Oecologia (2012) 168:335–342
DOI 10.1007/s00442-011-2105-y

PHYSIOLOGICAL ECOLOGY - ORIGINAL PAPER

N_2 fixation estimates in real-time by cavity ring-down laser absorption spectroscopy

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Received: 15 May 2011 / Accepted: 2 August 2011 / Published online: 31 August 2011

Abstract The most common currency for estimating N_2 fixation is acetylene reduction to ethylene. Real-time estimates of nitrogen fixation are needed to close the global nitrogen budget and these remain a critical gap in both laboratory and field experiments. We present a new method for continuous real-time measurements of ethylene production: Acetylene Reduction Assays by Cavity ring-down laser Absorption Spectroscopy (ARACAS). In ARACAS, air in the headspace of an incubation chamber is circulated with a diaphragm pump through a cavity ring-down ethylene spectrometer and back to the incubation chamber. This paper describes the new approach and its benefits compared to the conventional detection of ethylene by flame ionization detector gas chromatography. First, the detection of acetylene reduction to ethylene is non-intrusive and chemically non-destructive, allowing for real-time measurements of nitrogenase activity. Second, the measurements are made instantaneously and continuously at ppb levels, allowing for observation of real-time kinetics on time intervals as short as a few seconds. Third, the instrument can be automated for long time periods of measurement. Finally, the technique will be widely accessible by the research community as it can be readily adapted to most existing acetylene reduction protocols and is based on a modestly priced, commercially available instrument. We illustrate its use for measuring N_2 fixation using two species, the diazotrophic bacterium *Azotobacter vinelandii* and the lichen *Peltigera praetextata.* We also discuss potential limitations of the approach, primarily the implications of leaks in the analyzer, as well as future improvements.

Keywords Acetylene reduction · Cavity ring-down spectroscopy · N_2 fixation · Method development · ARACAS

Communicated by Evan DeLucia.

Electronic supplementary material The online version of this article (doi:10.1007/s00442-011-2105-y) contains supplementary material, which is available to authorized users.

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Introduction

Dinitrogen (N_2) fixation, the biological reduction of atmospheric N_2 to ammonium, is the dominant natural pathway for new nitrogen that enters most ecosystems. It influences plant growth and carbon budgets at local, regional and global scales (Cleveland et al. 1999; Evans and Johansen 1999; Galloway et al. 2004; Houlton et al. 2008; Menge and Hedin 2009; Reed et al. 2010; Sprent and Sprent 1990; Vitousek et al. 1997). The activity of the enzyme responsible for N_2 fixation, nitrogenase, is influenced by a wide variety of environmental factors, including light level, temperature, moisture and trace metal availability. However, current understanding of N_2 fixation kinetics remains limited, in part because enzymatic properties cannot be easily measured in real time.

Gas chromatography using flame ionization detection (GC-FID) has been the most widely used method for estimating rates of N_2 fixation since the discovery of acetylene (C_2H_2) reduction by nitrogenase more than 4 decades ago (Dilworth 1966; Hardy et al. 1968; Koch and Evans 1966; Koch et al. 1967; Schollhorn and Burris 1966; Stewart et al. 1967). Today, GC-FID monitoring of acetylene reduction assays (ARA) is still the method of choice in a wide range of ecosystems (Barron et al. 2009; Belnap 2002; Billings et al. 2003; Cerna et al. 2009; Cusack et al. 2009; Davis et al. 2010; DeLuca et al. 2008; Hara et al. 2009; Kitajima et al. 2009; Marcarelli and Wurtsbaugh 2009; Matzek and Vitousek 2003; Pinto-Tomas et al. 2009; Silvester 1989; Vitousek 1994). GC-FIDs are widely used because of their low-cost, simplicity and good sensitivity. However, field operation of GC-FID is often impractical, in part because of issues of portability but also because of susceptibility to changes in environmental variables such as humidity and temperature. Moreover, the finite number of discrete aliquots available for GC-FID analyses of ARA leads to a tradeoff between incubation time and sampling frequency. Finally, GC-FID's detection limit for ethylene (C_2H_4) is not suitable for N_2 fixers with low nitrogenase activity. Although the sensitivity of GC-FID measurements can be improved by preconcentration of C_2H_4 using adsorbent materials, the large volume required for this approach is generally not applicable for many ARA incubations.

The limitations of C_2H_2 reduction monitoring by GC-FID prompted the development of a photoacoustic spectral instrument 15 years ago for near real-time dynamic measurements (te Lintel Hekkert et al. 1998; Zuckermann et al. 1997). This instrument has led to new insights into N_2 fixation dynamics (Staal et al. 2001, 2007), but the method is technically intensive, and the analyzer, which has a response time of a few minutes, is not commercially available and has, to our knowledge, only been used by its developers.

