Comparative Assessment of Nitrogen Fixation Methodologies, Conducted in the Oligotrophic North Pacific Ocean

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Resolution of the nitrogen (N) cycle in the marine environment requires an accurate assessment of dinitrogen (N₂) fixation. We present here an update on progress in conducting field measurements of acetylene reduction (AR) and ¹⁵N₂ tracer assimilation in the oligotrophic North Pacific Subtropical Gyre (NPSG). The AR assay was conducted on discrete seawater samples after an incubation period of 3 to 4 h. The ¹⁵N₂ tracer measurements compared the addition of ¹⁵N₂ as a gas bubble and dissolved as ¹⁵N₂ enriched seawater. On all sampling occasions and at all depths, a 2- to 6-fold increase in the rate of ¹⁵N₂ assimilation was measured when ¹⁵N₂-enriched seawater was added to the seawater sample compared to the addition of ¹⁵N₂ as a gas bubble. In addition, we show that the ¹⁵N₂-enriched seawater can be prepared prior to its use with no detectable loss (<1.7%) of dissolved ¹⁵N₂ during 4 weeks of storage, facilitating its use in the field. The ratio of C₂H₄ production to ¹⁵N₂ assimilation varied from 7 to 27 when measured simultaneously in surface seawater samples. Collectively, the modifications to the AR assay and the ¹⁵N₂ assimilation technique present opportunities for more accurate and high frequency measurements (e.g., diel scale) of N₂ fixation, providing further insight into the contribution of different groups of diazotrophs to the input of N in the global oceans.

The commonly applied methods to measure the rates of marine N₂ fixation include the acetylene reduction (AR) assay (4, 7, 12, 36) and the ¹⁵N₂ assimilation technique (30). The AR assay relies on the preferential reduction of acetylene (C₂H₂) to ethylene (C₂H₄) by nitrogenase, instead of reducing N₂ to NH₃ (34). The most extensive applications of the AR assay at sea include the measurement of C₂H₄ production by Trichodesmium colonies via gas chromatography (6, 7) and the use of an online system incorporating a laser photoacoustic detector (37, 42). However, the AR assay has not escaped criticism during the past 4 decades of its use, predominantly due to potential indirect effects of C₂H₂ on microbial metabolism and the reliability of the factor used to extrapolate rates of C₂H₄ production to rates of N₂ fixation (14, 16, 21, 36). An additional concern, especially for oligotrophic seawater samples, is the need to concentrate the microbial biomass, either by shipboard filtration or net tows, in order to obtain a detectable signal of C₂H₄ production (6, 12, 37). The filtration process can impose undesired effects on the microbial population due to cell damage (1, 13). In comparison, the ¹⁵N₂ assimilation technique follows the net incorporation of the ¹⁵N₂ tracer into cellular biomass after a predetermined incubation period (2, 17, 26). Because the ¹⁵N₂ technique has a lower detection limit than the AR assay, it is the preferred method for the oligotrophic marine environment (30). Indeed, ¹⁵N₂ assimilation has been used to describe rates of N₂ fixation at Station (Stn) ALOHA (A Long-term Oligotrophic Habitat Assessment) located at 22°45'N, 158°W in the North Pacific Subtropical Gyre (NPSG) (9, 11). However, it has recently been demonstrated that the method used to add ¹⁵N₂ to the seawater sample can affect the measured rate of N₂ fixation (28). The authors of that study found that the addition of ¹⁵N₂ as a gas bubble underestimated N₂ fixation because the gas bubble introduced does not attain equilibrium within the incubation period, causing an unknown and time-dependent ¹⁵N/¹⁴N ratio for the N pool.
equilibrated headspace gas using a reduced gas analyzer that incorporates a reducing compound photodetector. Importantly, no preconcentration of microbial biomass is required. Additional key methodological aspects of the AR assay are also documented, including the saturation of nitrogenase with C$_2$H$_2$ and the importance of blank control treatments. The AR assay measurements were conducted alongside rates of $^{15}$N$_2$ assimilation, whereby $^{15}$N$_2$ was added to seawater samples in both gaseous form and as $^{15}$N$_2$-enriched seawater. We describe the preparation and storage of $^{15}$N$_2$-enriched seawater for use on oceanographic cruises, making its use in the field more time efficient. The resulting advances to the AR assay and $^{15}$N$_2$ assimilation method described here allow more accurate quantification of N$_2$ fixation and increase the ability to conduct time-resolved rate measurements in the oligotrophic ocean.

**MATERIALS AND METHODS**

The fieldwork was conducted on multiple expeditions to the NPSG between October 2010 and December 2011. Seawater samples were collected during Hawaii Ocean Time-series (HOT) cruises to Stn ALOHA and an additional oceanographic cruise located ~50 nautical miles to the north of Stn ALOHA (25°N, 157°30'W).

