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A Simple, High-Precision, High-Sensitivity Tracer Assay for N_2 Fixation

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We describe a simple, precise, and sensitive experimental protocol for direct measurement of N_2 fixation using the conversion of $^{15}N_2$ to organic N. Our protocol greatly reduces the limit of detection for N_2 fixation by taking advantage of the high sensitivity of a modern, multiple-collector isotope ratio mass spectrometer. This instrument allowed measurement of N_2 fixation by natural assemblages of plankton in incubations lasting several hours in the presence of relatively low-level (ca. 10 atom%) tracer additions of $^{15}N_2$ to the ambient pool of N_2. The sensitivity and precision of this tracer method are comparable to or better than those associated with the C_2H_2 reduction assay. Data obtained in a series of experiments in the Gotland Basin of the Baltic Sea showed excellent agreement between $^{15}N_2$ tracer and C_2H_2 reduction measurements, with the largest discrepancies between the methods occurring at very low fixation rates. The ratio of C_2H_2 reduced to N_2 fixed was 4.68 ± 0.11 (mean ± standard error, $n = 39$). In these experiments, the rate of C_2H_2 reduction was relatively insensitive to assay volume. Our results, the first for planktonic diazotroph populations of the Baltic, confirm the validity of the C_2H_2 reduction method as a quantitative measure of N_2 fixation in this system. Our $^{15}N_2$ protocols are comparable to standard C_2H_2 reduction procedures, which should promote use of direct $^{15}N_2$ fixation measurements in other systems.

Nitrogen fixation, the process by which organisms use N_2 as a substrate for growth, can provide an important source of nitrogen to nutrient-poor ecosystems. Although a wide variety of bacteria and cyanobacteria are known to fix N_2, the rates of N_2 fixation measured in oligotrophic water columns have typically been quite low relative to the apparent N demand of the ecosystem (5, 17, 20). The rate of N_2 fixation can be measured directly using ^{15}N tracer techniques, and studies of diazotrophy in the early 1960s relied on this tracer method (reference 1 and references therein). Over the last several decades, the tedium and complexity of the mass spectrometric analysis required in tracer experiments and the rather low levels of ^{15}N enrichment that usually result from short-term $^{15}N_2$ incubations have tended to discourage the direct measurement of aquatic N_2 fixation using tracer techniques. The introduction of the C_2H_2 reduction method in the late 1960s (16, 30) led to a surge in research on aquatic N_2 fixation (3, 4). The C_2H_2 reduction assay provides a sensitive measure of N_2 fixation, is easily adapted for field studies, and requires relatively simple experimental equipment and instrumentation. Nonetheless, it is an indirect assay of N_2 fixation, relying on the reduction of C_2H_2, a substrate analog of N_2. Although comparisons between C_2H_2 reduction and $^{15}N_2$ fixation rates in many ecosystems have shown good agreement with theoretical reduction ratios (C_2H_2:$N_2 \approx 3$), this is not always the case (26). Direct calibration of the C_2H_2 reduction assay is generally recommended in each system considered, though only a minority of studies to date have included such comparisons.

The availability of high-precision isotope ratio mass spectrometers now makes it feasible to carry out $^{15}N_2$ tracer incubations rapidly and with minimal disruption of the system. Here we describe an experimental protocol that exploits this high analytical sensitivity in making direct measurements of N_2 fixation by planktonic organisms in the central Baltic Sea.

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MATERIALS AND METHODS

We carried out a suite of N_2 fixation experiments in the Gotland Basin in the central Baltic Sea (57°17′N, 20°05′E) during the 1994 GOBEX (stands for Gotland Basin Experiment) cruise of the F/S *Alexander von Humboldt* (15 to 28 July 1994). Our sampling strategy and methods will be described in detail elsewhere. We collected surface water samples using a bucket or a CTD-rosette, which also provided samples spanning the upper water column. To test the sensitivity of the ^{15}N method over a wide range of activity levels, we conducted experiments with natural water samples as well as with concentrated samples of cyanobacteria collected in vertical tows of a 64-μm-mesh plankton net through the upper several meters of the water column.

All $^{15}N_2$ fixation experiments were done in parallel with C_2H_2 reduction measurements carried out by standard methods (4). Briefly, C_2H_2 reduction assays were conducted in 14- or 70-ml serum bottles, containing 10 or 50 ml of sample, respectively, or in 250-ml screw-top bottles fitted with Teflon-faced butyl-rubber stoppers and filled with 200-ml samples. All experiments were conducted under aerobic conditions. Acetylene was added to a final concentration in the headspace of about 15% (vol/vol). Subsamples of the headspace were removed at intervals over the assay period and analyzed for C_2H_4 production by flame ionization gas chromatography; all samples were corrected for C_2H_4 remaining in the liquid phase (4). C_2H_2 reduction experiments generally bracketed the $^{15}N_2$ uptake experiments, and rates were calculated over comparable periods.

$^{15}N_2$ fixation samples were incubated in 250-ml Pyrex bottles which were filled to overflowing before being carefully sealed with a septum cap (Teflon-lined butyl rubber). A gas-tight syringe was used to inject 0.5 ml of $^{15}N_2$ (99 atom% ^{15}N; CAMPRO Scientific) into the bottle and then to withdraw a 0.5-ml volume of solution to equalize the pressure across the septum. The sample bottles were gently mixed and then incubated either in a deck incubator covered with neutral-density screening to stimulate in situ light intensities or attached to an in situ array for periods ranging between 3 and 6 h. At the end of each experiment, the suspended particles in each bottle were collected by gentle vacuum filtration (pressure drop [ΔP] ≤ 25 cm Hg) through a 25-mm precombusted (450°C for 12 h) GF/F filter. The filters were frozen immediately and stored in a freezer (−20°C) while at sea.

