New Perspectives on Nitrogen Fixation Measurements Using $^{15}N_2$ Gas

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Recently, the method widely used to determine $^{15}N_2$ fixation rates in marine and freshwater environments was found to underestimate rates because the dissolution of the added $^{15}N_2$ gas bubble in seawater takes longer than theoretically calculated. As a solution to the potential underestimate of rate measurements, the usage of the enriched water method was proposed to provide constant $^{15}N_2$ enrichment. Still, the superiority of enriched water method over the previously used bubble injection remains inconclusive. To clarify this issue, we performed laboratory based experiments and implemented the results into an error analysis of $^{15}N_2$ fixation rates. Moreover, we conducted a literature search on the comparison of the two methods to calculate a mean effect size using a meta-analysis approach. Our results indicate that the error potentially introduced by an equilibrium phase of the $^{15}N_2$ gas is -72% at maximum for experiments with very short incubation times of 1 h. In contrast, the underestimation was negligible for incubations lasting 12–24 h (error is -0.2%). Our meta-analysis indicates that 84% of the measurements in the two groups will overlap and there is a 61% chance that a sample picked at random from the enriched water group will have a higher value than one picked at random from the bubble group. Overall, the underestimation of N_2 fixation rates when using the bubble method relative to the enriched water method is highly dependent on incubation time and other experimental conditions and cannot be generalized.

Keywords: $^{15}N_2$ fixation, enriched water method, bubble method, diazotrophs, meta-analysis

INTRODUCTION

Over the last few decades, the stable isotopic tracer $^{15}N_2$ was used to measure the production of diazotroph (N_2-fixer) biomass directly. This isotopic approach was first introduced by Burris and Miller (1941), but it was not until the 1990s that isotope ratio mass spectrometers (IRMS) were sensitive enough to measure low, open ocean low N_2 fixation rates. The protocol established by Montoya et al. (1996) has been widely used in the last two decades, yielding a large amount of N_2 fixation data, which are particularly abundant in the North Atlantic and North Pacific Oceans (Luo et al., 2012). Briefly, the method consists of adding a volume of $^{15}N_2$ gas into a seawater sample,

which is incubated for a given period (on deck or in situ), and finally terminated by filtration through glass fiber filters. The filters are later analyzed by IRMS to determine the amount of $^{15}N_2$ transferred from the aqueous phase to the particulate cell material. Montoya et al. (1996) introduced the calculation of the N_2 fixation rates using a mass-balance approach:

$$N_2 \text{ fixation rate} (M\,L^{-3}\,T^{-1}) = \frac{V}{2}\overline{PN} \approx \frac{V}{2} \times \left(\frac{[PN]0 + [PN]f}{2} \right) \tag{1}$$

With V calculated as:

$$V(T^-1) = \frac{1}{\Delta t} \times \frac{(A_P Nf - A_P N0)}{(A_{N_2} - A_{PN})}$$

Where M refers to mole nitrogen fixed, L to the volume (liter) and T to the incubation time. A_{PN} is the ^{15}N atom % enrichment of the particulate nitrogen (PN) pool as measured by IRMS, at the beginning (t_0) and end (t_f) of an incubation period; A_{N2} is the ^{15}N atom % enrichment of the dissolved N_2 gas in the incubated seawater; [PN] is the concentration of PN at the end of the incubation [if (PN) is stable over the incubation time; if (PN) varies significantly overtime, an average of initial and final (PN) values is recommended for calculations, see (Montoya et al., 1996)]; and Δt is the duration of the incubation. The values of all the terms in Equation (1) are measured empirically with the exception of A_{N2}. The latter term is theoretically calculated based on the volume of $^{15}N_2$ injected and the initial concentration of N_2 dissolved in seawater based on its temperature and salinity and the N_2 solubility equations of Weiss (1970) which have recently been revised by Hamme and Emerson (2004). This theoretical calculation assumes that isotopic equilibration of the $^{15}N_2$ bubble with the dissolved N_2 already present in the incubation bottle is rapid and complete relative to the incubation period. Simple mass balance tracer equations assume a constant isotope enrichment of the source pool over the duration of the incubation (Fry, 2006) an assumption violated if equilibration of $^{15}N_2$ with seawater is slow or incomplete during the experimental incubation.

Recently, Mohr et al. (2010) reported experimental evidence for a time lag in $^{15}N_2$ equilibration with the surrounding seawater of up to 24 h, depending on a number of factors such as incubation bottle size, volume of $^{15}N_2$ injected, bottle shaking, and incubation temperature. Amongst other things, an observed mismatch between $^{15}N_2$ fixation rates and biomass-specific growth rates motivated Mohr et al. (2010) to re-evaluate the $^{15}N_2$ bubble method introduced by Montoya et al. (1996). Mohr et al. (2010) proposed a new experimental procedure involving preparation of seawater enriched with $^{15}N_2$, which provides a near instantaneous enrichment of the dissolved pool of N_2 in an incubation bottle. This "enriched water" approach resulted in a 2–6 fold increase of measured N_2 fixation rates in comparison to the $^{15}N_2$ "bubble" method (Großkopf et al., 2012; Wilson et al., 2012). To the best of our knowledge, only a few comparisons of the two methods have been published up to date (11 studies) with only two studies including time series observations (Mohr et al., 2010; Klawonn et al., 2015).

There are several reasons why the bubble method may underestimate true N_2 fixation rates: (1) temperature (high temperatures inhibit dissolution of gases), (2) the volume of $^{15}N_2$ gas injected, (3) the duration of the incubation, (4) the time at which the incubation starts relative to the onset of $^{15}N_2$ fixation, and (5) possible DOM coating of the $^{15}N_2$ bubble (Mohr et al., 2010; Klawonn et al., 2015). The enriched water method on the other hand, also seems to be impacted by the mode of incubation, i.e., incubation on deck vs. an in situ array (Wilson et al., 2012).

