NITROGEN ALLOCATION AND STORAGE PATTERNS IN *GRACILARIA TIKVAHIAE* (RHODOPHYTA)[1]

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ABSTRACT

Internal nitrogen pools in thalli of Gracilaria tikvahiae *McLachlan were examined in three experiments as a function of total nitrogen content of the thallus, nitrogen deprivation, and nitrogen resupply. Amino acids and proteins appeared to form the major nitrogen storage pools in* G. tikvahiae, *while DNA appeared to be relatively unimportant in this regard. Inorganic nitrogen in the forms of NH_4^+ and NO_3^- was found in the thalli; however, its contribution to the total nitrogen pools was small. Within the protein pool, the phycoerythrin pigments appear important as a source of nitrogen when thalli are initially becoming nitrogen limited. In general, there was an inverse relationship between the levels of nitrogen and the carbohydrate content of the algal thalli.*

Key index words: aquaculture; carbohydrate analysis; Gracilaria; *nitrogen supply; pigments; protein content*

An ecological adaptation by algae to N limitation appears to be the phenomenon of luxury consumption and storage of nitrogen. This storage can either be in forms of inorganic N (Chapman and Craigie 1977), or biochemical metabolites (Conover 1975, Wheeler and North 1980). Nitrogen storage is important from an ecological aspect, and also has ramifications in management techniques in algal aquaculture. Previous studies have shown that *Gracilaria tikvahiae* is able to take up ambient N very rapidly and store it for subsequent growth. This storage is reflected in thallus N contents (3–5%) which can be substantially higher than those (1.5–2%) indicating incipient N deficiency (Ryther et al. 1981). The intent of our investigations was to determine the patterns of N storage in *Gracilaria tikvahiae* and the major N pools involved.

MATERIALS AND METHODS

Three types of experiments were carried out to investigate N allocation patterns in *Gracilaria tikvahiae*. In all experiments, the seaweed was grown in air agitated suspended culture in 2.1 × 0.8 × 0.6 m deep concrete vaults under natural sunlight and at ambient water temperatures. In the first experiment, the relative proportions of biochemical constituents at different levels of total thallus N were examined. As described in detail elsewhere (Ryther et al. 1981), 2.5 kg portions of N deficient *Gracilaria tikvahiae* were immersed in nutrient enriched seawater (NH_4Cl, 1 mM) for 5 periods of time, ranging from one to 48 h. The portions were washed and then returned to unenriched seawater in culture vaults. The algae were harvested after 2 wk and weighed, and the nutrient exposure repeated. After 6 wk of these treatments, samples were harvested and rinsed in deionized water. During the experimental period, ambient N concentrations of incoming seawater ranged from 8–12 μM as NH_4^+ and NO_3^-, and temperatures from 16–35° C.

Triplicate samples (2–3 g) from each treatment were ground in 90% ethanol in a ground glass homogenizer, and allowed to extract for 4 d at −20° C without agitation. The samples were then centrifuged at 12 000 × *g* for 20 min, and the pellets were reextracted in 80% ethanol for 1 h. Following centrifugation, the ethanolic supernatants were combined and used for amino acid analysis (Rosen 1957). The pellets were washed with cold 0.2 M $HClO_4$ for 30 min, followed by centrifugation and washing of the pellets for 1 h at 50° C in 2:1 (v/v) chloroform : methanol. The pellets were further extracted in 0.2 M $HClO_4$ for 3 h at 75° C and the supernatants used for the determination of DNA (Kochert 1978). The final pellets were extracted without stirring overnight in 1 N NaOH and protein was determinated (Lowry et al. 1951) in the supernatant after centrifugation. Soluble nucleotides and RNA were not measured because of the high content of carbohydrates such as agar which interfere in the assay.

Fresh algal thalli were also used to determine phycoerythrin, chlorophyll *a*, and carotenoids. Phycoerythrin was extracted by

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TABLE 1. *The relationship of biomass production, pigments, and constituents such as amino acids, carbohydrates, DNA, and protein in relation to the thallus N content of* Gracilaria tikvahiae

