CHARACTERISATION OF NITROGEN STRESS RESPONSE GENES OF THE MARINE ALGA GRACILARIA GRACILIS

BY

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DECLARATION

I declare that this thesis is my own, unaided work. Experimental work discussed in this thesis was carried out under the supervision of Dr. V. Coyne of the Department of Molecular and Cell Biology, University of Cape Town.

Material presented here is all original work by the author and has not been submitted in this or any other form to another university. Where use has been made of research of others, it has been dully acknowledged in the text.

Signed by candidate

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December, 2003

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TABLE OF CONTENTS

ABSTRACT

Low environmental nutrient concentration is the main factor limiting natural production and success of *Gracilaria gracilis* cultivation in Saldanha Bay, South Africa and nitrogen is the single element of all the nutrients required by seaweeds that is most frequently limiting to growth. Biomass, relative growth rate, the concentration of nitrogen in the growth medium, the mean nitrogen uptake rate of the plant and the amount of nitrogen in the thallus were determined for nitrogen enriched and nitrogen deprived *G. gracilis* cultures grown in the laboratory. Knowledge of these factors is essential in order to gain a better understanding of the effect of nitrogen deprivation on *G. gracilis* growth and productivity. The relative growth rate and increase in biomass was always higher in *G. gracilis* cultured in nitrogen rich medium than in nitrogen deprived *G. gracilis*. However, a marked difference in growth rate between the two cultures only became apparent after 11 days of nitrogen starvation.

The ammonium that was present in the culture medium was consumed within the first 24 hrs of nitrogen starvation. Nitrate nitrogen levels increased for the first day, after which a marked reduction was observed until day 7 of nitrogen starvation, remaining extremely low thereafter. However, *G. gracilis* continued to grow using nitrogen stored in the tissues. The tissue C:N ratio began to increase exponentially after day 4 of nitrogen starvation indicating that consumption of thallus pigment as a nitrogen source had begun. Low percentage nitrogen, very high C:N values and an extremely low growth rate

indicated that *G. gracilis* cultured in the nitrogen depleted growth medium had become significantly nitrogen limited from day 11 onward.

Differential screening analysis was employed to determine the presence of differentially expressed genes in response to nitrogen deprivation in *G. gracilis*. Twenty seven upregulated cDNA fragments were identified and sequenced. Twenty two cDNA fragments were identical and displayed 90% homology to the amino acid sequence of an expressed sequence tag from *G. gracilis*. Analysis of the other cDNA fragments revealed sequence similarity at the amino acid level to an aspartate aminotransferase, a hypothetical protein from the red alga *Chondrus crispus* and an expressed sequence tag from another red alga *Porphyra yezoensis*. Two of the cDNA fragments possessed homology to proteins which are involved in the stress response of terrestrial plants following pathogen infection, drought, cold and dehydration stress.

Northern hybridisation analysis was performed to confirm that the cDNA fragments are truly upregulated in response to nitrogen deprivation. The three cDNA fragments that were tested only hybridised to mRNA isolated from *G. gracilis* deprived of nitrogen for 18 days, verifying that the cDNA fragments identified by differential screening do indeed represent genes that are up regulated in response to nitrogen starvation.

CHAPTER 1

INTRODUCTION

CONTENTS

Chapter 1

INTRODUCTION

1.1 BIOLOGY OF GRACILARIA

Red algae (Rhodophyta), as the name suggests, are characterised primarily by a rosy, purplish or reddish brown colour, attributable to the presence of the bilipigments phycocyanin, phycoerythrin and allophycocyanin. The basic classification of algae is done based on pigmentation, storage products, mode of reproduction and life history and the presence or absence of flagellate stages. Bold and Wynne (1985) noted that the division Rhodophyta could be distinguished from the other groups of macroalgae by the following combination of characteristics:

(i) the complete absence of flagellate states;

(ii) the presence of accessory photosynthetic pigments called phycobillins (phycoerythrin and phycocyanin);

(iii) the occurrence of non-aggregated photosynthetic lamellae or thylakoids within the chloroplasts;

(iv) floridean starch as a food reserve;

The red coloration of these plants is due to phycoerythrin that masks the green colour of chlorophyll a – the major photosynthetic pigment present in rhodophytes. Photo destruction of phycoerythrin may cause red algae to exhibit a wide range of colours including violet, brownish, yellow and greenish (Bold and Wynne, 1985).

Like all algae, *Gracilaria* species have a relatively simple organisation of plant bodies compared to terrestrial plants. Though multicellular, these macroalgae do not have

differentiated true leaf, stem and root structures. The family Gracilariaceae encompasses some of the world's most valuable agarophytes and accordingly has been the subject of much research in recent decades (Bird, 1995). *Gracilaria gracilis* and *Gracilariopsis longissima* to a lesser extent are already commercially utilised in South Africa, but many other species are potential targets for commercial exploitation (Anderson *et al.*, 1989; Wakibia *et al.*, 2001).

Oliveira and Plastino (1994) described the genus *Gracilaria* as, "Plants macroscopic, with thallus terete, compressed or flattened to foliose, branching dichotomous, alternate to irregular, little to extensively branched, structurally composed of a solid pseudo parenchyma, with large medullary cells and a gradual or sharp transition to smaller subcortical and cortical cells". The taxonomic position of the genus *Gracilaria* is (Bird and Kain, 1995):

Division	Rhodophyta
Class	Florideophycea
Order	Gracilariales
Family	Gracilariaceae

1.2 REPRODUCTION AND LIFE HISTORY

Both sexual and asexual modes of reproduction occur in most red algae. Asexual reproduction in some *Gracilaria* species involves the regeneration of a new thallus from a stipe fragment. This is a very important characteristic for farming seaweeds. Sexual reproduction (Fig 1.1) involves a non-motile male gamete (spermatia) and specialised female cells (carpogonia). Successful fertilisation results in the formation of a cystocarp. Carpospores are released and germinate into diploid phase

tetrasporophytes. Mature tetrasporophytes produce tetrasporangia that in turn result in tetraspores following meiosis (Bold and Wynne, 1985).

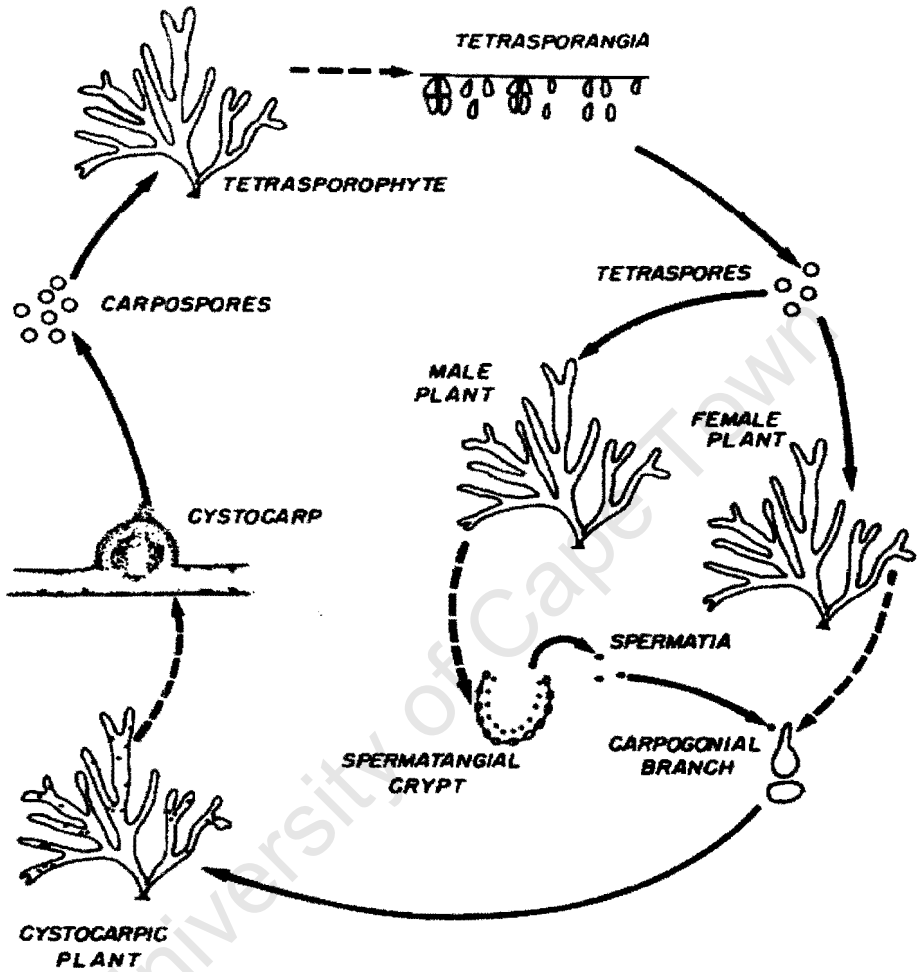


Fig. 1.1 A diagram of a *Polysiphonia*-type life history as reported in most species of *Gracilaria* (Oliveira and Plastino, 1994).

