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Nitrogen fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments

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ABSTRACT: Simultaneous assays of di-nitrogen fixation were carried out in sediments from Narragansett Bay, Rhode Island (USA) using the acetylene reduction technique and $^{15}N_2$ tracer. The ratio of moles of acetylene reduced to moles of ^{15}N recovered in ammonium and labile organic-N pools in estuarine sediments under anaerobic conditions ranged from approximately 10:1 to nearly 100:1. The ratio of acetylene reduced to ^{15}N fixed was always significantly greater than the theoretical 3:1 ratio. Rates of both acetylene reduction and $^{15}N_2$ fixation increased with the addition of labile organic carbon (as sucrose) to the sediments. Ratios of acetylene reduction:$^{15}N_2$ fixation in the sucrose-addition sediments (estimated to range between 12:1 and 27:1) were generally lower than those found in sediments without sucrose additions, but were always significantly greater than the theoretical 3:1 ratio. We caution that the use of the theoretical 3:1 ratio in assays of N_2 fixation by the acetylene reduction technique in anaerobic coastal marine sediments results in overestimates of N_2 fixation rates.

INTRODUCTION

The largest reservoir of nitrogen on earth, di-nitrogen gas (N_2), would be essentially inert in the biosphere were it not for the ability of certain bacteria and blue-green algae to reduce molecular nitrogen. These organisms contain a nitrogenase enzyme system capable of reducing the very stable di-nitrogen molecule to the amine ($-NH_2$) level, thereby introducing 'fixed' or combined nitrogen into the global nitrogen cycle. Since the productivity of many ecosystems, especially marine systems, is closely coupled to the availability of fixed nitrogenous compounds, nitrogen fixation may be an important factor contributing to the productivity of these systems. In central ocean gyres, for example, planktonic blue-green algae of the genus *Oscillatoria* supply a significant amount of fixed nitrogen to oligotrophic surface waters (Carpenter 1983). In coastal marine systems, nitrogen fixation (as measured by acetylene reduction) is reported to be an important N source in seagrass beds (Patriquin & Knowles 1972), salt marshes (Van Raalte et al. 1974, Whitney et al. 1975, Teal et al. 1979), and mangroves (Zuberer & Silver 1978), among others. For recent reviews of nitrogen fixation in marine benthic environments, see Capone (1983) and Howarth et al. (in press).

Quantitative assessment of nitrogen fixation in marine systems has been hampered by the lack of a simple, accurate assay for nitrogen fixation rates under natural or near-natural conditions. Direct determinations of fixation activity by decreases in N_2 concentrations are impossible with present technologies due to difficulties in detecting small changes in large reservoirs of N_2 gas. The same problems occur when attempts are made to detect increases in total fixed nitrogen due to N_2 fixation against the large background of living and non-living organic nitrogen present in waters and sediments. Unequivocal evidence for N_2 fixation is possible using ^{15}N-labelled N_2, but this technique is time-consuming and expensive. The simplest and most widely used method for determining N_2-fixation rates is the acetylene reduction assay. This technique is based on the finding that the nitrogenase enzyme also reduces acetylene to ethylene (Dilworth 1966, Schollhorne & Burris 1967). Numerous reviews have addressed the potential problems of the

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acetylene reduction assay (Hardy et al. 1973, Flett et al. 1976, David & Fay 1977, Buresh et al. 1980, Smith 1982, Taylor 1983, Payne 1984). Theoretically, the reduction of 3 moles of acetylene to ethylene is equivalent to the 6 electron transfer involved in the reduction of 1 mole of N_2 to ammonia. The 3:1 ratio has been widely employed to translate acetylene reduction rates into areal- or volume-based N_2-fixation rates. However, the wide range of ratios reported from concurrent $^{15}N_2$ and acetylene reduction measurements in a variety of pure cultures and natural systems demonstrates that the acetylene reduction assay should be calibrated with $^{15}N_2$ in any new system in which the assay is used. Such calibration measurements have been made in various marine environments including algal mats (Carpenter et al. 1978, Potts et al. 1978), and seagrass rhizospheres and sediments (Patriquin & Knowles 1972, Capone & Budin 1982). However, there are no reports of simultaneous measurements of acetylene reduction and $^{15}N_2$-fixation for anaerobic marine sediments.

The work described here was carried out in the context of an investigation of N cycling processes in nearshore subtidal sediments of Narragansett Bay, Rhode Island (USA). Our measurements of acetylene reduction rates in sediments from Narragansett Bay and our attempt to calibrate the acetylene reduction assay by direct measurements of $^{15}N_2$-fixation are reported here.

