

Nitrogen fixation on a coral reef

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Abstract

Acetylene reduction was used to assess nitrogen fixation on all major substrates at all major areas over a period of 1 to 6 yr (1980–1986) at One Tree Reef (southern Great Barrier Reef). Experiments using $^{15}\text{N}_2$ gave a ratio of 3.45:1.0 for C_2H_2 reduced: N_2 fixed. Acetylene reduction was largely light-dependent, saturated at 0.15 ml C_2H_2 per ml seawater, and linear over 6 h. High fixation was associated with two emergent cyanophyte associations, *Calothrix crustacea* and *Scytonema hofmannii*, of limited distribution. Subtidally, the major contribution to nitrogen fixation came from well-grazed limestone substrates with an epilithic algal community in the reef flat and patch reefs (3 to 15 nmol $\text{C}_2\text{H}_4 \text{ cm}^{-2} \text{ h}^{-1}$). Similar substrates from the outer reef slope showed lower rates. Nitrogen fixation on beach rock, intertidal coral rubble, reef crest and lagoon sand was relatively small (0.3 to 1.0 nmol $\text{C}_2\text{H}_4 \text{ cm}^{-2} \text{ h}^{-1}$). Seasonal changes in light-saturated rates were small, with slight reduction only in winter. Rates are also reported for experimental coral blocks (13 to 39 nmol $\text{cm}^{-2} \text{ h}^{-1}$) and for branching coral inside and outside territories of gardening damselfish (3 to 28 nmol $\text{cm}^{-2} \text{ h}^{-1}$). This work supports the hypothesis that the high nitrogen fixation on the reef flat and patch reefs of the lagoon (34 to 68 kg N $\text{ha}^{-1} \text{ yr}^{-1}$) is because these subtidal areas support highly disturbed communities with the greatest abundance of nitrogen-fixing cyanophyte algae. It is calculated from a budget of all areas that One Tree Reef has an annual nitrogen fixation rate of 8 to 16 kg N $\text{ha}^{-1} \text{ yr}^{-1}$.

reefs typically are surrounded by low-nutrient, low-productivity ocean waters but, paradoxically, the waters of coral reefs often have elevated nitrogen levels (Johannes et al. 1972, Hatcher and Hatcher 1981, Andrews and Muller 1983, Crossland and Barnes 1983), and their communities exhibit high primary productivity (Wanders 1976, Borowitzka et al. 1983, review by Lewis 1977). This paradox can be explained by the suppositions that (1) coral reef systems have efficient mechanisms for absorbing nitrogen sources from nutrient-poor ocean water (and for efficiently recycling nitrogen) (Lewis 1977) and/or (2) significant input of nitrogen from nitrogen-fixing organisms (Wiebe et al. 1975). The latter supposition has gained the widest support, and over the last decade there have been a number of reports of nitrogen fixation on coral reefs (Johannes et al. 1972, Mague and Holm-Hansen 1975, Webb et al. 1975, Wiebe et al. 1975, Burris 1976, Hanson and Gundersen 1977, Potts and Whitton 1977, Goldner 1980, Wilkinson and Sammarco 1983, Wilkinson et al. 1984). The organisms responsible for the fixation appear to be largely cyanobacteria, of which there are a great variety on the limestone substrata of coral reefs (Wiebe et al. 1975, Potts and Whitton 1977). Despite this evidence, the case for a substantial input of nitrogen from nitrogen fixation is still not proven. Data on seasonality and the quantitative abundance of nitrogen-fixing substrata on coral reefs are not available. In the present work a detailed survey of nitrogen fixation on a coral reef was performed to estimate the nitrogen input from nitrogen fixation, taking into account both temporal and spatial variations.

Introduction

With few exceptions, ocean waters are low in sources of nitrogen for plant growth (Ryther 1969, Sharp 1983). Associated with this fact is the low primary productivity of the majority of ocean waters (Ryther 1969). Tropical coral

Materials and methods

Sites

One Tree Reef (20°30'S; 152°06'E), at the southern end of the Great Barrier Reef, has been described in detail by Kinsey and Davies (1979). It is characterised by an emer-

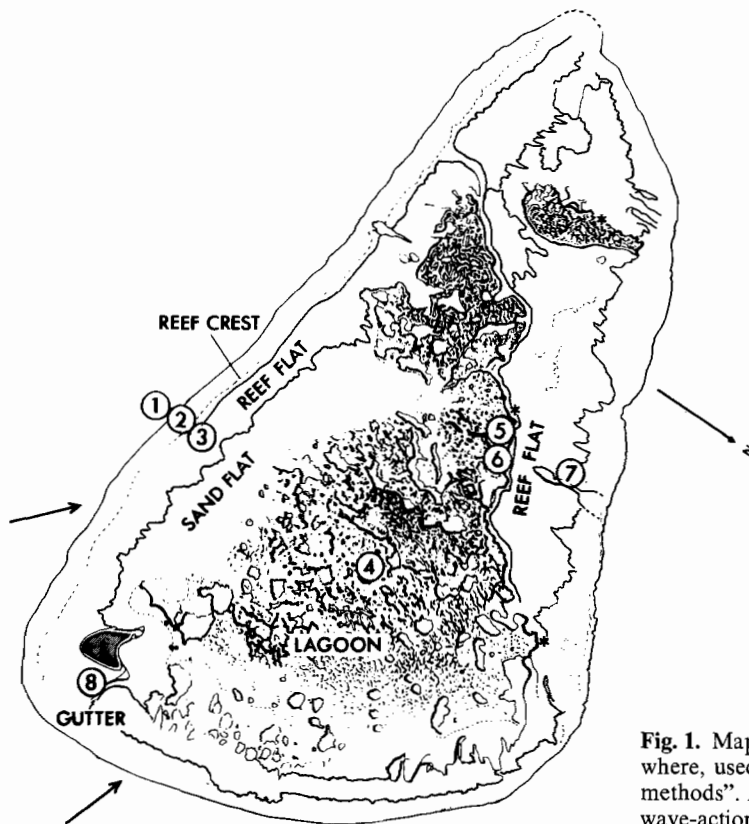


Fig. 1. Map of One Tree Reef showing sites along transect, and elsewhere, used in present work. Sites 1-8 are described in "Materials and methods". Arrows indicate direction of prevailing winds and consequent wave-action

gent crest (Fig. 1), which isolates a lagoon (mean depth = 4 m) studded with reticulated patch reefs, many of which reach the surface at low water, where they form a "pie-crust" of consolidated calcareous material, dominated by the crustose coralline algae *Porolithon onkodes* and *Fosliella* spp. Extensive reef flat occurs adjacent to the reef crest in many areas of the reef, particularly on the northern and southern sides.

