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EVALUATION OF ARGON-INDUCED HYDROGEN PRODUCTION AS A METHOD TO MEASURE NITROGEN FIXATION BY CYANOBACTERIA[1]

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The production of dihydrogen (H_2) is an enigmatic yet obligate component of biological dinitrogen (N_2) fixation. This study investigates the effect on H_2 production by N_2 fixing cyanobacteria when they are exposed to either air or a gas mixture consisting of argon, oxygen, and carbon dioxide (Ar:O_2:CO_2). In the absence of N_2, nitrogenase diverts the flow of electrons to the production of H_2, which becomes a measure of Total Nitrogenase Activity (TNA). This method of argon-induced hydrogen production (AIHP) is much less commonly used to infer rates of N_2 fixation than the acetylene reduction (AR) assay. We provide here a full evaluation of the AIHP method and demonstrate its ability to achieve high-resolution measurements of TNA in a gas exchange flow-through system. Complete diel profiles of H_2 production were obtained for N_2 fixing cyanobacteria despite the absence of N_2 that broadly reproduced the temporal patterns observed by the AR assay. Comparison of H_2 production under air versus Ar:O_2:CO_2 revealed the efficiency of electron usage during N_2 fixation and place these findings in the broader context of cell metabolism. Ultimately, AIHP is demonstrated to be a viable alternative to the AR assay with several additional merits that provide an insight into cell physiology and promise for successful field application.

Key index words: **cyanobacteria; nutrients; phytoplankton; nitrogen fixation; hydrogen; acetylene reduction; nitrogenase**

***Abbreviations*: H_2, dihydrogen; N_2, dinitrogen; Ar:O_2:CO_2, argon, oxygen, and carbon dioxide; ANA, Apparent Nitrogenase Activity; TNA, Total Nitrogenase Activity; AIHP, argon induced hydrogen production; AR, acetylene reduction**

Biological dinitrogen (N_2) fixation involves the reduction of N_2 to ammonia and under conditions of nitrogen (N) limitation, becomes a valuable source of fixed N to the ecosystem. Due to the ecological importance of N_2 fixation, understanding the structure and functioning of the nitrogenase enzyme has been at the forefront of scientific endeavors ever since it was conclusively shown that legumes can utilize N_2 in the 1880s (Hellriegel and Wilfarth, 1888, Nutman 1987). One of the key breakthrough moments in the scientific research on biological N_2 fixation was the identification of surrogate molecules with a triple bond that nitrogenase will reduce in addition to N_2. The list of these molecules includes cyanide, azide, nitrous oxide, and acetylene (Gallon and Chaplin 1987, Seefeldt et al. 2020). A series of seminal papers in the 1960s showed that the reduction of acetylene to ethylene provides an effective estimate of biological N_2 fixation in both field and laboratory samples (Koch and Evans 1966, Stewart et al. 1967, Hardy et al. 1968). Because ethylene can be readily quantified by gas chromatography, the ability to measure biological N_2 fixation suddenly became more accessible to the scientific community. Since then, the acetylene reduction (AR) assay has become a frequently used method from which putative N_2 fixing microorganisms have been identified and the influence of various growth parameters such as light, temperature, oxygen, and nutrients have been determined (e.g., Hutchins et al. 2007, Shi et al. 2012, Stal 2017). However, the ease of conducting the AR assay does not preclude some methodological issues and there have been several noteworthy precautions published about using this method (e.g., Flett et al. 1980, Giller 1987, Hyman and Arp 1987).

In this article, we revisit another facet of the nitrogenase enzyme that provides a valuable perspective on its function. All nitrogenase enzymes produce dihydrogen (H_2) during the reduction of N_2 to ammonia (Hoch et al. 1957). The most prevalent nitrogenase enzyme, the molybdenum (Mo) nitrogenase, produces one mole of H_2 produced for every mole of N_2 reduced to NH_3 (Eq. 1; Simpson and Burris 1984). Other nitrogenase enzymes, the vanadium (V) and iron (Fe-only) nitrogenases, produce higher quantities of H_2 for each mole of N_2 reduced (Bothe et al. 2010, Harwood, 2020). The production of H_2 during N_2 fixation is associated with the reduction of the N_2 molecule (Lowe and Thorneley 1984, Seefeldt et al. 2020).

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$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (1)$$

where hydrogen ion is H^+, electron is e^-, adenosine triphosphate is ATP, adenosine diphosphate is ADP, and inorganic phosphate is Pi.

The production of H_2 by nitrogenase can be used to infer rates of N_2 fixation. This is achieved by the complete removal of N_2 from the sample and substitution with a gas mixture consisting of argon, oxygen, and carbon dioxide ($Ar:O_2:CO_2$; Schubert and Evans 1976). In the absence of N_2, the nitrogenase enzyme diverts all of the electrons that would have been used to reduce N_2 to the production of H_2, which becomes a measure of Total Nitrogenase Activity (TNA; Fig. 1). The argon-induced hydrogen production (AIHP) method has been demonstrated to be an effective measure of nitrogenase activity (Schubert and Evans 1976, Phillips 1980, Saito et al. 1980, Hunt et al. 1987). However, the AIHP method was developed after the AR assay and this, together with the greater challenges of quantifying trace concentrations of H_2 compared to ethylene, caused the AIHP technique to be neglected despite some experimental advantages compared to the AR assay (Hunt and Layzell 1993). The AIHP technique is now mostly used to evaluate short-term yields of H_2 production from N_2 fixing cyanobacteria (e.g., Pinto et al. 2002, Bandyopadhyay et al. 2010, Masukawa et al. 2010) rather than being an assay for N_2 fixation.

Here, we revisit the analysis of H_2 production upon exposure to $Ar:O_2:CO_2$ to infer rates of N_2 fixation. The AIHP method is compared with the AR assay, and $^{15}N_2$ assimilation using cultures of N_2 fixing cyanobacteria. We discuss the advantages and disadvantages of the different approaches with particular focus on the merits of the AIHP method.