Thallus N (%)	Yield (g dry wt.·m^{-2}·d^{-1})	Amino acids (%)	Protein (%)	DNA (%)	Carbohydrate (%)	NH_4^+ (μmol·g^{-1})	NO_3^- (μmol·g^{-1})	Phycoerythrin (mg·g^{-1})	Chl *a* (mg·g^{-1})	Chl *a* : carotenoids	Phycoerythrin : total protein (%)
1.2	9.7	0.61	3.6	1.0	61.4	3.0	0.19	2.1	0.56	2.4	5.8
1.2	17.8	0.66	3.8	0.8	51.4	4.3	0.16	4.8	0.78	3.4	12.6
2.0	19.5	1.19	4.7	1.3	46.3	3.8	0.18	10.2	1.65	3.8	21.7
2.0	19.6	2.09	6.8	1.1	40.6	3.8	0.32	9.7	1.56	4.2	14.3
2.3	18.0	2.21	6.8	0.9	38.5	6.0	0.56	12.7	1.43	4.3	18.7

grinding the tissue (0.2 g) in cold 0.05 M PO_4^{3-} buffer (4° C), pH 6.7. The pellets from centrifugation were treated with cold 80% acetone to extract chlorophylls. Phycoerythrin was determined using the extinction coefficient of O'Carra (1965), chlorophyll by using that of Smith and Benitez (1955), and carotenoids by using the procedure of Jensen (1978).

A wet : dry weight ratio was determined after drying samples at 70° C to a constant weight. Carbohydrates were determined on dried plant material (DuBois et al. 1956) after extraction in 5% trichloroacetic acid for 3–4 h at 80–90° C. The percent thallus N was determined with a Perkin-Elmer elemental analyzer. Inorganic N was extracted from 2 g of dried plant material in 100 ml of 50% ethanol for 3 d, followed by determination of NH_4^+ and NO_3^- using standard techniques (Strickland and Parsons 1972).

In the second experiment, the changes in the biochemical constituents of N enriched thalli were examined as they gradually became N depleted. Thalli were grown for 6 weeks with once a week 24 h immersions in either NH_4Cl or $NaNO_3$ (1 mM) and $Na_2HPO_4 \cdot 7H_2O$ (1 mM). After the 24 h immersion, the enriched seawater was drained from the vault and ambient seawater circulated at one turnover per day. Ammonium enriched and NO_3^- enriched thalli were then stocked in vaults at 2.5 kg·m^{-2}. Unenriched seawater was circulated through the vaults at 1 turnover per day. Production was measured weekly, and samples were collected and frozen for later analyses of the same biochemical constituents as determined in experiment 1. During the experimental period, temperatures ranged from 20–33° C, and ambient N concentrations from 39 μM initially to 2 μM at the end of the experiment.

In the third experiment, the concentrations of pigments, amino acids, protein, and carbohydrates were examined when N starved seaweed was supplied with new N sources. Five treatments were used in two replicate trials: enrichment with NH_4^+ or NO_3^- in the light and in the dark, and a control with no enrichment. The N starved alga (1.5 kg) was immersed for 24 h in the same nutrient concentrations used in experiment 2. After 24 h, the enriched seawater was drained from the vaults, and ambient seawater circulated at 1 turnover per day. The alga held in darkness remained in the covered vaults throughout the course of the experiments. Samples were removed daily and frozen. Pigments were determined as described earlier. The protein, carbohydrate, and thallus N were determined on dried samples. Amino acids were determined following extraction of dried samples for 3 d in 80% ethanol. During the first experiment, temperature ranged from 8–18° C, while in the second, it ranged from 15–26° C. Ambient total N concentrations were in the range of 4–5 μM.

RESULTS

In the first experiment, increased thallus N (from 1.2 to 2.3%) was associated with a doubling of yields, and increased levels of amino acids and proteins (Table 1). The levels of carbohydrate decreased with increasing thallus N. Both NH_4^+ and NO_3^- in the thalli increased with the thallus N content. The concentration of NH_4^+ was an order of magnitude greater than that of NO_3^-. The DNA content did not change with differences in thallus N. A doubling of the thallus N was associated with six times more

TABLE 2. *Time course of changes in the production of biomass, thallus N content, and biochemical constituents of NH_4^+ and NO_3^- treated* Gracilaria tikvahiae *during N limitation.*