**AR assay.** The AR assay described here differs from the “typical” AR assay (see, for example, reference 4) in two main aspects. The first is with regard to sample preparation with C$_2$H$_2$ gas added in dissolved form to the seawater sample, followed by incubation of the seawater sample with no headspace, and subsequent quantification of the ensuing C$_2$H$_4$ gas in the whole sample. This differs from the routinely reported applications of the AR assay (4, 7, 12), whereby C$_2$H$_2$ gas is added to serum vials containing approximately two-thirds sample seawater and one-third headspace, and aliquots of the gas phase are analyzed for C$_2$H$_4$ concentrations at selected intervals during the sample incubation period or a predetermined endpoint. The second major methodological difference is that C$_2$H$_4$ concentrations are quantified by using a reducing compound photodetector in contrast with the more routinely utilized gas chromatography-flame ionization detector (GC-FID). Below, we describe the five steps of the modified AR assay: (i) preparation of the C$_2$H$_2$-enriched seawater, (ii) sample collection and incubation, (iii) extraction of dissolved C$_2$H$_4$ gas, (iv) quantification of the C$_2$H$_4$ concentrations, and (v) calculation of the C$_2$H$_4$ concentration.

(i) **Preparation of C$_2$H$_2$ enriched seawater.** The C$_2$H$_2$-saturated seawater for the AR assay was prepared ~1 h prior to use. To generate the C$_2$H$_2$ gas, 6 g of calcium carbide (CaC$_2$, Sigma) was added to 150 ml of deionized water in a 250-ml side-arm glass flask according to the following equation:

\[
\text{CaC}_2 + 2\text{H}_2\text{O} = \text{C}_2\text{H}_2 + \text{Ca(OH)}_2.
\]

The resulting C$_2$H$_2$ gas was transferred via the side-arm of the flask and 1/8-in. polytetrafluoroethylene (PTFE) tubing to the base of a secondary 1-liter glass flask that contained 300 ml of filtered (0.2-$\mu$m pore size) seawater collected from the sampling location. To enhance the mass transfer of C$_2$H$_2$ gas into the filtered seawater, the outlet tubing was fitted with a ceramic air stone diffuser resulting in the production of microbubbles. After purging the filtered seawater with C$_2$H$_2$, and shaking vigorously for 5 min, the C$_2$H$_2$-enriched seawater was stored in the dark until use. No measurements were made of C$_2$H$_2$ concentrations using the reducing compound photodetector since this would rapidly deplete the bed of mercuric oxide. Rather, we relied on empirical solubility studies showing that seawater saturated with C$_2$H$_2$ (1.6 ml of C$_2$H$_2$ per ml of water) contains 65 mM C$_2$H$_4$. (35) It should also be noted that C$_2$H$_2$ produced from calcium carbide does not result in pure C$_2$H$_4$ and contaminants is present, including C$_2$H$_4$. (22). These can potentially be decreased by scrubbing the C$_2$H$_2$ gas stream through an additional water flask (8); however, the presence of any level of contaminant C$_2$H$_4$ necessitates careful time zero measurements and blank controls, discussed in detail below.

(ii) **Sample collection and incubation.** Seawater samples were collected using a CTD-rosette and transferred to acid-washed, combusted, glass-stoppered 300-ml Wheaton bottles that were filled to two times overflowing. Subsequently, 20 ml of the seawater sample was removed from the Wheaton bottle using a pipettor and replaced with 20 ml of C$_2$H$_2$-enriched seawater, with a final C$_2$H$_2$ concentration of 4 mM. Replicate ($n = 3$) samples were immediately analyzed after the introduction of C$_2$H$_2$ enriched seawater to provide a time zero C$_2$H$_2$ concentration. Control samples, consisting of 0.2-$\mu$m-pore-size filtered seawater, were inoculated with 20 ml of C$_2$H$_2$-enriched seawater and analyzed in triplicate whenever measurements were conducted on seawater samples. Experimental and control treatments were incubated in deckboard incubators plumbed with flowing surface seawater and shaded using blue Plexiglas to 20% light intensity, approximately equivalent to a depth of 25 m in the water column. The typical incubation period was 3 to 4 h, although on a few occasions the incubation time was extended up to 8 h.

(iii) **Extraction of dissolved C$_2$H$_4$ gas from incubated samples.** To measure the dissolved C$_2$H$_4$ concentrations, a headspace analysis method was used to extract the C$_2$H$_4$ from seawater (Fig. 1). Samples of seawater from the Wheaton bottle were withdrawn into a 50-ml gas-tight, glass syringe (Perfektum). Syringes were rinsed at least twice with sample seawater, prior to withdrawing the final bubble-free sample for analysis. Accurate and reproducible volumes of sample seawater, typically 30 ml, are achieved by housing the 50-ml syringe within a custom-built syringe actuator (Fig. 1). The headspace gas, typically 10 ml, is added to the syringe via the bypass outlet of the analyzer, which passes the carrier gas (ultra-high-purity air) through a combustor providing a C$_2$H$_4$-free (<10 parts
per trillion) source of air. The water and gas phases in the syringe are then equilibrated at room temperature by vigorous shaking of the syringe for 3 min. The equilibrated headspace is subsequently injected into the gas analyzer inlet which incorporates a 0.2-μm-pore-size PTFE membrane hydrophobic filter (Acrodisc; Pall Life Sciences) to prevent the accidental injection of seawater. An internal 10-port switching valve is subsequently activated that allows 100 μl of the sample gas stream onto the chromatography columns described below. The total analytical time per sample ranges from 7 to 8 min.

