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Nitrogenase activity associated with *Codium* species from New Zealand marine habitats

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ABSTRACT

Nitrogenase activity, measured as acetylene reduction, was recorded at rates up to 1028 $nmol.h^{-1}.g^{-1}$ dry weight for *Codium adhaerens* (Cabr.) Ag. var. *convolutum* Dellow and *Codium fragile* (Sur.) Hariot subsp. *tomentosoides* (Van Goor) Silva collected from New Zealand habitats. In both species the ability to reduce acetylene is invariably associated with the presence of a heterocystous blue-green alga, *Calothrix* sp., epiphytic or embedded in the *Codium* thallus. A highly significant ($P < 0.001$) correlation between heterocyst frequency and nitrogenase activity was found.

Nitrogenase and net photosynthesis of the *Codium-Calothrix* system have different steady-state responses to light intensity, and the kinetics of the two processes also differ in that nitrogenase is slow to respond to illumination or darkening. Glucose additions to *Codium* did not significantly increase nitrogenase activity. Nitrogenase is relatively insensitive to oxygen tension over the range 0–1.0 atm (0–1.033 $kgf.cm^{-2}$) and still occurs at 1.5 atm (1.55 $kgf.cm^{-2}$); this condition is unique in all nitrogenase systems thus far reported. Collectively these facts suggest that *Calothrix* is the agent primarily responsible for nitrogenase activity in these *Codium* species.

INTRODUCTION

Although Stewart (1971) found no evidence of nitrogenase activity in the large algae of the Scottish coast, recent studies have indicated that marine epiphytic bacteria and blue-green algae may be important sources of fixed nitrogen (Carpenter 1972, Head & Carpenter 1975). Head & Carpenter (1975) reported significant nitrogenase activity associated with *Codium fragile* subsp. *tomentosoides* populations in Massachussetts, USA, which they attributed to rich populations of *Azotobacter* on the surface of the utricles. This *Codium* subspecies is a vigorous adventive in parts of Europe (Silva 1955) and North America (Bouck & Morgan 1957, Wood 1962, Taylor 1967) and has spread recently to New Zealand where it is still expanding its range (Dromgoole 1975).

The present work confirms that nitrogenase is associated with *Codium fragile* subsp. *tomentosoides* and extends the finding to another species, *Codium adhaerens* var. *convolutum*. In contrast to the earlier work, we believe the agent to be a species of *Calothrix* which is closely associated with the *Codium* tissues.

METHODS

Codium plants were collected at low tide from various places near Auckland (see Table 1) and taken to the laboratory in plastic bags containing seawater. Estimates of photosynthesis or nitrogenase activity were made within 24 h of collection unless otherwise indicated. Photosynthetic activity of the intact plants was estimated from changes in dissolved oxygen continuously monitored by a Beckman polarographic sensor in a closed perspex chamber (Dromgoole unpublished 1973). Photosynthetically active radiation (400–700 nm) at the surface of the *Codium* thallus is expressed as microEinsteins per square metre per second ($\mu E.m^{-2}.s^{-1}$).

Tests for nitrogenase activity were made using the acetylene reduction assay (Dilworth 1966). Whole plants were used in the preliminary survey of activities. In later experiments, to overcome the problems of heterogenous distribution of *Calothrix* in the *Codium* thallus, *Codium* was dissected into many pieces of about 5 mm diameter, and these were randomly distributed into 7 ml serum-capped bottles, normally five pieces per bottle. The bottles were then injected with 1 ml acetylene and incubated in front of fluorescent lamps (150 $\mu E.m^{-2}.s^{-1}$) at 26°c, the temperature being monitored by a thermistor probe inside the *Codium* thallus within one of the bottles. The gas was assayed after incubation using 0.1 ml injections into a Carle 9500 gas chromatograph fitted with a 1 m × 1.7 mm column of Porapak T at 70°c. Oxygen levels were produced by replacing the gas phase with argon by evacuation and adding oxygen to appropriate levels. Gas phases were checked on occasions by gas chromatography on a Carle 6500 thermal conductivity chromatograph.

