

Some aspects of the nitrogen nutrition and growth of
Gracilaria gracilis grown by suspended cultivation
in Saldanha Bay, South Africa.

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Abstract

For suspended cultivation of seaweeds in the open ocean, the selection of suitable sites and the optimization of cultivation methods for maximum productivity are of utmost importance. Growth of raft-cultivated *Gracilaria gracilis* in Saldanha Bay is known to be badly affected in late summer due to the persistence of warm oligotrophic water at the ocean surface which prevents replenishment of nutrients by upwelling. This study documents the extent of nitrogen starvation during this period and investigates some possible methods of managing this problem. Nitrogen content, C:N ratio and stable isotope abundances of C and N were obtained for material grown for varying lengths of time, material grown at various depths and material that had been fertilized prior to placement on the raft. The relationship between thallus colour and nitrogen content was also documented using a standard colour guide. The onset of nitrogen starvation was rapid, occurring after about two weeks on the raft. Growth was optimized about a meter below the water surface, possibly as a result of photoinhibition of photosynthesis at the surface. Nitrogen limitation ceased below about 2m growth depth. Prefertilization had no significant effect on yield after 4 weeks growth. Thallus colour showed a clear relationship with N content and may be a useful indicator of N status of cultivated *Gracilaria*. $\delta^{15}\text{N}$ was related to N content and increased rapidly when N limitation was removed. Stable isotope natural abundance measurements are a useful tool in the study of growth environment, but more controlled experimentation investigating the factors which influence these values is needed, particularly for macroalgae.

Introduction

Gracilaria is a commercially important red algal genus, many species of which are exploited for the production of the phycocolloid, agar (Critchley 1993). In recent years, the large and increasing international demand for *Gracilaria* as a raw material for agar production has necessitated a shift away from utilization of limited natural stocks towards cultivation of *Gracilaria* on a large scale (Oliveira *et al.* in press). Successful commercial *Gracilaria* farming ventures are currently established in many parts of the world including Chile, Venezuela, Namibia, Taiwan and China, and a wide variety of cultivation methods have been employed (Dawes 1995). These include intensive cultivation in tanks, cultivation in ponds and cultivation in the sea.

In recent years, cultivation in the open sea has gained much popularity as it allows for extensive production of *Gracilaria* without the necessity for constant monitoring and manipulation. It can be divided into two main techniques; bottom cultivation, where algae are planted directly onto the sea floor, and suspended cultivation, in which plants are suspended above the sea bottom on ropes or lines (Oliveira *et al.* in press). Commercially viable suspended cultivation has been, and continues to be employed successfully in countries such as Namibia and Venezuela. However, the additional costs of rafts and other farming infrastructure incurred by this method mean that, in order to be competitive, suspended cultivation must be more productive or produce better quality material than other, more cost-effective methods such as pond or bottom cultivation (Dawes 1995). Whilst this is possible, one of the drawbacks of cultivation in an open system is a lack of control over environmental conditions which may affect productivity (Oliveira *et al.* in press). Consequently, the selection of suitable sites for cultivation and the optimization of cultivation methods for maximum productivity are of utmost importance.

At Saldanha Bay on the west coast of South Africa, suspended cultivation of *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham on floated rafts is presently being investigated on a small scale with a view to future expansion of this

enterprise. Initial investigations into the potential of Saldanha Bay as a site for commercial farming have been encouraging with relative growth rates of over 4-5 % d⁻¹ occurring for most of the year (Anderson *et al.* 1996). However, researchers have identified several environmental constraints for mariculture in the area. Chief among these is a decreased availability of nutrients in the productive euphotic zone, which has been found to occur in Saldanha Bay during late summer. This is the result of southerly (onshore) winds or calm conditions which commonly persist for several weeks at a time during this period, maintaining a deep (5-10m) layer of warm oligotrophic water at the surface, and preventing the replenishment of surface nutrients by upwelling of cold nutrient rich water (R.J. Anderson pers. comm.). In the past, the persistence of these conditions for periods of a few weeks has been shown to drastically reduce growth of raft-cultivated *Gracilaria* (Anderson *et al.* 1996). This has been attributed to nitrogen limitation as a result of low nitrogen availability, and possibly, reduced nutrient uptake efficiency due to a lack of water movement resulting in the thickening of the boundary layer around the seaweed.

Although the nitrogen environment of *G. gracilis* in Saldanha Bay has been studied in detail (Smit 1998), the nitrogen status of raft grown material has, to date, not been directly assessed. The aim of the current experiment was to measure nitrogen contents and C:N ratios of raft-cultivated *Gracilaria* in late summer to assess the extent of N starvation during this period. Also investigated are:

The effect of depth on growth rate and nitrogen content

Previous investigations of optimum depth for *Gracilaria* growth have provided somewhat differing results. Experiments by Anderson *et al.* (1996) and Atkinson (1998) suggest that growth and yield of Saldanha *G. gracilis* is maximized when plants are suspended as close as possible to the surface of the water. Other researchers have reported reduced growth of *G. gracilis* very close to the water surface which may be the result of photoinhibition (Molloy 1992, Wakibia 1999). For the purposes of agar production, productivity is not the only consideration. Also important are agar content, which increases under nutrient limitation (N and P), and agar quality (gel strength) which commonly improves under nutrient replete conditions (Lewis and

Hanisak 1996). In the current study, growth rate and nitrogen content was measured in material grown along a gradient of depth in order to gauge the relative importance of light and nitrogen limitation at different depths.

The effect of prefertilization on long term yield

Because nutrient conditions often limit growth of *G.gracilis* on the rafts, it was considered that it may be possible to increase initial growth rate (and thus final yield) by prefertilizing stocking material in a nitrogen rich medium prior to placement on the rafts. This possibility is investigated here in light of the fact that *G.gracilis* has been found to be able to store sufficient nitrogen to grow for a week at maximum rates in nitrogen-free water (Smit *et al.* 1997).

The relationship between thallus N content and colour

It has been suggested that a lightening in the colour of *Gracilaria* is an indication of a lack of available nitrogen which has necessitated the utilization of pigment proteins (phycoerythrins) as a nitrogen source (Lapointe and Ryther 1979). The relationship between thallus colour and nitrogen content is documented using a standard colour guide. If a clearly observable relationship is found to occur, colour could be used to rapidly assess the nutrient status of seaweeds in cultivation.

Stable light isotope ratios of N and C in Gracilaria grown under different conditions

Natural abundances of stable carbon and nitrogen isotopes have been widely used by ecologists in the study of the uptake and assimilation of nutrients by marine microalgae (see Goericke *et al.* 1994, Owens & Watts 1998), but relatively little work has focused on macroalgae. At Saldanha Bay, two previous studies have used nitrogen isotopes to demonstrate the use, by macroalgae, of nitrogen derived from fish waste which is released periodically into the bay (Monteiro *et al.* 1998 for *Ulva lactuca*, Smit 1998 for *G. gracilis*). $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of algae may also provide information about physiological responses to the growth environment (e.g. light and nutrients)(e.g. Handely and Raven 1992, Goericke *et al.* 1994). In the present study, nitrogen and carbon isotopes were analysed in the hope that they would provide some insights into the growth environment of *G.gracilis*.

Study sites

Experimental material was collected from various sources between the 9/02 and the 7/04 1999. The majority of samples were collected at Saldanha Bay on the west coast of South Africa, about 100 km north of Cape Town (see Figure 1). Collection was from three different sources: an experimental raft at Small bay belonging to Marine and Coastal Management (A), a commercial raft in Big Bay, belonging to A.McLachlan (B) and a natural benthic population, occurring subtidally (depth \pm 5m) about 150 m off the northern shoreline in Small Bay. A few samples were also obtained at St Helena Bay, about 25 km north of Saldanha, from an experimental raft and the benthic population.

