Marine Biology 24, 109—119 (1974)

Nitrogen Fixation in the North Pacific Ocean

T. H. Mague, N. M. Weare and O. Holm-Hansen

Institute of Marine Resources, University of California, San Diego; La Jolla, California, USA

Abstract

Nitrogen fixation in the euphotic zone of the ocean was measured by C_2H_2 reduction and $^{15}N_2$ incorporation associated with *Trichodesmium* sp. and also with *Richelia intracellularis* occurring within the cells of *Rhizosolenia styliformis* var. *longispina*, and *R. cylindrus*. The vertical distribution of N_2 fixation activity, N_2-fixing species, particulate matter and dissolved nutrients was measured. The effects of light intensity, sample concentration, length of incubation, and nutrient enrichment on the rates of C_2H_2 reduction were determined. Estimates of the importance of N_2 fixation in adding previously uncycled nitrogen to the euphotic zone are given.

Introduction

In the marine environment, nitrogen is generally considered to be a limiting nutrient for the growth of phytoplankton (Institute of Marine Resources, 1971; Ryther and Dunstan, 1971), and can be a crucial factor determining productivity. Biological nitrogen fixation provides a source of new combined nitrogen for the nitrogen cycle. Consequently, organisms capable of nitrogen fixation have the potential for significantly affecting the nitrogen budget of their surroundings. However, the extent of biological nitrogen fixation in the oceans and its importance as a source of combined nitrogen in the euphotic zone is poorly understood. Some information is available on the rates and seasonal variation of nitrogen fixation in the supralittoral to sublittoral zones of sea-shores by bacteria in sediments (Brooks *et al.*, 1971; Patriquin and Knowles, 1972) and rock-encrusting blue-green algae (Stewart, 1964). Fixation by benthic blue-green alga on coral reefs has also been described (Bunt *et al.*, 1970, Johannes *et al.*, 1972). However, nitrogen fixation in coastal regions is not likely to affect the combined nitrogen levels in the upper mixed layer of deep oceanic water.

In the open ocean, bacteria capable of growth on agar medium with no added nitrogen have been found (Kawai and Sugahara, 1971), but the low populations of these bacteria (0 to 200 cells/l) and the possibility that they may have grown on contaminating combined nitrogen in the agar suggests that fixation by bacteria is relatively unimportant. However, little is known about *in situ* bacterial fixation, and conflicting reports exist (Pshenin, 1963; Maruyama *et al.*, 1970).

The organisms most likely to fix significant amounts of nitrogen in the ocean are the planktonic blue-green algae. These algae, some of which frequently occur in blooms, have generally been associated with tropical and sub-tropical waters (Baas Becking, 1951). Most physiological and ecological studies deal with the conspicuous *Trichodesmium* (= *Oscillatoria*; see Sournia, 1970) or *Richelia*, but more than 20 genera of blue-green algae are known in the marine phytoplankton. Nevertheless, studies of nitrogen fixation by blue-green algae in the open ocean have described only two genera: the filamentous, non-heterocystous *Trichodesmium* (Dudgale *et al.*, 1961, 1964; Goering *et al.*, 1966) and the filamentous, heterocystous *Dichothrix fucicola*, epiphytic on pelagic *Sargassum* (Carpenter, 1972).

Richelia intracellularis, a short-chain filamentous blue-green alga, is reported to be epiphytic, endophytic, or free-living; however, all but the endophytic forms are considered exceptional (Sournia, 1970). *R. intracellularis* occurs most frequently in the diatom *Rhizosolenia*, of which 10 species are reported to contain *R. intracellularis* as an endophyte (Sournia, 1970). Although *R. intracellularis* usually possesses terminal heterocysts, a morphological feature associated with the ability to fix nitrogen (Fay *et al.*, 1968), its capacity for nitrogen fixation has not been tested.

This paper describes nitrogen fixation associated with a dense population of *Rhizosolenia styliformis* var. *longispina* and *R. cylindrus* in the central North Pacific Ocean in June, 1972. The significance of this fixation to the overall nitrogen requirement of the phytoplankton in the area is discussed. C_2H_2 reduction activities for *Trichodesmium* are also reported.

Materials and Methods

For routine screening for the presence of N_2-fixing phytoplankton, samples were collected either in a 20 cm diameter, 35 μm mesh net towed vertically through the water column from 150 m depth, or in a 35 cm diameter, 153 μm mesh net towed obliquely through the water from the surface to 30 m and back to the surface. Oblique tows through the 30 to 60 m layer always collected fewer nitrogen-fixing organisms

than the surface to 30 m tow. For collecting large quantities of *Rhizosolenia* for physiological studies, a set of paired 0.5 m diameter, 102 μm mesh nets were towed vertically from 150 m to the surface. In all cases, the material was collected as a concentrated suspension in plastic cod-ends attached to the nets.

The suspended material from the net tows was either used directly, or concentrated by gentle filtration (maximum pressure differential of 125 mm Hg) onto 25 mm diameter GF/C glass-fiber filters (Whatman). For the depth-profile study, water samples were obtained in 30 l, PVC Niskin-type sampling bottles and concentrated by filtering 4 or 8 l aliquots through 47 mm diameter GF/C glass-fiber filters.

One ml of suspension or 1.0 ml of filtered seawater plus filter were put into 7 ml glass serum bottles and capped with septum-type, skirted rubber stoppers. One ml of purified grade C_2H_2, scrubbed of acetone by bubbling through water, was injected into the bottle with a disposable-type hypodermic syringe, and the excess pressure was released by piercing the stopper with a syringe needle. The resulting pC_2H_2 was approximately 0.14 atm. Removal of N_2 from the gas phase of the incubation vessel is not necessary when 0.1 atm or more of C_2H_2 is used (Stewart *et al.*, 1971).

Samples were incubated on deck in clear plastic boxes filled with flowing surface seawater and fitted with neutral-density filters calibrated in percent transmission of incident light. Except for the light-intensity study, all samples were incubated under a 50% transmission filter.

After 1 to 2 h incubation, 0.5 ml samples of the gas phase in each bottle were analyzed in a Varian model 1400 flame ionization gas chromatograph fitted with a 2 mm × 2 m column of Porapak N (Waters Associates, Inc.) held at 100 °C with N_2 as carrier gas. Control bottles were always included which contained filtered seawater or sample material killed with saturated $CuSO_4$. C_2H_4 production was quantitated by comparing peak heights, corrected for control background, to a standard curve prepared from known dilutions of C_2H_4 in air. The gas chromatograph was calibrated before each set of analyses with a reference C_2H_4-air mixture.

For time-course studies, 20 ml of sample suspension were put into a 120 ml glass bottle and closed with a septum type stopper. Ten ml of air were withdrawn from the bottle, and 15 ml of C_2H_2 were then injected. At the desired time intervals, 0.5 ml samples of the gas phase were withdrawn for analysis.

