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Heterogeneity of nitrogen fixation in the mesopelagic zone of the South China Sea

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Biological dinitrogen (N_2) fixation, the energetically expensive conversion of N_2 gas to ammonia, plays an essential role in balancing the nitrogen budget in the ocean. Accumulating studies show detectable N_2 fixation rates below the euphotic zone in various marine systems, revealing new insights of marine N_2 fixation. However, the reported rates are highly variable and frequently fall close to detection limits, raising the question of the ubiquity and significance of N_2 fixation in the global dark ocean. Using highly sensitive isotopic labeling incubation including a set of control incubations, we confirm the occurrence of mesopelagic N_2 fixation in the South China Sea. Interestingly, we consistently observed that ca. 30% of samples show a significant elevation of ^{15}N in the particulate nitrogen after incubation at most depths (200 - 1000 m). Although this approach does not allow accurate quantification of N_2 fixation rates, our data suggest the occurrence of dark N_2 fixation yet with highly heterogeneous signals in the mesopelagic zone of the South China Sea. A data compilation of reported N_2 fixation in the global dark ocean further reveals that such heterogeneity has also been observed elsewhere, unveiling the ubiquitous heterogeneity in mesopelagic N_2 fixation. Thus, we call for more observations to constrain mesopelagic N_2 fixation budgets and to understand the underlying mechanism for such heterogeneity.

KEYWORDS

nitrogen fixation, mesopelagic zone, heterogeneity, South China Sea, global compilation

1 Introduction

Biological nitrogen fixation (N_2 fixation) is an important source of new nitrogen to the open ocean by converting inert N_2 gas into bioavailable nitrogen (Jickells et al., 2017). N_2 fixation was long assumed to occur primarily within the euphotic zone where photoautotrophy can accommodate its high energetic cost (Zehr and Kudela, 2011). Nitrogenase is the enzyme responsible for N_2 fixation, and the highly conserved nitrogenase reductase gene (*nifH*) has been frequently targeted to detect diazotrophs in the ocean (Zehr and Capone, 1996). However, pervasive observations of *nifH*-containing non-photosynthetic microorganisms in the global ocean (Zehr et al., 1998; Moisander et al., 2008; Delmont et al., 2022) and recent studies demonstrating active N_2 fixation via non-cyanobacterial diazotrophs (Harding et al., 2022; Tschitschko et al., 2024) indicate the potential for non-cyanobacterial marine N_2 fixation, motivating broader investigations into the range of diazotrophs and their habitats, including in the mesopelagic zone (Bombar et al., 2016; Moisander et al., 2017; Benavides et al., 2018a).

Given the high energetic cost of N_2 fixation relative to the assimilation of nitrate (the major form of dissolved inorganic nitrogen (DIN) in the deep ocean; Falkowski, 1983; Zehr and Kudela, 2011), it seems counter-intuitive that this process occurs in nitrate-replete and energy-starved aphotic waters (Moisander et al., 2017). Several hypotheses have been proposed to explain the occurrence of mesopelagic N_2 fixation: (1) deep sea diazotrophs may lack the transporters necessary to assimilate or reduce nitrate (Karl et al., 2002; Bombar et al., 2016); (2) N_2 fixation may help cells maintain an ideal intracellular redox state (Bentzon-Tilia et al., 2015; Bombar et al., 2016); (3) nitrate could be depleted in aggregates as a result of microbial succession in densely packed microbial consortia (Dekas et al., 2009; Bombar et al., 2016; Chakraborty et al., 2021). Anaerobic microenvironments within particles or lower ambient oxygen concentrations at depth could also be suitable niches for diazotrophs because the high energetic cost largely comes from protecting nitrogenase from being inactivated by oxygen (Postgate, 1970; Paerl and Prufert, 1987; Großkopf and LaRoche, 2012; Farnelid et al., 2018; Riemann et al., 2022).

Several studies have detected N_2 fixation in the aphotic water column across a wide range of marine regimes including both hypoxic (Fernandez et al., 2011; Hamersley et al., 2011; Bonnet et al., 2013; Dekaezemacker et al., 2013; Farnelid et al., 2013; Loescher et al., 2014; Löscher et al., 2016; Gradoville et al., 2017; Chang et al., 2019; Selden et al., 2019, 2021b; Saxena et al., 2023) and oxygenated waters (Rahav et al., 2013, 2015; Benavides et al., 2015, 2016, 2018b; Weber, 2015; Gradoville et al., 2017; Mulholland et al., 2019; Shiozaki et al., 2023). Reported rates are highly variable, ranging from below the detection limit up to 35.9 nmol N L^{-1} d^{-1} in the oxygen minimum zone in the Eastern Tropical North Pacific (ETNP; Selden et al., 2019), but mostly fall below detection limits when they are assessed (Selden et al., 2021b). Despite the typically low volumetric rates, mesopelagic N_2 fixation can contribute up to 100% to integrated water column N_2 fixation rates (Benavides et al., 2016). As such, low rates matter for mesopelagic N_2 fixation, when integrated with the large volume of aphotic water, and thus, may contribute significantly to global nitrogen budget (Benavides et al., 2018a; Zehr and Capone, 2020).

However, owing to the low volumetric rates of mesopelagic N_2 fixation and low concentrations of particulate nitrogen (PN) in the ocean's interior, care must be taken to ensure that reported rates are representative and above the detection limits of analytical methods (Gradoville et al., 2017; Moisander et al., 2017; White et al., 2020). Mesopelagic N_2 fixation rates that are low or close to the detection limit could also be biased by processes other than N_2 fixation (e.g., isotopic fractionation during DIN or dissolved organic nitrogen assimilation), and inflated or deflated by errors in elemental analysis–isotope ratio mass spectrometry (EA-IRMS) at low PN amount (White et al., 2020). Thus, methods with sufficient $^{15}N_2$ enrichment together with controls and precise isotopic measurements are crucial. By far, EA-IRMS is still a major approach to determine the isotopic composition of PN. However, the recommended PN amount for EA-IRMS is 10 μg N, requiring large volume incubations (e.g. the averaged PN concentration of the mesopelagic water in our study was 0.07 μmol N L^{-1}, which results in a minimum volume of 10 L per sample for EA-IRMS) with high human and ship-board cost, limiting a broad measurement of mesopelagic N_2 fixation (Gradoville et al., 2017; White et al., 2020). Even the limited measurements show highly variable rates (Benavides et al., 2018b). Moreover, several studies find the change of ^{15}N before and after incubation is highly variable between replicate samples collected from the same depth, hindering the determination of a robust rate (Bonnet et al., 2013; Gradoville et al., 2017). However, it is also improper to conclude the absence of mesopelagic N_2 fixation when some of the replicates show reliable elevation of ^{15}N. Overall, the relatively few rate measurements performed up until now, their variability, and the fact that many observations are at or near the limit of analytical/computational detection result in ambiguous understanding of mesopelagic N_2 fixation and large uncertainties in their contribution to the global fixed N budget, calling for in depth investigation of this process (Moisander et al., 2017; White et al., 2020; Selden et al., 2021b).

