

# 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 (N<sub>2</sub>) fixation. We present here an update on progress in conducting field measurements of acetylene reduction (AR) and <sup>15</sup>N<sub>2</sub> tracer assimilation in the oligotrophic North Pacific Subtropical Gyre (NPSG). The AR assay was conducted on discrete seawater samples using a head-space analysis system, followed by quantification of ethylene (C<sub>2</sub>H<sub>4</sub>) with a reducing compound photodetector. The rates of C<sub>2</sub>H<sub>4</sub> production were measurable for nonconcentrated seawater samples after an incubation period of 3 to 4 h. The <sup>15</sup>N<sub>2</sub> tracer measurements compared the addition of <sup>15</sup>N<sub>2</sub> as a gas bubble and dissolved as <sup>15</sup>N<sub>2</sub> enriched seawater. On all sampling occasions and at all depths, a 2- to 6-fold increase in the rate of <sup>15</sup>N<sub>2</sub> assimilation was measured when <sup>15</sup>N<sub>2</sub>-enriched seawater was added to the seawater sample compared to the addition of <sup>15</sup>N<sub>2</sub> as a gas bubble. In addition, we show that the <sup>15</sup>N<sub>2</sub>-enriched seawater can be prepared prior to its use with no detectable loss (<1.7%) of dissolved <sup>15</sup>N<sub>2</sub> during 4 weeks of storage, facilitating its use in the field. The ratio of C<sub>2</sub>H<sub>4</sub> production to <sup>15</sup>N<sub>2</sub> assimilation varied from 7 to 27 when measured simultaneously in surface seawater samples. Collectively, the modifications to the AR assay and the <sup>15</sup>N<sub>2</sub> assimilation technique present opportunities for more accurate and high frequency measurements (e.g., diel scale) of N<sub>2</sub> fixation, providing further insight into the contribution of different groups of diazotrophs to the input of N in the global oceans.**

The biological conversion of dinitrogen (N<sub>2</sub>) gas into ammonia (NH<sub>3</sub>) is performed by a select group of organisms, termed diazotrophs. Examining the identity of diazotrophs present in the marine environment, determining their abundance, and understanding their physiological characteristics is vital to improving our understanding of the marine nitrogen (N) cycle. Equally important is quantifying the contribution of diazotrophs to the fixed pool of N in the global ocean, which is estimated to range from 100 to 200 Tg of N year<sup>-1</sup> (24). Calculating global N<sub>2</sub> fixation via field-based measurements (5, 25) results in lower estimates of global N<sub>2</sub> fixation compared to geochemical evidence such as nutrient stoichiometry and isotopic ratios (10, 20, 27). In part, this may derive from the methodologies used to measure N<sub>2</sub> fixation, a topic that forms the basis of the present study. Recently, there have been several model-based estimates on the specific contributions of individual diazotroph groups to the fixed pool of N (18, 29). The model outputs support field-based observations that the contribution of fixed N by unicellular N<sub>2</sub>-fixing cyanobacteria, including group A (termed UCYN-A) and *Crocosphaera* spp., can equal or even exceed the contribution of larger N<sub>2</sub>-fixing microorganisms, such as *Trichodesmium* and various heterocystous cyanobacteria (12, 31, 41).

The commonly applied methods to measure the rates of marine N<sub>2</sub> fixation include the acetylene reduction (AR) assay (4, 7, 12, 36) and the <sup>15</sup>N<sub>2</sub> assimilation technique (30). The AR assay relies on the preferential reduction of acetylene (C<sub>2</sub>H<sub>2</sub>) to ethylene (C<sub>2</sub>H<sub>4</sub>) by nitrogenase, instead of reducing N<sub>2</sub> to NH<sub>3</sub> (34). The most extensive applications of the AR assay at sea include the measurement of C<sub>2</sub>H<sub>4</sub> 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 C<sub>2</sub>H<sub>2</sub> on microbial metabolism and the reliability of the factor used to extrapolate

rates of C<sub>2</sub>H<sub>4</sub> production to rates of N<sub>2</sub> fixation (14, 16, 21, 36). An additional concern, especially for oligotrophic seawater samples, is the need to concentrate the microbial biomass, either by ship-board filtration or net tows, in order to obtain a detectable signal of C<sub>2</sub>H<sub>4</sub> production (6, 12, 37). The filtration process can impose undesired effects on the microbial population due to cell damage (1, 13). In comparison, the <sup>15</sup>N<sub>2</sub> assimilation technique follows the net incorporation of the <sup>15</sup>N<sub>2</sub> tracer into cellular biomass after a predetermined incubation period (2, 17, 26). Because the <sup>15</sup>N<sub>2</sub> technique has a lower detection limit than the AR assay, it is the preferred method for the oligotrophic marine environment (30). Indeed, <sup>15</sup>N<sub>2</sub> assimilation has been used to describe rates of N<sub>2</sub> 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 <sup>15</sup>N<sub>2</sub> to the seawater sample can affect the measured rate of N<sub>2</sub> fixation (28). The authors of that study found that the addition of <sup>15</sup>N<sub>2</sub> as a gas bubble underestimated N<sub>2</sub> fixation because the gas bubble introduced does not attain equilibrium within the incubation period, causing an unknown and time-dependent <sup>15</sup>N/<sup>14</sup>N ratio for the N pool.