To measure $^{15}N_2$ incorporation, samples were prepared as for C_2H_2 reduction, but the 7 ml bottles were flushed with a gas mixture containing 80% Argon, 20% O_2, and 0.03% CO_2. Two milliliters of the gas phase were then withdrawn, and 2.0 ml of 95 atom % $^{15}N_2$ were added with a hypodermic syringe to give a pN_2 of approximately 0.35 atm. $^{15}N_2$ was stored in a glass displacing flask fitted with a septum stopper for use on board ship (Burris, 1972). After incubation, samples were fixed with saturated $CuSO_4$ and the bottles were sealed with a self-curing silicone rubber compound. Upon return to the laboratory, the gas phases were analyzed for ^{15}N by mass spectrometry. The entire sample was digested by a micro-Kjeldahl procedure, and an aliquot was taken for ammonium analysis by a modified indophenol method. The remaining sample was distilled and converted with alkaline hypobromite to N_2 for mass spectrometry (Burris, 1972). N_2 fixation was calculated from the isotopic enrichment of the phytoplankton sample by the method of Stewart (1967).

For productivity measurements, the phytoplankton suspensions used for C_2H_2 reduction measurements were diluted 25-fold with seawater and incubated with 20 μc $H^{14}CO_3^-$ in 250 ml glass bottles for 3 h. The material then was filtered onto 25 mm glass-fiber filters, washed with filtered seawater, transferred to scintillation vials containing a toluene-based fluor solution, and counted on a liquid scintillation counter.

Particulate matter in the water samples was analyzed for chlorophyll *a* by fluorometry of 90% acetone extracts of filter-concentrated material (Holm-Hansen *et al.*, 1965), for nitrogen by the micro-Kjeldahl method of Holm-Hansen (1968), and for ATP by the luciferin-luciferase method of Holm-Hansen and Booth (1966). Urea was determined by the urease method of McCarthy (1970), and other dissolved nutrients were analyzed according to Strickland and Parsons (1968).

Results

The stations sampled for nitrogen-fixing activity are shown in Fig. 1 and the activities are listed in Table 1. Significant C_2H_2 reduction occurred in samples of net-tow material in which *Trichodesmium* was observed, and the specific activity increased more than 10-fold when colonies of *Trichodesmium* were carefully picked out and re-suspended in filtered seawater. The highest rates of C_2H_2 reduction were associated with samples abundant in *Rhizosolenia styliformis* and *R. cylindrus* containing *Richelia intracellularis*. *Rhizosolenia* was first encountered in abundance at Station 7, and active nitrogen fixation and abundant *R. styliformis* were found until June 19. From June 19 to June 28 other sampling operations were conducted north of Station 9 and, while *R. styliformis* was frequently observed in vertical net hauls through the upper 150 m, no C_2H_2 reduction measurements were made. At Station 10 on June 28, only a few fragments of *Rhizosolenia* were found in a layer at 40 to 50 m with little accompanying C_2H_2 reduction. However, a few *Trichodesmium* colonies of high specific activity were collected in a vertical net tow at Station 10.

Actively-fixing *Rhizosolenia* was again found in abundance in the upper 65 m of water at Station 12, but was not found further south at Station 13. During a

Table 1. *Distribution of nitrogen-fixation activity along transect from San Diego, California, to central North Pacific Ocean, 1972*

Station no.	Date	Type of sample	nM C_2H_4/μg chlorophyll *a*/h	Organisms responsible
1	June 8	Assorted net tow	0	—
2	June 9	Assorted net tow	0.30	?
3	June 10	Assorted net tow	0.35	?
4	June 11	Assorted net tow	0.35	*Trichodesmium*
5	June 12	Assorted net tow	0.47	*Trichodesmium*
5	June 12	Selected from net	6.64	12 *Trichodesmium* colonies
6	June 14	Selected from net	7.89	12 *Trichodesmium* colonies
7	June 15	Assorted net tow	17.5	*Rhizosolenia*
8	June 16	Assorted net tow	90.4	*Rhizosolenia*
9	June 17	Assorted net tow	40.2	*Rhizosolenia*
9	June 17	Assorted net tow	17.7	*Rhizosolenia*
9	June 17	Assorted net tow	21.8	*Rhizosolenia*
9	June 18	Filter concentrate	6.97	*Rhizosolenia*
9	June 19	Assorted net tow	52.9	*Rhizosolenia*
10	June 28	Filter concentrate	0.41	"Dying" *Rhizosolenia*
10	June 29	Filter concentrate	1.21	"Dying" *Rhizosolenia*
10	June 29	Selected from net	30.4	3 *Trichodesmium* colonies
11	June 30	Assorted net tow	0.67	?
12	July 2	Assorted net tow	31.4	*Rhizosolenia*
13	July 3	Filter concentrate	0.02	?

second sampling transect from Honolulu, Hawaii, to 25° S, 155° W and thence to Papeete, Tahiti, from July 10 to August 14, 1972, 28 vertical and oblique 150 μm mesh tows through the upper 150 m failed to

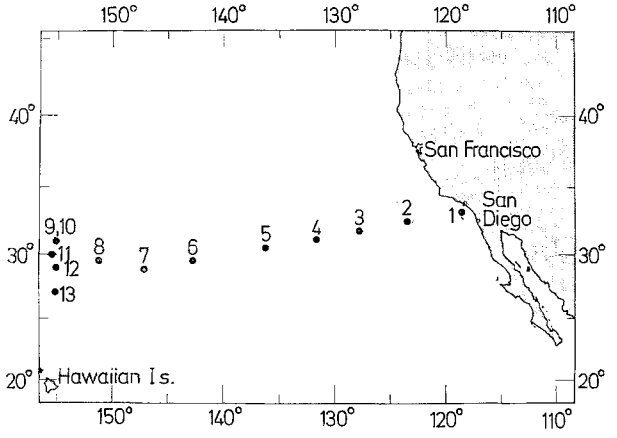

Fig. 1. Stations sampled for acetylene reduction measurements during cruise "CATO I" of the R.V. "Melville", June 8 to July 5, 1972

detect any *Trichodesmium* or *Rhizosolenia*, and there was no C_2H_2 reduction by samples of the net-tow material.

The depth profile of C_2H_2 reduction activity at Station 9 on June 18 is shown in Fig. 2A. Maximum activity occurred at 25 m, and is clearly separate from the chlorophyll maximum at 50 m (Fig. 2B). The distribution of *Rhizosolenia* containing *Richelia intracellularis* (Fig. 2C) was obtained at a station 10 miles north of Station 9 but, even here, the layer of *Rhizosolenia* lies within the zone of active C_2H_2 reduction. Although the distribution of phytoplankton species varied from station to station, *Rhizosolenia* spp. were much less abundant than other centric diatoms (*Hamiaulus hauckii* reached a maximum of 4000 cells/l at 40 m), and the phytoplankton maximum (217,000 cells/l) occurred at 60 m. Fig. 3 depicts the typical occurrence of *Richelia intracellularis* within the *Rhizosolenia* cell. *Richelia intracellularis* was observed in one or both ends of the diatom, usually as a pair of filaments, but occasionally singly. The basal heterocyst was always oriented towards the pointed tip of the diatom cell, although heterocysts in each end of a filament or two adjacent, basal heterocysts were rarely seen. *Rhizosolenia* spp. obtained during June appeared to be senescent and were often devoid of cytoplasm (Fig. 3A). In those few instances when metabolically-active cells were observed, chloroplasts streaming with the *Rhizosolenia* cytoplasm were seen to pass above and on either side of the *Richelia intracellularis* filament, indicating that the latter is located within the cytoplasm of the diatom.