Methods

Cultivation system

All cultivated material used in our analyses was grown by suspended cultivation in the sea on rope rafts. Although material was collected from three different source rafts of different sizes, these were fundamentally similar. Rafts consist of rectangular frames of polypropylene rope suspended at 0.4m depth and buoyed with empty plastic 2l coldrink bottles attached at short intervals along the frame. In order to keep the frame taut and prevent it from moving, it is anchored at the four corners and in the middle to lateral risers which are attached with chains to a heavy anchor. Seaweeds are threaded through "netlon" lines using wire hooks (see Dawes 1995) Stocking weight is low (<30g) to prevent self-shading and minimize competition for nutrients. The netlon lines are then tied across the frames at 0.75m intervals, parallel to the line of the prevailing wave direction. For more detail on the methods of suspended cultivation, see Dawes (1995).

Water temperature

Surface water temperatures (0.5m depth) were measured on submersible electronic recorders on the commercial raft (B) for the period of 10/03 to the 07/04/1999 (Fig 2).

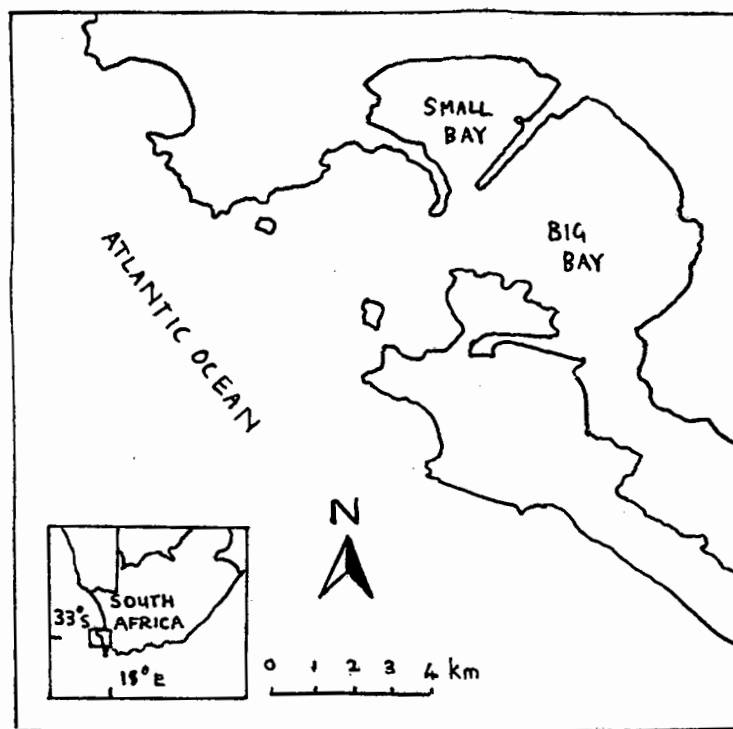


Figure 1: Map of Saldanha Bay showing the position of the Marine and Coastal management (A) and commercial (B) rafts.

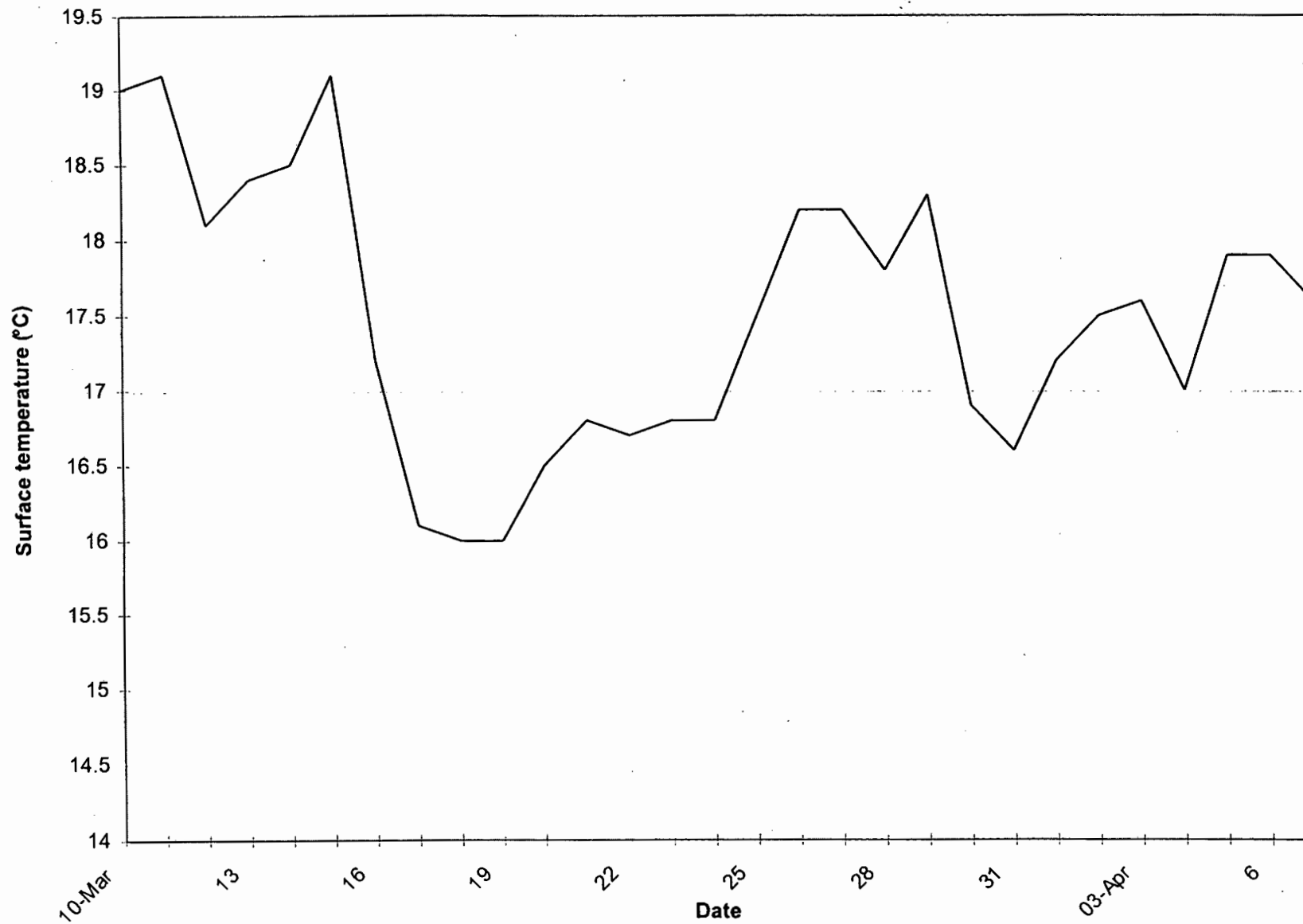


Figure 2: Average daily surface (0.5m) temperatures on the commercial raft for 10/03 to 07/04

Saldanha raft material

During late summer, a number of samples grown for various lengths of time on the commercial raft (B) were collected for analysis. In all cases, ropes were stocked initially with fresh benthic material from St Helena Bay. Material grown for 1, 2 and 4 weeks was harvested on the 9/2/99. Three samples were collected each from the 1 and 2 week old material, and six samples, three from the normal (paler) material and three from the darker material (which is trapped inside the netlons and not exposed to direct sunlight) were collected from the material grown for 4 weeks. On the 10/3/99, material grown for 5 and 6 weeks was harvested. As with the material grown for 4 weeks, 6 samples (3 paler and 3 darker) were collected for each of these growth periods.

Fertilization and depth tests

Fresh material from the Saldanha benthic population was used to set up a fertilization test on the commercial raft and a depth test on the MCM raft on the 10th March.

For the fertilization test, *Gracilaria* was allowed to soak for 20 hours in sea water fertilized with NH_4NO_3 (0.040g / l.) and $(\text{NH}_4)_2\text{SO}_4$ (0.032g / l). Four ropes were each stocked with 10 weighed tufts of between 20 and 25 g each (40 tufts in total) twenty of which were fertilized and the other twenty of which were not. Two samples each of the fertilized and unfertilized material were collected as controls before the ropes were placed on the rafts and plants were allowed to grow.