Here, we conducted N_2 fixation incubations in the mesopelagic South China Sea (SCS), the largest marginal sea in the western Pacific Ocean. The SCS is a representative oligotrophic marginal sea, although active N_2 fixation has been repeatedly reported in the euphotic zone of the SCS, mesopelagic N_2 fixation remains to be investigated in the SCS (Wu et al., 2024). Therefore, SCS is an ideal place to test the ubiquity of mesopelagic N_2 fixation. To better constrain the isotopic composition of PN under low PN amounts, we implemented an alkaline persulfate digestion (Knapp et al., 2005) coupled with a denitrifier method (the persulfate-denitrifier method hereafter; Sigman et al., 2001; Casciotti et al., 2002; McIlvin and Casciotti, 2011). This method requires only 1.4 μg N (or 100 nmol N) rather than the roughly 10 μg N required when using standard EA-IRMS analysis (White et al., 2020). We also conducted parallel $HgCl_2$-killed and tracer-free controls to account for non-diazotrophic signals possibly reflected in isotopic composition of PN. Using this new method, we were able to report robust mesopelagic N_2 fixation signals in individual samples yet with high variabilities between the replicated samples collected from

the same depth and between different depths and stations, reflecting detectable but not quantifiable mesopelagic N_2 fixation and its heterogeneity. Our new results add to the global data compilation, reveal that heterogeneity is an inherent nature of mesopelagic N_2 fixation, and call for more research and new approaches to account for such heterogeneity in estimating the rates and further its contribution to the global N budget.

2 Materials and methods

2.1 Field sampling

Water samples were collected from 4 stations during 2 cruises in the SCS aboard the *R/V Tan Kah Kee* in August 2018 (KK1806) and July 2019 (KK1905; Figure 1). Salinity, temperature, dissolved oxygen, fluorescence, and photosynthetically active radiation (PAR) data were obtained via a SeaBird conductivity-temperature-depth (CTD) profiler (SBE 911 plus). Water samples for incubations were obtained from a rosette equipped with 24 × 12-L-Niskin bottles.

2.2 N_2 fixation incubations

N_2 fixation was measured by the incorporation of $^{15}N_2$ into PN (Montoya et al., 1996). All incubations were conducted in the dark and at *in situ* temperature. Incubations were terminated after 24 to 96 hours by filtration of PN onto pre-combusted 25 mm 0.3 µm GF75 filters (Advantec) under <400 mbar. GF75 filters were used due to their efficiency in catching <0.7 µm diazotrophs (Bombar et al., 2018). All filters were preserved at – 80°C until isotopic composition analysis.

Samples were collected from 4 stations at different depths (K1: 5, 15, 30, 50, 75, 100, 200, 300 and 740 m; SEATS: 5, 15, 30, 50, 75, 100, 200, 300, 705, 1000, 2000 and 3800 m (bottom); SS1: 5, 15, 25, 50, 75, 100, 120, 150, 200, 300, 500, 700, 1000 and 4000 m (bottom); WXS: 5, 200 and 855 m; Figure 1). Sampling at each station included the oxygen minimum depth (OMD; K1: 740 m; SEATS: 705 m; SS1: 700 m; WXS: 855 m), although oxygen concentrations were never severely depleted (> 62 µM). Water samples from above 100 m were collected into acid-cleaned 1 L polycarbonate bottles (Nalgene) and those below 100 m were collected in acid-cleaned 4 L fluorinated polyethylene (FLPE) bottles. After sampling, bottles were kept in the dark using light-blocking black plastic bags until incubations began. Incubations above 200 m at the SEATS, K1 and SS1 stations were started at dusk (17:00 – 19:30). Supplementary Figure 1 provides a schematic plot of the different incubations conducted during both cruises.

During the August 2018 cruise, N_2 fixation was measured using the $^{15}N_2$-enriched seawater method (Mohr et al., 2010). However, this method reportedly reduces atom-% enrichment to 2-5% (White et al., 2020), requiring larger volumes of the enriched seawater for the incubations in environments with low N_2 fixation rates. In addition, large 0.2 µm-filtered enriched seawater (as much as 10% v/v) amendments dilute PN, and thus decrease the sensitivity of IRMS measurements. In contrast, the $^{15}N_2$ bubble method (Montoya et al., 1996), which we employed on the July 2019 cruise, does not dilute PN. However, this method can significantly underestimate N_2 fixation rates since the $^{15}N_2$ bubble can take between 3 to 15 hours to dissolve (Mohr et al., 2010; Wannicke et al., 2018; White et al., 2020). Our 24- to 96-hour incubations were designed to minimize the effect of delayed bubble dissolution as well as provide sufficient time for PN enrichment in ^{15}N (Wannicke et al., 2018). Still, our results should be considered as conservative

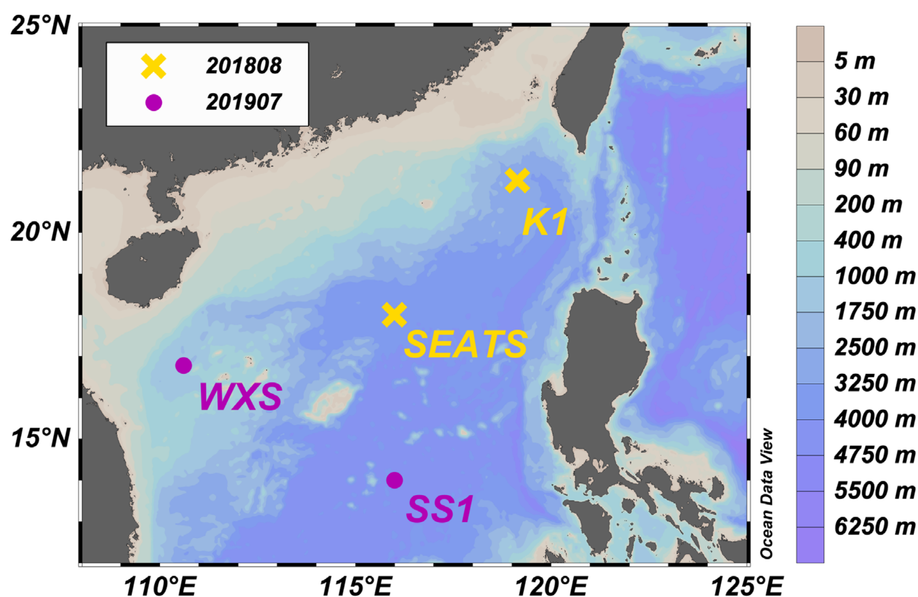

FIGURE 1
Station map of the two cruises in the SCS. Stations visited in August 2018 are shown in orange and those visited in July 2019 are shown in purple. Background color indicates bathymetry. The map was generated via Ocean Data View (Schlitzer, Reiner, Ocean Data View, odv.awi.de, 2020).

estimates considering the time for bubble dissolution, and possible cross-feeding and isotope dilution due to the long incubation periods. Details of our experimental design are shown in Table 1 and Supplementary Figure 1. The addition of $^{15}N_2$ in the incubation bottles was kept <10 ^{15}N-atom% for $^{15}N_2$-enriched seawater method and <20 ^{15}N-atom% for $^{15}N_2$ bubble method. Samples below 200 m were incubated to two destructively sampled time points: 48 and 96 h during the August 2018 cruise and 24 and 48 h during the July 2019 cruise.

