Toward Resolving Disparate Accounts of the Extent and Magnitude of Nitrogen Fixation in the Eastern Tropical South Pacific Oxygen Deficient Zone

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Toward resolving disparate accounts of the extent and magnitude of nitrogen fixation in the Eastern Tropical South Pacific oxygen deficient zone

Corday R. Selden, Margaret R. Mulholland, Brittany Widner, Peter Bernhardt, Amal Jayakumar

Abstract

Examination of dinitrogen (N₂) fixation in the Eastern Tropical South Pacific oxygen deficient zone has raised questions about the range of diazotrophs in the deep sea and their quantitative importance as a source of new nitrogen globally. However, technical considerations in the deployment of stable isotopes in quantifying N₂ fixation rates have complicated interpretation of this research. Here, we report the findings of a comprehensive survey of N₂ fixation within, above and below the Eastern Tropical South Pacific oxygen deficient zone. N₂ fixation rates were measured using a robust ¹⁵N tracer method (bubble removal) that accounts for the slow dissolution of N₂ gas and calculated using a conservative approach. N₂ fixation was only detected in a subset of samples (8 of 125 replicated measurements) collected within suboxic waters (< 20 μmol O₂ kg⁻¹) or at the oxycline. Most of these detectable rates were measured at nearshore stations, or where surface productivity was high. These findings support the hypothesis that low oxygen/high organic carbon conditions favor non-cyanobacterial diazotrophs. Nevertheless, this study indicates that N₂ fixation is neither widespread nor quantitatively important throughout this region.

Nitrogen (N) limits productivity across a vast expanse of the ocean’s surface (Moore et al. 2013). Consequently, N availability plays an important role in regulating ocean carbon cycling and global climate (Falkowski 1997; Karl et al. 2002; Deutsch et al. 2004). Unlike other important macronutrients such as soluble reactive phosphorus (SRP), reactive N (N₅) has biological sources and sinks capable of modulating the N₅ pool in response to environmental forcings. N₅ losses occur primarily in anoxic sediments and pelagic oxygen deficient zones where nitrate (NO₃⁻) respiration (denitrification) and anaerobic ammonium oxidation (anammox) are energetically favorable (Devol 2008). In contrast, the distribution and magnitude of oceanic dinitrogen (N₂) fixation, the prokaryote-mediated conversion of relatively unreactive N₂ gas to N₅, remain poorly constrained because diazotrophic groups are ecologically diverse and can be metabolically flexible (Zehr and Capone 2020).

The ocean’s largest pelagic oxygen (O₂) deficient zones occur in the Eastern Tropical North and South Pacific Oceans. Together, these account for roughly one-quarter of marine N₅ loss (DeVries et al. 2012). When denitrified waters surface, DIN is exhausted in advance of SRP, creating conditions thought to favor N₂ fixation (Deutsch et al. 2007; Weber and Deutsch 2014) because assimilation of ammonium and NO₃⁻, the primary forms of DIN available in the ocean, are typically less energetically-costly means of acquiring N (Falkowski 1983). The degree to which N₅ inputs and losses are spatially coupled is hypothesized to be a function of the availability of dissolved iron (Fe), a key cofactor in the N₂ fixation enzyme (Weber and Deutsch 2014; Bonnet et al. 2017). This mechanism is believed to play a major role in balancing the ocean’s N₅ inventory (e.g., Weber and Deutsch 2014).

Direct observations of N₂ fixation (e.g., Knapp et al. 2016; Knapp et al. 2018) suggest that N₅ inputs and losses are relatively decoupled due to Fe limitation in the Eastern Tropical South Pacific (Dekaezemacker et al. 2013; Weber and Deutsch 2014; Kondo and Moffett 2015). Nevertheless, and despite significant concentrations of DIN (> 1 μM), Eastern Tropical South Pacific waters harbor a diverse assemblage of
predominantly non-cyanobacterial diazotrophs (Fernandez et al. 2011; Bonnet et al. 2013; Löscher et al. 2014; Chang et al. 2019) reported to actively fix N₂ at low, but persistent, rates throughout the water column (e.g., Fernandez et al. 2011; Bonnet et al. 2013). If this pattern held true throughout the ocean’s interior, it would mean that sub-euphotic diazotrophs contribute a significant fraction (~6–32%) of the ocean’s N₁ inputs (Benavides et al. 2018). Moreover, the widespread occurrence of N₂ fixation under DIN-replete conditions would suggest that new N₁ inputs (via diazotrophy) are either less sensitive (see discussion in Bombar et al. 2016) to changes in the N₁ inventory (via denitrification/anammox) than hypothesized (e.g., Weber and Deutsch 2014), or that our conception of the feedback process between them is incomplete.

