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Influence of ammonium-N pulse concentrations and frequency, tank condition and nitrogen starvation on growth rate and biochemical composition of *Gracilaria gracilis*

Albertus J. Smit, Bruce L. Robertson & Derek R. du Preez
Department of Botany, University of Port Elizabeth, P.O. Box 1600, Port Elizabeth, 6000 South Africa

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Abstract

The growth rate of *Gracilaria gracilis* maintained in tanks at an abalone farm near Port Elizabeth, South Africa, was examined under various tank conditions and NH_4-N pulse frequencies and concentrations. This was accompanied by analyses of the components of the internal nitrogen pool. A maximum growth rate of ca. 35% wk^{-1} was obtained at 1200 μM NH_4-N. The alga was able to grow at non-nitrogen limited rates using only internal stored nitrogen to sustain growth for one week before the growth rate decreased to ca. 17% per week. NH_4-N pulse frequency did not affect growth rate but one pulse per week led to a marked decrease in total-N, protein, phycoerythrin and chlorophyll-*a* content. An increase in pulse frequency to two pulses per week doubled the protein content from 2.351 ± 0.143 to 4.453 ± 0.090% (per unit dry mass). Carbohydrate content was inversely related to nitrogen storage. The growth rate in fouled tanks was always lower than in clean tanks. It seems likely that seaweeds and diatoms colonising the tank sides reduced light reflected off the inside of a tank, thereby reducing the growth rate.

Introduction

The phenomenon of luxury consumption of available nutrients by algae is an ecological adaptation to nitrogen limitation. *Gracilaria* spp. have the capacity to take up and store nitrogen in excess of immediate requirement and use it during subsequent periods of nutrient deficiency to sustain growth for up to three weeks (Lapointe & Ryther, 1979; Ryther et al., 1981). Storage can be in the form of inorganic nitrogen (Chapman & Craigie, 1977) and/or metabolites such as proteins and pigments (Conover, 1975; Wheeler & North, 1980; Lapointe, 1985; Vergara et al., 1995; Lewis & Hanisak, 1996). Nitrogen storage is important ecologically, but also has important implications for nutrient management of cultivated seaweeds (Bird et al., 1982; Lapointe, 1985), such as providing the physiological basis for pulse-feeding.

Changes in the relative concentrations of components making up the internal nitrogen pools of seaweeds are often used to study nitrogen allocation and storage patterns under changing environmental conditions, and are used to gain an insight of the nutrient status of some cultivated algae (Friedlander & Dawes, 1985; Fujita, 1985; Lapointe, 1985; Vergara et al., 1995). In many macroalgae growth rates are reduced as soon as the total-N content of the thallus falls below the critical level of about 2% (Hanisak, 1979). The accumulation of polysaccharides such as agar and carrageenan as thallus nitrogen content decreases has also been documented (Neish et al., 1977; Chopin et al., 1985). Since nitrogen limitation affects both phycocolloid content and growth rate, the nitrogen requirements of cultivated seaweeds have to be determined depending on the end use of the seaweed. Biomass production depends on fast growth rates at high stocking densities. This is achieved by providing large amounts of nitrogen, but results in a decrease in phycocolloid content. In this case low agar or carrageenan yields are not important. Nitrogen supply to seaweeds cultivated for phycocolloid production is more critical. Here a balance has to be established between high (fast growth rate) and low (high agar yield) nitrogen supply.

The aim of this study was to investigate nitrogen storage and to determine the optimum nitrogen concentration and frequency of the nutrient pulse in order to maximise growth and production in outdoor tanks. This was achieved by conducting growth studies and examining the nitrogen allocation pattern of *G. gracilis* at an abalone aquaculture center near Port Elizabeth, South Africa. Since the seaweed tanks become fouled with nuisance algae and detritus when they are not maintained on a regular basis, the effect of tank cleanliness (i.e. clean or fouled tanks) on the growth rate was also evaluated.