The $^{15}N_2$-enriched seawater amendment used for the August 2018 incubations was made ≤24 h prior to the experiments following previous studies (Shiozaki et al., 2015; Lu et al., 2017, 2019). Seawater was filtered (0.22 µm membrane, Millipore), degassed (STERAPORE 20M1500A membrane, Mitsubishi Rayon Co., Ltd.) and stored in 2 L Tedlar Bags excluding bubbles. 20 mL $^{15}N_2$ gas (98.9%, Cambridge Isotope Laboratories) was injected and mixed manually until all bubbles dissolved. Either 100 mL or 400 mL of $^{15}N_2$-enriched seawater was added to 1 L or 4 L incubation bottles, respectively, resulting in $^{15}N_2$ enrichments of 6 to 10 or 7 to 9 ^{15}N-atom%, respectively (assuming complete $^{15}N_2$ dissolution). Due to limited water sample availability, seawater from the surface and 1000 m was collected in the SCS basin area prior to incubation sampling to make the $^{15}N_2$-enriched seawater used for 5 to 100 m and 200 to 3800 m incubation samples, respectively. Samples were filtered immediately after tracer addition to determine the initial (T_0) ^{15}N-atom% of the PN. Incubations were conducted for 24 h for waters ≤100 m and for 48 and 96 h for waters >100 m under the dark condition and *in situ* temperature (± 6°C for ≤100 m samples, and ± 2.5°C for all the other samples).

For the $^{15}N_2$ bubble method used on the July 2019 cruise, incubation bottles were filled with seawater excluding bubbles. Natural samples (considered as T_0) were filtered immediately after sampling before tracer addition. Either 1 mL or 10 mL of $^{15}N_2$ gas (98.9%, Cambridge Isotope Laboratories) equilibrated to atmospheric pressure was added through septum caps to the 1 L and 4 L bottles, respectively. Bottles were then inverted at least 10 times to enhance dissolution. Incubations were conducted in the dark and at *in situ* temperatures ±2.5°C for 24 h for waters <200 m (except the 5 m sample at WXS station, for which 48-hour incubations were also conducted) and for 24 and 48 h for waters ≥200 m.

2.3 Control experiments

Both tracer-free and $HgCl_2$-killed control incubations were conducted in August 2018 at 200 m, 300 m, 705 m, 1000 m, 2000 m, 3800 m depths at the SEATS station parallel to the N_2 fixation incubations. For tracer-free controls, 1 L polycarbonate bottles were fully filled with seawater without any $^{15}N_2$ addition and incubated as described above to determine any change in ^{15}N-atom% of PN over the course of incubations. For the killed controls, after adding 1 mL ca. 0.2 mol L^{-1} $HgCl_2$ solution, 100 mL $^{15}N_2$-enriched seawater was added to 1 L polycarbonate bottles to test whether abiotic processes could change the ^{15}N-atom% of PN over the course of incubations. Controls were incubated and PN samples were collected as described above.

2.4 PN concentration and ^{15}N-atom% determination

Filters collected as described above were freeze-dried and the PN they contained was subsequently oxidized to nitrate using the alkaline persulfate digestion method (Knapp et al., 2005; Yan et al., 2022). The concentration and isotopic composition of this nitrate were then determined via the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002; McIlvin and Casciotti, 2011) using a GasBench IRMS (Delta V, Thermo Scientific). Briefly, potassium peroxodisulfate ($K_2S_2O_8$, Merck, 105091) was recrystallized three times for purification, then mixed with sodium hydroxide (NaOH, Merck, 106498) and ultrapure water, forming peroxodisulfate reagent (POR) with 6: 6: 100 mass ratio of $K_2S_2O_8$: NaOH: Milli-Q water. Before each digestion experiment, POR blank was

TABLE 1 Experimental treatments used in this study.

Cruise	Station	Depth (m)	Treatment	Method	Replicates	Time Series (hours)	Filtered volume
Aug 2018	K1	5, 15, 30, 50, 75, 100		$^{15}N_2$-enriched seawater	2	0, 24	1 L
	K1	200, 300, 740, 1000		$^{15}N_2$-enriched seawater	4	0, 24	1 L
	SEATS	5, 15, 30, 50, 75, 100		$^{15}N_2$-enriched seawater	2	24	1 L
	SEATS	200, 300, 705, 1000, 2000, 3800	No tracer/$HgCl_2$	$^{15}N_2$-enriched seawater	4	0, 48, 96	1 L
Jul 2019	SS1	5, 15, 25, 50, 75, 100		$^{15}N_2$ gas bubble	3	0, 24	1 L
	SS1	120, 150		$^{15}N_2$ gas bubble	3	0, 24	4 L
	SS1	200, 300, 500, 700, 1000, 4000		$^{15}N_2$ gas bubble	3	0, 24, 48	4 L
	WXS	5, 200, 855		$^{15}N_2$ gas bubble	3	0, 24, 48	4 L

All incubations were in dark conditions. "No tracer" and "$HgCl_2$" denote $^{15}N_2$-tracer-free and $HgCl_2$-killed controls, respectively. Replicates indicate how many bottles were sampled at each time point.

determined by autoclaving POR solution at 105°C for 70 min, and their pH was adjusted to ca. 6 with 6 mol L^{-1} hydrochloric acid (HCl, Merck, 100317), after which nitrate concentrations were determined using the chemiluminescence method (Braman and Hendrix, 1989). The POR was only used if its N blank was below 2 μM N (Supplementary Figure 2A). A volume of 0.5 mL of POR was added to each sample, which was digested using the same procedure as POR blanks.

The N introduced by the POR was <1 nmol (<4% of the total sample N). The isotopic composition of blanks in each batch were reproducible. Likewise, the filter blanks contained 11.9 ± 2.7 nmol N (Supplementary Figure 2C), accounting for 14.0 ± 4.5% and 4.6 ± 3.3% of the N content of the samples in the August 2018 and July 2019 cruises, respectively. The low and reproducible contribution of the POR and filters reduced uncertainties, lowered detection limits for measured sample N, and can be subtracted as well.