1.3 HABITAT AND DISTRIBUTION

Red algae are often more abundant in deeper water than other seaweeds (Darley, 1982). No other photosynthetic organism has been found living at greater depths than the red algae (Hoek *et al.*, 1995). The major environmental factor limiting the distribution within these deep waters is turbidity, which determines photosynthetic ability. *Gracilaria* species are generally found attached to rocks or other solid substrata by a holdfast. Some species also grow in sandy areas (Rotmann, 1990). In South Africa red algae are found from mid tide to below the low tide mark (Chapman, 1970).

1.4 ENVIRONMENTAL FACTORS AFFECTING GRACILARIA GROWTH

Light is an important factor for growth in that it provides the initial energy for photosynthesis. It is also used as a signal for life processes such as reproduction and growth during the life cycle of seaweeds (Lobban, *et al.*, 1985). The ability of a plant to change its pigment ratios in order to maximise the absorption of light of a given wavelength is called complementary chromatic adaptation. Red algae are more adapted to the blue green light dominated, deeper, marine environment than other seaweeds. This is because they have the accessory pigment phycoerythrin that enhances the photosynthetic capacity in this low light environment (Darley, 1982). However, there is no enough experimental evidence to support the complementary chromatic adaptation and it is even argued that it be discarded (Dring, 1982, Gantt, 1990).

Metabolism in macroalgae is greatly influenced by temperature. Temperature is the major physical factor that determines latitudinal and seasonal distribution of seaweeds. As intertidal organisms, seaweeds have to adapt to the daily variation of temperature. High temperature tolerance limits are generally about 27-35°C (Darley, 1982).

Nitrogen is the single element of all the nutrients required by seaweeds that is most frequently limiting to growth. According to Lobban *et al.* (1985) the five major sources of nitrogen in the marine environment are:

(i) Nitrogen fixation by bacteria and blue green algae

(ii) Ammonium from bacterial decomposition

(iii) Deep water nitrates brought to the surface by physical advection

(iv) Atmospheric input of ammonia through rain

(v) Nitrogen input from sewage and fertilisers to coastal waters

Seaweeds are unable to use nitrogen gas that is abundantly present in the sea. Nitrate and ammonium are the two forms of nitrogen that can be used by most algal species. Deboer *et al.* (1978) have shown that ammonium yielded higher production than nitrate in some *Gracilaria* species. Light, water motion, age of plant and its nutritional history are some of the factors that determine the uptake rate of any nutrient. Salinity, desiccation, water motion and pollution are also important environmental factors affecting growth of *Gracilaria* species.

1.5 SIGNIFICANCE OF GRACILARIA

1.5.1 Ecological importance

As photosynthetic organisms seaweeds significantly contribute to the total primary productivity in marine waters. According to Harlin and Darley (1988) algal communities in oceans are more productive than any terrestrial community. Seaweeds are also ecologically important in that they provide food and suitable habitat for many organisms that need protection from waves, extreme heat and predators.

Seaweeds can also serve as efficient bio-indicators. Bio-indicators are organisms in the environment that infer, through changes in their biological systems, the conditions experienced in that environment (Wilson, 1994). *Gracilaria* species are particularly useful in this respect in that they have the ability to withstand large fluctuations of environmental factors such as light, temperature and salinity (Bird *et al.*, 1979). This characteristic can be important in coastal ecosystems with high effluents from aquaculture activities or that are impacted by other anthropogenically-derived nutrients. Not only are *Gracilaria* species used to assess water quality, but are useful in improving it as well. Significant reduction in nutrient levels can be achieved by stocking macroalgae in aquaculture ponds. In fact using plants such as seaweeds as natural biofilters is now considered as one of the few potential cost effective methods of domestic, agricultural and industrial effluent treatment (Jones *et al.*, 2002). Troell *et al.* (1997) estimated that 80% of the total nutrient losses from fish farms can be used by plants. Therefore integration of seaweeds with fish farms can reduce the negative impacts of eutrophication.

1.5.2 Commercial importance

For thousands of years seaweeds have been used both as animal and human food. As food supplements, macroalgae significantly improve yields from animals. It is even claimed that seaweed meals improve fertility and birth rate of animals (Chapman and Chapman, 1980). As human food, seaweeds are good sources of carbohydrates and proteins. They are also rich in vitamins and essential trace elements (Chapman and Chapman, 1980).

The most important economic use of *Gracilaria* species however, is in the agar industry. The most widely known application of agar is as a medium to culture microorganisms. Even though agar is also extracted from two other red algae (*Gelidium* and *Pterocladia* species.), agar from *Gracilaria* species has some characteristics that differentiate it from the agar obtained from *Gelidium* and *Pterocladia* species. In addition, these species do not grow as fast and as easily as *Gracilaria* when farmed (Abbot, 1988). Commercial applications of agar depend on its ability to gel, emulsify and stabilise. In the food sector, the ability of agar from *Gracilaria* species to combine with sugars and not lose gel strength makes it among the most expensive agars (Abbot, 1996). Agar can also be used in bakery, adhesives, cosmetics, textile dyeing and paper coating. Medicinally it can be applied in laxatives, ingredient for tablets and capsules and in radiological solutions as a suspending agent and anti-coagulants (Lewis *et al.*, 1988). Agar extracted from South African *Gracilaria* species serves extremely well in the manufacture of sweets (Chapman and Chapman, 1980).

1.6 STRESS RESPONSE IN PLANTS

1.6 1 Stress and stress response

Throughout their life time plants are subjected to a wide variety of environmental conditions, either favourable or adverse. Stress in living organisms can be defined as any external environmental factor capable of inducing a potentially harmful strain. A strain is any structural or physiological adjustment that results due to a stress. The latter involves a shift in metabolism that enables organisms to cope with the stress conditions. Levitt (1980) noted that stress acting on a plant could produce injuries in three different ways (Fig 1.2):

(i) Direct stress injury: An injury characterised by its speedy and irreversible appearance, induced by a stress that is beyond the endurance limit of the plant. It usually leads to instant death of cells. A sudden extremely low temperature that freezes protoplasm and produces loss of permeability can be a good example.

(ii) Indirect stress injury: This is not beyond the limit of the plant. But long exposure may result in changes in physical and chemical processes in the plant cell. Low temperatures that decrease the metabolic rate of the plant cell is an indirect stress.

(iii) Secondary stress injury: An injury produced by a strain that is the result of another stress. Desiccation stress due to high temperatures is an example of secondary stress injury.

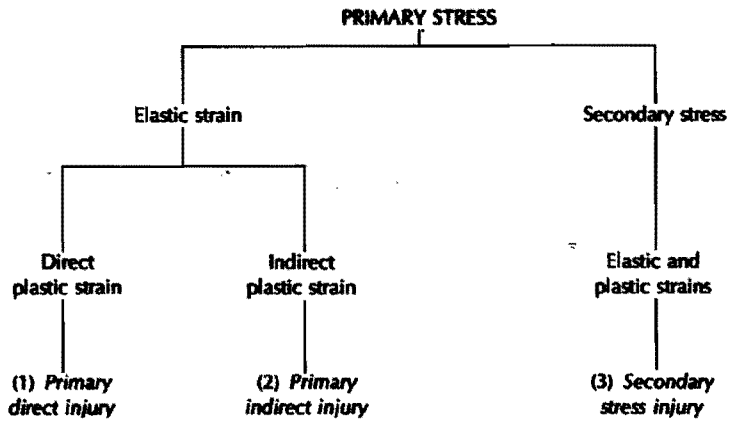


Fig. 1.2 Different kinds of injury produced in response to stress in plants (Levitt, 1980)

1.6.2 Stress Resistance

In response to the different stresses, plants show a wide range of stress resistance behaviour ranging from physiological processes to biochemical adjustments that involve production of new proteins. Levitt (1980) defined and outlined strategies of plant resistance to different types of stresses.

Up to a certain point a stress produces a completely reversible physical or chemical change. Such a strain is termed an elastic strain. However if a strain is irreversible, then it is referred to as a plastic strain. Two other important terms are avoidance and tolerance. Stress avoidance is stress resistance through the prevention of the penetration of the stress into tissues, whereas stress tolerance is decreasing or eliminating the strain in spite of allowing the stress to enter into tissues. Stress resistance strategies of plants are outlined in Fig. 1.3.