Materials and methods

Tank set-up

Six 45-L glass aquaria with white sides received filtered (10 μm) running seawater with a turnover rate of ca. 24 tank volumes per day. The light source was reduced daylight supplemented with 60 μmol photon m^{-2} s^{-1} from 40 watt Cool White (Philips) fluorescent tubes above the tanks. Compressed air supplied through a PVC pipe fixed to the bottom and along one side of each aquarium caused the seaweed to move in a rolling motion (day and night). Where appropriate, the aquaria were cleaned weekly by manually removing any fouling algae and detritus from sides and bottom. The initial stocking density was 2 kg m^{-2} (280 g seaweed per tank). Nitrogen was supplied as commercially available NH_4SO_4, and Maxiphos (commercial granular fertiliser – 105 g PO_4^- kg^{-1}) was used as source of phosphate. The molar ratio of NH_4-N to PO_4-P was kept at 10:1 for all experiments and treatments.

Effect of NH_4-N concentration on growth

Trial 1 determined the growth rate of *G. gracilis* at three NH_4-N concentrations, i.e. 400, 800 and 1200 μM. Twice weekly pulses of NH_4SO_4 were added to the tanks to obtain the following final NH_4-N concentrations: Tanks 1 and 2, 400 μM; 3 and 4, 800 μM; and 5 and 6, 1200 μM NH_4-N.

Nutrient pulses were added at night and water flow through the system was turned off for 12 h to allow time for nutrient uptake. The seaweed was harvested and weighed weekly, and restocked at the initial density. The specific growth rate (S.G.R.) was calculated and expressed as % weight increase wk^{-1}. Trial 2 was conducted at higher NH_4-N concentrations (800, 1200, 1600 μM).

Growth in nitrogen-limited seawater

Cultures received 1200 μM NH_4-N in two pulses per week. The seaweed was acclimatised to the high nutrient concentration for three weeks, after which all fertilisation ceased. The alga was allowed to grow in nitrogen-limited seawater (approximately 0.07 μM NH_4-N) for a further three weeks and the growth rate was recorded over this period. Biochemical and chemical analyses for carbohydrates, protein, total nitrogen, *r*-phycoerythrin, chlorophyll-*a* and carotenoids were analysed at the end of Week 6. The results of this experiment were used as the basis for determining the nutrient pulse frequencies used in a subsequent experiment and were compared with those from a treatment which received nitrogen at a concentration of 1200 μM NH_4-N for the duration of the experiment (six weeks).

Effect of NH_4-N pulse frequency on growth and nitrogen storage

NH_4-N pulses were varied from one to two pulses per week (2 tanks per treatment). The resulting NH_4-N concentration from each pulse was set at 1200 μM. All visible epiphytes were removed weekly to minimise competition for light and nutrients and the sides of the tanks were kept free of algae and other fouling material. The seaweed was allowed to acclimatise to the nutrient-pulse regime for three weeks. Specific growth rates for each treatment were determined at weekly intervals for six weeks, including the time of acclimatisation. At Weeks 4 and 5 samples were removed from each tank and analysed for carbohydrates, protein, total-N, *r*-phycoerythrin, chlorophyll-*a* and carotenoids.

Effect of tank cleanliness on growth rate, chemical and biochemical composition

In conjunction with the above experiment, algae in two tanks received 1200 μM NH_4-N in one pulse per week. The seaweed was acclimatised to the nutrient-pulse regime for the first three weeks of the experiment. The tanks were not cleaned and weed species (mainly *Enteromorpha* sp. and *Ectocarpus* sp.) colonised the tank sides and bottom. These weeds were allowed to accumulate detritus with time. Specific growth rates were determined weekly for five weeks (including acclimatisation) and at Weeks 4 and 5 samples were

Table 1. Analytical methods used for determination of selected chemical or biochemical constituents of *G. gracilis*

Protein extraction	Bird et al., 1982
Protein determination	Plummer, 1987
Carbohydrate extraction	Bird et al., 1982
Carbohydrate determination	DuBois et al., 1956
Total nitrogen	Bremner, 1965
Chlorophyll-*a* and carotenoid extraction and determination	Evans, 1988
r-Phycoerythrin	Beer & Eshel, 1985

taken from each tank for analyses of carbohydrates and nitrogen storage components as above. All visible epiphytes were removed weekly to minimise competition for light and nutrient between the epiphytes and the basiphyte.

Analytical methods

The wet:dry mass ratio and water content were determined by drying the seaweed to constant mass at 60 °C. Chemical and biochemical analyses on triplicate samples were according to the methods summarised in Table 1.