For the depth test, five ropes were stocked with approximately 25g tufts at 0.2m, 1.2m, 2.2m, 3.2 and 4.2m. These ropes were then suspended vertically from the commercial raft. After four weeks growth, samples were collected and weighed. Initial and final weights were used to calculate relative growth rate of seaweed using the formula:

$$\text{RGR} = \frac{100 \times \ln (W_f / W_i)}{n}$$

W_f = final weight,

W_i = initial weight

n = number of days

St Helena Bay material

On the 9/2/99 three samples of fresh benthic material were randomly collected from both Saldanha and St Helena bay for comparison. Three samples were also collected from the experimental raft at St Helena Bay on the 10/10/99.

Seaweed colour

Samples to be used in the analysis of the relationship between thallus colour and nitrogen content were collected from various different sources, usually in triplicate. The only criterion for use was that samples had been growing for at least two weeks so that their pigment contents had been allowed to acclimatize to the environment in which they had been placed. Samples in which colour was variable were separated into lighter and darker material. Colour of material was then quantified visually using the *Methuen Handbook of Colour* (Kornersrup and Wanscher 1984).

Isotope analysis and percentage C and N

All samples collected in the field were transported back to the lab in a cool-box, where large epiphytes and epifauna were removed as far as possible. Samples were then washed in a saline solution to remove small organisms such as isopods and diatoms, oven dried at 70° C and ground to a coarse texture using a pestle and mortar. At a subsequent date, samples were mechanically ground to a fine powder in a grinding mill.

Isotopic analysis was conducted at the UCT / FRD/ Goldfields Light Stable Isotope Facility. Quantities of finely ground material in the range of 1.8-2.2 mg were weighed, sealed in tin capsules and analysed on a Finnigan MAT 252 isotope ratio mass spectrometer with a Carlo-Erba NA 1500NC elemental analyser employed as a combustion unit. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios as well as total C and N percentage values were obtained simultaneously. Standards used were Merck gel and the laboratory reference gas was high purity nitrogen (99.995 %) calibrated against atmospheric N.

Results

Fertilization test

No significant differences in growth rate or N content were observable between fertilized and unfertilized plants, although a slight trend towards higher values in fertilized plants is apparent (Table 1). Analysis of percentage N content of the original fertilized and unfertilized material used to stock the rope indicated that the fertilized plants did have higher N contents (1.835 ± 0.09 compared to 1.4 ± 0.40), but because only two samples of each treatment were collected, the result was not significant. Isotope analysis of these samples also suggested that fertilized material was depleted in ^{15}N ($\delta^{15}\text{N} = 7.42 \pm 1.26$ compared to 9.075 ± 0.04) and enriched in ^{13}C ($\delta^{13}\text{C} = -13.72 \pm 0.30$ compared to $-14.12 \pm .060$), a trend which was still significant after 4 weeks of growth on the raft (Table 1).

Table 1: Growth, Nitrogen and isotope values for fertilized and unfertilized material grown for 4 weeks on the commercial raft at Saldanha Bay. n =10 except for RGR (n =20). Comparison of means is by Students t-test. Error values are 1S.D.

	RGR (%.day ⁻¹)	% N content	$\delta^{15}\text{N}$ (ppm)	$\delta^{13}\text{C}$ (ppm)
Fertilized	$6.462 \pm$	1.251 ± 0.27	9.498 ± 0.23	-10.078 ± 0.64
Unfertilized	$6.050 \pm$	1.217 ± 0.09	9.828 ± 0.38	-10.874 ± 0.58
PF vs UF	n.s.	n.s	p<0.05	p<0.05

n.s.= not significant

Raft Grown Material

Nitrogen contents and C:N ratios after varying lengths of growth on the raft are shown in Figures 3 and 4. Values for the stocking material for the 5 and 6 week material are included at 0 weeks. A clear decline in total N content of material is observed with increasing length of time on the raft. The decline is initially steep (between 0 and 2 weeks), becoming more gradual and apparently stabilising at about 5 weeks growth. C:N ratio shows the opposite trend to N, increasing as N limitation becomes more pronounced.

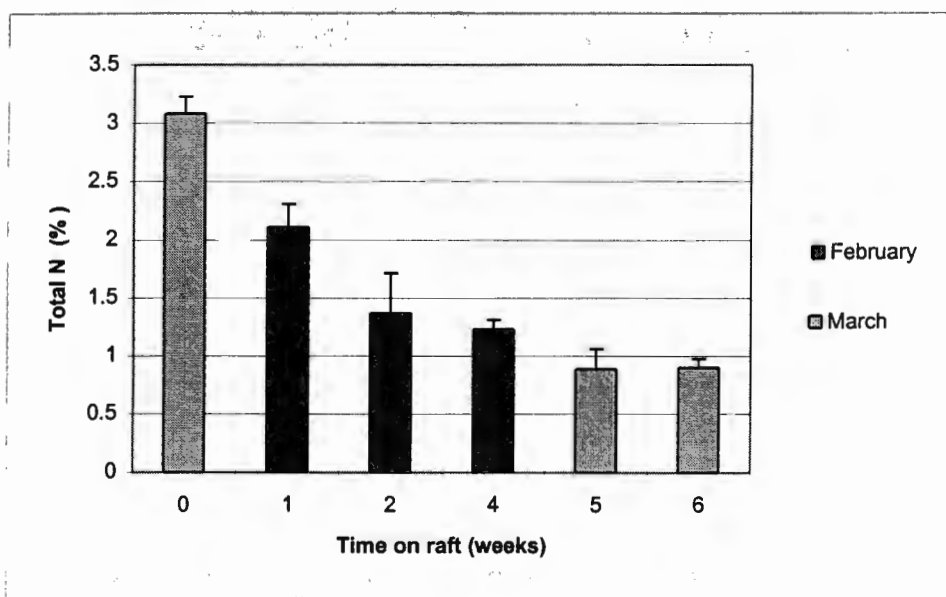


Figure 3: Thallus N content of plants grown for various lengths of time on the commercial raft in late summer (Feb-March). Material grown for 1,2 and 4 weeks was harvested on the 9 February, while material grown for 5 and 6 weeks was harvested on the 10 March. Error bars = + 1 SD

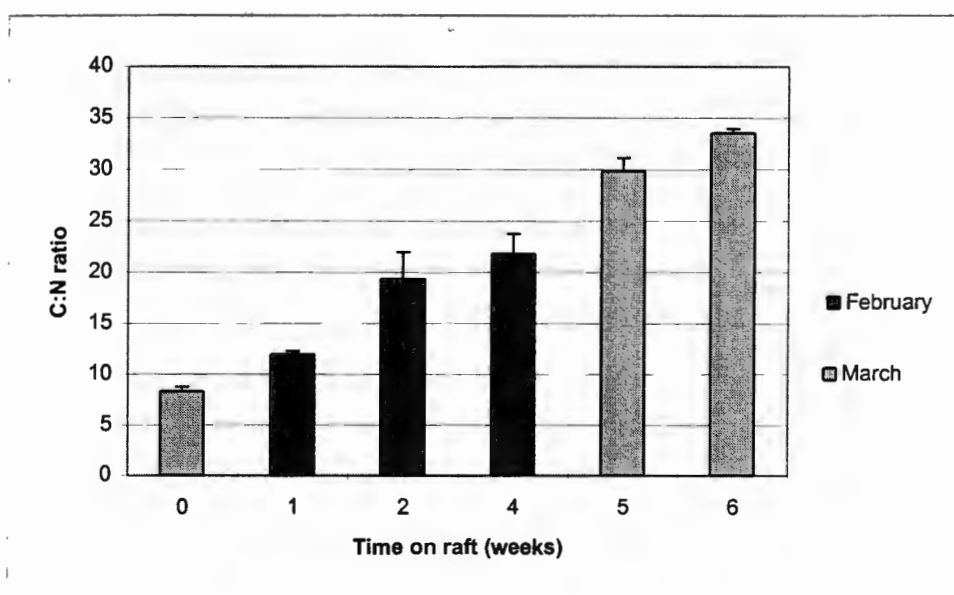


Figure 4. C:N ratio of plants grown for various lengths of time on the commercial raft in late summer (Feb-March). Material grown for 1,2 and 4 weeks was harvested on the 9/2/99, while material grown for 5 and 6 weeks was harvested on the 10/3/99. Error bars = + 1 SD