We present a new widely accessible, real-time method to monitor ARA by Cavity ring-down laser Absorption Spectroscopy (ARACAS). Spectral monitoring of the C_2H_2 reduction to C_2H_4 offers several benefits compared to conventional GC-FID approaches. It allows for continuous detection of ethylene evolution, requires no consumables other than calcium carbide and gastight bags for the production of acetylene, has a low operating cost, and is capable of detecting C_2H_4 non-intrusively and non-destructively. The method is also ideal for field deployment, as the detector is relatively compact and light weight (26.3 kg) and is operational within minutes of installation with minimal technical expertise and power requirements (90–120 VAC, 50/60 Hz, 220 VAC, 50 Hz, <300 W). The ability to perform measurements in the field should limit artifacts associated with sample handling and delays in processing.

Materials and methods

Description of ARACAS

The ARACAS method consists of a fully enclosed incubation chamber in which acetylene is reduced to ethylene, a surrogate reaction for N_2 fixation, at a rate monitored continuously by cavity ring-down spectroscopy (CRDS). In our experiments, 10% of air in the incubation chambers is replaced with calcium carbide-derived C_2H_2. Air in the headspace of the incubation chamber is circulated with a diaphragm pump into the CRDS module and back to the incubation chamber. Any vessel can be used for the incubation chamber, and most existing incubation designs will be readily adaptable to ARACAS, requiring only minor modifications. In our experiments, we used 500-ml Erlenmeyer flasks with side tubulation equipped with a septum for adding and collecting gas samples (Fig. 1). The flask was closed with a rubber stopper with PTFE tubing connected to the CRDS inlet and outlet. The total volume of our design, including the incubation chamber, optical cavity and tubing was ~660 ml based on the concentration change with sequential additions of known quantities of ethylene. The e-folding response time with the current setup is 1.65 min. However, the response time can be reduced to seconds with a smaller incubation chamber. In experiments with live bacterial cultures, the chamber flask was set on top of an orbital shaker for continuous stirring.

Ethylene contaminant in commercially available high-purity (prepurified) acetylene cylinders usually ranges from 5 to 12 ppm in a 10% acetylene incubation and rises substantially with increased storage time in the tank. Because of this potential contamination in commercial preparations, we produced C_2H_2 by reacting calcium carbide with water in gastight bags (see supplementary material). The ethylene contaminant is significantly reduced under such conditions (~1.9 ppm in a 10% acetylene incubation) and avoids potential spectral interference associated with the acetone solvent found in acetylene tanks (Zuckermann et al. 1997). By using calcium-carbide-derived C_2H_2, the leak of ethylene at the onset of ARA experiments was reduced by a factor of 2.6–6.3 (see "Discussion").

Cavity ring-down spectroscopy

In cavity ring-down spectroscopy (CRDS), light from a near-infrared laser is directed into a high-finesse optical cavity delimited by a set of mirrors. The laser is brought into resonance with the cavity, the intensity builds to a

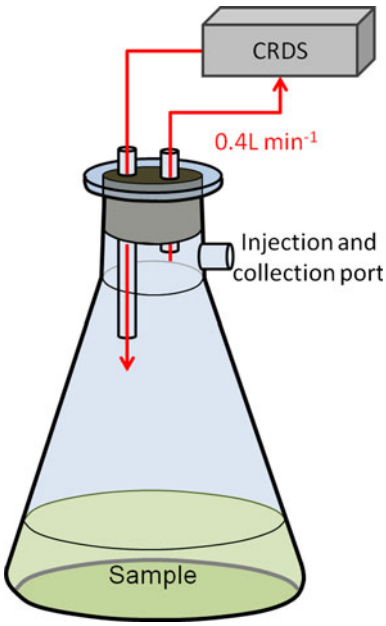

Fig. 1 Experimental design of ARACAS. N_2-fixers are incubated in an airtight chamber where 10% or some other quantity of the air is replaced with C_2H_2. A diaphragm pump circulates the headspace air (flow rate of 0.4 L min^{-1}) into the optical module and back into the incubation chamber in a closed loop. PTFE tubing connects the chamber to the CRDS. C_2H_4 measurements are taken at relatively high frequency ($\sim$0.2 Hz)

threshold level, and then the laser is turned off. The intensity of the light decays exponentially because of gas-phase absorption and reflectivity losses. The light decay (termed "ring-down") is monitored with a photodetector as the laser is tuned to specific wavelengths along the absorption profile of the particular spectroscopic line being measured for the target molecule. Small gas-phase molecules, such as C_2H_4, have an intrinsic infrared absorption spectrum. The decay rate at a given wavelength is a function of the concentration of the gas that absorbs at that particular wavelength and reflective losses (see Berden and Engeln 2009; Berden et al. 2000; Busch and Busch 1999, for a full description of the CRDS method). By tuning the laser to wavelengths at which the gas of interest does not absorb, the light decay rate intrinsic to the optical cavity (reflectivity losses) is estimated. The difference in ring-down times intrinsic to the optical cavity and at the wavelength absorbed by the gas of interest is proportional to the gas concentration. Compared to conventional absorption methods, CRDS has very low long-term drift because fluctuations in laser intensity do not affect the difference in ring-down times.