Weeks	Yield (g dry wt.·m^{-2}·d^{-1})	Thallus N (%)	Amino acids (%)	Protein (%)	DNA (%)	Carbohydrate (%)	NH_4^+ (μmol·g^{-1})	NO_3^- (μmol·g^{-1})	Phycoerythrin (mg·g^{-1})	Chlorophyll *a* (mg·g^{-1})	Chl *a* : carotenoids	Phycoerythrin : protein (%)
						NH_4^+ treated thalli						
0	—	5.1	10.0	14.3	0.8	23.9	84.8	1.7	24.1	1.5	6.3	16.8
1	13.8	3.9	5.3	12.0	1.0	25.9	18.4	2.4	19.9	1.4	5.6	16.6
2	12.0	2.6	3.8	10.5	0.9	26.9	18.0	0.5	18.1	1.1	5.5	17.2
3	13.5	1.8	2.7	7.7	0.9	27.6	7.5	0.4	7.4	0.7	3.4	9.6
4	9.3	1.4	1.3	6.1	1.1	43.7	9.4	0.2	6.6	0.6	2.2	10.8
5	4.9	1.3	1.1	4.8	1.1	39.6	—[a]	—[a]	3.5	0.4	2.2	7.3
6	1.3	1.1	1.3	4.8	1.3	48.0	6.6	0.1	—[b]	0.5	2.4	—[b]
						NO_3^- treated thalli						
0	—	2.8	2.3	10.2	0.7	26.1	19.1	14.5	19.6	1.4	6.4	19.2
1	9.3	2.4	2.2	9.4	0.9	32.9	12.4	0.6	13.7	1.2	4.4	14.6
2	9.3	1.8	2.4	8.0	1.0	42.5	11.3	0.4	12.6	0.7	4.7	15.8
3	4.9	1.4	2.9	4.9	1.0	46.8	8.3	0.3	6.4	0.5	2.9	13.1
4	9.3	1.0	0.9	3.6	1.0	46.7	9.8	0.2	3.8	0.4	1.7	10.6
5	4.9	1.0	1.0	3.0	1.0	54.6	7.9	0.2	3.5	0.3	2.1	11.6
6	3.7	0.9	0.8	2.7	0.9	50.7	7.3	0.2	—[b]	0.4	1.4	—[b]

[a] Sample was not included due to high background turbidity.
[b] Phycoerythrin concentration was too low to measure.

TABLE 3. *Changes in the percentage (dry wt. basis) of thallus N, amino acids, proteins, and carbohydrates of* Gracilaria tikvahiae *after immersion for 24 h in either NH_4^+ or NO_3^- enriched seawater in darkness or in light. Controls were held in ambient daylight. Day 1 represents the data for thalli immediately after the 24 h immersion in the N supplements, at which time the N supplement was drained and the thalli were returned to unenriched ambient seawater at one turnover per day.*

Days	Thallus N	Amino acids	Protein	Carbohydrate	Thallus N	Amino acids	Protein	Carbohydrate	Thallus N	Amino acids	Protein	Carbohydrate	Thallus N	Amino acids	Protein	Carbohydrate	Thallus N	Amino acids	Protein	Carbohydrate
	Control				NH_4^+ Light				NH_4^+ Dark				NO_3^- Light				NO_3^- Dark			
Trial 1																				
0	1.1	1.2	3.5	58.9	1.1	1.2	3.5	58.9	1.1	1.2	3.5	58.9	1.1	1.2	3.5	58.9	1.1	1.2	3.5	58.9
1	1.1	0.9	3.4	54.1	2.2	8.3	5.3	41.9	2.2	10.8	4.8	47.5	1.2	2.2	4.2	53.0	1.0	2.0	2.7	55.7
2	1.0	0.8	3.7	51.9	1.9	6.6	5.0	48.9	1.7	6.3	4.3	59.6	1.2	2.0	4.4	63.7	1.3	2.3	4.0	57.4
3	1.1	1.4	3.6	54.5	2.1	5.1	4.6	43.6	2.0	4.3	4.4	42.3	1.2	1.8	4.9	55.0	1.2	1.4	4.4	51.7
4	1.1	1.2	4.8	46.8	2.2	3.0	7.2	36.2	2.5	4.8	5.4	32.1	1.4	2.6	4.9	33.2	1.5	2.2	6.5	38.5
5	1.0	2.2	3.4	41.0	2.0	3.5	4.4	45.9	2.5	4.9	4.6	32.0	1.5	1.0	4.6	45.1	1.4	2.7	3.9	40.6
6	1.0	1.2	4.9	52.7	1.9	3.3	7.1	32.8	2.2	5.9	4.5	33.4	1.4	1.0	4.6	58.3	1.4	2.3	4.0	40.0
7	1.1	1.0	3.7	46.1	2.0	3.2	4.9	40.4	2.4	4.7	4.2	30.8	1.3	2.1	4.6	47.1	1.4	2.3	4.3	47.3
Trial 2																				
0	0.9	2.0	3.9	53.7	0.9	2.0	3.9	53.7	0.9	2.0	3.9	53.7	0.9	2.0	3.9	53.7	0.9	2.0	3.9	53.7
1	1.0	2.7	4.7	43.4	2.2	6.6	5.8	49.1	3.2	10.4	5.6	57.2	1.6	2.5	6.2	62.4	1.4	0.7	6.7	53.3
2	1.0	1.8	4.9	51.0	3.0	6.2	7.5	46.2	2.6	7.5	6.4	53.8	1.5	2.2	6.0	46.6	1.2	1.8	6.3	55.1
3	0.8	2.2	5.7	52.4	2.8	6.1	8.6	48.4	2.9	5.2	6.3	53.3	1.6	1.2	7.1	48.9	1.3	1.4	6.3	48.3
4	1.1	1.0	5.4	60.6	2.6	5.5	9.3	40.5	2.5	3.6	6.7	49.6	1.6	1.6	6.5	49.0	1.4	1.1	6.4	54.6
5	1.2	3.4	5.7	53.9	2.5	3.2	10.2	37.9	2.9	6.3	7.6	34.8	1.6	2.2	5.6	47.5	1.1	1.2	6.5	52.1
6	0.9	2.1	4.9	63.6	2.3	2.4	7.3	46.0	2.7	6.0	7.6	37.9	1.3	0.9	6.3	50.9	1.8	1.2	6.5	57.4
7	1.0	1.2	4.5	70.7	2.4	3.0	9.5	45.9	3.0	6.1	9.6	38.9	1.4	0.6	5.6	50.7	1.8	1.1	6.8	54.0