Adding to these factors, Großkopf et al. (2012) reported that underestimates of N_2 fixation rate are lower when the community is dominated by colonial cyanobacteria of the genus Trichodesmium and higher when diazotrophs other than Trichodesmium predominate (e.g., symbionts, unicellular cyanobacteria, and non-cyanobacterial diazotrophs). All these factors vary widely among published works, making a global recalculation of N_2 fixation very difficult (Großkopf et al., 2012). On the other hand, a number of authors have not found significant differences between the two methods (Mulholland et al., 2012; Shiozaki et al., 2015, not published personnel communication: Berman-Frank et al., Montoya et. al., Benavides and Wannicke et al., -the latter added as unpublished data set to the meta analysis-). Moreover, using $^{15}N_2$ enriched water has a number of drawbacks, including the potential introduction of unwanted dissolved constituents (nutrients, dissolved organic matter or trace metals; Klawonn et al., 2015) and the preparation of the labeled water, which is laborious compared to the injection of a gas bubble into an incubation bottle. Degassing of seawater might also alter seawater chemistry in undesirable ways (e.g., by altering dissolved inorganic carbon concentrations or pH), and the overall extent of the degassing affects the final $^{15}N_2$ enrichment of dissolved N_2 dissolution (Klawonn et al., 2015).

The aim of this study is twofold. Firstly, we used a laboratory experiment to determine the equilibrium time of $^{15}N_2$ in Seawater from the Baltic Sea and used these numbers for an error calculation. By doing so, we tried to generate a measure for the underestimation of $^{15}N_2$ fixation rates when using the bubble method.

Secondly, we applied a meta- analytical approach to evaluate results from published papers comparing both methods. Variability and heterogeneity of published $^{15}N_2$ fixation rates were estimated for different incubation times and a mean effect size over all studies was calculated. Finally, considerations are given for the bubble method and its use in future studies.

MATERIALS AND METHODS
Laboratory Experiment and Error Calculation
Dissolution of $^{15}N_2$ in Brackish Seawater
We tested the equilibration of $^{15}N_2$ with filtered seawater empirically using both the $^{15}N_2$ bubble method (Montoya et al., 1996) and the enriched water method (Mohr et al., 2010). The seawater used in these experiments was collected from the Baltic Sea at the pier off Heiligendamm (54° 8.55′ N, 11° 50.6′ E, salinity of 14) and filtered through 0.2 μm cellulose acetate membranes (Sartorius) using a peristaltic pump. For the bubble method, filtered seawater was then transferred to 1 L

polycarbonate Nalgene®bottles fitted with septum caps, taking care to eliminate all headspace from the filled bottles. We then added 1 ml $^{15}N_2$ gas (98%, Campro Scientific lot # EB1169V) by direct injection through the septum with overpressure released via a cannula. Bottles were gently mixed for 5 min.

We followed the protocol of Mohr et al. (2010) to test the enriched water method using degassed seawater. In brief, degassed seawater was prepared using vacuum in a 1.7 × 5.5 MiniModule (3M Liqui cel) attached to a peristaltic pump (TP4000 E—Economic, Thölen, Germany 950 mbar). We assessed the efficiency of degassing by determining dissolved O_2 concentrations in the degassed water by Winkler titration until O_2 concentration were below the detection limit. Thereupon, 1.1 liter of degassed water was transferred to a Tedlar bag (Dupont, USA), flushed with helium to ensure absence of air inside the bags to which 11 mL of $^{15}N_2$ gas (98%, Campro Scientific lot # EB1169V) was added. The bag was agitated for 5 min (in which the bubble did not disappear) at room temperature. 50 mL of the enriched water was added to each 1.1L incubation bottle filled air free with Baltic Sea water.

All bottles were incubated at 15°C on a horizontal shaker (10 rpm, IKA HS 501, USA) located in a walk- in incubator. Incubations were carried out in triplicate and lasted for 24 h. Replicate sets of bottles were sampled immediately ($t = 0$ h) and at 1, 2, 4, 8, 12, and 24 h after addition of $^{15}N_2$ for analysis of the ^{15}N atom% enrichment of dissolved N_2, for which duplicate sub-samples from each bottle were transferred headspace-free into 12 mL exetainers. Crimp-sealed exetainers were stored in the dark at 4°C for up to 3 days. The ^{15}N atom % enrichment of dissolved N_2 was analyzed by measuring the abundance and concentration of masses $^{29}N_2$ and $^{30}N_2$ using a manual method similar to the automated gas chromatography-isotope ratio mass spectrometry approach described in Holtappels et al. (2011). In brief, water samples were taken from sealed exetainers with a gas tight glass syringe and a subsample was injected on-column on a 2 m stainless steel packed Porapak Q column (Supelco) with a constant flow of He carrier. Water was removed from the sample cryogenically (liquid nitrogen) and oxygen was removed by passage through a column packed with copper wire heated to 650°C. After purification, the N_2 was introduced to a mass spectrometer through an open split interface (Conflo IV, Thermo Scientific) and analyzed on a Delta V Advantage (Thermo Scientific). After every 5th sample air a standard was introduced.

We calculated dissolved $^{15}N_2$ concentrations according to Dalsgaard and Thamdrup (2002). Results of the bubble method were taken as a basis for error estimation as described below.

Error Estimate for the Bubble Method During Isotopic Equilibration

We performed an error estimation to theoretically quantify the % difference in measured $^{15}N_2$ fixation rate considering an increasing time lag (T_i) between $^{15}N_2$ tracer addition and the beginning of diazotrophic $^{15}N_2$ fixation and the duration of fixation (T_f). The error was estimated relative to instantaneous isotopic equilibration of $^{15}N_2$ gas upon bubble injection.

This estimate is relevant in case of time delay between addition of the $^{15}N_2$ tracer and the active beginning of diazotrophic $^{15}N_2$ fixation and also applies for diazotrophs fixing continuously.