The particulate carbon, nitrogen, and phosphorus in the water column at Station 9 were highest at the depth of maximum nitrogen-fixation activity; however the maximum biomass (adenosine triphosphate, ATP) coincided with the chlorophyll peak where phyto-

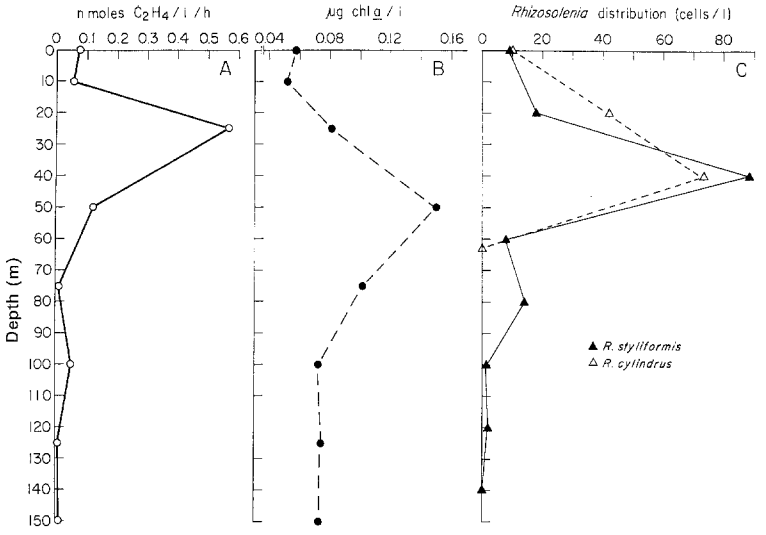

Fig. 2. Depth profile from Station 9, June 18, 1972. (A) Acetylene reduction by discrete water samples; (B) chlorophyll *a* concentration in the water column; (C) distribution of *Rhizosolenia* cells containing *Richelia intracellularis*

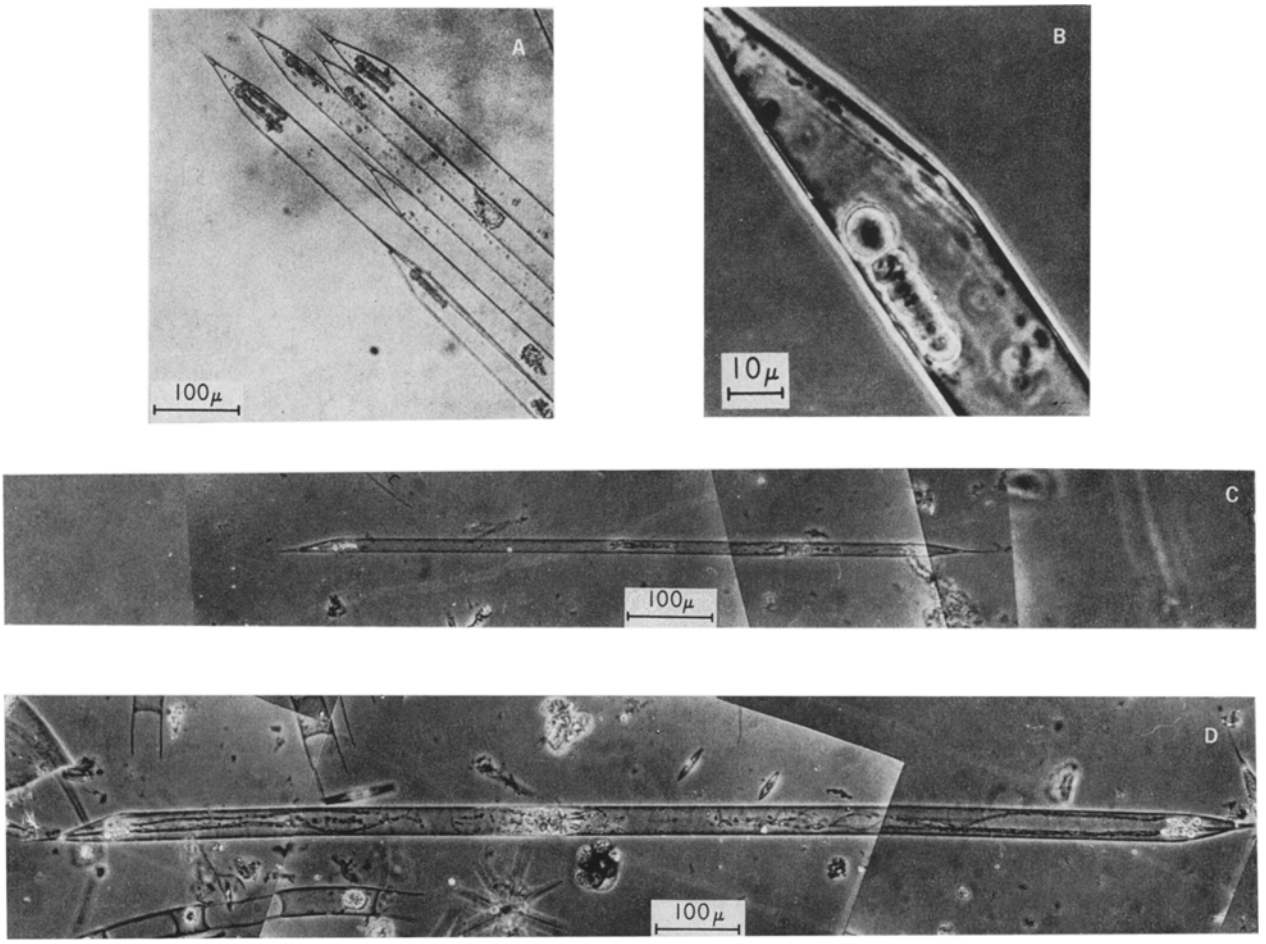

Fig. 3. *Rhizosolenia styliformis* var. *longispina* containing *Richelia intracellularis*. (A) Several cells clumped together to form "raft" — a typical configuration for samples collected from the area where active nitrogen fixation occurred (live material). (B) Higher magnification of the section of the diatom containing *Richelia intracellularis*. The terminal heterocyst is clearly visible as the enlarged cell at the top of the short chain of vegetative cells (formalin-preserved material). (C) and (D) Entire cells of *Rhizosolenia styliformis* showing variability in size of the diatom. Note that the larger cell contains more *Richelia intracellularis* (formalin-preserved material)

plankton were most abundant (Table 2). The total particulate carbon values vary considerably with depth, perhaps due to the presence in the sample of an occasional zooplankter. The large size of the *Rhizosolenia* cells prevents the use of the usual 150 μm mesh-netting pre-filter (Holm-Hansen *et al.*, 1966) to remove large debris. Concentrations of nitrate, nitrite, and phosphate in the upper 100 m at this station were all extremely low (less than 0.036 μM) or below the limit of detection (Table 3). Ammonium and urea, on the other hand, were considerably higher (0.014 to 0.433 μM) and may represent the only sources of combined nitrogen readily available for phytoplankton growth (Carpenter *et al.*, 1972). However, these concentrations of combined nitrogen must be insufficient to satisfy the nitrogen requirement of the phytoplankton population, since the nitrogenase enzyme is not synthesized under conditions of surplus fixed nitrogen.