Following nitrogenase activity tests, 3–4 subsamples of each *Codium* piece were examined by light microscopy for the presence of blue-green algae. The frequency of *Calothrix* heterocysts was determined by direct counting after teasing the samples apart and squashing gently with a coverslip. To determine if nitrogenase activity was stimulated by additions of a carbohydrate source, plants were incubated in the dark in seawater containing various concentrations of glucose.

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TABLE 1—Ranges of nitrogenase activity (as acetylene reduced) associated with *Codium* species. Collection localities and positions: a = Bastion Point, 36° 51′ S, 174° 49′ E; b = Parnell Reef, 36° 51′ S, 174° 48′ E; c = Half Moon Bay marina, 36° 53′ S, 174° 55′ E; d = Motuihe Island, 36° 49′ S, 174° 58′ E; e = Rakino Island, 36° 43′ S, 174° 47′ E; f = Piha, 36° 58′ S, 174° 28′ E. Plants (wet weight 1–10 g) were incubated for 1–1.5 h. ND = no detectable activity (i.e., < 0.1 nmol.g^{-1}.h^{-1}). Blue-green algae are recorded as present (+) or absent (—) in the samples

Species	Locality	Date (1976)	Nitrogenase (nmol.g^{-1}.h^{-1})	*Calothrix*	*Oscillatoria*	Replicates
C. adhaerens (Cabr.) Ag. var. *convolutum* Dellow	a	5 April	3-21	+	+	3
	b	4 March	20-193	+	+	10
	d	1 March	25-145	+	+	5
	e	15 March	4-37	+	+	15
C. fragile (Sur.) Hariot subsp. *tomentosoides* (Van Goor) Silva	a	1 April	67-240	+	+	5
	a	5 April	69-157	+	+	11
	b	11 March	37-288	+	+	5
	c	24 Feb.	ND	-	+	3
C. fragile (Sur.) Hariot subsp. *novae-zelandiae* (J. Ag.) Silva	f	22 March	ND	-	+	10

RESULTS

GENERAL DISTRIBUTION OF ACTIVITY

A survey of various species and sites indicated that nitrogenase is commonly associated with *Codium* species (Table 1). High activities were generally recorded for the introduced *C. fragile* subsp. *tomentosoides*. However, plants from Half Moon Bay marine were inactive; as the plants consisted only of the percurrent holdfast and lower stipe, this was attributed to scenescence. In the Bastion Point populations, the activity was low in the extremities of plants and at maximum in the upper stipe (Table 2).

This lack of uniformity may also account for the wide range of activities recorded in Table 1, as whole plants of varying size and age were used. Maximum activities were generally lower in *C. adhaerens* var. *convolutum*, but again showed a wide range. A population of ten mature samples of *C. fragile* subsp. *novae-zelandiae* collected from Piha were covered by a variety of epiphytic red, green and blue-green algae, but showed no detectable nitrogenase activity.

In all the samples tested the ability to reduce acetylene was associated with the presence of epiphytic hetereocystous blue-green algae belonging to the genus *Calothrix*. Most samples also contained an epiphytic *Oscillatoria* species, but this was not invariably linked with nitrogenase (Tables 1 and 2).

LIGHT REQUIREMENTS

Time-course studies showed that for material collected at 1130 h there was a linear ethylene production for at least 400 min without a lag period. However, for material collected at dawn (0600 h), there was a distinct lag of 2–3 h, indicating that light is required for activity.

This was confirmed by the finding that both nitrogenase and photosynthesis are increased by light at the lower intensities (Fig. 1). For *Codium adhaerens* the response curves for these two processes are quite different: nitrogenase showed a peak activity at 20–40 μE.m^{-2}.s^{-1}, whereas photosynthesis was still increasing at 100 μE.m^{-2}.s^{-1}. In *Codium fragile* subsp. *tomentosoides* the response to light of the two processes was rather similar: both increased up to 200 μE.m^{-2}.s^{-1} (Fig. 1).

The high light inhibition of nitrogenase activity in *C. adhaerens* was apparently not caused by exhaustion of inorganic carbon during the long preincubation period. Subsequent bicarbonate additions, whilst stimulating nitrogenase activity, did not alter the overall response pattern.