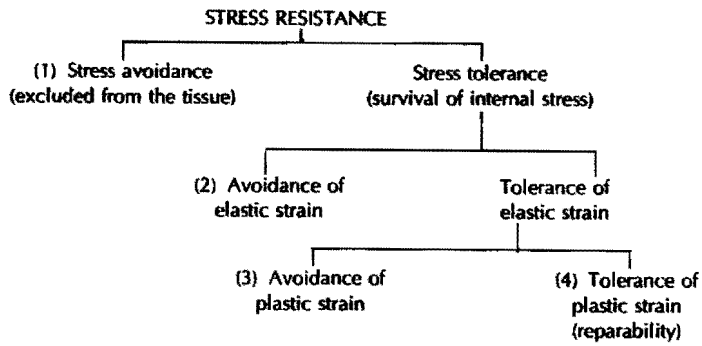


Fig. 1.3 Summary of possible mechanisms of resistance exhibited by plants in response to stress (Levitt, 1980)

Some of these stress resistance strategies may involve changes in gene expression and new protein synthesis. The following factors are important to understand changes in gene expression due to stressful conditions:

(i) How is the presence of stress recognised by the plant cell?

(ii) What genes are expressed?

(iii) What is the function of the genes expressed?

Plant cells recognise stress signals and transmit them in order to activate adaptive responses through signal transduction. This signalling system is complex in plants. In understanding signal transduction, it is important to identify which signalling pathway is followed when a cell is subjected to a specific stress. This is particularly difficult in plant cells because often a cell uses the same signalling system to respond to more than one type of stress. In fact Morgan (1979) suggested that there could be a universal gene set that is induced by most abiotic stresses. The absence of reliable phenotypes specific to the various environmental stresses further complicates the

studies (Zhu and Xiong, 2001). Several plant hormones such as abscisic acid (ABA), cytokinins (CKs), gibberellin (GA) and indole acetic acid (IAA) are involved in the signalling processes. Increased ABA levels and a decline in CKs (Table. 1.1) was observed in several nitrogen deficient plants (Morgan, 1979). Hormonal signals are then detected by specific receptors such as receptor like kinases (RLKs), two component histidine kinases and G-protein associated receptors. When activated, these receptors initiate or suppress a cascade to intracellularly transmit the signals they received. In this way they activate nuclear transcription factors to induce the expression of specific sets of genes (Zhu and Xiong, 2001). Morgan (1979) however suggested that plant hormones could directly induce protein synthesis with out receptors involved. As shown in Fig. 1.4, acclimation of a plant to stress via these processes may involve biochemical, functional or structural changes.

Table 1.1 Summary of effects of abiotic stresses on levels of several plant hormones (Morgan, 1979).

	Drought	Salt	Heat	Cold	Chilling	Low N/P
ABA	↑	↑	↑	↑	↑	↑
CKs	↓	↓	↓			↓
GA	↓			↓		
IAA	↓					

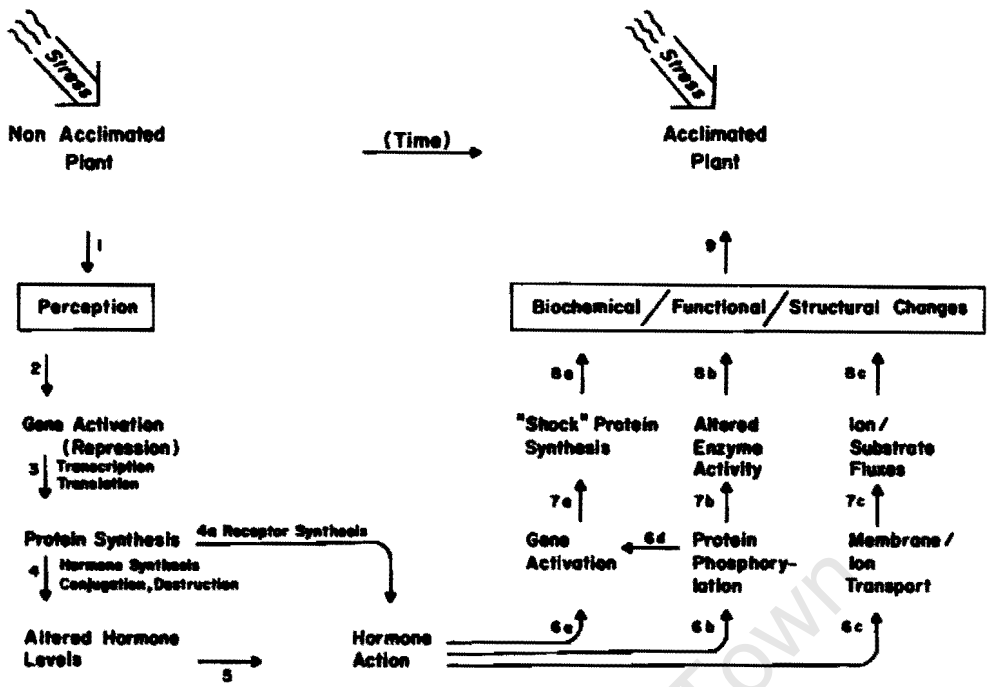


Fig. 1.4 Proposed model for role of plant hormones in acclimation to stress (Morgan, 1979).

1.7 NITROGEN ASSIMILATION PATHWAY IN GRACILARIA

The two most important forms of nitrogen available to seaweeds are nitrate and ammonium. For nitrate to be used by a plant tissue, it should first be reduced to ammonium. However when the rate of nitrate uptake is higher than the rate of nitrate reduction by nitrate reductase, nitrate is accumulated inside the plant cell. Plants differ in the amount of nitrate they can accumulate depending on species, age, time of the day and season (Hewitt and Smith, 1974).

Inokuchi *et al.* (2002) detailed the nitrogen assimilation pathway in macroalgae.

The first step of biochemical nitrogen assimilation in seaweeds is reduction of nitrate to nitrite catalysed by the enzyme nitrate reductase. Nitrate reductase is located in the cytosol of macroalgae. The reaction can be summarised as:

$$NO_3 + NAD(P)H + H^+ \rightarrow NO_2 + NAD(P)^+ + H_2O$$

Nitrite is then reduced to ammonium. Nitrite reductase is the enzyme that catalyses this reaction. In most plants nitrite reduction takes place in chloroplasts.

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$$

For a long time it was believed that glutamate is the first and direct product of ammonium assimilation via a single glutamate dehydrogenase catalysed reaction. However Lobban *et al.* (1985) have indicated that glutamine is the first product. Glutamine synthetase converts ammonium and glutamate to glutamine and the enzyme glutamate dehydrogenase does not have a major role in nitrogen assimilation.

$$\text{Glutamate} + NH_4 + ATP \rightarrow \text{Glutamine} + ADP + Pi + H_2O$$

Glutamate synthetase then transfers glutamine and 2-oxoglutarate (2-OG) to two molecules of glutamate.

$$\text{Glutamine} + \text{2-OG} + NAD(P)H \rightarrow 2\ \text{Glutamate} + NAD(P)^+$$

The synthesised glutamate can be used either as a source of glutamate for subsequent glutamine synthesis by glutamine synthetase or to donate its amino group to form aspartate. Aspartate aminotransferase catalyses the transamination reaction that produces aspartate.

$$\text{Glutamate} + \text{Oxaloacetate} \leftrightarrow \text{Aspartate} + \text{2-OG}$$

Asparagine is formed by the transfer of an amide group from glutamine by the enzyme asparagine synthetase.

$$\text{Glutamine} + \text{Aspartate} + \text{ATP} \leftrightarrow \text{Glutamate} + \text{Asparagine} + \text{AMP} + \text{PPi}$$

Glutamine, glutamate and aspartate produced by the above reactions are used as starting materials for the synthesis of organic compounds that contain nitrogen such as amino acids, nucleotides and chlorophylls. Some of the compounds used in the reactions (such as oxaloacetate) and ATP are generated from the tricarboxylic acid (TCA) cycle. This shows that nitrogen and carbon metabolism is co-ordinated. Nitrogen assimilation uses upto 55 % of the net plant carbon.

Apart from their role as nutrients essential for growth and development, nitrate and ammonium are involved as signaling molecules during stress. An important regulatory gene, ANR1, that is responsible for morphological response during stress in plants for example is nitrate induced (Zhang and Forde, 1998). Accumulation of mRNA of a number of carbon and nitrogen metabolic genes was also shown to be a result of nitrate functioning as a signal (Coruzzi and Bush, 2001).