Consider a seawater sample with an initial stable isotope composition of dissolved N_2, $N_i = \delta^{15}N_i$. For natural systems, this initial value will be very close to the global natural abundance of 0.366 at% ^{15}N. Into this sample, $^{15}N_2$ gas is injected and after a period of time (typically a couple of hours), the system reaches its equilibrium isotopic composition, $N_e = \delta^{15}N_{eq}$. The temporal development (**Figure 1**) of the ^{15}N enrichment in the dissolved N_2 pool is:

$$\delta^{15}N\,(t) = N_i + \Delta N(1 - e^{-bt}) \quad (2)$$

where $\Delta N = N_e - N_i$ is the difference between the equilibrium and initial isotopic compositions and b is an equilibration parameter with units of inverse time. The injection of $^{15}N_2$ gas has the consequence that $N_e > N_i$ and $\Delta N > 0$.

For $t \to \infty$, the equilibrium isotopic composition is reached and $N_e = N_i + \Delta N$. A general assumption of the bubble method is that fixation occurs with the dissolved N_2 pool at isotopic equilibrium. Any fixation that occurs before the system reaches equilibrium will contribute to error (underestimate) in the calculated rate, which is inversely proportional to $\delta^{15}N_2$. The simplest way to estimate the error is the integration of Equation 6 over the duration of fixation. Equation (2) can be rearranged to:

$$\delta^{15}N(t) = N_e - \Delta N e^{-bt} \quad (3)$$

which allows us to separate our experimental period into phases before and after the system reaches equilibrium. Following
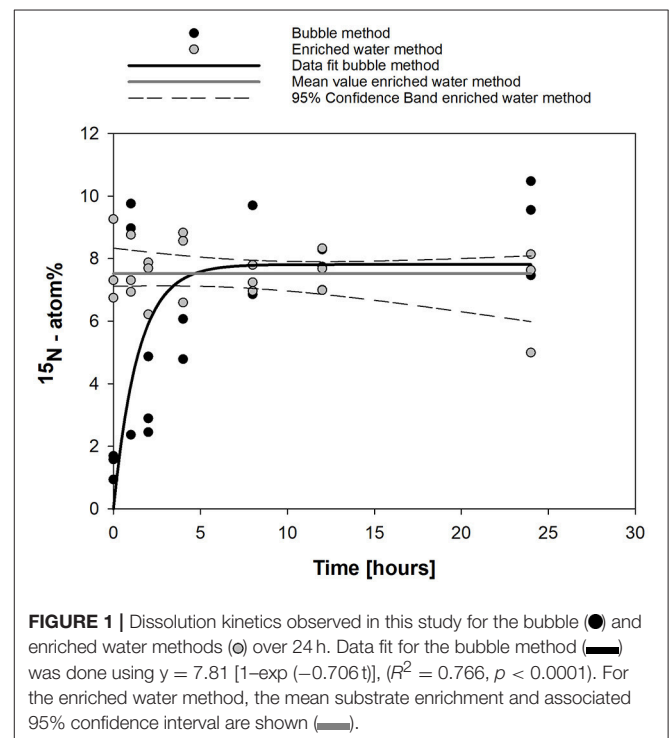
FIGURE 1 | Dissolution kinetics observed in this study for the bubble (●) and enriched water methods (◉) over 24 h. Data fit for the bubble method (━━) was done using y = 7.81 [1–exp (−0.706 t)], ($R^2 = 0.766$, $p < 0.0001$). For the enriched water method, the mean substrate enrichment and associated 95% confidence interval are shown (▬).

addition of $^{15}N_2$ to an incubation bottle, the mean $\delta^{15}N$ of the dissolved N_2 will increase and the average enrichment of the $^{15}N_2$ pool from the start of N_2-fixation to any time, t will be:

$$\langle \delta^{15}N \rangle = \frac{1}{T_f}\left[N_e \int_{T_i}^{T_f+T_i} dt - \Delta N \int_{T_i}^{T_f+T_i} e^{-bt} dt \right] \quad (4)$$

where the phase lag T_i is the difference in time between gas injection and the start of fixation, while T_f is the duration of N_2-fixation. For $T_i = 0$, the mixing of injected gas and the start of fixation are synchronized, as in the case of a diazotroph that fixes N_2 continuously through the day. Integration of Equation (4) yields an expression for the mean $\delta^{15}N_2$ during the period of active N_2-fixation T_f:

$$\langle \delta^{15}N \rangle = N_e + \frac{\Delta N}{bT_f}\left[e^{-b(T_f+T_i)} - e^{-bT_i} \right] \quad (5)$$

In Equation (5) the mean $\delta^{15}N_2$ value can be expressed as equilibrium composition and an error, ε, which represents the underestimation of the N_2-fixation rate:

$$\langle \delta^{15}N \rangle = \delta^{15}N_{eq} - \varepsilon \quad (6)$$

and the relative percent error, R, is then simply:

$$R(\%) = 100 * \frac{\varepsilon}{\delta^{15}N_{eq}} \quad (7)$$

Fitting Equation (7) to the observations (**Figure 1**) results in Ni = 0 atom%, Ne = 7.8 atom%, Δ N = 7.8 atom%, b = 0.71 h^{-1}.

Meta-Analysis

We obtained data for meta-analysis from published and unpublished sources (Supplementary Table 1). We conducted literature searches using ISI Web of Science and by personal communication. Data selection continued until September 2016. The studies included in our assessment all included a direct comparison of the enriched water and bubble methods with common samples. In all, 13 studies met the requirements for inclusion ($n = 368$ observations) along with two unpublished data sets (Supplementary Table 1, Supplementary Figure 1). $^{15}N_2$ fixation rates were either provided personally by authors, retrieved from tables published in supplemental material or manually digitized from figures in the published studies using the software WebPlotDigitizer (version 3.10) (Ankit Rohatgi, http://arohatgi.info/WebPlotDigitizer/).