We describe methodological developments to improve both the AR assay and the <sup>15</sup>N<sub>2</sub> assimilation technique. We demonstrate that the rates of C<sub>2</sub>H<sub>4</sub> production are quantifiable after incubations of 3 to 4 h by measuring the C<sub>2</sub>H<sub>4</sub> concentration in

Received 9 April 2012 Accepted 28 June 2012

Published ahead of print 6 July 2012

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doi:10.1128/AEM.01146-12

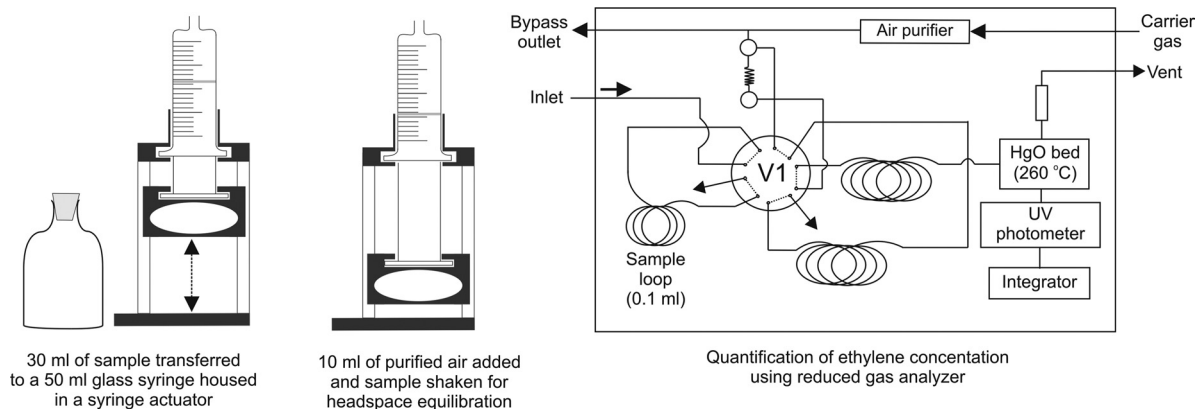


FIG 1 Schematic of the setup and analysis of dissolved ethylene ( $C_2H_4$ ) concentrations as part of the AR assay.

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  $C_2H_2$  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  $C_2H_2$  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  $C_2H_4$  gas in the whole sample. This differs from the routinely reported applications of the AR assay (4, 7, 12), whereby  $C_2H_2$  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  $C_2H_4$  concentrations at selected intervals during the sample incubation period or a predetermined endpoint. The second major methodological difference is that  $C_2H_4$  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  $C_2H_2$ -enriched seawater, (ii) sample collection and incubation, (iii) extraction of the dissolved  $C_2H_4$  gas, (iv) quantification of the  $C_2H_4$  concentrations, and (v) calculation of the  $C_2H_4$  concentration.

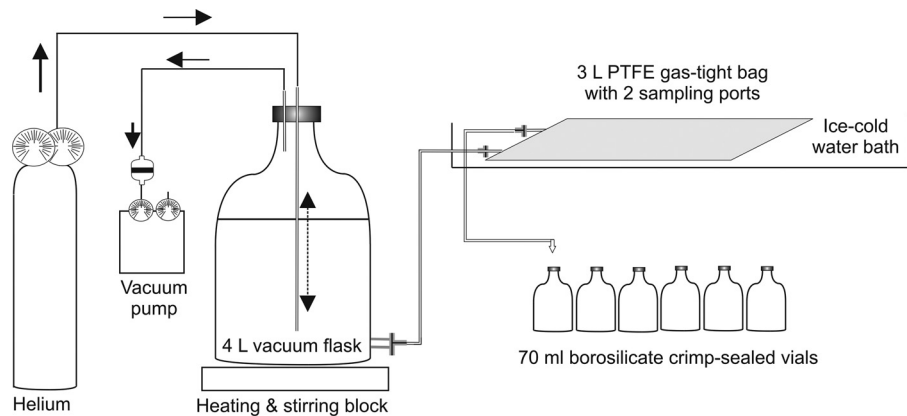
**(i) Preparation of  $C_2H_2$  enriched seawater.** The  $C_2H_2$ -saturated seawater for the AR assay was prepared ~1 h prior to use. To generate the  $C_2H_2$  gas, 6 g of calcium carbide ( $CaC_2$ ; Sigma) was added to 150 ml of deionized water in a 250-ml side-arm glass flask according to the following equation:



The resulting  $C_2H_2$  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  $C_2H_2$  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  $C_2H_2$  and shaking vigorously for 5 min, the  $C_2H_2$ -enriched seawater was stored in the dark until use. No measurements were made of  $C_2H_2$  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  $C_2H_2$  (1.6 ml of  $C_2H_2$  per ml of water) contains 65 mM  $C_2H_2$  (35). It should also be noted that  $C_2H_2$  produced from calcium carbide does not result in pure  $C_2H_2$  and contaminants are present, including  $C_2H_4$  (22). These can potentially be decreased by scrubbing the  $C_2H_2$  gas stream through an additional water flask (8); however, the presence of any level of contaminant  $C_2H_4$  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  $C_2H_2$ -enriched seawater, with a final  $C_2H_2$  concentration of 4 mM. Replicate ( $n = 3$ ) samples were immediately analyzed after the introduction of  $C_2H_2$  enriched seawater to provide a time zero  $C_2H_4$  concentration. Control samples, consisting of 0.2- $\mu$ m-pore-size filtered seawater, were inoculated with 20 ml of  $C_2H_2$ -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  $C_2H_4$  gas from incubated samples.** To measure the dissolved  $C_2H_4$  concentrations, a headspace analysis method was used to extract the  $C_2H_4$  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  $C_2H_4$ -free (<10 parts



**FIG 2** Schematic of the experimental setup for the preparation of seawater enriched with  $^{15}\text{N}_2$  gas. The experimental apparatus, including the filtered seawater, was purged with helium prior to vacuum degasification. The  $^{15}\text{N}_2$  gas was added to the filtered seawater after it was transferred to a gas-tight bag and cooled in an ice bath, and the  $^{15}\text{N}_2$ -enriched seawater was stored in the dark at  $4^\circ\text{C}$  in 70-ml crimp-sealed vials.