1.8 SALDANHA BAY

In the past the only place that yielded commercial quantities of *Gracilaria* in South Africa was Saldanha Bay (Anderson *et al.*, 1989). *Gracilaria gracilis* in this area is found cast up on beaches and sand banks or in deeper waters (Isaac, 1956). The biomass varies considerably in relation to heavy winds (Isaac, 1956). In summer (October-March) southeasterly winds bring about upwelling. This causes stratification where the warm (17-20°C) surface layer lies on top of a colder (10-12°C) bottom layer. The amount of nutrients in the surface water is extremely low. Nitrate nitrogen levels may be too low to detect at these times, while the bottom nutrient rich water may have as much as 20 μM nitrate and more than 3 μM phosphate (Anderson *et al*, 1996a). During winter, northerly winds de-stratify the water column causing it to become nutrient rich and uniformly cold with temperatures ranging from 10 to 14 °C (Anderson *et al*, 1996b).

The harvest of *G. gracilis* for industrial purposes in Saldanha Bay began just after the Second World War and was highly successful by the early 1970s. In 1973 for example, revenue of about one million rands was earned from the *G. gracilis* industry (Simons, 1977).

During 1974 an ore jetty and breakwater was constructed that divided Saldanha Bay into Small Bay and Big Bay. This resulted in changes in the physico-chemical dynamics of the bay. Since then, the growth of *G. gracilis* has been unstable and the average *G. gracilis* yield in 1988-1991 was lower by a factor of about 50 than the average yield before the construction of the jetty and breakwater (Simons, 1977). In

the late 1980s and again in 1996, a total collapse of the resource with no beach wash up was observed. Three possible reasons were postulated for the collapse:

(i) Severe grazing by numerous invertebrates and fish (Anderson *et al.*, 1993).

(ii) In the absence of utilizable carbon sources in the water column during summer, some epiphytic bacterial pathogens may metabolise the agar in the *Gracilaria* cell wall by producing agarase enzymes. This results in thallus bleaching and death of the algae (Jaffray and Coyne, 1996; Schroeder *et al*, 2003).

(iii) Prolonged extremely low nutrient levels with unusually windless conditions during summer may starve the *G. gracilis* plants of nitrogen and lead to poor growth and eventual death (Anderson *et al.*, 1996b).

In the past, Saldanha Bay has attracted attention as a potential site for a *G. gracilis* farming and agar industry. Even though two attempts at commercial farming were abandoned (Anderson *et al.*, 1989), Anderson *et al.* (1996b) have shown that cultivation of *G. gracilis* is technically feasible in Saldanha Bay and estimated that 11 hectares of cultivated *G. gracilis* would give the same annual yield as obtained from natural beds. Oligotrophic surface water during summer is likely to be the main environmental factor limiting production and success of *G. gracilis* cultivation to a large extent.

1.9 AIMS OF THIS STUDY

The objectives of this study were to determine the effects of nitrogen starvation stress on growth of *G. gracilis* and investigate the ability of the species to tolerate the stress.

Laboratory grown *G. gracilis* was subjected to nitrogen deprivation. However, since *G. gracilis* is capable of sustaining growth at rates close to maximum using only stored tissue nitrogen, internal tissue nitrogen, culture media nitrogen concentrations, plant nutrient uptake and requirement rates and their effect on growth were analysed in order to determine the effect of nitrogen limitation. Understanding the above parameters is beneficial for developing a sound strategy for farming *G. gracilis* as far as nutrient management is concerned.

The presence of differentially expressed *G. gracilis* genes in response to nitrogen deprivation was investigated. The identification and characterisation of these genes may lead to the genetic engineering of stress tolerance in *G. gracilis* species in the future. This is especially significant in environments such as Saldanha Bay where there is potential for mariculture and natural harvest of the economically important macroalga has been unstable.

CHAPTER 2

NITROGEN DEPRIVATION: EFFECTS ON GROWTH

CONTENTS

Chapter 2

NITROGEN DEPRIVATION: EFFECTS ON GROWTH

2.1 INTRODUCTION

The growth of *Gracilaria* species is greatly affected by nitrogen in the marine environment (Naldi and Wheeler, 2002). The relationship between growth rate and nitrogen is complicated and the two are not always correlated. To better understand the effect of nitrogen on plant productivity requires knowledge of:

(i) The concentration of nitrogen in the growth medium (Smit, 2002).

(ii) The nitrogen uptake rate by the plant (Naldi and Wheeler, 2002).

(iii) The amount of nitrogen in the thallus (Hanisak, 1979, Naldi and Wheeler, 1999).

(iv) The rate of assimilation of nitrogen in metabolic processes, which also depends on the activity of enzymes involved such as nitrate reductase (Thomas and Harrison, 1985, Smit, 2002).

Nitrogen uptake rate experiments in laboratories are usually performed by measuring the disappearance of nitrogen from the growth medium. Very low concentrations or total disappearance of nitrogen from the medium however does not necessarily mean that the plant is experiencing nitrogen deprivation stress. High growth rates of *G. gracilis* and other macroalga were observed at very low nitrogen concentrations for extended times (Bird *et al.*, 1982, Lapointe and Duke, 1984, Smit, 2002). This is due to the ability of the plant to store excess nitrogen during nitrogen rich conditions and use it when the nitrogen level in the environment drops. In these plants nitrogen can be stored as inorganic nitrate, amino acids, pigments or proteins. Many studies (Lignell and Pedersen, 1987, Andria *et*

al., 1999) have used plant tissue analysis to determine the nutrient status of a plant. Critical nutrient concentration, defined as the internal nitrogen concentration that just limits maximal growth, varies from species to species (Hanisk, 1979). In this study, nitrogen deprivation in *G. gracilis* plants is assessed by analysing the relationship between the concentration of nitrogen in the growth medium and thallus with the growth rate of the plant. No attempts were made to correlate nitrogen limitation with rate of nitrogen assimilation or enzyme activity.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection and acclimatisation

2.2.1 1 Sample collection

Gracilaria gracilis plants were obtained from Jacob's Bay abalone farm. Healthy looking thalli were selected. These were gently scrubbed and rinsed with deionized water to remove sediment and visible epiphytes. Thallus apical tips were excised with a blade to generate thallus fragments weighing 0.2 ± 0.05 g fresh weight.

2.2.1.2 Plant acclimatisation

Plant apical tips were preincubated in 2 litre Erlenmeyer flasks. One litre sterile Artificial Seawater (ASW) (Appendix A.1), with a salinity of 3.6% enriched according to Provasoli (1968) (Appendix A.1) was used as the growth medium. Artificial light was provided by fluorescent tubes to a light intensity of 100 μmol photons m^{-2} s^{-1} with a 16:8 light-dark cycle. Temperature was maintained at 15-17°C. Aeration was provided by passing compressed air filtered through a 0.22 μm Millipore filter through the growth medium. The medium was replaced every 48 hours. *G. gracilis* thallus fragments were allowed to acclimatise in these conditions for one week.

2.2.2 Growth analysis during nitrogen deprivation

2.2.2.1 Biomass determination

Growth rate experiments were performed in 500 ml Erlenmeyer flasks. Treatments were performed in triplicate and included nitrogen limited axenic, nitrogen limited xenic and

constant nitrogen control. For the nitrogen limited xenic experiment, four pre-weighed thallus fragments were added to flasks containing 500 ml ASW enriched with PES medium (1/3 strength) (Appendex A.1). Every seventh day PES medium lacking $NaNO_3$ and amended with $Fe_2(SO_4)$ instead of $Fe(NH_4)_2SO_4$, hereafter referred to as PES-N (Appendix A.1), was added to maintain nutrient concentrations. The nitrogen limited axenic experiment was set up exactly as the xenic experiment with a single exception. Antibiotic cocktail (Appendix A.2) was added one day before the start of the experiment and replenished every week thereafter. For the control experiment, PES medium (1/3 strength) was added every second day to flasks containing ASW and xenic thalli as described previously. For all the treatments, thallus fragments were weighed on days 2, 4, 6, 8, 10, 12, 14, 16 and 18 after commencing the experiment (T = 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18).