Interpretation of N₂ fixation rate data from the Eastern Tropical South Pacific and other mesopelagic systems has, however, been hampered by methodological issues associated with implementation of the 1⁵N₂ tracer incubation approach (Montoya et al. 1996) where diazotrophy activity is low (see discussion in White et al. 2020). Using a more conservative approach to quantifying N₂ fixation rates, we present a comprehensive examination of measurements from the Eastern Tropical South Pacific within the context of past work.

**Methods**

**Hydrographic data and sample collection**

Samples were collected in January 2015, while aboard the R/V Atlantis. Vertical profiles of temperature, salinity, photosynthetically-active radiation, dissolved O₂, and chlorophyll a fluorescence were obtained using a Sea-Bird SBE 11plus conductivity-temperature-depth (CTD) sensor package equipped with a model 43 dissolved O₂ sensor, a QSP200L Biospherical photosynthetically-active radiation sensor, and a WET Labs ECO-AFL chlorophyll a fluorometer. Samples for nutrient analysis were collected from Niskin bottles affixed to the CTD rosette and, within the O₂ deficient zone, from a pump profiling system. Samples for NO₃⁻ plus nitrite (NO₂⁻) and SRP were syringe-filtered through a Sterivex filter (0.2 μm). Filtrate was collected and stored upright in acid-washed polyethylene bottles at −20°C until analysis at Old Dominion University using an Astoria-Pacific autoanalyzer and standard colorimetric protocols (Parsons et al. 1984). NO₂⁻ samples were filtered by gravity through a 0.2 μm Millipore filter directly from the Niskin bottles into acid-washed Falcon tubes. These samples were analyzed immediately using a manual colorimetric method on a Shimadzu (UV-1800) spectrophotometer (Pai et al. 1990). The detection limits for NO₂⁻, NO₃⁻ + NO₂⁻, and SRP analyses were 0.02, 0.14, and 0.03 μM (3σ, n = 7), respectively.

Oxic water samples for N₂ fixation incubations and particulate N (PN) enrichment and mass were collected in 10 L carboys from Niskin bottles mounted to the CTD rosette. Incubations from the shallow oxic zone were conducted in clear, 1.2 L PETG bottles in triplicate. Duplicate water samples were also filtered at the initial time point onto pre-combusted (450°C, 2 h) 0.3 μm glass fiber filters (GF-75, Advantec MFS, Dublin, CA) to measure particulate carbon and PN concentrations and initial PN ¹⁵N enrichment. These samples were frozen and stored at −20°C until analysis at Old Dominion University (see below).

Incubation samples from below the suboxic layer were collected in triplicate directly from Niskin bottles into 4.3 L amber glass bottles. Particulate carbon/PN samples were collected as described above. Within the suboxic layer, samples were pumped directly from depth into He-flushed 4.3 L amber glass bottles using a submersible water pump affixed to a small CTD as described by Selden et al. (2019). To limit O₂ contamination, bottles were first filled with sample then submerged in a ~50 L tub of O₂ deficient water. Sample bottles were flushed continuously from the bottom to avoid back-flow from the tub until they had been filled three times over. With this setup, a roughly 0.5 m thick layer of continuously replenished low-O₂ water covered the bottles as they flushed, preventing atmospheric O₂ contamination and maintaining in situ temperature as samples were collected.

**N₂ fixation rate measurements**

**Incubation set-up**

N₂ fixation rates were determined using the bubble removal technique (e.g., Jayakumar et al. 2017), a modified version of the ¹⁵N₂ incubation-based assay of Montoya et al. (1996) that accounts for the slow dissolution time of N₂ gas (Mohr et al. 2010). In brief, approximately 1 or 4 mL additions (to 1.2 and 4.3 L incubation bottles, respectively) of pressurized, highly enriched (~99%, Cambridge Isotopes, Tewksbury MA) ¹⁵N₂ was added to PETG or glass incubation bottles. Prior to these additions, incubation bottles were filled completely and any air bubbles were removed. Additions were made using a gas-tight syringe (VICI Valco Instruments, Houston, TX) through a silicon septa cap that allowed for small changes in volume. Sample bottles were gently inverted for 15 min using a seesaw, as described by Selden et al. (2019), to increase the rate of gas dissolution. After mixing, the remaining gas bubble was removed using a syringe so that the ¹⁵N₂ enrichment of the seawater remained constant throughout the incubation period. Sample bottles were then incubated under approximately in situ light and temperature conditions.