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  $0.2\text{-}\mu\text{m}$ -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 injects  $100\ \mu\text{l}$  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  $\text{C}_2\text{H}_4$  concentrations.**  $\text{C}_2\text{H}_4$  concentrations were measured using a reduced gas analyzer (RGA; Peak Laboratories). The RGA has previously been used to quantify other reduced gases such as hydrogen and carbon monoxide (33, 39, 40) and was modified for the quantification of  $\text{C}_2\text{H}_4$  by installing two chromatographic columns—a precolumn packed with a Unibeads 60/80 mesh to trap water vapor and a 1/8-in. Unibeads 1S 60/80 analytical column to separate  $\text{C}_2\text{H}_2$  and  $\text{C}_2\text{H}_4$ . Furthermore, the analytical time program of the RGA was altered to divert the gas flow from the column after  $\text{C}_2\text{H}_4$  had eluted to ensure that the large pulse of  $\text{C}_2\text{H}_2$  did not reach the detector. One of the main advantages of using the RGA compared to a GC-FID is the high sensitivity to  $\text{C}_2\text{H}_4$  with a detection limit of 30 ppb. This high sensitivity is achieved by passing the sample gas stream over a bed of heated mercuric oxide and quantifying the resulting mercury vapor with a UV photodetector (Fig. 1). Calibration of the RGA was routinely conducted using serial dilutions of a  $10.3 \pm 0.1$  ppm of  $\text{C}_2\text{H}_4$  standard in  $\text{N}_2$  (Scott-Marrin, Riverside, CA).

**(v) Calculation of  $\text{C}_2\text{H}_4$  concentrations.** The measured concentration of  $\text{C}_2\text{H}_4$  in the equilibrated headspace was used to calculate the total dissolved  $\text{C}_2\text{H}_4$  concentration ( $\{\text{C}_2\text{H}_4\}_w$  in ml of  $\text{C}_2\text{H}_4$ /ml of  $\text{H}_2\text{O}$ ) remaining in the water after equilibration by

$$\{\text{C}_2\text{H}_4\}_w = 10^{-6} \beta m_a p \quad (2)$$

where  $\beta$  (ml of  $\text{C}_2\text{H}_4$ /ml of  $\text{H}_2\text{O}$ /atm) represents the Bunsen solubility coefficient of  $\text{C}_2\text{H}_4$  (3),  $m_a$  is the measured concentration of  $\text{C}_2\text{H}_4$  in the equilibrated headspace (in parts per million by volume), and  $p$  is atmospheric pressure (atm) of dry air. The  $\text{C}_2\text{H}_4$  concentration in the initial seawater ( $\{\text{C}_2\text{H}_4\}_{\text{aq}}$  in ml of  $\text{C}_2\text{H}_4$ /ml of  $\text{H}_2\text{O}$ ) was calculated, assuming mass balance, as follows:

$$\{\text{C}_2\text{H}_4\}_{\text{aq}} = (\{\text{C}_2\text{H}_4\}_w V_w + 10^{-6} m_a V_a) / V_w = 10^{-6} m_a (\beta p V_w + V_a) / V_w \quad (3)$$

where  $V_w$  is the water sample size (ml) and  $V_a$  is the volume of headspace air (ml), followed by the conversion of  $\{\text{C}_2\text{H}_4\}_{\text{aq}}$  to units in nM ( $[\text{C}_2\text{H}_4]_{\text{aq}}$ ) as follows:

$$[\text{C}_2\text{H}_4]_{\text{aq}} = 10^9 \times p \{\text{C}_2\text{H}_4\}_{\text{aq}} / (RT) \quad (4)$$

where  $R$  is the gas constant ( $0.08206\ \text{atm liter mol}^{-1}\ \text{K}^{-1}$ ) and  $T$  is temperature (K). After the  $\text{C}_2\text{H}_4$  concentrations in sample and control treatments were measured at both the beginning and end of the incubation period, the rate of  $\text{C}_2\text{H}_4$  production was calculated as follows:

$$(\text{C}_s^{\text{final}} - \text{C}_c^{\text{final}}) - (\text{C}_s^{t=0} - \text{C}_c^{t=0}) / \Delta t \quad (5)$$

where  $C$  represents the concentration of  $\text{C}_2\text{H}_4$  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.

**$^{15}\text{N}_2$  assimilation technique.** In conjunction with the AR assay, we also measured  $\text{N}_2$  fixation using the  $^{15}\text{N}_2$  assimilation technique. It was recently shown that the addition of  $^{15}\text{N}_2$  gas as a bubble resulted in the underestimation of rates of  $\text{N}_2$  fixation in cultures of *Crocospaera* (28). Therefore, the present work compared rates of  $^{15}\text{N}_2$  fixation when  $^{15}\text{N}_2$  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  $^{15}\text{N}_2$ -enriched seawater and the sample collection, preparation, and analysis.