phycoerythrin than the initial pigment levels. These N-enriched plants also had increased chlorophyll *a*, and chlorophyll *a* : carotenoid ratios. Below approximately 2% thallus N, the percent of phycoerythrin to protein decreased rapidly (Table 1).

In the second experiment, the amino acid content decreased rapidly as the NH_4^+ treated thalli became N deficient. The amino acid content also decreased with increasing N deficiency in the NO_3^- treated thalli; however, the changes were not as noticeable as in the NH_4^+ treated plants. After several weeks, the percentage of protein decreased very rapidly in both NH_4^+ and NO_3^- treated thalli as N limitation ensued. Production of new biomass also decreased with time, but only after a lag period of several weeks. Inorganic N as NO_3^- and NH_4^+ in the thalli decreased with time. In both the NH_4^+ and NO_3^- raised plants, internal NH_4^+ was typically in higher concentration than NO_3^-. The decrease in total thallus N was also accompanied by an increase in the percent carbohydrate in both treatments. As N limitation became greater, the thalli in both treatments also had lower levels of phycoerythrin, chlorophyll *a*, and lower chlorophyll *a* : carotenoid ratios. Below thallus N levels of ca. 1.8%, the percentage of phycoerythrin to protein began to decrease rapidly (Table 2).

The third experiment, which depicts the patterns of recovery from N deficiency, showed marked differences between fertilized plants and the controls in both replicate trials. The controls showed no increase in the biochemical constituents examined (Table 3, Fig. 1). The thalli enriched with NH_4^+ for 24 h showed measurable increases in total thallus N and protein, and a decrease in carbohydrate content. In both trials, the amino acid content increased over the first several days, then declined (Table 3). The patterns were similar for both dark held and light exposed algae. The content of phycoerythrin increased within 2 d after the plants were exposed to NH_4^+ in both the dark and light, while the chlorophyll *a* content did not begin to increase until several days later. Pigment levels appeared somewhat greater in thalli exposed to light (Fig. 1). The plants exposed to NO_3^- for 24 h showed changes in pigment patterns, amino acids, proteins and carbohydrates similar to those treated with NH_4^+, although the differences were not as marked (Fig. 1 and Table 3).