The N recovery (102 ± 3.6%) and reproducibility of this digestion method were determined using known amounts of urea and EDTA (Supplementary Figure 2B). $\delta^{15}N$ was -1.5 ± 0.3‰ for urea and -0.4 ± 0.1‰ for EDTA. These were not significantly different from $\delta^{15}N$ values acquired via EA-IRMS which were -2.0 ± 0.03‰ and -0.4 ± 0.05‰ for urea and EDTA, respectively (Supplementary Figure 2B). Both recovery rates and $\delta^{15}N$ values were stable between 90 and 2000 nmol of urea and EDTA (Supplementary Figure 2B), suggesting a negligible isotope fractionation effect during the digestion process. Together, the low blank and stable recovery of added N during the digestion experiment enabled a precise measurement of the concentration and isotopic composition of PN down to 90 nmol PN, which was the minimum tested value in this study. Although the minimum amount of N required for stable $\delta^{15}N$ using the denitrifier method can be as low as 5 nmol (Supplementary Figure 2D). However, in order to minimize biases caused by blanks of GF75 and POR (total 11.9 ± 2.7 nmol N), our recommended amount of PN for this method is ≥100 nmol.

The original ^{15}N-atom% and PN amount data obtained in this study are available in Supplementary Table 1.

2.5 Analytical considerations for dark N_2 fixation

Reliable N_2 fixation signals come from both sufficient PN amount for isotopic determination and the increase of ^{15}N-PN exceeding the minimum acceptable change. As mentioned above, our recommended amount of PN for the persulfate-denitrifier method is ≥100 nmol. However, not all the samples exceeded this recommended amount. Considering that more PN amount leads to less impact of blanks and more stable isotopic analysis by IRMS, we divide samples into 3 intervals according to the PN amount on the filters, i.e. <100 nmol N, 100 - 400 nmol N, and >400 nmol N. For each interval, we calculated averaged ^{15}N-atom% of PN and their standard deviations for T_0s from the N_2 fixation incubations that fall into each interval. Then, the minimum acceptable changes for N_2 fixation for each interval were set to be the averaged ^{15}N-atom% + 3 times the standard deviation. In this way, we account for both natural variability and analytical errors. We consider that N_2 fixation occurs when the ^{15}N-atom% enrichment of PN after incubation surpasses the minimum acceptable change. In addition, rates were only considered quantifiable when the averaged ^{15}N-atom% after incubation exceeded 10 times the standard deviation of replicates. As a result, even though we detected N_2 fixation signals, there is no quantifiable N_2 fixation rate below 200 m. Therefore, no rates were calculated here.

2.6 Data compilation

We collected available dark N_2 fixation rate data (depth ≥200 m) from previous studies listed in Table 2. We combined studies in similar geographical regions, and only included those with more than 5 data points for representative comparison. As a result, we compiled 8 studies, i.e. Gradoville et al. (2017) for ALOHA station in north Pacific subtropical gyre, Benavides et al. (2016) for Mediterranean Sea, Selden et al. (2019) for Eastern Tropical North Pacific, Bonnet et al. (2013) and Löscher et al. (2016) for Eastern Tropical South Pacific, Benavides et al. (2018b) for Western Tropical South Pacific, Shiozaki et al. (2023) for Eastern South Pacific, and Saxena et al. (2023) for Bay of Bengal. It is also worth noting that N_2 fixation rates from ALOHA were all below the minimum quantifiable rates (Gradoville et al., 2017). The data used for compilation are shown in Supplementary Table 2.

3 Results and discussion

3.1 Detectable but not quantifiable mesopelagic N_2 fixation in the South China Sea

We investigated mesopelagic N_2 fixation in the South China Sea basin from 200 m until around 4000 m bottom depths. In order to distinguish the N_2 fixation signal from other processes that might have resulted in ^{15}N-atom% change of PN, we conducted parallel control incubations with no tracer addition and with $^{15}N_2$ tracer but killed with $HgCl_2$ at SEATS station from 200 m to the bottom depth of 3800 m. Our results show that only two samples in all the samples of the control experiment (<3%) exceeded the minimum acceptable change of ^{15}N-atom% for PN, which is defined according to the T_0s in N_2 fixation incubations (Figure 2). This supports the applicability of the above defined minimum acceptable ^{15}N-atom% change, and also indicates that processes other than N_2 fixation had negligible effect on the ^{15}N-atom% of PN during the incubations. By comparison, more than 20% of the ≥200 m samples after parallel $^{15}N_2$ incubation had a ^{15}N-atom% of PN above the minimum acceptable change (Figure 3), showing the occurrence of active N_2 fixation at mesopelagic depths, but that could not be quantified due to the large variation between replicates. Moreover, statistical analyses show that ^{15}N-atom% of PN at T_1 and T_2 are significantly higher than those at T_0 (t-Test: samples assuming unequal variances, $p < 0.05$), whereas no significant difference was found in the control experiments (Figure 4). This further confirms

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TABLE 2 Compilation of N_2 fixation rate measurements conducted below the euphotic zone (mesopelagic N_2 fixation) using $^{15}N_2$ labeling techniques.