METHODS

Three experiments were conducted to compare rates of acetylene reduction with $^{15}N_2$ fixation. In all experiments homogenized sediment from the top 4 cm of sediment cores from Narragansett Bay was used. Sediments were collected north of Conanicut Island by SCUBA divers using plexiglass core tubing. Experiment I was carried out in December 1977 and Experiments II and III in April 1978. Detailed sediment characteristics in this area of the bay are described by Nixon et al. (1976) and Garber (1984). Briefly, the sediments are approximately 73% silt-clay and 19% sand with an organic C content of 0.61%. Pore-water ammonium concentrations in the top 4 cm are typically 100 to 250 µM. *Nepthys incisa* and *Nucula annulata* are the dominant macrofauna. Oxygen concentrations in the bottom waters of the bay are near saturation; however, below the top few mm of sediment, the pore waters are generally anoxic as indicated visually by the black sulfide coloration. Samples of sediment were either placed in 200 ml round-bottom flasks fitted with gas-tight ports or in 125 ml serum bottles fitted with rubber serum stoppers. Sediments were slurried with 15 ml seawater. Combining sediment from the top 4 cm of a number of cores and adding seawater to make a slurry destroys vertical *in situ* structure of the sediments. However, the purpose of the experiments described here was to make simultaneous measurements of acetylene reduction and $^{15}N_2$-fixation in sediment samples under the same conditions. Mixing and slurrying the sediments was done to decrease variability between samples and to facilitate a rapid and even distribution of $^{15}N_2$ or acetylene in the sediments.

Unlabelled N_2 was removed from sample flasks by flushing the slurry with a gas mixture of 20% O_2 and 315 ppm CO_2 in He (Experiment I) or 100% He (Experiments II and III). (Previous measurements had shown that incubating the sediments with oxygen had little or no effect on rates of acetylene reduction. Even when oxygen was present in the headspace over the sediments, it is likely that oxygen diffused into only the suface few mm of sediment [Seitzinger unpubl.].) The sediments were then incubated in the dark at room temperature (23 °C) with 0.1 atm acetylene or 0.25 atm of 99.9 atom % $^{15}N_2$ (Stohler Isotope Chemicals, Inc., Waltham, Massachusetts, USA). The appropriate amounts of acetylene and nitrogen gas were added to the samples with an He-flushed, gas-tight syringe after an identical volume of gas had been removed with an He-flushed, gas-tight syringe. The samples were shaken by hand initially and periodically throughout the experiments to equilibrate the gas and sediment slurry.

Experiment I. Five sediment samples (43 g wet sediment [gws] per sample) were incubated with acetylene and 3 were incubated with $^{15}N_2$ for 48 h. Using our previous estimates of acetylene reduction rates in these sediments, together with knowing the sensitivity of the mass spectrometer measurements, and assuming a ratio of 3 moles acetylene reduced to 1 mole of $^{15}N_2$ fixed, we calculated that in 48 h the amount of $^{15}N_2$ fixed as ammonium-N should have been 10 times greater than the minimum level of detection of the mass spectrometer. Ethylene production during the course of the experiment was determined by analyzing the gas phase from the acetylene-treated flasks after incubating 0, 6, 24, and 48 h. Fixation of $^{15}N_2$ was determined by measuring the incorporation of ^{15}N into ammonium and (amino acid + protein + hexosamine)-N fractions of the sediment after 48 h. The amount of ^{15}N fixed in the $^{15}N_2$-treated samples was compared to that in untreated controls.

Experiment II. The results of Experiment I indicated that incubations longer than 48 h were needed to produce detectable isotopic changes in the $^{15}N_2$-treated samples. Using data from Experiment I, we calculated that a 26 d incubation period should be sufficient. Therefore, 3 sediment samples were incubated with $^{15}N_2$ for 26 d, and then analyzed for $^{15}N_2$ fixation in the (ammonium + amino acid + protein + hexosamine)-

N pool. During this period the acetylene reduction rate was assayed in triplicate samples during Days 0 to 3, 10 to 12, and 21 to 24 from a suite of 9 sediment samples. When acetylene reduction rates were not measured, the sediment samples were incubated with He containing 0.25 atm unlabelled N_2. This was done to minimize exposure of the sediments to acetylene and to simulate conditions in the $^{15}N_2$-treated flasks during the long periods between acetylene reduction assays.