Once ashore, samples were dried at 60°C and then wrapped in tin cups (Heraeus CHN cups) and formed into pellets with a laboratory press. We used a Fisons EA1108 elemental analyzer to measure the concentrations of particulate nitrogen (PN) and particulate carbon and to convert organic N to N_2 for isotopic analysis. The gas stream from the elemental analyzer was passed through a split

interface, which injected a portion of the flow directly into the ion source of a Finnigan Delta-S mass spectrometer for the isotope ratio measurement. The N isotope ratio of each sample was measured continuously as the N peak from the elemental analyzer passed through the system (continuous-flow isotope ratio mass spectrometry). Each analysis was preceded by three injections of a working reference gas (ultra-high-purity N_2, $\delta^{15}N = -8.09‰$) and followed by a fourth injection to provide a highly accurate estimate of the difference in isotope ratios ($^{15}N/^{14}N$) between the sample and the reference gas. The difference in isotope ratios between standard injections provided a check on the internal precision of the mass spectrometer. Each batch of 20 to 30 samples included five to six organic standards (peptone and acetanilide) as a check on the state of the combustion and chromatography of the samples and to provide a calibration of the Fisons thermal conductivity detector used in measuring the N and C content of the sample. These organic standards also provide a measure of the overall precision of the analytical procedure: the standard deviation (SD) of the $\delta^{15}N$ values measured for a day's standards is typically 0.05‰ ($n = 5$). Both the reference gas and the organic standards have been intercalibrated with standards used at Harvard and Woods Hole and can be related to the isotopic composition of the proposed NBS primary isotopic references for N.

We carried out our measurements using an instrument and standards calibrated for natural abundance and low-level tracer measurements of ^{15}N content, which is usually expressed by the $\delta^{15}N$ convention:

$$\delta^{15}N(‰) = \left[\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{atmosphere}} - 1\right] \times 1{,}000 \quad (1)$$

where $(^{15}N/^{14}N)_{atmosphere} = 0.003676$ (i.e., 0.366 atom% [19]).

Note that $\delta^{15}N$ values are a linear transform of the isotope ratios $^{15}N/^{14}N$, representing the per mille difference between the isotope ratios in a sample and in atmospheric N_2. As noted above, the inherent precision of the isotopic analysis is ca. ±0.05‰, which is much smaller than the changes in isotopic enrichment typically resulting from N_2 fixation. For example, if 0.366 atom% ^{15}N is taken as the mean isotopic composition of atmospheric N_2, then a sample containing 0.466 atom% ^{15}N (i.e., 0.100 atom% excess) would have a $\delta^{15}N$ of 273‰. As noted above, the inherent precision of the isotopic analysis is ca. ±0.05‰, which is orders of magnitude smaller than the enrichment values typically resulting from N_2 fixation.

The isotope ratio $^{15}N/^{14}N$ is essentially equal to the mole fraction of ^{15}N [i.e., $^{15}N/^{14}N \approx {}^{15}N/(^{15}N/^{14}N)$] at low abundances but deviates significantly from the mole fraction of ^{15}N at enrichments exceeding ca. 5 atom%. In order to use a mass balance approach to determine N_2 fixation rates, we converted all $\delta^{15}N$ values to absolute abundance ratios (A) for use in our calculations as follows:

$$A(\text{atom\%}) = 100 \times \left(\frac{^{15}N}{^{15}N + {}^{14}N}\right) \quad (2)$$

The resulting value can be related directly to $\delta^{15}N$ values as follows:

$$A(\text{atom\%}) = 100 \times \left[\frac{(10^{-3}\delta^{15}N + 1)(^{15}N/^{14}N)_{atmosphere}}{1 + (10^{-3}\delta^{15}N + 1)(^{15}N/^{14}N)_{atmosphere}}\right] \quad (2a)$$

Calculations. For a short-term experiment, the mass- and volume-specific rates of N_2 fixation can be calculated if the following quantities are known: A_{N_2}, the ^{15}N enrichment of the N_2 available for fixation; A_{PN_0}, the ^{15}N enrichment of particulate N at the start of the experiment; A_{PN_f}, the ^{15}N enrichment of particulate N at the end of the experiment; and $[PN]_f$, the concentration of particulate N at the end of the experiment.

The isotopic composition of the N_2 pool available for fixation can be calculated if the initial concentration of N_2 and the size of the isotopic addition are both known. We used the equations provided by Weiss (32) to calculate the initial concentration of N_2 present, assuming equilibrium with the overlying atmosphere. The high sensitivity of the isotope ratio mass spectrometer makes it possible to use a relatively low-level ^{15}N enrichment in the N_2 pool, which can be achieved with a rather small addition of $^{15}N_2$ gas. In practice, we added a fixed quantity of $^{15}N_2$ (0.5 ml at ambient temperature and pressure) and calculated the enrichment independently for each bottle on the basis of its volume, the solubility of N_2, and the quantity of $^{15}N_2$ added. The resulting substrate enrichments (A_{N_2}) ranged between 12.6 and 13.0 atom% for different incubations.

