal.pone.0081265
- Codispoti, L. A. (2007). An oceanic fixed nitrogen sink exceeding 400 Tg N y⁻¹ vs the concept of homeostasis in the fixed-nitrogen inventory. *Biogeosciences* 4, 233–253. doi: 10.5194/bg-4-233-2007

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MB gathered N₂ fixation rates and made the calculations shown in the tables. MB, IB-F, SB, and LR wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00108/full#supplementary-material>

Table S1 | Error propagation analysis of aphotic N₂ fixation rates from various published and unpublished studies.

- Codispoti, L. A., Brandes, J. A., Christensen, J. P., Devol, A. H., Naqvi, S. W. A., Paerl, H. W., et al. (2001). The oceanic fixed nitrogen and nitrous oxide budgets: moving targets as we enter the anthropocene? *Sci. Mar.* 65, 85–105. doi: 10.3989/scimar.2001.65s285
- Dabundo, R., Lehmann, M. F., Treibergs, L., Tobias, C. R., Altabet, M. A., Moisaner, P. H., et al. (2014). The contamination of commercial ¹⁵N₂ gas stocks with ¹⁵N-labeled nitrate and ammonium and consequences for nitrogen fixation measurements. *PLoS ONE* 9:e110335. doi: 10.1371/journal.pone.0110335
- Eugster, O., and Gruber, N. (2012). A probabilistic estimate of global marine N-fixation and denitrification. *Global Biogeochem. Cycles* 26:4013. doi: 10.1029/2012GB004300
- Falkowski, P. G. (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO₂ in the ocean. *Nature* 387, 272–275. doi: 10.1038/387272a0
- Farnelid, H., Andersson, A. F., Bertilsson, S., Al-Soud, W. A., Hansen, L. H., Sørensen, S., et al. (2011). Nitrogenase gene amplicons from global marine surface waters are dominated by genes of non-cyanobacteria. *PLoS ONE* 6:e19223. doi: 10.1371/journal.pone.0019223
- Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., et al. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry* 70, 153–226. doi: 10.1007/s10533-004-0370-0
- Gradoville, M. R., Bombar, D., Crump, B. C., Letelier, R. M., Zehr, J. P., and White, A. E. (2017). Diversity and activity of nitrogen-fixing communities across ocean basins. *Limnol Oceanogr.* 62, 1895–1909. doi: 10.1002/lno.10542
- Großkopf, T., Mohr, W., Baustian, T., Schunck, H., Gill, D., Kuypers, M. M. M., et al. (2012). Doubling of marine dinitrogen-fixation rates based on direct measurements. *Nature* 488, 361–364. doi: 10.1038/nature11338
- Gruber, N. (2004). The dynamics of the marine nitrogen cycle and its influence on atmospheric CO₂ variations. *Ocean Carbon Cycle Clim.* 40, 97–148. doi: 10.1007/978-1-4020-2087-2_4
- Gruber, N. (2008). "The Marine Nitrogen Cycle: overview and challenges," in *Nitrogen in the Marine Environment*, eds D. G. Capone, D. A. Bronk, M. R. Mulholland, and E. J. Carpenter (Academic Press), 1–50.
- Gruber, N., and Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature* 451, 293–296. doi: 10.1038/nature06592
- Hamersley, M. R., Turk, K. A., Leinweber, A., Gruber, N., Zehr, J. P., Gunderson, T., et al. (2011). Nitrogen fixation within the water column associated with

- two hypoxic basins in the Southern California Bight. *Aquat. Microb. Ecol.* 63, 193–205. doi: 10.3354/ame01494
- Hewson, I., Moisander, P. H., Achilles, K. M., Carlson, C. A., Jenkins, B. D., Mondragon, E. A., et al. (2007). Characteristics of diazotrophs in surface to abyssopelagic waters of the Sargasso Sea. *Aquat. Microb. Ecol.* 46, 15–30. doi: 10.3354/ame046015
- Jayakumar, A., Al-Rshaidat, M. M. D., Ward, B. B., and Mulholland, M. R. (2012). Diversity, distribution, and expression of diazotroph *nifH* genes in oxygen-deficient waters of the Arabian Sea. *FEMS Microbiol. Ecol.* 82, 597–606. doi: 10.1111/j.1574-6941.2012.01430.x
- Jickells, T. D., Buitenhuis, E., Altieri, K., Baker, A. R., Capone, D., Duce, R. A., et al. (2017). A reevaluation of the magnitude and impacts of anthropogenic atmospheric nitrogen inputs on the ocean. *Glob. Biogeochem. Cycles* 31, 289–305. doi: 10.1002/2016GB005586
- Moisander, P. H., Benavides, M., Bonnet, S., Berman-Frank, I., White, A. E., and Riemann, L. (2017). Chasing after non-cyanobacterial nitrogen fixation in marine pelagic environments. *Front. Microbiol.* 8:1736. doi: 10.3389/fmicb.2017.01736
- Mohr, W., Großkopf, T., Wallace, D. W. R., and LaRoche, J. (2010). Methodological underestimation of oceanic nitrogen fixation rates. *PLoS ONE* 5:e12583. doi: 10.1371/journal.pone.0012583.t001
- Montoya, J. P., Voss, M., Kahler, P., and Capone, D. G. (1996). A simple, high-precision, high-sensitivity tracer assay for N₂ fixation. *Appl. Environ. Microbiol.* 62, 986–993.
- Rahav, E., Bar-Zeev, E., Ohayon, S., Elifantz, H., Belkin, N., Herut, B., et al. (2013). Dinitrogen fixation in aphotic oxygenated marine environments. *Front. Microbiol.* 4:227. doi: 10.3389/fmicb.2013.00227
- Riemann, L., Farnelid, H., and Steward, G. F. (2010). Nitrogenase genes in non-cyanobacterial plankton: prevalence, diversity and regulation in marine waters. *Aquat. Microb. Ecol.* 61, 235–247. doi: 10.3354/ame01431
- Sohm, J. A., Hilton, J. A., Noble, A. E., Zehr, J. P., Saito, M. A., and Webb, E. A. (2011). Nitrogen fixation in the South Atlantic Gyre and the Benguela Upwelling System. *Geophys. Res. Lett.* 38, 1–6. doi: 10.1029/2011GL048315
- Suter, E. A., Scranton, M. I., Chow, S., Stinton, D., Medina Faull, L., and Taylor, G. T. (2017). Niskin bottle sample collection aliases microbial community composition and biogeochemical interpretation. *Limnol. Oceanogr.* 62, 606–617. doi: 10.1002/lno.10447
- Turk-Kubo, K. A., Karamchandani, M., Capone, D. G., and Zehr, J. P. (2014). The paradox of marine heterotrophic nitrogen fixation: abundances of heterotrophic diazotrophs do not account for nitrogen fixation rates in the Eastern Tropical South Pacific. *Environ. Microbiol.* 16, 3095–3114. doi: 10.1111/1462-2920.12346
- Zehr, J. P. (2011). Nitrogen fixation by marine cyanobacteria. *Trends Microbiol.* 19, 162–173. doi: 10.1016/j.tim.2010.12.004
- Zehr, J. P., Carpenter, E., and Villareal, T. A. (2000). New perspectives on nitrogen-fixing microorganisms in tropical and subtropical oceans. *Trends Microbiol.* 8, 68–73. doi: 10.1016/S0966-842X(99)01670-4
- Zehr, J. P., Mellon, M. T., and Zani, S. (1998). New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. *Appl. Environ. Microbiol.* 64, 3444–3450.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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