(iv) Quantification of C\textsubscript{2}H\textsubscript{4} concentrations. C\textsubscript{2}H\textsubscript{4} concentrations were measured using a reduced gas analyzer (RGA; Peak Laboratories). The RGA has previously been used to quantify other reduced gases such as mercury vapor with a UV photodetector (Fig. 1). Calibration of the RGA was routinely conducted using serial dilutions of a 10.3 ppm of C\textsubscript{2}H\textsubscript{4} standard in N\textsubscript{2} (Scott-Marrin, Riverside, CA). The measured concentration of C\textsubscript{2}H\textsubscript{4} in the equilibrated headspace was used to calculate the total dissolved C\textsubscript{2}H\textsubscript{4} concentration ([C\textsubscript{2}H\textsubscript{4}]\textsubscript{aq} in ml of C\textsubscript{2}H\textsubscript{4}/ml of H\textsubscript{2}O) remaining in the water after equilibration by

\[
[C_{\text{2}H_{4}}]_{\text{aq}} = 10^{-6} \beta m_a p \tag{2}
\]

where \(\beta\) (ml of C\textsubscript{2}H\textsubscript{4}/ml of H\textsubscript{2}O/atm) represents the Bunsen solubility coefficient of C\textsubscript{2}H\textsubscript{4} (3), \(m_a\) is the measured concentration of C\textsubscript{2}H\textsubscript{4}, \(p\) is atmospheric pressure (atm) of dry air. The C\textsubscript{2}H\textsubscript{4} concentration in the initial seawater ([C\textsubscript{2}H\textsubscript{4}]\textsubscript{aq} in ml of C\textsubscript{2}H\textsubscript{4}/ml of H\textsubscript{2}O) was calculated, assuming mass balance, as follows:

\[
[C_{\text{2}H_{4}}]_{\text{aq}} = ([C_{\text{2}H_{4}}]_{\text{v}} V_w + 10^{-6} m_a V_a)/V_w = 10^{-6} m_a (BpV_w + V_a)/V_w \tag{3}
\]

where \(V_w\) is the water sample size (ml) and \(V_a\) is the volume of headspace air (ml), followed by the conversion of [C\textsubscript{2}H\textsubscript{4}]\textsubscript{aq} to units in nM ([C\textsubscript{2}H\textsubscript{4}]\textsubscript{aq}) as follows:

\[
[C_{\text{2}H_{4}}]_{\text{aq}} = 10^9 \times p[C_{\text{2}H_{4}}]_{\text{aq}}/(RT) \tag{4}
\]

where \(R\) is the gas constant (0.08206 atm liter mol\textsuperscript{-1} K\textsuperscript{-1}) and \(T\) is temperature (K). After the C\textsubscript{2}H\textsubscript{4} concentrations in sample and control treatments were measured at both the beginning and end of the incubation period, the rate of C\textsubscript{2}H\textsubscript{4} production was calculated as follows:

\[
\left( C_{\text{2}H_{4}}^{\text{final}} - C_{\text{2}H_{4}}^{\text{initial}} \right) - \left( C_{\text{2}H_{4}}^{\text{s}} \Delta T \right)/C_{\text{2}H_{4}}^{\text{final}} \right) / \Delta t \tag{5}
\]

where \(C\) represents the concentration of C\textsubscript{2}H\textsubscript{4} in the sample (s) and the control treatment (c), as calculated using equations 1 to 3, at the end (final) or beginning (t=0) of the incubation period.

**15N\textsubscript{2} assimilation technique.** In conjunction with the AR assay, we also measured N\textsubscript{2} fixation using the 15N\textsubscript{2} assimilation technique. It was recently shown that the addition of 15N\textsubscript{2} gas as a bubble resulted in the underestimation of rates of N\textsubscript{2} fixation in cultures of Crocosphaera (28). Therefore, the present work compared rates of 15N\textsubscript{2} fixation when 15N\textsubscript{2} gas was added either as a bubble or dissolved in seawater samples collected from discrete depths in the upper 50 m of the water column at Stn ALOHA. We describe below the preparation of the 15N\textsubscript{2}-enriched seawater and the sample collection, preparation, and analysis.