Experiment III. The effect of an additional energy source on rates of acetylene and $^{15}N_2$ reduction was examined by treating each of 9 replicate sediment slurries (30 gws + 20 ml seawater) in serum bottles with 7 ml of 0.58 M sucrose-in-seawater solution. An additional 6 replicate sediment slurries without sucrose were maintained as controls. All bottles were flushed with He for 10 min and fitted with serum stoppers. Three bottles with sucrose were incubated with 0.25 atm of $^{15}N_2$. The bottles were incubated for 6 d, after which ammonium-N and (amino acid + protein + hexosamine)-N were extracted from the sediment for $^{15}N/^{14}N$ analysis. The acetylene reduction rate was measured in the 12 remaining samples in suites of 4 bottles (2 with sucrose, 2 without sucrose) during Days 0 to 2, 3 to 5, and 0 to 5 of the $^{15}N_2$ incubation experiment. Prior to the acetylene reduction assay, the sediment samples to which acetylene was added on Day 3 were maintained during Days 0 to 3 in 0.25 atm unlabelled N_2 to simulate conditions in the $^{15}N_2$-incubation flasks.

Time course measurements of acetylene reduction. Twelve g of wet sediment were incubated with 0.1 atm acetylene in each of 21 serum bottles (38 ml) fitted with rubber serum stoppers. The samples were shaken by hand initially and periodically throughout the experiment to equilibrate the gas and sediment slurry. Time series samples of the gas phase were analyzed for ethylene concentration from 1 to 73.5 h after acetylene was added.

Controls. Control bottles consisting of seawater with and without added acetylene were assayed for ethylene production in each experiment. In addition, sediment controls without acetylene were assayed for ethylene production. Di-nitrogen in the gas phase above the water in the experimental bottles was monitored by gas chromatography (Seitzinger et al. 1980) to detect possible atmospheric contamination of the acetylene- and $^{15}N_2$-treated samples. Since the atmosphere surrounding the bottles contained 78 % N_2, increases in the N_2 content of the bottles would indicate atmospheric contamination. The sensitivity of the gas chromatographic analysis of N_2 in the headspace was sufficient to detect an increase of 2 % in the concentration of N_2. No increase was detected in any of these samples indicating that any dilution of $^{15}N_2$ by $^{14}N_2$ was negligible.

$^{15}N/^{14}N$ determinations. The biochemical pathway of N_2 fixation suggests that ^{15}N tracer originating in $^{15}N_2$ will move first into intracellular ammonium-N and then into amino acid and protein-N pools in living nitrogen-fixing organisms. From there it may move into other organisms grazing on these bacteria, be released into the pore water or enter the detrital N pool. Generally, the tracer-N may be recovered with sufficient sensitivity using techniques (e.g. Kjeldahl digestion) that extract ammonium and most forms of organic-N from the experimental system. However, the amount of nitrogen in living biomass and in recently formed detrital material in coastal marine sediments is small compared to the amount of poorly defined, and presumably refractory, humic-like organic-N compounds. Because fixed nitrogen should first appear in ammonium and the more labile organic-N forms such as amino acids, proteins and hexosamines, we extracted those components from the sediment total-N to improve our ability to detect the appearance of tracer ^{15}N.

Ammonium-N extraction. Sediment samples were shaken for 1 h at room temperature with 20 ml of 2N KC1 to extract exchangeable ammonium (Bremner 1965a) and ammonium dissolved in sediment pore waters. Ammonium-N in the extract was collected by steam distillation with MgO (Bremner 1965a) and analyzed separately (Experiment I) or combined with the other fractions (Experiments II and III).

Amino-, protein-, hexosamine-N. Proteins in the sediment were hydrolyzed into their constituent amino acids by refluxing sediment samples with 100 ml of 6N HC1 for 15 h under an atmosphere of N_2 (Whelan 1975). This procedure also converts hexosamine to ammonium (Bremner 1965b). The acid extracts were cooled to room temperature and filtered through acid-washed Whatman (GF/C) filters. Amino-N was then converted to ammonium by adjusting the pH of the hydrolyzate to 2.5 with citrate buffer and boiling it for 1 h with 0.5 g of ninhydrin (Sakata & Matsuo 1972). The hydrolyzate was made alkaline with 20 ml of 10 N NaOH and the resulting ammonium (originally present as amino-, protein-, and hexosamine-N) was steam distilled into 10 ml of boric acid indicator solution. The quantity of ammonium (which we will now refer to as 'labile organic-N' although it may also contain some fairly refractory compounds) was determined titrimetrically with 0.02 N or 0.005 N sulfuric acid using bromcresol green-methyl red indicator. After titration, the samples were acidified with 0.1 ml of 2N sulfuric acid, concentrated to a volume of about 4 ml by evaporation under a stream of ammonium-free air, and sealed in glass ampoules for $^{15}N/^{14}N$ determinations.

