Marine Biology 105, 1–8 (1990)

Marine Biology
International Journal on Life in Oceans and Coastal Waters

Nitrogen-fixation by cyanobacteria associated with *Codium fragile* (Chlorophyta): environmental effects and transfer of fixed nitrogen

V. A. Gerard *, S. E. Dunham, and G. Rosenberg **

Marine Sciences Research Center, State University of New York, Stony Brook, New York 11794-5000, USA

Abstract

N_2-fixation associated with the green macroalga *Codium fragile* subsp. *tomentosoides* (van Goor) Silva from Long Island, New York, USA, was attributable to several species of endophytic cyanobacteria. Rates of N_2-fixation ranged from 0.03 to 3.2 $\mu g\, N\, g^{-1}$ dry wt h^{-1} in freshly collected plants from several sites. Growth of the cyanobacteria appeared to be light-limited, due to the transmission of only 5 to 10% of incident light through the pigmented surface-layer of the macroalga. Daily irradiance was the most important factor determining both abundance of cyanobacterial cells and rate of N_2-fixation. The rate was also affected by instantaneous irradiance, and increased twofold from dark to ambient surface irradiance. Rates were reduced at low temperature (8 °C) but showed no temperature effect between 12° and 26 °C. External concentrations of dissolved inorganic nitrogen (DIN) up to 20 μM did not influence N_2-fixation rate, but long-term exposure to 60 $\mu mol\, l^{-1}\, d^{-1}$ of NH_4^+ caused a reduction in the rate. In *C. fragile* grown under high daily irradiance and low external DIN concentration, ~50% of the assimilated-N was attributable to N_2-fixation. However, chlorophyll *b* extracted from plants grown with $^{15}N_2$ showed an atom % excess ^{15}N of less than 0.1, suggesting that only a small proportion of the bacterially fixed-N was transferred to the seaweed. The association between *C. fragile* and its endophytic cyanobacteria appears to be based primarily on microhabitat suitability, rather than mutual metabolic dependence. It is doubtful that N_2-fixation by cyanobacteria is important to the ecological success of this seaweed species.

Introduction

N_2-fixing microorganisms occur in association with many macroalgae, including pelagic and benthic species of *Sargassum* (Carpenter 1972, Hanson 1977, Phlips et al. 1986), a variety of coral reef species (Capone et al. 1977, Penhale and Capone 1981), and *Enteromorpha* spp. on temperate sandflats (Bohlool and Wiebe 1978). The N_2-fixers in each of these associations were identified as epiphytic cyanobacteria. Perhaps the most unusual seaweed-cyanophyte association has been described for several species of *Codium*, specifically *C. fragile* and *C. adhaerens* from New Zealand (Dromgoole et al. 1978) and *C. decorticatum* from the southeast USA (Rosenberg and Paerl 1981). The thallus of this seaweed is composed of a surface layer of tightly-packed, photosynthetic utricles, surrounding a medulla of non-photosynthetic filaments. The cyanobacteria associated with *Codium* spp. are sometimes epiphytic, but are more typically located at the base of the utricles, i.e. are endophytic but extracellular. This microhabitat provides a low-O_2 environment, or reduced microzone, which is optimal for N_2-fixation (Rosenberg and Paerl 1981). However, N_2-fixation associated with *Codium* spp. is not always attributable to cyanobacteria. A heterotrophic bacterium, *Azotobacter* sp., which fixes N_2 aerobically, was identified as an epiphyte on *C. fragile* from the northeast USA, and credited with high rates of N_2-fixation associated with those plants (Head and Carpenter 1975).

Most researchers studying associations between seaweeds and N_2-fixing organisms have postulated that the seaweeds benefit by transfer of bacterially fixed-N to the host plant. N_2-fixation has been estimated to supply anywhere from 2 to 140% of the N-requirement of various seaweed-microbe associations (Carpenter 1972, Hanson 1977, Penhale and Capone 1981, Phlips et al. 1986). Estimates for *Codium* spp. (4 to 6%) fall at the low end of this range (Head and Carpenter 1975, Dromgoole et al. 1978, Rosenberg and Paerl 1981), but a particularly high efficiency of transfer might be predicted for endophytic symbionts. Transfer of fixed-N between symbiotic cyanobacteria and various macrophyte hosts, including lichens, bryophytes, aquatic ferns, and the angiosperm *Gunnera* spp., has been measured using ^{15}N and ^{13}N. In these associations, up to

* Please address all correspondence and requests for reprints to Dr Gerard at the State University of New York
** Present address: Département de biologie, Université Laval, Québec, Québec G1K 7P4, Canada

90% of the fixed-N is exuded by the cyanobacteria and taken up by the host (Silvester and Smith 1969, Stewart and Rodgers 1977, Stewart and Rowell 1977, Peters et al. 1980, Stewart et al. 1980, Rai et al. 1981, Meeks et al. 1985). N-transfer has also been extensively studied in associations between macrophytes and heterotrophic bacteria, particularly legume-rhizobia symbioses (reviewed by Schubert 1986). However, transfer of bacterially fixed-N has not been demonstrated in any seaweed-microbe association.

The present study examined N_2-fixation associated with *Codium fragile* subsp. *tomentosoides* (van Goor) Silva around Long Island, New York, USA. *C. fragile* was inadvertently introduced to this area in 1956 (Carlton and Scanlon 1985), and is now widespread and abundant at many sites. In order to evaluate the importance of N_2-fixation to the ecological success of this species around Long Island, we measured rates associated with plants from several sites, and determined the environmental conditions under which N_2-fixation was optimized. We also examined transfer of bacterially fixed-N to the seaweed host.