Before performing the meta-analysis, we determined the statistical dispersion and variability within the bubble and enriched water data sets. We calculated the mean absolute deviation (MAD) of each data set as follows:

$$MAD = \frac{1}{n}\sum_{i=1}^{n}|x_i - \bar{x}| \quad (8)$$

where n is the number of observations and $\bar{x}$ is the mean of the individual observations x_i.

Meta-analysis and meta-regression were conducted with R 3.1.2 using the "metafor" package (Viechtbauer, 2010), metafor package of R [http://www.metafor-project.org/], R Development Core Team 2013). To assess the two methods, we used the logarithmically transformed response ratio (Hedges et al., 1999), lnRR, where RR is the ratio of rates (R) measured with the enriched water and bubble methods (RR = R$_{enrichedwater}$:R$_{bubble}$), or the effect size of individual experiments), as well as the corresponding pooled standard deviation. All lnRR values were weighted by the reciprocal of their sampling variance, followed by a random effects model to compute the overall mean effect size, which is equivalent to Cohen's d parameter (Cohen, 1977). The random effects model, based on the DerSimonian-Laird estimator (DerSimonian and Laird, 1986) calculates the between-study variance (σ^2) and weights each study by the inverse sum of the individual study variance (v_i) and the between- study variance. Mean effect sizes (i.e., Cohen's d) were statistically significant different if their 95% confidence intervals did not overlap zero.

Apart from calculating the mean effect sizes for all 13 studies and 368 observations, we also divided observations into two groups: short incubation time (0–12 h) and long incubation times (24 h). For short incubation times three data sets were available (Mohr et al., 2010; Klawonn et al., 2015; Benavides and Wannicke et al., unpubl.,). Unfortunately, the experiments of Mohr et al. (2010) and Klawonn et al. (2015) lacked replication from single time points. To be able to include this data set in our meta-analysis, we pooled values collected at $t = 0$ to 12 h. Results from these publications for $t = 24$ h had to be omitted from the meta-analysis due to lack of replication. Furthermore, we added a subgroup analysis for each meta-analysis to determine mean effect sizes excluding unpublished data sets.

To explore heterogeneity in the meta-analysis, we calculated Cochrane Q-tests for heterogeneity (Cochran, 1954). A significant Q-statistic (p-value of <0.1) indicates that there is heterogeneity within the mean effect size and a greater variance among individual effect sizes than expected by sampling error. We furthermore, examined the sensitivity of the meta-analysis by examining publication bias (the probability that statistically significant ($p < 0.05$) results are more likely to be published than non-statistically significant results) using a contour-enhanced funnel plot (Peters et al., 2008; Supplementary Figure 2). The funnel plot represents a scatter plot of the effect estimates from individual studies against the standard error of the effect estimate. Specifically, a contour-enhanced funnel plots display the area of statistical significance on a funnel plot (Peters et al., 2008) to improve the correct identification of the presence or absence of publication bias. Publication bias would be expected when the usual funnel plot is asymmetrical.

Random-effects meta-regression analysis using a linear mixed-effects model was used to evaluate the association between incubation time and the mean effect size of $^{15}N_2$ fixation.

For interpretation of the meta-analysis we converted Cohen's d value to Cohen's U_3 parameter (Cohen, 1977) to give a measure of the degree of separation (i.e., % non-overlap) of data produced by the bubble and enriched water methods according to:

$$U_3 = \Phi(\delta) \tag{9}$$

where Φ is the cumulative distribution function of the standard normal distribution, and δ is the population value of Cohen's d.

In addition, we calculated the overlapping coefficient (OVL) of data from the two methods by converting Cohen's d using the following formula (Reiser and Faraggi, 1999)

$$OVL = 2\Phi\left(\frac{-|\delta|}{2}\right) \tag{10}$$

where Φ is the cumulative distribution function of the standard normal distribution, and δ the population Cohen's d.

The probability of superiority (CL) (Ruscio and Mullen, 2012) i.e., probability that a sample picked at random from the treatment group will have a higher score than a sample picked at random from the control group, was calculated the following (Ruscio, 2008)

$$CL = \Phi\left(\frac{\delta}{\sqrt{2}}\right) \tag{11}$$

where Φ is the cumulative distribution function of the standard normal distribution, and δ the population Cohen's d.

RESULTS

Experimental Dissolution of $^{15}N_2$ in Brackish Seawater

Dissolution of $^{15}N_2$ gas according to Montoya et al. (1996) resulted in a maximum ^{15}N atom% enrichment of 9.1 % (mean value for 24 h of incubation, **Figure 1**). Fitting of data resulted in an atom% enrichment of 7.8% (solid line, **Figure 1**). The time to reach 50% of the maximum ^{15}N atom% enrichment was 1.7 h and the shift from an exponential rise in dissolved ^{15}N enrichment to a plateau with only minimal changes in ^{15}N atom% enrichment (>60% of maximum) occurred after 4 h. After 8 h, 90% of ^{15}N atom% equilibration was reached. Injection of water pre-enriched with $^{15}N_2$ gas according to Mohr et al. (2010) resulted in a stable ^{15}N atom% enrichment over 24 h with a mean value of $7.5 \pm 0.9\%$ (**Figure 1**).

