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Nitrogen assimilation following NH_4^+ pulses in the red alga *Gracilariopsis lemaneiformis*: effect on C metabolism

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ABSTRACT: The short-term effect of external NH_4^+ availability on N assimilation, as well as on C metabolism, has been studied in the red alga *Gracilariopsis lemaneiformis*. This agarophyte seaweed showed a high capacity to take up external NH_4^+ and to channel it toward proteins. As in a previous study in *Corallina elongata*, it appears that N limitation redirected the flow of internal N toward non-pigmented proteins, whereas phycobiliprotein synthesis was preferentially stimulated with respect to other proteins in response to external NH_4^+ pulses. A possible mechanism by which chloroplastic protein synthesis is limited by external N availability more than cytosolic proteins could be involved. In relation to C metabolism, insoluble and soluble carbohydrates were mobilized in response to NH_4^+ assimilation at a non-saturating irradiance for photosynthesis of 80 µmol m^{-2} s^{-1}. The main change in C metabolism was not seen for total C. The principal effect was the flow of C among different compounds, from carbohydrates to organic N compounds (amino acids and proteins) during transient NH_4^+ assimilation.

KEY WORDS: Ammonia · Nitrogen assimilation · Carbon · Carbohydrates · Phycobiliproteins · *Gracilariopsis* · *Rhodophyta*

INTRODUCTION

The availability of N is important in assessing the physiological status of marine macroalgae. Given the importance of the agarophyte seaweeds, the changes in biochemical constituents following N addition have been extensively investigated in *Gracilaria* species in outdoor culture systems on a long-term basis (Bird et al. 1981, 1982, Lapointe 1981, Lapointe & Duke 1984, Levy & Friedlander 1990, Friedlander et al. 1991, Haglund & Pedersén 1993). However, little information exists regarding the biochemical processes that govern responses to NH_4^+ pulses on a short-term basis (hours) in red algae. In a previous study, it has been shown that N limitation redirects the flow of internal N compounds toward nonpigmented proteins, whereas phycobiliproteins (PBP) are synthesized in response to N addition in *Corallina elongata* (Vergara & Niell 1993). The aim of the present study was to examine the effect of NH_4^+ availability on N assimilation in *Gracilariopsis lemaneiformis*, an agarophyte red alga of potential use in aquaculture (Bird & Hinson 1993).

The source of N for this study was NH_4^+ instead of NO_3^-. NH_4^+ is an abundant source of N at intermittent pulses in coastal environments (Nixon & Pilson 1983). In natural populations of phytoplankton, uptake rates of N often exceed nitrogen requirements for growth (Caperon & Meyer 1972, Eppley & Renger 1974, Conway et al. 1976, McCarthy & Goldman 1979). Thus, the use of transient micropatches of N will allow balanced growth to be maintained in a fluctuating environment (McCarthy & Goldman 1979, Goldman & Glibert 1982), which is of importance for a perennial macrophyte (Ramus 1991). In addition, an interesting point is that the energetic demand for NH_4^+ uptake and assimilation is lower than for NO_3^-, as NH_4^+ does not have to

be reduced (Turpin 1991). Thus, the second objective of this study was to analyze the effect of NH_4^+ assimilation on C metabolism.

The interaction between C and N metabolism has been focused on unicellular algae, given the rapid and uniform responses of homogeneous cultures to changes in external control variables (Huppe & Turpin 1994). Multicellular organisms in contrast complicate the study of C-N interaction. In addition, red algae have peculiarities with respect to the end products of C metabolism. Unlike unicellular algae and green plants, red algae have a particular type of starch (floridean starch) which is located in the cytosol, and in agarophyte seaweeds such as *Gracilaria* species a certain amount of C is located in cell wall polysaccharides (Pueschel 1990). Along with agar and starch, red algae also have fairly high levels of floridoside. These features make the study of the effect of N assimilation on C partitioning paticularly interesting, since the different subcellular compartmentalization of C compounds in red algae may affect C-N interaction in a manner which is distinct from that which occurs in unicellular algae and green plants.

MATERIALS AND METHODS

Plant material and experimental design. *Gracilariopsis lemaneiformis* (Bory) Dawson Acleto et Folvik was collected at Kure beach (North Carolina, USA). The taxonomy of the genera *Gracilaria* and *Gracilariopsis* has been problematic (Fredericq & Hommersand 1989). This population has been recently analyzed and delineated with respect to other *Gracilaria* species by Goff et al. (1994). Algae were maintained in an outdoor culture tank in a CMSR facility. Apical tips were selected (4 to 5 cm length) and preincubated in N limitation for 24 h prior to the experiments. They have a low proportion of non-photosynthetic medullary tissue, and morphological variability is lower than in adult plants.

Experiments were conducted in artificial seawater (ASW; Woelkerling et al. 1983) with different initial pulses of NH_4^+ for 6 h, a control treatment without any N supply (0 M, Treatment 1, T1) and with 3 concentrations of NH_4^+ (50, 100 and 200 M, T2, T3 and T4, respectively). After that period, in a second phase, algae were transferred to N-limited conditions (ASW without N) for 48 h in a 14 h light : 10 h dark photoperiod cycle. Experiments were carried out in the laboratory in 1 l air-agitated flasks containing 0.44 g dry weight (DW) of algae at a temperature of 27°C (3 replicates per treatment). A non-saturating irradiance of 80 µmol m^{-2} s^{-1} was chosen as a condition of stimulation of PBP synthesis (photoacclimation), and as a condition of limitation in the supply of newly fixed C to meet N assimilation. The time course of both internal N compounds (PBP, soluble protein, internal NH_4^+, amino acid pool, and total N) and internal C compounds (total C, insoluble, and soluble carbohydrates) was monitored. Sampling times were 0, 2, 4 and 6 h, as well as 3, 5, 24 and 48 h after transferring algae to N limiting conditions.

