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Methodological and analytical improvement of the ISotopic Acetylene Reduction Assay for the assessment of complementary biological nitrogen fixation in low activity samples

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Understanding the enzymes responsible for biological nitrogen fixation in the natural environment is crucial for understanding the global nitrogen cycle. The isotopic acetylene reduction assay (ISARA) is currently one of the only ways to distinguish between nitrogenase enzymes and it involves measuring the $\delta^{13}C$ of ethylene generated via the reduction of acetylene. However, the classical method can only be applied to samples with ethylene concentrations >1,000 ppm which is limiting for environmental samples, where N_2 fixation activity is generally low resulting in a low headspace ethylene concentration (<300 ppm).

Here we describe an improved analytical method for analyzing $\delta^{13}C$ of ethylene using a homemade gas pre-concentration system and reproducible in-house standards developed from commercially available ethylene tanks. We also present a simple methodology using mutants of *Azotobacter vinelandii* (Mo-only and V-only nitrogenase) and the removal of headspace acetylene by chemical precipitation to easily scale the ISARA experiment from $\delta^{13}C$ to complementary nitrogenase contribution without the uncertainty and tediousness surrounding measurement of the source acetylene.

The new Low activity - ISARA (LISARA) method can now estimate contribution of complementary nitrogenase from environmental samples with as little as 10 ppm of ethylene. Updated limit of quantification for $\delta^{13}C$ of ethylene is < 2 ppm. Finally, we demonstrate the applicability of the method using samples with characteristically low N_2 fixation activity (termites, wood, leaf litter, soil, moss), with substantial contribution of complementary nitrogenase across multiple sites in the northeastern United States.

Our results expand our knowledge of the contribution of complementary nitrogenase to temperate ecosystems. The new methodology will allow broader access to the classical ISARA method for pure culture experiments and high activity samples through the outsourcing of $\delta^{13}C$ ethylene measurements, facilitating the study of complementary nitrogenases.