Location	Depth (m)	N_2 fixation rate (nmol N L^{-1} d^{-1})	Mesopelagic contribution to total %	Mesopelagic integrated NFR (μmol N m^{-2} d^{-1})	Method	Gas manufacturer	Reference
Hypoxic waters							
Southern California Bight	500, 885	0.7	ca. 30% (below DCM to 855 m)	55	$^{15}N_2$ bubble	98%, Sigma-Aldrich/Isotec	Hamersley et al., 2011
Eastern Tropical South Pacific	OMZ core (deepest 400 m)	BDL-3.5	ca. 90% (deepest level of 1 mmol L^{-1} O_2 to 400 m)	574 ± 294	$^{15}N_2$ bubble	99%, CAMPRO SCIENTIFIC	Fernandez et al., 2011
Baltic Sea	200	0.44 ± 0.26	[a]6% (Suboxic and anoxic area)	Not reported	$^{15}N_2$ bubble	98%, CAMPRO SCIENTIFIC	Farnelid et al., 2013
Eastern Tropical South Pacific	200-2000	BDL-0.6	87-90% (below the euphotic zone to 2000 m)	119-501	$^{15}N_2$ bubble	99% [f]EURISO-TOP	Bonnet et al., 2013
Eastern Tropical South Pacific	200	0.37	Not reported	Not reported	$^{15}N_2$ bubble	99% [f]EURISO-TOP	Dekaezemacker et al., 2013
Peruvian OMZ	200	0.4	Not reported	Not reported	$^{15}N_2$ bubble	98%, Sigma-Aldrich	Loescher et al., 2014
Eastern Tropical South Pacific	200-500	BDL-4.39	Not reported	150.6-628.7 (0-500 m)	$^{15}N_2$ enriched seawater	98% Cambridge Isotopes	Löscher et al., 2016
Pacific Northwest coastal upwelling system	600	BDL	Not reported	Not reported	$^{15}N_2$ enriched seawater	99% Cambridge Isotopes	Gradoville et al., 2017
Eastern Tropical South Pacific	200-350	BDL	Not reported	Not reported	Modified $^{15}N_2$ bubble	99% Cambridge Isotope Labs	Chang et al., 2019
Eastern Tropical North Pacific	200-3001	BDL-35.9	Not reported	Not reported	Modified $^{15}N_2$ bubble	99% Cambridge Isotopes	Selden et al., 2019
Eastern Tropical South Pacific	80-2500	[b]BDL-0.77	Not reported	Not reported	Modified $^{15}N_2$ bubble	99% Cambridge Isotopes	Selden et al., 2021b
Bay of Bengal	90-1500	BDL-0.35	0-100%	0-116.1	$^{15}N_2$ bubble	98% Cambridge Isotopes	Saxena et al., 2023
Without hypoxia							
Levantine Basin	250-500	0.01-0.24	37-75% (0.1% PAR to 500 m)	Not reported	$^{15}N_2$ bubble	Not mentioned	Rahav et al., 2013
Gulf of Aqaba	150-720	0.02-0.38	56% (0.1% light to 720 m)	Not reported	$^{15}N_2$ bubble	Not mentioned	Rahav et al., 2013
Gulf of Aqaba	200	0.2-0.3	Not reported	Not reported	$^{15}N_2$ bubble	Not mentioned	Rahav et al., 2015
Gulf of Mexico	330-538	[c]1.06 ± 0.24 × 10^{-5} h^{-1}	Not reported	Not reported	$^{15}N_2$ bubble	99% Cambridge Isotope	Weber, 2015

(Continued)

TABLE 2 Continued

Location	Depth (m)	N_2 fixation rate (nmol N L^{-1} d^{-1})	Mesopelagic contribution to total %	Mesopelagic integrated NFR (μmol N m^{-2} d^{-1})	Method	Gas manufacturer	Reference
Without hypoxia							
Solomon Seas	200-1000	BDL-0.35	[d]25% (200-1000 m)	Not reported	$^{15}N_2$ enriched seawater	98% [f]EURISO-TOP	Benavides et al., 2015
Bismarck Seas	200-1000	BDL-0.89	[d]25% (200-1000 m)	Not reported	$^{15}N_2$ enriched seawater	98% [f]EURISO-TOP	Benavides et al., 2015
Mediterranean Sea	200-2000	BDL-0.07	48-100% (below 0.01% PAR to 2000 m)	17.83-91.06	$^{15}N_2$ bubble	98.9% Cambridge Isotopes	Benavides et al., 2016
North Pacific Subtropical Gyre	200	BDL	Not reported	Not reported	$^{15}N_2$ enriched seawater	99% Cambridge Isotopes	Gradoville et al., 2017
Western Tropical South Pacific	200-800	0.05-0.68	[e]ca. 6-88% (200-800 m)	Not reported	$^{15}N_2$ bubble	98.9% [f]EURISO-TOP	Benavides et al., 2018b
Eastern South Pacific	200-3832	BDL-0.06	Not reported	Not reported	$^{15}N_2$ bubble	99.8% SI Science	Shiozaki et al., 2023

For studies without explicit depth of euphotic zone, only data ≥200 m were included. NFR, N_2 fixation rate; BDL, below detection limit; OMZ, oxygen minimum zone.
[a]Contribution of annual surface water NFR in Baltic Sea.
[b]If a limit of quantification is applied, then all detectable rates would be non-quantifiable.
[c]Shown in specific N_2 fixation rate.
[d]Of 5 to 70 m integration.
[e]Of photic zone integration.
[f]Subsidiary of Cambridge Isotope Laboratories.

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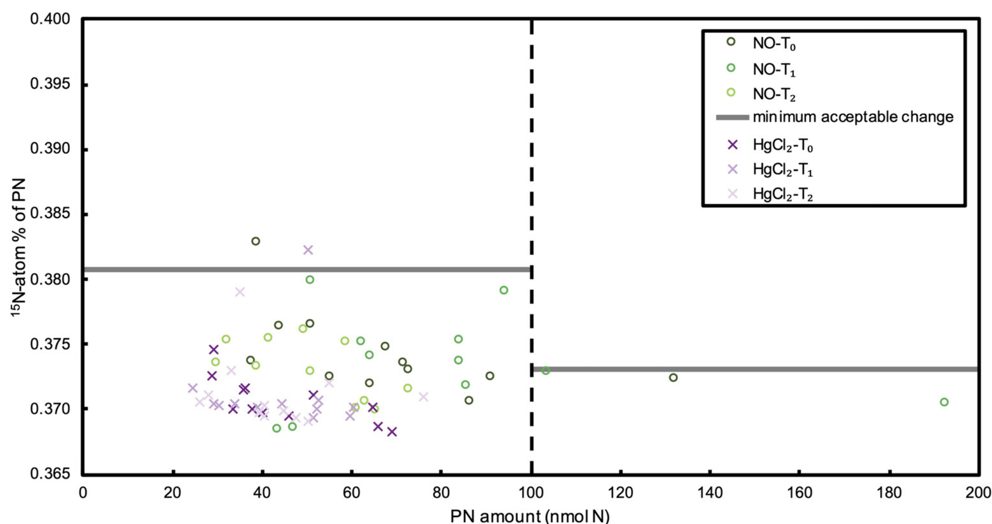

FIGURE 2
^{15}N-atom% of PN in no tracer (NO) and killed ($HgCl_2$) control incubations at SEATS station 200 - 3800 m. The x-axis indicates the PN amount on the sample filters, and the vertical dashed line indicates PN amount of 100 nmol N. Horizontal gray lines represent the minimum acceptable ^{15}N-atom% change for different intervals.

the robustness of N_2 fixation signals, and negligible effect of other processes on ^{15}N-atom% of PN.

