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CHAPTER 61

NITROGEN FIXATION RATES IN ANAEROBIC SEDIMENTS DETERMINED BY ACETYLENE REDUCTION, A NEW ^{15}N FIELD ASSAY, AND SIMULTANEOUS TOTAL N ^{15}N DETERMINATION

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INTRODUCTION

The acetylene reduction assay technique has been used extensively for detecting the presence of nitrogenase activity in a variety of aquatic environments (Peterson and Burris, 1976). Advantages of the method include its sensitivity, the portable and inexpensive apparatus required, and the rapid analysis of samples using gas chromatography (Hardy *et al.*, 1973). This method is generally preferred to the use of ^{15}N-enriched N_2, even though a number of investigators have stressed the importance of carrying out experiments with acetylene and $^{15}N_2$ simultaneously to obtain appropriate conversion factors (Burris, 1974; Peterson and Burris, 1976). However, it is now apparent that the validity of the acetylene reduction assay technique may be questionable when studying certain anaerobic environments, particularly those where bacterial communities using methane and lower hydrocarbons are present (Oremland and Taylor, 1975; de Bont, 1976a, 1976b; de Bont and Mulder, 1976).

After preliminary studies of several blue-green algae and photosynthetic bacteria communities along the northern coastline of West Germany, it was decided to develop methods for the simple and reliable measurement of nitrogen fixation rates in these semi-anaerobic and anaerobic populations.

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The following account describes a simplified $^{15}N_2$ reduction assay technique and a new method of estimating $^{15}N_2$ uptake by the direct combination of a CHN-O analyzer, gas chromatograph and mass spectrophotometer. Data are presented on conversion factors obtained by simultaneous estimations of acetylene and $^{15}N_2$ reduction, in laboratory-grown axenic cultures of blue-green algae and, also, in field populations.

METHODS

Algae

The heterocystous blue-green alga *Nostoc carneum* Agardh was used for laboratory experiments (Oldenburg Collection No. 02).

Nutrient Media

The nutrient solution of Allen and Arnon (1955) was used in modified form; major elements were used at one-quarter concentration; trace elements (minus vanadium) in full concentration; Fe (as EDTA complex) at 4 mg l^{-1}; K_2HPO_4 at 0.348 g l^{-1}, autoclaved separately.

Laboratory Culture

Axenic cultures were obtained by repeated streaking of algal suspensions on solid media (1% agar), and then isolation and transfer of single hormogonia into liquid media.

Stock culture aliquots of 0.1 ml were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of sterile liquid media. The flasks were then enclosed in a large clear polyethylene bag; the end was sealed around a rubber bung through which two glass tubes passed (inlet/outlet). The bag was flushed for five minutes with a gas mixture of 95% N_2/5% CO_2, sealed and placed in a light incubator such that the flasks received a light regime of 760 x 10^{17} Q m^{-2} sec^{-1} (Quanta Spectrometer, Techtum Instruments, Sweden), and a temperature of 32°C. The bag was flushed once daily (5 minutes) with the gas mixture.

After 7 days the contents of each flask was centrifuged at 3000 rpm (10 minutes), washed with sterile medium, re-centrifuged, resuspended in 100 ml sterile medium, and left at 32°C for 30 minutes. This procedure was repeated twice, and finally the centrifuged material from all flasks were combined in a single flask containing 100 ml of sterile medium. This flask was incubated under the above conditions for 24 hours.

Field Populations

In situ experiments were carried out at an intertidal mud- and sandflat on the southern coast of Wangerooge Island, 8 km off the German North Sea coast. The blue-green alga *Microcoleus chthonoplastes* Thuret forms a "Farbstreifen-Sandwatt" community (Schulz, 1936), together with the photosynthetic bacteria *Thiopedia* sp. and *Chromatium* sp. A detailed description of this community, together with physicochemical data on the sediments will be presented elsewhere (Krumbein, Potts and Rongen, in preparation).