RESPONSE TO ORGANIC CARBON

Repeated additions of 0.1% glucose to *Codium fragile* subsp. *tomentosoides* had no effect on the decline in nitrogenase activity in the dark following treatment in the light. Segments of *C. adhaerens*

TABLE 2—Ranges of nitrogenase activity (as acetylene reduced) associated with various parts of *Codium fragile* subsp. *tomentosoides*. Plants collected at Bastion Point on 23 March 1976. Samples (wet weight 1–5 g) were incubated for 1.5 h. ND = no detectable activity (< 0.1 nmol.g^{-1}.h^{-1})

Part of plant	Nitrogenase (nmol.g^{-1}.h^{-1})	*Calothrix*	*Oscillatoria*	Replicates
Apical tips	ND	-	-	3
Upper dichotomies	87-90	+	-	2
Lower dichotomies	20-245	+	-	3
Upper stipes	219-880	+	-	4
Holdfast	ND-17	+	-	2

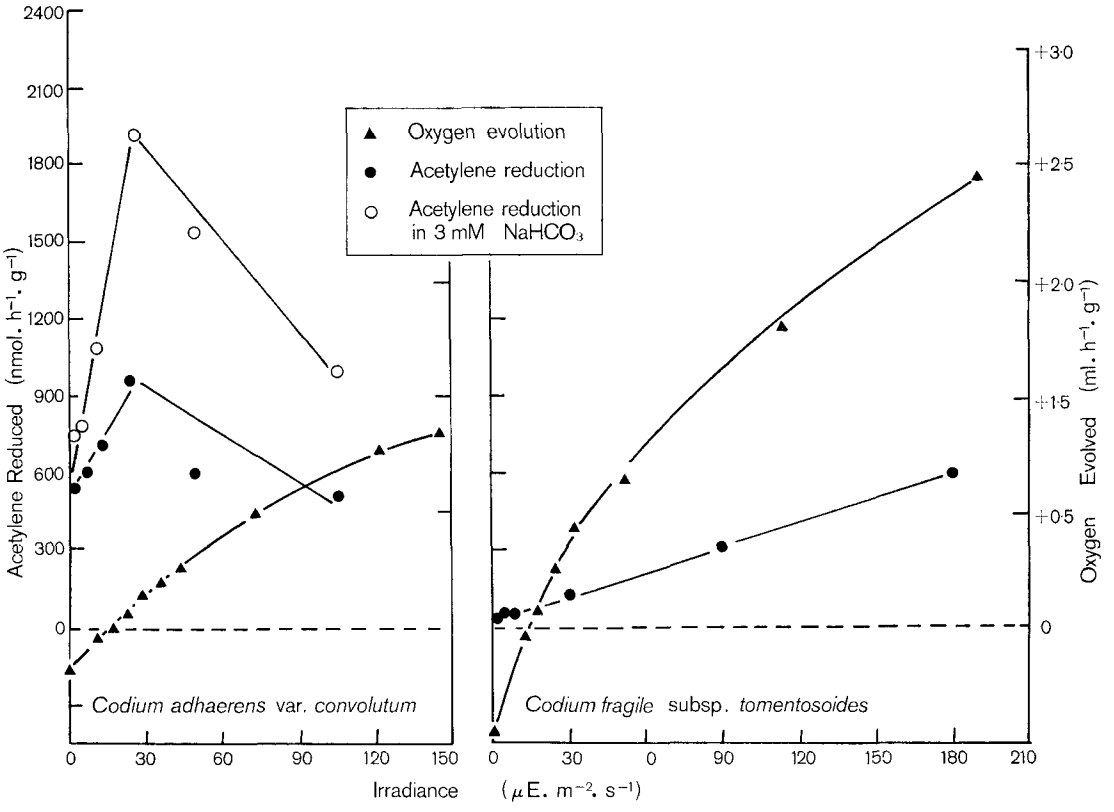

FIG. 1—Net photosynthesis (oxygen evolution) and nitrogenase activity (acetylene reduction) as a function of irradiance for two species of *Codium*; nitrogenase activity was determined after a 5–5½ h incubation following a 15 h pre-incubation in the light.

incubated in the dark in seawater containing a range of glucose concentrations also showed a gradual decline: there was no significant stimulation at any time, and no detectable acetylene reduction after 24 h (Table 3).