Total N values obtained from week 2 onwards are all well below the threshold value for nitrogen starvation (ca. 1.7%), reported by Smit *et al.* (1997). In contrast, average % N content of material grown on the St Helena Bay raft over the same period, was considerably higher than this value (Figure 5). It is apparent from Figure 5 that the Saldanha benthic population shows a similar degree of N starvation to the raft grown material, while the raft grown material and the benthic material from St Helena Bay is very N rich. $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (absolute) for Saldanha raft grown material are presented in Figure 6. No obvious trends are apparent, although $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ seem to track each other to some extent with $\delta^{15}\text{N}$ becoming more enriched when $\delta^{13}\text{C}$ becomes more depleted. The relationship between $\delta^{15}\text{N}$ and N content is shown in Figure 7. $\delta^{15}\text{N}$ increases with an increase in N content. The increase is initially rapid, becoming more gradual at higher N concentrations. $\delta^{13}\text{C}$ values did not show any apparent relationship with total N, C:N ratio or C content.

Nitrogen and thallus colour

Although the colour reproduction is not perfect, the broad relationship between thallus colour and nitrogen content is clear to see, with darker colours indicating more nitrogen rich material than paler colours (Figure 8). The transition between green-yellows and browns appears to occur somewhere between 0.8 and 1.6% total N and is indicated by bar 3F8. More experimentation would be needed to locate the precise range of N contents over which this transition takes place. Although not shown in colour, $\delta^{15}\text{N}$ also appears to show a good correlation with thallus colour (Figure 9), with the exception of bar 2C6, which represents material grown at the surface of the water (0.2m) in the depth test.

Depth test

Relative growth rates calculated for depth test material were highly variable for a given depth, a result which can be attributed in part, to a loss of material from the rope. However, a trend is still apparent (Figure 10). Growth was reduced at the surface (0.2m) relative to 1.2 m where growth was most rapid. Below this value, growth declined steadily with depth. Total N content was similar at 0.2m and 1.2m, increasing slowly but steadily with depth thereafter (Figure 11), while C:N ratio

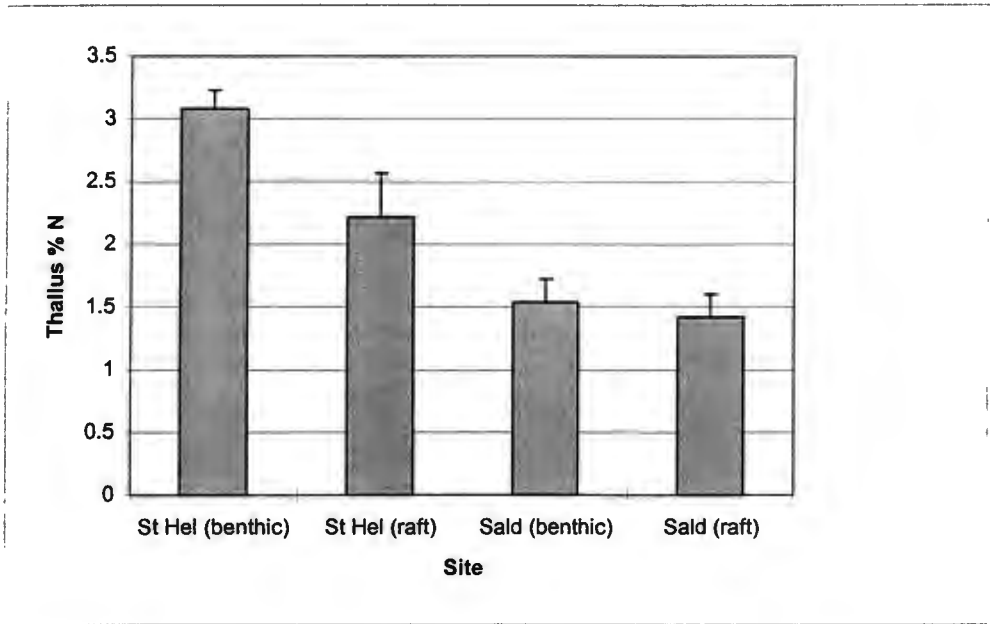


Figure 5: Thallus N contents of benthic and raft grown *G. gracilis* grown in St Helena Bay and Saldanha Bay during late summer. Raft material was grown for four weeks between 13/1 and 9/2/99. Error bars = ± 1 S.D

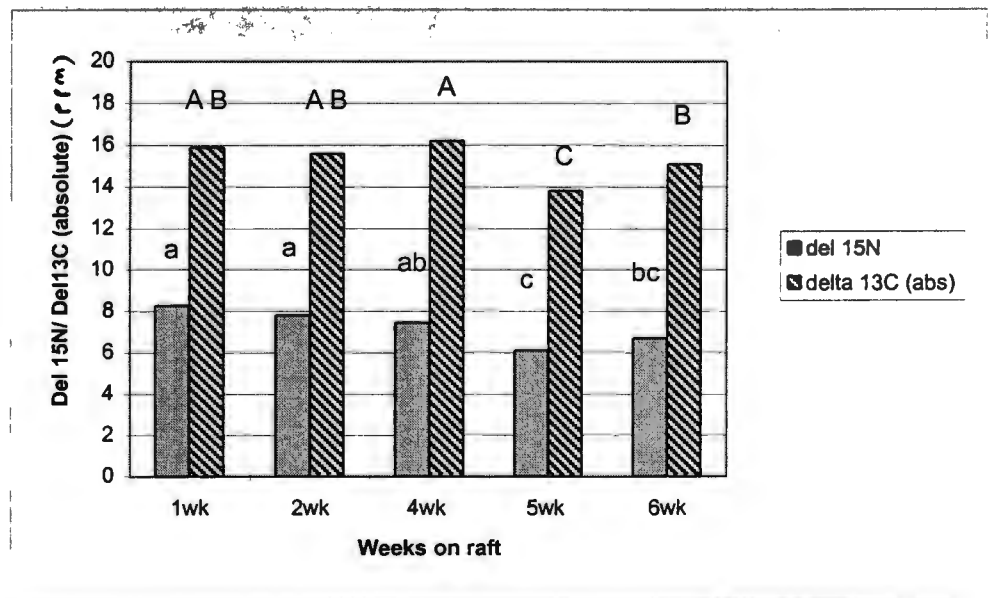


Figure 6: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (absolute) of material grown for various lengths of time on the commercial raft at Saldanha Bay in late summer (Feb-March). Material grown for 1,2 and 4 weeks was harvested on the 9/2/99, while material grown for 5 and 6 weeks was harvested on the 10/3/99. Different letters indicate significant differences at the 5 percent level (ANOVA and LSD post-hoc test)