Table 2. *Particulate material in water column for C_2H_2 reduction depth-profile of June 18 (Station 9)*

Depth (m)	Carbon (μg/l)	Nitrogen (μg/l)	Phosphorus (μg/l)	ATP (ng/l)
0	35.3	3.99	0.620	73
10	29.1	4.35	0.55	107
25	46.5	8.30	0.871	79
50	35.7	5.51	0.708	115
75	12.8	4.17	0.464	99
100	40.9	4.17	0.460	29
125	10.1	3.04	0.237	19
150	7.9	1.41	0.189	22

Table 3. *Dissolved nutrients for C_2H_2 reduction depth-profile of June 18 (Station 9). All concentrations are in μM/l*

Depth	NO_3^-	NO_2^-	PO_4^{---}	NH_4^+	Urea
0	<0.01	<0.01	<0.01	0.112	0.049
10	0.014	<0.01	0.03	0.196	0.260
25	0.019	<0.01	<0.01	0.056	0.367
50	0.019	<0.01	0.02	0.014	0.220
75	<0.01	<0.01	0.02	0.014	0.433
100	0.036	<0.01	<0.01	0.033	0.293
125	0.017	<0.01	0.06	0.161	0.179
150	0.635	0.018	0.08	0.077	0.027

Fig. 4. illustrates the need for short incubation times when using the C_2H_2 reduction assay. While the reaction is linear for up to 2 h (Curve A), activity declines soon after this. Crowding of organisms as a result of concentration may result in rapid loss of activity. However, the set of data in Fig. 4 show that the redilution of a sample does not increase specific activity. It is, therefore, likely that the measurements of C_2H_2 reduction activity by net-concentrated samples are indicative of *in situ* behavior. However, preparing samples by concentration onto glass-fiber filters caused a loss of activity (Table 4), probably as a result of breakage of the long and fragile *Rhizosolenia*.

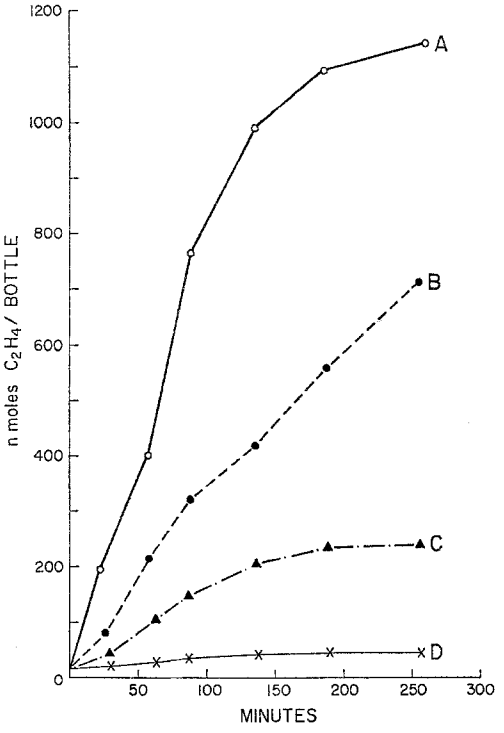

Fig. 4. Time course of acetylene reduction by 20 ml samples of net-concentrated *Richelia intracellularis*/*Rhizosolenia* spp. in the light. A: 13.8 μg, B: 6.9 μg, C: 3.5 μg, and D: 0.69 μg chlorophyll *a* per bottle

Table 4. *Rhizosolenia spp. Effect on activity of concentrating samples on glass-fiber filters*

Sample	nM C_2H_4/ml sample/h	
	Experiment I	Experiment II
1 ml net-tow material	0.140	0.176
11 ml net-tow material concentrated to 1 ml on filter	0.040	0.029

C_2H_2 reduction by *Rhizosolenia* is markedly light-dependent (Fig. 5). The sample darkened for 30 min before injection of C_2H_2 showed no significant reduction activity until the assay bottle was re-illuminated (Fig. 5A). C_2H_4 production then started within 7 min

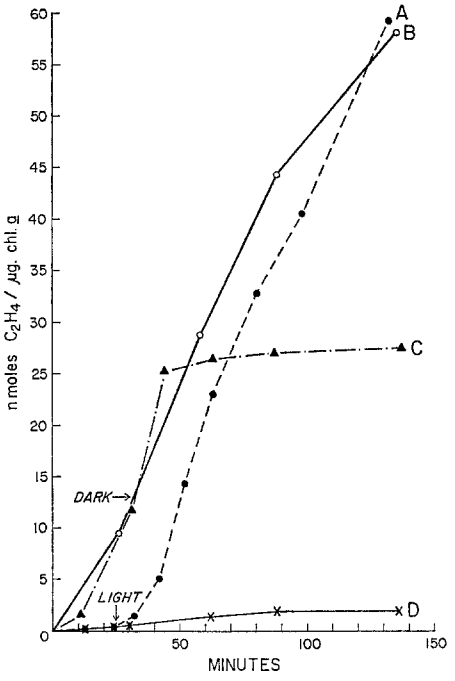

Fig. 5. Time course of response of acetylene reduction by *Richelia intracellularis*/*Rhizosolenia* spp. to light. A: Light after 25 min dark exposure; B: continuous light exposure; C: darkened after 33 min light exposure; D: continuous dark exposure. Light source was a high-intensity incandescent bulb providing 1.3×10^{16} quanta/sec/cm^2 between 400 and 700 nm at surface of incubation vessel

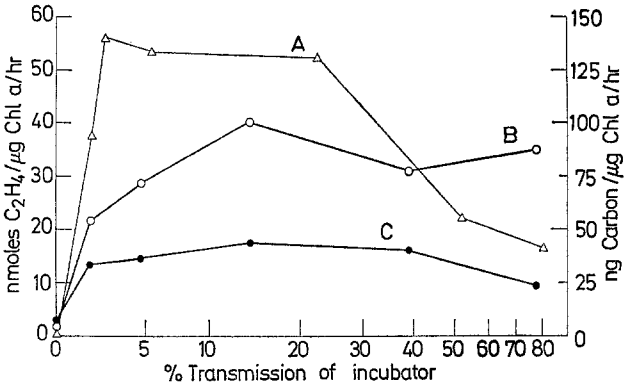

Fig. 6. Effect of light intensity on acetylene reduction and carbon fixation by net-concentrated samples of *Richelia intracellularis*/*Rhizosolenia*. A Net incorporation of ^{14}C into particulate material; B ethylene production, first experiment; C ethylene production, second experiment. Average incident sunlight on incubator was 1.3×10^{17} quanta/sec/cm^2 between 400 and 700 nm

and, after 17 min, had resumed the initial rate of the illuminated sample (Fig. 5B). The sample initially assayed in the light and darkened after 33 min (Fig. 5C) continued reduction at the initial rate for another 11 min before activity abruptly ceased. A sample darkened throughout the experiment showed very low C_2H_4 production (Fig. 5D).