MATERIALS AND METHODS

Experimental design. Our assessment of the AIHP method was conducted using laboratory-maintained cultures of known N_2 fixing cyanobacteria (termed 'diazotrophs'; Table 1). The cultures provided reproducible small volume samples that could be analyzed under controlled environmental settings with short incubation periods. Furthermore, the diazotrophs have varying physiologies and metabolic strategies for accommodating both N_2 fixation and photosynthesis. Their robust diel patterns in nitrogenase activity helped to evaluate short-term changes in H_2 production during a day–night cycle.

The cell abundances of *Crocosphaera* were made using an Attune Acoustic Focusing Flow Cytometer (Applied Biosystems by Life Technologies) with an excitation wavelength of 488 nm (Wilson et al. 2017). Enumeration of cell abundance for the filamentous cyanobacteria was conducted using an Imaging FlowCytoBot (IFCB) for diazotrophs *Anabaena, Gloeotrichia,* and *Nostoc.* Images of cell and filament length were acquired by the IFCB and the number of cells per filament calculated by dividing filament length by cell length. Cell abundances per ml were then calculated as the product of total filament counts and cells per filament. With regard to *Trichodesmium,* it was subsequently found that the IFCB focal path view was exceeded by filaments longer than 330 µm. Therefore, *Trichodesmium* cell abundances were computed from measured cell sizes to calculate C content · $cell^{-1}$ using the equation $C = 0.433 \times V^{0.863}$ (Verity et al. 1992) where C represents cell C content (pg) and V represents cell volume (μm^3) assuming cylindrical dimensions. A carbon: nitrogen ratio of 6.6 was used to convert C into N content which yielded 0.2 pmol N *Trichodesmium* · $cell^{-1}$. Cell abundances were subsequently derived by dividing the bulk particulate nitrogen values obtained from the $^{15}N_2$ assimilation measurements with the per cell N quota.

The trace gas measurements were conducted using a simple gas exchange flow-through system (Fig. 2). Such flow-through systems are often preferable to static closed incubations as they facilitate non-intrusive real-time monitoring of the effluent. The diazotroph cultures were transferred from their culture flasks to borosilicate glass vials. The sample volume ranged from 10 to 30 mL depending on the species and the vial size ranged from 15 to 40 mL. The vials were closed with two legged gray bromobutyl stoppers and made gas-tight with aluminum crimp-seals. By maintaining a headspace within the vial, there was no contact between the liquid culture and the stopper which can be toxic to microbiota

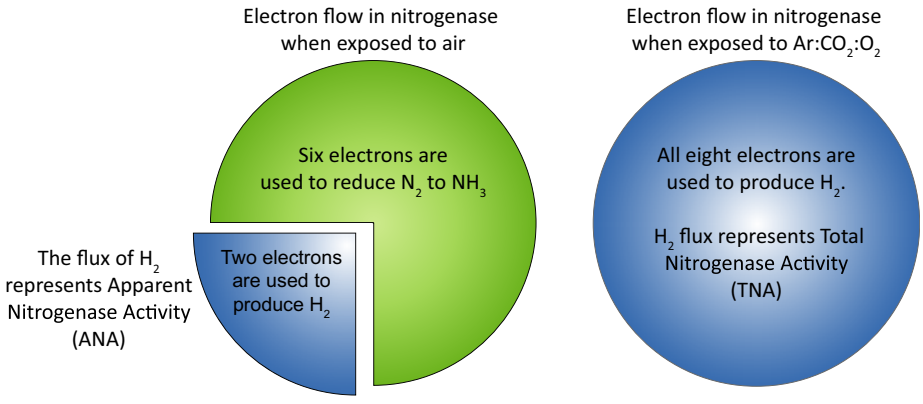

FIG. 1. Schematic representation of electron allocation by nitrogenase under air versus $Ar:O_2:CO_2$ for the Mo nitrogenase. H_2 production under air is referred to as Apparent Nitrogenase Activity (ANA), while H_2 production under $Ar:O_2:CO_2$ is referred to as Total Nitrogenase Activity (TNA). The recycling of H_2 via uptake hydrogenase prevents ANA from being a quantitative measurement of N2 fixation.

TABLE 1. List of cyanobacteria analyzed in this study with the relevant culture media that included modified BG11 (Stanier et al. 1971), SO (Waterbury and Willey 1988), and YBC-II (Chen et al. 1996).

Name and Strain	Medium	Morphology	Obtained from
Nostoc A10a	modified BG11	Filamentous heterocystous	DA Caron (USC)
Anabaena A1	modified BG11	Filamentous heterocystous	DA Caron (USC)
Gloeotrichia F1	modified BG11	Filamentous heterocystous	DA Caron (USC)
Trichodesmium IMS101	YBC-II	Filamentous nondifferentiated	EA Webb (USC)
Crocosphaera WH0401	SO	Unicellular	JP Zehr (UCSC)
Crocosphaera WH8501	SO	Unicellular	JP Zehr (UCSC)

The modification to BG11 media was the exclusion of nitrate.

(Niemann et al. 2015). The glass vials were held in a plexiglass rack within a benchtop incubator (Incu-Shaker; Benchmark Scientific) and temperatures were maintained at 25 ± 0.2°C. To avoid any light inhibition during the experimental period, the cultures were exposed to light levels of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ at wavelengths of 380–780 nm (Prime HD+; Aqua Illumination) on a 12:12 h square light: dark cycle that extended from 7 a.m. to 7 p.m. local time. The light levels were verified using an irradiance sensor (QSL-2100; Biospherical Instruments). During the analyses, the cultures were continually sparged at a flow rate of 13 $mL \cdot min^{-1}$. The continual sparging minimizes the intrusion of contaminant gases and facilitates the measurement of H_2 on the outflow, as described in previous work (Wilson et al. 2010). The outlet of the experimental glass flask was directly connected to the analyzers using 1/16" PEEK tubing. The tubing incorporated a small moisture trap consisting of indicating Drierite® to minimize the quantity of water vapor reaching the analyzer. Finally a hydrophobic filter (Acrodisc; Pall Life Sciences) was installed at the entry of the analyzer as a preventive measure against accidental injections of liquid.