Materials and methods

Experimental plants

Whole *Codium fragile* subsp. *tomentosoides* (van Goor) Silva plants were collected during September 1987, at four sites around Long Island, New York: Port Jefferson Harbor on the northern shore of central Long Island, Shinnecock Bay on the southern shore of eastern Long Island, Flanders Bay and Little Peconic Bay between the northern and southern forks of eastern Long Island. At the first two sites, plants were attached to the cobble/boulder substratum at 1 to 2 m below MLW (mean low water). Only unattached, drifting plants were collected at Flanders Bay. At Little Peconic Bay, plants were attached to cobbles, widely interspersed on a sand bottom at 0.5 m below MLW. Plants were collected again from Little Peconic Bay in May and September 1988. Prior to experimental use, plants were held in 170-l tanks in the greenhouse of Flax Pond Marine Laboratory, Long Island, with running seawater ($\sim 200\ l\ h^{-1}$) at ambient temperature and nutrient concentrations, at $\sim 50\%$ of surface irradiance, and with continuous aeration.

Estimation of N_2-fixation rates

Rates of N_2-fixation associated with individual branches of *Codium fragile* were estimated by acetylene reduction assay. In the standard assay, each branch (~ 10 cm long, 2 to 6 g wet wt) was placed in a stoppered 125-ml flask with 75-ml of filtered (6 μm) seawater. The seawater was enriched with 25% Provosoli's enriched seawater medium (PES/4) with no $NaNO_3$. The flask was injected with 10 ml of acetylene, giving 10 to 20% acetylene in the headspace, and incubated with continuous shaking at 12 °C and 200 μmol photons $m^{-2}\ s^{-1}$. Initial ethylene concentration was measured after 10 to 15 min, and final concentration was measured 1 to 2 h later. Ethylene concentrations were determined in 100 μl subsamples from the headspace, using a Perkin-Elmer gas chromatograph equipped with a 3 m Porapak R column and a flame ionization detector. A molar ratio of 3:1 was used to convert acetylene reduction to N_2-fixation (Hardy et al. 1968). Although the actual ratio may vary with different species and conditions (Turner and Gibson 1980), the theoretical 3:1 ratio has been consistently used in estimating N_2-fixation by cyanobacteria associated with macroalgae (Carpenter 1972, Capone et al. 1977, Hanson 1977, Bohlool and Wiebe 1978, Penhale and Capone 1981, Phlips et al. 1986), including *C. decorticatum* (Rosenberg and Paerl 1981).

Identification of N_2-fixing organisms

Freshly collected branches of *Codium fragile* that exhibited high N_2-fixation rates, as well as branches held in the laboratory for up to 4 mo, were examined for cyanobacteria using a Zeiss Universal epifluorescence microscope with filter sets for green light (# 48 77 12) and blue light (# 48 77 09 and LP 510 as the long-pass filter). Similar plant material was used to isolate and culture heterotrophic bacteria. The basal 5 cm of each branch was ground in sterile seawater with a mortar and pestle. The slurry was serially diluted, and 0.1-ml aliquots were plated onto solid medium, consisting of Rennie's combined carbon medium (Rennie 1981) made with artificial seawater (Guerinot and Colwell 1985). This N-deficient medium is selective for N_2-fixing bacteria. For the last two isolations, the chance of N-contamination in the medium was reduced by using noble agar and sterile low-N seawater. After a 5 d incubation period at 22° to 26 °C, bacterial strains were identified on the basis of colony color, size, and morphology. Individual colonies of each strain were transferred to tubes of semi-liquid media for 3 d, and assayed for N_2-fixation. In some cases, strips of solid medium containing bacterial colonies were inserted in tubes and assayed. During the first isolation, duplicate cultures were incubated and assayed both in air and anaerobically under N_2. As the duplicate cultures yielded the same results, subsequent incubations were done aerobically.

Environmental effects on N_2-fixation

To determine effects of daily irradiance on N_2-fixation associated with *Codium fragile*, branches were held for 2 wk under four irradiances (0.6, 1.2, 2.9, and 12 mol photons $m^{-2}\ d^{-1}$) on a 16 h light:8 h dark diel light cycle. All branches were taken from a single plant to reduce branch-to-branch variation, and were individually tagged for identification. Light was provided by cool-white fluorescent lamps, and was varied by layers of neutral-density screening. Irradiance in the photosynthetically active range (400 to 700 nm) was measured using a Biospherical Instruments quantum meter with a spherical sensor. For each irradiance, 5 branches were held in 3 l of filtered, enriched seawater (PES/4 with no $NaNO_3$), replaced weekly. Aeration provid-

ed continuous mixing, and temperature was maintained at 12 °C. N_2-fixation rates and shaken wet weight (±1% error) were measured for each branch at the beginning and end of the treatment period. Dry weight (48 h at 60 °C) and total N and C content (by CHN analyzer) were determined at the end of the experiment. The relative abundance of cyanobacteria was also determined for one branch from each treatment at the end of the experiment. A 2 mm thick cross-section was cut from the middle of the branch, the filaments of one quarter of the section were teased into a single layer, and individual cyanobacterial cells were counted under an epifluorescence microscope. Light transmitted through the pigmented outer layer of the macroalga was estimated by cutting a longitudinal section from each branch. The section was placed over the submerged, flat-plate sensor of a Skye quantum meter, and irradiance (PAR) was compared with and without the section.

