**Summary of N2 Fixation Town Hall at the 2018 Ocean Sciences Meeting in Portland, OR**

***Conveners:*** Angelicque White (Oregon State Univ.), Pia Moisander (Univ. Massachusetts, Dartmouth), Julie Granger (Univ. Connecticut)

***Objectives:*** The goal of the town hall meeting was to survey the community regarding perceived limitations and obstacles associated with current means of **(1)** estimating rates of N2 fixation with 15N2 tracer incubations (Montoya et al. 1996), and **(2)** uncovering the identity, abundance and activity of N2 fixing micro-organisms (diazotrophs) with current molecular techniques, especially quantitative PCR.

***List of attendees:*** A formal head count was not taken, but we estimated over 100 participants n the town hall. Of these participants, in addition to the working group, there were 33 town hall participants that requested to be included in future correspondence and interactions within the OCB N2 fixation working group. The emails for those participants are noted at the end of this document.

***Process:*** The town hall began with opening remarks (A), followed by two introductory talks (B). Attendees then self-selected into groups to discuss either the 15N2 method or abundance determination, and a questionnaire meant to serve as a basis for discussion was distributed. For the remainder of the town hall, groups reported back highlights of their discussions (C-D).

1. ***Opening remarks (Angelicque White) are paraphrased below:***

The input of the town hall participants as well as that of parties who completed the online or hard copy version of the N2 fixation survey ([link](https://goo.gl/forms/xpVmmeds7pgq8DQH2)) is summarized below.

Based on community consensus, a working group of invited and self-appointed members (N2 fixation working group: <https://www.us-ocb.org/n-fixation-working-group/>) will explore available techniques and data to develop a “living document” summarizing data-informed recommendations for “best practices” in N2 fixation studies. The document will put forth recommendations regarding best practices in **(1)** N2 fixation rates estimates with 15N2 tracer incubations and **(2)** evaluation of diazotroph identity, abundance and activity.

The “living document” is envisioned to enable iterative inputs from the community to publicize new insights pertinent to improvement of existing methodology and data analysis.

The working group will convene in Spring 2018 to generate an outline of the first iteration of the living document tentatively titled *Best Practices in Marine N2 Fixation Studies*.

1. **Brief presentations summarizing current practices and inherent limitations**
* *The Rate of Production: N2-Fixation Measurements.* Angelicque White (Oregon State Univ). ([link to slides](https://www.us-ocb.org/wp-content/uploads/sites/43/2018/05/TOWN_HALL_AWhite.pdf))
* *Advances in Understanding of the Activity and Diversity of Nitrogen-Fixing Organisms in the Marine Environment: Who Dunnit (and how fast are they doing it)*. Jonathan P. Zehr (UC Santa Cruz). (link to slides)
1. **N2 fixation rates from 15N2 tracer incubations: Methods and limitations**

Select participants discussed, among other topics, the following issues, concerns and areas for improvement. There are currently three distinct methods used by researchers to perform 15N2 tracer incubations:

1. *The traditional bubble method*: An aliquot of 15N2 gas is added to the bottles that are incubated directly (Montoya et al. 1996).
2. *The bubble release method*: An aliquot of 15N2 gas is added to incubation bottles, which are mixed for a time, after which the gas bubble is released to the atmosphere and replaced with water. The 15N2 enrichment in individual bottles in quantitated from subsamples taken at the final sampling (Klawoon et al. 2015).
3. *The enriched seawater method(s)*: 15N2 gas is added to degassed and/or filtered seawater, which is agitated to facilitate dissolution/equilibration. The 15N2 in the enrichments is measured, and an aliquot of 15N2-enriched water is then added to the incubation bottles (*e.g.*, Wilson et al. 2010, Klawoon et al. 2015).

Each of the above techniques has salient limitations and obstacles that contribute uncertainty in rate estimates. The N2 fixation community raised the following advantages and limitations singular to respective methods:

1. With respect to the *traditional bubble method*, dissolution of 15N2 gas is time-dependent and ultimately incomplete (Mohr et al. 2010), such that the 15N2 enrichment of the incubations is uncertain, leading to potentially erroneous rates. Some workers measure the final 15N2 enrichment, and interpolate to either a measured or initial 15N2 concentration in the incubations, in order to derive rate estimates (as discussed by select participants).
2. The *bubble release* method benefits from having a constant and quantitated 15N2 concentration throughout the incubation, in contrast to the traditional bubble method. However, the method involves the initial shaking of the incubation bottles, which has the potential to aggregate/disaggregate particles, and to disturb cells, thus potentially influencing measured rates.
3. The *enriched seawater method* also benefits from a constant and quantitated 15N2 concentration, and does not involve shaking the incubation bottles. However, the preparation of 15N2-enriched seawater has the potential to introduce contaminants *(e.g.,* trace metals, ammonium) to the incubations, and to modify O2 and DIC concentrations.