Statistical analyses

All statistical analyses used Statistica for Windows release 4.1 (StatSoft, Inc., 1993). Analysis of variance (ANOVA) was used to assess the main effects of nutrient pulse frequency and tank condition on the pooled data for growth rate and concentration of the biochemical components. No interactions were tested. Tukey's Honest Significant Difference (HSD) test was used as the *post hoc* test for comparisons of treatment means. The Pearson product-moment correlation was used in the correlation of selected variables (chemical and biochemical constituents), and where appropriate, lines were fitted to the data using the Distance Least Squares estimation technique. The probability level used to test the significance of the null hypothesis was $p = 0.05$.

Results

The effect of NH_4-N concentration on growth (Trial 1) is as shown in Figure 1. The specific growth rates are significantly higher for the seaweeds grown

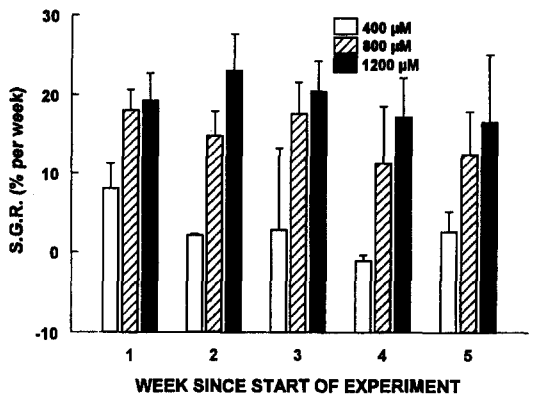

Figure 1. Specific growth rate of *G. gracilis* at three NH_4-N concentrations (Trial 1). Bars show mean values ± S.E.

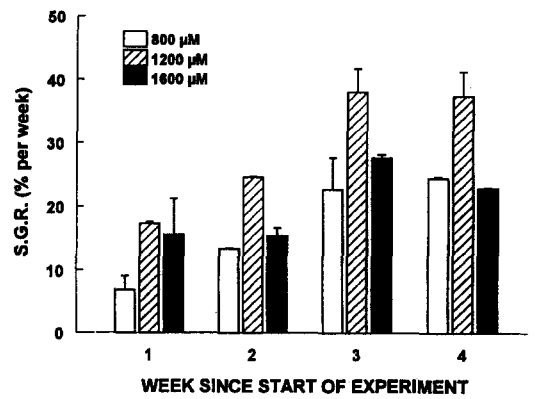

Figure 2. Specific growth rate of *G. gracilis* at three NH_4-N concentrations (Trial 2). Bars show mean values ± S.E.

at 800 and 1200 µM when compared to those grown at 400 µM NH_4-N ($p = 0.0006$ and $p = 0.0001$ respectively). Although the mean growth rate over five weeks is slightly higher at 1200 µM ($19.29 \pm 1.97\%$ wk^{-1}) than at 800 µM ($14.79 \pm 1.85\%$ wk^{-1}) it is not significantly so ($p = 0.2290$). Results from Trial 1 suggested that the optimum NH_4-N concentration of the nutrient pulse had not been reached.

Optimum NH_4-N concentration of the nutrient pulse is more obvious in Trial 2 as the growth rate of the seaweed is higher at 1200 µM than at 800 and 1600 µM (Figure 2). The differences in growth rates become significant ($p<0.01$) at Week 3 where the growth rates were 23.65 ± 2.05, 37.79 ± 2.17 and $25.28 \pm 1.39\%$ wk^{-1} for the alga grown at 800, 1200 and 1600 µM respectively.

Growth rate decreased very markedly with increasing nitrogen limitation (Figure 3). The seaweed was

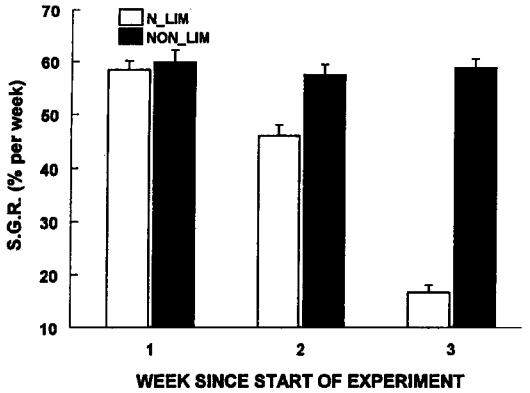

Figure 3. Specific growth rate of *G. gracilis* grown in nitrogen-limiting (N–LIM) and non nitrogen-limiting (NON–LIM) seawater. Mean values ± S.E. are shown.