A CRDS analyzer for C_2H_4 (model G1106) was recently developed by the company Picarro (Santa Clara, CA, USA) to monitor ethylene production associated with post-harvest fruit ripening (Fidric et al. 2003; Wahl et al. 2006). We have applied the instrument to estimate N_2 fixation based on real-time estimates of acetylene reduction. As described by the manufacturer, the instrument offers a high linearity in ethylene detection over a wide dynamic range. The Picarro C_2H_4 analyzer has a precision of 2 and 0.2 ppbv (3 σ) over 5 and 300 s, respectively, according to the instrument's specifications. The maximum drift of the instrument is $\pm$4 and 10 ppbv over 24 h and 1 month, respectively. Continuous high-speed wavelength scanning minimizes interference from other gas species in the detector and ensures high chemical specificity. The fast response time of the Picarro C_2H_4 analyzer, due to the small size of the detector cell (33 cc) and the high gas flow rate (0.4 l min^{-1}), is ideal for kinetic studies. The instrument is insensitive to changes in ambient temperature, and interferences from CO_2, methane and water. See Wahl et al. (2006) and the Picarro website (http://picarro.com/) for further description of the analyzer, including wavelength range and type of laser.

Comparison of the ARACAS method to GC-FID with standards

In order to test the linearity of the ethylene response and the potential for interference by acetylene, a series of C_2H_2/C_2H_4 mixes were analyzed and compared to GC-FID. C_2H_2 was derived from calcium carbide, and C_2H_4 standards (10, 100, 1,000 ppm with $\pm$1% analytical uncertainty) were obtained from Airgas (Raleigh, NC, USA) (mixture of 99.9% ethylene stock with UHP nitrogen). ARACAS measurements were obtained in real-time, while for GC-FID measurements, headspace aliquots were collected and stored in 11-ml vacuumed gas sampling septum vials (Exetainers; Labco, Buckinghamshire, UK) until analysis. GC-FID analyses were performed using a CP-3800 (Varian, Missisauga, ON, Canada) equipped with a Hayesep N 80-100 column (Hayes Separations, Bandera, TX, USA).

Results and discussion

Laboratory test of ARACAS

We found very close agreement between ARACAS and GC-FID over a wide range of acetylene/ethylene mix ratios (Fig. 2a). Based on these results, the decrease in acetylene concentrations during ARA incubations, which is orders of magnitudes smaller than the variations presented in Fig. 2a, is unlikely to influence the ethylene measurements.

CO_2 concentrations may vary substantially during prolonged incubations and may interfere with C_2H_4 measurements. Although the Picarro instrument is designed to avoid cross-talk with CO_2, the potential spectral interference of C_2H_4 measurements by CO_2 was also assessed.