**Preparation of 15N\textsubscript{2}-enriched seawater.** During a first comparative test, the 15N\textsubscript{2}-enriched seawater was prepared at sea by adding filtered (0.2-μm pore size) degassed (250 mbar for 30 min) seawater to a crimp-sealed glass vial and injecting an appropriate quantity (1 ml of 15N\textsubscript{2} gas per 100 ml of seawater) of 15N\textsubscript{2} gas through the septum. Vigorous shaking for ~30 min aided the complete dissolution of the bubble, and the 15N\textsubscript{2}-enriched seawater was then added to the seawater samples (10 ml of 15N\textsubscript{2}-enriched seawater per liter of seawater sample). Subsequent to this preliminary comparison, an alternative procedure was adopted whereby the 15N\textsubscript{2}-enriched seawater was prepared at the shore-based laboratory prior to the oceanographic cruise (Fig. 2). To prepare the 15N\textsubscript{2}-enriched seawater, 3.5 liters of filtered (0.2-μm pore size) seawater was added to a 4-liter acid-washed vacuum flask (Fig. 2). Ambient air was removed from the analytical apparatus by purging the system with helium at 5 lb/in\textsuperscript{2} (~500 ml min\textsuperscript{-1}) for 15 min. The seawater was placed under vacuum (250 mbar) for 60 min and transferred to a 3-liter gas-tight PTFE bag (Welch Fluorocarbon), which was cooled in a 4°C water bath. Subsequently, 40 ml of 15N\textsubscript{2} gas (98 atom%; Sigma-Aldrich) was injected via the sampling port and the gas-tight bag was physically agitated until the gas bubble was completely dissolved into the seawater (~10 min). Afterward, 70-ml borosilicate glass vials were filled from the gas-tight bag to two times overflowing using nylon tubing (4-mm outer diameter by 2-mm inner diameter; LeGris) and immediately crimp sealed with no headspace using Teflon-lined septa. The glass vials containing 15N\textsubscript{2}-enriched seawater were stored in the dark at 4°C until required for experimental purposes.
Assessment of Nitrogen Fixation Methods

Validation of $^{15}$N$_2$-enriched seawater. One of the major considerations for any analytical method is the ease of its application and although the $^{15}$N$_2$-enriched seawater was prepared at sea on one occasion, it was found to be more time efficient to prepare the $^{15}$N$_2$-enriched seawater on land prior to its use at sea. We therefore investigated the feasibility of storing the $^{15}$N$_2$-enriched seawater in crimp-sealed 70-ml borosilicate glass vials over a 4-week period by analyzing the dissolved $^{15}$N$_2$ content at weekly intervals using a membrane inlet mass spectrometer (MIMS) (23).

In brief, the MIMS provides rapid and accurate measurements of gas ratios by coupling semipermeable, microbore tubing with the inlet vacuum line of a quadrupole mass spectrometer. Reference measurements consisted of a 1-liter reservoir of filtered (0.2-$\mu$m pore size) surface seawater collected from Stn ALOHA. Instrument drift in the N$_2$/Ar ratio over the 4-week period was 0.6%. The analytical temperature for reference seawater and samples was kept constant at 25°C by immersing 1/16-in. stainless steel inlet tubing inside a water bath. The gases analyzed included those with a mass to charge ratio of 28, 30, and 32 (corresponding to $^{14}$N$_2$, $^{15}$N$_2$, and O$_2$), and they were detected sequentially using a repetitive cycle of 1.5 Hz. Replicate samples of prepared $^{15}$N$_2$-enriched seawater were analyzed for the loss of $^{15}$N$_2$ at weekly intervals over a 1-month period, comparing the ratio of mass 30 in $^{15}$N$_2$-enriched seawater/reference seawater.

Sample collection, inoculation, and incubation. Seawater samples were collected using a CTD-rosette from depths of 5, 25, and 45 m and subsampled into acid-washed and seawater-rinsed 4.3-liter polycarbonate bottles. Once the bottles were completely filled, 50 ml of seawater was removed and replaced with 50 ml of $^{15}$N$_2$-enriched seawater from the 70-ml crimp-sealed vials using a 50-ml glass syringe, resulting in a final $^{15}$N$_2$ enrichment of 1.5 atom%. The syringe was attached to a 15-cm length of 1/8-in. PTFE tubing which enabled the $^{15}$N$_2$-enriched seawater to be added below the neck of the 4.3-liter polycarbonate bottle. Bottles were carefully closed using septum closure caps (Thermo Scientific) with no headspace and inverted 20 times. In addition, replicate seawater samples which had been filled and capped with no headspace were injected with 3 ml of $^{15}$N$_2$ gas (98 atom%; Sigma-Aldrich) using a gas-tight syringe (SGE Analytical Sciences) through the septum cap into the bottle. The 3-ml $^{15}$N$_2$ gas-injected sample bottles were gently shaken, and then all of the bottles were incubated using either (i) a free-floating in situ array at three depths consisting of 5, 25, and 45 m (as described in reference 9) or (ii) deckboard incubators plumbed with surface seawater and shaded to 50, 25, and 10% of full sunlight to represent 5, 25, and 45 m.

Sample analysis and calculation of N Enrichment. At the end of the incubation period, the entire content of the 4.3-liter polycarbonate bottles was filtered onto combusted (450°C for 5 h) 25-mm glass fiber filters that were subsequently placed on combusted foil pieces in polystyrene petri dishes and stored frozen at -80°C until analysis. The combusted foil pieces were placed in polystyrene petri dishes and stored frozen at -80°C until analysis. The combusted foil pieces were placed in polystyrene petri dishes and stored frozen at -80°C until analysis. The combusted foil pieces were placed in polystyrene petri dishes and stored frozen at -80°C until analysis.