The major sites used, between 1980 and 1986, were set out along an approximate transect line that has been used previously (Goldman and Talbot 1976, Hatcher and Larkum 1983). These sites were (1) outer reef slope (south side), 4 m depth (below extreme low-tide level, spring tides); (2) reef crest (south side), exposed at approximately mid-tide level; (3) reef flat area (south side), 0 to 0.8 m (low-water mark, lagoon); (4) patch reef in centre of first lagoon (centre bommie), depth 1 m; (5) patch reef (microatoll: see Kinsey and Domm 1974, Hatcher and Larkum 1983) on north side (subsites at: 1 m depth, inside; 0.5 m, inside edge; 0 m, rim; 0.5 m, outside edge; 2.0 m, base of outside edge); (6) patch reef and old reef crest (comparison atoll: see Kinsey and Davies 1979) on north side (subsites at: 1.0 m depth, inside; 0.5 m, inside edge; 0 m at rim; 0.5 m, outside edge - inside edge of old reef crest); (7) long bank, pools at low water mark; (8) gutter (a shallow channel connecting the lagoon and the outer-reef waters at high tide).

Also, two small intertidal pools on beachrock were studied on the east side of One Tree Island. Measurements on lagoon sand were made at three sites: on the southern

prograding sand flat, near the centre of the first lagoon and in the "harbour" adjacent to the island.

Acetylene-reduction technique

Incubations were carried out in 227 ml glass jars with spring-loading perspex lids with a rubber injection port (Suba Seal). Rock chippings or other substrata (30 to 50 ml vol, 50 to 150 cm² surface area), immersed in 75 ml seawater from the sampling site, covered the floor of each vessel. Experiments were started by removal of 20 ml air and injection of 20 ml of C₂H₂ followed by swirling for several minutes. A submerged platform suspended at 0.5 m by floats at the surface caused gentle stirring, induced by wind and wave action. Gas samples were taken at 0, 2, 4 and 6 h using 3 ml evacuated tubes (Venoject) after swirling for several minutes. Ethylene production was assayed using a portable gas chromatograph (J.A.S Instruments, Melbourne) with a stainless steel column (60 × 0.32 cm, Porapak T) and a sensitive thermal conductivity detector for C₂H₄ detection (Figaro Gas Sensor). Routinely, 200 μl of sample gas was injected and the C₂H₄ peak was followed on a strip-chart recorder. The system was calibrated daily with known dilutions of C₂H₄ gas (Commonwealth Industrial Gases, Instrument Grade). The rate of C₂H₂ reduction (= C₂H₄ production) was calculated using the following equation (for an injection volume of 200 μl).

$$R = 5 \times d(v + wa) / t s,$$

where R = rate of C_2H_2 reduction ($nmol\ cm^{-2}\ h^{-1}$), x = height of C_2H_4 peak, d = $nmol\ C_2H_4$ per unit of peak of deflection for $200\ \mu l$ of calibration gas, v = volume of air space in container (ml), w = volume of seawater in vessel (ml), t = duration of experiment (h), s = surface area of exposed rock surface (cm^2), and a = Bunsen absorption coefficient for seawater at ambient temperature ($ml\ C_2H_4/ml$ seawater), as given below.

The following were the Bunsen absorption coefficients used ($ml\ C_2H_4/ml$ seawater for a partial pressure of C_2H_4 of $101.5\ kPa$) calculated from the International Critical Tables (1928):

20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	29 °C	30 °C
0.127	0.121	0.115	0.110	0.105	0.101	0.096	0.091	0.087	0.084	0.080

Experiments were carried out only on days when cloud cover was less than 50%. In over 90% of experiments cloud cover was zero. Light intensity varied seasonally. In mid-summer at noon, photon-flux density on the experimental samples was $2.34 \times 10^3\ \mu E\ m^{-2}\ s^{-1}$ and in midwinter it was 1.05×10^3 (Licor Quantum Meter LI-188B with 180° underwater PAR sensor LI-192SB). Water temperature at the incubation site varied seasonally from $18^\circ C$ in July to $28^\circ C$ in midsummer. The maximum change of temperature in any single experiment was less than $2\ C^\circ$.

The volume of the rock samples was measured by displacement of water. The exposed surface area of rock samples was measured directly: the major axes of individual elements were measured and surface areas was obtained using formulae for cylinders, spheres, spheroids, cones, paraboloids, etc. (Dahl 1973). Comparison between two workers using identical samples yielded estimates that differed by less than 5%; however, no attempt was made to assess surface irregularity and so these were minimum estimates.

For measurements on sand, the glass jars were filled on the surface with 100 ml seawater and 20 ml C_2H_2 , shaken, inverted underwater, the lid opened at the sediment surface and the jar gently pressed a few millimeters into the sand to effect a seal. At the end of the experiment (4 h) the jar was closed gently, shaken vigorously and the airspace sampled at the surface.

Coral blocks

Coral blocks of small dead specimens of *Porites lobata* were chiselled to an approximately hemispherical shape of ca. 6 cm radius, holes were drilled through the middle, and the surface areas and volumes were measured. Surface area was measured according to Hatcher (1981). The blocks were set out on a consolidated limestone bottom at Site 3 and were secured by stainless steel wire anchored to the bottom with underwater cement (Quickcrete, Selleys Chemical Co.). Acetylene reduction was measured using plastic boxes (1 400 ml; 560 ml seawater; 120 ml C_2H_2) with transparent lids modified to give a gas-tight seal.

^{15}N Techniques

$^{15}N_2$ was prepared from about 0.5 g $^{15}NH_4Cl$ in the field with alkaline hypobromite using the methods described by Burris (1976). A gas mixture comprising $^{15}N_2$, oxygen and argon in the ratio 0.35:0.2:0.45 (v/v/v) was then prepared by displacement of seawater from a volumetric flask using plastic syringes for gas transfer. Samples of the gas mixture used in exposures were taken to determine the enrichment of N_2 with ^{15}N by mass spectrometry.

For comparison of nitrogen reduction with C_2H_2 reduction, limestone substrates were first exposed to C_2H_2 as previously described and, after a period of re-exposure to air (ca. 30 min), the same sample was then exposed to the $^{15}N_2$ gas mixture described above. Exposure vessels were the same as those described above, or smaller Plexiglas vessels fitted with a removable, gas-tight lid and rubber septa (Suba seals) for gaseous transfer. Rock samples were extracted with 50 ml of 80% ethanol (v/v), the surface being scraped to a depth of about 1 cm to remove the majority of the living material. The bulked sample was centrifuged to obtain ethanol-soluble and ethanol-insoluble fractions. Kjeldahl digestion was carried out after removal of ethanol as described by Burris and Wilson (1957). The ethanol-insoluble fraction was treated with excess sulphuric acid before Kjeldahl digestion. Ammonia was steam-distilled into boric acid and titrated with dilute HCl as described by Bergersen (1980).

^{15}N measurements were made on the Micromass 903 mass-spectrometer at the Division of Plant Industry, CSIRO, Canberra, according to Bergersen and Turner (1983). The rate of nitrogen fixation was determined from the ^{15}N enrichment of samples corrected for the enrichment of nitrogen used in exposures (50 to 63% ^{15}N).