The argon induced hydrogen production (AIHP) method. To conduct the AIHP method, cultures were switched from ultra-high purity air to an argon gas mixture ($Ar:O_2:CO_2$) consisting of argon (80%), oxygen (20%), and carbon dioxide (0.04%; Air Liquide or Praxair). To ensure a high level of purity in the purge gas stream, all gases passed through a hopcalite scrubber (Peak Laboratories) maintained at 100°C to remove trace quantities of volatile gases including H_2. By always maintaining a constant flow rate (13 $mL \cdot min^{-1}$), the analytical setup mitigates the intrusion of N_2 when the sample is maintained under a headspace of $Ar:O_2:CO_2$ and avoids the accumulation of H_2, which might then be subject to leakage or contamination. The efficiency of sparging with $Ar:O_2:CO_2$ was evaluated by measuring the ratio of N_2 to Ar using membrane-inlet mass spectrometry (Kana et al. 1994).

Resulting concentrations of H_2 were quantified using a reduced gas detector (Peak Laboratories) that can measure sub-parts per billion levels of H_2 gas in bulk inert gases (Löffler and Sanford 2005). The reduced gas analyzer setup consisted of a mercuric oxide bed coupled to a reducing compound photometer. The reduction of heated mercuric oxide by H_2 gas liberated mercury vapor that was quantitatively detected using an ultraviolet absorption photometer located immediately downstream from the reaction site. The gas flow exiting the detector passed through an activated charcoal mercury scrubber before venting. Samples were injected onto the column via a fixed volume (1 mL) sample loop housed within a 2-position 10-port switching valve (Valco Instruments). The column had two packed 1/8" columns both of which were maintained at temperatures of 104°C. The first Unibeads 1S (60/80 mesh) column removed water moisture and the second Molecular Sieve 13X column separated the H_2 gas. The retention time for H_2 was 49 s with air as the carrier and 59 s with $Ar:O_2:CO_2$ as the carrier.

The H_2 analyzer was calibrated by dilution of a primary gas standard which had a H_2 concentration of 1.032 ppmv (Scott Marrin Inc.). Dilution is required because compressed H_2 gas standards are not available at values below 1 ppmv and the reduced gas analyzer will quantify H_2 several orders of magnitude below this value to 0.5 ppbv. The primary gas standard was diluted using ultra-clean air that passed through a hopcalite scrubber. By varying the relative flow rates of the H_2 standard and the ultra-clean air, while maintaining a total flow rate of 13 $mL \cdot min^{-1}$, the H_2 analyzer was calibrated under the same conditions as for the analysis of samples. The detector response was linear for the range of concentrations measured and the analytical precision based on the standard deviation of repeated injections of calibration gas standards was 2%–3%. Throughout the experimental period, the instrument drift was negligible (<0.1% difference in the calibration slope). The limit of quantification (LOQ) for the measurable concentration of H_2 was 0.03 pmol H_2, calculated as 10× the standard deviation of the intercept divided by the slope of the calibration curve. Blank controls consisted of culture media without cells and the working limit of quantification for rates of H_2 production, calculated as the blank signal plus

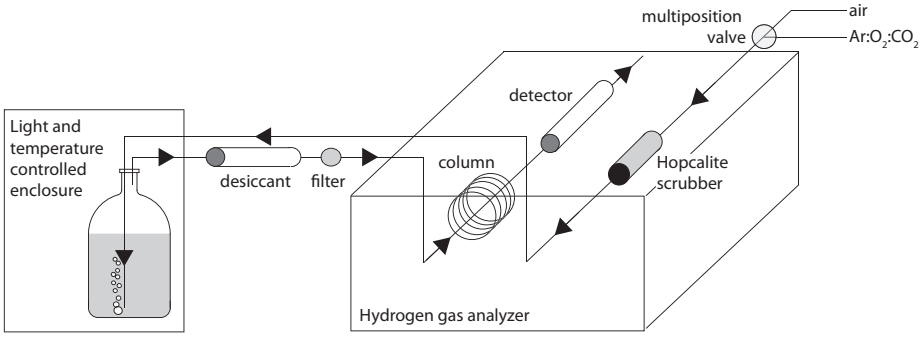

FIG. 2. Schematic of the gas flow-through system used in this study. The sparge gas was switched between air and $Ar:O_2:CO_2$ using the multi-position selector valve.

10× the standard deviation, ranged from 0.04 to 2.79 pmol $H_2 \cdot min^{-1}$. Measured concentrations of H_2 are presented as a H_2 production (fmol $H_2 \cdot cell^{-1} \cdot h^{-1}$).

The acetylene reduction (AR) assay. As part of this study, we also evaluated the strengths and weaknesses of the AIHP method against the strengths and weaknesses of the AR assay that is a frequently used method to estimate rates of N_2 fixation. Therefore, the two techniques were conducted simultaneously for the majority of our experiments. To generate the acetylene (C_2H_2), approximately 6 g of calcium carbide (CaC_2; Sigma) was added to 200 mL of deionized water in a 250 mL polycarbonate bottle. After displacing the ambient air headspace, C_2H_2 was transferred into a 3 L gas-tight PTFE bag (Welch Fluorocarbon) and subsequently sub-sampled using a 50 mL gas-tight syringe (SGE). During experimental measurements, a syringe actuator (Cole Parmer) was used to dispense C_2H_2 at a flow rate of 0.13 mL $\cdot$ min^{-1} into clean air with a total flow rate of 13 mL $\cdot$ min^{-1}, therefore achieving a 100-fold dilution. This 1% value is slightly lower than the original recommendation of 3%–10% (Hardy et al. 1968), but a clear signal was obtained at this concentration and a higher addition would have also increased the presence of contaminant gases (Hyman and Arp 1987).

The resulting ethylene (C_2H_4) was quantified using a reduced gas detector very similar to that described for H_2 gas. The differences for C_2H_4 were a smaller sample injection loop (0.1 mL volume), a higher oven temperature (145°C), and a longer Unibeads 1S 60/80 analytical column to aid in the separation of C_2H_4 and C_2H_2. The column was also backflushed after C_2H_4 had eluted to ensure that the large pulse of C_2H_2 did not reach the detector. The retention time for C_2H_4 was 41 s with air as the carrier. The C_2H_4 analyzer was calibrated by serial dilutions of a 10 ppmv ethylene standard in air (Scott Marrin), using the same technique as described for H_2. The analytical precision based on the standard deviation of repeated calibration samples was 2%–3%. The limit of quantification (LOQ) for the method was 0.047 pmol C_2H_4, calculated as 10× the standard deviation of the intercept divided by the slope of the calibration curve. Blank controls consisted of analyzing C_2H_4 concentrations in the C_2H_2 gas blended into the air for culture media without cells. A wide range of C_2H_4 concentrations was observed in the blanks, varying from 0.5 to >2 pmol $C_2H_4 \cdot mL^{-1}$ ($n = 13$). Only batches of C_2H_2 gas with a concentration of C_2H_4 less than 0.6 pmol $\cdot$ mL^{-1} were used for the AR assay.