For euphotic zone samples, incubation bottles were placed in on-deck incubators that were continuously flushed with surface seawater to maintain temperature. Appropriate light conditions, as determined using the CTD-mounted photosynthetically-active radiation sensor, were approximated using neutral-density screens. Samples collected below the euphotic zone were maintained in the dark, either in a walk-in cold van (~12°C) or refrigerator (~4°C), whichever more closely simulated ambient environmental conditions at the
depths samples were collected. For samples collected below the suboxic zone, incubation bottles were placed in a dark, walk-in refrigerator (~4°C), where they were incubated for ~48 h. All other samples were incubated for ~24 h.

Contamination (15N-labeled DIN) has been previously been reported for some commercially available 15N2 stocks (Dabundo et al. 2014; White et al. 2020). While the purity of the tracer stocks used here were not directly tested, we note that this issue has never been reported for 15N2 gas from Cambridge Isotope Laboratories. Additionally, after 24 and 48 h incubations, generally little 15N enrichment was detected in the particulate N pool (i.e., rates of N2 fixation were largely undetectable, see Results and Discussion). It is thus highly unlikely that our stocks were contaminated.

At the end of the incubation, aliquots (6 mL) were transferred to He-flushed 12 mL Exetainers™ using a gas-tight syringe (Hamilton 1000 series, Reno, NV) to determine the 15N enrichment of the N2 pool. To these samples, 50 μL of 50% w/v ZnCl2 (Thermo Fisher Scientific, Waltham, MA) was added to ensure the termination of microbial activity. The remaining sample was immediately filtered on 0.3 μm glass fiber filters (GF-75, Advantec MFS Inc, Dublin, CA). Filters were frozen and stored in sterile microcentrifuge tubes at −20°C until analysis at Old Dominion University. Exetainer samples were stored at room temperature until analysis at Princeton University. They did not undergo any significant pressure changes (e.g., from air shipping) during storage.

Sample analysis

15N2 gas samples were analyzed at Princeton University using a Europa 20–20 isotope ratio mass spectrometer (IRMS), following Jayakumar et al. (2017). 15N2 enrichment in sample incubations ranged from 2.22 to 8.57 atom-% (mean = 3.64 ± 0.04 atom-%, n = 159). Particulate samples, collected both from the environment (t = 0) and from incubation bottles (t = f), were dried (50°C for ~2 d) and then pelletized in tin discs at Old Dominion University. 15N enrichment of the PN pool and its mass were subsequently determined using a Europa 20–20 IRMS equipped with a automated N and carbon analyzer. Samples from initial (non-enriched) and final (potentially 15N tracer-enriched) time points were pelletized separately, stored in separate desiccators, and analyzed separately to avoid carry-over contamination.

The detection limit for PN mass was calculated separately for each run using 12.5 μg N ammonium sulfate standards (IA-RO45, SerCon, Cheshire, UK; 3σ, n = 7), which were calibrated using standards from the National Institute of Standards and Technology. The mean detection limit among natural abundance instrument runs (n = 8) in this study was 2.06 μg N. Since the accuracy of enrichment analysis diminishes at lower mass (White et al. 2020) and low N2 fixation rates are sensitive to small variations in 15N enrichment, we assumed a conservative lower linearity limit of 10 μg N based on instrument performance during the time samples were analyzed (Suppl. Fig. 1). This value is consistent with current “best practice” recommendations from the scientific community (White et al. 2020). If sample mass was below 10 μg N, 15N enrichment data from that sample was discarded. A standard curve (1.17–100 μg N) was also run each day to verify measurement linearity.

N2 fixation rate calculations

N2 fixation rates were calculated as described by Montoya et al. (1996):

$$\text{NFR} = \frac{A_{PN_{i-f}} - A_{PN_{i-o}}}{A_{N_2} - A_{PN_{i-o}}} \times \frac{[PN]}{\Delta t}$$ (1)

where $A_{PN_{i-f}}$, $A_{PN_{i-o}}$, and $A_{N_2}$ represent the atom-% 15N enrichment of the final and initial PN pool, and the incubation’s N2 pool, respectively. Incubation duration is denoted as Δt; [PN] is the mean concentration of PN across the incubation period. If a t = 0 measurement was not available from the exact location of the incubation water, then the final PN concentration and the mean $A_{PN_{i-o}}$ within either oxic or suboxic waters (whichever was appropriate) were used in the calculation in place of the [PN] and direct $A_{PN_{i-o}}$ measurement.