Sampling procedure and statistical analyses

A cost-benefit analysis (Underwood 1981) was carried out on the reef flat at Site 3, the region of highest nitrogen fixation (Table 1), to determine the optimum sampling procedure. Four subsites ($5 \times 5\ m$) were randomly selected in an area with 10 to 80% piecrust (consolidated dead coral substrate exposed at low water). At each subsite, five $1\ m^2$ quadrats were randomly taken from the possible 25, and within each quadrat seven random $25\ cm^2$ replicates were taken using a hammer and chisel (only hard substrates were included). Variance estimates and optimum number of replicates were derived from a nested analysis of variance of untransformed C_2H_2 reduction rates according to Underwood (1981). From the analysis (not shown) it was concluded that quadrats and replicates rather than subsites contributed to overall variation. To optimise costs, with a maximum number of 14 samples that could be analysed per day, the analysis showed that for each site on the reef 14 random quadrats from one $5 \times 5\ m^2$ area with only one replicate from each quadrat would give a standard error of mean C_2H_2 reduction of 21% of the overall mean. It was

decided to take two samplings at each site (on two different days), i.e., 28 replicates, since (a) it could not be assumed that every site would have the same variance as the reef flat at Site 3, and (b) this would allow a check of any day-to-day variation.

Analyses of variance were carried out, with a log-transformation comparing light intensities and C_2H_2 concentrations, but not the effect of time, as variation was homogeneous in this case.

For the analysis of sites and seasonal data, separate analyses of variance were first performed on data from each period, using a log-transformation. In these analyses, where repeat samplings were included as a factor, there was greater variation between repeats than between replicates within repeats; however, the variance ratios for repeats were low (all < 0.5). Thus, there were no consistent differences between repeats and the two means over 14 replicates for each site were the appropriate values to use in the final analysis for the transformed data. In addition, the range of residual mean squares from the analyses of the log-transformed data for each period indicated that all periods could be combined into a single analysis. Thus, in the final analysis, each site/period combination was considered as a "treatment" level and a variance ratio of 6.30 for treatments was obtained, which is highly significant ($P < 0.001$). Similarly, comparisons of C_2H_2 reduction rates on blocks with those on other substrata were made using an analysis of variance of log-transformed data over all periods, with each substratum/period combination being considered as a "treatment".

For comparison of coral types at Quadrat 2 (Site 3), there were different numbers of samples for each of the four substratum types out of the 14 samples taken at each period. Thus, a non-orthogonal analysis of variance was performed to examine differences between periods, between substratum types, and their interaction, again using log-transformed data.

General procedures

The areas of various substrate types at One Tree Reef were measured from aerial photographs. Areas were measured using a Lambda Instruments Licor LI-3000 area-measuring device. Dry weighing was carried out to constant weight at $80^\circ C$. Ash-free dry weight (AFDW) was determined by ashing at $400^\circ C$.

Results

Factors affecting rate of acetylene production

Using randomly-sampled, grazed limestone chippings, C_2H_2 reduction was linear during a 6 h period during the day, with a rate of $5.6 \text{ nmol cm}^{-2} \text{ h}^{-1}$ (Fig. 2); replicates which were darkened after three hours showed no signifi-

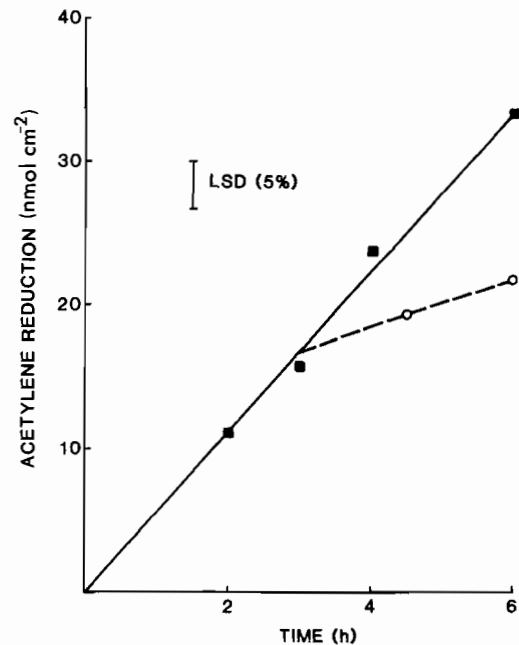


Fig. 2. Time-dependence and effect of darkness on acetylene reduction, measured on limestone substrata sampled randomly at Site 3, December 1981. Continuous line: samples left in light; dashed line: samples darkened at 3 h. LSD: least-significant difference

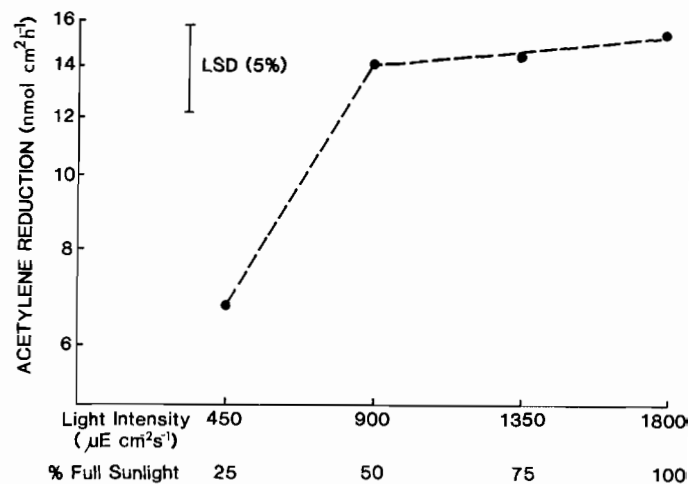


Fig. 3. Effect of light saturation on acetylene reduction, measured on limestone substratum collected randomly at Site 3 in February 1983

cant C_2H_2 reduction, although it is possible that a small amount of C_2H_2 reduction occurred shortly after darkening. Samples incubated at night, in the dark, showed no detectable C_2H_2 reduction over 6 h (data not shown).

The light-saturation level of C_2H_2 reduction, assessed using shade cloth and random rock samples on three consecutive cloudless days (Fig. 3), occurred at a photon flux rate of about $0.9 \times 10^3 \mu E \text{ cm}^{-2} \text{ s}^{-1}$. Thus, within the flux

rates of the experimental work (0.8×10^5 to $2.34 \times 10^5 \mu\text{E cm}^{-2} \text{s}^{-1}$), there would be no effect due to light reduction on cloudy days, except perhaps for a few experiments in mid-winter, and no light-inhibition due to high light intensities in the summer, with the possible exception of shade-adapted samples from deeper water.