At the end of the experiment, the rates of uptake can be calculated by a simple mixing model in which the final isotopic enrichment (A_{PN_f}) is taken to represent a mixture of the organic N present at the start of the experiment and the organic N added through N_2 fixation. Let $[PN]_o$, $[PN]_\Delta$, and $[PN]_f$ represent the initial, incremental, and final concentrations of PN in the experimental bottle such that

$$[PN]_f = [PN]_o + [PN]_\Delta \quad (3)$$

The final isotopic composition of PN can then be represented as the mass-weighted average of the ^{15}N enrichment of the original pool of PN and that of the PN added through N_2 fixation:

$$A_{PN_f} = \frac{([PN]_o)(A_{PN_0}) + ([PN]_\Delta)(A_{PN_f})}{[PN]_f} \quad (4)$$

Equations 3 and 4 can be combined to give an expression for the relative contribution of fixed N to the total pool of PN at the end of the experiment:

$$\frac{[PN]_\Delta}{[PN]_f} = \frac{A_{PN_f} - A_{PN_0}}{A_{N_2} - A_{PN_0}} \quad (5)$$

For a short-term experiment in which $[PN]_\Delta$ represents a small fraction of $[PN]_f$, this ratio will scale linearly with the turnover of N through N_2 fixation such that

$$V(T^{-1}) = \left(\frac{1}{\Delta t}\right)\left(\frac{[PN]_\Delta}{[PN]_f}\right) = \left(\frac{1}{\Delta t}\right)\left(\frac{A_{PN_f} - A_{PN_0}}{A_{N_2} - A_{PN_0}}\right) \quad (6)$$

where Δt is the duration of the experiment. In keeping with the notation commonly used in studies of N uptake, V represents the specific rate of uptake of N_2 by particles in the experimental bottle. The inverse of V provides an estimate of the turnover time for organic N solely on the basis of the input due to N_2 fixation.

Finally, the volumetric rate of N_2 fixation (ρ) is

$$\rho(M \cdot L^{-3} \cdot T^{-1}) = \frac{V}{2}\overline{[PN]} \quad (7)$$

where [PN] is the mean concentration of PN in the course of the experiment and the factor of 2 is necessary to convert from gram-atoms of N to moles of N_2. In practice, the concentration of PN typically changes little during a short-term experiment, and to a very good approximation,

$$\rho(M \cdot L^{-3} \cdot T^{-1}) \approx \frac{V}{2}[PN]_f \quad (7a)$$

In cases where the concentration of PN changes appreciably during an experiment, the mean concentration of PN during the incubation should be used instead:

$$\rho(M \cdot L^{-3} \cdot T^{-1}) = \frac{V}{2}\overline{[PN]} = \frac{V}{2\Delta t}\left(\int_0^t [PN]dt\right) \quad (7b)$$

In practice, detailed time courses of PN concentration may not be available, and equation 7b can be approximated by using the initial and final PN values as follows:

$$\rho(M \cdot L^{-3} \cdot T^{-1}) = \frac{V}{2}\overline{[PN]} \approx \frac{V}{2}\left(\frac{[PN]_0 + [PN]_f}{2}\right) \quad (7c)$$

RESULTS

We carried out a total of 42 experiments using either net-collected *Aphanizomenon* spp. suspended in surface seawater or natural plankton assemblages collected with buckets or Niskin-type bottles attached to a rosette. In all, 18 incubations were carried out under in situ conditions and 24 were carried out on deck under simulated in situ conditions. In each case, $^{15}N_2$ tracer and C_2H_2 reduction incubations were conducted in parallel with aliquots drawn from a single mixed sample. Our results show a highly significant ($P < 0.0001$) linear relationship between C_2H_2 reduction and $^{15}N_2$ fixation rate measurements (Fig. 1), with the largest departures from linearity occurring at low rates of fixation. Although the ratios of C_2H_2 reduction to N_2 fixation rates (C_2H_2/N_2) showed wide variation in the data set as a whole, much of this variability resulted from three samples incubated in the dark. For all other samples, the best-fit least-squares linear regression of C_2H_2 versus N_2 reduction rate yielded an estimate of 4.68 ± 0.11 mol of C_2H_2 reduced per mol of N_2 fixed (Fig. 1) ($\bar{X}$ ± standard error of the slope, $r^2 = 0.980$, $n = 39$). The ordinal intercept of the least-squares regression was not significantly different from zero ($P = 0.55$, by the Student two-tailed t test).

In contrast to $^{15}N_2$ experiments, C_2H_2 reduction measurements were conducted in assay vessels of several sizes. Comparison of assays of different sample and container sizes conducted in parallel and under similar conditions indicated that rates were relatively independent of incubation vessel size over a relatively broad range of volumes (Table 1). This suggests

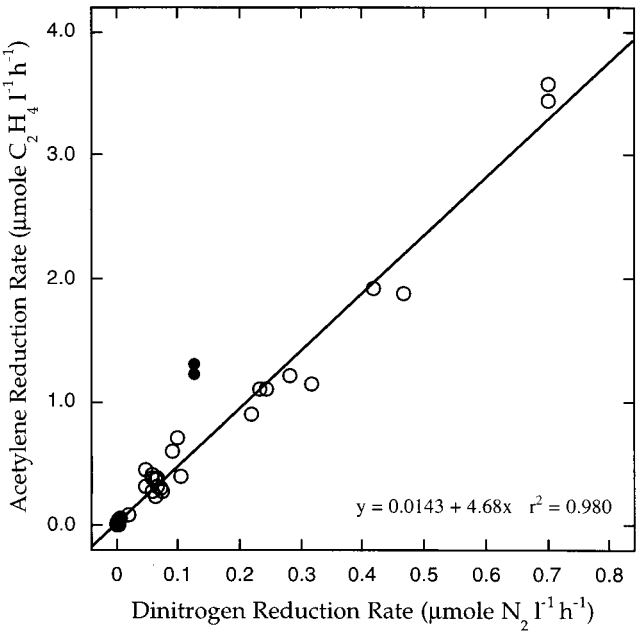

FIG. 1. Relationships between acetylene reduction and $^{15}N_2$ fixation rates measured for natural assemblages of phytoplankton and concentrated suspensions of net-collected *Aphanizomenon* spp. assayed in the Gotland Basin in July 1994. A best-fit least-squares line of regression (open circles) is shown for the data exclusive of samples incubated in the dark (filled circles).

that bottle size effects are minimal over the assay durations used in this study.