N2 fixation was considered detectable if $(A_{PN_{i-f}} - A_{PN_{i-o}})$ was greater than three times the standard deviation of seven 12.5 μg N standards run daily with enriched $(A_{PN_{i-o}})$ samples (Ripp 1996). To calculate minimum detectable rates i.e., detection limits, this minimum detectable enrichment value (mean = 0.0054 ± 0.0026 atom-% across 11 IRMS runs) was substituted for $(A_{PN_{i-f}} - A_{PN_{i-o}})$ in Eq. 1 (Jayakumar et al. 2017; White et al. 2020). Consequently, detection limits scale with PN concentration. In this study, the mean and median detection limit for N2 fixation rates were 3.00 and 1.09 nmol N L⁻¹ d⁻¹, respectively. All requisite information for calculating rates for each incubation is available on BCO-DMSO (http://www.bco-dmo.org/project/742492).

N2 fixation rates were considered detectable at a given location if 15N enrichment was detected in at least two replicate incubations. Where two replicates were deemed detectable but the third was not, a mean N2 fixation rate was calculated by forcing the undetectable rate to zero (Bonnet et al. 2013; Chang et al. 2019). Rate error was assessed by taking the standard deviation of rates from replicate incubations (Suppl. Text 1). These values are provided in Suppl. Table 1 alongside associated hydrographic data. To ensure that detectable changes in $A_{PN_{i-o}}$ were due to diazotrophy, control incubations were conducted at a subset of stations (Suppl. Text 2; Suppl. Table 2).

Results and discussion

Regional hydrography

Our study area encompassed both offshore and nearshore waters. Upwelling, visible as a decrease in sea surface temperature (Fig. 1A), was apparent near the Peruvian coast during the
study period (January 2015). These cooler waters were associated with elevated surface chlorophyll $a$ (Fig. 1B) and high surface nutrient concentrations. At the shallowest (< 750 m depth) and most nearshore stations, the concentrations of DIN and SRP exceeded 14 and 1 $\mu$M, respectively, in the upper 10 m (Sta. 13, 16 and 19; Suppl. Fig. 2A,B). Surface DIN and SRP concentrations were slightly lower at other nearshore stations (Sta. 12, 14, 15, and 18), and decreased to < 4 and < 0.7 $\mu$M, respectively, at most offshore stations. In addition to transporting macronutrients, upwelling can supply surface waters with Fe and other trace elements liberated from shelf sediments, provided that upwelled waters remain relatively reducing (Rapp et al. 2020).

Suboxia (< 20 $\mu$mol O$_2$ kg$^{-1}$) was detected at all stations. The suboxic layer shoaled to an average depth of 80 m among nearshore stations (12–19; Suppl. Fig. 2D). Here, the thermocline was shallower and stronger (Suppl. Fig. 2E). At offshore stations to the south (>$16.5^\circ$S, Sta. 14, 1 depth), and two from an offshore site (Sta. 1). Where detectable, N$_2$ fixation rates were low (0.18–0.13–0.77–0.003 nmol N L$^{-1}$ d$^{-1}$; Suppl. Table 1). By applying a conservative limit of quantification (Selden et al. 2019), calculated by propagating a minimum quantifiable change in $A_P$ (10s, $n = 7$ 12.5 $\mu$g standards; Ripp 1996) through Eq. 1, we assert that even the detectable N$_2$ fixation rates reported here cannot be accurately quantified. The quantification limits where N$_2$ fixation was detected ranged from 0.4 to 1.4 nmol N L$^{-1}$ d$^{-1}$ (Suppl. Table 1), representing an upper bound on N$_2$ fixation rates. In contrast, detection limits, i.e., the lower bounds for these rate measurements, ranged from 0.09 to 0.3 nmol N L$^{-1}$ d$^{-1}$ (Suppl. Table 1).

**Regional distribution of N$_2$ fixation**

We assessed N$_2$ fixation rates in 61 samples collected within the oxic (> 20 $\mu$mol kg$^{-1}$ O$_2$), euphotic waters, 59 samples collected within suboxic waters, and five samples collected in oxic waters beneath the O$_2$ deficient zone. Of these, N$_2$ fixation was detected in only eight samples (Figs. 1, 2, Suppl. Table 1), five from two stations at the southern end of our study site (Sta. 18 and 19, 5 depths), one from a particularly productive site (depth-integrated chlorophyll $a$: 36.7 mg m$^{-2}$, particulate C: 20–65 $\mu$M) slightly offshore (Sta. 14, 1 depth), and two from an offshore site (Sta. 1). The role of dissolved oxygen