^{15}N assimilation measurements. As part of the experiments, we wanted to ascertain the relationship between H_2 production when exposed to $Ar:O_2:CO_2$ and ^{15}N assimilation. A series of $^{15}N_2$ assimilation measurements were conducted at selected intervals throughout the day–night cycle. For the diazotrophs *Nostoc, Gloeotrichia, Anabaena*, and *Trichodesmium*, the ^{15}N assimilation measurements proceeded after measurements of H_2 with air and $Ar:O_2:CO_2$ as the purge gas. The same sub-sample of culture was used for all three measurements. The H_2 measurements lasted 30–40 min for each gas mixture and the $^{15}N_2$ measurements lasted 2 h. This was repeated 3–4 times throughout the day/night cycle for each microorganism. For *Crocosphaera*, measurements of $^{15}N_2$ assimilation were conducted in triplicate, simultaneous to the H_2 measurements. The diel measurements showed that N_2 fixation only occurred for a brief period lasting for 1–2 h and therefore, sequential measurements were not possible.

To conduct the rate measurements, a measured volume (5–7 mL) of $^{15}N_2$-enriched seawater was added to a measured volume (20–50 mL) of culture within a stoppered and crimp-sealed glass vial and incubated for period of 1.5-2 h. The incubation time and volumes of enriched seawater and culture were recorded for each experiment. Upon termination of the incubation, the entire contents of the vial were filtered onto a pre-combusted glass microfiber (Whatman 25 mm GF/F) filter and stored at −20°C. The filters were subsequently analyzed for total mass of N, and $\delta^{15}N$ composition using an elemental analyzer/isotope ratio mass spectrometer (Carlo-Erba EA NC2500 coupled with a ThermoFinnigan Delta Plus XP) at the Stable Isotope Facility, University of Hawaii. The ^{15}N-labeled N_2 gas was added in dissolved form using filtered seawater collected from the oligotrophic North Pacific Ocean. The initial atom % was calculated from the composition of nitrogen isotopes (N masses equivalent to 28, 29, and 30) in the $^{15}N_2$-enriched inoculum stock solution measured by membrane-inlet mass spectrometry (MIMS; Böttjer et al. 2017). It was recently highlighted that the N isotopes should be measured post-dilution rather than the stock solution (40× concentrated) to avoid overestimating the atom % enrichment (White et al. 2020). A single point calibration curve was determined for the MIMS using 0.2 μm filtered seawater collected from 22.75° N, 158° W in the oligotrophic North Pacific Subtropical Gyre and equilibrated with ambient air at 23°C.

RESULTS

A detailed examination of the AIHP method and the AR assay was conducted which included calibration procedures and control measurements. The reduced gas analyzers for both H_2 and C_2H_4 provided a linear response for the range of concentrations that were analyzed (Fig. 3). With regard to blank measurements, both assays had the capability of producing elevated quantities of H_2 or C_2H_4 when samples without cells, that is, only culture media, were analyzed (Fig. 3, B and D). For both C_2H_4 and H_2 measurements, the issues were relatively easy to diagnose and resolve. For H_2, the typical problem was elevated moisture in the setup which was resolved by drying the carrier lines and increasing the oven temperature to 195°C for several hours. For C_2H_4, a high blank value was due to contamination in the C_2H_2 and a 4-fold variation was observed across 13 test batches (Fig. 3B). The variable C_2H_4 content necessitated every batch to be tested for its C_2H_4 concentrations and, on some days, 3–4 batches of C_2H_2 were made before one was deemed acceptable for use by having a C_2H_4 concentration lower than 0.6 pmol $C_2H_4 \cdot mL^{-1}$ (Fig. 3B).

Another observation about the AIHP method related to switching the purge gas from air to $Ar:O_2:CO_2$. When a low volume of sample material was analyzed, switching from air to $Ar:O_2:CO_2$ could cause H_2 production to increase from below the LOQ to a measurable value. For example, this was observed with the heterocystous cyanobacteria (*Anabaena* and *Nostoc*) during the night period. This situation was avoided in future experiments by increasing the sample volume thereby increasing the H_2 yield (Table 2).

When air was used as the purge gas, H_2 production by diazotrophic cyanobacteria was always observed when a sample with sufficient biomass was

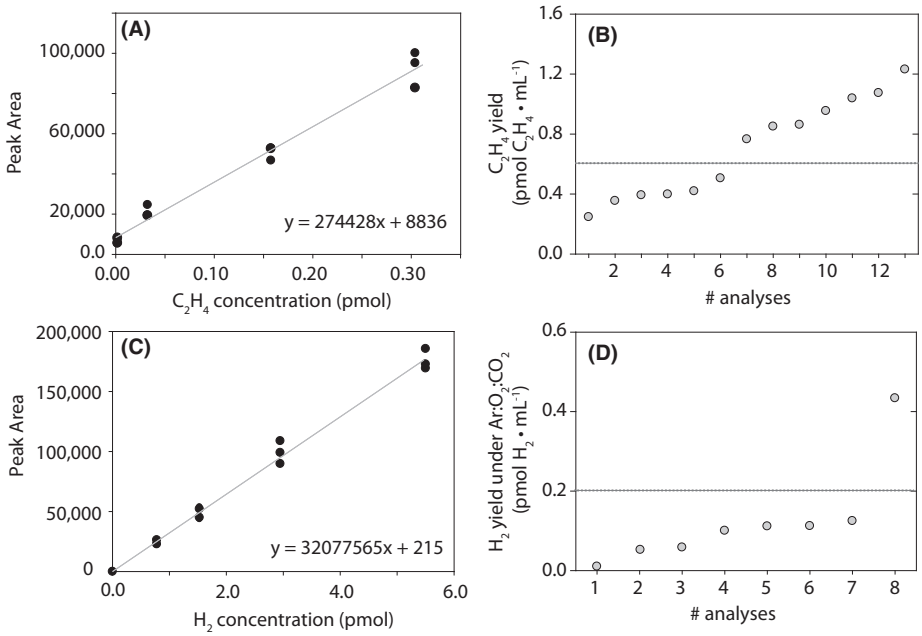

FIG. 3. Calibration curves and blank measurements of C_2H_4 (A) and (B) and H_2 (C) and (D). The dashed lines in (B) and (D) indicate our threshold values for determining acceptable blank values for C_2H_4 and H_2, respectively.