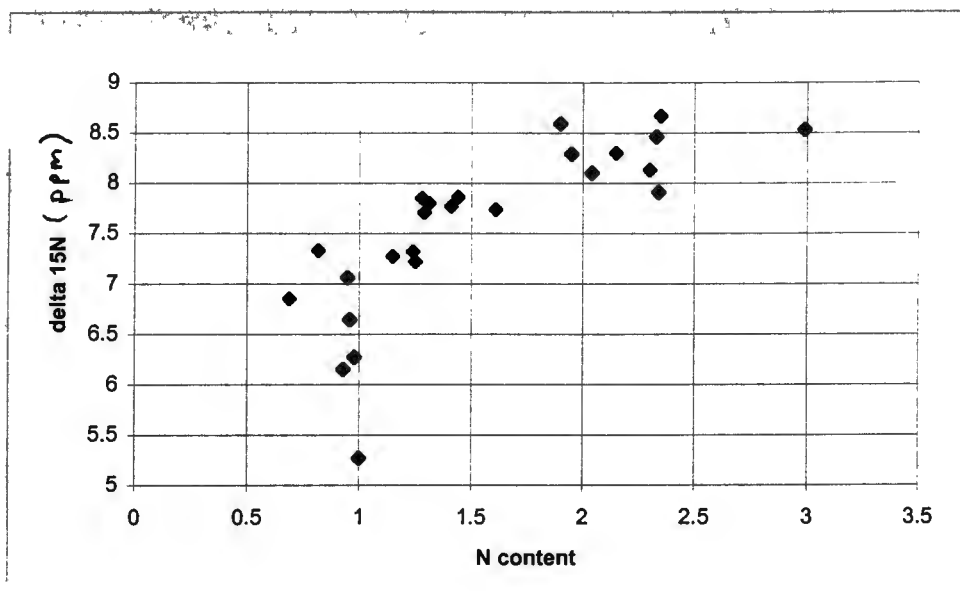


Figure 7: The relationship between $\delta^{15}\text{N}$ and total N for material grown on the commercial raft at Saldanha Bay in late summer

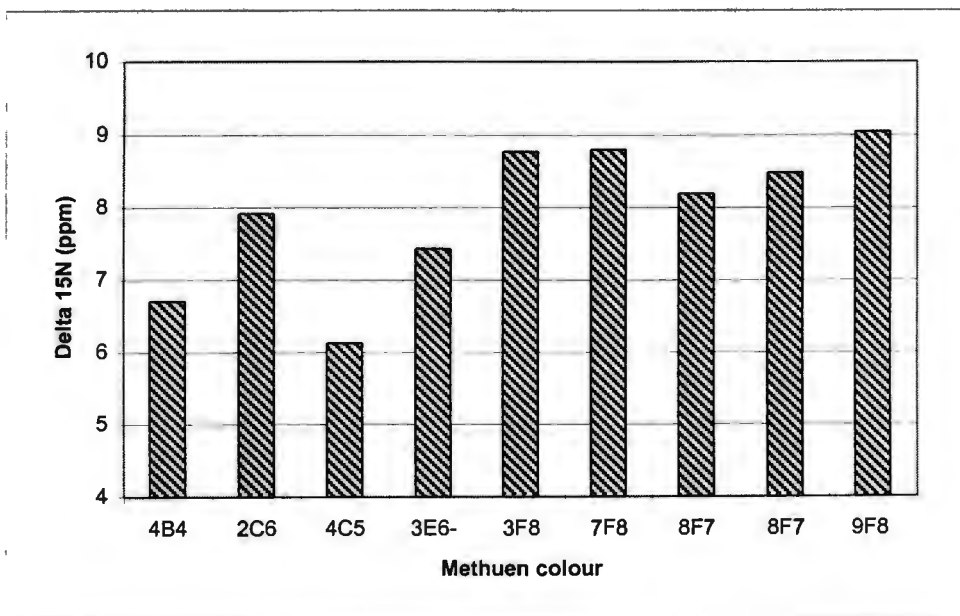


Figure 9: The relationship between thallus colour and $\delta^{15}\text{N}$ (‰). Samples are ranked by Methuen colour.

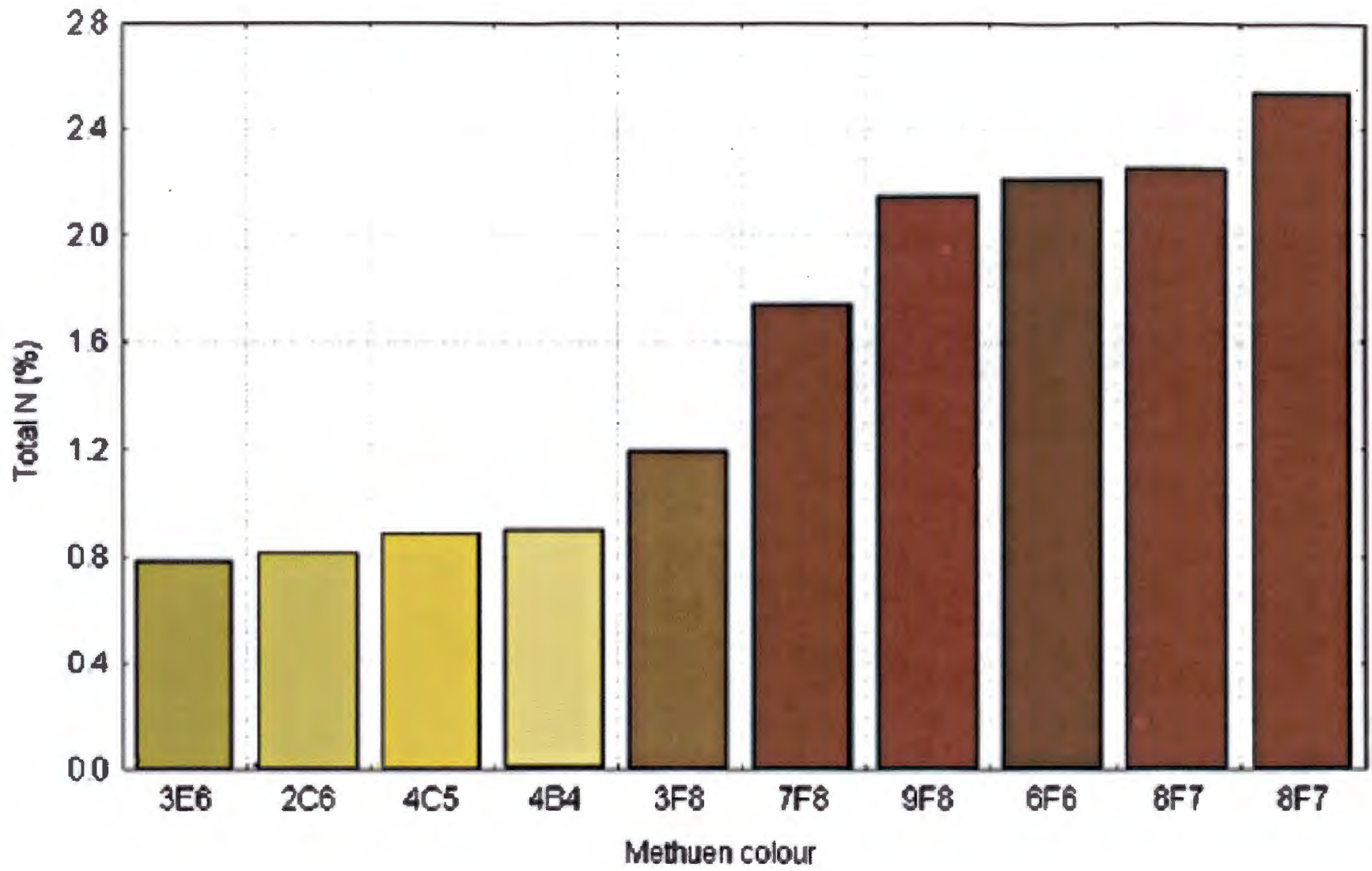


Figure 8: The relationship between thallus colour and total N content (%). Samples are ranked by N content and Methuen colour is indicated.