Analytical methods. Triplicate samples were ground in phosphate buffer 0.1 M pH 6.5 at 4°C, extracted overnight and then centrifuged (19 000 × *g*, 20 min). PBP [r-phycoerythrin (RPE) and r-phycocyanin (RPC)], and soluble protein concentrations were determined spectrophotometrically from the supernatant fraction. PBP were determined by using the chromatic equations of Beer & Eshel (1985) and soluble protein according to Bradford's method (1976). The pellet fraction was processed in 5% (w : v) trichloroacetic acid for 3 h at 80°C (Bird et al. 1982) and then centrifuged at 19 000 × *g* for 20 min to determine insoluble carbohydrate content according to the phenol-sulfuric acid method (Kochert 1978). In apical tips of *Gracilariopsis lemaneiformis*, the average levels of carbohydrates are lower than in adult plants of *Gracilaria* spp. (Bird et al. 1981, 1982), in which carbohydrates comprise about 30 to 40% DW (about 17% DW in our study). Indeed, in adult thalli of the agarophyte seaweed *Gelidium sesquipedale* (C : N ratio about 11), the concentration of insoluble carbohydrates is about 40 to 50% DW, accounting for 50% of total C and 90% of total carbohydrates (Vergara pers. obs.). In our study, insoluble carbohydrates only account for 15% of total C and 70% of total carbohydrates. Hence, apical tips of *G. lemaneiformis* may have a low concentration of cell wall polysaccharides, starch constituting the main fraction of C allocated in insoluble carbohydrates.

Protein was precipitated on the first supernatant fraction by adding trichloroacetic acid (0.37 M, final concentration) and sodium-deoxycholic acid (3.6 mM final concentration) (Clayton et al. 1988). After centrifuging (19 000 × *g*, 20 min), internal NH_4^+ and amino acid contents were determined from the supernatant according to Slawyk & MacIsaac (1972) and by the ninhydrin-ascorbate method (Pesez & Bartos 1974), respectively, and soluble carbohydrates (C compounds of low molecular weight) according to Kochert (1978). The previous precipitation of proteins in trichloroacetic acid and the high temperature developed during the assay in sulfuric acid will hydrolyze any oligosaccharide to their monomers.

Triplicate samples were also taken in order to determine the total amount of chlorophyll *a* (chl *a*). These samples were ground in ice-chilled neutralized acetone and extracted overnight. After centrifuging, chl *a* concentration was determined spectrophotometrically from the supernatant according to Talling & Driver

(1963). Carotenoids (in relative units) were determined according to Strickland & Parsons (1968).

Parallel triplicate samples were dried in an oven at 60°C for 48 h to determine total C and N content in a Perkin-Elmer 240-C elemental autoanalyzer. The fresh weight : dry weight relationship was also determined to normalize the results on a DW basis (g DW = 0.002 + 0.17 g FW; r = 0.96, n = 76).

Net rates of NH_4^+ uptake were calculated by measuring the disappearance of NH_4^+ from the medium in the 3 concentrations assayed. Several fitting procedures have been applied to estimate the kinetic parameters: 3 linear methods (Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf) and an iterative procedure (Kaleida Graph, Abelbeck Software, USA), since different methods will give different estimates of these parameters (Berges et al. 1994).

Cellular N flow equations. The net flow of N among intermediary N metabolites was determined taking into account that in our experimental conditions the main source of N would be external NH_4^+ and, therefore, internal NH_4^+ production via the nitrate and nitrite reductase pathway may be insignificant in comparison with the uptake rates of external NH_4^+. Even in the control treatment without N supply, 24 h preincubation in N limitation will reduce the internal NO_3^- pool to insignificant levels. The flow equations of N are the following:

$$F_1 = -d[NH_4^+]_{ext} = \text{uptake rate of } NH_4^+ \ (\mu\text{mol } NH_4^+ \ g^{-1} \text{ DW}) \quad (1)$$

and

$$d[NH_4^+]_{int} = X_1 = F_1 - F_2. \quad (2)$$

Then,

$$F_2 = F_1 - X_1 \ (\mu\text{mol } NH_4^+ \ g^{-1} \text{ DW}) \quad (3)$$

to the amino acid pool and

$$d[aa] = X_2 = F_2 - F_3. \quad (4)$$

Then,

$$F_3 = F_2 - X_2 \ (\mu\text{mol aa } g^{-1} \text{ DW}) \quad (5)$$

to the protein pool,

where F_1 is the uptake rate of NH_4^+, F_2 is the net flow of N between internal NH_4^+ and amino acids, and F_3 is the net flow of N between amino acids and proteins; the subscripts are ext = external, int = internal; and aa = amino acids.

These rates were calculated between successive periods of sampling. They represent net flows of N among intermediary N compounds. The sign defines the direction of the flow. They have not been normalized by time to appreciate graphically the flow of N during N limitation (24 and 48 h).

Statistics. Appropriate statistical analyses (2-way and 4-way ANOVA, Student's *t*-test) were applied to test the significance of the results (Sokal & Rohlf 1981).