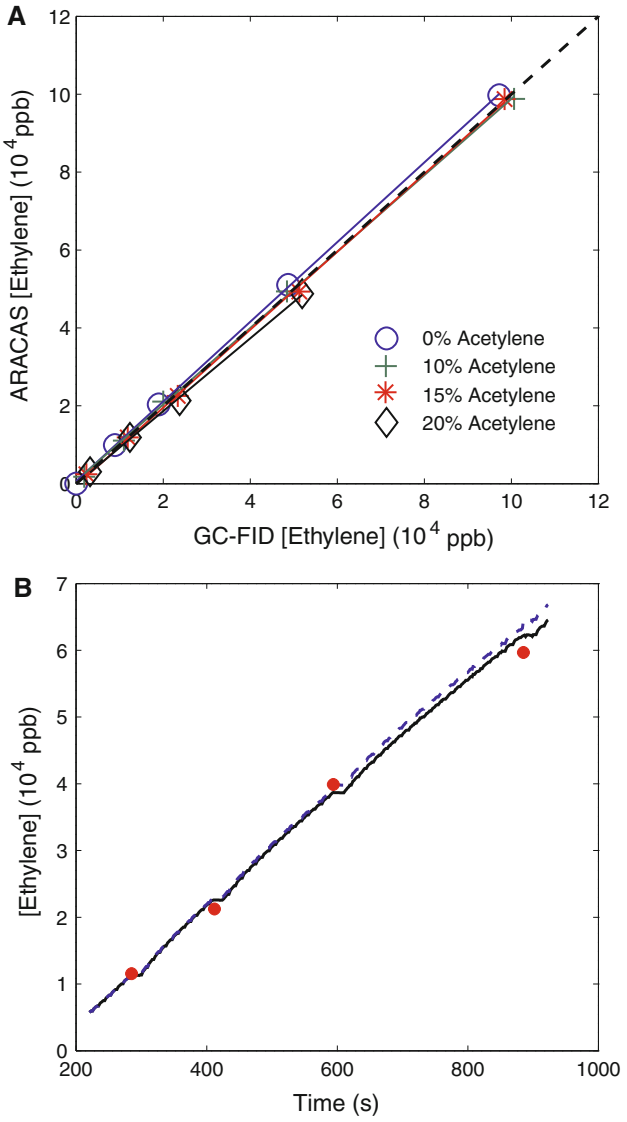

Fig. 2 **a** Comparison of ethylene concentration measurements by ARACAS and GC-FID at various acetylene concentrations. *Lines* represent Model II reduced major axis regression analyses. The slope of ARACAS versus GC-FID for the combined acetylene treatments is 1.000 ± 0.023 (95% CI), not significantly different from the identity slope, here represented by the *black dashed line*. **b** Estimates of N_2-fixation comparing ARACAS (*solid line*) to GC-FID (*points*) using an axenic culture of the N_2-fixing bacterium *A. vinelandii* (20 ml of culture, $OD_{620nm} = 0.268$, $\mu = 0.23\ h^{-1}$, 660-ml incubation flask). The small dips after the points are due to interruptions in mixing for sampling of the incubation chamber for GC-FID analyses. The slope of ARACAS versus GC-FID is 1.04 ± 0.21 (95% CI). In order to compare our ARACAS measurements with predictions of nitrogenase activity based on optical density change (see text), the ARACAS slope is corrected for collection of air samples for GC-FID analysis (*dashed line*), which slightly increases both the slope (from 85.4 to 88.0 ppb s^{-1}) and the linear least-square fit through the data (r^2 from 0.9989 to 0.9993). The correction for efflux (see "Discussion") is negligible; for example, at the highest values, the efflux is less than 1.5% of the acetylene reduction. The correction at the onset of the experiment is less than 0.1%

A known concentration of ethylene was measured under varying CO_2 concentrations. No interference in the reading was observed over a wide range of CO_2 concentrations (380 to 20,000 ppm) (<−3 ppb ethylene/1,000 ppm CO_2), which implies that the optical instrument's sensitivity to CO_2 is low or that the instrument's algorithm is adequately accounting for CO_2 interference. A small correction for the effect of varying humidity over the course of the experiment should be applied by, for example, normalizing the ethylene gas mixing ratio to a dry gas mixing ratio [i.e. $\text{Ethylene}_{dry} = \frac{\text{Ethylene}_{measured}}{(1-X_{H_2O})}$, where X_{H_2O} is the water vapor volume mixing ratio (v/v)].

Comparison of ARACAS method to GC-FID on live cultures

In order to test ARACAS with live cultures, N_2 fixation rates measured by ARACAS were compared to the conventional GC-FID on axenic cultures of *Azotobacter vinelandii* (OP, ATCC-13705), a free-living cosmopolitan N_2 fixer (see supplementary material for a description of the growth conditions of the bacterial culture). Bacterial suspensions were added to the incubation chamber and a headspace aliquot was replaced by an equivalent volume of C_2H_2 for a final air concentration of 10%. Discrete samples were collected from these experiments for comparison to GC-FID.

ARACAS measurements of culture-based N fixation show close agreement to GC-FID (Fig. 2b). The estimate of ethylene production of 88 ppb s^{-1} by ARACAS, corresponding to 2.13×10^{-7} nmol C_2H_4 $cell^{-1}$ min^{-1} under our experimental conditions, is also consistent with predictions based on the *A. vinelandii* growth rate as estimated by optical density change (1.54×10^{-7} nmol C_2H_4 $cell^{-1}$ min^{-1}; see supplementary information for details). The results also reveal negligible spectral interference from other gases, such as CO_2, which increased from 1,163 to 27,203 ppm over the course of the incubation.

ARACAS also provides continuous measurements for dynamic monitoring of N_2 fixation in real time (Fig. 3). For instance, rates of N_2 fixation by *A. vinelandii* in culture can be suppressed by the application of cold temperatures; pretreatment rates can then be restored by the removal of cold temperatures as measured in real time by ARACAS (Fig. 3a). Similarly, the influence of temperature on the relatively slow N_2 fixation rates in *Peltigera praetextata* lichens can be monitored continuously over long periods (Fig. 3b) (see supplementary material Fig. 1 for a picture of the *P. praetextata* sample). The experiments with *A. vinelandii* and *P. praetextata* demonstrate that the instrument can be used over a large dynamic range in