DISCUSSION

N_2 fixation in planktonic systems has been an area of active interest and research for the last several decades. N_2-fixing cyanobacteria contribute to eutrophication in lake systems (28) and are prominent in some marine coastal areas such as the Baltic Sea. Species of the nonheterocystous cyanobacterial genus *Trichodesmium* occur in the water column of the oligotrophic open ocean of the tropics and subtropics worldwide and may be important sources of N input in these severely N-limited systems (7, 10). The extensive body of research on aquatic N_2 fixation carried out during the last two decades has derived in large part from the availability of the C_2H_2 reduction method. In most cases, the measured rate of C_2H_2 reduction is converted to a rate of N_2 fixation by a theoretical reduction ratio (C_2H_2/N_2 = 3:1) derived from a consideration of the number of electrons needed to reduce C_2H_2 to C_2H_4 (2 e^-) and N_2 to NH_4^+ (6 e^-). Because the hydrogenase activity of nitrogenase is blocked by C_2H_2 but produces 1 mol of H_2 per mol of N_2 fixed, a theoretical ratio of C_2H_2/N_2 = 4:1 may be more appropriate (26). Significant departures from these theoretical ratios may occur, and C_2H_2/N_2 reduction ratios higher than 4:1 are generally interpreted to reflect greater H_2 production under N_2-fixing conditions relative to minimal H_2 evolution and an efficient reduction of C_2H_2 by nitrogenase under C_2H_2-reducing conditions. Elevated C_2H_2/N_2 reduction ratios may also reflect the excretion of dissolved organic nitrogen by N_2-fixing cyanobacteria (6, 14). Although the need for direct $^{15}N_2$ fixation measurements to calibrate the C_2H_2 reduction method is widely recognized, relatively few such studies have actually been carried out. To our knowledge, there have been no prior comparisons for the planktonic cyanobacteria of the Baltic Sea.

Previous direct intercomparisons of the C_2H_2 reduction method with $^{15}N_2$ uptake have been conducted with cultures and natural samples of cyanobacteria (Table 2). Stewart et al. (31) found very close agreement among three species of cyanobacteria, with an average molar ratio of C_2H_2 reduction to $^{15}N_2$ fixation of 3.2:1. Similarly, Jensen and Cox (18), measuring total N_2 uptake by membrane inlet mass spectrometry, found a ratio of 3.8:1 for a culture of *Anabaena variabilis*. Several studies have been conducted with concentrates or ambient waters of lake cyanobacteria. Peterson and Burris (25) reported an average C_2H_2/N_2 ratio of 4.4:1 for 24 comparisons using plankton from two Wisconsin lakes. A number of method intercomparisons have been conducted with the marine planktonic diazotroph *Trichodesmium* spp., yielding C_2H_2/N_2 reduction ratios between 1.9:1 (21) and roughly 6:1 (8, 9). More recently, several investigations have produced reduction ratios close to the theoretical value of 4:1 (11, 14, 29). In *Trichodesmium* spp. and perhaps other cyanobacteria, a significant fraction of recently fixed N may be released as dissolved organic nitrogen (6, 14), so the net rate of fixation of N_2 into cyanobacterial biomass may be substantially less than the gross rate of fixation measured by C_2H_2 reduction.

Several factors contribute to the relative paucity of direct calibrations of the C_2H_2 reduction assay. For some researchers, access to a suitable mass or emission spectrometer is likely to be a deciding factor. In addition, the tedium and complexity generally associated with previous $^{15}N_2$ reduction protocols may have thwarted all but the most stalwart investigators. In order to achieve sufficient enrichment of the product pool for analysis on single-collector mass spectrometers or emission spectrometers, researchers have often resorted to removing N_2 from the experimental system by sparging with inert gas or

TABLE 1. Effects of bottle size on C_2H_2 reduction rates by concentrated and unconcentrated samples of surface cyanobacteria in the central Baltic Sea

Plankton sample	C_2H_2 reduction (nmol of C_2H_2 ml^{-1} h^{-1}) in the following bottle size:			Incubation conditions
	14 ml	73 ml	250 ml	
Concentrated				
18 July 1994		3.44 ± 0.12 (n = 3)	3.58 ± 0.04 (n = 3)	On deck, 100% sun
		1.23 ± 0.06 (n = 3)	1.31 ± 0.004 (n = 3)	On deck, dark
19 July 1994		1.52 ± 0.01 (n = 3)	1.60 ± 0.09 (n = 2)	On deck, 100% sun
21 July 1994	0.80 ± 0.01 (n = 3)			In situ, surface
		1.15 ± 0.03		On deck, 100% sun
Unconcentrated				
19 July 1994		0.02 ± 0.02	0.026 ± 0.007 (n = 4)	On deck, 100% sun
25 July 1994	0.032 ± 0.005 (n = 3)		0.032 ± 0.007 (n = 2)	On deck, 100% sun

TABLE 2. Direct measurements of the C_2H_2/N_2 reduction ratios for various species and experimental systems