Error Estimate for the Bubble Method During Isotopic Equilibration

When using the bubble method, the rate of $^{15}N_2$ fixation will be systematically underestimated during the equilibration phase of $^{15}N_2$ gas with the dissolved pool of N_2 (**Figure 2A**). The overall magnitude of the underestimate during an incubation will depend on the length of the incubation and the timing and duration of $^{15}N_2$ fixation activity during the incubation period (**Figure 2B**), which in turn will reflect the nature (light depended or light independent) of the diazotrophic community present. The maximum error of −72% occurs when the time lag between
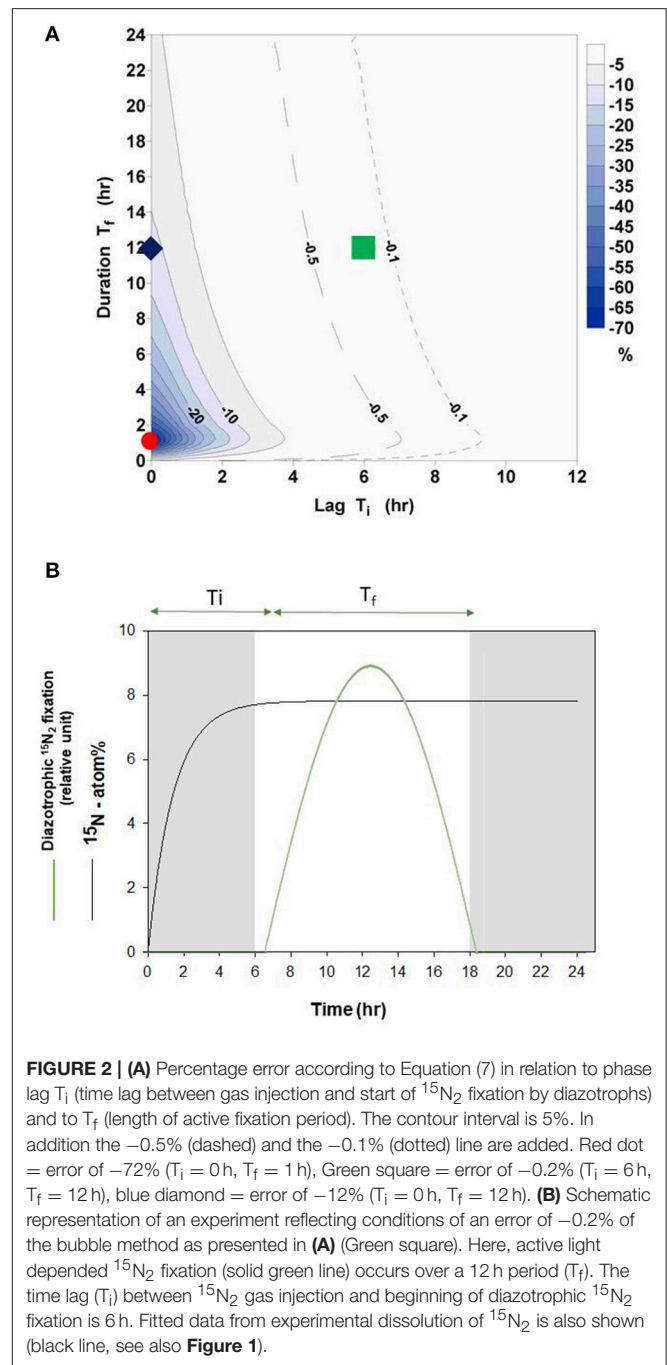
FIGURE 2 | (A) Percentage error according to Equation (7) in relation to phase lag T_i (time lag between gas injection and start of $^{15}N_2$ fixation by diazotrophs) and to T_f (length of active fixation period). The contour interval is 5%. In addition the −0.5% (dashed) and the −0.1% (dotted) line are added. Red dot = error of −72% ($T_i = 0$ h, $T_f = 1$ h), Green square = error of −0.2% ($T_i = 6$ h, $T_f = 12$ h), blue diamond = error of −12% ($T_i = 0$ h, $T_f = 12$ h). **(B)** Schematic representation of an experiment reflecting conditions of an error of −0.2% of the bubble method as presented in **(A)** (Green square). Here, active light depended $^{15}N_2$ fixation (solid green line) occurs over a 12 h period (T_f). The time lag (T_i) between $^{15}N_2$ gas injection and beginning of diazotrophic $^{15}N_2$ fixation is 6 h. Fitted data from experimental dissolution of $^{15}N_2$ is also shown (black line, see also **Figure 1**).

gas injection and start of the $^{15}N_2$ fixation is zero ($T_i = 0$ h) and the duration of $^{15}N_2$ fixation is 1 h ($T_f = 1$ h, red dot in **Figure 2A**). In contrast, a 12 h period of active fixation beginning 6 h after the injection of $^{15}N_2$ gas will result in an error of −0.2% (green square in **Figure 2A**, green solid line in **Figure 2B**, $T_i = 6$ h and $T_f = 12$ h).

When organisms are able to fix N_2 continuously, the error would be −12% (blue diamond in **Figure 2A**, $T_i = 0$ h and $T_f = 12$ h) over a 12 h incubation and −6% over a 24 h incubation ($T_i = 0$ h and $T_f = 24$ h).

Meta-Analysis

Variability among the replicates of individual studies was quite large as indicated by the standard deviation for the single studies and MAD of the two methods applied (**Figure 3**). Specifically, the MAD for the enriched water method is 7 and the MAD for bubble method is 5.

To determine a mean effect size (i.e., Cohen's d), we firstly resolved the response ratio (lnRR) of N_2 fixation for each study (**Figure 4A**). Subsequently, we calculated the overall mean effect size across all studies which resulted in a significant Cohen's d of 0.406 ± 0.110, ($p < 0.001$; **Figure 4B**). The mean effect size of ~0.4, detected in our analysis suggests that for a method comparison, 65 % of measurements from the enriched water group will be above the mean of the bubble group (Cohen's U_3, for subgroup analysis 73%) and that 84% of the measurements in the two groups will overlap (subgroup analysis 76%). In addition, there is a 61% chance that a sample picked at random from the enriched water group will have a higher value than one picked at random from the bubble group (subgroup analysis 66%).

A subgroup analysis excluding the unpublished data sets revealed a significant mean effect size of 0.631 ± 0.125 ($p < 0.001$).

We also performed a meta-regression to evaluate the influence of time on the overall mean effect size. No significant impact was detected (data not shown). Moreover, we checked for publication bias, which is expected when scatter of data in the funnel plot is asymmetric. In our analysis, assessment of the contour-enhanced funnel plot indicates an asymmetrical scatter of data and potential publication bias introduced by a lack of non-significant studies (Supplementary Figure 2).