Generation of ^{15}N-enriched N_2

Theory

The theoretical basis of the method is that relatively pure $^{15}N_2$ may be generated by the action of a concentrated solution of hypobromite (alkaline) with an ammonium salt containing ^{15}N.

$$2\ {}^{15}NH_3 + 3\ NaBrO \rightarrow 3\ NaBr + {}^{15}N_2\uparrow + 3\ H_2O$$
$$(\text{or } LiBrO \rightarrow LiBr)$$

Although this reaction is well-known and has been implemented by a number of investigators (for references, see Burris, 1974), the apparatus used has often been complex, fragile and expensive (*e.g.*, Porter and O'Deen, 1977). In the present method, vacutainers are used as reaction chambers in which to generate $^{15}N_2$.

Practical Applications

Two milliliters of a solution of $(^{15}NH_4)_2SO_4$ (27 g l^{-1}; 97.7% ^{15}N, MN-524 (Merck, Sharp and Dohme, Montréal), was injected with a gastight syringe into a 5-ml draw vacutainer (Becton and Dickinson, cat. no. 3206 U). This was followed by the injection of 1 ml of a concentrated hypobromite solution consisting of 40-ml NaOH solution (480 g l^{-1}), 50-ml KI solution (1.8 g l^{-1}), 10 ml Br_2. The vacutainer was then immediately inverted and shaken vigorously to ensure complete mixing of the reactants. After the effervescence had subsided, 100 µl of the gas phase in the vacutainer was removed with a gastight syringe to check the purity of the $^{15}N_2$ (See p. 757). This reaction was carried out a number of times in separate vacutainers to provide sufficient $^{15}N_2$ for assays (see p. 756, $^{15}N_2$ Reduction Assay).

To minimize any contamination with $^{14}N_2$ during the reaction, both the $(^{15}NH_4)_2SO_4$ and hypobromite solutions were

kept in 100-ml glass bottles, fitted with perforated screw caps and serum liners, and flushed with 99.9% Ar. All gastight syringes were flushed with Ar prior to use. The use of a concentrated alkaline solution ensured the absorption of any nitrogen oxides formed during the reaction.

$^{15}N_2$ Reduction Assay

Laboratory Assay

An attempt was made to develop an assay method which enabled a direct comparison with the acetylene reduction assay technique (see page 758, Acetylene Reduction Assays). Glass serum bottles (7 ml) with perforated serum caps were used for all incubations, and gases were introduced or removed through the rubber serum seal by gastight syringe.

Two-milliliter aliquots of the algal suspension from the Laboratory Culture (see page 754) were pipetted into the bottles and allowed to equilibriate for one hour. The caps were then secured and each bottle flushed with 30 ml of a gas mixture of 95% Ar/5% CO_2. Replicates were prepared for light and dark incubations and, in addition, several were used to check the purity of gases and temperature changes during the experiment.

After flushing with the Ar mixture, the bottles were allowed a further incubation period of 15 minutes. From separate vacutainers (see page 755, Practical Applications), 1 ml of $^{15}N_2$ was then taken with a gastight syringe (Ar-flushed), and injected into each bottle. One reaction chamber vacutainer was used for a single incubation bottle. The partial pressure of $^{15}N_2$ in each bottle was approximately 20%, the same as the pC_2H_2 used in the acetylene reduction assays (see page 758, Acetylene Reduction Assays). The bottles were then incubated for 2 hr at 32°C, under a light intensity of 760×10^{17} Q m^{-2} sec^{-1}. At the end of the incubation period, 100 µl of the gas phase in each bottle was removed with a gastight syringe (Ar-flushed) and analyzed using a mass spectrophotometer (see page 757, Laboratory Assay). The caps were then removed and the contents of each bottle immediately vacuum dried at 50°C. When completely dry, approximate 1-mg aliquots from each bottle (weighted to ± 0.1 µg, Sartorius Electronic balance) were analyzed as described in the Field Assay section below.