Fig. 6 shows the effect of varying light intensity on C_2H_2 reduction activity and photosynthetic $^{14}CO_2$ assimilation by *Rhizosolenia*. $^{14}CO_2$ uptake saturated at a lower intensity than C_2H_2 reduction, and was more severely inhibited at surface light-intensities. The material used in this experiment was collected in a vertical net tow from 150 m to the surface, so that it contained both light-adapted and dark-adapted cells. C_2H_2 reduction by this mixed population was highest at a light intensity 15% of that at the surface, an intensity which corresponds to a depth of 22 m. The depth profile experiment performed the next day (Fig. 2A), showed maximum C_2H_2 reduction activity at 25 m. This indicates that the cells were maximally adapted to fix nitrogen at the ambient light levels and were not rapidly circulating through the upper layers of water. Difficulty was experienced in maintaining the proper orientation of the 78% transmittance filter for the samples shown in Fig. 6, Curve B, so the experiment was repeated (Curve C), and it was shown that inhibition of acetylene reduction does continue at high light-intensities.

Table 5. *Rhizosolenia spp. Effect on activity of adding phosphate to samples*

µM PO_4^{---} added	Average nM C_2H_2/µg chlorophyll *a*/h
0	21.8
0.5	49.6
1.0	41.5
2.0	39.4
5.0	52.8
10	37.0
20	29.7
50	19.1

When samples of *Rhizosolenia* were enriched with orthophosphate 0.5 h before assaying for C_2H_2 reduction, activity was doubled by the addition of 0.5 to 5 µM phosphate but, at concentrations above 5 µM, activity declined and, at 50 µM, returned to the initial rate (Table 5).

To obtain a valid conversion factor for extrapolating C_2H_2 reduction to actual N_2 fixation, several parallel sets of samples (containing primarily *Rhizosolenia* spp.) exposed to either C_2H_2 or $^{15}N_2$ were assayed under identical conditions, except that the $^{15}N_2$ samples were generally incubated longer to increase isotope incorporation (Table 6). In only two experiments were the molar ratios of C_2H_2 reduced to N_2 fixed close to the theoretical ratio of 3.0:1 (based on a 6 e^- transfer for the conversion of N_2 to $2NH_3$ and a

Table 6. $^{15}N_2$ *fixation rates and* C_2H_2*:*N_2 *ratios in phytoplankton samples containing primarily Rhizosolenia spp.*

Experiment no.	Date (1972)	Number of replicates	Hours of incubation[b]	Average μg N fixed/mg sample N/h	Average nM N_2 fixed/sample/h	Average nM C_2H_2 reduced/sample/h[a]	Ratio C_2H_2:N_2
1[c]	June 16	2	4	0.073	0.23	12.9	56
2[d]	June 16	3	4	0.336	0.32	4.59	14
3[e]	June 16	3	4	0.539	0.88	3.42	3.9
4	June 19	5	2.2	0.286	0.907	11.6	13
5	June 29	2	11	0.023	0.0079	0.091	12
6	July 2	4	1.8	0.643	1.93	6.44	3.3

[a] In Experiments 1, 2, and 3, phytoplankton concentrations are not equal, so activities are not comparable.
[b] Refers to $^{15}N_2$ incubations only; C_2H_2 samples were incubated 1 to 2 h.
[c] Contained no nutrient additions.
[d] Contained 5 μM additional PO_4^{---}.
[e] Contained 15 μM additional PO_4^{---}.

2 e^- transfer for the reduction of C_2H_2 to C_2H_4). Specific rates of N_2 fixation calculated from ^{15}N incorporation ranged from 0.023 to 0.643 μg N fixed/mg sample N/h. However, the total sample included non-fixing phytoplankton which dilute the isotope enrichment of the nitrogen-fixing organisms. The rate of uptake of $^{15}N_2$ by blue-green algae and mixed phytoplankton samples under constant illumination has been shown to be constant for at least 30 h (Dugdale *et al.*, 1959; Stewart, 1967), but this has not been tested for marine phytoplankton. Our measurements of ^{15}N incorporation may be in error if N_2 fixation by *Rhizosolenia-Richelia* is as short-lived as C_2H_2 reduction (Fig. 4).

Discussion

Nitrogen fixation by planktonic organisms is a process extremely variable in space and time (Goering *et al.*, 1966; Horne and Goldman, 1972). The present study likewise shows the patchy distribution of active nitrogen-fixing phytoplankton in the central North Pacific Ocean during June, 1972, and the virtual absence of fixation along a subsequent transect through the South Pacific Ocean. In a large section of the eastern North Pacific, low rates of fixation were found, suggesting that the total amount of nitrogen added to the euphotic zone by fixation may be relatively unimportant for the total population of phytoplankton, but may be important for sustaining small numbers of the nitrogen-fixing phytoplankton. Under favorable conditions (as yet unknown) the phytoplankton proliferate to form the blooms of *Rhizosolenia* reported here, and of *Trichodesmium* such as that studied in the Sargasso Sea (Dugdale *et al.*, 1961). Sequential observations of C_2H_2 reduction activity at the same location (Stations 9 and 10) suggest that the bloom of *Rhizosolenia*, when first observed on June 17, was very active, but 10 days later had subsided; the small fragments in the 40 to 50 m layer (just above the thermocline) were probably disintegrating cells which had settled from the zone of maximum C_2H_2 reduction.

Certain considerations are important for meaningful C_2H_2 reduction assays. It is evident that the assay is only linear for approximately the first 2 h incubation. *Trichodesmium* colonies disintegrated within a few hours in assay bottles, and most of the C_2H_2 reduction activity was lost after 1 h. The cause for the loss of activity in the *Rhizosolenia* samples is not clear, for the cells were not visibly damaged after confinement for several hours. However, the eventual depletion of CO_2 and the build-up of O_2 in a confined, photosynthesizing sample would be expected to favor photorespiration (Jackson and Volk, 1970) which competes with nitrogen fixation for reductant and, thus, may lower the apparent rate of fixation (Lex *et al.*, 1972).

Other investigators have employed longer incubation times, ranging from 6 h (Carpenter, 1972) to 38 h (Dugdale *et al.*, 1964). Although longer incubation times are necessary to realize sufficient isotope enrichment when using $^{15}N_2$ as a tracer, one risks underestimating N_2-fixing potential by using excessive in cubation times and assuming constant activity. When the sensitive C_2H_2 reduction assay is employed, a 1 h incubation period should be sufficient, and the effects of prolonged confinement can be avoided.