Long-term effects of temperature or external DIN concentration on N_2-fixation were examined using the same experimental protocol as for daily irradiance. In these experiments, irradiance was held at 6 mol photons $m^{-2} d^{-1}$ (16 h light : 8 h dark). Temperature was varied by placing different treatments in temperature-controlled rooms at 8°, 12°, and 22°C. DIN concentration was varied by the daily addition of 0, 2, 20, or 60 μmol l^{-1} of NH_4Cl, or 20 μmol l^{-1} of $NaNO_3$. For the 60 μmol $l^{-1} d^{-1}$ treatment, 20 μmol l^{-1} of NH_4Cl was added 3× daily. At the end of a week of enrichment, NH_4^+ and NO_3^- concentrations were 8 and 91 μM, respectively, in the 20 μmol $l^{-1} d^{-1}$ treatments, and NH_4^+ concentration was 150 μM in the 60 μmol $l^{-1} d^{-1}$ treatment. The treatment with no N-enrichment ended with NH_4^+ and NO_3^- concentrations of 0.1 to 1 μM. Short-term effects of DIN concentration on N_2-fixation were determined by standard assay, using untreated branches from a single plant. Rates were measured for individual branches, then remeasured on the following day with different enrichments of NH_4Cl (0, 2, 20, and 60 μM) or $NaNO_3$ (20 μM) added to the seawater medium.

The effect of instantaneous irradiance on N_2-fixation was determined by comparing the rate at each irradiance to the light-saturated rate for the same branch, in order to compensate for branch-to-branch variation. Light-saturated rates were measured at 1 060 μmol photons $m^{-2} s^{-1}$ during the morning, and rates at 0, 60, 110, 250, 1 060, and 1 670 μmol photons $m^{-2} s^{-1}$ were measured later on the same day. Incubations at different irradiances were performed simultaneously to eliminate possible circadian-type effects. Acetylene reduction assays were conducted in serum-stoppered tubes, covered with 0 to 8 layers of neutral density screening or with aluminum foil, and held for 1 to 2 h in a shaking water bath at 12 °C. Light was provided by incandescent lamps, and irradiance was measured using a Skye quantum meter with a flat-plate sensor. Photosynthetic rates of 1 to 2 cm branch sections were determined under a similar range of irradiances using a Rank Bros. O_2-electrode, as described by Gerard (1988). "Saturating" irradiance, I_k, was determined for both N_2-fixation and photosynthesis as the maximum rate divided by the initial slope of the rate vs irradiance curve (calculated by linear regression analysis).

^{15}N-transfer experiment

Transfer of bacterially fixed-N to the macroalga was examined by growing actively fixing branches of *Codium fragile* under $^{15}N_2$. The atom % enrichment was then determined in extracted chlorophyll *b*, which occurs in green algae but not in cyanobacteria. Compounds common to both the macroalga and cyanobacteria were not examined, because cyanobacterial filaments could not be easily separated from the algal tissue. Replicate branches taken from a single plant were tagged, weighed, and assayed for N_2-fixation rates. Each branch was placed in a stoppered 125-ml flask with 75 ml of filtered, enriched seawater (PES/4 with no $NaNO_3$). The medium and headspace were flushed with mock air (0.04% CO_2, 22% O_2, 78% Ar) to remove N_2. Gas (30 ml) was then removed from the headspace and replaced with 30 ml of $^{15}N_2$ gas for experimental branches and with 30 ml of $^{14}N_2$ gas for control branches. All flasks were held for 1 wk at 12 mol photons $m^{-2} d^{-1}$ and 12 °C with continuous shaking. On the last day, irradiance was reduced to 1.5 mol photons $m^{-2} d^{-1}$, to promote chlorophyll *b* synthesis (Ramus et al. 1976). Three branches were assayed for N_2-fixation rates, and the remaining branches were weighed and frozen.

Chlorophyll *b* extraction was done under low light for three experimental branches grown under $^{15}N_2$ and three control branches. Each branch was ground in 100% acetone using a tissue homogenizer and ice jacket, and filtered through a GF/F glass fiber filter. Chlorophyll was transferred from the filtrate to ethyl ether, which was washed several times with distilled water and dried over Na_2SO_4, then evaporated to dryness and redissolved in acetone. Chlorophyll *b* was separated using kieselguhr paper (Jensen 1978), redissolved in acetone, checked for concentration and purity (no chlorophyll *a* contamination) by scanning spectrophotometry, and spotted on a baked GF/F filter. The amount of extract used was calculated to contain 10 to 15 μg N. The filter was enclosed in a discharge tube under vacuum, and the atom % ^{15}N was determined for each sample using a ^{15}N-analyzer as described by Fiedler and Proksch (1975). The atom % ^{15}N excess of branches grown with $^{15}N_2$ was calculated as the difference between the atom % ^{15}N of treated and control branches.

N-limitation of *Codium fragile*

DIN concentrations in seawater used for various assays and culture experiments were measured using an Autoanalyzer. For all experiments, ambient NH_4^+ concentration ranged from 0.2 to 7.2 μM, and $NO_3^- + NO_2^-$ concentration ranged from 0.1 to 3.2 μM. Growth of branches was assumed to be N-limited in all experiments, except in the long-term N-enrichment experiment. N-limited growth was confirmed by low tissue N-contents, which ranged from 0.3 to 1.5% of

dry weight. The critical N-content for N-saturated growth of *Codium fragile* is 1.9% (Hanisak 1979a). Only plants in the long-term N-enrichment treatment with 60 μmol l^{-1} d^{-1} had an average N-content above the critical level (3.3% of dry weight).

Statistical analyses

The statistical significance of effects of various experimental treatments on N_2-fixation rate was determined using two-way analysis of variance (ANOVA). Unless otherwise specified, the significance of the interactive effect of time (initial vs final rate) and treatment (e.g. irradiance, temperature, N-enrichment) is given. The null hypothesis was rejected at the 95% probability level.