Field Assay

The field assays were essentially the same as those used in the laboratory. Circular cores 5-mm deep were taken of the Farbstreifen-Sandwatt sediment and transferred carefully to the 7-ml serum bottles. Filtered sea water was then

added to bring the final volume of sediment plus sea water to about 2 ml. Assays were terminated by a 0.5-ml injection of concentrated formaldehyde. The bottles were then inverted to seal any puncture holes and returned to the laboratory for immediate analysis.

Purity of Gases

All gases and gas mixtures used above were of spectroscopy-grade quality (Messer. Griesheim GmbH). A number of experiments were carried out to check the purity of these gases and to also check sources of error in handling and transfer of gas phases.

Measurement of Total N and $^{15}N_2$ Uptake

Quality of $^{15}N_2$ and Percent Excess in Gas Mixtures

One-hundred-µl gas samples were removed from reaction chamber vacutainers as well as incubation bottles and analyzed using a Varian MAT 111 Gas Chromatograph/Mass Spectrophotometer (GC/MS) system with accessory Varian Mat Kompensograph and Oscillofil. Masses 28, 29 and 30 were measured.

Analysis of Algal and Sediment Samples

A new method was developed and tested not only for the generation of $^{15}N_2$ but also for measuring the total N and ^{15}N content of each sample simultaneously, using a direct combination of a Carlo-Erba CHN-O Elemental Analyzer 1104 and the Varian MAT 111. Each 1-mg sample was pyrolyzed at 1060°C under addition of oxygen. The gases (N_2, CO_2 and H_2O) were separated by GC (Poropak Q) and the chromatograms were integrated on an automatic integrator-calculator. The outlet of the GC channel of the CHN-analyzer was directly connected by means of stainless steel capillaries (1/16 in. outer diameter) and a simple swagelok adapter to the slit separator of the Varian MAT GC/MS system. The carreer gas flow was 30 ml min^{-1} helium. We measured the masses of 28, 29 and 30, respectively, at a scanning speed of 100 masses sec^{-1}. For a single sample, we recorded at least 10 mass spectra. The error was ± 0.02% at ^{15}N concentrations of 0.5%.

Thus for each mg dry weight of the sample, it was possible to measure directly and without further manipulation of the gases (a) total N and (b) the masses 28, 29 and 30. This system was easily disconnected so that the normal gas analyses could be carried out as described in Quality of $^{15}N_2$ and Percent Excess in Gas Mixtures. No Kjeldahl N-analyses and no gas vacuum line was thus needed.

Acetylene Reduction Assays

The method given by Potts and Whitton (1977) was used, modified to the incubation times and partial gas pressures as described previously. For field assays, controls were run with Ar-flushed sediments, as well as those not flushed. In addition to dark controls, replicates were incubated with DCMU (7 μM), and 0.5-ml additions of glucose solution (100 g l^{-1}).

Ethylene and acetylene were measured in 1-ml samples using a Varian Series 3700 Gas Chromatograph-CDS 111 Data system. The chromatographic column was packed with carbon molecular sieve (CMS 60/80, Serva International) and maintained at 190°C. The FID detector was operated at 170°C. Nitrogen was used as carrier gas, at a flow rate of 40 ml min^{-1}.

Pigment Analyses

Samples of the Farbstreifen-Sandwatt community were collected for pigment analysis. Pigments were extracted in 95% methanol using the methods given in Potts (1977). Chlorophyll *a* and bacteriochlorophyll *a* were both estimated.

RESULTS

Table I shows the purity of the $^{15}N_2$ generated in vacutainers and, also, after transfer to a second or third vacutainer. Each value is the mean of five readings.