There appears to be no dramatic effect from the crowding of *Rhizosolenia* during net concentration as a result of mutual shading or nutrient depletion, since the activity was approximately proportional to the phytoplankton concentration in each sample (Fig. 4). However, concentration of such large and fragile cells as *Rhizosolenia* on a filter pad evidently results in damage and decreased activity (Table 4). The light requirement for C_2H_2 reduction and the speed of the response to changes in illumination (Fig. 5) are important factors to consider when comparing data from successive stations. The interrelationship of nitrogen fixation and photosynthesis has been established by Fogg and Than-Tun (1960) in studies of *Anabaena*

cylindrica cultures. The strong inhibition of CO_2 fixation by high light-intensities (Fig. 6) is similar to that observed in primary-productivity measurements (Steele, 1964). High light-intensities also inhibit C_2H_2 reduction. This effect, which has been described for fresh-water phytoplankton (Horne and Fogg, 1970; Granhall and Lundgren, 1971) is not surprising, since photosynthetic energy and reductant required by nitrogen fixation may be diverted to photorespiration which is stimulated by high light-intensities (Lex *et al.*, 1972). Estimates of *in situ* rates of fixation are necessarily based on ambient, and therefore variable, light levels. Our results suggest that comparative measurements should be made under constant illumination at intensities which do not limit either carbon or nitrogen fixation. The inhibitory effect of high light-intensities should also be considered in making measurements of N_2 fixation when a deck incubator is to be used; some samples of *Rhizosolenia* used in this study lost all activity after 30 min exposure to full midday sunlight, even though surface sea-water temperatures were maintained.

The rapid response of C_2H_2 reduction to light suggests that *in situ* N_2 fixation would closely follow the relative intensity of incident light. Thus, the use of a 10 h day at constant fixation activity for calculating daily N contribution may be a close approximation in 14 h summer days. The low rates of C_2H_2 reduction observed in the dark (Fig. 5) may be due to insufficient dark-pre-incubation to deplete photosynthetic reductant and energy reserves (Weare and Benemann, 1973). In addition, a slow rate of dark N_2 fixation may exist (Fay, 1965), supported by organic substrates present in the seawater or the diatom cytoplasm. Dark samples of fresh-water phytoplankton sometimes fix as much N_2 as light samples (Horne and Fogg, 1970).

Simultaneous measurements of N_2 incorporation and C_2H_2 reduction in the literature give widely variable ratios for these two substrates. C_2H_2:N_2 reduction ratios vary from 0.54 to 8.4 in legume nodules, 2.8 to 3.6 in laboratory cultures of blue-green algae, and 2.5 to 6.0 in cell-free extracts of bacteria (Stewart *et al.*, 1968; Fisher and Brill, 1969; Jeng *et al.*, 1969; Bergersen, 1970; Mague and Burris, 1972; Hardy *et al.*, 1973). The ratios obtained from North Pacific phytoplankton (Table 6) are quite high and variable. We have selected a ratio of 10 as representative of the majority of samples.

A possible explanation for this variation is suggested by Experiments 1, 2 and 3 in Table 6, where one large phytoplankton sample was enriched with two different concentrations of orthophosphate. Unenriched samples had a C_2H_2:N_2 ratio of 56, whereas 5 μM phosphate decreased the ratio to 14, and 15 μM phosphate nearly attained the theoretical ratio of 3.0. C_2H_2 reduction, unlike $^{15}N_2$ incorporation, is a measure of enzyme activity under ambient conditions, and the product of the reduction, C_2H_4, is released from the enzyme surface to diffuse out of the cell. In contrast, the fixation of ^{15}N into cellular materials requires the utilization of NH_4^+, the enzyme product. There is no evidence for product inhibition of nitrogenase, but if phytoplankton growth is limited by insufficient phosphate, a readily available source of nitrogen may not be utilized. With excess phosphate, the full potential capacity of the enzyme system, as measured by C_2H_2 reduction, may be realized for N_2 fixation. Hardy *et al.* (1973) have reviewed several lines of evidence for suspecting that C_2H_2 and N_2 do not behave identically as enzyme substrates. Clearly, more information is needed to properly evaluate this possibility.

Despite the apparent low rate of N_2 reduction, the specific activities of fixation, up to 0.643 μg N/mg N/h, compare favorably to specific activities of planktonic blue-green algae in eutrophic lakes (0.003 to 1.9 μg N/mg N/h; Dugdale and Dugdale, 1965; Horne and Fogg, 1970), of *Trichodesmium* from the tropical Atlantic Ocean (0 to 1.38 μg N/mg N/h; Goering *et al.*, 1966), and of *Dichothrix* epiphytic on *Sargassum* (0 to 16 μg N/mg N/h; average, 4.4; Carpenter, 1972).

Lack of phosphate appears to limit even potential nitrogenase activity (Table 5), since phosphate enrichment of *Rhizosolenia* samples caused an increase in acetylene reduction. A similar effect was observed with phosphorus-starved cultures of *Anabaena flos-aquae* (Stewart *et al.*, 1970), in which 2 μM phosphate gave maximum stimulation of C_2H_2 reduction. We observed the maximum response between 0.5 and 5.0 μM phosphate, while 50 μM seemed to approach an inhibitory effect. This is reasonable, considering the extremely low ambient levels of phosphate (often less than 0.01 μM) in the upper mixed layer in the central North Pacific Ocean.

The depth profile (Fig. 2) allows estimation of actual *in situ* rates of fixation in relation to the total phytoplankton biomass. The maximum activity observed (0.565 nM C_2H_4/l/h) is equivalent to 1.6 ng N incorporated/l/h (assuming a C_2H_2:N_2 ratio of 10) or 16 ng N/l/10-h day. This agrees well with values obtained by Goering *et al.* (1966) for *Trichodesmium* in the Atlantic Ocean (0 to 7.9 ng N/l/h; average, 1.65), although at one station they reported rates of 323 ng N/l/h for a concentrated surface bloom of this alga. From Table 2, the total particulate nitrogen at 25 m was 8.30 μg N/l. Assuming a specific growth rate of 0.02 to 0.1 doublings/day (J. Sharp, unpublished data) fixation could supply 2 to 10% of the total nitrogen requirement. In addition, Eppley *et al.* (1973) found carbon assimilation rates of up to 50 μg C/μg chlorophyll *a*/12-h day for near-surface phytoplankton. From Fig. 2, nitrogen assimilation rates range up to 6.97 nM C_2H_4/μg chlorophyll *a*/h, or 0.195 μg N/μg chlorophyll *a*/day. Assuming a C:N assimilation ratio of 5:1 (Eppley *et al.*, 1973), fixation would supply approximately 2% of the daily nitrogen requirement. Although the average C:N ratio in the particulate

material in the water column at Station 9 was 6.2, the data of Fig. 6 show maximum C and N assimilation rates of 132 ng C/μg chlorophyll *a*/h and 28.5 nM C_2H_4/μg chlorophyll *a*/h, which is equivalent to a ratio of 1.65:1; this indicates greater nitrogen fixation than would be necessary for the concommitant carbon uptake. However, the carbon assimilation is much lower than the 2.7 μg C/μg chlorophyll *a*/h reported for North Pacific phytoplankton by Eppley *et al.* (1973).