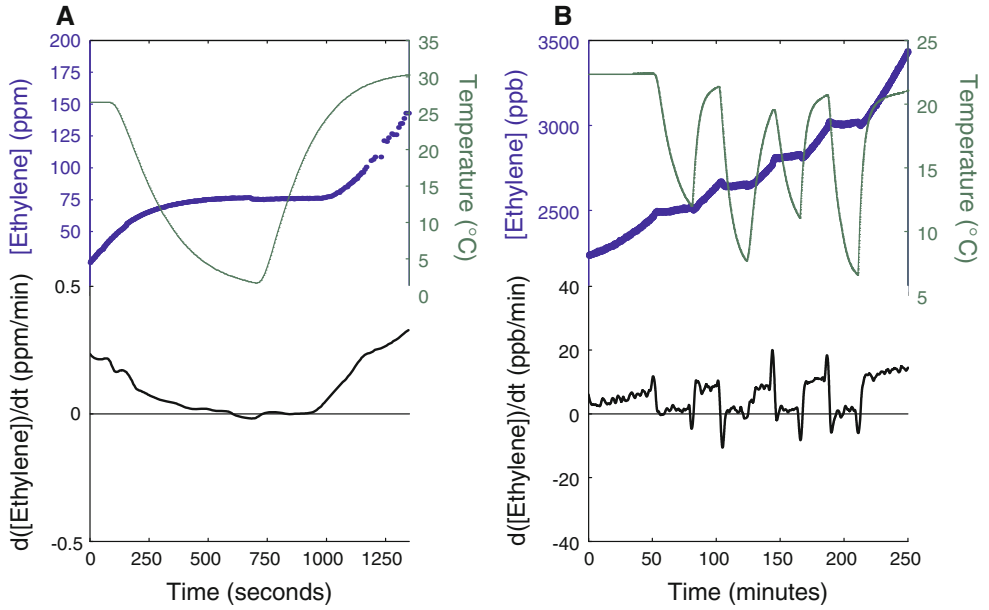

Fig. 3 ARACAS kinetics. **a** ARACAS with axenic culture of *A. vinelandii* as a function of incubation temperature (90 ml of culture, OD620 nm = 0.16, $\mu = 0.24\ h^{-1}$, 660-ml incubation flask). **b** ARACAS measurements of the influence of temperature on N_2 fixation on a sample *Peltigera praetextata* lichen (dry weight of 1.2 g). *Thick* and *thin curves* in *upper panels* represent ethylene concentration and incubation temperature, respectively. The lichen was illuminated with 948 lumens, color temperature of 2,700 K and incubated in the dark for 24 h prior to the experiment. In both experiments, the incubation chamber was thermo-regulated with a water jacket and the temperature was continuously monitored using a temperature probe (Hobo Tidbit waterproof datalogger). The *lower panels* represent the time-derivative of the ethylene concentration averaged by loess with a span of 70 and 300 in (**a**) and (**b**), respectively (Cleveland 1979). As expected for both species, when the temperature drops, nitrogenase activity is inhibited (ethylene production drops to zero), and is restored with a rise in temperature. The spikes in the signal are most likely artifacts associated with changeover periods. ARACAS detects subtle variations in N_2 fixation, and over long periods of time (note the ppm and seconds vs. ppb and minutes scales in **a** and **b**, respectively)

ethylene concentrations (ppb to ppm) with high sensitivity over long periods of incubation.

Theoretical considerations: ARACAS kinetics

The ethylene analyzer employs a four-stage diaphragm pump and is designed for flow-through operation rather than closed-loop operation. It is therefore not designed as a leak-tight system. Thus, acetylene is lost from the experimental system through two processes, reduction to ethylene and efflux (i.e., leaks). Despite competitive inhibition by N_2, acetylene reduction by nitrogenase is adequately described by Michaelis–Menten kinetics (Denison 1989). Acetylene efflux from the system can be approximated by first-order kinetics. The time-rate of change of acetylene concentration (A) is therefore a function of the acetylene efflux and reduction according to the following equation:

$$\frac{dA}{dt} = -k_{\text{eff}}A - \frac{V_{\max}A}{K_{\text{m}} + A}$$

where k_{eff} (s^{-1}), $V_{\max}$ (here in ppb s^{-1}) and K_{m} (here in ppb) are the efflux rate constant, the Michaelis–Menten maximum reaction rate, and the half-saturation constant, respectively. Similarly, the time-rate of change of ethylene concentration (E) is a function of ethylene efflux and acetylene reduction:

$$\frac{dE}{dt} = -k_{\text{eff}}E + \frac{V_{\max}A}{K_{\text{m}} + A}.$$

Efflux rate constants for ethylene and acetylene are assumed to be identical. Based on these calculations (Fig. 4), the large reduction in ethylene accumulation at high efflux is not due to subsaturation of the nitrogenase enzyme, as the acetylene concentration is still significantly greater than the half-saturation constant, but is associated with efflux of produced ethylene. Over the short term of our incubations, the production of ethylene is approximately zeroth-order with respect to the acetylene concentration. As the ethylene concentration increases, the efflux becomes an increasingly important factor, with ethylene production remaining relatively constant because the change in acetylene saturation of nitrogenase is negligible during our relatively short incubations. Changes in nitrogenase saturation due to acetylene efflux could occur during long incubations.