Results

Codium fragile collected from all four sites around Long Island, New York, during September, 1987, exhibited measurable N_2-fixation, but the rates of different populations varied by two orders of magnitude (Table 1). Plants from Little Peconic Bay had consistently high N_2-fixation rates and were used in subsequent experiments.

Microscopic examination revealed that three species of cyanobacteria were consistently associated with *Codium fragile* having high N_2-fixation rates. *Microcoleus lyngbyaceus* (Kutzing) Crouan, a non-heterocystous species, was most abundant. Two heterocystous species, *Calothrix crustacea* Schousboe and Thuret and *Scytonema hofmannii* C. Agardh, were less abundant. These three species were found at the base of the utricles, extending outward between the utricles and inward into the medulla, in freshly collected plants and in plants held in culture for up to 4 mo. The density of cyanobacteria was highest in the old, basal portions and lowest in the young, distal portions of branches. Eighteen strains of heterotrophic bacteria were also isolated from *C. fragile*. Most of the strains were isolated during two or more of the four separate isolations. Nine of the strains were cultured both aerobically and anaerobically. Although they were able to grow on N-deficient medium, none of the strains exhibited measurable N_2-fixation.

Of the environmental factors examined, daily irradiance had the greatest effect on N_2-fixation. Rates varied more than sixfold in *Codium fragile* branches grown for 2 wk under daily irradiances ranging from 0.6 to 12 mol photons m^{-2} d^{-1} (Fig. 1). Differences among treatments were statistically significant ($F=5.2$, $p<0.01$) and were significantly correlated ($r=0.999$, $p<0.01$) with variation in the relative abundance of cyanobacteria (Fig. 1). Branches grown at the highest irradiance had the highest concentration of cyanobacterial cells observed during the study – estimated to be ~20% of total biomass.

Variation in instantaneous irradiance caused a twofold change in N_2-fixation, with rates in the dark being ~50% of the light-saturated rate (Fig. 2). The "saturation" irradiance, I_k, of N_2-fixation was 134 μmol photons m^{-2} s^{-1}, somewhat higher than the I_k of photosynthesis which was 80 μmol photons m^{-2} s^{-1} (Fig. 2). Because only 5 to 10% of incident light penetrated the utricle layer of the macroalga ($n=20$, mean=7.4, SE=0.3), N_2-fixation was saturated when the cyanobacteria were exposed to ~10 μmol photons m^{-2} s^{-1}.

Temperature appeared to influence the rate of N_2-fixation associated with *Codium fragile* only toward the low end of the seasonal range around Long Island (0° to 28 °C). N_2-fixation rates were similar in branches acclimated at 12° and 22 °C, but were reduced at 8 °C (Fig. 3). Although the difference between temperature treatments was not statistically significant ($F=2.7$, $p>0.05$), the data suggest that N_2-fixation would be negligible at winter temperatures.

Table 1. *Codium fragile*. Rates of N_2-fixation associated with plants from four sites around Long Island, New York. Values are mean ± SE (n)

Collection site	Date	N_2-fixation (μg N g^{-1} dry wt h^{-1})
Port Jefferson Harbor	30 Sep. 1987	0.04 ± 0.01 (4)
Shinnecock Bay	30 Sep. 1987	0.16 ± 0.04 (4)
Flanders Bay	30 Sep. 1987	0.03 ± 0.01 (4)
Little Peconic Bay	30 Sep. 1987	3.17 ± 1.82 (4)
	12 May 1988	1.31 ± 0.08 (20)
	6 Sep. 1988	1.63 ± 0.10 (20)

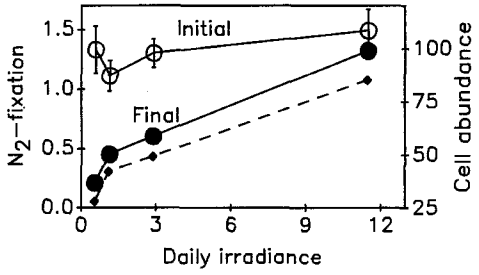

Fig. 1. *Codium fragile*. Initial (o) and final (•) rates of N_2-fixation (μg N g^{-1} dry wt h^{-1}) associated with branches grown for 2 wk at four daily irradiances (mol photons m^{-2} d^{-1}). Values are mean ± 1 SE, $n=5$. ♦: Relative abundance of cyanobacterial cells at the end of the growth period

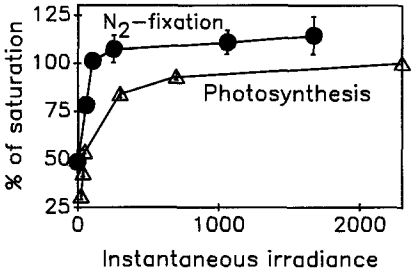

Fig. 2. *Codium fragile*. Rates of N_2-fixation and net photosynthesis vs instantaneous irradiance (μmol photons m^{-2} s^{-1}). Rates are expressed as % of light-saturated rates. Values are mean ± 1 SE, $n=3$ to 6

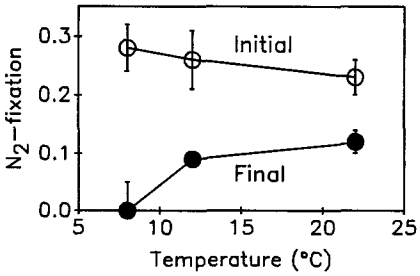

Fig. 3. *Codium fragile.* Initial (o) and final (•) rates of N_2-fixation (μg N g^{-1} dry wt h^{-1}) associated with branches grown for 2 wk at three temperatures (8°, 12° and 22 °C). Values are mean ± 1 SE, $n=5$