The effect of C_2H_2 concentration using dead *Acropora* sp. samples from Site 3 (Fig. 4) indicated that C_2H_2 reduction was saturated below 0.10 ml C_2H_2 per ml seawater (cf. Burris 1976). No inhibition of C_2H_2 reduction rate was found up to nearly 0.3 ml C_2H_2 per ml seawater. A standard concentration of 0.23 ml C_2H_2 per ml seawater was used for all experiments.

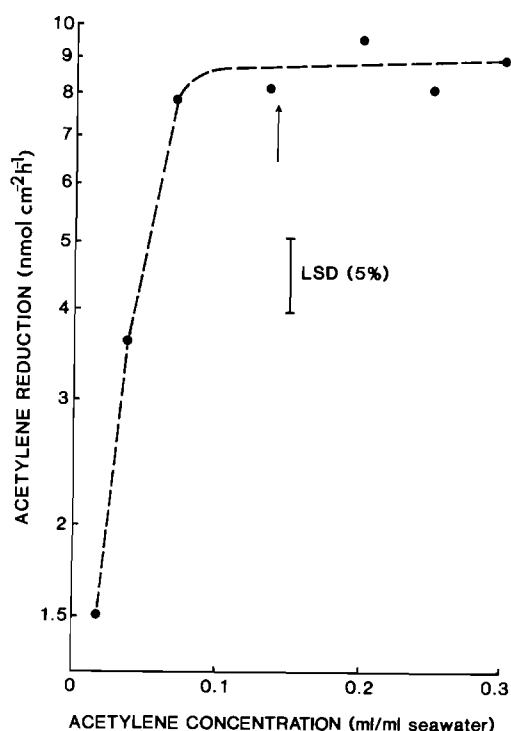


Fig. 4. Effect of acetylene concentration in seawater on acetylene reduction, measured on branching *Acropora* sp. rubble at Site 3 in February 1983. Arrow shows acetylene concentration used by Burris (1976)

Ratio of rates of nitrogen and acetylene reduction

The average ratio obtained for six exposures was 3.45 (Table 1), which falls within the range of previous published values for blue-green algae of 1.9 (Burris 1976) to 4.8 (Stewart et al. 1968, Peterson and Burris 1976). The material used here was from the rocks found above lagoon low water on the reef flat (Site 3) with a high density of *Calothrix crustacea* (Table 2). Thus, use of this conversion ratio for C_2H_2 reduction provided a reasonably valid measure of the rate of nitrogen fixation occurring in the rock samples. Furthermore, the experiment shows that a previous exposure to C_2H_2 had little or no effect on the subsequent rate of nitrogen assimilation, as would be anticipated by the linear rates of C_2H_2 reduction observed over the period of 6 h (Fig. 2).

Analysis of sites and seasonal effects

The results of a survey of C_2H_2 reduction of major substrata and areas are summarized in Table 2. On heavily-grazed limestone substrata, C_2H_2 reduction varied between sites and can be summarized as follows. The reef slope outside the reef crest (1 to 10 m depth) had low to medium rates (Tables 2 and 3); the reef crest, both "bare" rocks and surfaces covered with algal turf, had low rates (with the exception of the gelatinous masses mentioned below); the reef flat had medium to high rates; patch reefs in the lagoon had low to high rates; beach rock and coral rubble around One Tree Island generally had low rates, except where the *Scytonema hofmannii* community, mentioned below, occurred. All these substrata supported a microscopic epilithic algal community (EAC: see Hatcher and Larkum 1983), in which the following cyanobacteria were common: *Calothrix crustacea* Schousboe and Thuret, *Microcoleus lyngbyaceus* (Kütz) Crouan, *Anabaina oscillarioides* Bory, *Anacystis marina* Drouet and Daily, *Entophysalis conferta* Drouet and Daily, *Nostoc spumigena* (Mertens) Drouet, *Oscillatoria lutea* C. Agardh, *Oscillatoria submembranacea* Ard. and Straff., *Schizothrix mexicana* Gomont, *Schizothrix calcicola* (C. Ag.) Gomont, and

Table 1. Relative rates of nitrogen and acetylene reduction ($\text{pmol min}^{-1} \text{flask}^{-1}$) of six samples taken at Site 3 at One Tree Reef in July 1982. $^{15}\text{N}_2$ fixation rate represents total fixation of dinitrogen gas based on rate of ^{15}N fixation

Sample	Excess ^{15}N (at. %)	Total N (μg)	$^{15}\text{N}_2$ fixation rate	C_2H_4 production rate	Ratio
(1) Rock	0.025	2 940	427	886	2.07
(2) Rock	0.042	2 280	494	2 868	5.81
(3) Algal mat	0.019	400	25	86	3.44
(4) Algal mat	0.019	340	21	62	2.95
(5) Rock	0.0025	3 990	297	1 076	3.62
(6) Rock	0.0020	9 990	206	584	2.83
Mean					3.45

Table 2. Some representative values of acetylene reduction for major substrata and various zones of One Tree Reef. Acetylene reduction rate given as mean \pm standard error, with number of replicates in parentheses

Zone/association	Depth	Date	Common cyanophytes	C ₂ H ₂ reduction (nmol cm ⁻² h ⁻¹)
Outer reef slope	5 m	12. XII. 1980	<i>Microcoleus lyngbyaceus</i> <i>Calothrix crustacea</i>	4.5 \pm 0.9 (14)
	2 m	14. XII. 1981	<i>Microcoleus lyngbyaceus</i> <i>Calothrix crustacea</i>	2.3 \pm 0.5 (14)
Reef crest	Intertidal	10. XII. 1980	<i>Microcoleus lyngbyaceus</i>	20.1 (3)
Brown mass on algal turf	Intertidal	14. XII. 1981	<i>Microcoleus lyngbyaceus</i>	51.2 (5)
Rubble zone, with filamentous green algae	Intertidal	11. IV. 1980	<i>Calothrix crustacea</i> <i>Oscillatoria submembranacea</i> <i>Schizothrix (mexicana?)</i> <i>Microcoleus lyngbyaceus</i>	41.7 (3)
Red algal turf	Intertidal	12. IX. 1980	<i>Microcoleus lyngbyaceus</i>	0.45 (3)
Mixed algal turf	Intertidal	7. XII. 1981	<i>Microcoleus lyngbyaceus</i>	1.1 \pm 0.32 (14)
Reef flat				
Limestone substrate	1 m	10. IX. 1980	<i>Calothrix crustacea</i>	11.0 \pm 3.1 (14)
Coral boulder	Intertidal	12. IX. 1980	<i>Calothrix crustacea</i>	43.5 \pm 4.5 (14)
Lagoon				
Clean sand	1 m	1. I. 1985	<i>Microcoleus lyngbyaceus</i>	0.35 \pm 0.09 (7)
Sand with algal film	1 m	1. I. 1985	<i>Microcoleus lyngbyaceus</i>	0.83 \pm 0.31 (7)
Beach rock				
Grey-black rubble (consolidated)	Intertidal	5. XII. 1981	None abundant (<i>Entophysalis deusta</i>)	0.42 \pm 0.08 (7)
As above with macroscopic cyanophytes	Intertidal	6. XII. 1981	<i>Scytonema hofmannii</i>	82.0 (3)
As above	Intertidal	10. VII. 1984	<i>Scytonema hofmannii</i>	21.6 \pm 4.2 (8)

Spirulina subsalsa Oersted (all species *sensu* Drouet: see Humm and Wicks 1980). Other algae on these limestone surfaces were similar to those found previously (Hatcher and Larkum 1983, Larkum 1988).