The community also perceives additional limitations and uncertainties associated with some or all of the above methods: (a) The limits of detection and mass spectrometric linearity of 15N PON measurements are either not reported or not considered; (b) The mass of PON has a disproportionate influence on rate measurements, but this quantity is highly variable among replicates (*see* Montoya et al. 1996); (c) The collection and storage of 15N2 gas samples has not been tested or codified; (d) Membrane Inlet Mass Spectrometry (MIMS) calibration to a single air standard – in order to estimate 15N2 gas enrichments – has not be verified; (e) The collection of initial and final15N PON controls is not standard, although there is mounting evidence that the natural abundance 15N of PON can change drastically during incubations; (f) The time of initiation and the duration of incubations varies among workers; (g) Testing the 15N ammonium and nitrate contamination of 15N2 gas stocks *(see* Dabudo et al. 2014) is impractical and can be costly; (h) The analytical errors associated with inherent analyses are not well defined, rendering accurate propagation of experimental error difficult (as per Montoya et al. 1996); (i) 15N2 bubble release can prove costly.

Moving forward, town hall participants concurred that collation of pertinent information, supporting data, and recommendations in a “best practices” document could help alleviate some of these obstacles in relatively short order (*e.g.,* reporting detected 15N ammonium contamination for specific lots of 15N2 gas in the “living document,” recommendation to report initial and final controls of 15N PON in publications, recommendation on a standardized length of field incubations). However, other obstacles will require additional laboratory or field tests (*e.g.,* standardization of MIMS measurements, test of 15N2 sample collection and preservation protocols) and/or reaching consensus on the interpretation of some types analytical data (*e.g.*, identifying the lower limit of quantitation of 15N PON on various Elemental Analyzer-Isotope Ratio Mass Spectrometers). Importantly, the “living document” will iterate on previous efforts to standardize 15N2 measurements during a workshop in Kiel (2012; [link to document](https://www.us-ocb.org/wp-content/uploads/sites/43/2018/03/Kiel_Workshop_Report.pdf)), and from tests of different methods of introducing 15N2 investigated by Mahaffey, Rees and Kitidis (2013; [link to document](https://www.us-ocb.org/wp-content/uploads/sites/43/2018/03/15N2_Plymouth_Feb2013-Mahaffey-Rees-Kitidis.pdf)), and from published studies (*e.g.*, Klawoon et al. 2015, Bombar et al. 2018).

Other methods and tracers to estimate N2 fixation rates that are complementary to 15N2-incubation rate estimates include: Underway H2 gas measurements (Moore et al. 2009; Wilson et al. 2013), underway acetylene gas reduction measurements (Cassar et al. 2018), natural abundance 15N mass balances from 15NO3- and sediment trap 15N (Knapp et al. 2005). These will not be discussed specifically in this forum.

**Molecular tools to interrogate N2-fixing communities: Methods and limitations**

In the most commonly used approaches, unraveling the identity, abundance and activity of N2-fixing microorganisms by DNA and RNA amplification involves the following techniques:

1. Detection of the presence of *nifH* genes based on degenerate and/or targeted PCR primers
2. Estimates of *nifH* gene copy abundances of individual diazotroph groups by quantitative PCR
3. Evaluation of activity from detection of reverse transcribed *nifH* mRNA by qPCR using primers targeting individual diazotroph groups

The group discussed several sources of uncertainty in the qPCR methods and our ability to compare data across studies, as well as predict organism abundances from the qPCR data. The issues below were discussed during the town hall and during the conference week.