Studies of ^{15}N-labelled nitrate, ammonia, and urea uptake during this same cruise provide another basis for comparing the significance of nitrogen fixation. With a total combined nitrogen uptake of 0.05 to 1 μg N/l/day (J. Sharp, unpublished data), the maximum N_2 fixation rates observed at 25 m (0.016 μg N/l/day) could account for 1.6 to 30% of the combined nitrogen uptake. Integration of the C_2H_2 reduction-data from Station 9 indicates a total fixation rate of approximately 800 μg N/m²/day. The total rate of photosynthetic CO_2 reduction in the water column was approximately 100 mg C/m²/day (unpublished data), which requires a daily input of about 20 mg fixed N/m²/day, assuming a C:N ratio of 5. Our figures of 0.8 mg N/m²/day thus supply only 4% of the total daily N requirement, but N_2 fixation may be of greater significance than this value would indicate. Fixed nitrogen can be repeatedly recycled within the euphotic zone by processes of active excretion, cell lysis, zooplankton grazing, etc. It is not possible to estimate the ecological importance of this nitrogen fixation in regard to primary productivity until we know more about (a) advection of fixed nitrogen into the euphotic zone from nutrient-rich deep water, (b) input of fixed nitrogen via excretion by migrating populations of zooplankton and fish, (c) loss of fixed nitrogen from the euphotic zone, either through passive sinking or by grazing. Part of our present program in the north Pacific Gyre is directed at determining the rates of these processes.

While it is possible to make gross estimates of nitrogen budgets from information such as this, it is thus far impossible to determine whether some of the nitrogen fixed by certain phytoplankton is immediately available to others through excretion of nitrogenous compounds (Fogg, 1966), or only by regeneration after death or grazing. At 25 m, where highest N_2 fixation occurred, the nitrogen and carbon in the particulate fraction were higher than at other depths, although this fraction was lower in phytoplankton (chlorophyll *a*) and total biomass (ATP). It is attractive to assume that the increased nitrogen content is a result of fixation. However, in a mixed population, it is possible that the nitrogen-fixing phytoplankton are considerably enriched in nitrogen, but that this is masked by the more abundant non-fixing species. That the particulate carbon maximum does not correspond to the biomass maximum is further evidence to support the observations that the *Rhizosolenia* cells were senescent and in a post-bloom condition. The high particulate phosphorus content at 25 m may reflect the continued healthy state of the blue-green alga, some species of which are known to store polyphosphate (Stewart and Alexander, 1971).

Attempts to culture *Richelia intracellularis* either within *Rhizosolenia* or separately were unsuccessful, so unequivocal proof that *Richelia intracellularis* itself is responsible for N_2 fixation is not yet available. However, samples of several *Rhizosolenia* species obtained from the North Central Pacific in February, 1973, which did not contain *Richelia*, did not reduce C_2H_2. Fixation by bacteria associated with the diatom/blue-green alga combination is also not likely, since fixation is markedly light-dependent and takes place under aerobic conditions. Even if the blue-green alga is solely responsible for fixation, it may nonetheless benefit from metabolites of the diatom host and may supply combined nitrogen in return. Some blue-green algae can grow heterotrophically and fix N_2 (Fay, 1965; Watanabi and Yamamoto, 1967) but, in our samples, *Richelia intracellularis* was actively photosynthesizing as demonstrated by micro-autoradiography using $H^{14}CO_3^-$ (Weare *et al.*, in preparation).

We also have confirmation of nitrogen fixation by *Trichodesmium*, and the evidence strongly suggests that it is the alga which fixed N_2, despite the lack of heterocysts. Colonies of *Trichodesmium* which were individually separated from other phytoplankton and re-suspended in filtered water showed active C_2H_2 reduction (Table 1). Additional observations have shown that C_2H_2 reduction by *Trichodesmium* is short-lived, and ceases even prior to disintegration of the algal colonies. Possible bacterial fixation within the colonies would most likely increase upon lysis of algal cells and liberation of protoplasmic contents.

Summary

1. Nitrogen fixation was found associated with *Trichodesmium* sp. and *Richelia intracellularis* in the central North Pacific Ocean during June, 1972.

2. *Richelia intracellularis* occurred as an endophyte in *Rhizosolenia* spp. distributed throughout the upper 100 m, but maximally at 25 m.

3. Nitrogen fixation was measured by $^{15}N_2$ assimilation and C_2H_2 reduction, but the ratio of N_2 fixed: C_2H_2 reduced was not constant. Fixation was stimulated by phosphate.

4. C_2H_2 reduction by *Richelia intracellularis* was light dependent, but was partially inhibited at surface-light-intensities.

5. The rate of C_2H_2 reduction by samples *in vitro* was constant for only 2 h before activity was lost.

6. Fixation might provide 2 to 5% of the daily nitrogen requirement of the standing phytoplankton population, although some data indicate up to 30%.

Acknowledgements. We wish to thank F. C. Mague for expert assistance with work performed at sea, and E. L. Venrick and F. M. Reid for identification and counting of phytoplankton samples. Support was provided by National Science Foundation Grant GV-27110 and U.S. Atomic Energy Commission Contract No. AT(11-1)GEN 10, P.A. 20.

Literature Cited

Baas Becking, L. G. M.: Notes on some Cyanophyceae of the Pacific region. Proc. Sect. Sci. K. ned. Akad. Wet. (Sect. C) **54**, 213—225 (1951).

Bergersen, F. J.: The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Aust. J. biol. Sci. **23**, 1015—1025 (1970).

Brooks, R. H. Jr., P. L. Brezonik, H. D. Putnam and M. A. Keirn: Nitrogen fixation in an estuarine environment: the Waccasassa on the Florida Gulf Coast. Limnol. Oceanogr. **16**, 701—710 (1971).

Bunt, J. S., K. E. Cooksey, M. A. Heeb, C. C. Lee and B. F. Taylor: Assay of algal nitrogen fixation in the marine subtropics by acetylene reduction. Nature, Lond. **227**, 1163—1164 (1970).

Burris, R. H.: Nitrogen fixation — assay methods and techniques. *In:* Methods in enzymology. Vol. 24. Photosynthesis and nitrogen fixation. Part B, pp 415—431. Ed. by S. P. Colowick and N. D. Kaplan. New York: Academic Press 1972.

Carpenter, E. J.: Nitrogen fixation by a blue-green epiphyte on pelagic *Sargassum*. Science, N.Y. **178**, 1207—1209 (1972).

—, C. C. Remsen and S. W. Watson: Utilization of urea by some marine phytoplankters. Limnol. Oceanogr. **17**, 265—269 (1972).

Dugdale, R. C. and V. A. Dugdale: Nitrogen metabolism in lakes. III. Tracer studies of the assimilation of inorganic nitrogen sources. Limnol. Oceanogr. **10**, 53—57 (1965).

— —, J. Neess and J. Goering: Nitrogen fixation in lakes. Science, N.Y. **130**, 859—860 (1959).

—, J. J. Goering and J. H. Ryther: High nitrogen fixation rates in the Sargasso Sea and the Arabian Sea. Limnol. Oceanogr. **9**, 507—510 (1964).

—, D. W. Menzel and J. H. Ryther: Nitrogen fixation in the Sargasso Sea. Deep Sea Res. **7**, 297—300 (1961).

Eppley, R. W., E. H. Renger, E. L. Venrick and M. M. Mullin: A study of plankton dynamics and nutrient cycling in the Central Gyre of the North Pacific Ocean. Limnol. Oceanogr. **18**, 534—551 (1973).