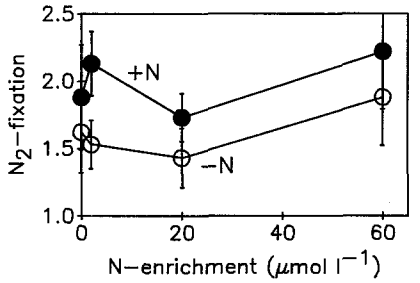

Fig. 4. *Codium fragile.* Rates of N_2-fixation (μg N g^{-1} dry wt h^{-1}) determined for individual branches before (o) and after (•) addition of NH_4^+ to assay medium. Values are mean ± 1 SE, $n=4$

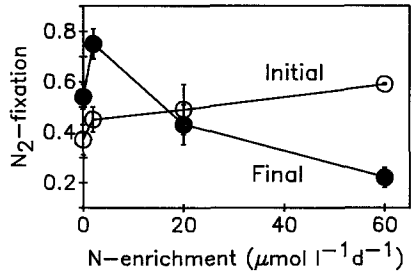

Fig. 5. *Codium fragile.* Initial (o) and final (•) rates of N_2-fixation (μg N g^{-1} dry wt h^{-1}) associated with branches grown for 2 wk with different NH_4^+ enrichments. Values are mean ± 1 SE, $n=4$

External DIN influenced N_2-fixation only after prolonged exposure to high concentrations of NH_4^+. Plants grown in unenriched seawater exhibited no significant effect on N_2-fixation rate when assayed in seawater enriched with up to 60 μmol l^{-1} NH_4^+ (Fig. 4, $F=0.1$, $p>0.05$). In contrast, branches grown with 60 μmol l^{-1} d^{-1} NH_4^+ exhibited significantly lower N_2-fixation rates than branches grown with lower DIN enrichments, even when assayed in low-N seawater (Fig. 5, $F=9.5$, $p<0.01$). N_2-fixation rates were reduced even further ($n=5$, mean = 0.01 μg N g^{-1} dry wt h^{-1}, SE = 0.003) when these branches were assayed in seawater containing 60 μM NH_4^+. The apparently enhanced N_2-fixation rates at low NH_4^+ concentrations (Figs. 4 and 5) were not significantly different from rates of unenriched treatments ($F=1.3$ and 0.4, respectively, for long-term and short-term enrichments, $p>0.05$). Long- or short-term enrichment with NO_3^- at 20 μmol l^{-1} d^{-1} or 20 μmol l^{-1}, respectively, gave results similar to NH_4^+ enrichment at those levels (Figs. 4 and 5), i.e. had no significant effect on N_2-fixation.

N_2-fixation accounted for up to 50% of total N-assimilation by the *Codium fragile*-cyanobacteria association in experimental culture. This high proportion was estimated for branches held under high irradiance (12 mol photons m^{-2} d^{-1}) for 2 wk with no N-enrichment. Their average specific growth rate was 3% d^{-1} (wet weight basis), and their light-saturated N_2-fixation rates at the beginning and end of the experiment averaged 1.4 μg N g^{-1} dry wt h^{-1} (Fig. 1). Assuming that the rate during the 8 h daily dark period was half the light-saturated rate (Fig. 2), mean daily N_2-fixation was 28 μg N g^{-1} dry wt. Daily N-assimilation averaged 55 μg N g^{-1} dry wt, calculated from initial and final dry wt (0.24 and 0.47 g per branch, respectively) and N-content (0.38 and 0.26% of dry wt, respectively). The remainder of the assimilated nitrogen could be accounted for by uptake of ambient NO_3^- and NH_4^+ in the seawater. This estimate of daily N_2-fixation may be high if N_2-fixation rates in the dark were overestimated due to the short incubation period (Dromgoole et al. 1978). However, if N_2-fixation was assumed to cease entirely during the dark period, N_2-fixation still accounted for 40% of total daily N-assimilation (23 μg N g^{-1} dry wt).

In the ^{15}N-enrichment experiment, *Codium fragile* held under high irradiance and low ambient DIN maintained even higher rates of N_2-fixation (3.3 and 3.4 μg N g^{-1} dry wt h^{-1}, respectively, at the beginning and end of the experiment) than branches held under similar conditions in the daily irradiance experiment (Fig. 1). The atom % ^{15}N in chlorophyll *b* averaged 0.59 (SE = 0.03) for experimental branches and 0.50 for control branches (SE = 0.008). Thus, chlorophyll *b* from the experimental branches grown with $^{15}N_2$ had an atom % excess of 0.09.

Discussion

Several lines of evidence indicated that photosynthetic cyanobacteria, rather than heterotrophic bacteria, are the primary N_2-fixing organisms associated with *Codium fragile* around Long Island, New York. First, high N_2-fixation rates were correlated with high densities of cyanobacteria. Second, N_2-fixation rates were strongly light-dependent. Finally, we were unable to isolate heterotrophic bacteria with measurable rates of N_2-fixation from *C. fragile* branches exhibiting high rates. This *C. fragile*-cyanobacteria association is more similar to associations between cyanobacteria and *Codium* spp. from New Zealand and the southeastern USA (Dromgoole et al. 1978, Rosenberg and Paerl 1981) than to the *C. fragile-Azotobacter* sp. association described for populations around Woods Hole, Massachusetts (Head and Carpenter 1975).