2.2.2.2 Relative Growth Rate

The Relative Growth Rate (μ), which is the percentage increase in fresh weight per day, was calculated using the following equation.

$$\mu = [100 (\ln (N_t / N_o)] / t$$

where:

N_t = weight at time t

N_o = initial weight

t = time

2.2.3 Growth media nitrogen and total tissue nitrogen analyses

Six flasks were set up (Fig. 2.1) as described for the nitrogen deprivation experiment (section 2.1.2.1). On days 0, 2, 4, 7, 11 and 18 after commencing the experiment, (T = 0, 2, 4, 7, 11 and 18), a single flask was removed. Growth medium samples (20 ml) from this flask were stored in duplicate at –20°C. A portion of thallus fragments (approximately 0.5 g) was dried (70°C, 48 hrs), powdered using a mortar and pestle and stored in an oven (60°C). The remaining thallus fragments were flash frozen in liquid nitrogen and stored at –70°C. Extra *G. gracilis* thallus fragments that were not used in the growth analysis (2.2.2) were used to determine the proportion of dry to fresh weight by weighing before and after drying (70°C, 48 hrs). These were subsequently used to calculate the mean nitrogen requirement (2.2.4).

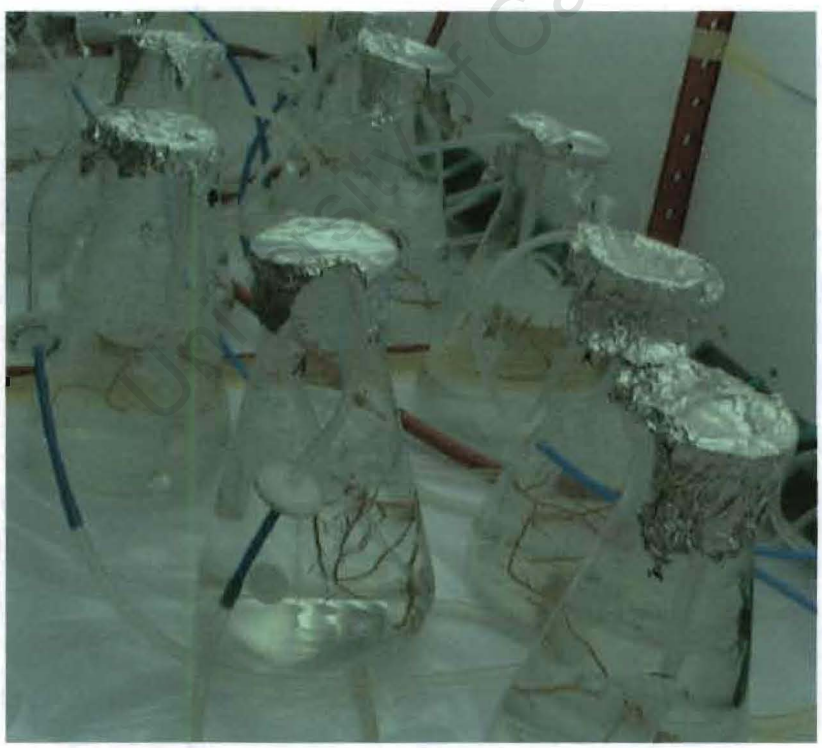


Fig. 2.1 Photograph showing the experimental setup of flasks.

2.2.3.1 Nitrate and ammonium nitrogen concentration

To determine the levels of nitrogen used by the plant and the concentration of nitrogen available in the medium, nitrate (NO_3^-N) and ammonium (NH_4^+N) concentration measurements were performed. Nitrate and ammonium concentrations in the growth medium samples (section 2.2.3) were determined using a Quickchem FIA+ 8000 series auto analyser (Lachat instruments, USA). These analyses were performed for the xenic and axenic nitrogen deprived treatments.

2.2.3.2 Total thallus nitrogen

Total thallus nitrogen content was determined in xenic *G. gracilis* cultured in nitrogen deprived growth medium. Total carbon and nitrogen measurements were performed on thallus fragments that were dried and powdered using mortar and pestle (section 2.2.3) in triplicate using a CHNS-932 analyser (Leco Co-orp, st Joseph, MI, USA).

2.2.4 Mean nitrogen requirement and uptake rate

The mean nitrogen requirement was estimated according to the equation described by Andria, *et al.*, (1999):

$$[\mu mol\ N\ (g\ FW)^{-1}\ d^{-1}] = (N_{int}) \,.\, (DW : FW) \,.\, (\mu) / AW_N$$

Where:

N_{int} is the total thallus nitrogen

DW : FW is the ratio between the dry and fresh weight of the thalli

μ is the mean relative growth rate expressed as d^{-1}

AW_N is the nitrogen atomic weight

The nitrogen uptake rate was determined by measuring the amount of nitrogen depleted from the medium and dividing it by the average fresh weight of *G. gracilis* thalli within a given culture period (number of days). It is expressed as μmol N $(g\ FW)^{-1}\ d^{-1}$.

2.3 RESULTS

2.3.1 Sample collection and acclimatisation

All the *G, gracilis* thalli displayed a healthy appearance having a dark red colour at the end of the acclimatisation period (Fig.2.2).

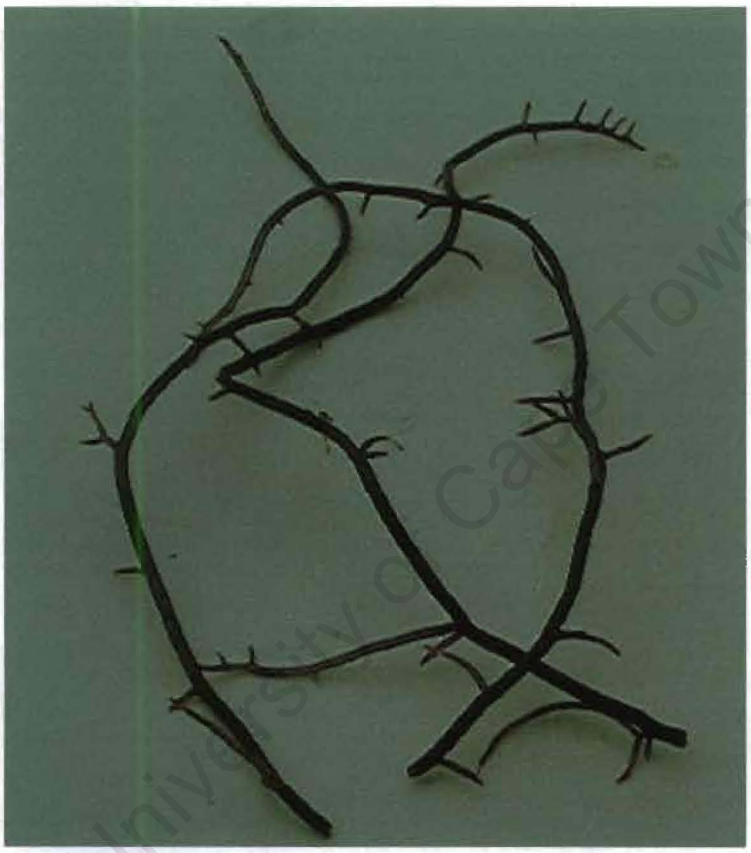


Fig. 2.2 *Gracilaria gracils* appearance after acclimatisation for 7 days.

2.3.2 Growth analysis during nitrogen deprivation

Changes in the biomass of *Gracilaria gracilis* over the experimental period is shown in Fig 2.3. Biomass increased with a doubling time of about 6-8 days in both the control and nitrogen limited xenic plants. The biomass doubling point was never reached in the nitrogen limited growth trial with axenic *Gracilaria*. The relative growth rates (RGR) of axenic *G. gracilis* cultured in nitrogen limited media were much lower than that of the control and the xenic plants grown in nitrogen limited media over the course of the growth study (Table 2.1). The growth rate of *G. gracilis* in nitrogen rich media was only 12.6-26.7% higher than that of plants (xenic) cultured in nitrogen limited media until day 12 (Fig 2.4). After that period the difference increased to 112%. About 7 days after the start of the experiment, xenic plants in the nitrogen starved media began to lose their dark red colour, taking on a yellowish green colour (Fig 2.5), while the control and axenic plants retained their colour and healthy appearance.