RESULTS

AR assay samples and control treatments. A typical AR measurement from near-surface (25 m) seawater collected from the oligotrophic NPSG (Fig. 3) highlights several important considerations when conducting the assay. First, at the beginning of the incubation period (time zero) dissolved C$_2$H$_4$ concentrations were always measurable, exceeding the analytical detection limit of 5 pmol liter$^{-1}$ by several orders of magnitude.

In this particular example, at time zero the dissolved C$_2$H$_4$ concentrations measured 37 nmol of C$_2$H$_4$ liter$^{-1}$ (Fig. 3). At the end of the 4-h incubation period (time final), the blank/biological signal ratio decreased to 80%, when a significant difference was measured between the control and sample treatment (one-way analysis of variance [ANOVA], $P = 0.036$ and $P < 0.05$). The biological C$_2$H$_4$ production associated with this particular seawater sample, as calculated per equation 5, was 2.7 nmol of C$_2$H$_4$ liter$^{-1}$ h$^{-1}$. An additional important observation is the 12% increase in C$_2$H$_4$ concentrations in the control treatment during the 4 h of incubation period (Fig. 3). The nonbiological production of C$_2$H$_4$ was investigated further, and an increase was repeatedly observed, even when the filtered seawater controls were amended with mercuric chloride (HgCl$_2$; 200 $\mu$g of saturated HgCl$_2$ in 300 ml of seawater sample) and regardless of whether samples were incubated in the light or dark. The abiotic production of C$_2$H$_4$ during the AR assay has previously been reported, due to reactions with the serum cap liners often used to crimp-seal glass vials (38). The observation of increasing C$_2$H$_4$ concentrations in glass Wheaton bottles highlights the importance of quantifying the C$_2$H$_4$ concentration at time zero in both the samples and control treatments and also reporting the blank to biological signal ratio for the appropriate time points of the experimental period (as previously highlighted by reference 14).

Following the analytical procedures described above, the AR assay was conducted on surface seawater samples collected at 25 m in the oligotrophic NPSG on multiple occasions between March and October 2011 (Table 1). Concentrations of C$_2$H$_4$ at the end of the incubation period ranged from 42 to 67 nmol liter$^{-1}$, with an overall analytical precision of ± 0.05 nmol liter$^{-1}$ and the blank/signal ratio at the end of the incubation period ranged from 67 to 82%. The corresponding rates of C$_2$H$_4$ production varied by an order of magnitude ranging from 0.46 nmol liter$^{-1}$ h$^{-1}$ (incubation period: 2100 to 0600, 19 September) to 4.7 nmol liter$^{-1}$ h$^{-1}$ (incubation period: 1000 to 1400, 20 July).
Determining the quantity of C$_2$H$_2$ added to seawater sample. A major consideration of the AR assay is the quantity of C$_2$H$_2$ added to the seawater samples. The addition of low C$_2$H$_2$ concentrations will insufficiently saturate nitrogenase (42); however, complete saturation of nitrogenase will inhibit N$_2$ fixation, resulting in N starvation and production of new nitrogenase (36). Balancing these considerations has resulted in C$_2$H$_2$ typically added in N starvation and production of new nitrogenase (36). Balloon were conducted with a 10% addition of C$_2$H$_2$-saturated seawater (4). We measured the effect of C$_2$H$_2$ concentrations on the complete saturation of nitrogenase will inhibit N$_2$ fixation, resulting in N starvation and production of new nitrogenase (36). Balancing these considerations has resulted in C$_2$H$_2$ (4). We measured the effect of C$_2$H$_2$ concentrations on the rate of C$_2$H$_2$ production in seawater samples by increasing the volume of C$_2$H$_2$ added from 2 to 10%, equivalent to C$_2$H$_2$ concentrations of 1.3 to 6.5 mM, respectively. Overall, a nearly 2-fold increase was observed in the rate of C$_2$H$_2$ production, from 2.7 nmol liter$^{-1}$ h$^{-1}$ at 2% (vol/vol) to 4.5 nmol liter$^{-1}$ h$^{-1}$ at 10% (vol/vol) (Fig. 4). All subsequent measurements of C$_2$H$_2$ production were conducted with a 10% addition of C$_2$H$_2$-saturated seawater.

Incubation period. During both day and night sample incubation periods, the increase in C$_2$H$_4$ production rate was linear over an 8-h period (Fig. 5). The minimum length of the incubation period is dictated by the quantity of time required to measure a significant increase in the quantity of C$_2$H$_4$ produced relative to the control treatments. We advise restricting the AR assay incubation period to a few hours since the presence of C$_2$H$_2$ can adversely affect other metabolic pathways and the wider microbial community (32).

$^{15}$N$_2$ assimilation. In addition to conducting the field assessment of the $^{15}$N$_2$ tracer technique, the methods used to prepare and store the $^{15}$N$_2$-enriched seawater were also assessed. A mass-to-charge ratio of 30, corresponding to $^{15}$N$_2$, was measured in $^{15}$N$_2$-enriched seawater/reference seawater over a 4-week period to determine any loss of $^{15}$N$_2$ in the $^{15}$N$_2$-enriched seawater. Over the storage period, the coefficients of variation averaged 1.7%, and no decrease was detected within this range of variation (Fig. 6).