Organism(s)	Exptl system	Reference	Exptl condition(s)					C_2H_2/N_2 ratio (mean ± SD) (n)[a]
			Bottle size (ml)	Sample size (ml)	$^{15}N_2$ addition (ml) (% ^{15}N)	Notes	Δt (h)	
Three species of cyanobacteria	Lab cultures	31	25	5	5 (45)	Ar + O_2 mix	ca. 0.5	3.2
Anabaena variabilis	Lab culture	18	7	4.5	None	15% N_2 in Ar	ca. 0.3	3.8 ± 0.29 (5)
Aphanizomenon spp. and *Anabaena* spp. plus *Gloeotrichia* sp.	Lake Mendota, Little Arbor Vitae Lake	25	6	1	ND[b] (75–80, final ^{15}N enrichment)	1,000× preconcn, 36% N_2 in Ar	0.5	4.4 ± 0.27 (12)
Aphanizomenon spp. and *Anabaena* spp.	ELA[c] no. 226, 227, and 304	15	120	30	NA[d]	10× preconcn, unconcn	NA	7.5 ± 0.2 (83)
				50				
Three *Aphanizomenon* spp. and *Anabaena* spp.	ELA no. 226, 227, and 261	13	120	120	4 (95)	NA	<5	2.58 ± 0.28 (10)
			50	30	1 (95)			
Rhizosolenia spp.	Central Pacific	22	7	1	2 (95)	Net samples, Ar + O_2 mix	1.8–11	9.29 ± 5.34 (11)
Trichodesmium spp.	Sargasso Sea	8	NA	NA	NA	Net samples		ca. 6 (18)
Trichodesmium spp.	Central Pacific	21	7	1	2 (95)	Net samples, Ar + O_2 mix	1.5	1.91
Trichodesmium spp.	Caribbean	9	25	10	10 (99)	Net samples, Ar + O_2 mix	3	7.22 ± 4.4[e] (21)
Trichodesmium spp.	Bay of Bengal and South China Sea	27	NA	NA	NA	NA	NA	2.85 ± 0.18 (4)
Trichodesmium spp.	Bermuda	29	360	350	10, in headspace	Diver-collected colonies	1–2.5	4.1 ± 1.6 (3)
Trichodesmium spp.	Caribbean	14	125	125	ND (ca. 30, final ^{15}N enrichment)	Net samples	2	3.4

[a] With the exception of the study by Jensen and Cox (18), who used a membrane leak mass spectrometer to monitor steady-state N_2 consumption by a cyanobacterial culture, all of these ratios were measured by $^{15}N_2$ tracer techniques.
[b] ND, not determined.
[c] ELA, experimental lakes area.
[d] NA, information not available.
[e] Means and SDs were calculated with data in Table 4 of reference 9.

applying a vacuum before the addition of highly enriched $^{15}N_2$. These manipulations are generally not part of the C_2H_2 reduction assay, resulting in a divergence in experimental conditions for the two methods. For example, removal of the ambient N_2 pool is also likely to affect the concentration of other dissolved gases, including O_2, which may affect the activity of nitrogenase.

Our experimental approach differs from older $^{15}N_2$ tracer assays for N_2 fixation in several respects. The major analytical improvement over older methods is the use of a multiple-collector isotope ratio mass spectrometer to carry out the ^{15}N measurements with very high sensitivity and precision. This effectively reduces the incubation time needed to accumulate sufficient tracer in the biomass for a reliable measurement of ^{15}N abundance. In addition, the high sensitivity of the isotope measurement reduces the quantity of tracer necessary in the source pool of N_2. By using a relatively small addition of label (10 to 15 atom% ^{15}N), the handling and physical disruption of the system can be minimized. Finally, by using an automated, continuous-flow isotope ratio mass spectrometer, we greatly reduced the time and costs associated with sample preparation and analysis.

Error analysis. A number of factors may influence our final experimental estimate of the N_2 fixation rate. We have estimated the potential importance of each experimental and analytical factor by examining the effects of typical variations on the final rate estimates of V and ρ derived from a model data set representing a typical 5-h incubation. We will begin by considering the errors associated with each of the individual terms in equations 6 and 7 and then consider the contributions of these errors to the overall uncertainty in evaluating V and ρ.

Incubation time (Δt). Sample filtration typically requires several minutes, during which N_2 fixation may continue in the unfiltered sample. This will typically lead to an uncertainty of no more than 10 min (3.3%) in the actual duration of the experimental incubation.

Isotopic composition of PN (A_{PN_0} and A_{PN_f}). As noted above, the precision of the mass spectrometric measurement of standards is ±0.05‰ (SD), or $\pm 1.82 \times 10^{-5}$ atom%. We commonly observe greater variation between replicate analyses of natural samples (23, 24), as well as a decrease in precision for analysis of small samples (≤ ca. 1 μmol of N). For PN samples containing 2 to 4 μmol of N, the overall precision of the isotopic measurement is typically better than ±0.2‰ (SD), or $\pm 7.29 \times 10^{-5}$ atom%.

Substrate isotopic composition (A_{N_2}). We calculated the isotopic composition of the N_2 pool available for fixation using the equations of Weiss (32) relating the Bunsen coefficient for dissolution of N_2 in seawater to temperature and salinity. The calculated solubility is relatively insensitive to small errors in temperature and salinity. For example, a 1°C change in temperature (15 ± 1°C) and a 1‰ change in salinity (7 ± 1‰) alter the equilibrium concentration of N_2 by 1.8% and 0.7%, respectively. Varying both temperature and salinity simultaneously (±1°C and ±1‰) produces an overall change of 2.5% in the concentration of N_2. Other sources of error in our calculation of A_{N_2} arise from uncertainties in the sample volume and the volume of the spike of $^{15}N_2$ added to the incu-

TABLE 3. Contributions of different sources of error to the overall uncertainty in the value of V (h^{-1})[a]