RESPONSE TO OXYGEN

Nitrogenase was relatively insensitive to oxygen tension (Table 4). In the upper part of Table 4 the results for pO_2 levels from 0–0.40 atm (0.413 kgf.cm^{-2}) are presented as a ratio of an initial rate in air; because there is the initial lag described above, all subsequent rates are significantly higher. However, the results are consistent in that there is little effect of oxygen except for a possible slight reduction at the lowest level. The same samples were then regassed in groups of five at pO_2 levels of 0 atm, 0.40 atm and 0.80 atm (0, 0.413, and 0.826 kgf.cm^{-2}). There was no significant effect of oxygen after 70 min. Higher pO_2 levels were tested with material which had been illuminated to eliminate the initial lag (lower part of Table 4). Although there was a reduction in activity at pO_2 of 1 atm, there was still some activity present at 1.5 atm after 200 min. This experiment was further assayed after 20 h at the highest pO_2, when significant ethylene production above background was still present.

ORGANISM RESPONSIBLE

Nitrogenase activity and the presence of *Calothrix* sp. on the *Codium* were invariably associated (Tables 1 & 2). Further experiments (Fig. 2) showed a highly significant ($P < 0.001$) linear correlation between frequency of *Calothrix* heterocysts and acetylene reduction level ($r = 0.84$ and 0.96 for *Codium fragile* subsp. *tomentosoides* and *C. adhaerens* respectively). Thus, the alga is probably responsible for most of the acetylene reduction observed.

Microscopy showed that *Calothrix* was sometimes epiphytic on the outer utricle surfaces, in contact with the external medium, or more commonly endophytic (i.e., lying between the appressed utricle surfaces, and thus inside the *Codium* thallus but external to the *Codium* cells). The morphology of *Calothrix* was variable, possibly because of its varying position, and its exact taxonomic status is

TABLE 3—Effects of various concentrations of glucose on mean rates of nitrogenase activity (as acetylene reduced) during a dark incubation of *Codium adhaerens*. Three replicates (wet weight 0.5–1.0 g) were used for each treatment. ND = Not detectable (i.e., < 0.01 nmol.h^{-1})

Glucose (%)	Activity (nmol.h^{-1})		
	3h	6h	24h
Nil	0.40	0.45	ND
0.05	0.31	0.23	ND
0.10	0.30	0.20	ND
0.20	0.46	0.33	ND
0.50	0.30	0.25	ND

TABLE 4—Effects of oxygen tension on nitrogenase activity (C_2H_2 reduction) of *Codium adhaerens*. Triplicate tissue samples assayed in air (1 h) and then transferred to various pO_2 levels. Results expressed as a mean ratio of the initial rate in air. In the lower section of this table, tissue samples had been illuminated before testing, to reduce the initial lag

pO_2	Ratio of initial rate		
	160 min	250 min	430 min
0	1.65	1.80	1.95
0.08	1.95	1.86	2.20
0.15	1.75	1.75	1.70
0.25	2.04	2.18	2.30
0.40	2.04	2.24	2.28

pO_2	Ratio of initial rate (200min)
0	0.67
0.20	0.66
0.40	0.90
1.00	0.47
1.50	0.15

uncertain. However, its general form and the dimensions of the trichome agree closely with *C. scopulorum* (Weber & Mohr) C. Ag., but younger plants could be assigned to *C. confervicola* (Roth) C. Ag., which has been recorded as parasitic on larger New Zealand marine algae (Chapman 1956).

The *Codium* plants also housed an epiphytic population of *Oscillatoria* sp., the presence of which was not correlated with nitrogenase activity (Tables 1 & 2). Isolates of *Oscillatoria* on a nitrogen-free medium were incapable of acetylene reduction. Attempts to isolate the *Calothrix* into a variety of media were unsuccessful; when present, the *Oscillatoria* sp. invariably dominated.