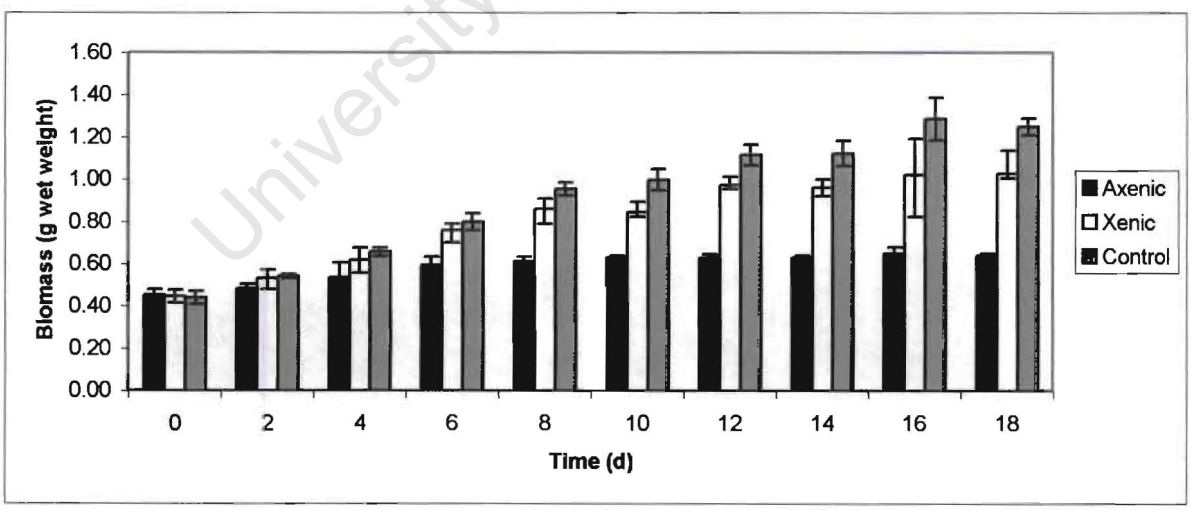


Fig. 2.3 Mean biomass of *G.gracilis* during the experimental period. Error bars indicate standard error. Each bar represents the mean of three experiments.

Table 2.1 Relative growth rate (μ) of axenic and xenic *G. gracilis* grown in nitrogen limited media and *G. gracilis* (control) grown in nitrogen rich media.

Treatment	Day 0-4	Day 5-8	Day 9-12	Day 13-18
Axenic	4.2	3.1	0.8	0.3
Xenic	8.1	8.3	3.1	1.3
Control	10.0	9.3	3.9	2.8

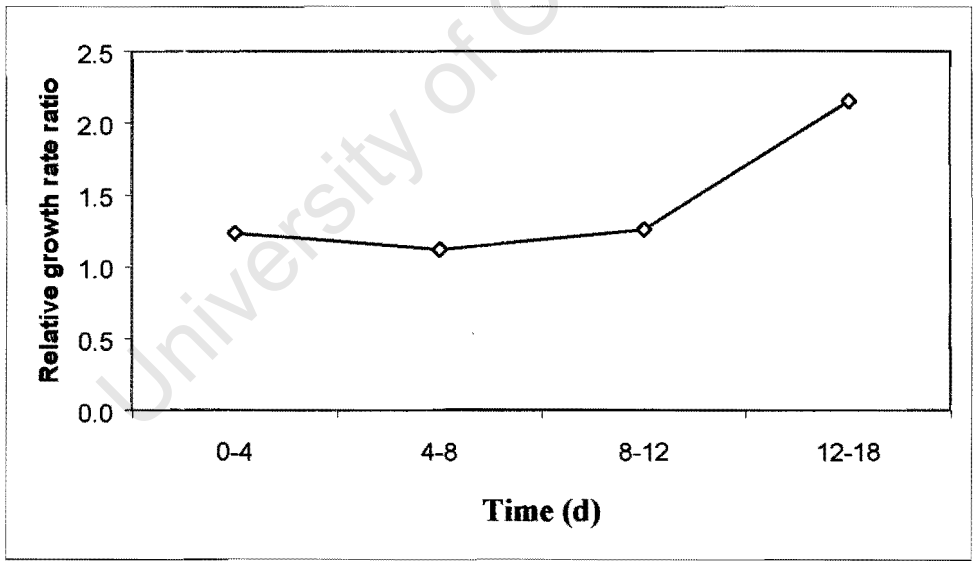


Fig. 2.4 Relative growth rate ratio of xenic *G. gracilis* grown in nitrogen rich media to plants cultivated in nitrogen deprived media.

a. b.

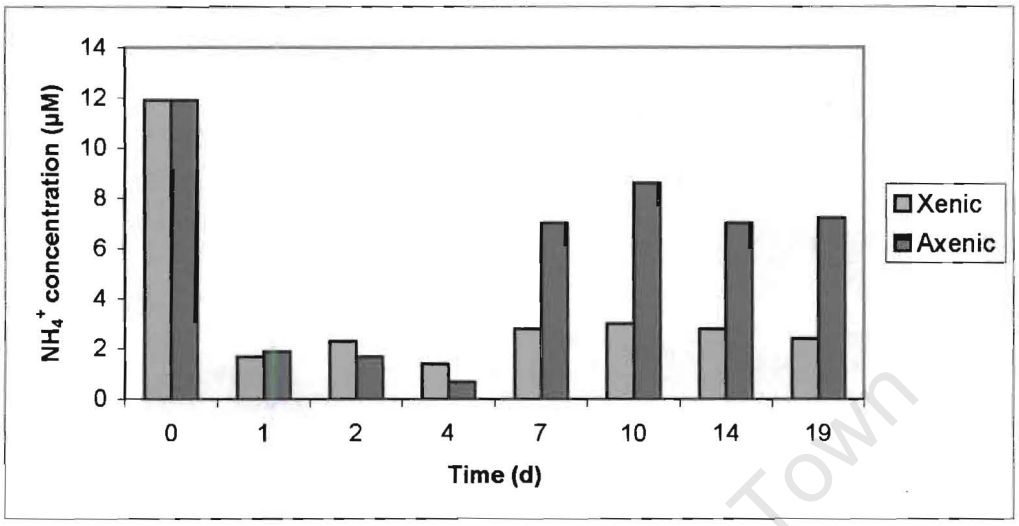
Fig. 2.5 Appearance of (a) axenic and (b) xenic *Gracilaria gracilis* after cultivation in nitrogen limited media for eight days.

2.3.3 Growth media nitrogen and total tissue nitrogen analyses

2.3.3.1 Nitrate and Ammonium nitrogen concentration

The ammonium concentration in the nitrogen deprived media dropped from 11.9 to approximately 2 µM within the first 24 hrs in both the xenic and axenic cultures (Fig. 2.6 a) and remained close to that level for the rest of the experiment in the xenic plant culture media. An increase in ammonium levels was observed in the axenic plant culture media from day 9 onwards. Nitrate levels increased one day after the commencement of the experiment after which a marked reduction was observed until day 7. Thereafter, the nitrate levels remained extremely low (Fig.2.6 b).

a)



b)

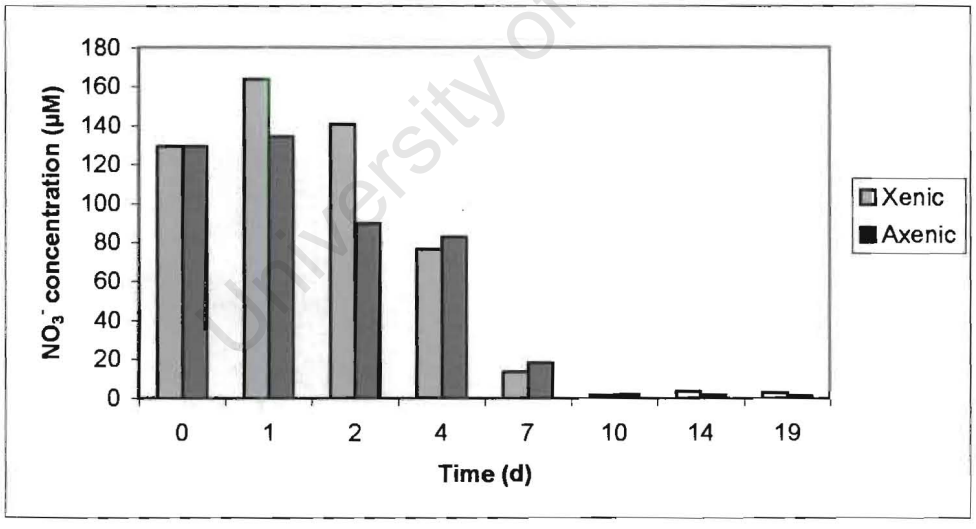
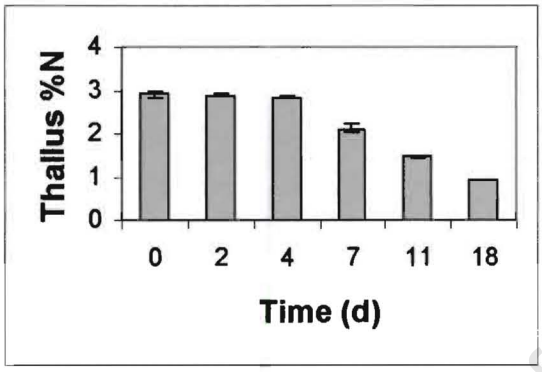


Fig. 2.6 Concentration of (a) ammonium and (b) nitrate in the growth medium of axenic and xenic *G. gracilis*.