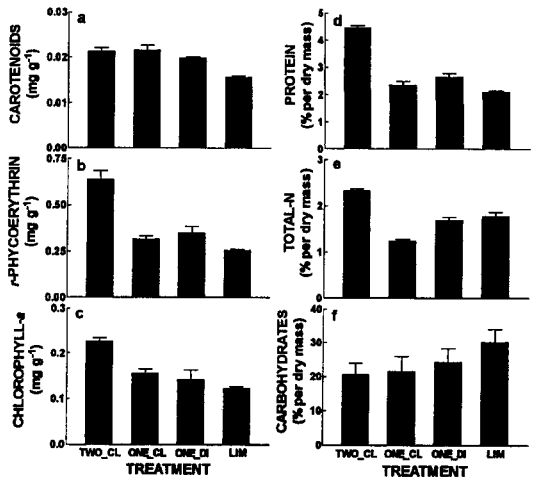

Figure 4. Variation in biochemical and chemical composition of *G. gracilis*; (a) carotenoids, (b) *r*-phycoerythrin, (c) chlorophyll-*a*, (d) protein, (e) total-N and (f) carbohydrates. TWO–CL, two nitrogen pulses per week/clean tanks; ONE–CL, one nitrogen pulse per week/clean tanks; ONE–DI, one nitrogen pulse per week/fouled tanks; LIM, nitrogen-limited seaweed. Mean values ± S.E. are shown.

able to grow at non-nitrogen limited rates of 50–60% wk^{-1} using only internally stored nitrogen to sustain growth for one week and thereafter the rate decreased to approximately 17% wk^{-1}. The growth rate for the non-nitrogen limited treatment remained constant over that period at about 60% wk^{-1}.

Tank cleanliness and pulse frequency have a marked effect on nitrogen storage and allocation of *G. gracilis* (Figure 4a–f). The concentration of carotenoids (Figure 4a) did not seem to be influenced by the pulse frequency or tank cleanliness since there were no significant differences between the clean tanks receiving two nitrogen pulses per week (TWO–CL), the clean tanks receiving one pulse per week (ONE–CL) and the fouled tank receiving one pulse per week (ONE-DI) ($p<0.05$ in all cases). The concentration of carotenoids extracted from nitrogen-limited *G. gracilis* (LIM) is significantly lower than both TWO–CL and ONE–CL ($p=0.0076$ and $p=0.0069$ respectively).

Both *r*-phycoerythrin (Figure 4b) and chlorophyll-*a* (Figure 4c) showed similar trends with respect to pulse frequency and tank cleanliness. The concentration of these two pigments was significantly lower for ONE–CL and ONE–DI when compared to TWO–CL ($p < 0.05$), however there was no significant difference in concentration of *r*-phycoerythrin and chlorophyll-*a* between ONE–CL and ONE–DI ($p>0.05$). When the pulse frequency was increased from one to two pulses per week there was a corresponding doubling in *r*-phycoerythrin content (0.636 ± 0.048 and 0.316 ± 0.018 mg g^{-1} for TWO–CL and ONE–CL respectively). The concentrations of both *r*-phycoerythrin and chlorophyll-*a* were lower in the nitrogen-limited treatment (LIM) than in TWO–CL ($p<0.05$).

As in the case with *r*-phycoerythrin, the increase in pulse frequency from one to two pulses per week resulted in the doubling of protein content (Figure 4d), from 2.351 ± 0.143 to 4.453 ± 0.090% (per unit dry mass). Tank cleanliness did not affect the protein content since there was no significant difference between ONE–CL and ONE–DI ($p=0.5678$). The protein content was significantly lower for LIM when compared to TWO–CL but not so when compared to ONE–CL and ONE–DI. Overall, total nitrogen (Figure 4e) content followed the same trends as *r*-phycoerythrin and protein.

Carbohydrate content (Figure 4f) did not differ significantly between any of the treatments ($p>0.05$ in all cases). Visual examination of the treatment means however, suggests the trend was the inverse of that exhibited by total nitrogen, i.e. the amount of carbohydrates synthesised increased with increasing nitrogen-limitation.