Seven of the eight locations (of 125 total) where N$_2$ fixation was detected were within suboxic waters. At all but one of these (Sta. 18, 100 m), NO$_2^-$ concentrations exceeded 0.5 $\mu$M, suggesting functional anoxia (Thamdrup et al. 2012). The
Table 1. Volumetric N₂ fixation rates (NFRs) reported from the Eastern Tropical South Pacific oxygen deficient zone (ODZ) and neighboring waters.*

| Reference       | Date        | Oceanic Niño Index | Depth range                  | Mean NFR or range (nmol N L⁻¹ d⁻¹) | Sample collection | Method | Filter size | Incub. vol. (L) | Min. filter mass (µg N) | Min. detectable enrichment | APN₂,₆ ± measured directly? |
|-----------------|-------------|--------------------|------------------------------|------------------------------------|--------------------|--------|-------------|------------------|--------------------------|--------------------------------|-------------------------------|
| Fernandez et al. 2011 | Oct/ Nov 2005 -0.3 | Surface | 0.89±0.08 (n=17) | Niskin** | TBM | 0.7 GF/F | 2 | Not reported | Not reported | Not reported |
|                 | Feb 2007 | 0.7 | Upper oxycline | 0.075±0.07 (n=8) | | | | | | | |
|                 |            | Surface | 0.66±0.7 (n=10) | | | | | | | | |
|                 |            | Upper oxycline | 1.71±1.03 (n=17) | | | | | | | | |
|                 |            | Upper ODZ (<400 m) | 1.27±1.2 (n=13) | | | | | | | | |
| Bonnet et al. 2013 | Feb/ Mar 2010 | 1.3 | Surface – 2000 m | BDL – 0.80 (n=40) | Niskin** | TBM | 0.7 GF/F | 4.5 | 3.8** | 0.0005 atom-%, (3σ, n=10 IAEA reference samples) | No*** |
|                 | Mar/ Apr 2011 | -0.8 | Surface – 2000 m | BDL – 0.26±0.12 | | | | | | | |
|                 |            | Below ODZ (400–2000 m) | 0.00±0.01–0.21±0.13 | | | | | | | | |
| Dekaezemacker et al. 2013 | Feb/ Mar 2010 | 1.3 | Surface – 200 m | <0.06–0.88 | Niskin** | TBM | 0.7 GF/F | 4.5 | 2.9** | 0.0005 atom-%, (3σ, n=10 IAEA reference samples) | Surface only |
|                 | Mar/ Apr 2011 | -0.8 | Surface – 200 m | BDL – 0.87 | | | | | | | |
| Löscher et al. 2014 | Jan/ Feb 2009 | -0.8 | Surface – ODZ (40–350 m) | <24.8±8.4 | Niskin Pump** | TBM | 0.7 GF/F | 2 | -3*** | Not reported | Yes |
| Knapp et al. 2016 | Feb/ Mar 2010 | 1.3 | Surface – 180 m | 0.25±0.11–0.54±0.72** | Niskin | TBM | 0.7 GF/F | 4 | 10 | Not reported | Yes |
|                 | Mar/ Apr 2011 | -0.8 | Surface – 150 m | 0.15±0.16–0.41±0.15*** | | | | | | | |
| Löscher et al. 2016 | Nov/ Dec 2012 | 0.0 | Surface – ODZ | 2.2±3.6 | Niskin or pump | ESM*** | 0.7 GF/F | 4.5 | 3.9 | 0.0005 atom-% | Yes |

(Continues)
Table 1. Continued

| Reference          | Date    | Mean NFR or range (nmol N L\(^{-1}\) d\(^{-1}\)) \(^{†} \) | Sample collection Method\(^{§} \) | Filter size | Incub. vol. (L) | Min. filter mass (\(\mu g N \)) | Min. detectable enrichment | \(A_{PN=0} \) measured directly? |
|--------------------|---------|-------------------------------------------------------------|-----------------------------------|-------------|-----------------|-------------------------------|-------------------------------|-------------------------------|
| Chang et al. 2019  | Jul 2011| 0.18±0.28                                                   | Niskin** BRM                      | 0.7 GF/F    | 2.5             | 2                             | 0.0025 (3\(\sigma\), \(n\)=7 12 \(\mu g N \) standards) | Yes                           |
| This study         | Jan 2015| BDL – 0.52††††††                                          | Niskin BRM                        | 0.3 GF75    | 1.2             | 10                            | Mean=0.0054 ±0.0026 atom-% (3\(\sigma\), \(n\)=7 12.5 \(\mu g N \) standards) | Yes†††††                        |
|                    |         | BDL – 0.77††††††                                          | Pump‡‡ BRM                        | 4.3         |                 |                               |                              |                               |
|                    |         | BDL                                                        | Niskin‡‡ BRM                      | 4.3         |                 |                               |                              |                               |

*Geographic distribution of studies displayed in Fig. 3.