Table I
The Purity of Generated $^{15}N_2$ in Vacutainers

Reaction Vacutainer	Transfer to 2nd Vacutainer
84.5% ± 0.8%	75.5% ± 0.8%
82.0% ± 0.8%	75.5% ± 0.8%
82.6% ± 0.8%	76.0% ± 0.8%

The percentage purity of $^{15}N_2$ as measured on the mass spectrophotometer (MS) is shown in Table II, and compared when the gastight syringe was flushed with Ar prior to removing a gas sample or not flushed before sampling. A comparison of the Ar purity (contamination with air) from several different sources is shown in Table III.

Table II
Effects on the Purity of $^{15}N_2$ by Ar Flushing of Gastight Syringes

$^{15}N_2$% in Initial Vacutainer	Transfer to MS Syringe Ar Flushed	Transfer to MS Not Ar Flushed
85.3% ± 0.8%	83.0% ± 0.8%	60.0% ± 0.8%

Table III
The Purity of Argon Used in Experiments

Direct From Cylinder	From Football Bladder	From Glass Bottle[a] (to over pressure)
99.9% ± 0.5%	84.5% ± 3.6%	95.5% ± 0.7%

[a]Used in field assays.

The results of all acetylene and $^{15}N_2$ reduction assay experiments with *Nostoc carneum* are given in Table IV, and a set of representative $^{15}N_2$ data in Table V. Total N is for samples analyzed on the CHN-O analyzer, rates have been expressed as n*M* N_2 fixed per mg N/hr.

Table VI shows the mean chlorophyll *a* and bacteriochlorophyll *a* concentration in four different areas of surface sediment, in the region of *in situ* C_2H_2 and $^{15}N_2$ reduction assay experiments. A summary of these rates is shown in Table VII. Simultaneous rates of C_2H_2 and $^{15}N_2$ reduction in the light for the *M. chthonoplastes*, *Thiopedia* sp., *Chromatium* sp. community are compared in Table VIII.

DISCUSSION

Two electrons are required to reduce C_2H_2 to C_2H_4, and six for the reduction of N_2. This relationship has persuaded many authors to adopt the theoretical conversion factor of one-third N_2 reduced per acetylene reduced, when expressing nitrogen fixation rates in terms of acetylene reduced. However, experimentally determined factors appear to vary between different experimental systems; Hardy *et al.* (1973) summarized much of the literature and gave a range in factors between 2 and 25. Deviations from the theoretical value of 3 are explained in terms of the greater solubility

Table IV
Simultaneous Acetylene and $^{15}N_2$ Reduction Assays with *Nostoc carneum*

Assay	n	Total N in Aliquot (mg)	Average n*M* C_2H_4 (mg N^{-1} h^{-1})	Average n*M* N_2 (mg N^{-1} h^{-1})	Conversion Factor $(C_2H_4/N_2)^a$
1	5	0.0194	301	83.77	3.64
2	5	0.0193	355	70.18	5.06
3	6	0.0270	220	69.72	3.16
4	5	0.0291	183	75.02	2.44
5	6	0.0211	448	73.14	6.13

[a]Mean conversion factor = 4.1 [n]number of N and ^{15}N-replicate measurements.

Table V

Representative Data from an Initial $^{15}N_2$ Reduction Assay Experiment with *Nostoc carneum*

Aliquot wt (mg)	Total N (mg)	%N	[a]atom % ^{15}N excess	Total N Fixed (μg)	n*M* N_2 Reduced (mg N^{-1} h^{-1})
0.8950	0.01771	1.97	0.0761	0.02657	53.6
0.6350	0.01198	1.89	0.0998	0.02276	67.9
0.5510	0.00899	1.63	0.0957	0.01708	67.9
0.8690	0.01696	1.95	0.0894	0.03053	64.2
0.6580	0.01174	1.78	0.0767	0.01761	53.8
0.5730	0.01081	1.79	0.1208	0.02594	85.7
0.6060	0.01015	1.67	0.0966	0.01929	67.8
0.9380	0.02036	2.17	0.0751	0.03054	53.5
0.6610	0.01216	1.84	0.0659	0.01582	46.4
0.8230	0.01645	1.99	0.0712	0.02302	50.0

[a]Corrected for enrichment of $^{15}N_2$ gas used, and naturally occurring ^{15}N in samples.