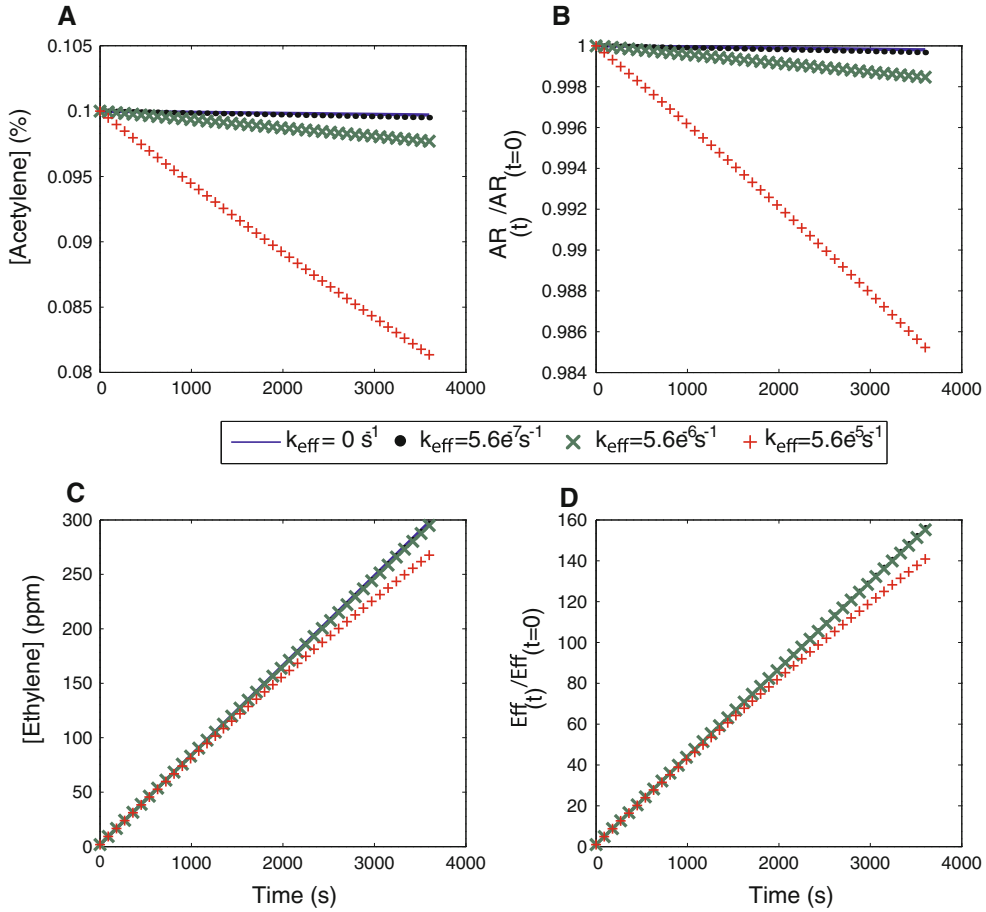

Fig. 4 Model predictions of acetylene reduction (*AR*) and efflux (*Eff*) with various efflux rate constants. Acetylene is lost from the system through efflux and reduction to ethylene (**a**). Ethylene is produced by acetylene reduction and lost through efflux (**c**). The *solid*, *dotted*, *x-marked*, and *cross-marked curves* efflux rate constants correspond to $AR_{(t=0)}/Eff_{(t=0)}$ of ∞, 7.7×10^4, 7.7×10^3 and 7.7×10^2, respectively. An airtight system has an infinitesimally small efflux/reduction ratio (*solid curve*). The *x-marked curve* represents the leak rate constant of our current experimental system. Whereas the ethylene production is relatively unaffected by loss of acetylene from the system through efflux and reduction (**b**), the ethylene efflux increases rapidly with increasing ethylene concentration (**d**). Estimates of N_2 fixation rates under our experimental conditions are not significantly influenced by the decreasing acetylene concentration because the nitrogenase enzyme remains at saturating or quasi-saturating acetylene concentrations. However, at low N_2 fixation rates, the negative bias associated with ethylene efflux from the system must be taken into account. K_m of 0.007 was used in the model (Christiansen et al. 2000). The ordinary differential equations describing the time evolution of acetylene and ethylene (see text) were numerically solved by the Runge–Kutta method with variable time step

However, because of the advantageous ppb detection level of the instrument, long incubations are in most cases unnecessary. For very low N_2 fixation rates ($\sim$ppb min^{-1}), our experiments and theoretical considerations outlined above show that the method would benefit from reduction or complete elimination of leaks. One can limit the leak rate by (1) gas tightening the instrument (i.e. reducing k_{eff}) and/or (2) decreasing the initial contaminant ethylene concentration (as described in "Materials and Methods"). To limit the efflux of both ethylene and acetylene during ARACAS measurements, known leak points in the system were modified as described in the supplementary material. These physical modifications reduced the leak rate by $\sim$45% (see below).

Determination of leak rate

Although the leak in our system was small, it could be significant when nitrogenase activity is low. Assuming an ethylene contaminant concentration of 1,900 ppb (see above), we estimated the leak rate to be $\sim 0.63 \pm 0.05$ ppb min^{-1} for our current experimental configuration (see supplementary material for details). Any N_2 fixation measurements by ARACAS within this order of magnitude should be corrected for efflux and interpreted with caution. Because the leak is reproducible, the ARA measurements can be corrected for ethylene loss from the system (Fig. 5). For further accuracy, the leak rate may be estimated for each experiment by measuring the evolution of ethylene