3.2 Mesopelagic N_2 fixation shows great heterogeneity

With detectable mesopelagic N_2 fixation signals revealed by ^{15}N-atom% of PN, we also observed great variation in those signals. The ^{15}N-atom% of PN in T_1 and T_2 samples are obviously more variable than those in control experiments, while the variation in T_0s are similar across different treatments (Figure 4), being indicative of highly variable mesopelagic N_2 fixation between different stations, depths, and even between the replicated samples collected from the same depths. Of course, the difference between stations might be also partly affected by the different incubation settings between the two cruises, while variabilities within replicates and between depths are not subject to such incubation differences. It is worth noting that most of the ^{15}N-atom% change of the mesopelagic samples after incubation were still within the minimum acceptable value, suggesting sporadic occurrence of N_2 fixation. However, while ^{15}N-atom% of PN in T_1 and T_2 are significantly higher than those in T_0 in N_2 fixation incubations, we observed no significant difference between ^{15}N-atom% of PN in T_1 and T_2 (Figure 4). This is also likely due to the heterogeneity in the

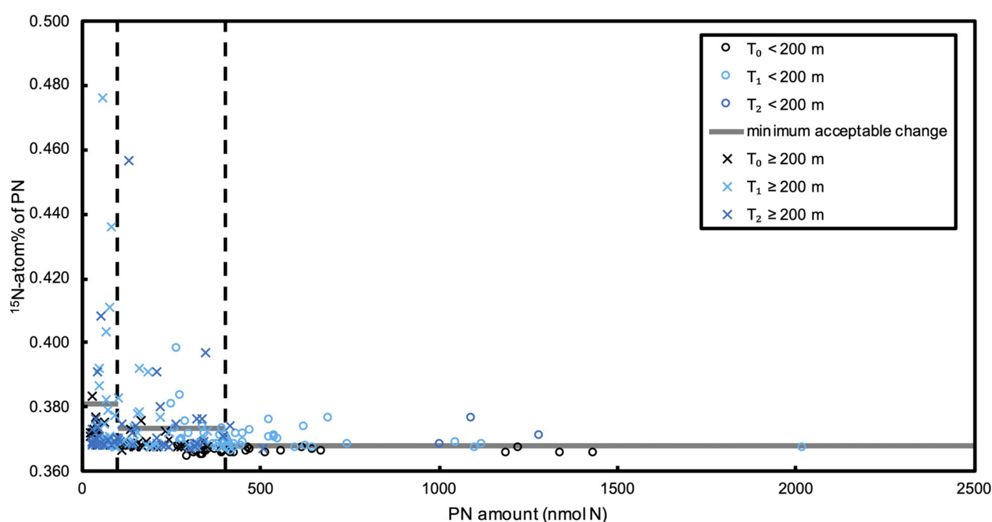

FIGURE 3
^{15}N-atom% of PN in N_2 fixation incubations at all incubation depths. The x-axis indicates the PN amount on the sample filters, and the vertical dashed lines indicate PN amount of 100 and 400 nmol N. Horizontal gray lines represent the minimum acceptable ^{15}N-atom% change for different intervals.

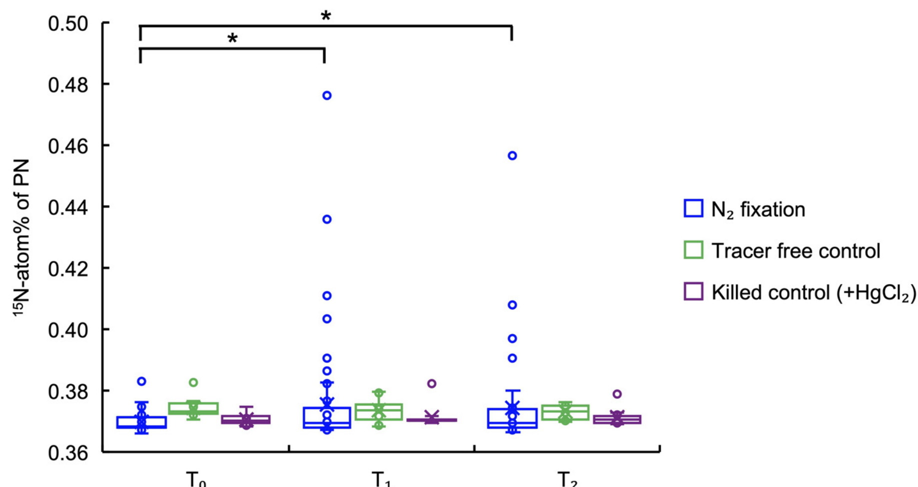

FIGURE 4
Box plot of ^{15}N-atom% of PN ≥200 m under N_2 fixation (blue), tracer free control (green), and killed control (+$HgCl_2$, purple) incubations. Black solid lines with asterisks (*) on top represent statistically significantly different groups, i.e. T_1s and T_2s in N_2 fixation incubations are significantly different from T_0s (t-Test: samples assuming unequal variances, $p < 0.05$).

abundance and composition of N_2-fixing microorganisms, which results in large variation of ^{15}N-atom% in both T_1 and T_2 samples, and leads to a lack of significant differences in the time series incubation. The high heterogeneity of mesopelagic N_2 fixation observed here is also consistent with previous observations showing high heterogeneity of marine particles in composition, which may provide variable conditions for N_2 fixation (Iversen and Lampitt, 2020; Ho et al., 2022; Comstock et al., 2024). Based on the high variation in ^{15}N-atom% between the replicated samples, we further speculate uneven distribution, community composition and activities of N_2-fixing microorganisms in mesopelagic waters.

We then quantified the frequency of samples showing significant increase of ^{15}N-atom% in replicates after incubations to see whether there are any vertical patterns. Generally, the result showed that samples from <200 m depths had a higher possibility of catching N_2 fixing signals compared to greater depths (≥200 m, Figure 5). This could be due to the presence of cyanobacterial diazotrophs (e.g. UCYN-B) that fix nitrogen in the dark or non-cyanobacterial diazotrophs that do not directly require light for energy or both (Chen et al., 2019; Turk-Kubo et al., 2023; Masuda et al., 2024). Only between 15 - 75 m (corresponding to the depth range with highest N_2 fixation in the South China Sea; Lu et al., 2019; Wen et al., 2022) did we find incubations in which all of the replicates showed detectable N_2 fixation. However, even in the euphotic zone, there were many depths at which some of the replicates showed no detectable N_2 fixation, reinforcing that the heterogeneity of N_2 fixation extends to the euphotic zone. The lack of detectable N_2 fixation in some euphotic zone incubations may also be due to dark incubation conditions limiting autotrophic N_2 fixation by cyanobacteria, or the low isotope sensitivity caused