**Preparation of  $^{15}\text{N}_2$ -enriched seawater.** During a first comparative test, the  $^{15}\text{N}_2$ -enriched seawater was prepared at sea by adding filtered ( $0.2\text{-}\mu\text{m}$  pore size) degassed (250 mbar for 30 min) seawater to a crimp-sealed glass vial and injecting an appropriate quantity (1 ml of  $^{15}\text{N}_2$  gas per 100 ml of seawater) of  $^{15}\text{N}_2$  gas through the septum. Vigorous shaking for  $\sim 30$  min aided the complete dissolution of the bubble, and the  $^{15}\text{N}_2$ -enriched seawater was then added to the seawater samples (10 ml of  $^{15}\text{N}_2$ -enriched seawater per liter of seawater sample). Subsequent to this preliminary comparison, an alternative procedure was adopted whereby the  $^{15}\text{N}_2$ -enriched seawater was prepared at the shore-based laboratory prior to the oceanographic cruise (Fig. 2). To prepare the  $^{15}\text{N}_2$ -enriched seawater, 3.5 liters of filtered ( $0.2\text{-}\mu\text{m}$  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  $5\ \text{lb/in}^2$  ( $\sim 500\ \text{ml min}^{-1}$ ) 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  $4^\circ\text{C}$  water bath. Subsequently, 40 ml of  $^{15}\text{N}_2$  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 ( $\sim 10$  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  $^{15}\text{N}_2$ -enriched seawater were stored in the dark at  $4^\circ\text{C}$  until required for experimental purposes.

**Validation of  $^{15}\text{N}_2$ -enriched seawater.** One of the major considerations for any analytical method is the ease of its application and although the  $^{15}\text{N}_2$ -enriched seawater was prepared at sea on one occasion, it was found to be more time efficient to prepare the  $^{15}\text{N}_2$ -enriched seawater on land prior to its use at sea. We therefore investigated the feasibility of storing the  $^{15}\text{N}_2$ -enriched seawater in crimp-sealed 70-ml borosilicate glass vials over a 4-week period by analyzing the dissolved  $^{15}\text{N}_2$  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- $\mu\text{m}$  pore size) surface seawater collected from Stn ALOHA. Instrument drift in the  $\text{N}_2/\text{Ar}$  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  $^{14}\text{N}_2$ ,  $^{15}\text{N}_2$ , and  $\text{O}_2$ ), and they were detected sequentially using a repetitive cycle of 1.5 Hz. Replicate samples of prepared  $^{15}\text{N}_2$ -enriched seawater were analyzed for the loss of  $^{15}\text{N}_2$  at weekly intervals over a 1-month period, comparing the ratio of mass 30 in  $^{15}\text{N}_2$ -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  $^{15}\text{N}_2$ -enriched seawater from the 70-ml crimp-sealed vials using a 50-ml glass syringe, resulting in a final  $^{15}\text{N}_2$  enrichment of 1.5 atom%. The syringe was attached to a 15-cm length of 1/8-in. PTFE tubing which enabled the  $^{15}\text{N}_2$ -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  $^{15}\text{N}_2$  gas (98 atom%; Sigma-Aldrich) using a gas-tight syringe (SGE Analytical Sciences) through the septum cap into the bottle. The 3-ml  $^{15}\text{N}_2$  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  $^{15}\text{N}$  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 -20°C for transport. To quantify the  $^{15}\text{N}_2$  enrichment of particulate material, samples were dried at 60°C for ~24 h and pelleted before analysis with an elemental analyzer-isotope ratio mass spectrometer (EA-IRMA Carlo Erba NC2500 coupled to a Thermo-Finnigan Delta S) by the Stable Isotope Facility, University of Hawaii.

$\text{N}_2$  fixation rates were determined from the particulate N (PN) and  $\delta^{15}\text{N}$ -PN content of incubated samples, the  $^{15}\text{N}$  atom% enrichment, ambient dissolved  $\text{N}_2$  concentration, and the  $\delta^{15}\text{N}$ -PN for control (unamended) samples. The  $\delta^{15}\text{N}$ -PN of control samples (described as the  $\text{AP}_{\text{PN-initial}}$  in equation 6 below) was provided by natural abundance PN concentration and  $\delta^{15}\text{N}$ -PN at a depth of 25 m in the water column at Stn ALOHA. Atmospheric  $\text{N}_2$  gas is used as the reference standard ( $^{15}\text{N}/^{14}\text{N} = 0.0036765$ ,  $\delta^{15}\text{N} = 0\text{‰}$ ). After the isotope mass balance calculation, the rates of  $\text{N}_2$  fixation rates were expressed as PN by including a factor of 2 (as described in reference 30):

$$V(t^{-1}) = (\text{AP}_{\text{PN-final}} - \text{AP}_{\text{PN-initial}}) / (\text{AP}_{\text{N}_2} - \text{AP}_{\text{PN-initial}}) \cdot 1 / \Delta t \cdot \text{PN}_{\text{final}} / 2 \quad (6)$$

where AP is the atom%  $^{15}\text{N}$  of the sample (e.g.,  $\text{PN}_{\text{final}}$ ) or substrate (i.e.,  $\text{N}_2$ ) pool and  $\Delta t$  is the length of the sample incubation period.

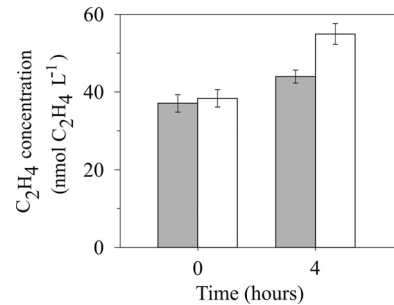


FIG 3  $\text{C}_2\text{H}_4$  concentrations in whole seawater (white bars) and control treatments (0.2- $\mu\text{m}$ -pore-size filtered seawater) (gray bars) at time zero and after 4 h of incubation in deckboard incubators.