analyzed at the appropriate time of day (i.e., when nitrogenase was active; Table 2). The quantity of H_2 produced under air varied between microorganisms with maximum production occurring in *Trichodesmium* and *Anabaena* (8 fmol $H_2 \cdot cell^{-1} \cdot h^{-1}$). Previous studies identified *Crocosphaera* as having extremely low rates of H_2 production under air (Wilson et al. 2010) and these measurements were not repeated during this study. An increase in H_2 production was always observed upon exposure to $Ar:O_2:CO_2$ (Table 2). With the exception of *Gloeotrichia*, there was a consistent 15-fold increase when switching from air to $Ar:O_2:CO_2$ (Fig. 4). For *Trichodesmium*, *Nostoc*, and *Anabaena*, H_2 production increased by a factor of 15 ± 3 with the total range in values extending from 8–20. In comparison, $Ar:O_2:CO_2$ had less of an effect on H_2 production from *Gloeotrichia* with a 4-fold increase observed and the total range extending from 2.7–4.7.

We initially questioned whether the total replacement of air with $Ar:O_2:CO_2$ would disrupt N_2 fixation. The complete removal of N_2 contrasts sharply with the AR assay where C_2H_2 was added at 1% vol/vol. However, the AIHP method successfully recorded a complete diel pattern of nitrogenase activity for each microorganism. On one occasion the AIHP method was extended beyond a 24 h period and no H_2 production was observed when the second period of nitrogenase activity should have begun. The diel patterns of H_2 production with $Ar:O_2:CO_2$ as the purge gas largely resemble those achieved with the AR assay (Fig. 5). Some differences were observed between the results from the two techniques such as the ~1 h offset in the maximal rates observed in *Crocosphaera* (Fig. 5a) and the rate of increase during the daytime as shown by *Nostoc* (Fig. 5c). To verify the robustness of the H_2 signal, the purge gas was switched from $Ar:O_2:CO_2$ to air, and then back to $Ar:O_2:CO_2$ again (Fig. 5c). It was observed that H_2 production resumed at the same rate, indicating that the diel pattern was not influenced by the removal of N_2. Overall, the diel patterns observed in Figure 5 support our overall understanding of cellular physiology for these particular microorganisms. For example, *Crocosphaera* had maximal rates during the dark period (Fig. 5A) and *Trichodesmium* had maximal rates during the light period (Fig 5B). The two other microorganisms, *Nostoc* and *Gloeotrichia*, had high rates of nitrogenase activity during the light period and low rates during the dark period (Fig. 5, C and D). There is no C_2H_4 production data for the *Gloeotrichia* diel experiments due to excessively large blank values for those measurements. Since N_2 fixation by cyanobacteria is dependent either directly or indirectly on light-derived energy, varying the light intensity is a straightforward test of whether N_2 fixation is actually being measured. Switching off the lights during the light period decreased, but did not completely eliminate, H_2 production in *Nostoc*. In contrast, H_2 production during the daytime was completely eliminated in *Trichodesmium* when the lights were switched off (Fig 6a).

The diel patterns of H_2 production indicate that exposure to $Ar:O_2:CO_2$ did not negatively impact N_2 fixation for timescales less than 24 h. However, it is

TABLE 2. H_2 production by diazotrophic cyanobacteria when exposed to air or Ar:O_2:CO_2 reported in units of fmol $H_2 \cdot cell^{-1} \cdot h^{-1}$.

Diazotroph culture	Time of day	H_2 prod. air	H_2 prod. Ar: O_2:CO_2	$^{15}N_2$ assim.
[1]*Trichodesmium IMS101*	12:15	6.6	108.9	34.8
[1]*Trichodesmium IMS101*	14:00	6.0	118.5	48.8
[2]*Trichodesmium IMS101*	11:00	4.6	72.6	19.8
[2]*Trichodesmium IMS101*	12:00	5.4	95.3	20.8
[2]*Trichodesmium IMS101*	13:00	8.2	113.7	13.6
[3]*Trichodesmium IMS101*	11:30	3.8	31.5	27.7
[3]*Trichodesmium IMS101*	12:45	3.1	36.4	36.5
[3]*Trichodesmium IMS101*	14:00	3.8	44.6	32.5
Nostoc	13:30	5.8	88.1	17.6
Nostoc	17:30	7.7	116.4	23.8
Nostoc	20:15	3.2	42.5	11.6
Anabaena	11:00	1.8	30.5	29.7
Anabaena	14:30	2.6	48.3	18.1
Anabaena	15:30	0. 9	15.6	7.9
Anabaena	21:30	0.4	4.2	11.1
Anabaena	22:30	0.2	3.1	n/d
Gloeotrichia	11:10	5.7	26.5	25.0
Gloeotrichia	12:15	3.1	13.4	16.8
Gloeotrichia	13:15	2.6	7.8	14.6
Gloeotrichia	14:15	7.7	20.7	26.3
Gloeotrichia	15:30	1.5	5.9	16.1
Gloeotrichia	20:45	n/d	1.5	6.4
Gloeotrichia	21:45	0.1	1.8	4.5
Crocosphaera WH8501	01:00	n/m	5.9	1.6
Crocosphaera WH0401	01:00	n/m	4.2	0.5

The $^{15}N_2$ assimilation values were achieved as static incubations and are also reported in units of fmol $H_2 \cdot cell^{-1} \cdot h^{-1}$. The superscript text for *Trichodesmium* indicates 3 different sampling dates. n/m = not measured and n/d = not detected. The light: dark cycle extended from 7 a.m. to 7 p.m.