Apart from the EAC, a number of macroscopic, cyanobacterial communities or associations could be recognised at One Tree Reef. The most notable of these were:

(1) Gelatinous masses (ca. 100 cm²) over algal turf communities on the reef crest and, more rarely, elsewhere. These contained much *Microcoleus lyngbyaceus* but also a number of other cyanobacteria and, in addition, many bacteria and animals and much detritus. They generally had a high rate of C₂H₂ reduction (Table 2), but were of limited distribution, only very rarely occurring on randomly sampled substrata.

(2) A blue-green film on emergent, storm-tossed coral boulders above lagoon low-water level on the reef flat. *Calothrix crustacea* occurred abundantly on the boulders, often as a unialgal community. High rates of C₂H₂ reduction were always associated with this material (cf. Mague and Holm-Hansen 1975, Wiebe et al. 1975, Potts and Whitton 1977).

(3) A macroscopic coating of *Scytonema hofmannii* C. Agardh, which occurred in a narrow zone (near high-water

mark) on beach rock adjacent to One Tree Island and along the eastern crest (cf. Burris 1976, Potts and Whitton 1977). This community was most widespread from September to November, and was considerably reduced in the summer (December to February). The highest reduction rates were recorded for this community (Table 2).

All three communities were of extremely limited distribution compared with the EAC community.

A number of macroalgae exhibited the capacity to reduce C₂H₂ (cf. Capone et al. 1977, Goldner 1980), presumably through the presence of cyanobacterial epiphytes, of which *Schizothrix calcicola* (C. Agardh) Gomont was the most common. Even the highest rates, 145 nmol g dr. wt⁻¹ h⁻¹ for the spring (September to November) bloom of *Laurencia intricata* Lamouroux, were comparatively low compared with those of hard substrates.

Soft-bottom sand substrates were sampled in the first lagoon, and exhibited very low fixation rates even when covered with a visible layer of *Microcoleus lyngbyaceus* (Table 2).

Sponges and ascidians with prokaryotic algal symbiont associations showed negligible rates of C₂H₂ reduction, never exceeding the background rates of the substrates on which the associations grew or the seawater in which they

Table 3. Spatial and temporal variation of acetylene reduction rates ($\text{nmol cm}^{-2} \text{h}^{-1}$) for limestone substrata at sites arranged along an approximate transect across One Tree Reef (see “Materials and methods – Sites”) and for five sampling periods. Values in parentheses are means of log-transformed acetylene-reduction rates [$\log_{10}(10 \times \text{rate})$]; standard error of difference between means = 0.147. Where no data appear, site was not visited at that period

Site (Fig. 1)	Feb.	May	Aug.	Sep.	Dec.
1 Outer reef slope	1.96 (1.175)				
2 Rubble zone inside crest		0.74 (0.785)	5.70 (1.686)		
3 Reef flat					
Quadrat 1	10.07 (1.891)	5.06 (1.661)	5.77 (1.644)	2.36 (1.283)	3.70 (1.522)
Quadrat 2	9.00 (1.891)	3.70 (1.503)	6.57 (1.734)	3.53 (1.441)	7.32 (1.817)
Piccrust on reef flat		3.74 (1.499)	5.92 (1.698)		
4 Centre bommie					4.68 (1.626)
5 Microatoll					
Inside	14.58 (2.014)	4.51 (1.571)	5.92 (1.708)	4.07 (1.491)	7.95 (1.847)
Inside edge		1.34 (1.032)	2.94 (1.362)		5.74 (1.656)
Piccrust		1.27 (1.037)	2.09 (1.237)	4.99 (1.564)	5.20 (1.633)
Outside edge			1.90 (1.171)		5.04 (1.601)
Base of outside edge	4.42 (1.545)		3.70 (1.430)		3.73 (1.514)
6 Comparison atoll					
Inside		3.66 (1.505)	6.28 (1.678)		5.17 (1.630)
Inside edge			3.53 (1.344)		
Piccrust			1.25 (1.014)		
Outside edge			1.96 (1.093)		
7 Long bank			2.94 (1.278)	8.66 (1.774)	
8 Gutter					
Tidal pools	3.35 (1.424)	1.21 (1.026)	4.08 (1.510)	4.11 (1.425)	6.52 (1.653)
Green slimy zone			3.69 (1.510)		

Table 4. Comparison of acetylene reduction rates ($\text{nmol cm}^{-2} \text{h}^{-1}$) for different types of substratum on reef flat at One Tree Reef (Site 3, Quadrat 2). Means are over eight measurement periods between February 1984 and January 1986, and in each case are adjusted for period differences. DF: degrees of freedom; SS: sum of squares; MS: mean square

Analysis of variance				
Source of variation	DF	SS	MS	F
Periods (unadjusted for substrata)	7	4.5924	0.6560	22.85***
Substrata (adjusted for periods)	3	3.1645	1.0548	36.74***
Periods \times substrata	20	0.4348	0.0217	0.76 ^{NS}
Residual	81	2.3256	0.0287	
Total	111	10.5173		
Mean acetylene reduction rates				
Substratum	Acetylene reduction	Log acetylene reduction \pm SE		
Old dead coral	7.63	0.822 \pm 0.028		
Recently dead coral	12.98	1.040 \pm 0.040		
Rubble on floor of flat	9.10	0.885 \pm 0.036		
Piccrust	4.14	0.547 \pm 0.029		

*** Significant at $P < 0.001$; NS: not significant

were incubated (0.02 to $0.1 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-2} \text{ h}^{-1}$). The associations tested were *Prochloron* sp. in *Lissoclinum patella*, *Didemnum molle*, *Diplosoma virens*; *Synechocystis trididemni* in a sponge (Cox et al. 1985) and *Oscillatoria spongeliae* in the sponge *Dysidea herbacea* (Larkum et al.

1987). Experiments were carried out under a gaseous atmosphere of air or nitrogen and in full sunlight or shade.

Seasonality and site effects of C_2H_2 reduction were studied at five periods over 10 mo along a transect across One Tree Reef (Fig. 1 and “Materials and methods – Sites”). The results are shown in Table 3. Little seasonality was evident: a reduction in rates in September (late winter) was significant at some sites, and some of the May and August values of the microatoll subsites were significantly lower than the December values. Highest rates were found in February. In terms of sites, the data show that substrata within the small atolls of the patch reef system were generally comparable to those of the reef flat. However, substrata in deeper water outside the atolls had lower rates generally, as did the piccrust of these atolls (in agreement with the results on “Comparison of coral types”, below).