RESULTS

Uptake of NH_4^+

External NH_4^+ was taken up linearly throughout time, being exhausted in the lowest pulse of NH_4^+ applied (Fig. 1). No net NH_4^+ excretion was observed after transferring algae to N-limited conditions (second phase) nor in the control treatment without any NH_4^+ supply (first phase). The uptake process, normalized by culture density, resulted in a typical saturation kinetic with a high capacity (high V_{max}) and a low affinity (high K_s) in comparison with other macroalgae (O'Brien & Wheeler 1987), although a certain degree of variability exists when estimating the kinetic parameters according to different methods (Berges et al. 1994; Table 1). Thus, the ratio V_{max}/K_s ranged from 1.2 to 1.7. These values are far removed from those reported in *Enteromorpha prolifera* (from 13 to 20; O'Brien & Wheeler 1987). However, we were less interested in these parameters than in knowing the amount of N taken up by the algae following the pulses of NH_4^+. Thus, the specific NH_4^+ uptake rate was, in the most favorable treatment (pulse of 200 M NH_4^+), up to 9 times greater than in steady-state conditions (assuming a growth rate of 5% d^{-1} and the internal N content) (Table 2).

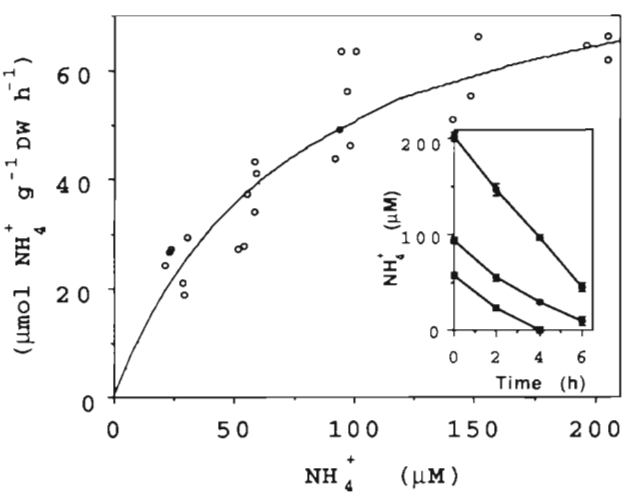

Fig. 1. *Gracilariopsis lemaneiformis*. Representation of the NH_4^+ uptake rate (µmol NH_4^+ g^{-1} DW h^{-1}) vs external NH_4^+ concentration (µM). Inset: time course of NH_4^+ depletion (µM) in seawater at different initial NH_4^+ pulses (0, 50, 100 and 200 µM)

Table 1 *Gracilariopsis lemaneiformis*. Estimates of kinetic parameters of NH_4^+ uptake in red alga according to several fitting procedures

Equation used	V_{max} (µmol N g^{-1} DW h^{-1})	K_s (µM NH_4^+)	V_{max}/K_s	r
Iterative procedure	89	73	1.2	0.92
Hanes-Woolf (S/V against S)	89	75	1.2	0.93
Lineweaver-Burk ($1/V$ against $1/S$)	75	52	1.4	0.85
Eadie-Hofstee (V against V/S)	68	40	1.7	0.64

Table 2. *Gracilariopsis lemaneiformis*. Specific uptake rates of NH_4^+ during transient NH_4^+ assimilation in red alga for 6 h. Accumulation factor also indicated with respect to maintenance uptake rate (0.0021 h^{-1}, assuming a growth rate about 5% d^{-1} and the internal N content). $F_2:F_1$ indicates the ratio of net amino acid synthesis with respect to NH_4^+ uptake; $F_2:F_3$ the ratio between net amino acid and net protein synthesis; $F_3:F_{3max}$ the ratio of protein synthesis with respect to the maximum rate observed

NH_4^+ pulse:	0	50	100	200
Specific uptake rate (h^{-1})	0	0.0076	0.0113	0.0207
Accumulation factor	0	3.6×	5.4×	9.8×
$F_2:F_1$	–	0.84	0.94	0.98
$F_2:F_3$	0.08	0.68	1.08	1.20
$F_3:F_{3max}$	0.25	0.55	0.58	1.00

Internal N and C:N ratio

Because of the high uptake capacity of external NH_4^+, internal N content increased in response to NH_4^+ pulses, decreasing in the control treatment without N supply after 6 h (Fig 2). Afterwards, in N-limited conditions, internal N decreased in all treatments as a result of growth (applying the equation $dN/dt = -\mu N$, in the absence of an external N source, growth rates ranging from 5 to 10% d^{-1} explain this drop in N concentration, result not shown). Differences in internal N concentration among the treatments were maintained after 24 and 48 h of N limitation. The C:N ratio was the mirror image of N, since variability in this ratio was dominated markedly by N content in our experimental conditions. Once it was known that N was accumulated in the tissue, the next question was which compounds contained this N.