On five occasions between October 2010 and September 2011, comparisons of the $^{15}$N$_2$ assimilation methods were conducted, adding $^{15}$N$_2$ tracer to sampled seawater incubations either as a gas bubble or in dissolved form. On two occasions (20 July and 30 August), samples were incubated using an in situ floating array (Fig. 7). Rates of $^{15}$N$_2$ assimilation were significantly greater (one-way ANOVA, P < 0.001) when $^{15}$N$_2$ tracer was added to the seawater sample in dissolved form, averaging 4.6 ± 0.9 nmol of N liter$^{-1}$ day$^{-1}$ (±represents the standard deviation, n = 18) compared to an overall average of 2.7 ± 0.8 nmol of N liter$^{-1}$ day$^{-1}$ when $^{15}$N$_2$ tracer was added as a gas bubble. On two occasions (20 July and 29 September), samples were incubated in deckboard incubators (Fig. 8). Similar to the seawater samples incubated using the in situ array, the rates of $^{15}$N$_2$ assimilation were significantly greater (one-way ANOVA, P < 0.001) when $^{15}$N$_2$ tracer was added to the seawater sample in dissolved form (overall average of 5 ± 1.3 nmol of N liter$^{-1}$ day$^{-1}$) compared to the addition of a gas bubble (overall average of 1.9 ± 1.1 nmol of N liter$^{-1}$ day$^{-1}$).

On 20 July 2011, replicate samples collected from 5, 25, and 45 m, were incubated simultaneously using the in situ array and deckboard incubators. When the $^{15}$N$_2$ tracer was added as a bubble, there was no significant difference (one-way ANOVA, $P = 0.98$) between samples incubated using the in situ array and deckboard incubator. In contrast, when the $^{15}$N$_2$ tracer was added in dissolved form, there was a weak significant difference (one-way ANOVA, $P = 0.044$ and $P < 0.05$) between the in situ array and deckboard incubator, with a 30% increase in the depth-integrated (0 to 45 m) $^{15}$N$_2$ assimilation rates in the deckboard-incubated samples (Table 2). We speculate that the difference between deckboard and in situ incubations was primarily due to a daytime increase (ca. 2 to 3°C) in seawater temperature in the deckboard incubators, which was measured at the start of the cruise, together with irradiance, but not continuously logged. We therefore recommend continuously monitoring deckboard incubator condi-

### Table 1 Measurements of AR and $^{15}$N$_2$ assimilation, determined using $^{15}$N$_2$-enriched seawater, for water column samples collected from 25 m at Stn ALOHA between July and September 2011

<table>
<thead>
<tr>
<th>Date</th>
<th>Incubation period (hourly range)</th>
<th>rate (nmol of N liter$^{-1}$ h$^{-1}$)</th>
<th>rate (nmol of N liter$^{-1}$ h$^{-1}$)</th>
<th>15N$_2$/N$_2$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 July 2011</td>
<td>Day (1000–1400)</td>
<td>1.1 ± 0.8</td>
<td>0.15 ± 0.01</td>
<td>18</td>
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<tr>
<td></td>
<td>Night (2200–0500)</td>
<td>0.7 ± 0.2</td>
<td>0.22 ± 0.09</td>
<td>7</td>
</tr>
<tr>
<td>30 Aug 2011</td>
<td>Day (1200–1500)</td>
<td>2.0 ± 0.1</td>
<td>0.22 ± 0.09</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Night (2200–0600)</td>
<td>1.0 ± 0.2</td>
<td>0.22 ± 0.10</td>
<td>16</td>
</tr>
<tr>
<td>9 Sept 2011</td>
<td>Day (10:00–14:30)</td>
<td>2.1 ± 0.1</td>
<td>0.08 ± 0.02</td>
<td>27</td>
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<tr>
<td></td>
<td>Night (2200–0600)</td>
<td>3.4 ± 0.1</td>
<td>0.22 ± 0.10</td>
<td>16</td>
</tr>
<tr>
<td>13 Sept 2011</td>
<td>Day (1000–1400)</td>
<td>0.9 ± 0.2</td>
<td>0.08 ± 0.02</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Night (2200–0600)</td>
<td>1.3 ± 0.4</td>
<td>0.10 ± 0.01</td>
<td>13</td>
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<tr>
<td>19 Sept 2011</td>
<td>Day (11:00–13:30)</td>
<td>4.0 ± 0.5</td>
<td>0.26 ± 0.05</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Night (2100–0600)</td>
<td>0.5 ± 0.2</td>
<td>0.04 ± 0.02</td>
<td>13</td>
</tr>
</tbody>
</table>