We furthermore, did separate meta- analysis for observations with short incubation times (0–12 h) and long incubation times (24 h only, Supplementary Figures 3A,B). The overall mean effect size of the three studies with short incubation time was not significant with a Cohen's d of 0.057 ± 0.188, ($p > 0.1$) (Supplementary Figure 3A). Excluding the unpublished data set of Benavides and Wannicke et al., revealed a non-significant mean effect size of 0.557 ± 0.382 ($p > 0.1$). Furthermore, the meta- analysis for long incubation times of 24 h resulted in a Cohen's d of 0.406 ± 0.101 ($p < 0.001$) (Supplementary Figure 3B). Excluding unpublished studies resulted in a mean effect size of 0.68 ± 0.136 ($p < 0.001$).

We found no significant correlations with ocean province or temperature in exploring which factors might influence the mean effect size (data not shown).

DISCUSSION

We combined a theoretical examination of the error associated with the equilibration time of the bubble of $^{15}N_2$ gas and a meta-analysis of published and unpublished sets of N_2-fixation measurements comparing both methods. Our findings allows us to detect mean differences in rate estimates and provide critical insight into the strengths and weaknesses of the two experimental approaches.

Error Estimation of Bubble Method

Our error estimation of the bubble method during a 24 h experiment reveals a negligible error of −0.2% assuming a diazotroph community that fixes only during 12 h daytime and starting of nitrogen fixation 6 h after the injection of $^{15}N_2$ gas i.e., the addition was done 6 h before sunrise. Considering that the error introduced by using a gas-tight syringe to inject $^{15}N_2$ gas of ±1% (according to the manufacturer, Hamilton USA), the error introduced by using bubble injection is insignificant. The error introduced by using the bubble method will increase to −6% when diazotrophs fix continuously over 24 h (assuming there is no time lag between bubble injection and start of active fixation). Overall, it is important to adjust incubation times relative to the onset of active N_2 fixation (which is in turn is depended on the dominating diazotrophs present), as has been indicated before (Mohr et al., 2010; Wilson et al., 2012).

Meta-Analytical Comparison of the Enriched Water and Bubble Method

Statistical dispersion, as represented by the MAD from the mean value, appears to be higher in the data set based on the enriched water method (mean MAD of all studies 7) than in the data set of studies that used the bubble method (mean MAD of all studies 5). That is, measured N_2 fixation rates appear to be more consistent when determined using the bubble method. A larger dispersion of data in experiments using the enriched water method might be introduced in by the process of preparing the enriched water for later usage, i.e., degassing water of different volumes and varying accuracy of degassing. In addition, Wilson et al.(2012) presented evidence for a dispersion of data in experiments using the enriched water method, which was connected to abiotic factors influencing the incubation itself. They noted an interesting contrast between the two methods when comparing samples incubated at sea using either an *in situ* array or shipboard incubators. Specifically, they found that the enriched water method produced depth-integrated N_2-fixation estimates that were 30% greater when samples were incubated aboard ship in deck incubators than when incubated on an *in situ* array. In contrast, depth-integrated rates measured using the bubble method were not significantly different between incubations carried out on deck and on an *in situ* array. The 30 % difference among replicates of the enriched water method also overlaps with the divergence of the enriched water and bubble methods referred to by Mohr et al. (2010) and Wilson et al. (2012) in their methods assessments.

Our meta- analysis revealed a large congruence in the estimates of $^{15}N_2$ fixation rate produced using the two experimental methods. The 84% overlap of rate estimates make it very difficult at this stage to estimate any sort of a global factor to quantify the degree of underestimation of $^{15}N_2$ fixation rates when using the bubble method. Our literature review moreover revealed that a thorough comparison over a 24-h cycle is needed with only three studies on sort incubation times of 0–12 h. A larger comparative analysis is clearly necessary especially in view of the elevated dispersion (i.e., larger MAD)
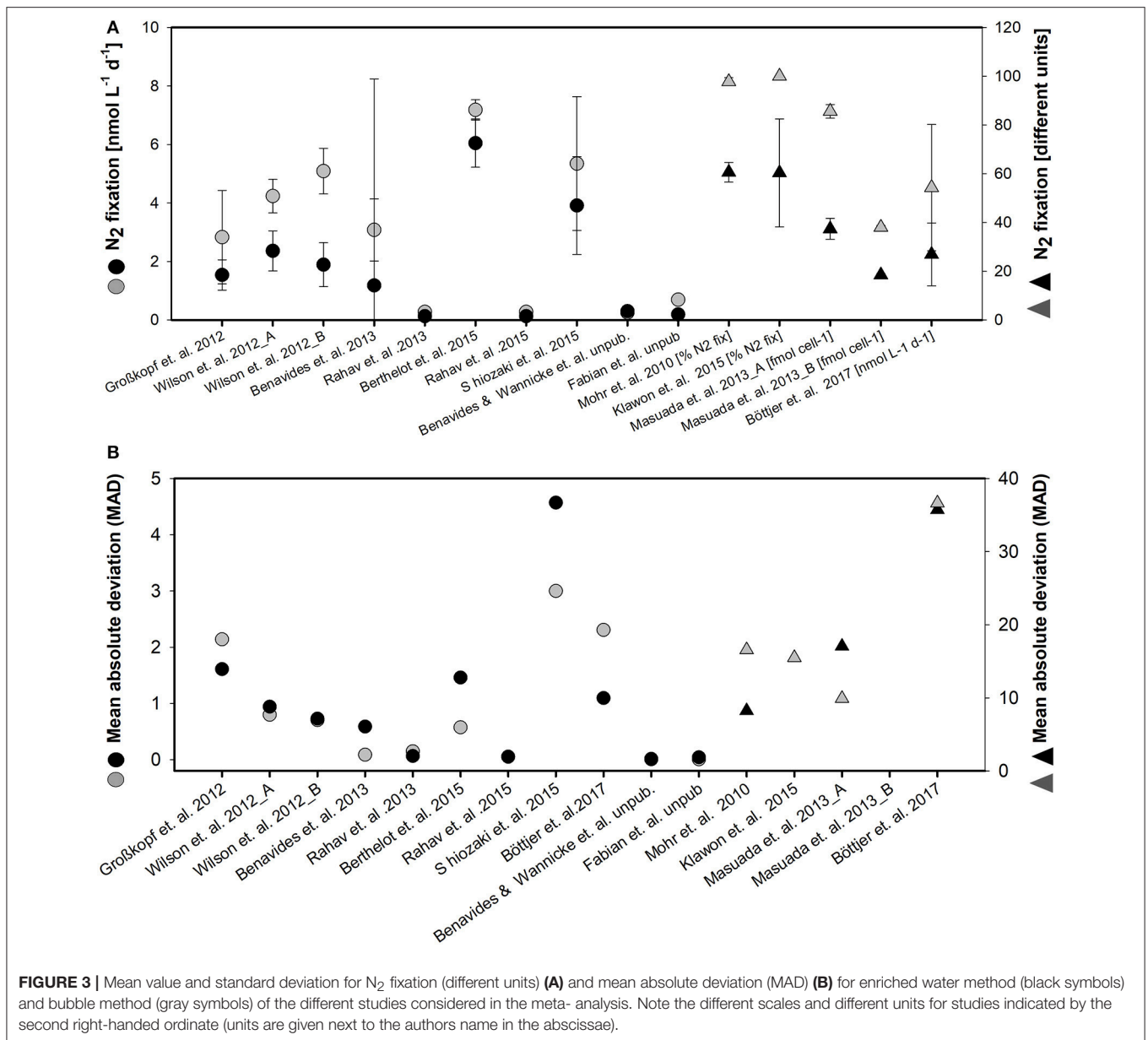
FIGURE 3 | Mean value and standard deviation for N_2 fixation (different units) **(A)** and mean absolute deviation (MAD) **(B)** for enriched water method (black symbols) and bubble method (gray symbols) of the different studies considered in the meta- analysis. Note the different scales and different units for studies indicated by the second right-handed ordinate (units are given next to the authors name in the abscissae).