Fay, P.: Heterotrophy and nitrogen fixation in *Chlorogloea fritschii*. J. gen. Microbiol. **39**, 11—20 (1965).

—, W. D. P. Stewart, A. E. Walsby and G. E. Fogg: Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature, Lond. **220**, 810—812 (1968).

Fisher, R. J. and W. J. Brill: Mutants of *Azotobacter vinelandii* unable to fix nitrogen. Bact. Proc. p. 148 (1969).

Fogg, G. E.: The extracellular products of algae. Oceanogr. mar. Biol. A. Rev. **4**, 195—212 (1966).

— and Than-Tun: Interrelations of photosynthesis and assimilation of elementary nitrogen in a blue-green alga. Proc. R. Soc. Lond. (Ser. B) **153**, 111—127 (1960).

Goering, J. J., R. C. Dugdale and D. W. Menzel: Estimates of *in situ* rates of nitrogen uptake by *Trichodesmium* sp. in the tropical Atlantic Ocean. Limnol. Oceanogr. **11**, 614—620 (1966).

Granhall, V. and A. Lundgren: Nitrogen fixation in Lake Erken. Limnol. Oceanogr. **16**, 711—719 (1971).

Hardy, R. W. F., R. C. Burns and R. D. Holsten: Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. **5**, 47—81 (1973).

Holm-Hansen, O.: Determination of particulate organic nitrogen. Limnol. Oceanogr. **13**, 175—178 (1968).

— and C. R. Booth: The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. **11**, 510—519 (1966).

—, C. J. Lorenzen, R. W. Holmes and J. D. H. Strickland: Fluorometric determination of chlorophyll. J. Cons. perm. int. Explor. Mer **30**, 3—15 (1965).

—, J. D. H. Strickland and P. M. Williams: A detailed analysis of biologically important substances in a profile off southern California. Limnol. Oceanogr. **11**, 548—561 (1966).

Horne, A. J. and G. E. Fogg: Nitrogen fixation in some English lakes. Proc. R. Soc. Lond. (Ser. B) **175**, 351—366 (1970).

— and C. R. Goldman: Nitrogen fixation in Clear Lake, California. I. Seasonal variation and the role of heterocysts. Limnol. Oceanogr. **17**, 678—692 (1972).

Institute of Marine Resources: Eutrophication in coastal waters: nitrogen as a controlling factor. U.S. Environmental Protection Agency, Water Pollution Control Research Series: 16010 EHC, December, 1971.

Jackson, W. S. and R. J. Volk: Photorespiration. A. Rev. Pl. Physiol. **21**, 385—432 (1970).

Jeng, D. Y., T. Devanthan and L. E. Mortenson: Components of cell-free extracts of *Clostridium pasteurianum* as required for acetylene reduction and N_2 fixation. Biochem. biophys. Res. Commun. **35**, 625—633 (1969).

Johannes, R. E., J. Alberts, C. D'Elia, R. A. Kinzie, L. R. Pomeroy, W. Sottile, W. Wiebe, J. A. Marsh, Jr., P. Helfrich, J. Maragos, J. Meyer, S. Smith, D. Crabtree, A. Roth, L. R. McClosky, S. Betzer, N. Marshall, M. E. Q. Pilson, G. Telek, R. I. Clutter, W. D. DuPaul, K. L. Webb and J. M. Wells, Jr.: The metabolism of some coral reef communities: a team study of nutrient and energy flux at Eniwetok. Bioscience **22**, 541—543 (1972).

Kawai, A. and I. Sugahara: Microbiological studies on nitrogen fixation in aquatic environments. III. On the nitrogen fixing bacteria in offshore regions. Bull. Jap. Soc. scient. Fish. **37**, 981—985 (1971).

Lex, M., W. B. Silvester and W. D. P. Stewart: Photorespiration and nitrogenase activity in the blue-green alga, *Anabaena cylindrica*. Proc. R. Soc. Lond. (Ser. B) **180**, 87—102 (1972).

Mague, T. H. and R. H. Burris: Reduction of acetylene and nitrogen by field-grown soybeans. New Phytol. **71**, 275—286 (1972).

McCarthy, J. J.: A urease method for urea in sea water. Limnol. Oceanogr. **15**, 309—313 (1970).

Maruyama, Y., N. Taga and O. Matsuda: Distribution of nitrogen-fixing bacteria in the central Pacific Ocean. J. oceanogr. Soc. Japan **26**, 360—366 (1970).

Patriquin, P. and R. Knowles: Nitrogen fixation in the rhizosphere of marine angiosperms. Mar. Biol. **16**, 49—58 (1972).

Pshenin, L. W.: Distribution and ecology of *Azotobacter* in the Black Sea. *In:* Marine microbiology, pp 383—391. Ed. by C. H. Oppenheimer. Illinois: C. C. Thomas 1963.

Ryther, J. H. and W. M. Dunstan: Nitrogen, phosphorus and eutrophication in the coastal marine environment. Science, N.Y. **171**, 1008—1013 (1971).

Sournia, A.: Les cyanophycées dans le plancton marin. Annls Biol. (Ser. 4) **9**, 63—76 (1970).

Steele, J. H.: A study of production in the Gulf of Mexico. J. mar. Res. **22**, 211—222 (1964).

Stewart, W. D. P.: Nitrogen fixation by Myxophyceae from marine environments. J. gen. Microbiol. **36**, 415—422 (1964).

— Nitrogen turnover in marine and brackish habitats. II. Use of ^{15}N in measuring nitrogen fixation in the field. Ann. Bot. (N.S.) **31**, 385—407 (1967).

— and G. Alexander: Phosphorus availability and nitrogenase activity in aquatic blue-green algae. Freshwat. Biol. **1**, 389—404 (1971).

—, G. P. Fitzgerald and R. H. Burris: Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. **62**, 336—348 (1968).

— — — Acetylene reduction assay for determination of phosphorus availability in Wisconsin lakes. Proc. natn. Acad. Sci. U.S.A. **66**, 1104—1111 (1970).

—, T. Mague, G. P. Fitzgerald and R. H. Burris: Nitrogenase activity in Wisconsin lakes of differing degrees of eutrophication. New Phytol. **70**, 497—509 (1971).

Strickland, J. D. H. and T. R. Parsons: A practical handbook of seawater analysis. Bull. Fish. Res. Bd Can. **167**, 1—311 (1968).

Watanabi, A. and Y. Yamamoto: Heterotrophic nitrogen fixation by the blue-green alga *Anabaenopsis circularis*. Nature, Lond. **214**, p. 738 (1967).

Weare, N. M., F. Azam, T. H. Mague and O. Holm-Hansen: Autoradiographic studies of the marine phycobionts *Rhizosolenia* and *Richelia*. (In preparation).

— and J. R. Benemann: Nitrogen fixation by *Anabaena cylindrica*. I. Localization of nitrogen fixation in the heterocysts. Arch. Mikrobiol. **90**, 323—332 (1973).

First author's address: Dr. T. H. Mague
Institute of Marine Resources
University of California, San Diego
P.O. Box 1529
La Jolla, California 92037
USA

Date of final manuscript acceptance: October 12, 1973. Communicated by J. Bunt, Miami