†Oceanic Niño Index (https://origin.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ONI_v5.php) is the three-month running mean (period leading up to and including given cruise) sea surface temperature anomaly (based on 30 year mean within area from 5°N to 5°S and 120°W to 170°W). Positive (> 0.5) and negative (< −0.5) values indicate El Niño and La Niña events, respectively.

‡†††† ‡BDL indicates values that are below the reported detection limit.

‡TBM, BRM, and ESM refer to the traditional bubble method, the bubble removal method, and the enriched seawater method, respectively. See White et al. (2020) for detailed descriptions and comparison.

§\(A_{PN=0} \) refers to \(^{15}\)N-PN atom-% enrichment.

**Care taken to avoid O₂ contamination in low-O₂ samples by filling evacuated gas-tight bags.

††In order to achieve a minimum mass of 10 \(\mu g N \) (a reasonable minimum for most instruments; White et al. 2020), ambient PN concentration would need to be > 0.36 \(\mu M \) given a filtration volume of 2 L (as reported by the authors). While mean PN concentrations in the region within the upper 400 m typically exceed this threshold, lower values are often observed, particularly away from coastal upwelling and < ~150 m (Chang et al. 2019; this study; Knapp et al. 2016).

‡‡Care taken to avoid O₂ contamination in low-O₂ samples by flushing and filling bottles from the bottom.

§§Bottles shaken after \(^{15}\)N₂ addition to increase rate of gas dissolution.

¶¶IRMS linearity verified via Fisher test \((p<0.01)\).

***\(A_{PN=0} \) assumed to be in equilibrium with atmospheric N₂ (0.3663 atom-%).

†††Authors reported that most samples exceeded 10 \(\mu g N \).

‡‡‡Authors reported “reproducibility” (\(\sigma\)) as equal to 0.0001 atom-%.

††††The mean \(^{15}\)N-PN enrichment in the upper water column was used as \(A_{PN=0} \) for N₂ fixation rate calculations.

‡‡‡‡Enriched seawater collected from the same depth and location as sample water.

†††††If a limit of quantification (10\(\sigma\), \(n\)=7 12.5 \(\mu g N \) standards) is applied, then all detectable rates would be considered too low to quantify (< 0.4–1.4 nmol N L\(^{-1}\) d\(^{-1}\)).

‡‡‡‡‡Mean \(^{15}\)N-PN enrichment within oxic waters was used as \(A_{PN=0} \) when direct measurement was not available (e.g., when mass of collected sample was insufficient).

‡‡‡‡‡‡Mean \(^{15}\)N-PN enrichment within suboxic (< 20 \(\mu mol kg^{-1}\)) waters used as \(A_{PN=0} \) when direct measurement was not available (e.g., when mass of collected sample was insufficient).
eighth and final detectable rate occurred along a shallow oxycline (Sta. 1, 80 m). Our observation that N₂ fixation is restricted to the upper oxycline/O₂ deficient zone is consistent with prior reporting of a broad N₂ fixation rate peak (< 0.4 nmol N L⁻¹ d⁻¹) across the oxycline and upper O₂ deficient zone at nearshore stations (Löscher et al. 2014). Similarly, Chang et al. (2019) observed an increase in nifH (a requisite gene for N₂ fixation) concentrations within the O₂ deficient zone, and Löscher et al. (2014) noted that the majority of the nifH sequences that they recovered were from within low O₂ waters.

Theoretical calculations suggest that N₂ fixation may offer a slight energetic advantage over NO₃⁻ assimilation in low O₂ waters where the cost of shielding nitrogenase from oxidative damage is minimized, provided that the organism is capable of recycling electrons (via an uptake hydrogenase) and efficient respiration (Großkopf and LaRoche 2012). Additionally, it has been proposed that some microbes may use nitrogenase as an electron sink as a mechanism for balancing intracellular redox state (e.g., Bombar et al. 2016). These claims are supported by experimental work with Baltic Sea proteobacteria (Bentzon-Tilia et al. 2015). Bentzon-Tilia et al. (2015) observed that all of their isolates increased N₂ fixation at low O₂ concentrations (- 4-40 μM). Moreover, one isolate, an α-proteobacterium (Rhodopseudomonas palustris) closely related to sequences found near our study site (Chang et al. 2019), enhanced its diazotrophic activity upon the addition of ammonium (a reduced N-compound).