Flow of N

From the external NH_4^+ uptake data and the time course of the concentrations of internal NH_4^+ and amino acids, we calculated the net flow of N that was established in the pathway of assimilation of N from external NH_4^+ to proteins. The F_2 rate (net flow of N between internal NH_4^+ and amino acids) showed a parallel evolution to the uptake rate of NH_4^+ (F_1) during NH_4^+ assimilation (0 to 6 h), indicating that the main fraction of external NH_4^+ taken up from seawater was channeled toward amino acids (Fig. 3, Table 3). In addition, the F_3 rate (net flow of N between amino acids and proteins) also showed a high covariation with F_2 rate. These flows revealed that the main part of NH_4^+ was directed toward proteins. Thus, internal NH_4^+ levels remained fairly constant, and amino acids were accumulated only slightly in the highest pulse of NH_4^+ applied (Table 3). No transient increases of internal NH_4^+ nor amino acids were observed during the

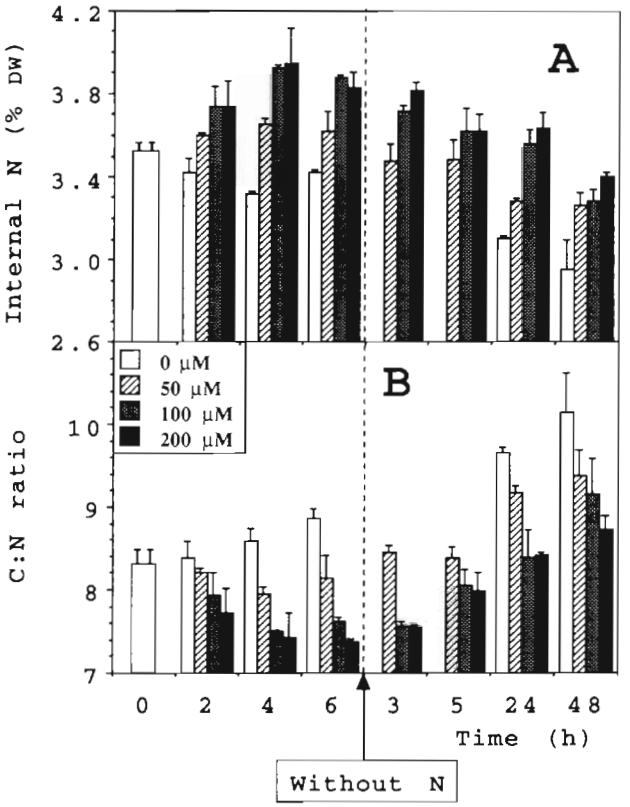

Fig. 2. *Gracilariopsis lemaneiformis*. Time course evolution of (A) internal N content (% DW) and of (B) C:N ratio in the different pulses of NH_4^+ applied

Table 3. *Gracilariopsis lemaneiformis*. Initial concentrations and responses of internal NH_4^+, amino acids, and F_1, F_2 and F_3 rates to NH_4^+ availability (4 treatments, T1 to T4) at 6 h and at 24 and 48 h after transferring algae to N-limiting conditions (without N supply). SD in parentheses

Time	NH_4^+ (μM)	Amino acids (μmol g^{-1} DW)	Internal NH_4^+ (μmol g^{-1} DW)	F_1	F_2 (μmol g^{-1} DW h^{-1})	F_3
Initial value:		241 (33)	52.7 (6.7)			
6 h	T1 (0)	172 (19)	46.4 (3.7)	0	1.1	12.6
	T2 (50)	189 (50)	74.7 (1.7)	22.3	18.6	27.3
	T3 (100)	254 (19)	65.8 (6.8)	33.1	30.9	29.0
	T4 (200)	300 (13)	60.1 (4.1)	61.0	59.7	50.0
24 h N-limited	T1 (0)	194 (9)	49.9 (6.8)	0	−0.2	−1.4
	T2 (0)	168 (26)	52.3 (1.0)	0	0.9	1.8
	T3 (0)	120 (20)	67.2 (3.5)	0	−0.1	3.5
	T4 (0)	263 (35)	54.9 (6.0)	0	0.2	1.8
48 h N-limited	T1 (0)	233 (33)	54.9 (6.0)	0	−0.2	−1.8
	T2 (0)	201 (5)	78.4 (4.4)	0	−1.0	−2.5
	T3 (0)	168 (36)	53.1 (9.2)	0	0.6	−1.4
	T4 (0)	306 (36)	45.8 (10.6)	0	0.4	−1.4

period of time analyzed (2 and 4 h, data not shown). In the control treatment without N supply, a net protein synthesis was observed (25% of V_{max}, Table 2), which caused a reduction in the levels of internal NH_4^+ and in particular of amino acids. Afterwards, when algae were transferred to N-limited conditions (second phase), there were net synthetic and degradative flows of N in the different treatments, but both were 1 order of magnitude lower than during transient NH_4^+ assimilation (0 to 6 h) (Table 3). These degradative flows may explain the increase of internal NH_4^+ and amino acids in the absence of an external N source.

Photosynthetic pigments

As the main fraction of external NH_4^+ was directed toward proteins, the next step was to determine in which kind of protein this N was located. The response of both RPE and RPC was dependent on N supply (Table 4). For simplicity, only results after 6 h, and 24 and 48 h after transferring algae to N limitation are shown. Whereas RPE concentration decreased in the control treatment without N supply, there was an effective RPE synthesis in response to the initial pulses of NH_4^+ after 6 h. RPC was also

Table 4. *Gracilariopsis lemaneiformis*. Initial values and responses of photosynthetic pigments to NH_4^+ availability (4 treatments, T1 to T4) at 6 h and at 24 and 48 h after transferring algae to N-limiting conditions (without N supply). SD in parentheses. RPE: r-phycoerythrin; RPC: r-phycocyanin