## 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  $\text{C}_2\text{H}_4$  concentrations were always measurable, exceeding the analytical detection limit of 5 pmol liter<sup>-1</sup> by several orders of magnitude. In this particular example, at time zero the dissolved  $\text{C}_2\text{H}_4$  concentrations measured 37 nmol of  $\text{C}_2\text{H}_4$  liter<sup>-1</sup> (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  $\text{C}_2\text{H}_4$  production associated with this particular seawater sample, as calculated per equation 5, was 2.7 nmol of  $\text{C}_2\text{H}_4$  liter<sup>-1</sup> h<sup>-1</sup>. An additional important observation is the 12% increase in  $\text{C}_2\text{H}_4$  concentrations in the control treatment during the 4 h of incubation period (Fig. 3). The nonbiological production of  $\text{C}_2\text{H}_4$  was investigated further, and an increase was repeatedly observed, even when the filtered seawater controls were amended with mercuric chloride ( $\text{HgCl}_2$ ; 200  $\mu\text{l}$  of saturated  $\text{HgCl}_2$  in 300 ml of seawater sample) and regardless of whether samples were incubated in the light or dark. The abiotic production of  $\text{C}_2\text{H}_4$  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  $\text{C}_2\text{H}_4$  concentrations in glass Wheaton bottles highlights the importance of quantifying the  $\text{C}_2\text{H}_4$  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  $\text{C}_2\text{H}_4$  at the end of the incubation period ranged from 42 to 67 nmol liter<sup>-1</sup>, with an overall analytical precision of  $\pm 0.05$  nmol liter<sup>-1</sup> and the blank/signal ratio at the end of the incubation period ranged from 67 to 82%. The corresponding rates of  $\text{C}_2\text{H}_4$  production varied by an order of magnitude ranging from 0.46 nmol liter<sup>-1</sup> h<sup>-1</sup> (incubation period: 2100 to 0600, 19 September) to 4.7 nmol liter<sup>-1</sup> h<sup>-1</sup> (incubation period: 1000 to 1400, 20 July).

**TABLE 1** Measurements of AR and  $^{15}\text{N}_2$  assimilation, determined using  $^{15}\text{N}_2$ -enriched seawater, for water column samples collected from 25 m at Stn ALOHA between July and September 2011<sup>a</sup>

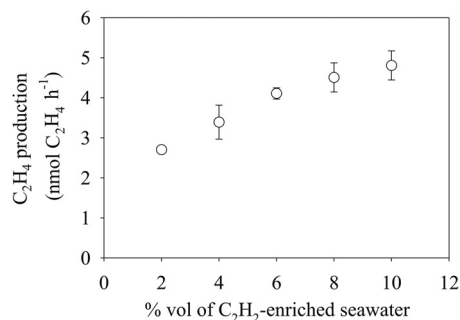
Date	Incubation period (hourly range)	Mean $\pm$ SD		$\text{C}_2\text{H}_4/\text{N}_2$ ratio
		$\text{C}_2\text{H}_4$ production (nmol of $\text{C}_2\text{H}_4$ liter $^{-1}$ h $^{-1}$ )	$^{15}\text{N}_2$ assimilation rate (nmol of N liter $^{-1}$ h $^{-1}$ )	
20 July 2011	Day (1000–1400)	4.7 $\pm$ 0.3	0.15 $\pm$ 0.01	18
	Night (2200–0500)	0.7 $\pm$ 0.2		
30 Aug 2011	Day (1200–1500)	2.0 $\pm$ 0.1	0.22 $\pm$ 0.09	7
	Night (2200–0600)	1.0 $\pm$ 0.2		
9 Sept 2011	Day (1030–1430)	2.1 $\pm$ 0.1	0.08 $\pm$ 0.02	27
	Night (2200–0600)	3.4 $\pm$ 0.1	0.22 $\pm$ 0.10	
13 Sept 2011	Day (1000–1400)	0.9 $\pm$ 0.2	0.08 $\pm$ 0.02	12
	Night (2200–0600)	1.3 $\pm$ 0.4	0.10 $\pm$ 0.01	
19 Sept 2011	Day (1100–1330)	4.0 $\pm$ 0.5	0.26 $\pm$ 0.05	16
	Night (2100–0600)	0.5 $\pm$ 0.2	0.04 $\pm$ 0.02	

<sup>a</sup> The length of the sample incubation period is reported for the AR assay in parentheses, whereas it was either 12 or 24 h for  $^{15}\text{N}_2$  assimilation. Both  $\text{C}_2\text{H}_4$  production and  $^{15}\text{N}_2$  assimilation are reported as hourly rates ( $n = 3$ ).

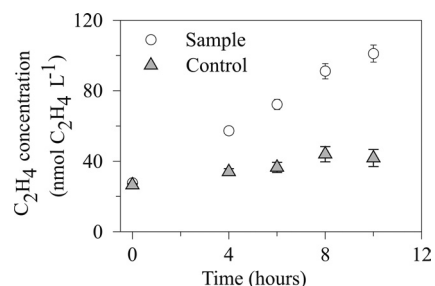
#### Determining the quantity of $\text{C}_2\text{H}_2$ added to seawater sample.

A major consideration of the AR assay is the quantity of  $\text{C}_2\text{H}_2$  added to the seawater samples. The addition of low  $\text{C}_2\text{H}_2$  concentrations will insufficiently saturate nitrogenase (42); however, complete saturation of nitrogenase will inhibit  $\text{N}_2$  fixation, resulting in N starvation and production of new nitrogenase (36). Balancing these considerations has resulted in  $\text{C}_2\text{H}_2$  typically added in gaseous form to the headspace of a sample at 10 to 20% (vol/vol) (4). We measured the effect of  $\text{C}_2\text{H}_2$  concentrations on the rate of  $\text{C}_2\text{H}_4$  production in seawater samples by increasing the volume of  $\text{C}_2\text{H}_2$  added from 2 to 10%, equivalent to  $\text{C}_2\text{H}_2$  concentrations of 1.3 to 6.5 mM, respectively. Overall, a nearly 2-fold increase was observed in the rate of  $\text{C}_2\text{H}_4$  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  $\text{C}_2\text{H}_4$  production were conducted with a 10% addition of  $\text{C}_2\text{H}_2$ -saturated seawater.