Reagent blanks consisting of 30 ml of artificial seawater were simultaneously run through the entire procedure. The efficiency of the acid hyrolysis was checked by comparing the nitrogen yield of bovine serum albumin (BSA) with its estimated composition. The efficiency of the amino-N to ammonium-N conversion was checked by analyzing the amino acid content of each sediment hydrolyzate using fluorescamine (North 1975) and comparing this with the ammonium recovered by the reaction with ninhydrin.

$^{15}N/^{14}N$ determinations. Ammonium-N was converted to N_2 for isotopic analysis by reaction with sodium hypobromite *in vacuo* (Bremner 1965c, Fiedler & Proksch 1975). The isotopic composition of the resulting N_2 was determined using a Micromass 602C double collector mass spectrometer. The ^{15}N content of the sample was expressed as atom% ^{15}N using the standard equation

$$\text{atom\% } ^{15}N = 100\ (2R + 1) \qquad (1)$$

where $R = {}^{14}N^{14}N/{}^{15}N^{14}N$, i.e. the ratio of ion currents produced in the mass spectrometer at mass positions 28 and 29 (Fiedler & Proksch 1975). The accuracy and precision of the mass spectrometric determination of R is a function of sample size and ^{15}N enrichment. For the instrument used here, there was an inverse relation between R and sample size at similar ^{15}N enrichments. We corrected for this effect by analyzing standards of known isotopic composition over the range of sample sizes and enrichments encountered here. Even without such corrections the difference in ^{15}N enrichment between samples exposed to $^{15}N_2$ and untreated controls must represent the amount of fixed ^{15}N when all other experimental variables (especially sample size and preparation) are constant. Coefficients of variation for R at levels similar to those encountered in this study can be expected to fall in the range of 0.1 to 0.5% (Garber 1982).

Gas chromatography. Gas samples (1 ml) from the air space above the acetylene-incubated sediment samples were injected directly into a Hewlett Packard Model 5731 gas chromatograph fitted with a flame ionization detector. The acetylene and ethylene were separated with a 3 mm × 2 m column of Poropack R at 60 °C with a carrier gas (N_2) flow rate of 25 ml min^{-1}. A standard of 10 ppm ethylene in N_2 was used to calibrate the detector. Before sampling all bottles were hand shaken for 60 s.

RESULTS

Experiment I

Rates of ethylene production ranged from 44 pmoles gws^{-1} h^{-1} during the first 6 h to 60 pmole gws^{-1} h^{-1} for sediments incubated for 2 to 4 d (Table 1). At the same time, we found no detectable increase in the ^{15}N enrichment of either the ammonium-N or labile organic-N fractions of the sediments during the 48 h incubation in the presence of $^{15}N_2$ (Table 2).

Table 1. Rates of ethylene production by Narragansett Bay sediments incubated with 0.1 atm acetylene. Incubation temperature ~23 °C. Samples were incubated with 0.25 atm $^{14}N_2$ when acetylene reduction measurements were not being made

Experiment	Incubation interval	Ethylene production[a] (pmol gws^{-1} h^{-1})	
I	0 – 6 h	46, 44	
	0–24 h	58, 58	
	0–48 h	60, –[b]	
II	0 – 3 d	67, –[b], 69	
	10–12 d	78, 62, 56	
	21–24 d	51, 67, 82	
		With sucrose	Without sucrose
III	0 – 2 d	436, –[b]	47, 43
	3 – 5 d	12.7×10^3, 17.1×10^3	108, 83
	0 – 5 d	8.2×10^3, 9.9×10^3	82, 74

[a] Each value = production rate of single sediment sample
[b] Sample lost

Experiment II

Ethylene production in Narragansett Bay sediments incubated with acetylene for various lengths of time during a 25 d period averaged 67 pmole gws^{-1} h^{-1}; we found no obvious trend in ethylene production during the course of the experiment. During the same 25 d period the average increase in the isotopic composition of ammonium plus labile organic-N fractions in the sediments was 0.01 atom% ^{15}N (Table 2).

Experiment III

Sediments supplied with sucrose as an additional source of organic carbon exhibited large increases in both acetylene reduction rates (Table 1) and $^{15}N_2$ fixation (Table 2) compared to sediment without sucrose. The rate of ethylene production in sediments with sucrose addition peaked during Days 3 to 5 of the experiment and were about 160 times greater than that found in sediments without added sucrose. At the same time, an average increase of 0.641 atom% ^{15}N was found in the ammonium plus labile organic-N fraction in sediments supplied with sucrose and $^{15}N_2$.