While the observed distribution of detectable N₂ fixation rates reported here supports the idea that low O₂ concentrations may favor diazotrophy, the limited range and low rates observed suggest that diazotrophs active in the Eastern Tropical South Pacific O₂ deficient zone are unlikely to represent a significant source of N₂ locally.

The role of organic carbon availability

Most detectable N₂ fixation rates, and all observed within suboxic waters, occurred at stations where coastal upwelling (Fig. 2A) drove high productivity, as indicated by surface chlorophyll a concentrations (Fig. 2B). At Sta. 14 and 19, depth-integrated chlorophyll a concentrations exceed 100 mg m⁻² in the euphotic zone—approximately double the average value observed among all stations. Particulate carbon concentrations, indicative of microbial abundance, tended to be high where N₂ fixation was detectable relative to measurements within the O₂ deficient zone at other stations (Suppl. Fig. 3). However, this difference was not statistically significant (Wilcoxon Rank Sum, n₁ = 8, n₂ = 40, U = -1.69, p = 0.092).

Several lines of evidence suggest that diazotrophic activity in deep waters is subject to variability in labile organic carbon inputs (which occurs mainly via photosynthetic production in surface waters). (1) Within the Eastern Tropical South Pacific O₂ deficient zone, nifH genes/transcripts largely group with methylo-trophic and heterotrophic bacteria (e.g., Löscher et al. 2014; Turk-Kubo et al. 2014; Chang et al. 2019), including known sulfate-reducers (Bonnet et al. 2013). However, recent work has demonstrated that many marine microbes are more metabolically flexible than previously thought (e.g., Füssel et al. 2017). Thus, diazotrophs that are known heterotrophs may be capable of utilizing different energy acquisition strategies. (2) Direct addition of organic carbon substrates to incubation bottles frequently enhances N₂ fixation rates in O₂ depleted waters (e.g., Bonnet et al. 2013; Löscher et al. 2014).
Interpretation of low rates

Overestimation

The potential for low N\textsubscript{2} fixation rates in deep, sub-euphotic environments (Moisander et al. 2017; Benavides et al. 2018) has instigated reconsideration of how to apply the \textsuperscript{15}N\textsubscript{2} tracer assay in pelagic environments (White et al. 2020). N\textsubscript{2} fixation rate methodology in the Eastern Tropical South Pacific O\textsubscript{2} deficient zone has therefore varied (Table 1), complicating comparisons between studies. Rates may be overestimated when the natural abundance of \textsuperscript{15}N in the PN pool (i.e., $A_{PN,0}$) is assumed rather than measured, when the mass of PN on the filter is too low (e.g., < 10 µg N), and/or detection
limits are not calculated based on instrument performance (see White et al. 2020 for comprehensive discussion).

Some studies assume that $A_{\text{PN}t=0}$ is in equilibrium with atmospheric $N_2$ (0.3663 atom-%). This is a poor assumption within and around denitrified waters and in many environments where the dissolved $N_2$ pool is highly dynamic; we observed a mean $A_{\text{PN}t=0}$ of $0.3692 \pm 0.0018$ atom-% ($n = 146$) throughout all surveyed waters at the time of our study. The difference between these values would have constituted detectable enrichment in about one-third of our IRMS runs in this study. Moreover, the mean natural abundance of $^{15}N$ in PN is typically greater in suboxic waters than in the overlying waters (Chang et al. 2019; this study; Voss et al. 2001). Consequently, the use of surface $A_{\text{PN}t=0}$ measurements for incubations conducted with suboxic water may result in the overestimation of rates from deeper waters. In January 2015, $A_{\text{PN}t=0}$ was significantly different between suboxic (0.3700 ± 0.0016 atom-%, $n = 44$) and oxic waters (0.3690 ± 0.0017 atom-%, $n = 94$; one-way ANOVA, $df = 136$, $F = 11.85$, $p = 0.0002$ based on 10,000 random permutations). These values are consistent with those reported by Chang et al. (2019) in July 2011, suggesting low seasonal/interannual variability.