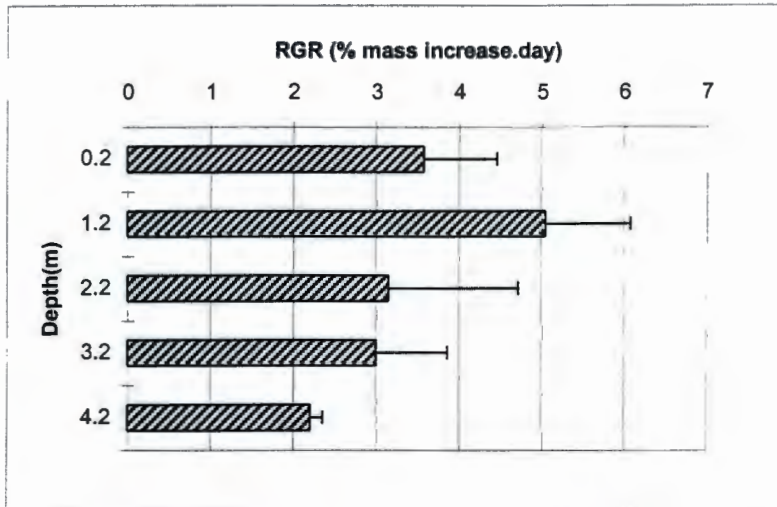


Figure 10: Relative growth rates (RGR) of plants grown at different depths. Error bars are 1 SD.

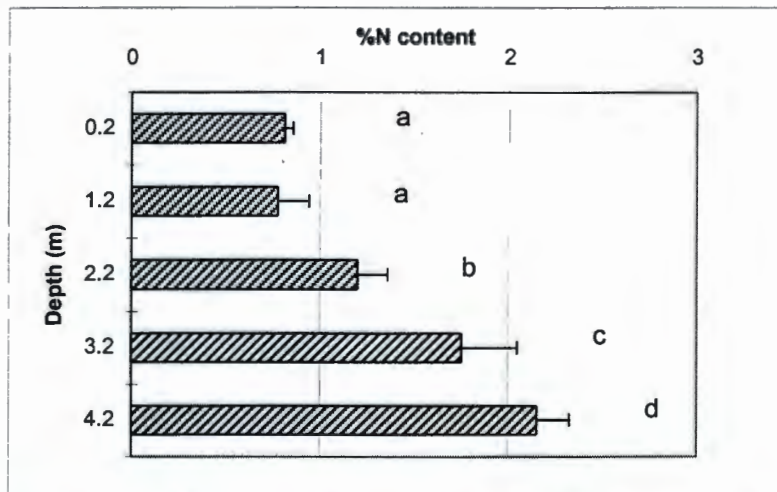


Figure 11: N content of material grown at different depths. Error bars are 1 SD. Different letters indicate significant differences at the 5 percent level (ANOVA and LSD post-hoc test)

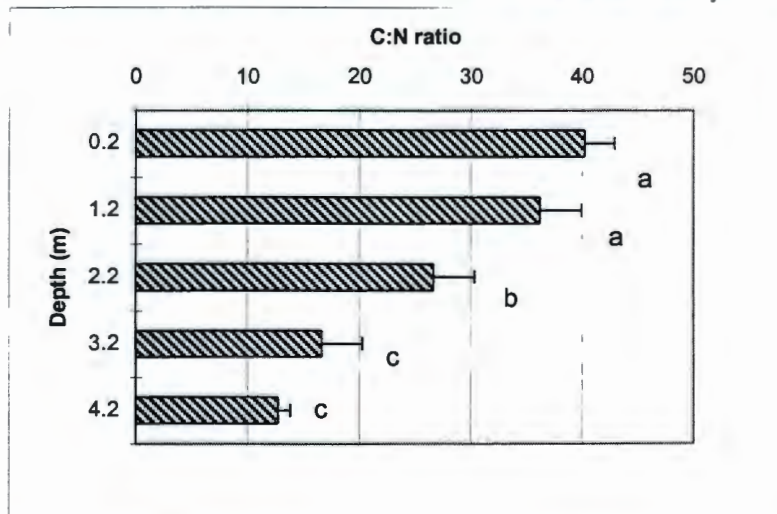


Figure 12: C:N ratio of material grown at different depths. Error bars are 1 SD. Different letters indicate significant differences at the 5 percent level (ANOVA and LSD post-hoc test)

showed a steady rise with increasing depth (Fig 12).

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values are shown in Figure 13. Material grown near the surface (0.2m and 1.2m) is significantly depleted in ^{15}N relative to deeper grown material. $\delta^{13}\text{C}$ shows a different response with material grown at 1.2m and 2.2m being significantly enriched in ^{13}C relative to surface grown and deep grown (3.2 and 4.2 m) material. $\delta^{15}\text{N}$ shows a similar relationship with total N to that observed in the raft grown material. At low N, a slight increase produces a large change in $\delta^{15}\text{N}$. At higher total N contents, $\delta^{15}\text{N}$ increases more gradually with increasing N (Figure 14). Mean $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ at a given depth are correlated with mean RGR ($R^2 = 0.88$ and 0.89 respectively). However, because RGR was very variable in part due to loss of material, all regressions of RGR use the mean for a given depth, making them less convincing due to a lack of data points.

Discussion

N content of raft grown material

It is clear from the results of this experiment that raft grown *G. gracilis* at Saldanha Bay (Big bay) experiences severe nitrogen limitation during late summer. In *G. tikvahiae*, growth becomes N-limited below about 2% total N content (Hanisak 1990, Lewis & Hanisak 1996) and controlled experiments conducted by Smit *et al.* (1997) suggest that the corresponding value for *G. gracilis* is around 1.7 %. In the current study conducted during late summer, all plants grown in Saldanha Bay for two weeks or more had total N contents well below either of these threshold values. Smit *et al.* (1997) grew nutrient replete *G. gracilis* in nitrogen-free sea water and observed a decrease in total-N content from about 2.3 to 1.7% over a three week period, with growth rate becoming N-limited after approx. 1 week. If anything, N contents of samples measured in the current experiment were reduced even more rapidly, decreasing from a value of 2.1 ± 0.2 to 1.37 ± 0.3 between 1 and 2 weeks. The increasing C:N ratios with time spent on the raft were for, the most part, a simple function of decreasing N content, as C content of samples varied very little.

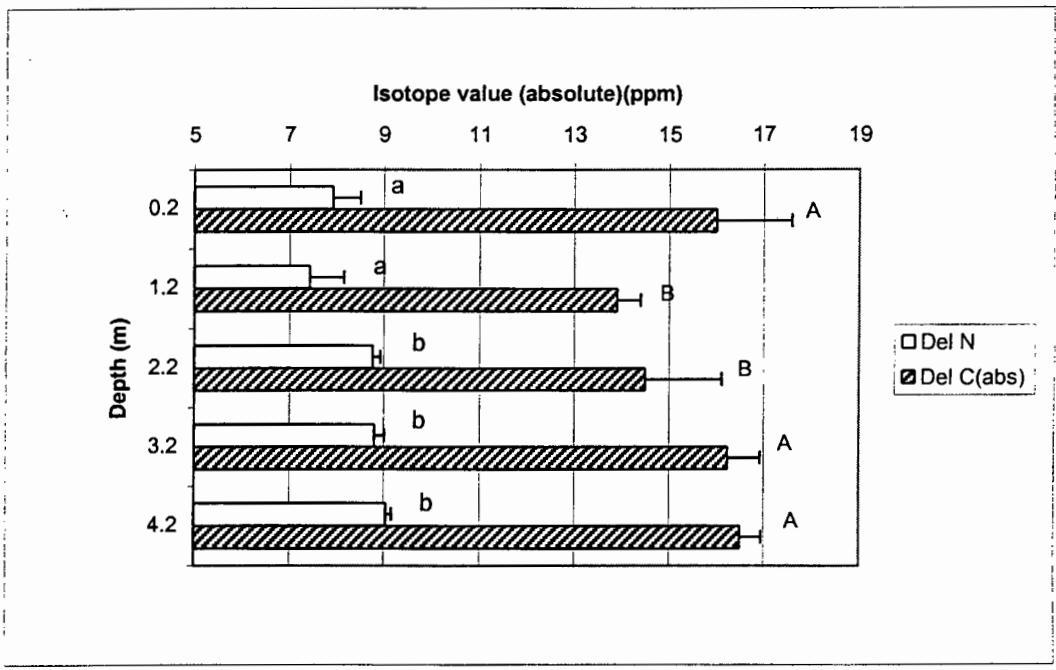


Figure 13: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (absolute) of material grown at different depths. Error bars are 1 SD. Different letters indicate significant differences at the 5 percent level (ANOVA and LSD post-hoc test)