1.3250	0.14178	10.7	0.0000 (atom % ^{15}N = 0.360)[b]	

[b]Control sample from a culture grown in the presence of combined nitrogen.

Table VI
Concentrations of Chlorophyll *a* and Bacteriochlorophyll *a* from the Surface Sediments of the Farbstreifen-Sandwatt Community of *M. chthonoplastes*, *Thiopedia* sp. and *Chromatium* sp.

Area	n	$\bar{x}$ µg chl *a* cm^{-2}	$\bar{x}$ µg Bchl *a* cm^{-2}	Ratio
a	4	145	12.2	11.9:1
[1]b	4	184	12.4	14.8:1
c	4	245	18.3	13.4:1
d	4	123	18.3	6.7:1

[1]Experimental area.

Table VII
Simultaneous Acetylene and $^{15}N_2$ Reduction Assays in the Surface Sediment of the Farbstreifen-Sandwatt Community of *M. chthonoplastes*, *Thiopedia* sp. and *Chromatium* sp.

Assay	Average Total N (mg)	L nM C_2H_4 (mg N^{-1} h^{-1})	D nM C_2H_4 (mg N^{-1} h^{-1})	G nM C_2H_4 (mg N^{-1} h^{-1})	DCMU nM C_2H_4 (mg N^{-1} h^{-1})	L nM N_2 (mg N^{-1} h^{-1})
1	2.373	687	____	514	856	146
2	2.189	745	____	557	928	130
3	1.476	1104	442	826	1376	204
4	1.303	1250	____	936	1560	182
5	2.769	589	515	440	734	126
6	2.009	811	____	607	1011	168

L = light assay; D = dark assay; G = with glucose in light; DCMU = with DCMU in light.

Table VIII
Conversion Factors Obtained from the Comparison of C_2H_2 and $^{15}N_2$ Reduction Rates in the Surface Sediment of the Farbstreifen-Sandwatt Community

n*M* C_2H_4 mg N^{-1} h^{-1}	n*M* N_2 mg N^{-1} h^{-1}	Conversion Factor (C_2H_4/N_2)[a]
637	146	4.71
745	130	5.73
1104	204	5.41
1250	182	6.87
589	126	4.67
811	168	4.83

[a]Mean rate = 5.37.

of acetylene in water, and the depression of H_2 production in the presence of acetylene (Hardy and Burns, 1971; Benemann and Weare, 1974). As a result, experimentally determined factors are closer to 4 (Burris, 1974). In a study of natural populations of lake phytoplankton (*Aphanizomenon* sp., *Anabaena* sp., *Gloeotrichia* sp.) Peterson and Burris (1976) found a conversion factor of 4.4 (4.2-4.8). This is close to the factor of 4.1 found in the present study with axenic laboratory-grown cultures of *Nostoc carneum*. The rates of acetylene reduction found for this species are similar to those found by Stewart (1967.1968), for *N. muscorum* and *Nostoc* sp., *i.e.*, 0.100 n*M* C_2H_4 mg dry wt^{-1} min^{-1}, 0.152 n*M* C_2H_4 mg dry wt^{-1} min^{-1} and 0.144 n*M* C_2H_4 mg dry wt^{-1} min^{-1}, respectively. The latter two rates have been calculated assuming a factor of 0.08 for N to dry weight, although as Table V shows, the percentage N in laboratory cultures of *N. carneum* was closer to 2%, suggesting the cultures to be nitrogen-starved before the commencement of assays.