DISCUSSION AND CONCLUSIONS

In contrast to the conclusions of Head & Carpenter (1975), who attributed *Codium* nitrogenase activity to epiphytic *Azotobacter*, we suggest that in the New Zealand species of *Codium* nitrogenase activity is caused by an epiphytic/endophytic population of *Calothrix*. Our reasoning is: (i) intact plants or plant parts lacking *Calothrix* were unable to reduce acetylene, and (ii) there was a highly significant correlation between heterocyst frequency and acetylene reduction activity for both *Codium* species tested.

The dissimilarity of the light intensity curves for photosynthesis and acetylene reduction in *C. adhaerens* suggests that at least in this species acetylene reduction is not closely linked with *Codium* photosynthesis. The two processes also differ markedly in kinetics in both *Codium* species, in that nitrogenase shows a lag of several hours after extended dark treatment and a slow decline lasting 24 h or more after light treatment; the equivalent transients for evolution of photosynthetic oxygen in *Codium* are shorter, the maximum being 12 min for the dark-light induction lag (F.I.D., unpublished observations). Thus, the two processes appear to be relatively independent. Acetylene reduction in free-living blue-green algae is similarly slow to respond to dark–light changes (Bennett unpublished 1975).

The pronounced inhibition of nitrogenase activity in high light intensities in *C. adhaerens* and the absence of a similar effect in *C. fragile* subsp. *tomentosoides* (Fig. 2) samples was perhaps caused by an adaptation of *Calothrix* to the light regime characteristic of the two sample sites: *C. adhaerens* shows a marked preference for shaded rock faces (Dellow 1953) and the samples were taken from a south-facing vertical rock face beneath the Orakei Wharf; *C. fragile* samples were taken from a horizontal rock face exposed to full sunlight at low water.

Nitrogenase systems of heterotrophic bacteria characteristically show a rapid and marked stimulation by added carbohydrate (Knowles 1977). The insensitivity to additions of glucose suggests that these organisms do not contribute to the nitrogenase activity in the *Codium* plants. The finding is, however, compatible with the lack of marked stimulation of most blue-green algae to exogenous carbohydrate (Stewart 1973).

The acetylene reduction activity associated with *Codium* is unique in all nitrogenase systems thus far reported in that it is relatively insensitive to oxygen partial pressures up to 1 atm (1.033 kgf.cm^{-2}) and it still occurs at 1.5 atm (1.55 kgf.cm^{-2}). The association between *Codium* and *Calothrix* is also unique in that it is the only symbiosis in which the nitrogen fixing organism is closely associated with photosynthetic tissue. In bacterial associations, the diazotroph is in roots or nodules far from photosynthetic sites. The closest approach to the *Codium*

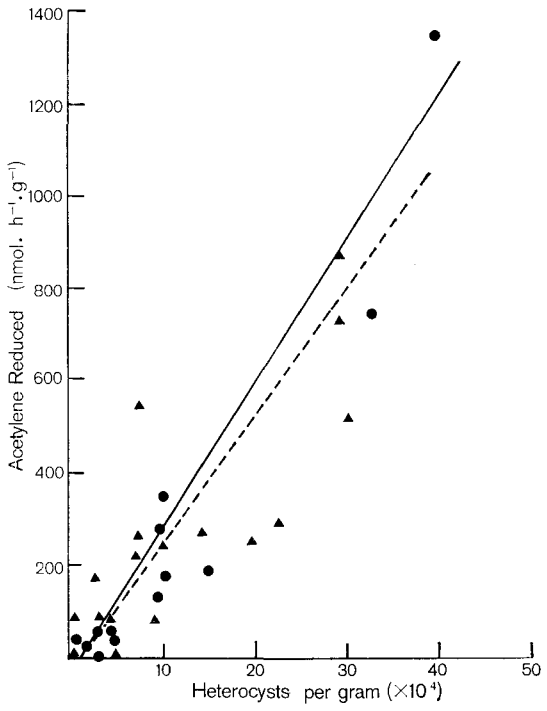

FIG. 2—Acetylene reduction as a function of *Calothrix* heterocyst frequency in tissue samples of *Codium fragile* subspecies *tomentosoides* (circles, dashed line) and *Codium adhaerens* (triangles, solid line).