**Incubation period.** During both day and night sample incubation periods, the increase in  $\text{C}_2\text{H}_4$  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  $\text{C}_2\text{H}_4$  produced relative to the control treatments. We advise restricting the AR assay incubation period to a few hours since the presence of  $\text{C}_2\text{H}_2$  can adversely



**FIG 4** Rates of  $\text{C}_2\text{H}_4$  production in oligotrophic, near-surface (25 m) seawater samples incubated for 3 h at different  $\text{C}_2\text{H}_2$  concentrations.



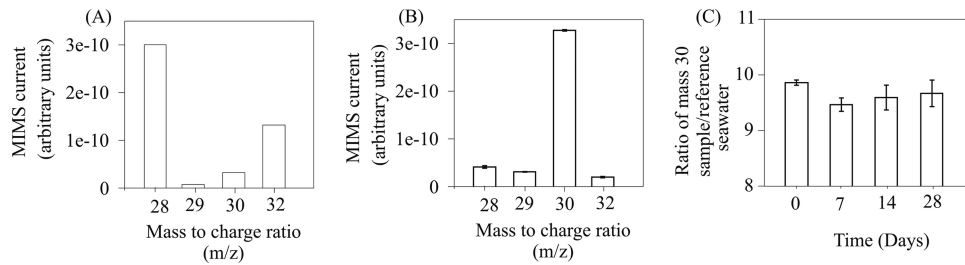
**FIG 5** Linear response of  $\text{C}_2\text{H}_4$  concentrations measured over an 8-h incubation period in surface seawater incubations (open circles) compared to filtered seawater controls (gray triangles).

affect other metabolic pathways and the wider microbial community (32).

**$^{15}\text{N}_2$  assimilation.** In addition to conducting the field assessment of the  $^{15}\text{N}_2$  tracer technique, the methods used to prepare and store the  $^{15}\text{N}_2$ -enriched seawater were also assessed. A mass-to-charge ratio of 30, corresponding to  $^{15}\text{N}_2$ , was measured in  $^{15}\text{N}_2$ -enriched seawater/reference seawater over a 4-week period to determine any loss of  $^{15}\text{N}_2$  in the  $^{15}\text{N}_2$ -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  $^{15}\text{N}_2$  assimilation methods were conducted, adding  $^{15}\text{N}_2$  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  $^{15}\text{N}_2$  assimilation were significantly greater (one-way ANOVA,  $P = <0.001$ ) when  $^{15}\text{N}_2$  tracer was added to the seawater sample in dissolved form, averaging  $4.6 \pm 0.9$  nmol of N liter $^{-1}$  day $^{-1}$  ( $\pm$  represents the standard deviation,  $n = 18$ ) compared to an overall average of  $2.7 \pm 0.8$  nmol of N liter $^{-1}$  day $^{-1}$  when  $^{15}\text{N}_2$  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  $^{15}\text{N}_2$  assimilation were significantly greater (one-way ANOVA,  $P = <0.001$ ) when  $^{15}\text{N}_2$  tracer was added to the seawater sample in dissolved form (overall average of  $5 \pm 1.3$  nmol of N liter $^{-1}$  day $^{-1}$ ) compared to the addition of a gas bubble (overall average of  $1.9 \pm 1.1$  nmol of N liter $^{-1}$  day $^{-1}$ ).

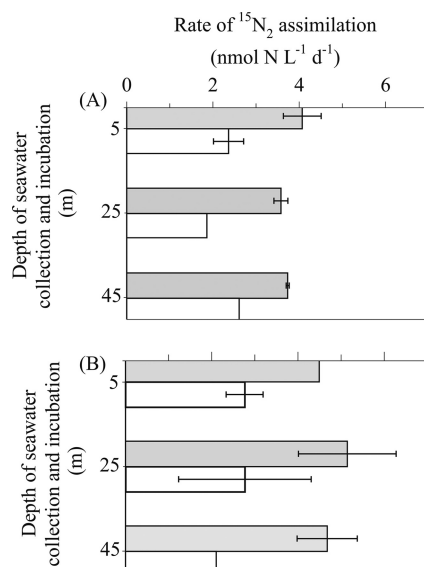
On 20 July 2011, replicate samples collected from 5, 25, and 45 m, were incubated simultaneously using the *in situ* array and deckboard incubators. When the  $^{15}\text{N}_2$  tracer was added as a bubble, there was no significant difference (one-way ANOVA,  $P = 0.98$ ) between samples incubated using the *in situ* array and deckboard incubator. In contrast, when the  $^{15}\text{N}_2$  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)  $^{15}\text{N}_2$  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-



**FIG 6** Analysis of the storage capability of <sup>15</sup>N<sub>2</sub>-enriched seawater. The mass-to-charge ( $m/z$ ) 28, 29, 30, and 32 peak areas as measured by membrane inlet mass spectrometry (MIMS) are shown for reference seawater (A) and samples of <sup>15</sup>N<sub>2</sub>-enriched seawater (B) with a <sup>15</sup>N<sub>2</sub> atom% enrichment of 12 ml liter<sup>-1</sup>. (C)  $m/z$  ratio corresponding to <sup>15</sup>N<sub>2</sub> in <sup>15</sup>N<sub>2</sub>-enriched seawater/reference seawater over a 4-week period.

tions with a temperature/light logger (e.g., HOBO Pendant, Onset, MA) or conducting *in situ* analyses.