Significant rates of C_2H_2 and $^{15}N_2$ reduction were detected in the *Microcoleus chthonoplastes*, *Thiopedia* sp., *Chromatium* sp. community, associated with anaerobic marine sediments. The rates of acetylene reduction are similar to those found by Potts and Whitton (1977) for a Farbstreifen-Sandwatt community dominated by the nonheterocystous blue-green alga *Hyella balani*, in the intertidal zone of the lagoon of Aldabra Atoll, Indian Ocean. The rates found by these authors for communities of *M. chthonoplastes* in similar

habitats were somewhat lower than those of the present study; however, these authors carried out assays without first flushing with Ar. It is not possible to state with certainty which of the dominant species in the Farbstreifen-Sandwatt community is responsible for the observed rates of nitrogen fixation. The greater abundance of *M. chthonoplastes* as demonstrated by microscopic study and pigment analysis (Table VI), as well as depression of acetylene reduction rates in the dark and insignificant or no stimulation of rates in the presence of glucose, indicates that nitrogen fixation is mostly associated with this species. The somewhat higher rates observed in sediment samples incubated with DCMU, also suggest that nitrogen fixation was stimulated by lower oxygen levels, and in part possibly due to photosynthetic bacteria. However, Gallon *et al.* (1975) have shown in studies with a species of *Gloeocapsa* (nonheterocystous blue-green alga), that levels of DCMU which completely inhibited oxygen production had little effect on the ability to reduce acetylene, and reduction in the light in the presence of DCMU was approximately 30% greater than that in the dark. Recently, Cohen *et al.* (1975) demonstrated that the nonheterocystous filamentous blue-gree alga *Oscillatoria limnetica* Lemmerman could use H_2S as the sole electron donor in the photoassimilation of CO_2 with photosystem I, when photosystem II was inhibited with DCMU. At Wangerooge Island, E_h measurements from the Farbstreifen-Sandwatt community ranged from +295 mV at the surface, to -232 mV just below the layer of photosynthetic bacteria. In addition, the sediment had a strong smell of H_2S. It is therefore possible that the nonheterocystous *M. chthonoplastes* is able to use H_2S as an electron donor and to assimilate CO_2 and maintain rates of acetylene reduction when incubated in the presence of DCMU.

Another possible explanation of our field fixation data may be that in the interior of *Microcoleus* bundles, lower photosynthetic activity coupled with higher nitrogenase activity occurs comparable to the specialization between normal cells and heterocysts in heterocysteous blue-green algae. This on the other hand would imply material transfer between different trichomes. These assumptions remain speculative until further experiments have been carried out with this community. (See also Krumbein and Cohen, 1977).

The conversion factor determined by simultaneous C_2H_2 and $^{15}N_2$ light reduction assays in the *M. chthonoplastes* community was 5.4, significantly higher than that found in laboratory experiments with *Nostoc carneum* using the same assay methods. This shows that a factor of 3, if applied to this system, would give a substantial error (80%)and overestimate rates of N_2 fixation.

There appear to be no reports of simultaneous C_2H_2 and $^{15}N_2$ reduction assays with anaerobic marine sediments, yet these are essential in view of the increasing interest in the study of these environments. Oremland and Taylor (1975) have shown that acetylene can inhibit methanogenesis in some marine sediments, yet they were unable to state if this would over- or underestimate nitrogen fixation rates. A number of recent papers also question the validity of the acetylene reduction technique when applied to these systems (*e.g.*, de Bont and Mulder, 1975).

The main facts we recollect from the study of literature and our own preliminary laboratory and field data concerning the question of estimating N fixation in the laboratory and in nature have already been summarized by Burris (1974) and Peterson and Burris (1976). Burris (1974) wrote: "Remarkably few have felt compelled to determine a proper conversion factor."