In the treatments where the tanks were kept clean (Figure 5) the growth rate during Weeks 1–3 for tanks receiving one pulse per week (ONE–CL) is slightly higher than that receiving two pulses per week (TWO–CL). The total nitrogen content of the seaweed is 2.328 ± 0.039 and 1.236 ± 0.041% (per unit

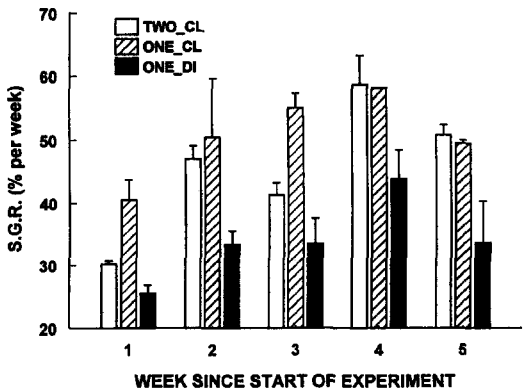

Figure 5. Specific growth rate of *G. gracilis* at two nutrient pulse frequencies and tank conditions: TWO–CL, ONE–CL and ONE–DI as in Figure 4. Bars show the mean values ± S.E.

dry mass) for TWO–CL and ONE–CL respectively. The data were analysed with and without the acclimatisation period (Weeks 1–5 and 4–5 respectively) and in both cases no significant difference was found in growth rate between cultures grown with one or two pulses per week (TWO–CL vs ONE–CL – Weeks 1-5: $p = 0.3955$; Weeks 4–5: $p = 0.9810$). However, the growth rate of *G. gracilis* grown in fouled tanks receiving one pulse per week (ONE–DI) is significantly lower than both the clean tank treatments over the five week period (TWO–CL vs ONE–DI: $p = 0.0232$; ONE–CL vs ONE–DI: $p = 0.0309$) (Figure 5).

The following significant correlations were obtained from the correlation analyses (Table 2): chlorophyll-*a* and *r*-phycoerythrin, protein and *r*-phycoerythrin, protein and chlorophyll-*a*, carbohydrates and chlorophyll-*a*, total nitrogen and *r*-phycoerythrin, total nitrogen and chlorophyll- *a*, total nitrogen and protein ($p<0.05$). Carotenoid content showed no correlation with any of the other biochemical components. Although carbohydrate content was only significantly correlated to chlorophyll-*a*, the other insignificant *r*-values obtained for carbohydrates were always negative, once again indicating the inverse relationship of the polysaccharide to the individual components responsible for nitrogen storage.

The concentration of each of the components of the internal nitrogen pool is given in Table 3. The carotenoids are least responsible for nitrogen storage which explains why they remain relatively constant between the various treatments. The largest sinks for nitrogen are proteins and *r*-phycoerythrin.

Discussion

For a commercial seaweed farm to be economically viable, cultivation must be continuous and the system easily and cheaply operated (Bidwell et al., 1985). Furthermore, it should be cheaper than harvesting from natural populations. The most cost effective form of seaweed tank cultivation is possible in mariculture effluent water where running seawater is readily available and no fertilisation is needed. It is possible however, that the ambient nutrient levels in the effluent water is too low to sustain the high seaweed densities required for biomass production, in which case external nutrient sources are needed. In this case the amount of nutrients to be added to provide maximal growth has to be determined.

The concentration of NH_4-N used in this study falls within the range of concentrations commonly used in other studies. The highest NH_4-N concentration used by Pickering et al. (1993) for 30 g *Gracilaria* sp. in 30 L seawater was 143 μM while D'Elia & DeBoer (1978) used 50 μM for 7 g seaweed cultures in 6.4 or 8.3 L cylinders. On the other hand, Ryther et al. (1981) used concentrations as high as 2000 μM for 5 kg *Gracilaria* sp. in 300 or 500 L tanks while Lapointe (1985) maintained 300 g *Gracilaria* sp. in 14 L of seawater with NH_4-N concentrations ranging from 250–200 μM. In this study *G. gracilis* grew maximally at 1200 μM NH_4-N. Although the S.G.R. decreased at 1600 μM NH_4-N, no signs of ammonium toxicity were evident.