of $^{15}N_2$ fixation rates measured using the enriched water method.

As Großkopf et al. (2012) have pointed out, there is an indication for a species-specific potential for underestimation of N_2 fixation rates using the bubble method, especially when buoyant diazotrophs are presented. Thus, in habitats dominated by filamentous species like *Trichodesmium* or *Nodularia,* underestimation seems to be less severe compared to habitats dominated by unicellular species (UCYN, e.g., Zehr et al., 1998), as well as non-diazotrophic *Bacteria* and *Archaea* (e.g., Riemann et al., 2010). In the latter, incubation times have to be adjusted to the equilibration time of $^{15}N_2$ when using the bubble method. Alternatively, as proposed in the sub-chapter below (see "Experimental Recommendations") the determination of the final $^{15}N_2$ (i.e., substrate) enrichment in the

incubation bottle enables a concerted calculation of N_2 fixation rates.

Dealing With Unpublished Data-Sets

In our analysis, we have included two data sets that are currently unpublished (Benavides and Wannicke et al., Fabian et al., Supplementary Table 1). The literature dealing with the inclusion of unpublished data in meta-analysis clearly recommends inclusion of this sort of "gray literature" (e.g., Cook et al., 1993; McAuley et al., 2000). Exclusion from meta-analysis can lead to exaggerated overestimation of treatment effects (e.g., Cook et al., 1993; McAuley et al., 2000). This is also reflected in our analysis of funnel plot symmetry, where studies displaying no significant effect (Cohen's d of ~ 0) are under-represented and/or missing in our analysis. Overall, publication bias is the greatest
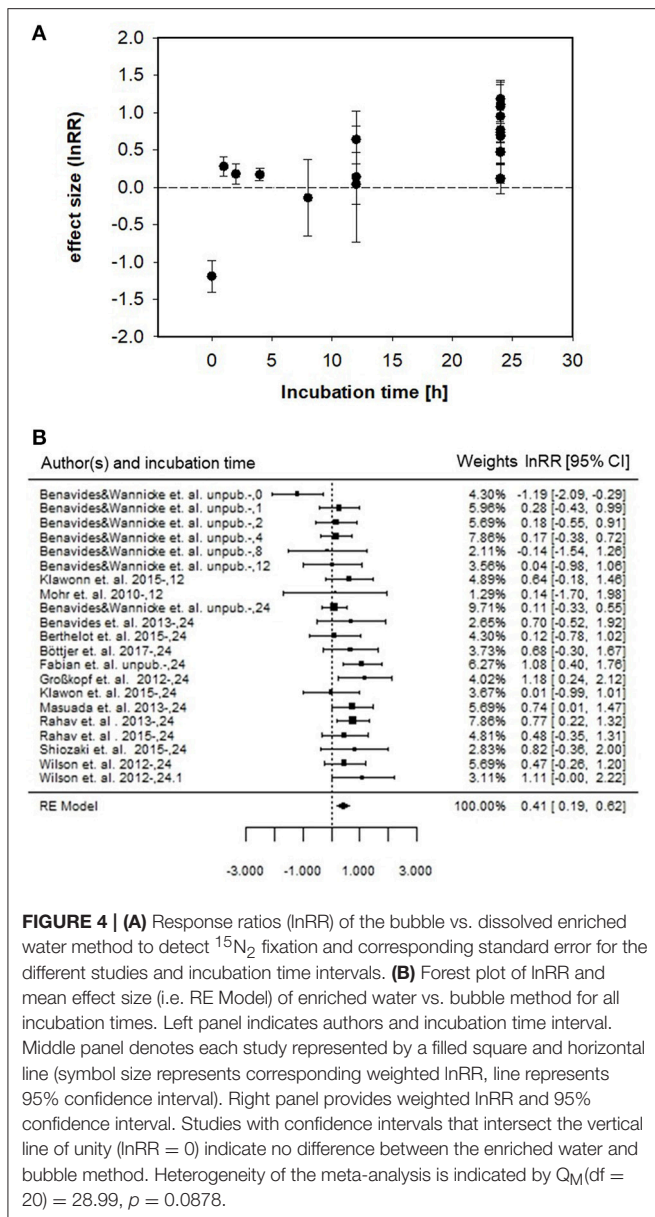
FIGURE 4 | (A) Response ratios (lnRR) of the bubble vs. dissolved enriched water method to detect $^{15}N_2$ fixation and corresponding standard error for the different studies and incubation time intervals. **(B)** Forest plot of lnRR and mean effect size (i.e. RE Model) of enriched water vs. bubble method for all incubation times. Left panel indicates authors and incubation time interval. Middle panel denotes each study represented by a filled square and horizontal line (symbol size represents corresponding weighted lnRR, line represents 95% confidence interval). Right panel provides weighted lnRR and 95% confidence interval. Studies with confidence intervals that intersect the vertical line of unity (lnRR = 0) indicate no difference between the enriched water and bubble method. Heterogeneity of the meta-analysis is indicated by Q_M(df = 20) = 28.99, p = 0.0878.