The cyanobacteria associated with *Codium fragile* are found primarily below the pigmented, photosynthetic surface layer of the macroalga. As demonstrated for cyanobacteria associated with *C. decorticatum* (Rosenberg and Paerl 1981), they are probably located in a reduced microzone with low O_2-concentration. This microhabitat is also characterized by low and predominantly green light, since only

5 to 10% of incident irradiance is transmitted through the pigmented layer of the macroalga. Reduced pigment content in *C. fragile* acclimated to high-light (Ramus et al. 1976) might enhance transmittance or light quality somewhat, although thallus absorptance was found to be independent of pigment concentration in this species (Ramus 1978). The correlation between cell abundance and daily irradiance (Fig. 1) indicated that cyanobacterial growth was light-limited. At high daily irradiance, the growth rate was sufficiently enhanced to allow a cell abundance 3 × higher than that at the lowest irradiance, despite the simultaneous enhancement of macroalgal growth (specific growth rate, calculated from initial and final wet wt and dry content, was 43 × higher at 12 than at 0.6 mol photons $m^{-2}d^{-1}$). The strong dependence of cyanobacterial growth on daily irradiance suggests that photosynthesis is the primary energy source, rather than heterotrophic uptake of organic carbon compounds exuded by the macroalga. This is further supported by the lack of stimulation of N_2-fixation by glucose addition in other *Codium* spp.-cyanophyte associations (Dromgoole et al. 1978, Rosenberg and Paerl 1981).

Results of the present study indicated that the abundance of cyanobacterial cells was the most important determinant of N_2-fixation rates associated with *Codium fragile*, in agreement with the conclusion of Phlips et al. (1986) for N_2-fixation associated with *Sargassum* spp. Average daily irradiance, therefore, is probably the most important environmental factor affecting N_2-fixation rates associated with *C. fragile* in situ. Site-specific variation in daily irradiance may largely account for variation in N_2-fixation rates (Table 1). Plants at Little Peconic Bay, the population with consistently high rates, were probably exposed to higher light levels than plants at the other three sites, by virtue of the shallower depth and the light-colored sand substratum. Seasonal variation in daily irradiance probably also causes variation in N_2-fixation rates. Rates should be low during the low-light period in winter, and may be further reduced by low temperature (Fig. 3). Site-specific and seasonal variation in external DIN concentration do not appear to influence N_2-fixation associated with *C. fragile* in situ, because combined-N at naturally occurring concentrations ($< 20\ \mu M$) had no significant effect in laboratory experiments (Figs. 4 and 5).

In *Codium fragile* grown at high daily irradiance and low ambient DIN concentrations, ~50% of assimilated-N was attributable to N_2-fixation. However, the results of the ^{15}N-experiment suggest that little of the fixed-N was transferred to the macroalga. After 2 wk of treatment in the daily irradiance experiment, bacterially fixed-N made up ~15% of total N-content in the high-light plants. A similar proportion would be predicted for plants used in the ^{15}N experiment, which were held under similar conditions for 1 wk and exhibited 2 × higher rates of N_2-fixation. If 100% of the fixed-N was transferred from the cyanobacteria to the macroalga and equally distributed among various nitrogenous compounds, the atom % ^{15}N excess in chlorophyll *b* would have been 15. Chlorophyll *b* might be expected to have an even higher level of labelling, because photosynthetic pigments undergo rapid and continuous turnover in algae. The half-life of chlorophyll *b* in *Chlorella pyrenoidosa* was estimated to be only 50 min (Grumbach et al. 1978), and *C. fragile* held at low irradiance tripled its chlorophyll *b* content in a week (Ramus et al. 1976). However, the atom % ^{15}N excess of chlorophyll *b* extracted from the experimental *C. fragile* was less than 0.1. Thus, it appears that only a small proportion of the bacterially fixed-N was transferred to the macroalga. While it is possible that transfer of a higher proportion would have been found under different environmental conditions, over a longer period of time, or in other nitrogenous compounds, the results suggest that N_2-fixation is not a major N-source for the seaweed host.

Much of the N_2-fixation associated with *Codium fragile* must be used to support the N-requirements of the cyanobacteria. The N-content of free-living cyanobacteria ranges from 0.7 to 9% of dry weight (Fogg et al. 1973, Atkinson and Smith 1983), with actively growing cells at the high end of the range (Gerloff and Skoog 1954). The critical N-content for N-saturated growth of *Microcystis aeruginosa*, for example, is 4% of dry weight (Gerloff and Skoog 1954). In the *C. fragile* association, therefore, the critical N-content of cyanobacteria is probably about 2 × higher than that of the macroalga (1.9% of dry weight, Hanisak 1979a). The cyanobacteria associated with *C. fragile* may have a particularly high N-requirement, due to their need to maintain high concentrations of phycobiliproteins for photosynthesis under low and predominantly green light. Phycoerythrin content has been shown to increase in cyanobacteria in response to green light or reduced irradiance (Hattori and Fujita 1959a, b, Marsac and Tandeau 1977). N-starvation caused reduced concentrations of phycocyanin (Stal and Krumbein 1985), while N-addition stimulated phycoerythrin production (Hattori and Fujita 1959b). In contrast to the bleached appearance of N-starved cells (Stal and Krumbein 1985), the cyanobacteria associated with *C. fragile* appeared yellow when excited with blue light and bright red when excited with green light under the epifluorescence microscope, indicating high concentrations of phycoerythrin. N_2-fixation is probably the major N-source for these cyanobacteria. The tightly packed utricles at the surface of the macroalga must greatly restrict exchange between external seawater and the extracellular fluids within the thallus. Uptake by the macroalga probably further limits external combined-N reaching the cyanobacteria, because *C. fragile* is characterized by efficient uptake of NH_4^+ and NO_3^- (Hanisak and Harlin 1978).