1. *DNA/RNA extraction efficiency and losses during extraction:* This issue elicited the most discussion in the town hall group. Participants agreed that there are losses of DNA (and RNA) during extraction, and such losses are variable across methods used. There is also concern about DNA extraction efficiency changing across variable sample matrixes (e.g., from coastal to oceanic waters). Participants suggested that the best way forward would be to recommend the use of typical silica column-based nucleic acid extraction protocols that are known to be efficient in removing DNA inhibitors. The caveat is that this approach also results in the greatest DNA losses. In oceanographic studies, when sampling low biomass surface waters, less stringent methods could be sufficient in many waters and would result in higher DNA yields (lower DNA losses), yet in the case of patchy blooms and when processing materials from the Deep Chlorophyll Maximum, potential PCR inhibition is likely an issue. Overall, silica column-based purification is currently the best option to ensure sample comparability within and across studies.

2. *Generation and running standards and reporting detection limits:* There is a high level of uncertainty when it comes to equivalency of gene copies to cell numbers. Notably, some work has been done with *nifH* as well as other genes (e.g., *Prochlorococcus* research) studying the cell vs. gene copy equivalency question, and these published and unpublished data could help assess the question as to whether it might be possible to correct measured gene copies to represent actual gene copies. For many *nifH* targets, these studies are not possible because there are no cultivated representatives, and such relationships (e.g., cell to gene copy) may be organism-specific. We are aware of some researchers who have collected these types of data and intend to contact them personally (some have already agreed to share data), and we'll continue to look for any other studies that have tested this question with oceanographic samples. Additional testing may be warranted with unicellular diazotrophs that can be cultivated and flow sorted.

There are different methods among researchers as to how the gene copy abundances are standardized in qPCR. Standards can be synthetic fragments of DNA, cloned inserts in plasmids, or synthetic plasmids with specific insert sequence. Plasmid standards may or may not be linearized. Although most agree that different standard types may yield variability in the standards produced, some argue that it does not matter what standard type is used, since numbers will be relative anyway. Individual studies could thus have flexibility to use different qPCR standardization methods, still allowing good within-study relative comparisons across samples. However, if the community were to accept a more universal approach to standards, the cross-study comparisons should improve. It should also be pertinent to recommend reporting the level of quantification and detection for qPCR assays with any published data. Providing best practices for reporting detection limits and levels of quantification would be optimal. Most researchers do not seem to report these, yet understanding what values represent background vs. actual gene copies would be critical when drawing conclusions from these ecological studies to ecosystem-level functions and budgets.

3. *Standard data interpretation practices:* Participants who are familiar with qPCR approaches agreed that these approaches provide relative abundances at best, and there are a number of other caveats that are often ignored. The recommended practices should provide guidance on data interpretation.

4. *Accessibility of primer and probe information and target sequences:* Participants agreed that development of a database for efficient distribution of primer-probe information would be welcome in the field. Such a database/library of primers and probes would be envisioned to function similarly to what is currently available for 16S rRNA-based Fluorescent In Situ Hybridization probes (probebase.net). This online resource would list all known *nifH* primers and probes that have been tested, along with a link to the sequence and the original accession number. Additional information archived should include reported amplification efficiencies, a link to the original reference, and potential availability of the plasmid standard in an external plasmid repository. Any future studies using a particular primer probe set could be linked to the library.

*5. Other considerations:* It was also pointed out that reagent lot numbers should be reported for key steps in the protocols. The workers should also consider the potential influence of diel cycles in DNA and RNA data, as well as lab-to-lab and person-to-person variability in handling DNA and RNA and conducting the assays. An inter-lab comparison exercise in overall qPCR protocols might elucidate the extent to which such differences translate to reported gene copy numbers across studies.

 Other methods under development include digital PCR, and potential new applications of amplicon sequencing. The latter could potentially be used as a semi-quantitative method with spiked internal standards. Neither of these two approaches is common as of yet in quantification of diazotroph targets, although amplicon sequencing is becoming a common tool in characterizing the overall *nifH* community composition.

**References**

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***Feedback and Next Steps:*** In addition to summarizing the input gained from prior teleconferences and discussions at the Ocean Sciences town hall, we have compiled results from the distributed questionnaire ([link](https://docs.google.com/forms/d/1yvoqSpjZrLDgJeuqJqpCYTVN1g4yHMSVt1uOeeD3t4o/edit?usp=sharing), n=16 responses). All of this community input will be used to form the basis for a follow-up meeting of the OCB N2 fixation working group scheduled to be held in Boston, MA on May 31-June 1, 2018.