Table 2. Recovery of ^{15}N in ammonium and labile organic-N pools of Narragansett Bay sediment slurries before and after exposure to 0.25 atm of 99.9 atom % $^{15}N_2$

Experiment	Sample no.	Ammonium-N			N recovered (µg-at N)	Labile organic-N		^{15}N fixed (nmoles $^{15}N_2$)
		NH_4-N recovered (µg-at N)	Atom % 15 N			Atom % 15 N		
			Initial	Final		Initial	Final	
I[a]	1	6.8	0.376	0.369	1083	0.37136[c]	0.37131	< 3.5
48 h	2	5.1	0.356	0.378	999	0.37126[c]	0.37111	< 3.5
	3	5.8	0.366	0.385	970	ND	0.37150	< 3.5
	Mean ± SD	5.9 ± 0.8	0.366 ± 0.011	0.377 ± 0.009	1017 ± 59	0.37131 ± 0.00014	0.37131 ± 0.00019	
II[b]	1	Ammonium combined with labile organic-N			905	0.37110[d]	0.37418[d]	13.2
26 d	2				920	0.3713[d]	0.39389	104.4
	3				926	ND	0.37570	20.3
	Mean ± SD				917 ± 11	0.37124 ± 0.00017	0.3813 ± 0.011	
III[b]	1	Ammonium combined with labile organic-N			903	0.37230	1.052	3067
6 d with	2				934	0.37253	1.026	3220
sucrose	3				860	ND	0.926	2380
	Mean ± SD				899 ± 37	0.37241	1.013 ± 0.075	

[a] Sample = 43 g wet sediment; [b] sample = 30 g wet sediment; [c] mean of duplicate determinations; [d] mean of triplicate determinations; ND: no data

Time series measurements of ethylene production

There was no lag in the onset of ethylene production (Fig. 1). The rate of ethylene production decreased gradually up to 73.5 h.

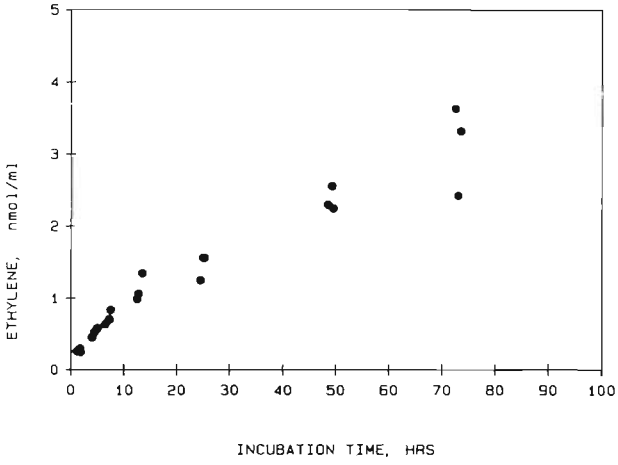

Fig. 1. Time course of ethylene production from Narragansett Bay sediment incubated with 0.10 atm acetylene. Each point represents the ethylene concentration in the incubation bottle from an individual sediment sample

Controls

No ethylene was detected in any of the samples that were not supplied with acetylene.

We estimated that the recovery of ammonium-N from protein by acid hydrolysis and reaction with ninhydrin was approximately 97 %. This calculation was based on the reported amino acid sequence (Brown 1975) and by comparing the ammonium-N yield from 23 mg of BSA (3276 µg ammonium-N) with the predicted N content of 23 mg of BSA (3377 µg N). In addition, our recovery of ammonium-N from amino acids liberated from the sediment hydrolyzates was in close agreement with the amount predicted from the fluorescamine estimate of free amino acids in the hydrolyzate. Based on the yield of ammonium-N after hydrolysis and ninhydrin reaction, the protein-N content of Narragansett Bay sediments was estimated to be approximately 0.5 mg N g^{-1} dry weight. The total organic nitrogen content of these sediments was approximately 2 mg N g^{-1} dry weight (Garber 1984); protein therefore comprised about 22 % of the sediment total organic-N.