The mass of PN collected on the filter at the end of the incubation is also crucial for accurate instrument detection. When sample mass is too low, typically below ~10 $\mu g$ N, isotope ratio measurements often drift (Suppl. Fig. 1; White et al. 2020). Either positive drift in $A_{\text{PN}t=0}$ or negative drift in $A_{\text{PN}t=1}$ may falsely inflate $N_2$ fixation rates. Even when mass is sufficiently high, analytical variability may affect the relative difference between $A_{\text{PN}t=0}$ and $A_{\text{PN}t=1}$, necessitating the determination of a minimum detectable difference in $A_{\text{PN}}$ based on instrument variability (e.g., 3σ of standards) from which a detection limit can be calculated (White et al. 2020). As variability is a function of mass (Suppl. Fig. 1; White et al. 2020), a conservative minimum detectable difference would be one based on the variability of standards at the lower end of the sample mass range. Where reported, minimum detectable differences in enrichment for Eastern Tropical South Pacific studies vary by ~10x (Table 1). We note that calculating $N_2$ fixation rate uncertainty as the standard deviation of rates from replicate incubations does not constrain variability in $A_{\text{PN}t=0}$, complicating inter-comparisons of low rates (Suppl. Text 1).

**Underestimation**

$N_2$ fixation rates may be underestimated because of slow $^{15}N_2$ equilibration (Mohr et al. 2010) when employing the traditional bubble method (Montoya et al. 1996), inappropriately large filter pore-size in systems with small diazotrophs (Bombar et al. 2018), drift in $A_{\text{PN}}$ measurements at low mass (see above), and/or inhibition of low $O_2$-adapted organisms by $O_2$ contamination during sampling. Löscher et al. (2014) found that $O_2$ exposure (10 $\mu M$) in low $O_2$ incubations reduced the abundance of $nifH$ associated with some non-cyanobacterial diazotrophs. While $O_2$ exposure is minimized by collecting samples using a submersible pump (as deployed in this study), even minor shifts in $O_2$ and substrate availability, temperature and pressure can alter the activity of $O_2$ deficient zone microbial communities (Stewart et al. 2012). Most studies from the Eastern Tropical South Pacific have attempted to address the issue of $O_2$ contamination (Table 1); however, the efficacy of different approaches and the effect of $O_2$ intrusion on $N_2$ fixation rates in suboxic waters, has not been assessed.

**Summary**

Given the issues outlined here, we proffer that some previous studies in the Eastern Tropical South Pacific may have overestimated $N_2$ fixation rates there. Depth-integration of rates near the analytical detection limit can compound this issue, making it essential that error is accurately calculated and propagated (Suppl. Text 1). Such overestimation of measured rates could resolve the discrepancy observed in the Eastern Tropical South Pacific between previously reported values and those predicted from the abundance of dominant proteobacterial diazotroph groups (Turk-Kubo et al. 2014).

In addition to the methodological differences outlined above (Table 1), discrepancies among $N_2$ fixation rates in surface waters between this and previous studies may also be attributable to temporal (see discussion above) and geographic differences among these studies; Bonnet et al. (2013), Dekaezemacker et al. (2013) and Knapp et al. (2016) all focused on the periphery of the South Pacific Subtropical Gyre, further offshore than this study (Fig. 3). However, given that sub-euphotic diazotrophs typically respond to organic matter inputs suggestive of heterotrophic carbon limitation (e.g., Bonnet et al. 2013; Löscher et al. 2014), we consider it unlikely that sub-euphotic $N_2$ fixation rates would be higher further offshore than beneath relatively more productive upwelling waters. Ultimately, differentiating detectable and undetectable $N_2$ fixation rates with sensitivity has far-ranging implications for our understanding of oceanic $N$ budgets and $N_2$ fixation in marine systems.

**Conclusions**

Our conservative evaluation contradicts previous reports of low but persistent $N_2$ fixation rates throughout the Eastern Tropical South Pacific $O_2$ deficient zone (Table 1), which are often cited in support of the hypothesis that $N_2$ fixation is widespread in the ocean’s interior (e.g., Benavides et al. 2018). Instead, our work suggests that $N_2$ fixation in this region is sparse and restricted to low $O_2$ waters in the upper $O_2$ deficient zone. Our findings support the idea that low $O_2$ conditions (e.g., Großkopf and LaRoche 2012; Bombar et al. 2016) and high surface productivity (e.g., Löscher et al. 2014) may favor $N_2$ fixation by non-cyanobacterial diazotrophs despite significant concentrations of ambient DIN. However, $N_2$
fixation in the Eastern Tropical South Pacific does not appear to be a quantitatively important source of N₂, either locally or globally. Quantifying the contribution of deep waters, where metabolic rates are low, to global biogeochemical cycles will ultimately require more sensitive discernment of biological signals from noise as we push the boundaries of analytical detection.

Data availability statement
Data presented here are available in the Supporting Material as well as on BCO-DMO (http://www.bco-dmo.org/project/742492).

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