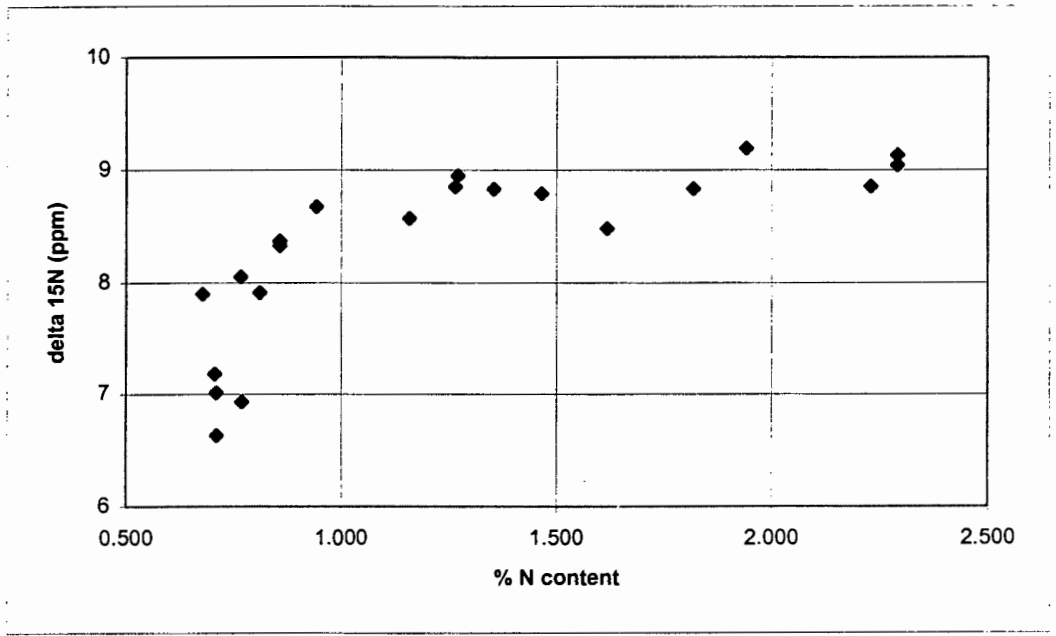


Figure 14: The relationship between $\delta^{15}\text{N}$ and total N for material grown at different depths.

N-limited growth over the experimental period can be linked to the thermal stratification which is a regular feature of Saldanha Bay for much of the ten month upwelling season (August to May). Stratification is particularly pronounced during the 3 month period from January to March, when our experiments were conducted. Because *G.gracilis* is able to store sufficient N for approximately a week of non N-limited growth (Smit *et al.* 1997), productivity of this species is affected if these oligotrophic conditions persist at the surface for periods longer than this. Our results indicate that this was the case for much of the experimental period. Water temperatures are a useful indicator of this persistence of this condition; temperatures above 17°C generally indicate oligotrophic conditions while those below 12°C indicate nutrient-rich water (Monteiro & Brundritt, unpublished results). Unfortunately, water temperature data for this period are not available to corroborate our observations. However, the generally high water temperatures for the period immediately following the harvesting of raft-grown material (Figure 2) indicate that temperatures above 17°C were probably the norm during the growth period, as stratification during March and April is, if anything, usually less pronounced than in January and February.

Growth is likely to be limited not only by the scarce supply of nutrients, but also by the lack of water movement during this period, which is needed to facilitate efficient uptake of nutrients (Anderson *et al.* 1996) and dissolved inorganic carbon (DIC) (Parker 1982). Lack of water movement may also result in reduced concentration of DIC in the surface water. Thus late summer is a problem period for *Gracilaria* cultivation at Saldanha Bay.

The very low N values obtained for the Saldanha benthic population on the 9/2 (Figure 3) indicate that N starvation is being experienced down to depths below 5m . In January 1993, large-scale die-off of natural beds at around 5m deep occurred (Anderson *et al.* 1996) presumably for this reason. The higher nitrogen content of St Helena Bay benthic material cannot be compared with that of the Saldanha Bay, as the St Helena population is deeper than its Saldanha Bay counterpart. However, although it would not be wise to make a statement based on values obtained for a single point in time, the high N content of material from the experimental raft at St Helena Bay,

suggests that this area may be more suited to *Gracilaria* cultivation during late summer. The relatively high nutrient levels and rapid growth rates obtained in a St Helena Bay pilot study by Wakibia (1999) support this suggestion. However, greatly reduced growth rates were observed during a period of increased water temperature around February, indicating that nutrient starvation may also be a factor in St Helena Bay at certain times of the year (Wakibia 1999).

Prefertilization test

Considering the fact that *G.gracilis* is only able to rely on stored N reserves for about a week of non-N-limited growth, it was perhaps unsurprising that prefertilization of stocking material did not provide any significant long-term growth benefits, although growth rates of fertilized plants were probably boosted initially. The fact that the isotope signature of the fertilizer was still significant after 4 weeks growth is interesting, and gives an indication of the low rates of N uptake and turnover during the experimental period.

Depth Test

Due to the highly variable growth rates and losses of material in the depth test, it would be unwise to place too much emphasis on the result. The question of optimum depth for cultivation is an interesting one, however. Growth rates would generally be expected to decrease with depth, as light becomes more and more limiting. Several experiments by Anderson *et al.* (1996) at Saldanha Bay, found growth rates of *G.gracilis* on rafts to be maximized when plants were grown very close to the surface (0.2m) with significantly reduced growth rates occurring at 0.5m or deeper. They attributed this result to increased nutrient exchange at the surface, facilitated by water movement. In contrast, several studies on *G.gracilis* have found growth rate to be negatively affected near the surface, with optimum growth rates being observed between 0.5 and 2m (Molloy 1992, Wakibia 1999). The explanation usually offered is that growth is limited by photoinhibition near the surface, due to the high levels of incident light received here. This seems conceivable, especially as *G.gracilis* is a sub-tidal species, not naturally adapted to high light levels.

Support for this explanation was provided by the finding of Molloy (1992) that the

optimum depth for growth rate varied according to season, with optimum growth occurring at deeper levels (around 1.2m) during the summer when irradiance levels were high, than in winter (around 0.5m). These findings agree very well with our study, which was also conducted in late summer, when irradiance levels are generally high. It is thus suggested that photoinhibition of photosynthesis in Saldanha *G.gracilis* may be a factor, particularly in summer. However, this is difficult to reconcile with the results obtained by Anderson *et al.* (1996) whose depth tests were also conducted at this time of year. One possible explanation that might warrant further investigation, is that growth response to surface irradiance levels varies according to the stocking material used. Perhaps material which has previously been acclimatized to high light levels is less susceptible to photoinhibition than the benthic material used in the current experiment.

Total nitrogen levels of seaweeds grown in the current experiment provide no evidence that nutrition (at least N nutrition) of raft-grown *Gracilaria* is improved at the surface due to water movement, as N content at 1.2m was not significantly different from that recorded at 0.2m. There are two main factors that are likely to contribute to the observed increase in N content with depth. Firstly, levels of available N, mainly in the form of NO_3^- may be expected to show a gradual increase with depth, as the concentration of nutrients supplied by entrainment through the thermocline increases. This factor is unlikely to be important in our investigation however, as entrainment is generally very minimal in late summer as discussed earlier. The primary explanation is likely to be related to luxury consumption and storage of N (Lapointe and Ryther 1979) by deeper growing, non-N-limited plants. Material grown below a certain threshold depth is limited by light availability before N becomes limiting. N uptake thus exceeds requirements for growth and can be stored in a number of different forms including tissue NO_3^- and phycobiliprotein pigments (Lapointe and Duke 1994, Lewis and Hanisak 1996). Material grown nearer the surface is subject to saturating light conditions such that N becomes the limiting factor for growth stored N needs to be utilized in order to maximize growth (Lapointe and Ryther 1979, Lapointe and Duke 1984). Growth rate of *Gracilaria* under light and nutrient limitation is thus a parabolic shaped function of C:N ratio (or N content)

(Lapointe and Duke 1994). Lapointe and Duke (1994) calculated for *G. tikvahiae* that the transition zone between light limitation and N limitation where growth was maximized was indicated by a C:N ratio of somewhere in the region of 13. Applying this to our C:N results, it would indicate that light limitation is only taking place at 4.2m and possibly 3.2m, while plants growing above this depth are N-limited. However, based on the observation that N content in our experiment is significantly increased at 2.2m, and assuming this is due to luxury consumption, it seems likely that this transition zone is indicated by a significantly higher C:N ratio in our experiment (in the region of 25 to 30). This is also supported by the fact that the fastest growth rate occurred at 1.2m.