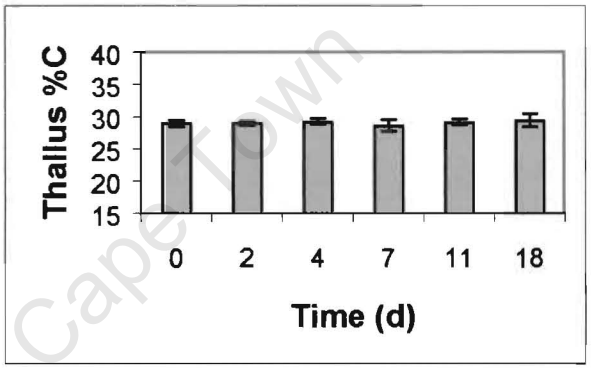
2.3.3.2 Total thallus nitrogen

The percentage tissue nitrogen, carbon and C:N ratios of xenic *G. gracilis* cultured in nitrogen limited growth medium are displayed in Figure 2.7. The carbon content remained constant during the experimental period, whereas nitrogen levels started to drop from day 4 onwards. This is highlighted by the almost exponential increase in C:N after day 4.

a)



b)



c)

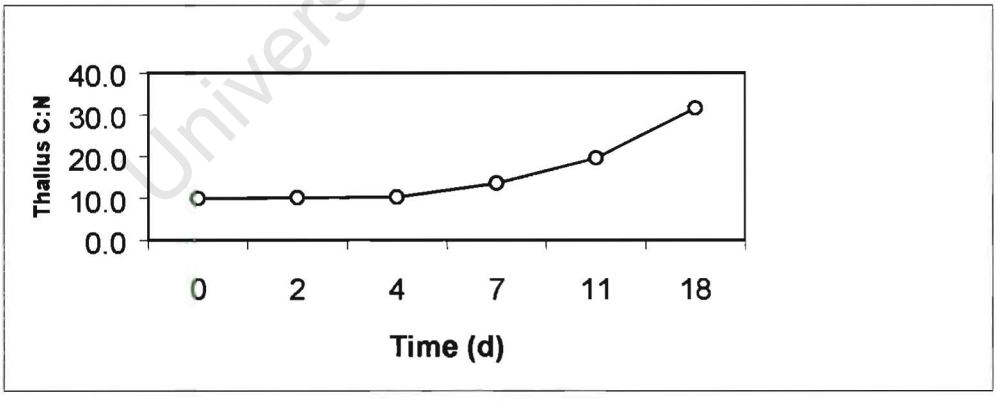


Fig. 2.7 (a) Percentage dry mass nitrogen, (b) percentage dry mass carbon and (c) C:N ratio in nitrogen deprived xenic *G. gracilis* thallus.

2.3.4 Mean nitrogen requirement and uptake rate

The mean fresh to dry weight ratio was 8.4 and was subsequently used to calculate the mean nitrogen requirement. The mean nitrogen uptake and requirement rates of *G. gracilis* grown in the nitrogen limited culture media are shown in Fig. 2.8. The mean nitrogen requirement rate at the commencement of the experiment when the medium was relatively nitrogen rich was 13.8 μmolN/g $FW^{-1}.day^{-1}$and decreased to 0.7 on day 18. The nitrogen uptake rate was well above the requirement rate until about day 12. Minimal uptake of nitrogen was observed after that period.

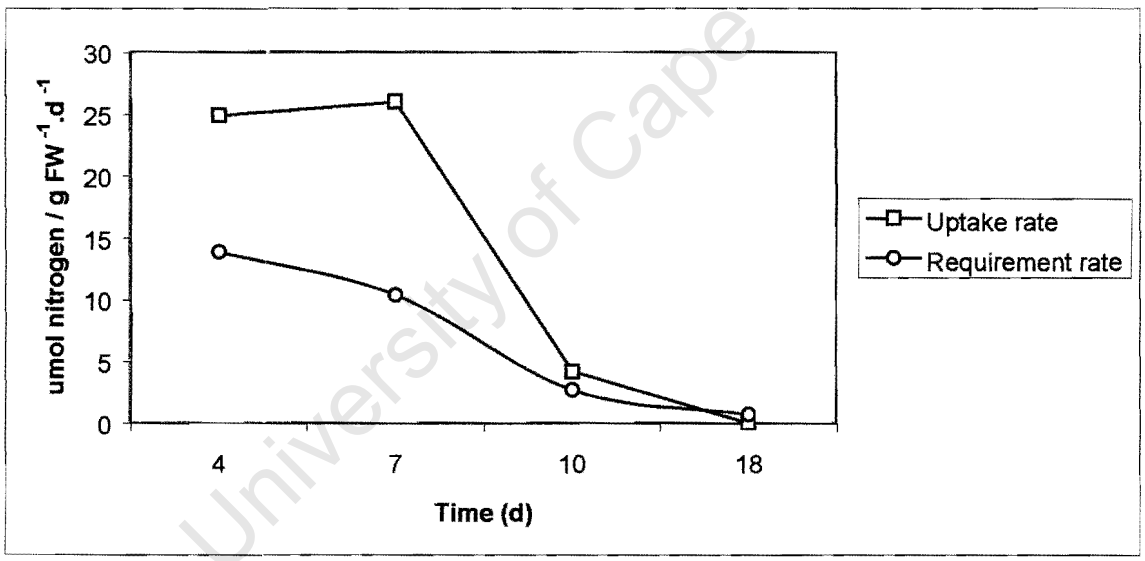


Fig. 2.8 Mean nitrogen uptake and requirement rates of xenic *G. gracilis* grown in the nitrogen limited media.

2.4 DISCUSSION

Both biomass and specific growth rate in seaweeds increase with increasing external nitrogen concentrations (Rosenberg and Ramus, 1982). However this relationship is not linear and is true only until a certain saturation point is reached. Hanisak (1979) has shown that the specific growth rate saturation point occurs at a NO_3^-N concentration of 68 μM for some green seaweed species. In the present study the concentration of nitrogen in the growth medium was higher than the growth rate saturation point until about days 4-7 in the nitrogen limited cultures. Even though the relative growth rate of *Gracilaria gracilis* in the nitrogen rich media was higher than that in the nitrogen-limited cultures during this period, the difference was not as significant as that observed after this period. This may indicate that for about the first week the thalli obtained almost all their nitrogen from the growth medium even in the nitrogen limited culture. That the mean nitrate uptake rate exceeded the mean requirement rate in the nitrogen limited growth medium during the above mentioned period further enforces this observation (Fig. 2.8). The mean nitrogen requirement rate at the beginning of the experiment, when the culture medium was relatively nutrient rich (13.9 $\mu mol.g^{-1}.day^{-1}$) was slightly lower than the 15 $\mu mol.g^{-1}.day^{-1}$ that Andria *et al.* (1999) calculated for *Gracilaria gaditana* species. Past nutritional history, light, species and other factors influence a plant's nutrient uptake and requirement rates (Naldi and Wheeler, 2002) and may explain the variation observed.

The ammonium present in the culture medium was consumed within the first 24 hours, emphasizing *G. gracilis*' preference for ammonium over nitrate as a source of nitrogen. Since nitrate has to be reduced to ammonium prior to assimilation, this could be a

strategy by which the plant increases its metabolic efficiency. Smit (2002) has shown that *G. gracilis* cultured at temperatures lower than 20°C has a higher affinity for NH_4^+N than NO_3^-N and this preference persists as long as the concentration of ammonium in the culture is above 5 μM. During this study the ammonium concentration was higher than 5 μM for the first 24 hours only. During this time an increase in nitrate levels was observed. Fujita *et al.* (1988) demonstrated that the release of inorganic nitrogen from the green alga *Ulva rigida* to the growth medium that occurs when nutrient release rates exceed nutrient uptake rates commenses after 30 min of culture in nitrogen rich media. Bacteria could also be responsible for the increase in nitrate levels. This, however, is unlikely as an increase in NO_3^-N was also observed in the axenic cultures, albeit to a lesser extent (an increase of 5 μM as compared to 34 μM for the xenic culture (Fig. 2.6)). From day one the alga utilised NO_3 (the only nitrogen source available in the medium) and within less than ten days it was almost completely consumed. It is very difficult to explain the increase in ammonium levels observed in the axenic cultures during the last ten days of the experiment. Since the growth rate of these plants was very low (Fig. 2.3, Table 2.1) and since they were probably not using stored nitrogen, it is possible that the NH_4^+ release rate exceeded the uptake rate resulting in a net release of ammonium. It is likely that the nitrate absorbed and subsequently reduced to ammonium within the first seven days was the source of the ammonium released into the culture medium.