threat to the validity of meta-analysis, because combining only the identified published studies uncritically, may lead to an incorrect, unusually one sided, conclusion.

General Experimental Considerations for Future Nitrogen Fixation Measurements

A number of experimental factors have a strong influence on the precision and accuracy of the determination of N_2-fixation rates. Firstly, the sensitivity of any experiment using $^{15}N_2$ depends on the amount of tracer added to the dissolved pool of N_2. For example, addition of 1 mL of $^{15}N_2$ per liter of sample will produce an equilibrium enrichment of ∼5–10 atom% ^{15}N, depending on the size of the ambient pool of N_2. In contrast, the procedure proposed by Großkopf et al. (2012) results in an enrichment of only 2 atom%. Since N_2

availability does not limit N_2-fixation activity, greater additions may easily be realized to increase the substrate labeling, thereby increasing the sensitivity of the rate measurement. This is especially important in systems where rates are expected to be low, for example in aphotic deep waters. In general, we recommend adding sufficient $^{15}N_2$ to raise the ^{15}N content of the dissolved N_2 pool to 9–10 at% as noted by Montoya et al. (1996).

Secondly, the natural variability of $\delta^{15}N$ of the particulate nitrogen (PN) pool sets a lower limit to rate measurements. If the variability in $\delta^{15}N$ of PN is high at the start of the incubation (t_0) and the final increase in $\delta^{15}N$ values of the PN in the incubation bottles is low due to low N_2 fixation rates, then N_2-fixation activity may not be detectable. For example, Wasmund et al. (2015) have nicely explored this issue, discussing $^{15}N_2$ fixation rate measurements in the Benguela upwelling region where they compared initial (t_0) and final $\delta^{15}N$ measurements of samples incubated with $^{15}N_2$. The mean values of the two batches of filters differed only by 0.9‰, leading Wasmund et al. (2015) to conclude that $^{15}N_2$ fixation rates were too low to resolve with the tracer method. Nowadays, mass spectrometers clearly perform analytical precisions of 0.2‰ and better. Therefore, the detection limit of enriched PN is well below 4‰, as original proposed in the paper by Montoya et al. (1996).

Finally, the two experimental approaches differ fundamentally in the degree and nature of experimental manipulation of the sample. The bubble method involves minimal handling (a thourough mixing of water and gas bubble after injection has to be guaranteed, by using e.g., a continuously rotating) and perturbation of the system, but can lead to a systematic underestimate of N_2-fixation rate if a significant fraction of the overall activity during the experiment occurs during the isotopic equilibration phase. In contrast, the enriched water method requires extensive processing in advance to prepare the $^{15}N_2$-labeled water used to inject tracer into the experimental bottle as described by Klawonn et al. (2015) and Mulholland et al. (2012). Ideally, the enriched water should be obtained from the same location and depth as the experimental sample, which imposes a significant cost in time and handling while setting up the experiment, with the added risk of contamination with ammonium, DON, or other dissolved species during handling. If the enriched water is prepared in advance from artificial seawater or water obtained at a different station, it represents an addition of alien water and dissolved species and should be minimized in volume to less than a few percent of the bottle volume. The potential for contamination and the degree to which the addition of enriched water may affect estimates of N_2 fixation rates are very difficult to constrain but can clearly compromise the reliability of the final rate estimates. Another approach using the bubble addition followed by the removal of the bubble after only few hours and retrieval of a subsample for determination of $^{15}N_2$ atom% enrichment requires the tedious determination of the $^{15}N_2$ atom% enrichment for each incubation bottle (Jayakumar et al., 2017). Although this approach is easy to handle, it requires efficient handling and subsequent stable isotope analysis.

Our dissolution experiments investigating the isotopic equilibration in seawater along with the theoretical error calculation both suggest that incubation times longer than about 6 h are minimally affected by the equilibration of the added $^{15}N_2$ gas and the dissolved pool of N_2 in the experimental bottle (**Figures 3A, 4A**). In practical terms, 24 h incubations are frequently recommended and used because these experiments integrate over a full day/night cycle.

A final recommendation to improve the accuracy of N_2 fixation measurements and potentially help resolve the source of variability among replicates is collection and preservation of a water sample from each experimental bottle for determination of the final $^{15}N_2$ (i.e., substrate) enrichment. This would improve the accuracy of the enriched water, as well as the bubble method, both of which typically rely on solubility calculations to estimate the size of the ambient pool of N_2, which in turn determines the actual $^{15}N_2$ enrichment of the dissolved pool.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the study. Laboratory and analytical work was conducted by NW, MB, JM, and TD. Data search and meta- analysis was performed by NW and MB.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00120/full#supplementary-material

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