Time	NH_4^+ (μM)	RPE (mg g^{-1} DW)	RPC (mg g^{-1} DW)	Chl *a* (mg g^{-1} DW)	Carotenoids : chl *a* (rel. units mg^{-1} chl *a*)	RPE : chl *a* (mg mg^{-1})
Initial value		5.17 (0.20)	0.37 (0.01)	0.68 (0.05)	0.65 (0.01)	7.60
6 h	T1 (0)	4.27 (0.60)	0.35 (0.06)	0.67 (0.04)	0.63 (0.01)	6.37
	T2 (50)	5.83 (0.34)	0.42 (0.03)	0.70 (0.07)	0.62 (0.01)	8.33
	T3 (100)	6.15 (0.12)	0.48 (0.02)	0.74 (0.09)	0.64 (0.01)	8.31
	T4 (200)	6.79 (0.26)	0.47 (0.03)	0.81 (0.03)	0.60 (0.01)	8.38
24 h N-limited	T1 (0)	5.28 (0.53)	0.46 (0.03)	0.65 (0.03)	0.58 (0.01)	8.12
	T2 (0)	5.92 (0.28)	0.41 (0.03)	0.67 (0.02)	0.58 (0.01)	8.84
	T3 (0)	6.59 (0.71)	0.42 (0.02)	0.81 (0.02)	0.55 (0.01)	8.14
	T4 (0)	7.12 (0.05)	0.47 (0.04)	1.15 (0.07)	0.53 (0.01)	6.19
48 h N-limited	T1 (0)	5.13 (0.21)	0.39 (0.02)	0.76 (0.01)	0.58 (0.01)	6.75
	T2 (0)	6.01 (0.06)	0.39 (0.04)	0.79 (0.02)	0.52 (0.01)	7.61
	T3 (0)	6.44 (0.58)	0.40 (0.06)	1.02 (0.07)	0.48 (0.03)	6.31
	T4 (0)	6.36 (0.56)	0.45 (0.02)	1.00 (0.03)	0.52 (0.01)	6.36

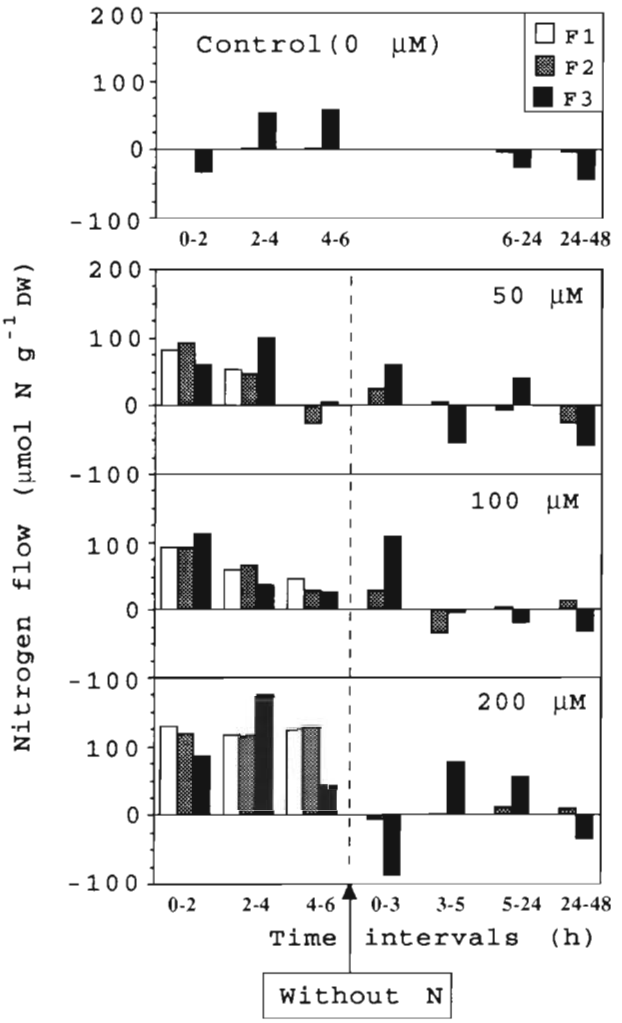

Fig. 3. *Gracilariopsis lemaneiformis*. Nitrogen flow in different NH_4^+ treatments at different time intervals. F_1: NH_4^+ uptake rate (µmol N g^{-1} DW). F_2: net flow of N between internal NH_4^+ and amino acids (µmol N g^{-1} DW). Values greater than zero indicate net amino acid synthesis; in contrast, values less than zero indicate net amino acid degradation. F_3: net flow of N between amino acids and proteins (µmol N g^{-1} DW h^{-1}). Values greater than zero indicate net protein synthesis; in contrast, negative values show net protein degradation

affected by N availability but to a lesser extent than RPE was. Chl *a* was also less affected than RPE by N limitation. The ratio carotenoids : chl *a* decreased throughout the experiment. This drop was larger in N-pulsed algae than in the N-limited treatment (0 M). The ratio RPE chl *a* decreased in response to N limitation after 6 h, unlike N-pulsed treatments. Afterwards, during the N-limited period, this ratio was not affected by initial N pulses as it was during the previous phase of N assimilation.

Proteins

In contrast to PBP, soluble protein did not show a clear trend of response to NH_4^+ pulses after 6 h (Table 5). In the case of nonpigmented soluble protein (estimated as the difference between soluble protein and PBP; Vergara & Niell 1993), there was even more protein in the control treatment without N supply than in the rest of the treatments after 6 h (Table 5). Nonpigmented protein was only affected by N availability 48 h after the addition of NH_4^+ pulses.

As a consequence of the different response of PBP and soluble protein, the PBP:SP ratio increased in response to NH_4^+ pulses after 6 h, decreasing in the control treatment without a N supply. Afterwards, in N-limited conditions, differences decreased among all the treatments (Table 5).