Parameter (X)	Value	SD	$\partial V/\partial X$	Error contribution (SD × $[\partial V/\partial X]$)^2	% Total error	Summary for V	V (h^{-1})
Δt	5	0.16667	-4.658×10^{-4}	6.028×10^{-9}	55.23	Mean	2.329×10^{-3}
A_{N_2}	12.841462	0.37426	-1.867×10^{-4}	4.883×10^{-9}	44.74	Variance	1.091×10^{-8}
A_{PN_0}	0.366365	7.2932×10^{-5}	-1.585×10^{-2}	1.335×10^{-12}	0.01	SD	1.045×10^{-4}
A_{PN_f}	0.511651	7.2932×10^{-5}	1.603×10^{-2}	1.367×10^{-12}	0.01	*CV*	4.49%
Δt	5	0.16667	-1.052×10^{-5}	3.076×10^{-12}	37.05	Mean	5.261×10^{-5}
A_{N_2}	12.841462	0.37426	-4.218×10^{-6}	2.492×10^{-12}	30.02	Variance	8.301×10^{-12}
A_{PN_0}	0.366365	7.2932×10^{-5}	-1.603×10^{-2}	1.366×10^{-12}	16.46	SD	2.881×10^{-6}
A_{PN_f}	0.369646	7.2932×10^{-5}	1.603×10^{-2}	1.367×10^{-12}	16.47	*CV*	5.48%
Δt	5	0.16667	-4.677×10^{-6}	6.076×10^{-13}	15.85	Mean	2.338×10^{-5}
A_{N_2}	12.841462	0.37426	-1.874×10^{-6}	4.922×10^{-13}	12.84	Variance	3.834×10^{-12}
A_{PN_0}	0.366365	7.2932×10^{-5}	-1.603×10^{-2}	1.367×10^{-12}	35.65	SD	1.958×10^{-6}
A_{PN_f}	0.367823	7.2932×10^{-5}	1.603×10^{-2}	1.367×10^{-12}	35.66	*CV*	8.37%

[a] The first three columns list the experimental parameters used in the calculation of V, their nominal values, and their estimated errors (SD). The fourth column gives the value of the partial derivative of V with respect to each parameter, evaluated with the values in the second column. The absolute and relative contributions of each individual parameter to the overall variance of V are listed in the fifth and sixth columns, respectively. Finally, the rightmost column presents summary statistics for V. We repeated the suite of calculations for different dinitrogen fixation rates ($\delta^{15}PN_f$ = 400, 10, and 5‰) to illustrate the reduction in overall precision and the increased importance of error associated with the isotopic measurement at very low fixation rates.

bation bottle. We measured the volume of each sample bottle used in this study and used that volume in calculating the actual isotopic enrichment resulting from the spike. Use of the average bottle volume would result in typical errors in volume of ca. 1 to 2 ml, leading to an error of less than 1% in the calculated enrichment of the N_2 pool. Although we did not attempt to measure the volume of gas delivered by the syringe, the manufacturer (Hamilton) claims an accuracy and precision of ±1% for its syringes. We conservatively estimate that all of these factors together are unlikely to lead to an error greater than 3% (i.e., ca. 0.37 atom%) in our estimate of A_{N_2}.

Concentration of PN ([PN]). The relative internal precision of the Fisons elemental analyzer is better than 0.2% of the signal (Fisons EA1108 manual), but replicate analyses of standards typically show substantially greater variation. Daily calibration of the instrument reduces the analytical variability to less than 4% (SD), which we take as an upper limit to the error associated with the PN measurement.

Overall error in V and ρ. We used standard methods for calculating the propagation of errors (2) to assess the overall uncertainty in our estimates of N_2 fixation. We performed this calculation using realistic values for A_{N_2}, A_{PN_f}, A_{PN_0}, Δt, and [PN] along with the error estimates described above. We conservatively treated all of our error estimates as SDs, yielding upper limits for the SDs associated with V and ρ.

We conservatively estimate the precision of V to be ±4.5% (CV = SD/$\bar{X}$). For typical experiments with relatively high N_2 fixation rates, the primary sources of error in V are the uncertainties in Δt and A_{N_2} (Table 3). At very low fixation rates ($\delta^{15}PN_f$ < ca. 10‰), the overall experimental error increases to 8.4% (CV) and the analytic error associated with the mass spectrometer becomes significant (Table 3).

Because of the additional measurement of PN required in its calculation, the error associated with ρ (CV = 6.0%) is somewhat higher than the error in V (Table 4). At high N_2 fixation rates, the PN concentration measurement is the most important source of error, though Δt and A_{N_2} also make significant

TABLE 4. Contributions of different sources of error to the overall uncertainty in the value of ρ (μmol of N_2 $liter^{-1}$ h^{-1})[a]