All three species of cyanobacteria associated with *Codium fragile* also occur as free-living forms in benthic or floating mats, and *Calothrix crustacea* is found in association with many species of seaweeds (Humm and Wicks 1980). Their association with *C. fragile* does not seem to be as strongly interdependent as some other plant-cyanobacterial associations. Cyanobacteria associated with certain lichens, bryophytes, ferns, and angiosperms are physiologically modified in such a way as to limit their assimilation and enhance their exudation of fixed-N, so that up to 90% is transferred to the host (Stewart and Rodgers 1977, Stewart

and Rowell 1977, Stewart et al. 1980, Meeks et al. 1985). In some cases, these symbiotic cyanobacteria may also lose their ability to fix carbon photosynthetically, and depend on the host as a source of organic carbon to support heterotrophic growth (Silvester 1976, Rodgers and Stewart 1977, Peters et al. 1980). The cyanobacteria in these associations are typically N-depleted (characterized by low concentrations of phycobiliproteins) and slow-growing. In contrast, the cyanobacteria associated with *C. fragile* do not appear N-starved and are capable of rapid growth at high daily irradiance.

The cosmopolitan association between *Codium* spp. and cyanobacteria seems to be based on habitat suitability, rather than on mutual metabolic dependence. N_2-fixation by free-living cyanobacteria is stimulated by physical structuring, which allows formation of reduced microzones, and a sufficient energy source (Paerl 1985). The unusual thallus structure of *Codium* spp. provides a physically stable, reduced microzone below the photosynthetic surface layer (Rosenberg and Paerl 1981). Light seems to be the primary energy source and the primary factor limiting cyanobacterial growth. Conditions favoring high rates of N_2-fixation may be necessary for existence in this microhabitat, due to the limited amount of external DIN reaching the endophytic cyanobacteria. In the case of *C. fragile*, the macroalga does not appear to benefit greatly from association with the cyanobacteria if, as results of the present study suggested, only a small fraction of the fixed-N is transferred to the host. This proportion may be higher in other species. For example, *C. decorticatum* with actively fixing cyanobacteria exhibited higher concentrations of internal dissolved-N than non-fixing plants (Rosenberg and Paerl 1981), but the proportion of N in macroalgal and cyanobacterial tissues was not determined. The proportion of fixed-N transferred to the host may also be higher under environmental conditions which are more limiting to cyanobacterial growth than to N_2-fixation.

Overall, results of the present study suggest that association with N_2-fixing cyanobacteria is not important to the ecological success of *Codium fragile* as an introduced species around Long Island. As concluded by Hanisak and Harlin (1978), this seaweed probably depends on efficient uptake of external nutrients, as well as on luxury consumption and storage of nutrients (Hanisak 1979a), to maintain N-saturated growth under all but the most limiting conditions (Hanisak 1979b). As the same conditions that result in N-limitation of *C. fragile* – high daily irradiance and high temperature – also enhance growth of the symbiotic cyanobacteria, high rates of N_2-fixation are needed to support cyanobacterial N-requirements.

Acknowledgements. We thank H. Lyman for the kieselguhr paper, J. Walsh for assistance with PI measurements, and E. J. Carpenter, J. Schubauer, and T. Villareal for critical review of the manuscript. The research was supported by Grant # NA86AADSG045 from the New York Sea Grant Institute to V. A. Gerard and D. G. Capone.

Literature cited

Atkinson, M. J., Smith, S. V. (1983). C:N:P ratios of benethic marine plants. Limnol. Oceanogr. 28: 569–574

Bohlool, B. B., Wiebe, W. J. (1978). Nitrogen-fixing communities in an intertidal ecosystem. Can. J. Microbiol. 24: 932–938

Capone, D. G., Taylor, D. L., Taylor, B. F. (1977). Nitrogen fixation (acetylene reduction) associated with macroalgae in a coral-reef community in the Bahamas. Mar. Biol. 40: 29–32

Carlton, J. T., Scanlon, J. A. (1985). Progression and dispersal of an introduced alga: *Codium fragile* ssp. *tomentosoides* (Chlorophyta) on the Atlantic coast of North America. Botanica mar. 28: 155–165

Carpenter, E. J. (1972). Nitrogen fixation by a blue-green epiphyte on pelagic *Sargassum*. Science, N. Y. 178: 1207–1208

Dromgoole, F. I., Silvester, W. B., Hicks, B. J. (1978). Nitrogenase activity associated with *Codium* species from New Zealand marine habitats. N. Z. Jl mar. Freshwat. Res. 12: 17–22

Fiedler, R., Proksch, G. (1975). The determination of nitrogen-15 by emission and mass spectrometry in biochemical analysis: a review. Analytica chim. Acta 78: 1–62

Fogg, G. E., Stewart, W. D. P., Fay, P., Walsby, A. E. (1973). The blue-green algae. Academic Press, London

Gerard, V. A. (1988). Ecotypic differentiation in light-related traits of the kelp *Laminaria saccharina*. Mar. Biol. 97: 25–36

Gerloff, G. C., Skoog, F. (1954). Cell contents of nitrogen and phosphorus as a measure of their availability for growth of *Microcystis aeruginosa*. Ecology 35: 348–353

Grumbach, K. H., Lichtenthaler, H. K., Erismann, K. H. (1978). Incorporation of $^{14}CO_2$ in photosynthetic pigments of *Chlorella pyrenoidosa*. Planta 140: 37–43

Guerinot, M. L., Colwell, R. R. (1985). Enumeration, isolation, and characterization of N_2-fixating bacteria from seawater. Appl. envir. Microbiol. 50: 350–355

Hanisak, M. D. (1979a). Nitrogen limitation of *Codium fragile* ssp. *tomentosoides* as determined by tissue analysis. Mar. Biol. 50: 333–337