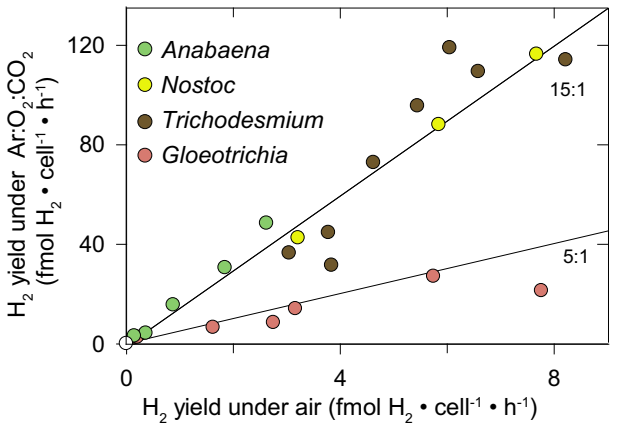

FIG. 4. Comparison of H_2 production by the diazotrophic cyanobacteria under air versus Ar:O_2:CO_2. The solid lines highlight a relationship of 5:1 and 15:1 for H_2 production.

also possible that the reverse could occur and the argon gas mixture stimulated a positive impact. For example, an increase in nitrogenase activity might result from the cell not receiving any N_2. To test this, we switched the flow of purge gas between air and Ar:O_2:CO_2 repeatedly to ensure that the measurements were repeatable (Fig. 6B). If N_2 starvation causes an increase in H_2 production under Ar:O_2:CO_2, then varying the length of time that a cell is without N_2 should cause differences in H_2 production. As shown by our experiments, we did not observe any effect and the diel patterns of H_2 production were reproducible (Fig. 6B).

In addition to the AIHP method and AR assay, measurements were also conducted of cell-specific $^{15}N_2$ assimilation in order to compare with H_2 production under Ar:O_2:CO_2. The rates of $^{15}N_2$ assimilation varied between microorganisms with the highest rates observed by *Trichodesmium* (49 fmol $N \cdot cell^{-1} \cdot h^{-1}$), followed by *Anabaena* (30 fmol $N \cdot cell^{-1} \cdot h^{-1}$) and *Nostoc* (24 fmol $N \cdot cell^{-1} \cdot h^{-1}$) during the light period (Table 2). In comparison, the lowest rates were obtained for *Crocosphaera* (1 fmol $N \cdot cell^{-1} \cdot h^{-1}$). The day and night patterns of $15N_2$ assimilation recorded in the cynaobacteria cultures broadly corresponded to diel patterns of H_2 production under Ar:O_2:CO_2. For example, $^{15}N_2$ assimilation rates were 2–3 times lower during the night time than the daytime for the heterocystous cyanobacteria. Compiling all the measurements of $^{15}N_2$ assimilation and H_2 production under Ar:O_2:CO_2 reveals an overall average mol H_2: mol N ratio of 3.2 (n = 24) with a range from 0.2 to 13 (Fig. 7).

DISCUSSION

The ubiquitous production of H_2 by diazotrophic cyanobacteria is consistent with the scientific understanding that H_2 production is an inherent component of N_2 fixation (Schubert and Evans 1976, Phillips, 1980, Wilson et al. 2010). The replacement of air with Ar:O_2:CO_2 using a flow-through experimental system demonstrated the AIHP method successfully achieves real-time measurements of N_2 fixation. There have been previous reports that the addition of either Ar:O_2:CO_2 or acetylene causes a decline in nitrogenase activity after approximately 1 h (Hardy et al. 1968, Hunt and Layzell 1993, Vessey 1994). This has been attributed to the adverse effects on nitrogenase and, consequently, it has been recommended to temporally restrict measurements to within 1 h after the initiation of the experiment. The diel profiles obtained in this study (Fig. 5) did not reveal a short-term decline in nitrogenase activity with either the addition of Ar:O_2:CO_2 or C_2H_2. The reason for the difference between the previous observations and this study is not known. It is likely that this study benefited from

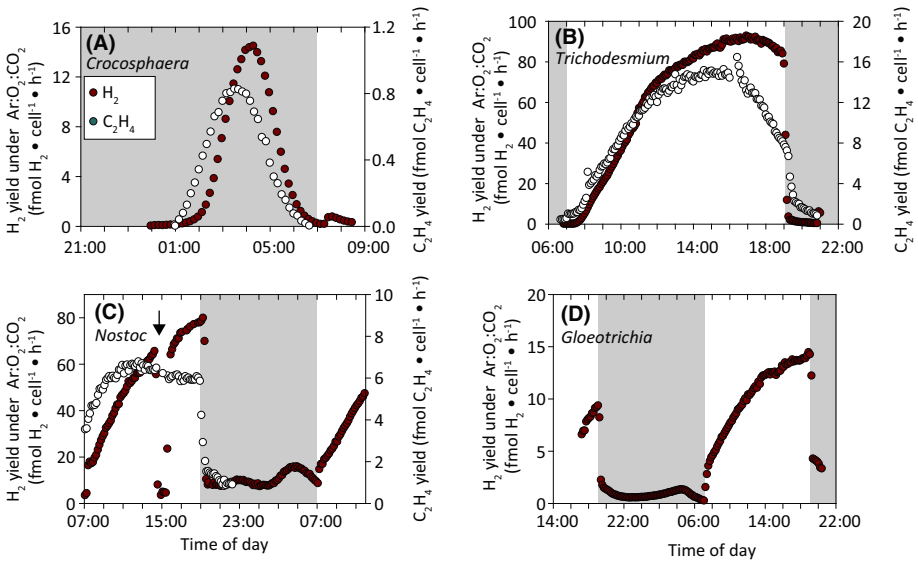

FIG. 5. Diel periodicity in N_2 fixation for four diazotrophic cyanobacteria: *Crocosphaera* (A) *Trichodesmium* (B), *Nostoc* (C), and *Gloeotrichia* (D). The H_2 and C_2H_4 measurements were conducted simultaneously. The measurements were made autonomously and samples were exposed to either Ar:O_2:CO_2 or air with 1% acetylene. The acetylene reduction assay was unsuccessful for the *Gloeotrichia* culture (see text for details). The arrow in (C) indicates when the purge gas was switched from Ar:O_2:CO_2 to air for a 15 min period to verify that an absence of N_2 was not influencing the observed measurements. The gray shaded background represents dark periods (lights off) from 7 p.m. to 7 a.m. in all experiments.