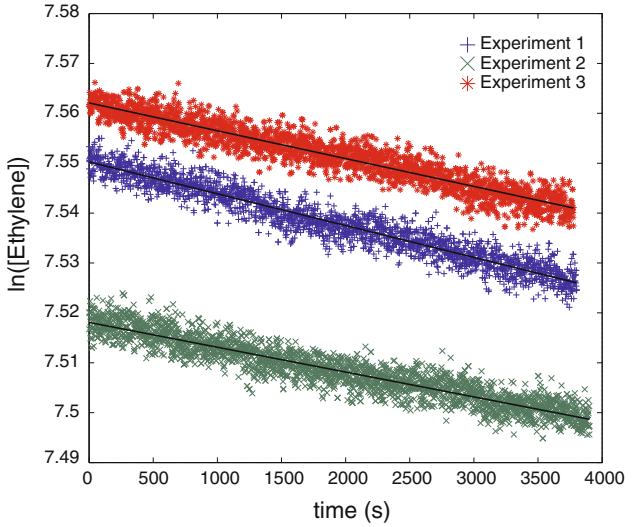

Fig. 5 Natural log of ethylene concentration (ppb) as a function of time for incubation chamber of 660 ml total volume as quantified in triplicate experiments. The average slope is $5.6 \times 10^{-6} \pm 0.4\ s^{-1}$ (±SE). *Solid black lines* are Model I least-squares linear regressions. See supplementary material for a description of the leak rate calculations

before N_2 fixers are added to the incubation chamber, or at the end of the experiment by quantitatively inhibiting the nitrogenase activity. For comparison, rates of non-symbiotic N_2 fixation in Panama soil (Barro Colorado Island) range from 3 ppb/min to 100 ppb/min (~25 g of leaf litter incubated in a similar size incubation chamber) (Barron et al. 2009). We are currently working on reducing the physical leak and contaminant of ethylene further. Moreover, the manufacturer, Picarro, is also planning to produce a leak-proof instrument in the future.

Conclusions

Because closing the global nitrogen budget depends on a better characterization of N_2 fixation kinetics, new analytical techniques are needed that provide affordable, sensitive, and continuous in situ assessments of nitrogenase activity. Here, we have described such a new method for real-time and high-resolution monitoring of acetylene reduction assays.

Despite the many advantages of ARACAS technology, conventional GC-FID may still be desirable in some cases. GC-FID allows for ethylene measurements on small samples. On the other hand, ARACAS provides continuous measurements for kinetic studies of C_2H_4 production at ppb levels. In addition, ARACAS is portable and very stable compared to GC-FID. By performing measurements in the field, perturbation artifacts associated with sample handling are reduced.

Our method could potentially be used with chambers sitting on top of soil, water, or other media if leaks can be measured accurately, or using a flow-through equilibration system (Zuckermann et al. 1997). Furthermore, the higher sensitivity of our approach allows for shorter incubations and more dilute cultures, limiting potential artifacts associated with longer incubations (e.g., changes in CO_2 and O_2 concentrations that inhibit nitrogenase activity) (Minchin et al. 1983). By providing insights into the kinetic properties of the nitrogenase enzyme, ARACAS should improve our understanding of the mechanisms governing the biogeochemistry of nitrogen fixation in a wide range of terrestrial and aquatic environments.

Acknowledgments We would like to thank François Lutzoni (Duke) and Brendan Hodkinson (Duke) for collection and identification of the lichen sample, and Aaron Van Pelt (Picarro) for helpful discussions. This work was supported by the National Science Foundation (DEB Ecosystems #1050227) (N.C. and R.B.J.) and the Canadian Research Chair in Terrestrial Biogeochemistry (J.P.B.). We acknowledge additional support from the Office of the Provost, Department of Biology, and Center on Global Change at Duke University.

References

Barron AR, Wurzberger N, Bellenger JP, Wright SJ, Kraepiel AML, Hedin LO (2009) Molybdenum limits nitrogen fixation in tropical forest soils. Nat Geosci 2:42–45

Belnap J (2002) Nitrogen fixation in biological soil crusts from southeast Utah, USA. Biol Fertil Soils 35:128–135

Berden G, Engeln R (2009) Cavity-ring down spectroscopy: techniques and applications. Wiley-Blackwell, Hoboken

Berden G, Peeters R, Meijer G (2000) Cavity ring-down spectroscopy: experimental schemes and applications. Int Rev Phys Chem 19:565–607

Billings SA, Schaeffer SM, Evans RD (2003) Nitrogen fixation by biological soil crusts and heterotrophic bacteria in an intact Mojave Desert ecosystem with elevated CO_2 and added soil carbon. Soil Biol Biochem 35:643–649

Busch KW, Busch MA (eds) (1999) Cavity ring-down spectroscopy: an ultratrace-absorption measurement technique. Oxford University Press, Washington, DC

Cerna B, Rejmankova E, Snyder JM, Santruckova H (2009) Heterotrophic nitrogen fixation in oligotrophic tropical marshes: changes after phosphorus addition. Hydrobiologia 627:55–65

Christiansen J, Cash VL, Seefeldt LC, Dean DR (2000) Isolation and characterization of an acetylene-resistant nitrogenase. J Biol Chem 275:11459–11464

Cleveland WS (1979) Robust locally weighted regression and smoothing scatterplots. J Am Stat Assoc 74:829–836

Cleveland CC et al (1999) Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. Glob Biogeochem Cycles 13:623–645