Bird et al. (1982) and Lapointe & Duke (1984) showed that *G. tikvahiae* has the ability to grow on internally stored nitrogen at non-nitrogen limiting rates for up to four weeks before its growth rate decreased. The equivalent period for *G. gracilis* in this study is one week. After three weeks in nitrogen-free seawater the total-N content decreased from about 2.3 to 1.7%. Increasing nitrogen limitation was accompanied by the loss of the red colouration. Over the experimental period of three weeks the colour changed from dark red/brown to an almost straw colour, and corresponded to the reduction in *r*-phycoerythrin content from ca. 0.64 to 0.25 mg g^{-1} (wet). Similar results have been obtained by Lapointe (1981). In red algae, phycobiliproteins may account for up to 60% of the total soluble cellular protein (Bogorad, 1975, Rosenberg & Ramus, 1982b) and therefore represent a large nitrogen reserve. In this study support for this is provided by the significant positive correlation found between soluble protein and *r*-phycoerythrin at different nitrogen pulse concentrations and frequencies.

Table 2. Summary of correlation analyses for the components of the internal nitrogen pool in *G. gracilis*. Significant correlations are marked with an * (p=0.05)

	r-Phycoerythrin	Chlorophyll-*a*	Protein	Carbohydrates	Total-N
Carotenoids	$r = 0.3460$	$r = 0.446$	$r = 0.1718$	$r = -0.3045$	$r = -0.0662$
	$p = 0.189$	$p = 0.083$	$p = 0.525$	$p = 0.251$	$p = 0.808$
r-Phycoerythrin		$r = 0.7534$*	$r = 0.7632$*	$r = -0.1620$	$r = 0.7166$*
		$p = 0.001$	$p = 0.001$	$p = 0.549$	$p = 0.002$
Chlorophyll-*a*			$r = 0.7925$*	$r = -0.6665$*	$r = 0.6496$*
			$p = 0.000$	$p = 0.005$	$p = 0.006$
Protein				$r = -0.3926$	$r = 0.7725$*
				$p = 0.133$	$p = 0.000$
Carbohydrates					$r = -0.1978$
					$p = 0.463$

Table 3. Contribution of selected biochemical and chemical components to internal nitrogen storage in *G. gracilis*. Data shown for cultures grown in clean tanks receiving two nitrogen pulses per week (TWO_CL) only

	n	Mean	± S.E.
Protein (%)	8	4.15	0.09
r-Phycoerythrin (mg g^{-1})	8	0.64	0.05
Chlorophyll-*a* (mg g^{-1})	8	0.23	0.01
Carotenoids (mg g^{-1})	8	0.02	0
Total N (%)	4	2.33	0.04

While the amount of protein is halved under severe nitrogen limitation after three weeks, chlorophyll-*a* concentration also decreases, although to a lesser extent. The ratio of *r*-phycoerythrin:chlorophyll-*a* decreased from 2.8 to 2.1. According to Lapointe (1981), the decreased ratio of phycoerythrin:chlorophyll-*a* points on a unique growth strategy for *Gracilaria* spp. During high light and rapid growth the pigments may reduce nitrogen limitation by releasing nitrogen to sustain growth, whereas during low light, slow growth periods, they may serve as a nitrogen sink, increasing the amount of light harvesting pigments. For example, in natural systems where peaks in inorganic nitrogen occur as short pulses rather than seasonal maxima, some members of the genus are able to take advantage of the transiently available nitrogen to increase the amount of pigments. This temporary increase in pigment content results in an enhanced photosynthetic capacity until tissue nitrogen levels have to be depleted to sustain growth (Rosenberg & Ramus, 1982b).

Amino acids make up the second largest part of the metabolites responsible for nitrogen storage (Bird et al., 1982), but their content was not determined in this study. Very little nitrogen is stored as DNA, ammonium or nitrate (Bird et al., 1982) and these were therefore also omitted from this study. Although the analyses made in these experiments do not represent a complete fractionation of the nitrogen pool, they give an indication of the relative size of the various compartments. Results of the analyses indicating the relative importance of the various constituents of the internal nitrogen pool are very similar to those obtained by Bird et al. (1982) and Friedlander & Dawes (1985). The order of importance (Table 3) is: proteins (most important), *r*-phycoerythrin, chlorophyll-*a* and the carotenoids (least important).