*The length of the sample incubation period is reported for the AR assay in parentheses, whereas it was either 12 or 24 h for $^{15}$N$_2$ assimilation. Both C$_2$H$_4$ production and $^{15}$N$_2$ assimilation are reported as hourly rates (n = 3).
Comparing the addition of $^{15}$N$_2$ in gaseous or dissolved phase. It was considered whether the addition of 50 ml of exogenous seawater to a 4.3-liter polycarbonate bottle (which represents a 1.2% [vol/vol] addition) could have stimulated N$_2$ fixation. Therefore, on 7 November 2011, a third treatment was included in addition to the comparison of $^{15}$N$_2$ in gaseous or dissolved phase. The third treatment consisted of 50 ml of degassed, but non-$^{15}$N$_2$-enriched seawater that was added to seawater samples prior to the injection of the $^{15}$N$_2$ gas bubble. No significant difference was observed in the rate of $^{15}$N assimilation when amended with a bubble (2.1 ± 0.6 nmol of N liter$^{-1}$ day$^{-1}$) compared to the bubble plus degassed, nonenriched seawater (1.3 ± 0.2 nmol of N liter$^{-1}$ day$^{-1}$) (one-way ANOVA, $P = 0.096$ and $P > 0.05$). In contrast, the rate of $^{15}$N$_2$ assimilation in seawater samples inoculated with dissolved $^{15}$N$_2$ averaged 6.5 ± 0.4 nmol of N liter$^{-1}$ day$^{-1}$ and were significantly higher than the $^{15}$N$_2$ gas bubble injected treatments (one-way ANOVA, $P = 0.003$ and $P < 0.05$).

**Comparison of AR assay and $^{15}$N$_2$ assimilation.** On eight separate occasions, the AR assay was conducted alongside rate measurements of $^{15}$N$_2$ assimilation conducted using $^{15}$N$_2$-enriched seawater (Table 1). The incubation period differed for the two analyses, ranging from 3 to 8 h for the AR assay and either 12 or 24 h for the $^{15}$N$_2$ tracer. Expression of C$_2$H$_4$ production and $^{15}$N$_2$ assimilation incubated with $^{15}$N$_2$-enriched seawater as an hourly rate measurement revealed a positive relationship (Pearson correlation, $r = 0.83$, $P = 0.017$) with the ratio of C$_2$H$_4$ production to $^{15}$N$_2$ assimilation ranging from 7 to 27.

**DISCUSSION**

Measuring N$_2$ fixation in the oligotrophic open ocean is essential for a comprehensive understanding of the marine N cycle and the role of diazotrophs in upper-water-column biogeochemistry. This study reports on the specifics of two conventional methods frequently used to measure N$_2$ fixation in the marine environment: the $^{15}$N$_2$ assimilation technique and the AR assay. A common theme in the currently applied protocols of the AR assay and the $^{15}$N$_2$ assimilation is the issue of gas equilibration. It has previously been demonstrated that without sufficient agitation of the sample, dissolved C$_2$H$_4$ resulting from the reduction of C$_2$H$_2$ does not equilibrate with the gas phase (14). Similarly, it was recently shown that the addition of $^{15}$N$_2$ in gas form does not completely equilibrate with the liquid phase, even over a 24-h incubation period (28). The loss of signal makes data comparison between separate studies difficult and presents a serious drawback when estimating the contribution of diazotrophs to the fixed pool of N over global ocean basins. To avoid the addition of $^{15}$N$_2$ and C$_2$H$_2$ in gaseous form, we present here a modified protocol for the AR assay and describe the preparation and use of $^{15}$N$_2$-enriched seawater. Both methods were tested on several oceanographic cruises in the oligotrophic open ocean environment of the NPSG between October 2010 and September 2011, with particular attention paid to the time efficiency and practicality of the protocols.

With respect to the AR assay, we found that the RGA provides accurate and reproducible measurements of dissolved C$_2$H$_4$. Two inherent features of the RGA which aid the analysis of C$_2$H$_4$ are the redirection of the carrier gas flow after C$_2$H$_4$ has eluted from the column avoiding the C$_2$H$_2$ reaching the detector bed and the installation of a primary chromatographic column to handle the high water vapor content of the sample. Installation of a water-impermeable filter prior to the analyzer inlet also prevents the accidental injection of seawater. Use of the RGA at sea is facilitated by the ease of transport and setup, requiring a single compressed gas cylinder to supply the carrier gas flow. The only custom-built
surement of C2H4, particularly for field samples that contain
represents a valuable tool for obtaining a rapid and sensitive mea-
the best of our understanding, these C2H2 gas cylinders are no
C2H2," which refers to gas cylinders of compressed C2H2 (37). To
minimize the background C2H4 (26), and 1.9 to 9.3 (30). The reasons for the discrepancies be-
tween the theoretical and observed ratios have previously been
an underestimation of 15N2 assimilation rates when adding 15N2 as
A ratio of 4:1 has also been suggested since this incorporates the H2
production to a rate of N2 fixation, multiple simultaneous AR assay
and 15N2 assimilation measurements should be conducted, as pre-
viously recommended (4, 15, 19, 30).