DISCUSSION

Increasing the time of exposure of nitrogen deficient *Gracilaria tikvahiae* to NH_4^+ enriched solutions resulted in increased production and higher thallus N content (Ryther et al. 1981). The analyses in our first experiment revealed that most of the increased thallus N occurred as protein, although some of it also occurred as amino acids. As the DNA content did not vary greatly during the first two experiments, its contribution to internal N increases and storage appears to be minimal. Inorganic forms of N also did not appear to contribute much to the total N content of the thalli. Our results were similar to those of Wheeler and North (1980) who found that thallus NO_3^- did not contribute greatly to the total N content of juvenile *Macrocystis* sporophytes. Increases in the chlorophyll *a* content, and the chlorophyll *a* : carotenoid ratios with increases in cellular N are well known for algae (Antia et al. 1963, Fogg 1965); however, the low levels of chlorophyll

indicate that these pigments do not contribute greatly to the N content of *Gracilaria tikvahiae*. The contribution of phycoerythrin to the protein pool when the thallus contained >2% N was ca. 15–20%.

A similar pattern of nitrogen distribution was found in the second experiment. Amino acids formed a large proportion of the biochemical constituents in the NH_4^+ raised plants. The rapid decrease in amino acid content at the onset of nitrogen deficiency suggests that amino acids form the initial pool of nitrogen metabolized when ambient nitrogen concentrations become reduced, and therefore plays a major function in N storage. The protein content decreased more slowly and did not reach half its initial content until the third week, while amino acids decreased by half in the first week. The sustained yields of both NH_4^+ and NO_3^- raised plants during the initial 3–4 weeks suggests that they were able to draw on internal N sources to meet their growth requirements. Thallus N eventually declined, presumably due to growth and lack of sufficient ambient N sources to maintain the initial high thallus N contents. The NO_3^- raised plants did not show the same large decrease in the amino acid pool, probably because the N content of these plants was initially lower. Both amino acids and proteins did decrease with the onset of N limitation, again indicating that these pools are primary sources of nitrogen during increasing limitation. Neither NH_4^+ raised nor NO_3^- raised thalli contained large amounts of inorganic N as NH_4^+ or NO_3^-. In both treatments, these forms of N decreased rapidly within the first week, pointing to rapid utilization of any internal inorganic N available. It is interesting to note that in both treatments, NH_4^+ contributed more to the total inorganic N pool than NO_3^-. In the NO_3^- raised thalli, this could represent NO_3^- reduced to NH_4^+ for metabolic assimilation and suggests that some steady state pool of NH_4^+ may exist within *Gracilaria tikvahiae*. Carbohydrate content increased in the first two experiments with increasing N limitation, suggesting that newly fixed C was stored in nonaminated forms due to the inavailability of inorganic N. In general, NH_4^+ raised thalli showed a greater yield of biomass than did NO_3^- raised thalli as was noted by DeBoer et al. (1978), and appeared to be capable of storing more N, as demonstrated by the greater N content of nitrogenous biochemical constituents. The chlorophyll *a* content and chlorophyll *a* : carotenoid ratios also decreased with the ensuing N limitation during the second experiment. As noted earlier, pigments are sensitive to the N status of the algae and probably declined in content due to growth and a lack of sufficient ambient N for continued synthesis of new chlorophyll. Phycoerythrin levels also decreased as N limitation became greater. The percent of phycoerythrin to total protein decreased particularly when the thallus N content fell below 1.8%. Perhaps at incipient N limitation these pigments may be preferentially utilized from the protein pool for the N requirements of continued