The amino acid content decreased rapidly with the onset of nitrogen limitation in *G. tikvahiae* (Bird et al., 1982). This rapid decrease in amino acid content suggests that the amino acids form the initial pool of nitrogen metabolised when ambient nitrogen concentration is reduced, and therefore plays a major function in nitrogen storage (Bird et al., 1982). The protein and *r*-phycoerythrin content decreased more slowly than the amino acids, but at a seemingly equal rate (Laycock & Craigie, 1977). Here, *G. gracilis* metabolises *r*-phycoerythrin at a faster rate than protein during nitrogen limitation, but it appears that both these are sources of nitrogen during nitrogen deficiency. Protein is the most important source of nitrogen to the seaweed since more protein is present compared to *r*-phycoerythrin.

Rotem et al. (1986) found that when growth rate was limited by nutrient availability, the floridean starch content increased with respect to that in an enriched culture medium, rising with increasing irradiance. Agar content is also higher under nitrogen-deficient conditions and it therefore seems that when growth

is limited by nitrogen deficiency, there is an increased flow of photosynthates towards polysaccharide synthesis (Rotem et al., 1986), but during nitrogen-sufficient growth, protein and pigment synthesis predominates (Lapointe & Ryther, 1979).

Results from this study agree with those obtained by Chapman & Craigie (1977), Bird et al., (1982), Rosenberg and Ramus (1982a) and Rotem et al. (1986) in indicating that carbohydrates show the opposite response to nitrogen limitation than do protein, *r*-phycoerythrin and chlorophyll-*a*.

This suggests that newly fixed carbon is stored as non-aminated compounds due to the unavailability of inorganic nitrogen (Bird et al., 1982). For this reason the C:N ratio increases with increasing nitrogen limitation (Lapointe & Duke, 1984). Dawes et al., (1977) found that the protein:carbohydrate ratio was proportional to the growth rate in *Eucheuma* sp. In this study, inverse correlations of carbohydrates with all components of the internal nitrogen pool were obtained, although not always significant (Table 2).

In nitrogen-starved unicellular algae, the rapid uptake of NH_4-N is accompanied by an increase in respiration at the expense of endogenous carbohydrates that are not present in nitrogen-replete cells (Syrett, 1956a). In the Rhodophyta the accumulation of polysaccharides in nitrogen-starved seaweeds has also been shown (Chapman & Craigie, 1977; DeBoer, 1978; Bird et al., 1982; Rosenberg & Ramus, 1982a; Lewis & Hanisak, 1996). Nitrogen-starved *G. tikvahiae* assimilates more NH_4-N following exposure to daylight than when held in the dark (Ryther et al., 1981), confirming the observation of Syrett (1956a, b) that the rapid assimilation by nitrogen starved algae depends on carbohydrate reserves and ceases when those reserves are depleted. It is clear that the light and nitrogen requirements of *Gracilaria* spp. will greatly affect agar yield and quality, as well as growth rate. In general, agar content is lower at fast growth rates (Bird et al., 1981; Buschmann et al., 1994). Since it is not possible to simultaneously obtain a fast growth rate and high agar content, it is essential that the critical balance between growth rate and agar content is found if the aim is to produce agar cost effectively. On the other hand, with seaweed production as food for invertebrates, large doses of nitrogen to the cultures will significantly increase biomass production.

Different pulse feeding frequencies in this study led to marked changes in the sizes of the individual components of the internal nitrogen pool, but did not significantly affect specific growth rate. The lower concentration of protein, *r*-phycoerythrin and chlorophyll-*a* in the treatment receiving one pulse per week suggests that use was made of internal nitrogen stores to sustain growth. The treatment receiving two pulses per week had a much higher concentration of the compounds responsible for nitrogen storage, and it is evident that less use was made of the metabolites to provide nitrogen for growth. Here more inorganic nitrogen (NH_4-N) was used since it was readily available. The fact that there was no noticeable increase in growth rate when pulse frequency was increased from one to two pulses per week meant that the algae were not nitrogen limited at any stage. Carbohydrate content seemed to increase when the pulse frequency was halved, but this increase was insignificant.