the analytical setup which employed a gaseous flow-through system. This is considered preferential to the incubation of discrete batch samples (Layzell et al. 1984, Hunt and Layzell 1993). The gaseous flow-through system also prevents an accumulation of H_2 which would presumably be more susceptible to oxidation via uptake hydrogenase. All nitrogenase-containing microorganisms contain uptake hydrogenase to mitigate the loss of chemical energy (Bothe et al. 1980, Tamagnini et al. 2002). Reassimilation of H_2 produced under a headspace of Ar:O_2:CO_2 would ultimately cause TNA to be underestimated. However, the robust diel signals (Fig. 5) and the consistent response to experimental manipulation (Fig. 6) do not appear to support any substantial increase in H_2 metabolism when cells are exposed to Ar:O_2:CO_2.

The maintenance of diel patterns in N_2 fixation despite the absence of N_2 reflects the strong transcriptional regulation of the nitrogenase enzyme (Dixon and Kahn 2004). Nitrogenase is also regulated on the metabolic level in addition to transcriptional control. For example, switching off the light energy supply during the photoperiod will cause nitrogenase to either partially (e.g., *Anabaena*) or completely (e.g., *Trichodesmium*) switch off. The addition of dissolved inorganic nitrogen (ammonium or nitrate) also deactivates N_2 fixation in *Trichodesmium* (e.g., Capone et al. 1990, Wilson et al. 2010). The most common post-translation control mechanism of nitrogenase is ADP-ribosylation of the Fe protein (Zumft and Castillo 1978, Nordlund 2000, Huergo et al. 2012). It is not known whether this ADP-ribosylation mechanism would also cause the deactivation of nitrogenase at the onset of the second diel period in the presence of Ar:O_2:CO_2. Since ADP-ribosylation is a reversible process and N_2 fixation was reactivated upon switching the gas flow to an air supply for a 1 h period, it is not an unreasonable explanation, but requires further testing.

The diel patterns of H_2 production differed in some instances from the AR assay. The most prominent differences were the 1 h difference in maximum rates of N_2 fixation for *Crocosphaera* and the daytime increase of H_2 production that were most evident in *Nostoc* (Fig. 5C). The continual increase of H_2 during the daytime is particularly noteworthy as it occurred in all daytime N_2 fixers and was not altered by the temporary provision of air (Fig. 5c). Further insight would be obtained by experimentally depleting the cells of light energy and subsequently measuring H_2 production under full light. An alternative experimental approach would be to limit N supply for 1–2 d and then provide N_2. It is anticipated that when cells are maintained under energy- or nutrient-limited conditions and then are exposed to high light or the provision of N_2, nitrogenase activity is maximized causing a more rapid increase in H_2 production. Ultimately, the diel patterns and the accompanying tests of its consistency validate the AIHP method as an insightful tool for assessing nitrogenase activity and its physiological controls. The advantages to using Ar:O_2:CO_2 compared to C_2H_2 is that the gas mixture is available as a high purity compressed gas. In contrast, C_2H_2

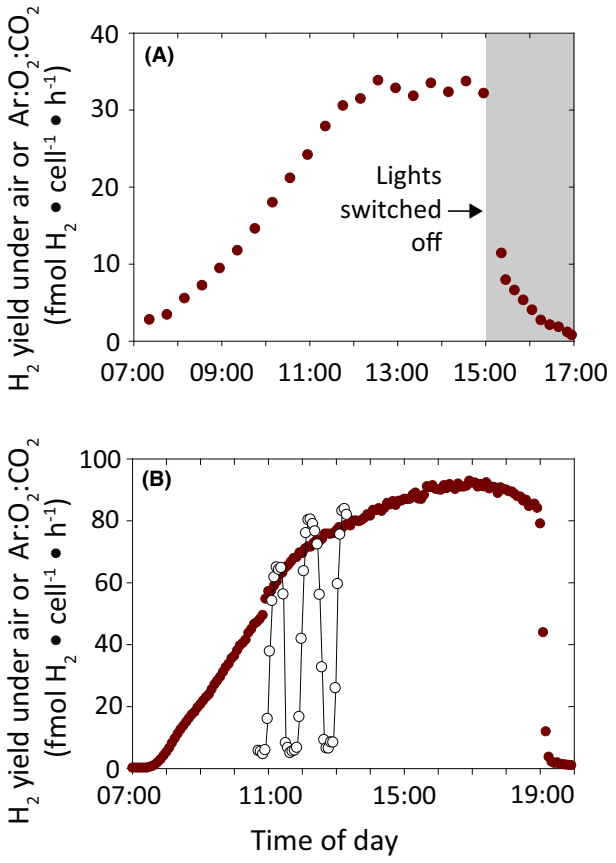

FIG. 6. H_2 production by *Trichodesmium* when purged with Ar:O_2:CO_2 and subject to manipulations (A) Lights switched off as indicated by the gray shading (B) Switching between Ar:O_2:CO_2 and air. The open symbols are the oscillation in H_2 production when the purge gas is switched from air to Ar:O_2:CO_2 (solid black arrows) and from Ar:O_2:CO_2 to air (dashed black arrows). The solid symbols represent the diel pattern of N_2 fixation obtained under a continuous headspace of Ar:O_2:CO_2 and is shown for comparison.