DISCUSSION

The primary objective of these experiments was to compare acetylene reduction rates with $^{15}N_2$-fixation rates in anoxic coastal marine sediments and secondly to estimate *in situ* rates of N_2 fixation in those sediments. Our results underscore the necessity of carrying out such calibrations and the inadequacy of applying the theoretical ratio of 3 moles ethylene produced for each mole of N_2 fixed in coastal marine sediments. All 3 calibration experiments showed that this ratio was far greater than 3:1, higher, in fact, than ratios reported for other marine benthic systems (Table 3). Note however, that many of the systems listed in Table 3 are aerobic environments and, as such, may not be directly

Table 3. Mean or range of ratios of C_2H_2 reduction to N_2 fixation in various benthic marine systems

System	$C_2H_2 : N_2$ values and ranges reported	Source
Algal mats		
Salt marsh, aerobic	3.6 (average)[a]	Carpenter et al. 1978
Intertidal mud and sand flat, aerobic	4.7– 6.9[a]	Potts et al. 1978
Seagrasses		
Zostera marina		
Sediments within 3 mm of rhizome, anaerobic, glucose-amended	0.5– 6.2[b]	Patriquin & Knowles 1972
Syringodium filiforme		
Sediments within 3 mm of rhizome, aerobic	2.6–15.5[b]	Patriquin & Knowles 1972
washed rhizomes, aerobic	0.8–10.5[b]	Patriquin & Knowles 1972
Thalassia testudinum		
Sediment and macerated tissue, glucose-amended, aerobic	2 – 3.3[b]	Patriquin & Knowles 1972
Zostera marina		
Rinsed roots and rhizomes, anaerobic	1.7– 4.1[a]	Capone & Budin 1982, Capone 1983
Subtidal sediments		
Narragansett Bay, Rhode Island	12 –94	Present study
Silt-clay, anaerobic		
Sucrose-amended, anaerobic	13 –27	Present study

[a] Reported, [b] Calculated from reported data

comparable to the anaerobic muds examined here. In addition, we found that the ratio may be influenced by the availability of labile organic carbon.

In Experiment I the average rate of acetylene reduction was 53.2 pmol $(gws)^{-1}$ h^{-1}, or 110 nmol $(43\ gws)^{-1}$ $(48\ h)^{-1}$. If the ratio of acetylene reduced to $^{15}N_2$ fixed had been 3:1, then 36.6 nmol $^{15}N_2$ $(43\ gws)^{-1}$ $(48\ h)^{-1}$ would have been fixed. If all the $^{15}N_2$ fixed were in the ammonium pool, ^{15}N enrichment of this ammonium would have been 1.590 atom %. Similarly, if all the $^{15}N_2$ fixed were converted to labile organic-N at the end of the experiment, the ^{15}N enrichment in that fraction would have been 0.3784 atom % ^{15}N, very near our detection limit. However, we were unable to detect an increase of ^{15}N at the end of the 48 h incubation in either the ammonium- or labile organic-N fractions. Allowing conservative estimates for the sensitivity of the mass spectrometer of 0.03 atom % and 0.0005 atom % ^{15}N for the analyses of ammonium and labile organic-N, respectively, (the difference is due to the very small sample available for the ammonium determinations), we calculate that less than 3.5 nmoles of $^{15}N_2$-N had been fixed in the 43 g sample during the 48 h incubation. This is less than 10 % of that predicted based on the acetylene reduction rate measured and a 3:1 ratio. Using the average acetylene reduction rate in the experiment, we calculated that the ratio of acetylene reduced to $^{15}N_2$ fixed during this experiment must, therefore, have been greater than 31:1 or measurable amounts of ^{15}N would have been recovered in the sediment.

In Experiment II the average rate of acetylene reduction measured at various times thoughout the experiment was 66.5 pmol $(gws)^{-1}$, or 1245 nmol $(30\ gws)^{-1}$ $(26\ d)^{-1}$. This corresponds to 413 nmol $^{15}N_2$ fixed $(30\ gws)^{-1}$ $(26\ d)^{-1}$ if the ratio of acetylene reduced to $^{15}N_2$ fixed was 3:1. This would correspond to an ^{15}N enrichment in the ammonium plus labile organic-N pool of 0.4606 atom % ^{15}N. Actual ^{15}N enrichment measured in the ammonium plus labile organic-N pool in Experiment II were markedly lower and ranged from 0.37418 to 0.39389 atom % ^{15}N (Table 2). These increases corresponded to the fixation of between 13 and 104 nmoles $^{15}N_2$ per 30 gws during the 26 d incubation. The ratios of moles of ethylene produced to moles of $^{15}N_2$ fixed in the 3 replicate flasks were 12:1, 61:1, and 94:1.