Comparison of coral types

In an experiment carried out at eight periods over 2 yr on the reef flat at Site 3, significant differences were found between C_2H_2 reduction activity on four types of substratum (Table 4). For each of 14 samples of coral at each period, C_2H_2 reduction was measured and the type of substratum noted. As analysis of variance showed that differences between means for substratum types (adjusted for periods) were highly significant and the interactions were not significant (Table 4), comparison of substratum types was made between means over all periods. The adjusted mean-

Table 5. Rates of acetylene reduction ($\text{nmol cm}^{-2} \text{h}^{-1}$) for various substrata at Site 3 at various periods between 1983 and 1986. Values in parentheses are means of log-transformed acetylene-reduction rates; standard errors of differences apply to these means. Where no data appear, samples were not taken at that time

Substrata (<i>n</i> = replicates)	1983	1984					1985		1986
	Dec.	Feb.	May	July	Sep.	Dec.	May	Sep.	Jan.
Control area (14)	5.00 (0.671)	4.30 (0.588)	2.96 (0.425)	4.92 (0.610)	9.20 (0.908)	7.80 (0.838)	9.86 (0.920)	9.16 (0.913)	13.78 (1.103)
Coral blocks (6)	13.40 (1.119)	19.71 (1.252)	18.64 (1.266)	15.10 (1.158)	12.40 (1.087)	33.54 (1.489)	34.42 (1.473)	33.13 (1.516)	27.84 (1.441)
	[3 mo]	[3 mo]	[6 mo]	[9 mo]	[3 mo]	[5 mo]	[10 mo]	[14 mo]	[18 mo]
		18.81 (1.267)			16.26 (1.207)	39.58 (1.590)	32.69 (1.491)		37.67 (1.565)
		[30 mo]			[12 mo]	[15 mo]	[24 mo]		[30 mo]
Natural blocks (6)						12.68 (1.099)			16.73 (1.211)
Non-garden <i>Acropora aspersa</i> (7)	10.18 (0.987)	3.74 (0.511)	7.68 (0.872)	4.06 (0.596)	5.14 (0.703)	7.89 (0.885)	9.16 (0.959)	16.43 (1.203)	27.63 (1.435)
Garden <i>Acropora aspersa</i> (7)	12.72 (1.068)	5.57 (0.672)	2.86 (0.427)	3.03 (0.456)	3.28 (0.505)	6.62 (0.788)	6.82 (0.827)	10.54 (1.001)	13.26 (1.110)
Standard errors of differences between means:									
						0.068			
						0.088			
						0.084			
						0.105			
						0.101			
						0.097			

transformed C_2H_2 reduction rates and their standard errors (Table 4) show that C_2H_2 reduction in recently dead coral was significantly greater than that in old dead coral and in rubble, and these in turn were significantly greater than reduction in the piecrust substratum.

Coral blocks

Acetylene reduction rates on coral blocks were high after 3 to 6 mo and continued at a high rate for up to 30 mo (Table 5). The table also compares rates with rates from randomly collected substrata at a control site in the same area (Quadrat 2, Site 3, Fig. 1). For up to 30 mo the coral blocks were largely colonized by microscopic algae (see Hatcher and Larkum 1983, for typical algal species) which included a number of cyanobacterial genera of which the following were heterocystous types: *Calothrix crustacea*, *Anabaina oscillarioides*, *Spirulina subsalsa* and *Anacystis marina*. Algal biomass on these blocks was relatively high: 6 to 8 mg AFDW cm^{-2} after 3 mo (cf. 5 to 6 mg AFDW cm^{-2} for damselfish gardens). After 12 mo, crustose coral-line algae became dominant, but nevertheless cyanobacteria were still common.

As a further control, C_2H_2 reduction was measured twice on natural blocks (dead *Porites lobata*) of approximately the same size and shape as the artificial blocks, ly-

ing unattached on the bottom in the same area. The experiment was performed twice and, on both occasions, the rates were significantly lower than those of artificial blocks (Table 5). The age of the natural blocks could not be ascertained. Since their algal flora was similar to those of natural surfaces they were likely to be over 6 mo old. The surface of natural blocks was rugose, whereas that of the artificial blocks was smooth.

Damselfish gardens

Damselfish gardens were sampled in *Acropora aspersa*, a branching coral which forms 0.5 to 1.0 m high thickets in the same area as Quadrat 1 on the southern reef flat. The garden substrates generally had rates not significantly different from the control area, although in 2 out of 9 samplings the rates were significantly different (Table 5). The non-garden material had rates similar to or higher than garden material and the control area, although the trends for the two latter treatments were not always consistent at each sampling. The gardens contained mainly red algae (*Polysiphonia* spp., *Centroceras* spp., *Lophosiphonia* spp.) with some *Enteromorpha clathrata* (Roth) J. Agardh, *Sphacelaria novae-hollandiae* Sonder, and cyanobacteria, of which *Microcoleus lyngbyaceus* was the most common. Algal biomass was stable for bimonthly samplings during

1984, with a lowest value in July of 5.1 ± 1.8 and a highest value in January of 5.9 ± 1.1 mg AFDW cm^{-2} (mean \pm SD, $n=6$, for each sampling). The gardens were dominated by the gardening damsel fish *Eupomacentrus nigricans*, but territories were shared with and aggressively defended by two other damsel fish, *Dischistodus notophthalmus* and *D. perspicillatus*. *D. notophthalmus* also had separate gardens in some instances; however, only gardens dominated by *E. nigricans* were sampled.

Discussion

Substratum type, sites and nitrogen fixation

There is wide variability of nitrogen-fixation rates on natural substrata for a coral reef. However, the transect across the reef showed relatively little variation in rates for the epilithic algal community (EAC) when substrata were taken randomly at each site (Table 3). Significantly lower values were found for the pic crust substrata in four out of six samplings (Table 3), and this was supported by the analysis shown in Table 4. For the two patch reefs (microatoll and comparison atoll) there was some indication of highest values (but only at some seasons) on substrata on the floor bottom inside the atolls as compared with their sides. The deeper substrata (1 to 2 m below lagoon low-water) on the outside of these patch reefs had somewhat lower rates. Recently dead coral had high rates of nitrogen fixation (Table 4). It is factors such as these that give rise to the relatively high variance for random samplings at any site.

The low values found for the outer reef slope (Tables 2 and 3) are in agreement with data reported by Wiebe et al. (1975) but not with that of Wilkinson et al. (1984). Other substrata had a much larger range of rates compared to the EAC (Table 2), but none of the substrata showing high rates were widely distributed.