Carbon variables

Insoluble and soluble carbohydrate concentration decreased in response to NH_4^+ assimilation after 6 h (Table 6). Internal C content also decreased in response to N assimilation in the highest pulse of NH_4^+ applied (unpaired *t*-test, $p = 0.048$, with respect to the initial C concentration). However, the main balance was the mobilization of C from carbohydrates to meet N assimilation. It was indicated by the decrease in the ratios insoluble and soluble carbohydrates : total C (ICH:TC and SCH:TC) during N assimilation in *Gracilariopsis lemaneiformis* (Table 6).

Afterwards, in N limited conditions, internal C was not affected by the previous N treatments. Soluble carbohydrates also recovered initial values but not insoluble carbohydrates, indicating the faster turnover of soluble carbohydrates as compared with insoluble carbohydrates. In contrast, in N-limited algae (control) the levels of both insoluble and soluble carbohydrates after 48 h were higher than in the initial conditions.

DISCUSSION

Effect of NH_4^+ pulses on N assimilation

The results obtained after applying pulses of NH_4^+ of different magnitude reveal a high capacity to take up a large amount of NH_4^+ and to channel it toward proteins in *Gracilariopsis lemaneiformis*. Neither amino acids nor internal NH_4^+ were transiently accumulated, at least within the sampling period. In fact, in the highest pulse of NH_4^+ applied (200 µM), with respect to an overall net flow of 366 µmol N g^{-1} DW through the amino acid pool over 6 h, the net accumulation of

Table 5. *Gracilariopsis lemaneiformis*. Initial values and responses of phycobiliproteins (PBP), soluble protein (SP) and PBP:SP ratio to NH_4^+ availability (4 treatments, T1 to T4) at 6 h and at 24 and 48 h after transferring algae to N-limiting conditions (without N supply). SD in parentheses

Time	NH_4^+ (µM)	PBP (mg g^{-1} DW)	SP (mg g^{-1} DW)	Nonpigmented SP (mg g^{-1} DW)	PBP:SP (%)
Initial value		5.54 (0.20)	25.8 (1.3)	20.2 (1.4)	21.5 (1.6)
6 h	T1 (0)	4.62 (0.65)	29.0 (1.4)	24.3 (0.7)	15.9 (2.0)
	T2 (50)	6.25 (0.34)	26.3 (2.4)	20.1 (2.2)	23.7 (1.0)
	T3 (100)	6.63 (0.13)	25.1 (0.5)	18.4 (0.4)	26.5 (0.5)
	T4 (200)	7.26 (0.29)	29.2 (0.5)	21.9 (0.3)	24.9 (0.8)
24 h N-limited	T1 (0)	5.75 (0.50)	28.7 (2.4)	23.0 (1.4)	20.0 (0.3)
	T2 (0)	6.34 (0.31)	31.6 (2.3)	25.2 (1.8)	20.1 (1.4)
	T3 (0)	7.01 (0.72)	28.4 (2.8)	21.4 (2.4)	24.7 (1.3)
	T4 (0)	7.58 (0.55)	31.8 (0.5)	24.2 (0.4)	23.9 (1.5)
48 h N-limited	T1 (0)	5.52 (0.16)	27.1 (1.8)	21.6 (1.1)	20.3 (0.4)
	T2 (0)	6.40 (0.07)	29.1 (0.6)	22.7 (0.4)	22.0 (0.3)
	T3 (0)	6.84 (0.64)	29.0 (2.4)	22.1 (1.9)	23.6 (0.2)
	T4 (0)	6.81 (0.41)	31.5 (2.5)	24.7 (2.4)	21.6 (0.8)

amino acids was only 16% of the net flow (59 µmol aa g^{-1} DW; Table 3). Transient internal NH_4^+ or amino acid accumulation, if any, will occur within a period of less than 2 h. The accumulation of these metabolites has been observed during N assimilation (Dortch 1982, Fujita et al. 1988). In microalgae, the transient increase of the amino acid pool, particularly glutamine (Flynn et al. 1989), occurs within a matter of minutes (Zehr et al. 1988, Vanlerberghe et al. 1992). The high capacity of microalgae to incorporate and assimilate an intermittent pulse of NH_4^+ allows them to maintain balanced growth in a patchy environment, because the importance of an enhanced N uptake depends on the coupling between the uptake and the incorporation of N into new cell material (Collos 1986).

Once it had been determined that the main fraction of the incorporated N was directed toward proteins, it was interesting to see what kind of protein, if any, is preferentially synthesized in response to N pulses. The covariation of different kinds of protein is revealed in the ratio PBP:SP. This index is a good indicator of the selective response of the red pigments as a N reserve.

Table 6. *Gracilariopsis lemaneiformis*. Initial values and responses of internal C variables to NH_4^+ availability (4 treatments, T1 to T4) at 6 h and at 24 and 48 h after transferring algae to N-limiting conditions (without N supply). SD in parentheses. The ratios ICH:TC (insoluble carbohydrates : total carbon) and SCH:TC (soluble carbohydrates : total carbon) have been calculated assuming an average composition of $(CH_2O)_n$ for carbohydrates. Thus, C content represents 40% of the carbohydrate concentration on a weight basis [12 g C per 30 g carbohydrate for 1 mol of a (CH_2O) unit]