Hanisak, M. D. (1979b). Growth patterns of *Codium fragile* ssp. *tomentosoides* in response to temperature, irradiance, salinity, and nitrogen source. Mar. Biol. 50: 319–332

Hanisak, M. D., Harlin, M. M., (1978). Uptake of inorganic nitrogen by *Codium fragile* subsp. *tomentosoides* (Chlorophyta). J. Phycol. 14: 450–454

Hanson, R. B. (1977). Pelagic *Sargassum* community metabolism: carbon and nitrogen. J. exp. mar. Biol. Ecol. 29: 107–118

Hardy, R. W. F., Holsten, R. D., Jackson, E. K., Burns, R. C. (1968). The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. Pl. Physiol., Wash. 43: 1185–1207

Hattori, A., Fujita, Y. (1959a). Formation of phycobilin pigments in a blue-green alga, *Tolypothrix tenuis*, as induced by illumination with colored lights. J. Biochem., Tokyo 46: 521–524

Hattori, A., Fujita, Y. (1959b). Effect of pre-illumination on the formation of phycobilin pigments in a blue-green alga, *Tolypothrix tenuis*. J. Biochem., Tokyo 46: 1259–1261

Head, W. D., Carpenter, E. J. (1975). Nitrogen fixation associated with the marine macroalga *Codium fragile*. Limnol. Oceanogr. 20: 815–823

Humm, H. J., Wicks, S. R. (1980). Introduction and guide to the marine bluegreen algae. John Wiley and Sons, New York

Jensen, A. (1978). Chlorophylls and carotenoids. In: Hellebust, J. A., Craigie, J. S. (eds.) Handbook of phycological methods. Cambridge University Press, Cambridge, p. 59–70

Marsac, N. T., Tandeau, N. (1977). Occurrence and nature of chromatic adaptation in cyanobacteria. J. Bacteriol., Tokyo 130: 82–91

Meeks, J. C., Enderlin, C. S., Joseph, C. M., Chapman, J. S., Lollar, M. W. L. (1985). Fixation of $[^{13}N]N_2$ and transfer of fixed nitrogen in the *Anthoceros-Nostoc* symbiotic association. Planta 164: 406–414

Paerl, H. W. (1985). Microzone formation: its role in the enhancement of aquatic N_2 fixation. Limnol. Oceanogr. 30: 1246–1252

Penhale, P. A., Capone, D. G. (1981). Primary productivity and nitrogen fixation in two macroalgae-cyanobacteria associations. Bull. mar. Sci. 31: 164–169

Peters, G. A., Ray, T. B. Mayne, B. C., Toia, R. E. Jr. (1980). *Azolla-Anabaena* association: morphological and physiological studies. In: Newton, W. E., Orme-Johnson, W. H. (eds.) Nitrogen fixation. University Park Press, Baltimore, p. 293–309

Phlips, E. J., Willis, M., Verchick, A. (1986). Aspects of nitrogen fixation in *Sargassum* communities off the coast of Florida. J. exp. mar. Biol. Ecol. 102: 99–119

Rai, A. N., Rowell, P., Stewart, W. D. P. (1981). $^{15}N_2$ incorporation and metabolism in the lichen *Peltigera aphthosa* Willd. Planta 152: 544–552

Ramus, J. (1978). Seaweed anatomy and photosynthetic performance: the ecological significance of light guides, heterogeneous absorption and multiple scatter. J. Phycol. 14: 352–362

Ramus, J., Beale, S. I., Mauzerall, D., Howard, K. L. (1976). Changes in photosynthetic pigment concentration in seaweeds as a function of water depth. Mar. Biol. 37: 223–229

Rennie, R. J. (1981). A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27: 8–14

Rodgers, G. A., Stewart, W. D. P. (1977). The cyanophyte-hepatic symbiosis. I. Morphology and physiology. New Phytol. 78: 441–458

Rosenberg, G., Paerl, H. W. (1981). Nitrogen fixation by bluegreen algae associated with the siphonous green seaweed *Codium decorticatum*: effects on ammonium uptake. Mar. Biol. 61: 151–158

Schubert, K. R. (1986). Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. A. Rev. Pl. Physiol. 37: 539–574

Silvester, W. B. (1976). Endophyte adaptation in *Gunnera-Nostoc* symbiosis. In: Nutman, P. S. (ed.) Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge, p. 521–538

Silvester, W. B., Smith, D. R. (1969). Nitrogen fixation by *Gunnera-Nostoc* symbiosis. Nature, Lond. 224: 1231

Stal, L. J., Krumbein, W. E. (1985). Isolation and characterization of cyanobacteria from a marine microbial mat. Botanica mar. 28: 351–365

Stewart, W. D. P., Rodgers, G. A. (1977). The cyanophyte-hepatic symbiosis. II. Nitrogen fixation and the interchange of nitrogen and carbon. New Phytol. 78: 459–471

Stewart, W. D. P., Rowell, P. (1977). Modifications of nitrogen-fixing algae in lichen symbioses. Nature 265: 371–372

Stewart, W. D. P., Rowell, P., Rai, A. N. (1980). Nitrogen metabolism in symbiotic cyanobacteria. In: Stewart, W. D. P., Gallon, J. R. (eds.) Nitrogen fixation. Academic Press, New York, p. 239–277

Turner, G. L., Gibson, A. H. (1980). Measurement of nitrogen fixation by indirect means. In: Bergersen, F. J. (ed.) Methods for evaluating biological nitrogen fixation. John Wiley & Sons, New York, p. 111–138

Date of final manuscript acceptance: January 5, 1990.
Communicated by J. Grassle, Woods Hole