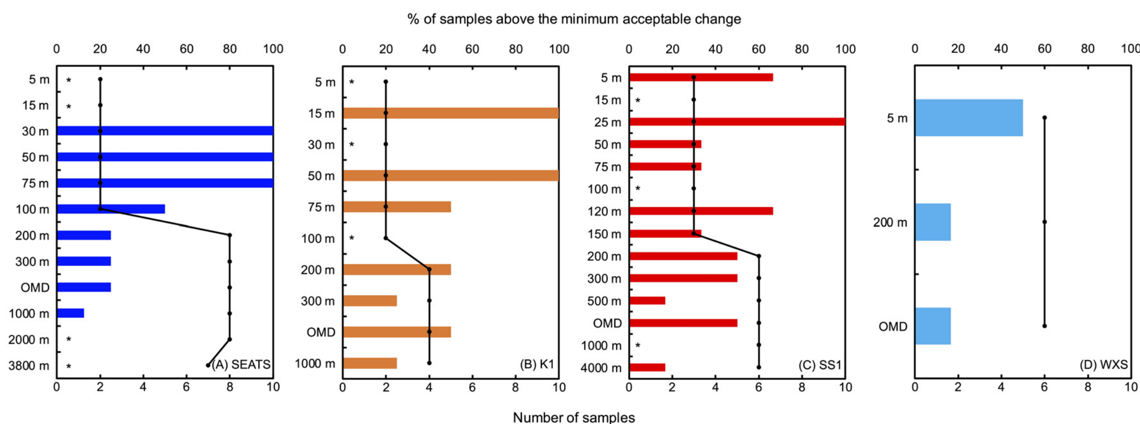

FIGURE 5
Proportion of ^{15}N-atom% of PN after incubation above the minimum acceptable change in SEATS **(A)**, K1 **(B)**, SS1 **(C)** and WXS **(D)** stations (bar plots). Scatter plots indicate how many points were sampled after incubations, i.e. the sum of T_1 and T_2 (if sampled) replicates. Bar plots indicate the percentage of sampled ^{15}N-atom% of PN surpassed the minimum acceptable change. Asterisks (*) indicate that all of the ^{15}N-atom% of PN after incubations was below the minimum acceptable change (0%).

by high PN concentrations (White et al., 2020). In fact, at 5 m in WXS station, the only <200 m depth time course, we observed significant linear production of ^{15}N-PN from N_2 fixation even though only T_2 replicates passed the minimum acceptable change (Supplementary Figure 3). This suggests that our conservative calculation of the detection limit for N_2 fixation may be masking measurable N_2 fixation. At mesopelagic depths, no depth had detectable N_2 fixation signals in >50% of replicates, while we consistently observed N_2 fixation signals in 1 - 3 of the 4 - 8 replicates (12.5 - 50%) between 200 m and 1000 m, except for 1000 m at SS1 station that had no replicate with N_2 fixation signal. Interestingly, we observed that around 30% of replicates at most depths exhibited detectable N_2 fixation signals, an observation, which was surprisingly stable regardless of the different methods and replicates we used. The stably low frequency of detectable N_2 fixation signals, therefore, reveals the inherent nature of heterogeneity in mesopelagic N_2 fixing microorganisms. It seems that no matter how many replicates we have, with the huge heterogeneity observed, the widely accepted quantification criteria, i.e. 10 times of the standard deviation of replicates, cannot be met (White et al., 2020). Unfortunately, we haven't found a way yet to overcome this heterogeneity for robust rate calculation. However, with the recurrence of detectable N_2 fixation signals, we suggest the occurrence of mesopelagic N_2 fixation in the SCS despite that the rates were not quantifiable due to the large variation of ^{15}N-atom% between the replicated samples. More studies are needed to understand the heterogeneity of mesopelagic N_2 fixation and its causes, and further how to obtain representative rates under such heterogeneity. In contrast, below 1000 m (2000 m and 3800 m in SEATS station and 4000 m of SS1 station), only one sample showed an increase of ^{15}N-atom% above the minimum acceptable change, implying no detectable dark N_2 fixation at greater depths than 1000 m in the SCS. To date, N_2 fixation at the deep ocean (>1000 m) was only observed in several studies conducted in the Eastern Tropical North Pacific (Selden et al., 2019), Eastern South Pacific (Bonnet et al., 2013; Shiozaki et al., 2023), Mediterranean Sea (Benavides et al., 2016), and the Bay of Bengal (Saxena et al., 2023).

3.3 Global data compilation

To compare dark N_2 fixation rates measured in the global ocean, we further compiled mesopelagic N_2 fixation rates from 8 studies reported globally, which revealed a great heterogeneity across different geological regimes (Table 2; Figure 6). Reported N_2 fixation rates ranged between below detection limit to 35.9 nmol N L^{-1} d^{-1} (Table 2), revealing great variability among studies and ocean basins. More than 80% of compiled N_2 fixation rates were below 0.2 nmol N L^{-1} d^{-1} with around one third reported as below detection limit. High rates of >2 nmol N L^{-1} d^{-1} were observed occasionally in the Eastern Pacific (Figure 6), mostly associated with low oxygen conditions (Löscher et al., 2016; Selden et al., 2019). However, high rates are extremely patchy with the vast majority of mesopelagic N_2 fixation rates in the oxygen minimum zones still comparable to those in the oxygenated areas. This indicates that the low oxygen condition favors the activity of diazotroph, but the occurrence of dark N_2 fixation is likely regulated by multiple factors rather than oxygen only.

Locally, reported mesopelagic N_2 fixation rates can also vary across magnitudes even within a single study (Table 2). For