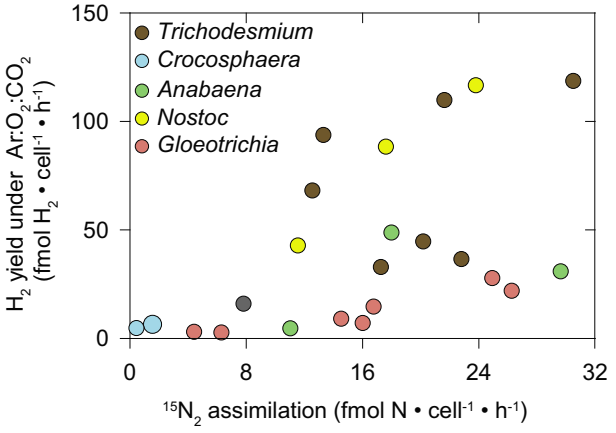

FIG. 7. Comparison of $^{15}N_2$ assimilation with H_2 production under Ar:O_2:CO_2 for the five cultures of cyanobacteria analyzed in this study.

needs to be produced for each experiment and tested for C_2H_4 contamination (Fig. 3). We do recognize, however, that using C_2H_2 generated from CaC_2 avoids the need for unwieldy compressed gas cylinders in the laboratory. Another advantage of using Ar:O_2:CO_2 is that it removes all N_2, whereas the most common application of the AR assay involves adding the acetylene at 10% vol/vol, which could contribute to analytical variability.

In addition to measuring N_2 fixation, there is a second role for the AIHP method which does not exist for the AR assay. As previously discussed, H_2 production under Ar:O_2:CO_2 represents a measurement of TNA while H_2 under air reflects Apparent Nitrogenase Activity (ANA; Fig. 1). Comparison of these two processes permits the calculation of the relative efficiency of nitrogenase, known as the electron allocation coefficient and calculated as 1-[ANA/TNA] (Schubert and Evans 1976). In our experiments, switching the purge gas from air to Ar:O_2:CO_2 caused H_2 production to increase 4-fold in *Gloeotrichia* and 15-fold for the other cyanobacteria analyzed in this study. The electron allocation coefficients are 0.72 for *Gloeotrichia* and 0.93 for the other cyanobacteria. To the best of our knowledge, the most comprehensive comparisons of H_2 production under air and argon were conducted on twenty-two individual legumes with an overall electron allocation efficient of 0.55 reported after removing two outlier values (Schubert and Evans 1976). This electron allocation coefficient for legumes was verified by a more recent study which reported a value of 0.59 (Fischinger and Schulze 2010). Therefore, the N_2 fixing cyanobacteria analyzed in this study operate the nitrogenase enzyme system at a greater efficiency with respect to H_2 than legume root nodules. This might not be surprising given the difference between the subterranean habitat of a legume and the aerated environment of an aquatic photoautotroph. H_2 oxidation will consume O_2 and therefore, provide some respiratory protection for the O_2-sensitive nitrogenase (Bothe et al. 2010). Such considerations of cellular energy required to sustain N_2 fixation led to the hypothesis that microorganisms (e.g., *Crocosphaera*) which rely on respiratory-derived energy for nitrogenase, emit less H_2 than microorganisms (e.g., *Trichodesmium*; Wilson et al. 2012) which supply nitrogenase with light-derived energy.

The degree to which real-time techniques (the AIHP method and the AR assay) provide an accurate measure of N_2 fixation is of high scientific importance and there is a substantial number of publications about the use and relevance of a 3:1 or 4:1 ratio with regards to converting the AR assay to N_2 fixation (e.g., Witty and Minchin 1988, Wilson et al. 2012). This study followed in the path of many previous studies by basing an evaluation of the accuracy of the AIHP method on a comparison with $^{15}N_2$ assimilation (e.g., Mague et al. 1977, Vessey

1994, Moisander et al. 1996, Mulholland et al. 2004, Saiz et al. 2019). The comparison revealed an overall average ratio of 2.6 for TNA:$^{15}N_2$ assimilation, in contrast to the theoretical ratio of 4 (Table 2), with considerable variability, both within and between species. This study indicates the AIHP method does not more accurately reflect the true rate of N_2 fixation than the AR assay. Therefore, consistent with the AR assay, the AIHP method is best employed in comparative studies and to elucidate temporal and spatial patterns in N_2 fixation that are verified by additional observations. Multiple independent measurements of N_2 fixation are advocated for when a high level of confidence is required, such as growth rates derived from an increase in cell abundance or biomass when laboratory cultures are grown in N-free media (e.g., Mulholland et al. 2004, Mohr et al. 2010). This interpretation of the H_2 data and recommendations for use of the AIHP method is appropriately cautious and it is possible that the AIHP method is more accurate than what is apparent from a comparison with $^{15}N_2$ assimilation as these methods have very different experimental procedures and analyses.

Another caution about inferring rates of N_2 fixation directly from the AIHP method without knowledge of the sample type or verification using rates of $^{15}N_2$ assimilation is that the quantity of H_2 produced during N_2 fixation varies between the nitrogenase isozymes. For each molecule of N_2 reduced to NH_3, the Mo nitrogenase produces 1 molecule of H_2, the V nitrogenase produces 3 molecules of H_2, and the Fe-only nitrogenase produces 7 molecules of H_2 (Bothe et al. 2010, Harris et al. 2019). The Mo nitrogenase is the most widespread nitrogenase with the two alternative nitrogenases typically used during periods of Mo limitation (Harwood, 2020). In the future, the AIHP method could be used to quantify the activation of alternative nitrogenases in culture-based studies (Oda et al. 2005) and also help identify instances of active V and Fe-only nitrogenases in environmental settings (Bellenger et al. 2020).

CONCLUSIONS

Our evaluations demonstrate the AIHP method is a valuable tool to determine rates of N_2 fixation. The technique was demonstrated on laboratory-maintained cultures of aquatic diazotrophic cyanobacteria and in the near future, the AIHP method will be tested in the marine environment. The comparison of H_2 production under air versus $Ar:O_2:CO_2$ provides a relative measure of the efficiency of the nitrogenase system with minimal perturbation to the cells. Such analyses can be conducted alongside photosynthetic and respiration measurements to establish how diazotrophic cyanobacteria meet the energetic costs of operating nitrogenase in an oxygenated environment. Ultimately, we predict that greater use of the AIHP method will lead to increased understanding of diazotroph cell physiology and environmental controls.

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AUTHOR CONTRIBUTIONS

S. Wilson: Methodology (equal); project administration (equal); writing-original draft (equal). **M. Caffin**: Methodology (equal); writing–original draft (equal). **A. White**: Methodology (equal); writing–-original draft (equal). **D. Karl**: Methodology (equal); writing–original draft (equal).

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