Parameter (X)	Value	SD	$\partial\rho/\partial X$	Error contribution (SD × $[\partial\rho/\partial X]$)^2	% Total error	Summary for ρ	ρ (μmol $liter^{-1}$)
Δt	5	0.16667	-6.988×10^{-3}	1.356×10^{-6}	30.76	Mean	3.494×10^{-2}
A_{N_2}	12.841462	0.37426	-2.801×10^{-3}	1.099×10^{-6}	24.92	Variance	4.409×10^{-6}
A_{PN_0}	0.366365	7.2932×10^{-5}	-2.377×10^{-1}	3.005×10^{-10}	0.01	SD	2.100×10^{-3}
A_{PN_f}	0.511651	7.2932×10^{-5}	2.405×10^{-1}	3.076×10^{-10}	0.01	*CV*	6.01%
$[PN]_f$	30	1.2	1.165×10^{-3}	1.953×10^{-6}	44.30		
Δt	5	0.16667	-1.578×10^{-4}	6.921×10^{-10}	24.16	Mean	7.892×10^{-4}
A_{N_2}	12.841462	0.37426	-6.326×10^{-5}	5.606×10^{-10}	19.57	Variance	2.864×10^{-9}
A_{PN_0}	0.366365	7.2932×10^{-5}	-2.404×10^{-1}	3.074×10^{-10}	10.73	SD	5.352×10^{-5}
A_{PN_f}	0.369646	7.2932×10^{-5}	2.405×10^{-1}	3.076×10^{-10}	10.74	*CV*	6.78%
$[PN]_f$	30	1.2	2.631×10^{-5}	9.966×10^{-10}	34.79		
Δt	5	0.16667	-7.015×10^{-5}	1.367×10^{-10}	12.90	Mean	3.508×10^{-4}
A_{N_2}	12.841462	0.37426	-2.812×10^{-5}	1.107×10^{-10}	10.45	Variance	1.059×10^{-9}
A_{PN_0}	0.366365	7.2932×10^{-5}	-2.405×10^{-1}	3.075×10^{-10}	29.03	SD	3.255×10^{-5}
A_{PN_f}	0.367823	7.2932×10^{-5}	2.405×10^{-1}	3.076×10^{-10}	29.03	*CV*	9.28%
$[PN]_f$	30	1.2	1.169×10^{-5}	1.969×10^{-10}	18.58		

[a] Data are as explained in Table 3.

TABLE 5. Effects of experimental conditions on the limits of detection by the C_2H_2 reduction assay[a]

Exptl condition					Background C_2H_4 = 0		Background C_2H_4 = 50	
Flask size (ml)	GPV (ml)	LPV (ml)	Liquid/gas ratio	SC (25°C)	Total C_2H_4 (pmol)	N_2 fixation (nmol liter^{-1} h^{-1})	Total C_2H_4 (pmol)	N_2 fixation (nmol liter^{-1} h^{-1})
14	1	13	13.0	2.404	24	0.092	120	0.462
	4	10	2.50	1.270	51	0.254	254	1.270
	9	5	0.556	1.060	95	0.954	477	4.770
73	1	72	72.0	8.776	88	0.061	439	0.305
	23	50	2.17	1.235	284	0.284	1,420	1.420
250	5	245	49.0	6.292	315	0.064	1,573	0.321
	50	200	4.00	1.432	716	0.179	3,580	0.895

[a] We assumed an experimental temperature of 25°C and used typical combinations of flask size, gas phase volume (GPV), and liquid phase volume (LPV). For each combination, we calculated the solubility correction factor (SC) as well as the total quantity of C_2H_4 required to produce a gas phase concentration equal to the limit of detection by gas chromatography, with no background C_2H_4 or with double the background concentration of C_2H_4 (50 pmol/ml of C_2H_2). The N_2 fixation rate was calculated assuming a C_2H_2/N_2 reduction ratio of 4:1.

contributions. At lower rates, the isotope measurement error increases in importance, yielding an overall precision of ±9.3% (*CV*) at very low fixation rates. Here again, the error associated with the mass spectrometer makes a significant contribution only at very low N_2 fixation rates.

Sensitivities of the $^{15}N_2$ and C_2H_2 reduction assays. The inherent sensitivity of our tracer assay is a function of the incubation time (e.g., Δt = 5 h) and the smallest change in δ^{15}PN that we can confidently attribute to N_2 fixation. We can estimate the limit of detection for V by using equations 6 and 7a to calculate the N_2 fixation rate associated with a small change in the ^{15}N enrichment of PN. For example, if we take 4‰ as the minimum acceptable change in the δ^{15}N of PN (i.e., a change of 0.00146 in A_{PN}), then we can measure a specific N_2 fixation rate of $V = 2.046 \times 10^{-5}$ h^{-1} in a 5-h experiment (Table 3).

In contrast, the minimum detectable volumetric rate of N_2 fixation (ρ) is inversely related to the concentration of PN. Although this result is perhaps counterintuitive, it arises because our isotopic measurements provide a direct index to the specific rate of incorporation of ^{15}N into particles, $V(T^{-1})$. The conversion of this specific rate to a volumetric rate ($M \cdot L^{-3} \cdot T^{-1}$) requires multiplication by [PN], so a high PN concentration acts to dilute the isotopic signature from N_2 fixation and effectively increases the minimum rate we can measure. In our Gotland Basin experiments, PN concentrations were typically ca. 30 μmol of N liter^{-1}, yielding a limit of detection of 0.31 nmol of N_2 liter^{-1} h^{-1} (Table 4). In a more oligotrophic system, the absolute sensitivity of our method would be higher. For example, the same specific rate of N_2 fixation corresponds to a volumetric rate of 0.010 nmol of N_2 liter^{-1} h^{-1} for a sample in which [PN] = 1 μmol of N liter^{-1}. In general, the limit of detection in a 5-h experiment will be:

$$\rho_{\text{minimum, 5 h}} = 2.046 \times 10^{-5}\ \text{h}^{-1} \times \frac{[\text{PN}]}{2} \qquad (8)$$