Accuracy of techniques

Despite the criticism of Flett et al. (1976), the present method, employing an incubation vessel with a relatively large airspace, works well, as judged by repeatability and linearity with time, if the contents of the container are stirred vigorously, both after C_2H_2 injection and before an air sample is taken for C_2H_4 determination, if the container is agitated during the experimental period, and if due allowance is made for the volume of the airspace and the residual C_2H_4 in solution. As shown here, linear rates can be obtained from 0 to 6 h. The lag in C_2H_4 production noted by a number of other workers (Wiebe et al. 1975, Hanson and Gundersen 1977, Wilkinson et al. 1984) probably resulted from inadequate stirring during the experimental period (see Flett et al. 1976). While Flett et al. pointed out that greater sensitivity can be achieved by reducing the air-

space in the system, the effect of a small airspace on C_2H_4 concentration in the airspace becomes pronounced only at airspaces $< 30\%$. Caution should be exercised in following Flett et al., since photosynthetic oxygen production could lead to inhibition of nitrogen fixation. Rates as high as $1.5 \mu\text{mol O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ have been measured for epilithic algal communities on coral reefs (Borowitzka et al. 1983), and with a small airspace the oxygen concentration could rapidly increase to levels where inhibition of nitrogen fixation, already present at air-saturation levels (Burris 1976), is significantly enhanced.

The experimentally determined ratio of C_2H_2 reduced to nitrogen (3.45) compares well with previously published values for blue-green algae in the range 1.9 (Burris 1976) to 4.8 (Stewart et al. 1968, Peterson and Burris 1976). There is no published evidence that such variation is due to real differences. More likely, the variation reflects the considerable errors of calibration. Consequently, the use of an experimentally determined ratio, rather than the theoretical ratio of 3.0 (assuming no hydrogen evolution) or 4.0 (assuming one H_2 evolved by nitrogenase per N_2 reduced), does not guarantee improved accuracy, especially when the variance of the mean is large, as here. The main benefit of a demonstration of comparable activity using $^{15}\text{N}_2$ or C_2H_2 lies in its confirmation that nitrogen fixation is indeed taking place and that the experimental procedures employed are satisfactory.

As a result of the cost-benefit analysis ("Materials and methods – Sampling procedure and statistical analyses") two repeats of 14 replicates were taken for each experiment at any given site and time; the two repeats being separated by 1 to 10 d. Analysis of these repeats indicated that, although often similar to one another, there is overall greater variability between the repeats than within them. This suggests that there are factors which cause changes in the nitrogen fixation at a given site from day to day. Identification of these factors has not been attempted. They might be related to site/situation-dependent factors such as water movement, light history, grazing, levels of inorganic nitrogen in the water and temperature, or they might be related to some variable feature of the assay such as degree of agitation or temperature. In regard to the second alternative, the assay technique was designed to provide as constant conditions as possible (within the limitations of the method) in terms of light, temperature and stirring. Inspection of the data revealed no obvious association between variable repeats and any of these factors.

Seasonal effects

Seasonal variation was generally not discernible against the high background variation, although there are indications of lower rates in the autumn and winter (Tables 3 and 5). Further work at Site 3 reef flat (Larkum 1988) has confirmed this trend. Wilkinson et al. (1985) also found a significant reduction on coral plates in mid-winter.

Midday temperatures varied from 20°–24 °C in winter to 27°–30 °C in summer. On two brief occasions in mid-winter the midday temperature dropped to 18.0° and 17.6 °C, respectively. On both occasions the rates of C₂H₂ reduction were exceptionally low. On the second occasion (1985), the control site (Quadrat 2, Site 3) was measured and gave a mean (\pm SD, $n = 14$) of 2.30 ± 1.36 nmol C₂H₄ cm⁻² h⁻¹. This was repeated five days later at a temperature of 22.0 °C, when a mean of 4.92 ± 3.22 nmol C₂H₄ cm⁻² h⁻¹ was recorded. These values are significantly different (Student's *t*-test at $P < 0.01$). The results indicate that 18° to 22 °C may be a critical temperature range for nitrogen fixation on coral reefs.

Agents of nitrogen fixation

Two unialgal samples of cyanophytes (both heterocystous) fixed nitrogen at high rates (*Calothrix crustacea*, *Scytonema hofmannii*), in accord with previous reports (Mague and Holm-Hansen 1975, Wiebe et al. 1975, Burris 1976, Potts and Whitton 1977), and these same algae were abundant on substrates that exhibited high rates of nitrogen fixation. However, there were numerous other cyanophytes, some heterocystous, present. It is therefore possible that one of the other heterocystous algae was the major agent of nitrogen fixation. This may be true for the gelatinous masses found on the reef crest and elsewhere, which reduced C₂H₂ at high rates and yet did not contain either of the above species. It is, however, possible that other non-heterocystous cyanophytes also fixed nitrogen, either in a semi-anaerobic layer of the epilithic algal community, or the gelatinous masses (Kallas et al. 1983), or even under aerobic conditions (Kallas et al. 1983). Potts and Whitton (1977) found evidence for nitrogen fixation by some non-heterocystous cyanophytes, including *Hyella balani*. Although a similar species (*Entophysalis deusta* = *Hyella balani*) occurs abundantly on beach rock and intertidal rubble at One Tree Reef, such substrata were found to have relatively low nitrogen fixation rates (Table 2), even when rewetted. These results, therefore, agree with those of Crossland and Barnes (1976) who found low rates of C₂H₂ reduction associated with unidentified endolithic cyanophytes of coral skeletons. A contribution from photosynthetic bacteria cannot be ruled out (Potts and Whitton 1977).

Disturbance and nitrogen fixation

Coral skeletons, produced by predation by the crown of thorns starfish *Acanthaster planci* displayed enhanced nitrogen-fixation levels after 3 to 6 mo (Larkum 1988) and so did coral blocks after 3 to 6 mo (Table 5). On both these substrata the abundance of cyanophytes, especially *Calothrix crustacea*, was high. This evidence supports the hypothesis that disturbance maintains high nitrogen fixation on coral reefs by providing new space for colonization by ni-

trogen-fixing cyanophytes which form part of an early successional stage (cf. Wilkinson et al. 1984, 1985). Later successional stages, such as crustose coralline algae (e.g. "piecrust") or dense stands of macroalgae (e.g. the reef crest) or overgrowth by sedentary animals such as corals, sponges and ascidians, may then lead to a reduction in the levels of cyanophytes and, consequently, of nitrogen fixation. Thus, disturbances such as fish grazing, storms and cyclones, *A. planci* predation and summer extremes of temperature and insolation may all serve to enhance nitrogen fixation. Disturbance of a similar kind has been previously invoked to account for the presence of the epilithic algal community (EAC) and its high productivity (Wanders 1977, Borowitzka et al. 1978, 1983, Hatcher and Larkum 1983).

Our damselfish observations do not support the hypothesis that lack of grazing within damselfish territories leads to lower rates of nitrogen fixation (Wilkinson and Sammarco 1983, Wilkinson et al. 1985).