Colour and N

Past studies have found that levels of pigment proteins are often closely correlated with N content (Bird *et al.* 1981, Lapointe and Ryther 1979). The strategy of increasing concentrations of pigment proteins at low levels of irradiance does not only allow *Gracilaria* to store excess N, but it also provides another benefit by increasing quantum efficiency (the efficiency with which the minimal light received is used) due to increased levels of light harvesting pigments (Lapointe and Ryther 1979). As pigment proteins such as phycoerythrin in red algae are largely responsible for determining the colour of seaweeds, changes in the concentrations of these pigments according to N availability cause lightening or darkening in seaweed colour. This relationship between seaweed colour and N content is often reported (Lewis and Hanisak 1996, Lapointe and Ryther 1979, Bird *et al.* 1981), however, to our knowledge no-one has ever tried to quantify the relationship as we have done here. The results suggest that seaweed colour could be used very effectively to assess nitrogen status of cultivated *G.gracilis* in the field. Naturally, there is some variation in colour for a given N content but this could be taken into account by analysis of a large number of samples so that a range of N contents could be calculated for a given colour. The small number of samples in the current experiment makes it impossible to pinpoint the range of N values over which the colour change from yellow-green to brown occurs. This is likely to be the most important region of the chart as a change to brown is presumably due to the production of phycoerythrins. If the production of

phycoerythrins occurs directly in response to the availability of N in excess of that needed for growth, then brown would indicate nutrient replete material and yellow or green would indicate nitrogen starved material.

Nitrogen isotopes

Our $\delta^{15}\text{N}$ results are interesting but interpretation is hindered by a lack of controls and treatments. In order to make confident statements about our data, the effects of nutrients and light on $\delta^{15}\text{N}$ would first need to be tested independently under controlled conditions. However, a number of possible explanations may be offered for the patterns observed in our data.

Nitrogen content is likely to be the most important factor accounting for variation in $\delta^{15}\text{N}$. This statement is strengthened by the fact that a similar relationship was observed between these two variables for both raft-grown and depth test material (Figures 7 and 14). Previous studies on marine phytoplankton have shown that, under light-limiting conditions, $\delta^{15}\text{N}$ of algae resembles that of the combined N source (Wada 1980 cited in Handely and Raven 1992). However, when nutrients are non-limiting, substantial fractionation of N may occur during uptake and assimilation. This seems to be the case with *G.gracilis*. In the depth test, the switch from nutrient-limited to non-nutrient-limited material between 1.2 and 2.2m corresponds with a large increase in $\delta^{15}\text{N}$ in the same interval. The rapid increase in $\delta^{15}\text{N}$ is even more apparent in the two plots of N against $\delta^{15}\text{N}$ (Figures ~~6~~⁷ and ~~12~~¹⁴), apparently occurring in the region of 0.7 to 1 % N. If this increase is an accurate indication of the disappearance of N-limitation, it would suggest that the threshold for N limitation is much lower than that reported by Smit *et al.* (1997), occurring in the region of 0.7 to 1 % total N. Interestingly, the switch in the colour of material from green-yellows to browns occurs over approximately the same range of N contents, due to luxury consumption and storage of N in the form of phycobiliproteins. Macko *et al.* (1987) found that $\delta^{15}\text{N}$ of protein in the brown macroalgae *Fucus* and *Ascophyllum* was approximately 3.2 to 3.9 ‰ higher than the value for total N. It is thus suggested that the increase in $\delta^{15}\text{N}$ when available N is in excess of that required for growth may be at least partly as a result of the storage of N in the form of protein. The initially rapid increase would be

caused by the sudden creation of protein with very different $\delta^{15}\text{N}$ and this increase would level out when total $\delta^{15}\text{N}$ reached a value close to that of protein $\delta^{15}\text{N}$, after which only a gradual increase would be observed with increasing N. This suggestion is supported by the relationship found between colour and $\delta^{15}\text{N}$ in the current experiment. However, in order to properly investigate this suggestion, determination of protein content and compound specific analysis of the protein component of *G.gracilis* would be required.

A second factor which might influence $\delta^{15}\text{N}$, but to a lesser extent than N content, is the source of dissolved organic nitrogen (DIN) utilized by the alga. Under natural conditions, the main sources of N used by algae are nitrate (NO_3^-) and ammonium (NH_4^+) (Hanisak 1990). The isotopic signature produced by the usage of these two different sources is influenced by two factors; the $\delta^{15}\text{N}$ of the source and the fractionation factor associated with the uptake of that source. Fractionation factors for NO_3^- and NH_4^+ uptake are poorly known for macroalgae (A.J. Smit pers. comm.). However, dissolved NH_4^+ is usually significantly enriched relative to NO_3^- (e.g. Cifuentes *et al.* 1989) and may thus be expected to produce a higher $\delta^{15}\text{N}$ value.

Within the genus *Gracilaria*, different species have been found to show preferences for either ammonium or nitrate (Hanisak 1990). In controlled experiments, Smit (1998) found that *G. gracilis* has a higher affinity for NO_3^- than NH_4^+ . In our depth test, a preference for one form of N over the other could have played a role in determining $\delta^{15}\text{N}$. Under N-limiting conditions, both forms of N would be expected to be utilized. However, when N was non-limiting (as indicated by darker thallus colour), *G.gracilis* may have taken up NO_3^- in preference to NH_4^+ , causing the resulting $\delta^{15}\text{N}$ values in nutrient replete material to be reduced.

Carbon isotopes

Because the current study is concerned primarily with nitrogen nutrition, $\delta^{13}\text{C}$ is not considered here in much detail. Interpretation of $\delta^{13}\text{C}$ results is made extremely difficult by the number of possible factors which may influence these values including light, nutrient availability, temperature and pH (Johnston and Kennedy 1998).

As discussed for nitrogen, utilization of different dissolved inorganic carbon (DIC) sources could also be responsible for some of the variation in $\delta^{13}\text{C}$. Whereas certain species are only able to utilize CO_2 as a carbon source, resulting in very negative $\delta^{13}\text{C}$ values in the region of -30 ‰ (Raven *et al.* 1995), *G. gracilis* has been shown to utilize both CO_2 and HCO_3^- producing average $\delta^{13}\text{C}$ values of about -15 to -17 ‰ (A.J. Smit pers. comm.). The $\delta^{13}\text{C}$ values measured in the current study range from about -10 to -17 ‰ which may indicate greater usage of the latter form. Preference for one of these sources may occur under non-limiting C concentrations and this might influence $\delta^{13}\text{C}$ values.

In marine algae, the primary cause of isotopic fractionation of C is photosynthetic fixation of dissolved inorganic carbon (DIC) (Raven *et al.* 1995). Thus, the main factor explaining $\delta^{13}\text{C}$ is often the rate of fixation which is related to growth rate (Goericke *et al.* 1994). Higher growth rates result in more fractionation of substrate, and higher $\delta^{13}\text{C}$ values. The correlation between RGR and $\delta^{13}\text{C}$ observed in the present study indicates that this is likely to be the case for *G. gracilis*. However, as with all our isotope data, further controlled experimentation would first be needed in order to accept or reject this hypothesis.

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