For comparison, we can estimate the sensitivity of the C_2H_2 reduction assay on the basis of the minimum quantity of C_2H_4 typically detectable by gas chromatography, ca. 1 pmol of C_2H_4. For a gas injection of 100 μl of gas at 25°C, this corresponds to a concentration of 0.244 ppmv C_2H_4. In a typical experiment (e.g., Δt = 5 h, volume incubated = 10 ml, total bottle volume = 14 ml), followed by a headspace equilibration at 25°C, a C_2H_4 concentration of 0.244 ppmv would result from a reaction rate of 1.02 nmol of C_2H_4 liter^{-1} h^{-1}, or an N_2 fixation rate of 0.25 nmol of N_2 liter^{-1} h^{-1} (Table 5). The absolute sensitivity of the C_2H_2 reduction assay can be improved by increasing the liquid phase-to-gas phase ratio in the incubation bottle (4, 12). For example, incubating 13 ml of sample and a 1-ml gas phase would reduce the limit of detection by C_2H_2 reduction to 0.09 nmol of N_2 liter^{-1} h^{-1}. However, both these projections assume no background C_2H_4. In practice, both commercially available C_2H_2 and that prepared from CaC_2 typically contain an appreciable concentration of C_2H_4. This background will compromise the absolute sensitivity of the C_2H_2 reduction method (Table 5). For example, in the presence of a background of 1.19 ppmv C_2H_4 (50 pmol/ml), typical of commercial preparations, a rate of 1.27 nmol of N_2 liter^{-1} h^{-1} with the assay format used in this study, or 0.46 nmol of N_2 liter^{-1} h^{-1} with a volume-optimized assay, would be required to double background levels over the 5-h time course we used. Thus, in a eutrophic system like the central Baltic Sea, the $^{15}N_2$ and optimized C_2H_2 reduction assays have similar limits of detection. In a more oligotrophic system, our tracer assay has a significantly higher absolute sensitivity than the standard C_2H_2 reduction method.

Applications. An immediate and obvious application of our procedure is the direct determination of the relationship between C_2H_2 reduction and N_2 fixation rates. More often than not, the theoretical ratio (either 3:1 or 4:1; see references 4 and 26) is assumed to apply in field studies using the C_2H_2 reduction assay. While many direct comparisons have shown good agreement with theoretical C_2H_2/N_2 reduction ratios, a variety of studies have shown large deviations from the theoretical value (Table 2). Hence, where quantitative estimates of N_2 fixation are required and C_2H_2 reduction is the method of choice, direct calibrations with $^{15}N_2$ reduction are strongly recommended (4, 12). The procedures described above greatly simplify the field application of $^{15}N_2$ reduction measurements and, in fact, make the protocols for the two assays more directly comparable.

In addition to its use in calibrating the C_2H_2 reduction assay, direct measurement of $^{15}N_2$ fixation is well suited to a variety of other experimental studies. The C_2H_2 reduction and $^{15}N_2$ fixation assays actually provide information on different aspects of nitrogen fixation in aquatic ecosystems. Since C_2H_2 is a substrate analog of N_2 and is generally added at saturating concentrations, the C_2H_2 reduction assay provides an estimate of the total, or gross, activity of nitrogenase in a system. In contrast, the $^{15}N_2$ fixation assay directly quantifies the net conversion of N_2 into biomass. The minimal handling required by our protocol makes it feasible to carry out a large number of experiments at sea, either on deck or in situ. Although the

C_2H_2 reduction assay is best suited to providing real-time rate measurements and detailed time series of N_2 fixation rates, the $^{15}N_2$ tracer assay offers a number of distinct experimental advantages that complement those of the C_2H_2 reduction method. (i) The $^{15}N_2$ tracer assay provides a direct estimate of the contribution of N_2 fixation to the production of biomass by the planktonic community; no conversion factors are needed, as is the case in converting C_2H_2 reduction rates to N_2 fixation rates. (ii) The C_2H_2 reduction assay generally employs a headspace to facilitate withdrawal of a gas sample from the incubation bottle. Accounting for the partitioning of C_2H_2 and C_2H_4 between the gas and liquid phases in the system introduces additional sources of error in the calculation of C_2H_2 reduction rates. The presence of a headspace may also contribute to bottle effects, and some agitation of the sample is generally needed to equilibrate the headspace with the fluid volume. The $^{15}N_2$ tracer assay is a measurement of the movement of ^{15}N into the particulate matter within an incubation bottle; the gas volume within the bottle is minimal and the experiment is terminated by filtration. In addition, N_2 fixation by different components of the plankton can be assayed by postincubation sorting and/or size fractionation of the incubation volume. (iii) In eutrophic systems with high biomass concentrations, the sensitivity of the tracer method is comparable to that of the C_2H_2 reduction assay. In oligotrophic systems characterized by low biomass and very low concentrations of diazotrophs, the tracer method is significantly more sensitive than the C_2H_2 reduction assay.

An additional benefit of the ^{15}N tracer method is its potential for direct tracking of the movement of fixed N into other biologically active pools. For example, ^{15}N added as N_2 can be monitored in diazotrophs and subsequently in heterotrophic components of the planktonic food web. In the central Baltic, our experimental conditions produce changes on the order of 100 to 400‰ in the $\delta^{15}N$ of the bulk pool of suspended particles, a large isotopic signal that is relatively easy to follow into other biologically active pools of N. The rates of both N_2 fixation and grazing by zooplankton on diazotrophs can be measured directly through time-course measurements of the $\delta^{15}N$ of suspended particles and zooplankton incubated in the presence of $^{15}N_2$. Similarly, the recent development of methods for the isotopic analysis of dissolved organic N provides a tool for assaying the production of dissolved organic N by N_2 fixers directly (14).

Finally, the stable isotope tracer approach can be easily extended to studies of autotrophic diazotroph growth and primary production by incubating samples in the presence of both $^{15}N_2$ and $^{13}C\text{-}HCO_3^-$. A number of commercially available continuous-flow isotope ratio mass spectrometry systems are capable of measuring the isotopic composition of both C and N from a single sample, allowing coupled measurements of both N and C fluxes in diazotrophs. These experiments can also be extended to monitor the movement of diazotroph C and N into other components of the food web and may represent the most efficient experimental approach for studying the contribution of N_2 fixation to elemental cycling in planktonic systems.

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