The present work was meant to clarify the situation that many data are presently based on field acetylene reduction assays, and converted into nitrogen fixation values per volume or surface unit by using arbitrary and theoretical conversion factors. Using the more rapid method of direct combination of N-determination with the mass determination of ^{15}N-compounds and the rapid and reliable field method of generating $^{15}N_2$ directly in the field, we were able to compare acetylene reduction rates with $^{15}N_2$ reduction and incorporation rates by measuring both the changes in the gas phase and the relative masses of N within the samples at the same time. The laboratory data and the field data we collected are reproducible and in agreement with the few reports on factors obtained in laboratory experiments using pure cultures and field samples. This suggests that within the limits of the method, our field data on anaerobic sediments are also reliable. The conversion factors of Burris and Peterson (1976) from an aerobic plankton community ranged from 2.7 to 6.5 (C_2H_2 reduced/N_2 reduced). Our field data on an anaerobic sediment with its complicated aspects of inhibition and/or enhancement of acetylene reduction and fluctuating environmental conditions range from 4.67 to 6.87 (C_2H_2 reduced/N_2 reduced). Besides naturally occurring influences on the rates, the greatest source of error in the simplified $^{15}N_2$-assay is contamination of the $^{15}N_2$ with $^{14}N_2$. However, provided a standard technique is followed in flushing syringes, incubation bottles and argon containers, this can be kept to a minimum. The new method of estimating total N and $^{15}N_2$ uptake simultaneously and of generating $^{15}N_2$ in the field is accurate, rapid (one sample every ten minutes in the CHN-O analyzer-MS combination) and much easier to use than the Kjeldahl method combined with MS.

The problem lies elsewhere. As has been discussed, several sources of error exist in the use of direct or theoretical conversion factors in transferring acetylene reduction data to N fixation. This inconvenience is further increased in anaerobic sediments (Oremland and Taylor, 1975; de Bont and Mulder, 1976). Though our conversion data obtained in the laboratory and field experiments, respectively, varied even less than in other reported comparisons of the two methods, we conclude that accurate conversion factors cannot be given so far even if linearity is observed in the two assay methods (Peterson and Burris, 1976). The conversion "factors" reported in literature vary from 2-25, while our conversion rates in laboratory and field experiments varied much less than that and were always higher than the theoretical value of 3. Therefore, it still seems better to calibrate the acetylene reduction assay in the laboratory and in the field with a few parallel assays using the ^{15}N method.

A conversion factor of 4.1 or 5.4, as in our study, is still more meaningful than the theoretical value which is almost never reached in the field. On the other hand, it is necessary to further refine the field assay described here as well as the new methods described for simultaneous N and ^{15}N determination. In environments in which it is essential to quantify the nitrogen cycle it may be advisable to use the new ^{15}N method exclusively instead of the environmentally influenced conversion rates.

SUMMARY AND CONCLUSIONS

Two new methods for the simultaneous measurement of acetylene reduction and ^{15}N reduction are presented here:

1. a new laboratory and field assay method for simultaneous C_2H_2 reduction and $^{15}N_2$ reduction by generating $^{15}N_2$ directly in the field with simple equipment; and

2. a new method for estimating total N simultaneously with $^{15}N_2$ uptake rates using a direct on-line combination of a CHN-O Elemental Analyzer with gas chromatography and mass spectrometry.

Simultaneous rates of acetylene reduction and $^{15}N_2$ reduction were measured by using these methods with laboratory-grown axenic culture of *Nostoc carneum* and an anaerobically growing field population of *Microcoleus chthonoplastes*. The conversion "factors" obtained were 4.1 and 5.4, respectively. Simultaneous estimations of the acetylene reduction and $^{15}N_2$ reduction are considered essential in the study of N_2 fixation in semi-anaerobic and anaerobic environments with populations

of photosynthetic prokaryotes associated with chemoheterotrophic anaerobic prokaryotes. In order to be able to differentiate between the latter, it is also necessary to use glucose to enhance heterotrophic activity, DCMU to block oxygenic photosynthesis and to analyze for biomass and chlorophylls of the photosynthetic prokaryotes present in the environment.

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