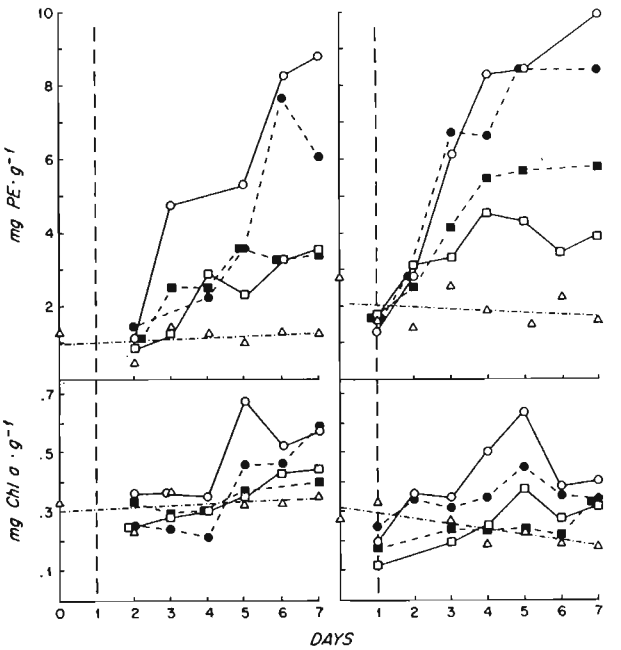

FIG. 1. Changes in the phycoerythrin and chlorophyll *a* content of *Gracilaria tikvahiae* after immersion for 24 h in either NH_4^+ or NO_3^-, in darkness or in light. Open symbols represent the light treatments, dark symbols the dark treatments. Circles represent the thalli receiving the NH_4^+ treatments, squares the NO_3^- treatments and triangles the controls with no fertilization. The dashed vertical line at day 1 represents the time at which the immersions were ended. Graphs on the left are for trial 1, those on the right for trial 2. A linear regression line was calculated for the controls.

algal growth. In general, the changes in the thallus compositions in *G. tikvahiae* during increasing N deficiency were similar to those found in phytoplankton (Antia et al. 1963).

Recovery from N limitation appeared to be more complete after immersion of the thalli in NH_4^+ enriched seawater than NO_3^- enriched seawater. This recovery occurred both in the light and in darkness. The amino acid content rose rapidly the first day, followed by an increase in protein, indicating that newly assimilated NH_4^+ is stored as amino acids. The dark-held thalli had a larger content of amino acids after 24 h than those held in the light. Dark-held thalli also had lower levels of carbohydrate, suggesting its utilization to supply the carbon necessary for increased amino acid and protein synthesis. Such carbohydrate utilization for assimilation of newly available NH_4^+ is common in other plants (Kanazawa et al. 1970, Platt et al. 1977). The increase in total N content and in amino acids and proteins was less in NO_3^- exposed plants in either the light or in darkness. The absence of any strong effects of NO_3^- might be due to low NO_3^- uptake rates. As Ryther et al. (1981) found much slower rates of NO_3^- uptake than NH_4^+ uptake in similar experiments, it appears that some rate limiting step may be responsible for the differences between NO_3^+ and NH_4^+ treated plants.

Changes in pigments during the N recovery process revealed several interesting features. Again, exposure to NH_4^+ enriched seawater led to greater levels of phycoerythrin than did exposure to NO_3^-. The effects of the two N species were less noticeable with respect to chlorophyll *a*, possibly because of lower N requirements for synthesis of this pigment. Phycoerythrin was not resynthesized as quickly as the total protein pool which increased in content within one day. This suggests that there may be a priority established for the synthesis of certain types of proteins over others of less immediate need. The development of phycoerythrins in the dark, and their more rapid development than chlorophyll, further suggests a N storage function for phycoerythrins in addition to a light harvesting role, a conclusion reached by others (LaPointe 1981, Gantt 1981).

Data indicate that N allocation and storage in *Gracilaria tikvahiae* occurs primarily in amino acid and protein pools. DNA appears to be unimportant with respect to N storage. The development of N limitation in *G. tikvahiae* appears to initially stimulate the utilization of inorganic N and low molecular weight amino acids, followed by the use of more complex N-containing macromolecules. Among the macromolecules, the phycoerythrins may be acting as an initial N source within the protein pool, particularly when thallus N content falls below 2 percent, the beginning of N deficiency (Ryther et al. 1981).

These data have important implications for aquacultural management. The ability of *Gracilaria tikvahiae* to store N and use it for subsequent growth suggests that 'pulse fertilization' can produce high growth rates without some of the disadvantages of continuous fertilization, as suggested by Ryther et al. (1981). The greater growth rates and N storage capacity of *G. tikvahiae* supplied with NH_4^+ rather than NO_3^- fertilization has important consequences if other algae display similar patterns. Juvenile sporophytes of *Macrocystis* did not store N readily when enriched with NO_3^- (Wheeler and North 1980). Attempts to raise algae by the use of deep oceanic seawater highly enriched in NO_3^- may therefore be less effective than other methods of enrichment. The recycling and use of effluent liquids rich in NH_4^+ as from methane digesters has been shown to stimulate the production of new biomass as well as inorganic N fertilizers (Hanisak 1981). Such recycling and pulse feeding of nutrients may be more effective in a seaweed energy farm than the continual pumping of deep water nutrients.

The authors thank Dr. John Ryther for critical comments in experimental design and in the preparation of the manuscript, and Mr. Blake Faulkner for seawater nutrient analysis. The research was supported by the Harbor Branch Foundation and by SERI contract number XR-9-8133-1. This article is Harbor Branch Contribution No. 271.

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