Rates of ethylene production and $^{15}N_2$ fixation were greatly enhanced in sediments to which sucrose was added (Experiment III) indicating an energy limitation for nitrogenase activity. Enhanced acetylene reduction and N_2 fixation rates with organic carbon additions have been noted in numerous other studies (Knowles & Wishart 1977, Zuberer & Silver 1978, Capone & Budin 1982). The amount of $^{15}N_2$ fixed during the 6 d incuba-

tion with sucrose was more than 10 times that found during the 26 d incubation without sucrose. The rate of ethylene production varied in sucrose-amended sediments. However, taking the average of the values found during the 0 to 5 d incubation as a reasonable estimate of the ethylene production rate, we calculated that 39 µmoles of ethylene would have been produced by the 30 gws in 6 d. The ratio of acetylene reduced to $^{15}N_2$ fixed would therefore have ranged from 12:1 to 16:1. For comparison, similar calculations using the ethylene production rates for the 3 to 5 d incubation with sucrose gave acetylene reduced to $^{15}N_2$ fixed ratios of 20:1 to 27:1. Either way, the ratios are 4 or more times greater than the theoretical ratio of 3:1 and appear to be lower than those found in sediments without a source of labile organic carbon.

The reason for the high ratios of acetylene reduced to $^{15}N_2$ fixed in Narragansett Bay sediments is not clear. Factors that could contribute to our results include: (1) the concentration of N_2 which saturates nitrogenase; (2) transfer of $^{15}N_2$ fixed into the refractory organic nitrogen pool in the sediments; (3) depression of H_2 production in the samples incubated with acetylene; (4) increased nitrogenase synthesis in the acetylene treatments due to nitrogen starvation; (5) endogenous ethylene production; (6) ethylene production from acetylene mediated by non-nitrogen fixing organisms. Each of these possibilities is discussed below.

(1) Maximum rates of $^{15}N_2$ fixation in sandy loam (Knowles et al. 1973) and in *Anabaena* cultures (Jensen & Cox 1983) occur at a pN_2 of about 0.5 atm. The $p^{15}N_2$ in our experiments was 0.25 atm and, as such, the rates of $^{15}N_2$ fixation we measured may have been less than maximum rates. However, it is unlikely that the rates of $^{15}N_2$ fixation in our experiments were reduced by even 50% of maximum rates since half-saturation values for intact cells are in the range of 0.01 to 0.095 atm N_2 (Strandberg & Wilson 1967). At 0.25 atm N_2, Knowles et al. (1973) and Jensen & Cox (1983) found that N_2 fixation rates were about 85% of the maximum rate. If the rate of $^{15}N_2$ fixation in our experiments at 0.25 atm N_2 was similarly decreased, the measured ratio of acetylene reduced to $^{15}N_2$ fixed would have increased to only 3.5:1.

(2) We extracted ammonium and amino acids, proteins and hexosamine for ^{15}N analysis from the more refractory nitrogen compounds. It is possible that some of the $^{15}N_2$ fixed was in the refractory pool which would lead to an underestimate of the amount of $^{15}N_2$ fixed. However, most of the $^{15}N_2$ fixed would have had to be converted to refractory forms to account for the ratios measured. For instance, if the ratio of acetylene reduced to $^{15}N_2$ fixed was actually 3:1, then 85% of the $^{15}N_2$ fixed would have had to be transfered to the refractory pool for the measured ratio to be 20:1. It seems unlikely that such a high percentage of $^{15}N_2$ fixed would be lost to the refractory nitrogen pool during the relatively short course of these experiments, but the possibility cannot be entirely ruled out.

(3) Electrons are transferred by nitrogenase to reduce both N_2 to ammonium and protons to H_2 (Hardy et al. 1971). In the presence of acetylene almost all of the electrons are transferred to acetylene and H_2 evolution is depressed (Rivera-Ortiz & Burris 1975, Schubert & Evans 1976, Paerl 1982). Schubert & Evans (1976) predicted that in samples where acetylene completely suppressed hydrogen production, the ratio of acetylene reduced to N_2 fixed would be 6:1.