An additional experiment was performed to ensure the validity of comparing the addition of <sup>15</sup>N<sub>2</sub> 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<sub>2</sub> fixation. Therefore, on 7 November 2011, a third treatment was included in addition to the comparison of <sup>15</sup>N<sub>2</sub> in gaseous or dissolved phase. The third treatment consisted of 50 ml of degassed, but non-<sup>15</sup>N<sub>2</sub>-enriched seawater that was added to seawater samples prior to the injection of the <sup>15</sup>N<sub>2</sub> gas bubble. No significant difference was observed in the rate of <sup>15</sup>N assimilation when amended with a bubble ( $2.1 \pm 0.6$  nmol of N liter<sup>-1</sup> day<sup>-1</sup>) compared to the bubble plus degassed, nonenriched seawater ( $1.3 \pm 0.2$  nmol of N liter<sup>-1</sup> day<sup>-1</sup>) (one-way ANOVA,  $P = 0.096$  and  $P > 0.05$ ). In contrast, the rate of <sup>15</sup>N assimilation in seawater samples inoculated with dissolved <sup>15</sup>N<sub>2</sub> averaged  $6.5 \pm 0.4$  nmol of N liter<sup>-1</sup> day<sup>-1</sup> and were significantly higher than the <sup>15</sup>N<sub>2</sub> gas bubble injected treatments (one-way ANOVA,  $P = 0.003$  and  $P < 0.05$ ).



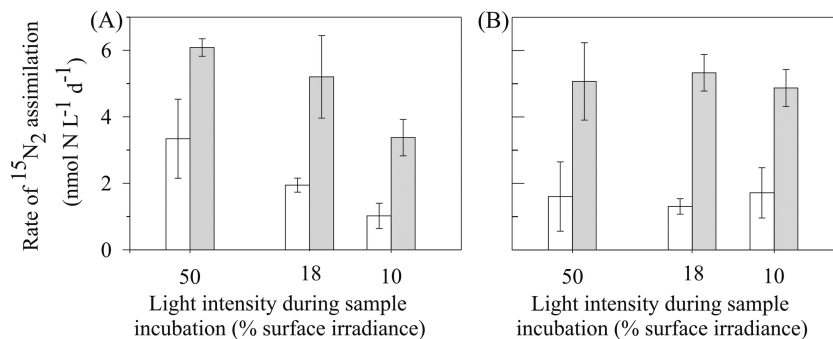
**FIG 7** Depth profiles of N<sub>2</sub> fixation rates at Stn ALOHA incubated *in situ* for 24 h using a free-drifting array on two separate occasions: 20 July 2011 (A) and 31 August 2011 (B). The <sup>15</sup>N<sub>2</sub> tracer was either added as a gas bubble (open bars) or in dissolved form (gray bars). Error bars where shown represent standard deviation ( $n = 3$ ).

**Comparison of AR assay and <sup>15</sup>N<sub>2</sub> assimilation.** On eight separate occasions, the AR assay was conducted alongside rate measurements of <sup>15</sup>N<sub>2</sub> assimilation conducted using <sup>15</sup>N<sub>2</sub>-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 <sup>15</sup>N<sub>2</sub> tracer. Expression of C<sub>2</sub>H<sub>4</sub> production and <sup>15</sup>N<sub>2</sub> assimilation incubated with <sup>15</sup>N<sub>2</sub>-enriched seawater as an hourly rate measurement revealed a positive relationship (Pearson correlation,  $r = 0.83$ ,  $P = 0.017$ ) with the ratio of C<sub>2</sub>H<sub>4</sub> production to <sup>15</sup>N<sub>2</sub> assimilation ranging from 7 to 27.

## DISCUSSION

Measuring N<sub>2</sub> 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<sub>2</sub> fixation in the marine environment: the <sup>15</sup>N<sub>2</sub> assimilation technique and the AR assay. A common theme in the currently applied protocols of the AR assay and the <sup>15</sup>N<sub>2</sub> assimilation is the issue of gas equilibration. It has previously been demonstrated that without sufficient agitation of the sample, dissolved C<sub>2</sub>H<sub>4</sub> resulting from the reduction of C<sub>2</sub>H<sub>2</sub> does not equilibrate with the gas phase (14). Similarly, it was recently shown that the addition of <sup>15</sup>N<sub>2</sub> tracer 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 <sup>15</sup>N<sub>2</sub> and C<sub>2</sub>H<sub>2</sub> in gaseous form, we present here a modified protocol for the AR assay and describe the preparation and use of <sup>15</sup>N<sub>2</sub>-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<sub>2</sub>H<sub>4</sub>. Two inherent features of the RGA which aid the analysis of C<sub>2</sub>H<sub>4</sub> are the redirection of the carrier gas flow after C<sub>2</sub>H<sub>4</sub> has eluted from the column avoiding the C<sub>2</sub>H<sub>2</sub> 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



**FIG 8** N<sub>2</sub> 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 <sup>15</sup>N<sub>2</sub> tracer was either added as a gas bubble (open bars) or in dissolved form (gray bars). Error bars represent the standard deviation ( $n = 3$ ).