system is found in *Azolla-Anabaena* and the *Richelia-Rhizosolenium* associations. In the former, the alga is in a cavity connecting via a pore which allows free oxygen diffusion; in the latter, the host is a unicell and thus a large surface area is available for oxygen exchange. However, in the *Codium-Calothrix* system, the host thallus is composed of a great number of compressed utricles which provide considerable resistance to the outward diffusion of oxygen, and gas bubbles form in the tissues during active photosynthesis. Thus it is not surprising to find that a nitrogen-fixing organism embedded in this tissue is physiologically adapted to high oxygen tensions.

Although it is difficult to compare acetylene reduction rates based on heterocyst numbers, the rates for *Codium-Calothrix* are much higher than quoted elsewhere. Fay (1969) and Jewell & Kooliosoriya (1970) indicate a rate of 3.6 nmol.h^{-1} per 10^5 heterocysts for *Anabaena cylindrica*, Silvester (1975) gives 3.6 nmol.h^{-1} per 10^4 heterocysts for the *Gunnera-Nostoc* association, and in the present study we found a mean activity of 3.6 nmol.h^{-1} per 10^3 heterocysts. Thus *Codium-Calothrix* has an extremely high efficiency, but part of the discrepancy may be caused by the larger heterocysts of *Calothrix* (up to 50% greater in volume than those of *Anabaena cylindrica*) and also by the difficulty of counting heterocysts. However, the consistency of the rates we have obtained for this relationship leads us to believe it is correct, and the conclusion drawn is that the micro-environment produced in *Codium* is able to stimulate high nitrogenase levels or facilitate high nitrogenase efficiency.

The nitrogenase activities associated with *Codium* may be of some ecological significance: both species are dominant in the sublittoral fringe zone of sheltered waters. The average dry weight of the standing crop of *C. adhaerens* in this zone at Parnell Reef was 1400 g.m^{-2}. Calculated nitrogen fixation (acetylene reduction) rates for this material based on laboratory activities and a 12 h day average 8.7 mg.m^{-2}.d^{-1} (maximum 209 mg.m^{-2}.d^{-1}). *C. fragile* subsp. *tomentosoides* is a seasonal dominant with a peak standing crop of 1200 g.m^{-2} in December. Calculated rates for this species average 18.5 mg.m^{-2}.d^{-1} (maximum 133 mg.m^{-2}.d^{-1}). Actual fixation rates in both species may be in excess of these figures if activity continues in the dark.

The embedded *Calothrix* populations could provide *Codium* with combined nitrogen compounds. Other free-living and symbiotic blue-green algae are known to liberate ammonia and amides, and some species of green macroalgae can utilise organic nitrogen compounds (Lewin 1955, Berglund 1969). Head & Carpenter (1975) calculate that the fixation rates they found could account for 2–7% of the *Codium*'s nitrogen requirements per day. Using their figures for the C : N ratio of *Codium fragile* (mean = 16) we calculate (using the data in Fig. 2) that the *Calothrix* fixation could similarly supply 6% of the required nitrogen. However, as Head & Carpenter (1975) pointed out, such calculations are probably an underestimate as recycling of newly fixed nitrogen is very likely.

The extent to which the *Calothrix-Codium* association is specific is not known. The general morphology of the epiphyte is similar to *C. scopulorum* (Weber & Mohr) C. Ag., which is a free-living form often abundant on Auckland shores on muddy rocks, near high water mark. However, attempts to maintain the epiphyte in culture were not successful, suggesting some degree of specificity. There are several records of *Calothrix* species which are entirely or largely restricted to one or two species of macrophytic algae, e.g., *C. parasitica* endophytic in *Nemalion* and *Liagora* (Fremy 1933, Feldmann 1958); *C. chapmanii* endophytic in *Enteromorpha torta* (Lami & Meslin 1959). Further work is necessary to identify the *Calothrix* associated with *Codium* and to determine its degree of dependence on the host.

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