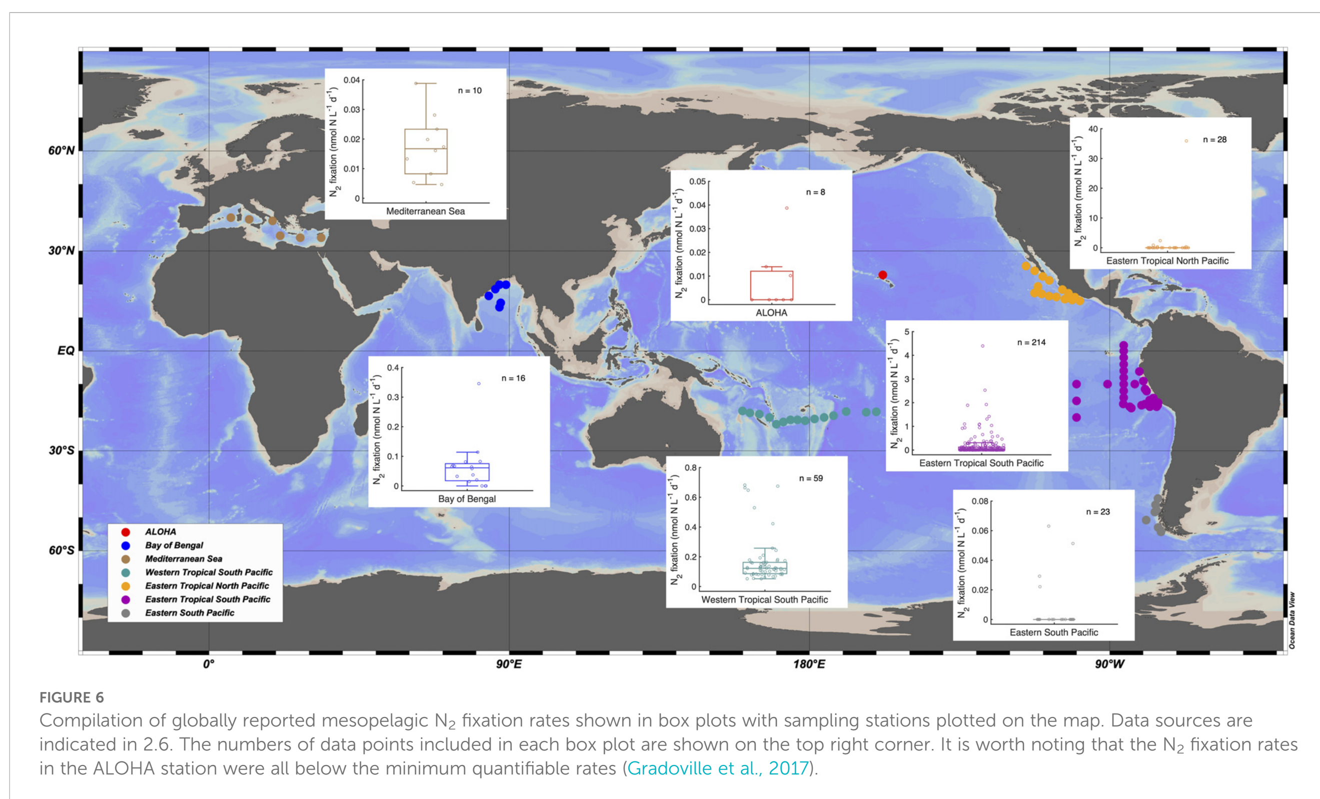

FIGURE 6
Compilation of globally reported mesopelagic N_2 fixation rates shown in box plots with sampling stations plotted on the map. Data sources are indicated in 2.6. The numbers of data points included in each box plot are shown on the top right corner. It is worth noting that the N_2 fixation rates in the ALOHA station were all below the minimum quantifiable rates (Gradoville et al., 2017).

example, the rates in the Eastern Tropical North Pacific varied from below detection limit to 35.9 nmol N L^{-1} d^{-1}, yielding no statistical differences with any other regions in Figure 6, possibly due to the high variability. Localized heterogeneity in N_2 fixation was also reported for epipelagic oceans, which might be caused by variabilities in environmental parameters (Messer et al., 2015; Selden et al., 2021a). Similarly, mesopelagic N_2 fixation might also be regulated by various local diazotrophic communities and environmental conditions, such as sinking particles, bioavailability of dissolved organic matter, dissolved oxygen, and eddies (Löscher et al., 2016; Selden et al., 2019; Chakraborty et al., 2021). Therefore, heterogeneity of mesopelagic N_2 fixation reveals the coincidence of N_2 fixing microorganisms and the environmental conditions that allow them to fix N_2.

Temporal dynamics of mesopelagic N_2 fixation are seldomly reported due to limited observations. In terms of seasonal variabilities, Hamersley et al. (2011) reported highest mesopelagic N_2 fixation rates in winter mixed period in Southern California Bight, while contrasting results were observed from Gulf of Aqaba and Levantine Basin with higher rates in the stratified periods than the mixed periods (Rahav et al., 2013, 2015). Interannual variabilities of mesopelagic N_2 fixation were only reported for the most extensively studied site, Eastern Tropical South Pacific, which might be largely related to upwelling intensity (Selden et al., 2021b).

The great heterogeneity between studies might also come from the different methods used for rate determination (Selden et al., 2021b), as N_2 fixation measurements developed rapidly during the past decades (White et al., 2020). Therefore, unifying and standardizing the measurements for future research are crucial for intercomparison of the results. Even though we were not able to apply, we are prone to the modified bubble method (Klawonn et al., 2015) as injecting large amounts of $^{15}N_2$ as bubbles are applicable with minor disturbance to the samples and stable $^{15}N_2$ enrichments throughout the incubation.

Even with low rates, mesopelagic N_2 fixation holds the potential to contribute significantly to the global N budget when integrated with the large volume of mesopelagic oceans. For example, even though the rates in the Mediterranean Sea were all below 0.04 nmol N L^{-1} d^{-1}, the integrated rates of mesopelagic N_2 fixation were comparable to those in the euphotic zone (Benavides et al., 2016). However, the integrated rates are often obtained via a limited number of rate measurements integrated by vertically hundreds of meters. Even though the mesopelagic ocean is not as dynamic as the euphotic ocean, it is far from homogenous (Robinson et al., 2010; Baltar et al., 2012; Rigonato et al., 2023). Special care should be taken to avoid over- or under-extrapolation. Therefore, the best practice for obtaining representative rates and robust extrapolation into the global N budget under such heterogeneity needs to be established.

4 Conclusion and future perspectives

With carefully designed control incubations and sophisticated measurements, we were able to detect mesopelagic N_2 fixation in the South China Sea down to 1000 m depth. We consistently observed that around 30% of the replicates at mesopelagic depths exhibited an N_2 fixation signal, although the high variability prevented the quantification of representative rates. Detectable signals also present great variability across depths and stations. Global data compilation also shows regional and global heterogeneity in mesopelagic N_2 fixation with variations of up to three orders of magnitude. Therefore, we conclude that heterogeneity is the inherent nature of mesopelagic N_2 fixation, which prevents us from concrete rate determination as well as the extrapolation into integrated rates and global budgets.

Studies of mesopelagic N_2 fixation are still scarce, which limits our understanding of global mesopelagic N_2 fixation. Most of the studies are from the Pacific Ocean, and there are, as yet, no such studies carried out in the Atlantic ocean and polar regions. The temporal and spatial distribution of mesopelagic N_2 fixation is still poorly understood. Future research should explore mesopelagic N_2 fixation in not yet covered geographical areas, and further focus on the temporal variabilities in regions of high rates, which will shed more light on the pervasiveness of mesopelagic N_2 fixation globally. In addition, coastal waters undergoing intensified deoxygenation (Breitburg et al., 2018; Zhang et al., 2023) may also be a new niche for non-cyanobacterial diazotroph. Further, developing standardized methods and calculations is also of urgent need to comprehend the heterogeneous rates and develop reliable budgets of mesopelagic N_2 fixation globally. On the other hand, the mechanisms and environmental factors influencing mesopelagic N_2 fixation remain to be elucidated. For example, dissolved organic matter has been assumed to affect mesopelagic N_2 fixation, while the results are not consistent between different studies and the underlying mechanism remains hard to reconcile (Benavides et al., 2015; Selden et al., 2019). Lastly, further studies should be conducted to establish the link between observed rates and corresponding diazotrophs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

SW: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. XW: Writing – review & editing, Supervision, Conceptualization. MD: Writing – review & editing, Formal analysis. XC: Writing – review & editing, Investigation. CS: Writing – review & editing, Investigation. MB: Writing – review & editing, Investigation. SB: Writing – review & editing, Investigation. CL: Writing – review & editing, Investigation. MRH: Writing – review & editing, Investigation. MM: Writing – review & editing, Investigation. XY: Writing – review & editing, Investigation. SK: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Conflict of interest

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2024.1495649/full#supplementary-material

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