feature of the analytical setup was the hand-held syringe actuator (Fig. 1), which may also be commercially available. When conducting the AR assay in the field, the most consistent measurements were obtained when analyzing three to four samples with a sample incubation period of 4 h. It is recommended to avoid having samples waiting to be processed due to the continuing biological and potentially abiotic production of C<sub>2</sub>H<sub>4</sub> 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 measurement of C<sub>2</sub>H<sub>4</sub>, particularly for field samples that contain mixed community assemblages of diazotrophs. This work demonstrates that no preconcentration of biological material is required when dissolved C<sub>2</sub>H<sub>4</sub> concentrations are quantified using the RGA, excluding the potential of harming the N<sub>2</sub>-fixing microorganisms during the filtration process. Regardless of whether C<sub>2</sub>H<sub>4</sub> 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 C<sub>2</sub>H<sub>4</sub> (<0.2 ppm) by using “clean C<sub>2</sub>H<sub>2</sub>,” which refers to gas cylinders of compressed C<sub>2</sub>H<sub>2</sub> (37). To the best of our understanding, these C<sub>2</sub>H<sub>2</sub> gas cylinders are no longer commercially available. Finally, we recommend before commencing extensive field measurements, an initial set of C<sub>2</sub>H<sub>4</sub> rate measurements are conducted at a range of C<sub>2</sub>H<sub>2</sub> concentrations, e.g., 5, 10, and 20% (vol/vol), to demonstrate the response of nitrogenase enzyme within the ambient microbial community to various levels of C<sub>2</sub>H<sub>2</sub>.

An additional issue regarding the AR assay concerns the use of a stoichiometric conversion factor to obtain an estimate of N<sub>2</sub> 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 C<sub>2</sub>H<sub>4</sub> production to be converted into a rate of N<sub>2</sub> fixation. A theoretical ratio of 3:1 is often cited, based on the difference between two hydrogen ions required to reduce C<sub>2</sub>H<sub>2</sub> to C<sub>2</sub>H<sub>4</sub> and six hydrogen ions needed to reduce N<sub>2</sub> to 2NH<sub>3</sub>. A ratio of 4:1 has also been suggested since this incorporates the H<sub>2</sub> evolution associated with the reduction of a N<sub>2</sub> molecule (4). A wide range of C<sub>2</sub>H<sub>4</sub>/<sup>15</sup>N<sub>2</sub> 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 between 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 C<sub>2</sub>H<sub>4</sub> production to a rate of N<sub>2</sub> fixation, multiple simultaneous AR assay and <sup>15</sup>N<sub>2</sub> assimilation measurements should be conducted, as previously recommended (4, 15, 19, 30).

With respect to the <sup>15</sup>N<sub>2</sub> assimilation technique, the results demonstrate that introducing the <sup>15</sup>N<sub>2</sub> gas into the seawater sample, either as a gas bubble or dissolved in sterile seawater, causes significant differences in the quantity of <sup>15</sup>N recovered in particulate form (Fig. 7 and 8). The issue of <sup>15</sup>N<sub>2</sub> gas solubility was recently demonstrated for laboratory cultures of the diazotroph *Crocospheera watsonii* (28), and we now extend the observation of an underestimation of <sup>15</sup>N<sub>2</sub> assimilation rates when adding <sup>15</sup>N<sub>2</sub> as a gas bubble to open ocean seawater samples composed of mixed diazotrophs assemblages. We demonstrate here that the addition of <sup>15</sup>N<sub>2</sub> gas in dissolved form increases the recovery of <sup>15</sup>N compared 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 N<sub>2</sub> fixation over an 11-year period, from 1989 to 2001, derived from the isotopic composition of sinking material exceeded rates of <sup>15</sup>N<sub>2</sub> assimilation measured on five occasions by 40 to 80% (11). This previous study concluded that their <sup>15</sup>N<sub>2</sub> assimilation tracer measurements, as conducted by the addition of N<sub>2</sub> gas in a bubble, were underrepresenting N<sub>2</sub> fixation rates in the upper 0 to 100 m of the water column. A more recent study of N<sub>2</sub> fixation at Stn ALOHA over a 3-year period measured depth-integrated (0 to 100 m) rates of <sup>15</sup>N<sub>2</sub> assimilation of 150 μmol of N m<sup>-2</sup> day<sup>-1</sup> in the summer months (July to September) (9), which is within the range of surface N<sub>2</sub> fixation (100 to 400 μmol of N m<sup>-2</sup> day<sup>-1</sup>) reported by Deutsch et al. (10). The rates of <sup>15</sup>N<sub>2</sub> assimilation measured here by adding <sup>15</sup>N<sub>2</sub> in dissolved form are 2- to 3.5-fold higher than the rates of

**TABLE 2** Depth-integrated (0 to 45 m) rates of N<sub>2</sub> fixation as measured by <sup>15</sup>N<sub>2</sub> assimilation by adding the <sup>15</sup>N<sub>2</sub> gas in dissolved and bubble form<sup>a</sup>

Date	Mode of incubation	<sup>15</sup> N <sub>2</sub> assimilation rate (μmol of N m <sup>-2</sup> day <sup>-1</sup> )	
		Bubble	Dissolved
20 July 2011	Array	82	150
20 July 2011	Incubator	87	198
30 Aug 2011	Array	104	195
29 Sept 2011	Incubator	59	206

<sup>a</sup> The seawater samples were incubated either using an *in situ* array or in a shipboard incubator.

$^{15}\text{N}_2$  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.

## ACKNOWLEDGMENTS

We thank the HOT scientists and staff for field-work assistance, in particular chief scientists Susan Curless and Craig Nosse, and also the captain and crew of the R/V *Kilo Moana* for their support. We also thank Daniela del Valle for help with running the membrane inlet mass spectrometer and Brian Popp for his advice and help with the  $^{15}\text{N}_2$  analysis.

The Gordon and Betty Moore Foundation supported this research through the Marine Microbiology Investigator program, and the National Science Foundation supported this research through the Center for Microbial Oceanography: Research and Education (C-MORE; EF0424599) (D.M.K.), the Hawaii Ocean Time-series (HOT OCE-0926766, D.M.K. and M.J.C.), and grant OCE-0850827 (M.J.C.).

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