Lapointe (1985) and Pickering et al. (1993) found that pulse frequency had a greater influence than pulse concentration on the growth of *G. chilensis*. Pulse frequency became important over time scales similar to the time the seaweed can use internal stores of nitrogen to sustain growth. If the seaweed can sustain growth for one week by using its internal nitrogen stores (*G. gracilis* in these experiments), there should be no significant difference in specific growth rate of *Gracilaria* sp. grown at pulse frequencies of one and two pulses per week, which is indeed the case. However, if a treatment which received one pulse every two weeks is compared with a treatment receiving one or two pulses a week, a difference in growth rate would be expected. This is confirmed by Pickering et al. (1993) and results from this experiment are in close accordance with their findings.

According to Pickering et al. (1993) the length of time *G. chilensis* can sustain growth on its internal nitrogen stores should be roughly the same as the doubling time of its biomass, as it would represent the time taken for percentage thallus nitrogen to halve from 3% (nitrogen-replete) to 1.5% (nitrogen-starved) during growth without the addition of nitrogen to the culture medium. Results from this study, suggest *G. gracilis* is nitrogen-replete at ca. 2.3% and nitrogen-starved at ca. 1.7% total-N. In this case the nitrogen storage time is approximately the time it takes for the biomass to increase by a factor of 1.35, which is substantially less than that of *G. chilensis*.

Lapointe (1985) compared the growth of *G. tikvahiae* with treatments having the same nitrogen flux (flux = flow rate * nutrient concentration) but at different concentrations and frequencies and found that there were no significant differences. This suggests that the growth is a function of the total amount of

nitrogen being supplied, irrespective of the frequency and concentration of the pulses. For this reason Lapointe and Ryther (1979) have suggested that nitrogen flux is perhaps the single most important parameter for determining nitrogen-seaweed yield relationships. Pickering et al. (1993) on the other hand found that growth rate was only a function of nitrogen flux about a certain critical frequency of pulses. In each set of treatments with the same nitrogen flux, growth was slower when there was only one pulse per 14 days. It is thus important to bring the time the seaweed can sustain growth on internal nitrogen stores into account when the pulse frequency is determined.

Competition for light and nutrient was prevented by weekly removal of epiphytes throughout the duration of the experiments. Epiphytes like *Enteromorpha* sp. are able to grow on internally stored nitrogen for 8–10 days (Fujita, 1985), similar to *G. gracilis* in this study. This rendered pulsed nutrient supply as a method of epiphyte control ineffective. Nutrient pulsing can only be effective if the epiphyte's internally stored nitrogen becomes depleted sooner than that of its host. Pulsed nutrient supply would also be ineffective in a polyculture system where effluent water is used to sustain growth. Harlin et al. (1978) showed that *Enteromorpha* spp. can scavenge nitrate at concentrations of as low as 0.5 μM. It is reasonable to assume that under conditions of continuous nutrient supply, epiphytes will thrive even if ambient concentrations of NO_3-N or NH_4-N are low.

The effect of tank condition on the growth of *G. gracilis* was marked. The growth rate in fouled tanks was ca. 27% lower than in clean tanks. In general there was no significant difference in protein, *r*-phycoerythrin, chlorophyll-*a*, carotenoid and carbohydrate content between the algae grown in clean tanks at one nutrient pulse per week and those grown in fouled tanks at one pulse per week. It is therefore suggested that the reduction in growth rate in the fouled tanks containing *Enteromorpha* sp. and *Ectocarpus* sp. on the side walls was not the result of competition for nutrients. Nuisance algae growing on the tank sides accumulated detritus with time and light reflected off the sides of the tanks was thus reduced. This caused the *G. gracilis* to be light limited, resulting in the reduced growth rate. The decreased growth rates obtained here are similar to those obtained by Lignell et al. (1987) where *G. secundata* grown in tanks with a black bottom had a lower growth rate than that grown in tanks with white bottoms. They found that tank colour significantly affects the light field experienced by the algae.

A better understanding of the nutrient requirements and uptake kinetics of seaweeds is needed when optimising agar or biomass production from tank-based mariculture systems. Together with detailed biochemical analyses, studies based on growth and uptake kinetics will enable more precise predictions regarding the possible effects of various sources of nutrients (both phosphorus and nitrogen) as well as effluent water on the growth of seaweeds maintained in tanks. This study has shown that the biochemical analysis for nitrogen containing metabolites is useful to indicate effects of different concentrations and pulses of nitrogen on *Gracilaria* spp. which is not always detected when production or growth rate is studied on its own. Similar experiments will be needed to assess the phosphorus requirements of *G. gracilis* in tank cultivation.

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