From the evidence that coral blocks maintained a typical EAC and high rates of fixation up to 30 mo, in contrast to skeletons killed by the crown of thorns starfish (Larkum 1988), and at variance with nitrogen fixation on natural blocks, we conclude that these blocks did not simulate natural substrates. Several differences from natural substrates can be suggested: the blocks were anchored on the bottom at approx 1 m depth, where much coarse sand accumulated and during storms much sand abrasion would have taken place; the blocks were smooth, which might have affected algal colonization and fish grazing; the blocks were separate from other coral/limestone stands in the area, and might have been subjected to increased grazing by schools of fish. Partial support for these effects comes from the evidence that exposed bedrock in the centre of the microatoll and control atoll had high rates of nitrogen fixation and that "natural" blocks (of indeterminate age, rugose surface and loose on the bottom) had much lower rates of nitrogen fixation.

Nitrogen-fixation budget

The budget for nitrogen fixation on One Tree Reef (Table 6), has been adjusted for slightly lower (80%) rates in the three winter months (June–August) and for light-saturated rates, as follows: 8 h for 3 mo (June–August), 12 h for 3 mo (December–February), and 10 h for the remaining 6 mo. The reef-flat and patch-reef substrates have a high level of annual nitrogen fixation (34 to 68 kg N ha⁻¹ yr⁻¹), together accounting for 80 to 85% of the total nitrogen fixation on One Tree Reef. The mean nitrogen fixation for the whole reef was considerably lower (8 to 16 kg N ha⁻¹ yr⁻¹). No such detailed budget has been attempted previously, but the above rates are one to two orders of magnitude less than estimates of early workers (Mague and Holm-Hansen 1975, Webb et al. 1975, Wiebe et al. 1975, Potts and Whitton 1977, Paerl et al. 1981). They are in fairly general agreement with work from the Great Barrier

Table 6. Budget sheet of nitrogen fixation at One Tree Reef. C₂H₂ reduction rate is based on summer data from Tables 2, 3, 5

Region (1)	C ₂ H ₂ reduction rate (nmol cm ² h ⁻¹) (2)	Nitrogen fixation rate ^a (kg ha ⁻¹ yr ⁻¹) (3)	Ratio of actual to projected area ^b (4)	Corrected nitrogen fixation rate ^c (kg ha ⁻¹ yr ⁻¹) (5)	Total available projected area ^d (ha) (6)	Total amount N ₂ fixed annually (kg) (7)
Reef slope	2–4.5	4.5–10.2	2	9.0–20.4	75	675–1 530
Reef crest	1–2	2.3–4.5	1.2	2.8–5.4	50	140–270
Reef flat	5–10	11.4–22.7	3	34.2–68.1	135	4 617–9 194
Patch reefs	5–10	11.4–22.7	3	34.2–68.1	70	2 394–4 767
Lagoon sand	0.4–0.8	0.9–1.8	1	0.9–1.8	380	342–684
Beach rock	0.3–0.6	0.7–1.4	1.5	1.0–2.0	80	80–160
					Total	8 248–16 605 ^e

^a Conversion based on ratio of C₂H₂:N₂ fixed of 3.45:1.0 and a total number of 2 920 light-saturated hours annually (light-saturated hours per day: summer 9, spring/autumn 8, winter 7); winter rate was adjusted to 0.8 of summer rate to allow for temperature effects

^b Based on original (unpublished) data and measurements of Hatcher (1981), using technique of Dahl (1973)

^c Nitrogen fixation rate (projected area basis) corrected for substratum tortuosity by taking the product of nitrogen fixation rate (Column 3) and ratio of actual to projected area (Column 4)

^d Projected area of limestone substratum derived from actual projected area of zone and % limestone substratum for each region as follows: reef slope, reef flat, patch reefs = 50%; reef crest, lagoon sand = 100%

^e For One Tree Reef with a total area of 1 050 ha, mean annual fixation of nitrogen is thus 7.9 to 15.8 kg ha⁻¹

Reef (Burris 1976, Wilkinson et al. 1984). Despite this downwards revision, these rates still compare favourably with many agricultural systems (Postgate 1982), except for symbiotic nitrogen fixation in legumes grown in monoculture.

Nitrogen fixation and nitrogen turnover

The epilithic algal community of shallow, well-grazed substrates provides the major input of nitrogen from nitrogen fixation on One Tree Reef (Table 6, Column 7). Presumably, the fixed nitrogen is efficiently recycled within this community, but fish grazing and detrital processes must lead to significant mineralization and production of dissolved inorganic nitrogen (DIN). Previous studies have shown a correlation between areas of high nitrogen fixation and increased levels of DIN in adjacent waters (Johannes et al. 1972, Webb et al. 1975, Wiebe et al. 1975, Wilkinson et al. 1984). Neither we (unpublished results) nor Hatcher and Hatcher (1981) found high levels of DIN (ammonium, nitrate and nitrite) in water ebbing downstream of the southern reef flat at One Tree Reef. Thus, any mineralization of organic nitrogen is efficiently recycled within a short time. The high values of Hatcher and Hatcher (1981) for tidal pools near One Tree Island might be explained for the present work as remineralization of nitrogen fixed by the *Scytonema hofmannii* communities which were seasonally abundant adjacent to these pools. However, further work of Hatcher and Frith (1985) indicates high DIN levels in the water close to the eastern reef crest. Their suggestion that high nitrogen fixation in this region led to high mineralization is not supported by the present work. The substrata in this area (beach rock exposed at low tide) were found to have low nitrogen fixation activity apart from the *S. hofmannii* communities, which

covered relatively small areas at restricted periods of the year. It seems more likely that organic material swept up onto this emergent reef crest by the prevailing swell supports a detrital system in which much mineralization occurs, stimulated by the aerobic conditions.

The high estimates of early workers suggested that nitrogen fixation would provide all the nitrogen requirements for a coral reef (Paerl et al. 1981). More recent work (Burris 1976, Wilkinson et al. 1984, present results) showing rates an order of magnitude lower, makes this more doubtful. The detailed budget for Kaneohe Bay, Hawaii (Hanson and Gundersen 1977) indicated that nitrogen fixation accounted for only 1% of primary productivity. However, it was for a eutrophically polluted bay dominated by phytoplankton production. From the present data, from primary production estimates of Larkum, Day and Borowitzka (in preparation), and from C:N ratios, Koop and Larkum (in preparation) have calculated mass-balance estimates which show that nitrogen fixation provides between 9 and 14% of the annual nitrogen requirement on One Tree Reef. This is a significant contribution (and the contribution is larger for the epilithic algal communities of the reef flat), but it is not clear at present whether this input is sufficient to make up for annual losses of nitrogen, principally those of denitrification (Goering 1983) and output to the surrounding ocean. Further studies of nitrogen turnover and flushing rates for One Tree Reef are needed to resolve this issue.

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