Time	NH_4^+ (µM)	TC (% DW)	ICH (% DW)	SCH (% DW)	ICH:TC (g C/g C) × 100	SCH:TC	SCH:TCH (%)
Initial value		29.3 (0.4)	11.8 (0.5)	5.0 (0.3)	16.1	6.8	29.7
6 h	T1 (0)	30.3 (0.2)	11.9 (1.3)	5.7 (0.9)	15.6	7.5	32.5
	T2 (50)	29.4 (0.4)	12.0 (2.1)	5.3 (0.1)	16.3	7.1	30.3
	T3 (100)	29.5 (0.2)	8.2 (1.6)	4.1 (0.3)	11.0	5.6	33.7
	T4 (200)	28.3 (0.6)	9.3 (1.2)	4.0 (0.3)	13.2	5.7	30.2
24 h N-limited	T1 (0)	30.0 (0.2)	12.5 (0.6)	5.3 (0.4)	16.7	7.1	29.8
	T2 (0)	30.1 (0.2)	11.9 (1.5)	5.1 (0.4)	15.8	6.7	29.8
	T3 (0)	29.8 (0.7)	10.9 (1.0)	4.8 (0.2)	14.6	6.5	30.8
	T4 (0)	30.5 (0.8)	9.9 (1.3)	4.7 (0.2)	12.9	6.2	32.5
48 h N-limited	T1 (0)	29.8 (0.1)	13.4 (0.3)	6.1 (0.4)	18.0	8.2	31.3
	T2 (0)	30.6 (0.4)	12.2 (0.7)	6.1 (0.5)	16.0	7.9	33.1
	T3 (0)	30.0 (1.0)	11.3 (1.6)	6.0 (0.2)	15.1	8.1	34.9
	T4 (0)	29.6 (0.4)	9.7 (0.3)	5.6 (0.7)	13.1	7.6	36.7

This role is not only fulfilled by indicating an increase or a decrease of PBP content in response to N supply or limitation. The process of storage is indicated by the selective variation of PBP with respect to other proteins. The PBP:SP ratio increased in response to N pulses, decreasing in the control without N. In the control treatment without N supply, in which 25% of the maximum rate of protein synthesis was observed (Table 2), the PBP content decreased, unlike nonpigmented proteins. In this case the ratio $F_2:F_3$ was 0.08, indicating that 92% of the protein synthesis was supported by preexisting amino acids (Table 2). The increase of the NH_4^+ supply implied a higher contribution of net amino acid formation to protein synthesis. In these conditions, PBP became the main protein synthesized. Afterwards, in N-limited conditions, differences in the PBP:SP ratio decreased among all the treatments, suggesting that N was partitioned differently during transient N assimilation and during N limitation (Table 5).

With respect to other photosynthetic pigments, RPC was less affected by N availability than RPE, which agrees with previous results (Fredriksen & Rueness 1989, Vergara & Niell 1993). The ratio carotenoids:chl *a* decreased throughout time. The relatively low irradiance applied during the experiment could account for the decrease in photoprotective pigments. However, this ratio was higher in the control treatment (N-limited algae) than in N-pulsed algae. The smaller decrease in N limited algae is in accordance with the increase of this ratio caused by N limitation (Plumley et al. 1989).

Differential protein synthesis

From the results obtained in *Corallina elongata* (Vergara & Niell 1993) and in this study, it appears that N limitation redirects the flow of internal N compounds toward the synthesis of nonpigmented proteins, whereas after an N pulse PBP synthesis is stimulated with respect to other proteins. These results raise the question: what is the mechanism which causes PBP to be more affected by N supply than other proteins. The mechanism underlying this selective response could be related to a more general behavior of chloroplast proteins. Several authors have pointed out that chloroplast proteins may be more affected by N limitation than nuclear-encoded proteins (Coleman et al. 1988, Kolber et al. 1988, Falkowski et al. 1989). It seems that during N limitation, plastid protein synthesis is affected at the translational level (Plumley & Schmidt 1989, Taylor 1989) as a result of a reduced level of amino acids (Zehr et al. 1988). External N is assimilated in amino acids through the GS-GOGAT pathway (Turpin & Harrison 1978, Cullimore & Sims 1981, Syrett 1981, Zehr & Falkowski 1988) which is located primarily in the chloroplast (Fisher & Klein 1988). During N limitation, cytosolic tRNAs may bind amino acids before these amino acids are transported into the chloroplast. Thus, cytosolic protein synthesis will be less affected by N limitation than plastid protein synthesis. Conversely, during transient N assimilation following a N pulse, protein synthesis in the chloroplast may be saturated and will be more important than cytosolic protein synthesis. Thus, PBP could act as a N reserve in the context of a more general response of chloroplast proteins to N limitation. In agreement with this hypothesis, the selective response of the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) to N availability has been shown in microalgae (Falkowski et al. 1989, Geider et al. 1994) and in red algae (Lapointe & Duke 1984, Ekman et al. 1989, García-Sánchez et al. 1993), where both Rubisco subunits are chloroplast-encoded proteins (Valentin & Zetsche 1989). This attractive hypothesis, proposed by Falkowski et al. (1989), merits closer examination, and further investigation is need to elucidate the existence of such a mechanism in red algae.

Effect of N assimilation on C metabolism

The second objective of this study was to determine the effect of N assimilation on C metabolism, as N assimilation is a photosynthetic-dependent process, the supply of reducing power and energy and of C skeletons to fix N in amino acids (see Huppe & Turpin 1994 for a recent revision). It is known that transient N assimilation stimulates the flow of C through glycolytic and respiratory pathways (Bassham et al. 1981, Ohmori et al. 1984, Turpin et al. 1988). Therefore, the addition of NH_4^+ promotes the mobilization of C in cyanobacteria and microalgae (Kanazawa et al. 1983, Miyachi & Miyachi 1987, Di Martino Rigano et al. 1991, García-González et al. 1992).