(4) Acetylene reduction rates often change as a function of the time of exposure to acetylene. David & Fay (1977) found that the rate of acetylene reduction gradually increased and peaked at 2 to 6 h in cell suspensions grown in nitrogen-free media. They attributed the increasing rate of acetylene reduction to de-repression of nitrogenase synthesis caused by nitrogen depletion. Higher and constant rates of acetylene reduction following a lag period of several hours to several days have been reported for some marine sediments (Teal et al. 1979, Capone & Carpenter 1982, Capone & Budin 1982). Patriquin & Knowles (1972) found that rates of acetylene reduction in some sediments decreased over the 3 to 4 d incubations they used. If the rate of acetylene reduction changes during an incubation, the rate of nitrogen fixation predicted will vary with the time period chosen for the calculations. We carried out time course measurements of acetylene reduction ranging from 1 to 73.5 h in Narragansett Bay sediments (Fig. 1). No lag period was found, but rates of acetylene reduction did decrease gradually with time. If lag periods reflect increased nitrogenase synthesis due to nitrogen starvation, the lack of a lag period may reflect the absence of nitrogen starvation. This seems likely since concentrations of ammonia in pore waters of these sediments are generally 100 to 250 µM (Garber 1982). The rates of acetylene reduction we measured over periods of 2 to 3 d during the ^{15}N experiments may actually be low; shorter incubation times (3 to 6 h) would have led to even higher ratios of acetylene reduced to $^{15}N_2$ fixed.

$^{15}N_2$ fixation rates might be expected to change due to containment and isolation of samples for fairly long periods of time: incubation times with $^{15}N_2$ ranged from 48 h to 26 d. While we were not able to make time series measurements of $^{15}N_2$ fixation, we did make repeated measurements of acetylene reduction rates throughout the experiments. The acetylene samples were incubated with 0.25 atm $^{14}N_2$ during the days when acetylene reduction was not measured to simulate conditions in the $^{15}N_2$ incubated samples as closely as possible. If, indeed, acetylene reduction rates are a

measure of N_2 fixation, then changes in $^{15}N_2$ fixation during the course of the experiment should be reflected in changes in acetylene reduction rates. Acetylene reduction rates stayed fairly constant throughout Experiments I and II, suggesting no change in $^{15}N_2$ fixation rates during the experiments. In Experiment III, samples were incubated with sucrose and acetylene reduction rates (and $^{15}N_2$ fixation rates) did increase probably due to increased bacterial growth with the added organic carbon source.

(5) Many species of bacteria can produce ethylene in the absence of acetylene (Primrose 1979). However, ethylene can also be oxidized in the absence of acetylene and thus measurements of endogenous ethylene production in control sediments without added acetylene may underestimate the gross rate of ethylene production. In the presence of acetylene, ethylene oxidation is blocked, thus leading to an overestimation of acetylene reduction rates (DeBont 1976, Witty 1979). Thus, while we found no accumulation of ethylene in the absence of acetylene, it is possible that there was a substantial amount of endogenous ethylene production that was not oxidized but accumulated in the presence of acetylene and that lead to a gross overestimation of acetylene reduction rates. Experiments using ^{14}C-acetylene are required to substantiate this.

In summary, a combination of all of the above factors may have contributed to the anomalous ratios found in Narragansett Bay sediments. However, it seems unlikely that undersaturation of nitrogenase with N_2, depression of H_2 production, nitrogen starvation, or the production of refractory organic nitrogen compounds were significant contributors. The contribution of endogenous ethylene production to the ratios reported here remains unresolved. Another unanswered question concerns the production of ethylene from acetylene by non-nitrogen fixing organisms. We were unable to find any evidence suggesting the occurrence of this process in anaerobic sediments. In fact, we do not know the exact reason for the high ratios of ethylene production to $^{15}N_2$ fixation. However, our data clearly demonstrate that N_2 fixation did indeed occur in these sediments and, regardless of the cause, the amount of $^{15}N_2$ fixed was considerably less than what we would have predicted based on the measured rates of acetylene reduction.

Depth profiles of acetylene reduction rates measured in Narragansett Bay sediments indicate that approximately 5 µmoles ethylene m^{-2} h^{-1} are reduced in the top 10 cm during summer. Applying the range of conversion factors found in the above calibration experiments, 12:1 to 94:1, gives a range of N_2-fixation rates of 0.8 to 0.1 µg-at N m^{-2} h^{-1}, respectively. Regardless of which conversion factor is used, the N_2-fixation rates are small and account for less than 1 % of the inputs of N to Narragansett Bay from precipitation, riverine and sewage inputs (~100 µg-at N m^{-2} h^{-1}; Nixon 1981).

We conclude that the ratio between the rate of ethylene production in the presence of acetylene and the rate of N_2 fixation in coastal marine sediments under anoxic conditions can be highly variable and significantly different from the theoretical 3:1 molar ratio. Use of the theoretical ratio to convert rates of acetylene reduction to N_2 fixation rates for anoxic coastal marine sediments may result in significant overestimates of the magnitude of N_2-fixation occurring in coastal marine sediments.

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