With respect to the 15N2 assimilation technique, the results
demonstrate that introducing the 15N2 gas into the seawater sam-
ple, either as a gas bubble or dissolved in sterile seawater, causes
significant differences in the quantity of 15N recovered in partic-
ulate form (Fig. 7 and 8). The issue of 15N2 gas solubility was
recently demonstrated for laboratory cultures of the diazotroph
Crocosphaera watsonii (28), and we now extend the observation of an underestimation of 15N2 assimilation rates when adding 15N2 as
gas bubble to open ocean seawater samples composed of mixed
diazotrophs assemblages. We demonstrate here that the addition of 15N2 gas in dissolved form increases the recovery of 15N com-
pared to the addition of a gas bubble. Our findings help to resolve an existing conundrum in the upper oceanic N cycle at Stn
ALOHA, whereby indirect estimates of N2 fixation over an 11-year
period, from 1989 to 2001, derived from the isotopic composition of sinking material exceeded rates of 15N2 assimilation measured
on five occasions by 40 to 80% (11). This previous study con-
cluded that their 15N2 assimilation tracer measurements, as con-
ducted by the addition of N2 gas in a bubble, were underrepresent-
N2 fixation rates in the upper 0 to 100 m of the water column.
A more recent study of N2 fixation at Stn ALOHA over a 3-year
period measured depth-integrated (0 to 100 m) rates of 15N2 as-
similation of 150 μmol of N m⁻² day⁻¹ in the summer months
(July to September) (9), which is within the range of surface N2
fixation (100 to 400 μmol of N m⁻² day⁻¹) reported by Deutsch et
al. (10). The rates of 15N2 assimilation measured here by adding
15N2 in dissolved form are 2- to 3.5-fold higher than the rates of

feature of the analytical setup was the hand-held syringe actuator
(Fig. 1), which may also be commercially available. When con-
ducting the AR assay in the field, the most consistent measure-
ments were obtained when analyzing three to four samples with a
sample incubation period of 4 h. It is recommended to avoid hav-
ing samples waiting to be processed due to the continuing biolog-
cal and potentially abiotic production of C2H4 in the sample (15).

As concluded on previous occasions (4, 14, 30), the AR assay
represents a valuable tool for obtaining a rapid and sensitive mea-
surement of C2H4, particularly for field samples that contain
mixed community assemblages of diazotrophs. This work dem-
onstrates that no preconcentration of biological material is re-
quired when dissolved C2H4 concentrations are quantified using
the RGA, excluding the potential of harming the N2-fixing micro-
organisms during the filtration process. Regardless of whether
C2H4 is added in gaseous or dissolved phase to the sample, it is
recommended to report the blank to signal ratio for all time points
when conducting the AR assay. Previous studies have been able to
minimize the background C2H4 (<0.2 ppm) by using “clean
C2H4,” which refers to gas cylinders of compressed C2H4 (37). To
the best of our understanding, these C2H4 gas cylinders are no
longer commercially available. Finally, we recommend before commencing extensive field measurements, an initial set of C2H4
rate measurements are conducted at a range of C2H4 concentra-
tions, e.g., 5, 10, and 20% (vol/vol), to demonstrate the response
of nitrogenase enzyme within the ambient microbial community
to various levels of C2H4.

An additional issue regarding the AR assay concerns the use of
a stoichiometric conversion factor to obtain an estimate of N2
fixation. Since the development of the AR assay, there has been
considerable research on the possibility of a universal conversion
factor enabling the rate of C2H4 production to be converted into a
rate of N2 fixation. A theoretical ratio of 3:1 is often cited, based on
the difference between two hydrogen ions required to reduce
C2H4 to C2H2 and six hydrogen ions needed to reduce N2 to 2NH3.
A ratio of 4:1 has also been suggested since this incorporates the H2
evolution associated with the reduction of a N2 molecule (4). A
wide range of C2H4/15N2 assimilation ratios have previously been
observed, ranging from 0.93 to 7.26 (6), 6.7 to 11.6 (19), 3.3 to 56
(26), and 1.9 to 9.3 (30). The reasons for the discrepancies be-
tween the theoretical and observed ratios have previously been
discussed at length (14, 16, 19) and demonstrate that it is unwise to
assume a fixed conversion factor. Prior to converting C2H4 pro-
duction to a rate of N2 fixation, multiple simultaneous AR assay
and 15N2 assimilation measurements should be conducted, as pre-
viously recommended (4, 15, 19, 30).

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15N2 in dissolved form are 2- to 3.5-fold higher than the rates of

![FIG 8 N2 fixation rates on surface seawater samples at Stn ALOHA incubated in deckboard incubators at 3 different light intensities for 24 h on two separate occasions: 20 July 2011 (A) and 27 September 2011 (B). The 15N2 tracer was either added as a gas bubble (open bars) or in dissolved form (gray bars). Error bars represent the standard deviation (n = 3).](6522_aem.asm.org)
\(^{15}\text{N}_2\) assimilation when \(^{15}\text{N}_2\) is added as a gas bubble. Therefore, the present study supports previous findings (28) that conducting \(^{15}\text{N}_2\) assimilation incubations using \(^{15}\text{N}_2\)-enriched seawater will reduce existing imbalances in the fixed pool of N in the surface seawater of the oligotrophic ocean.

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**REFERENCES**