The decrease in internal C content in response to the highest pulse of NH_4^+ applied after 6 h could be evidence of NH_4^+-mediated inhibition of photosynthetic carbon fixation (Elfiri & Turpin 1986, Elfiri et al. 1988), since this drop was further alleviated in N-limited conditions (24 and 48 h).

With respect to carbohydrates, their measurement quantifies the C allocated in compounds of C metabolism (cell wall polysaccharides, starch, soluble C compounds), whereas total C not only includes these compounds but also the C allocated in organic N compounds (amino acids and proteins). Thus, the ratios ICH:TC and SCH:TC are indicative of the C partitioning between different metabolic pathways. The decrease in carbohydrate content and in the ratios

ICH:TC and SCH:TC denoted the mobilization of C during N assimilation in *Gracilariopsis lemaneiformis* (Table 6). The mobilization of C from carbohydrates is not only achieved by means of the high uptake and assimilation of NH_4^+ into amino acids and proteins but is also a consequence of the limitation in the supply of photosynthetic C, as has been pointed out in the cyanobacterium *Anacystis nidulans* (García-González et al. 1992). Hence, saturating irradiances would diminish the effect of mobilization of C during transient NH_4^+ assimilation. Afterwards, in N-limited conditions (second phase), soluble carbohydrates but not insoluble carbohydrates recovered to initial levels, indicating a more persistent effect of the initial NH_4^+ addition on C accumulation in insoluble compounds after 48 h of N limitation.

Interaction C-N

As a consequence of the preferential flow of C toward C compounds (cell wall polysaccharides, starch) or toward amino acid synthesis, and, on the other hand, of the preferential flow of N toward different kinds of protein, it is likely that several ratios relating C and N metabolism will respond with different sensitivity to the NH_4^+ supply. These ratios are shown in Table 7 and have been normalized to 100 % of initial values to allow a comparison among them. All of these ratios showed an inverse covariation with an external NH_4^+ supply, and the differences among NH_4^+ pulses decreased throughout time. However, the ratios using carbohydrates showed larger differences than those of total C. With respect to N, the ratios normalized by PBP showed more sensitivity to the NH_4^+ supply than those normalized by total N or soluble protein. The effect of the initial NH_4^+ pulses still persisted after 48 h, although these ratios showed a different degree of sensitivity.

Table 7. *Gracilariopsis lemaneiformis*. Several ratios normalized to 100% of initial value relating C and N metabolism, as a function of NH_4^+ availability after 6 h and after transferring plants to N-limited conditions. Differences between 200 and 0 µM NH_4^+ treatments also indicated. ICH: insoluble carbohydrates; PBP: phycobiliproteins; SP: soluble protein

Time	NH_4^+ (µM)				
	0	50	100	200	200 – 0
6 h					
C:SP	92	98	104	85	7
C:N	107	98	92	89	18
C:PBP	126	90	85	74	52
ICH:SP	89	99	71	70	19
ICH:N	103	99	63	65	38
ICH:PBP	121	90	58	60	61
24 h N-limited					
C:SP	92	84	92	85	7
C:N	116	110	101	101	15
C:PBP	100	91	81	77	23
ICH:SP	95	82	83	68	27
ICH:N	121	108	97	81	40
ICH:PBP	102	88	73	61	41
48 N-limited					
C:SP	96	92	91	83	13
C:N	122	113	110	105	17
C:PBP	103	91	84	83	20
ICH:SP	108	91	85	67	31
ICH:N	136	112	91	86	50
ICH:PBP	114	90	78	67	47

Table 8. *F*-values of a 4-way ANOVA of the different ratios normalized from Table 6, in relation to: (1) initial pulse of NH_4^+; (2) variable of C (C or insoluble carbohydrates); (3) variable of N (soluble protein, N or phycobiliproteins); (4) time (6 h, 24 h N-limited and 48 h N-limited, which is indicative of a change in the supply of N). All *F*-values significant at $p < 0.001$, unless indicated as ns

Source of variation	df	MS	*F*	p
(1) External NH_4^+	3	3017.4	615.8	
(2) Variable of C (C)	1	1020.0	208.2	
(3) Variable of N (N)	2	1528.6	312.0	
(4) Time (T)	2	309.0	63.1	
$NH_4^+ \times C$	3	508.5	103.8	
$NH_4^+ \times N$	6	215.4	44.0	
$NH_4^+ \times T$	6	50.7	10.3	
$C \times N$	2	4.3	0.9	ns
$C \times T$	4	124.3	25.4	
$N \times T$	4	316.2	64.5	
$NH_4^+ \times C \times N$	6	5.8	1.2	ns
$NH_4^+ \times C \times T$	6	68.1	13.9	
$NH_4^+ \times N \times T$	12	59.6	12.2	
$C \times N \times T$	4	3.6	0.7	ns
$NH_4^+ \times C \times N \times T$ (error)	12	4.9		

Table 7 resembles the typical structure of an analysis of variance (ANOVA). All the factors [(1) the magnitude of the NH_4^+ pulse, (2) the variable of C, (3) the variable of N, and (4) time, as a condition of NH_4^+ supply (6 h) or N-limitation (24 and 48 h without N)] had a significant effect on these ratios (Table 8). Although the C:N ratio is widely used to assess the physiological status of macroalgae (Atkinson & Smith 1983, Hanisak 1990, Duarte 1992, Vergara et al. 1993), other ratios show more sensitivity to NH_4^+ availability, these differences being an indication of the close interaction between C and N metabolism during transient N assimilation in red algae.

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