Cusack DF, Silver W, McDowell WH (2009) Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. Ecosystems 12:1299–1315

Davis SC et al (2010) Comparative biogeochemical cycles of bioenergy crops reveal nitrogen-fixation and low greenhouse gas emissions in a *Miscanthus* × *giganteus* agro-ecosystem. Ecosystems 13:144–156

DeLuca TH, Zackrisson O, Gundale MJ, Nilsson MC (2008) Ecosystem feedbacks and nitrogen fixation in boreal forests. Science 320:1181

Denison RF (1989) Implications of competitive-inhibition in the acetylene-reduction assay for dinitrogen fixation. Ann Bot 64: 167–169

Dilworth MJ (1966) Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim Biophys Acta 127:285–294

Evans RD, Johansen JR (1999) Microbiotic crusts and ecosystem processes. Crit Rev Plant Sci 18:183–225

Fidric BG, Provencal RA, Tan SM, Crosson ER, Kachanov AA, Paldus BA (2003) Bananas, explosives, and the future of cavity ring-down spectroscopy. Optics Photonics News 14:24–29

Galloway JN et al (2004) Nitrogen cycles: past, present, and future. Biogeochemistry 70:153–226

Hara S, Hashidoko Y, Desyatkin RV, Hatano R, Tahara S (2009) High rate of N_2 fixation by east Siberian cryophilic soil bacteria as determined by measuring acetylene reduction in nitrogen-poor medium solidified with gellan gum. Appl Environ Microbiol 75:2811–2819

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) Acetylene-ethylene assay for N_2 fixation-laboratory and field evaluation. Plant Physiol 43:1185–1207

Houlton BZ, Wang YP, Vitousek PM, Field CB (2008) A unifying framework for dinitrogen fixation in the terrestrial biosphere. Nature 454:U327–U334

Kitajima S, Furuya K, Hashihama F, Takeda S, Kanda J (2009) Latitudinal distribution of diazotrophs and their nitrogen fixation in the tropical and subtropical western North Pacific. Limnol Oceanogr 54:537–547

Koch B, Evans HJ (1966) Reduction of acetylene to ethylene by soybean root nodules. Plant Physiol 41:1748–1750

Koch B, Evans HJ, Russell S (1967) Reduction of acetylene and nitrogen gas by breis and cell-free extracts of soybean root nodules. Plant Physiol 42:466–468

Marcarelli AM, Wurtsbaugh WA (2009) Nitrogen fixation varies spatially and seasonally in linked stream-lake ecosystems. Biogeochemistry 94:95–110

Matzek V, Vitousek P (2003) Nitrogen fixation in bryophytes, lichens, and decaying wood along a soil-age gradient in Hawaiian montane rain forest. Biotropica 35:12–19

Menge DNL, Hedin LO (2009) Nitrogen fixation in different biogeochemical niches along a 120 000-year chronosequence in New Zealand. Ecology 90:2190–2201

Minchin FR, Witty JF, Sheehy JE, Muller M (1983) A major error in the acetylene-reduction assay—decreases in nodular nitrogenase activity under assay conditions. J Exp Bot 34:641–649

Pinto-Tomas AA et al (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science 326:1120–1123

Reed SC, Townsend AR, Cleveland CC, Nemergut DR (2010) Microbial community shifts influence patterns in tropical forest nitrogen fixation. Oecologia 164:521–531

Schollhorn R, Burris RH (1966) Study of intermediates in nitrogen fixation. Fed Proc 25:710

Silvester WB (1989) Molybdenum limitation of asymbiotic nitrogen fixation in forests of pacific northwest America. Soil Biol Biochem 21:283–289

Sprent JL, Sprent P (1990) Nitrogen fixing organisms: pure and applied aspects. Chapman and Hall, London

Staal M, Lintel-Hekkert ST, Harren F, Stal L (2001) Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: a comparison between batch incubation and on-line monitoring. Environ Microbiol 3:343–351

Staal M et al (2007) Nitrogen fixation along a north-south transect in the eastern Atlantic Ocean. Limnol Oceanogr 52:1305–1316

Stewart WDP, Fitzgera Gp, Burris RH (1967) In situ studies on n_2 fixation using acetylene reduction technique. Proc Natl Acad Sci USA 58:2071–2078

te Lintel Hekkert ST et al (1998) Laser photoacoustic trace gas detection, an extremely sensitive technique applied in biological research. Instrum Sci Technol 26:157–175

Vitousek PM (1994) Potential nitrogen fixation during primary succession in Hawaii volcanoes national park. Biotropica 26: 234–240

Vitousek PM et al (1997) Human alteration of the global nitrogen cycle: sources and consequences. Ecol Appl 7:737–750

Wahl EH et al (2006) Ultra-sensitive ethylene post-harvest monitor based on cavity ring-down spectroscopy. Optics Express 14: 1673–1684

Zuckermann H et al (1997) On-line monitoring of nitrogenase activity in cyanobacteria by sensitive laser photoacoustic detection of ethylene. Appl Environ Microbiol 63:4243–4251