The growth rate of axenic *G. gracilis* in the nitrogen limited medium was extremely low. The axenicity of *G. gracilis* was maintained by adding antibiotic cocktail every seven days. Since Jaffray (1998) has shown that antibiotic cocktail generates axenic *G. gracilis*

that has no bacterial epiphytes when tested on marine agar over a 10-day period, it is likely that the number of bacteria was very low in the axenic *G. gracilis* culture medium for the entire experimental period. In this nitrogen-starved environment, the absence of bacteria that normally compete for nutrients had a negative effect on the growth rate of the alga. Microorganisms are not always harmful to seaweeds. Provasoli and Pintner (1980) have shown that axenically grown green algae exhibit abnormal morphology. Many epiphytic bacteria produce growth regulators, cell binding factors and even vitamins essential for macroalgal growth (Lobban *et al.*, 1985). Holmstrom *et al.* (2002) have noted that bacteria enhance spore settlement and development of algae.

Because of the aforementioned absence of microorganisms, or for some other unknown reason, the *G. gracilis* failed to use the reserve nitrogen mainly stored as phycoerythrin (Bold and Wynne, 1985) for growth and consequently was able to maintain the dark red colour. This can clearly be seen when compared to the nitrogen limited xenic plants (Figure 2.5).

Due to the abnormal growth displayed by axenic *G. gracilis* in nitrogen limited media, it was decided that further investigations of tissue nitrogen content and analysis of differential gene expression in response to nitrogen limitation would be performed only on xenic *G. gracilis* samples.

The difference between the mean nitrogen uptake rate and the mean requirement rate started to fall abruptly from day 7 onwards in the N-limited xenic cultures (Fig. 2.8). The

growth rate of these plants was, however, only 21.1% lower than the nitrogen enriched control plants between days 7 to 12. About the same difference in growth rate was also observed during the first four days when the N-limited culture medium was still relatively nutrient rich. Some other *Gracilaria* species have shown equal growth rates when grown in nitrogen enriched and unsupplemented seawater for 6 days, maintaining a C:N value of 10 (Lapointe and Ryther, 1979). Nitrogen is mainly stored as pigments and amino acids in seaweed tissues. In this study the plants began to use stored tissue nitrogen between days 4 and 7 (Fig.2.7 a). Many studies have shown that *Gracilaria* species maintain relatively high growth rates for a relatively long time during periods of nitrogen starvation (Lignell and Pedersen, 1987, Smit *et al.*, 1997). In some seaweed species, the growth is reduced when the total nitrogen content of the thallus falls below 1.9 %. The C:N ratio at this critical nutrient concentration is 13.5 (Hanisak, 1979). In the present study this point would be reached between days 7 and 11 (Figure 2.7 c). Lapointe and Ryther (1979) have suggested that C:N values greater than 10 generally denote nitrogen limitation, even though values may vary from species to species. The low percentage nitrogen (0.9-1.4), very high C:N values (19.5-31.5) and very low growth rates (Table 2.1) all indicate that *G. gracilis* was severely nitrogen limited between days 11 and 18.

The stress due to nitrogen starvation is also reflected by the unhealthy appearance and loss of dark red pigmentation of the alga (Fig. 2.5). As already mentioned, nitrogen in red algae is mainly stored in pigments. In some *Gracilaria* species the red pigment phycoerythrin alone makes up one third of the total protein (Rosenberg and Ramus, 1982). The plant regained its dark red colour following re-supply of nitrogen within 4 - 7

days after the nitrogen starvation experiment ended, proving the importance of the red pigment in nitrogen storage.

Many studies have associated stresses due to environmental factors such as salinity and light intensity with diseases in seaweeds (Largo *et al.*, 1995, Uyenco *et al.*, 1981). Jaffray and Coyne (1996) have postulated that extreme environmental conditions in Saldanha Bay in summer induce bacterial diseases in *G. gracilis*. These unfavourable conditions are nutrient starvation and increased water temperature. The disease symptoms, described by Jaffray and Coyne (1996) as lesion formation and tip bleaching were not observed over the 18 days of nitrogen deprivation. Since water temperature in this study was maintained at 15-17°C, it may be that both increased temperature and nitrogen starvation occurring simultaneously may be necessary for epiphytic bacteria to induce disease in this *Gracilaria* species. Nutrients other than nitrogen, such as phosphate, may also play a role.

CHAPTER 3

INVESTIGATION OF G. GRACILIS GENES EXPRESSED IN RESPONSE TO NITROGEN DEPRIVATION

CONTENTS

Chapter 3

INVESTIGATION OF G. GRACILIS GENES EXPRESSED IN RESPONSE TO NITROGEN DEPRIVATION

3.1 INTRODUCTION

Different techniques have been employed in several studies to explore differential gene expression in plants in response to pathogen infection and environmental stress (von Stein *et al.*, 1997; Breyne and Zabeau, 2001). One approach to identifying differentially expressed genes is to use techniques such as RT-PCR to clone genes homologous to those already characterised in other plant species and investigate their expression patterns by northern hybridization analysis. Recently, PCR based techniques that do not require prior knowledge of any nucleotide sequence have been developed (Liang and Pardee, 1992; Vos *et al.*, 1995; Pearson *et al.*, 2001; Happe and Kaminisiki, 2002). These include differential display, SSH (subtraction suppression hybridization) and cDNA-AFLP (amplified fragment length polymorphism).

The differential display method involves amplification of cDNA sequences from subsets of mRNA by reverse transcription and PCR. The amplified cDNA fragments from different tissues are separated on a denaturing polyacrylamide gel and differences in banding patterns are analysed.

SSH is based on a specific form of PCR that allows exponential amplification of cDNAs that differ in abundance, while amplification of sequences of identical abundance is suppressed. Happe and Kaminsiki (2002) used SSH to isolate differentially expressed genes in response to anaerobic stress in the green macroalga *Chlamydomonas reinhardtii*.

The cDNA-AFLP technique was applied with success by Jaffray and Coyne (1998) to identify genes expressed in response to pathogen infection in G. *gracilis.*

Despite the fact that all these methods have been successfully used to isolate differentially expressed genes, they all have some specific drawbacks. These drawbacks include bias towards abundant mRNA molecules, low reproducibility of band patterns and identification of false positive clones. In the present study differential expression of *G. gracilis* genes in response to nitrogen deprivation is investigated by performing differential screening analysis on full-length cDNA library synthesised using a technique developed by CLONTECH laboratories, Inc. This technique, unlike most other cDNA synthesis methods, produces full length cDNA that contains the complete 5' end of the mRNA and therefore enables one to perform a more representative analysis of the mRNA present in *G. gracilis* at different stages of nitrogen limitation.

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3.2 MATERIALS AND METHODS

All media and solutions used in this study are listed in Appendix A.

3.2.1 RNA extraction for northern blot analysis and RT-PCR

RNA was extracted from thallus samples subjected to nitrogen limitation for various time periods (T = 0, 2, 4, 7, 11 and 18 days). RNA extracted from thallus fragments obtained on day 0 (T = 0) was used as a control, representing a nitrogen enriched sample. A small fragment (approximately 5 cm) of flash frozen thallus (see section 2.1.6) was ground to a very fine powder in a mortar using a pestle and liquid nitrogen. Total RNA was extracted using the Trizol method (Invitrogen Life Technologies). Trizol (750 µl) was added to the ground powder in an Eppendorf tube and the sample was vortexed for 5 min and incubated for 5 min at room temperature. Chloroform (200 µl) was added and the sample incubated for 3 min at room temperature. The sample was centrifuged for 15 min at 12 000 x g at 4°C. RNA was recovered from the aqueous phase by the addition of 500 µl of isopropanol followed by a 10 min incubation at room temperature. The precipitate was pelleted by centrifugation for 5 min, washed with 70% ethanol and dried briefly. The RNA was re-suspended in 50 µl DEPC-water for 5 min at 55°C and stored at -70°C.

The integrity of the RNA was determined by resolving on a 1.1% agarose gel. Agarose gel electrophoresis was performed in 1 X TAE running buffer (Appendix A.3) as described by Sambrook *et al.* (1989). RNA was visualised on a 254 nm uv transilluminator.

3.2.2 Full-length cDNA synthesis

Full-length cDNA synthesis was performed using the Creator™ SMART™ cDNA Library Construction kit (CLONTECH laboratories, Inc.). Full-length cDNA synthesis, cloning and subsequent blotting was performed only on RNA obtained from *Gracilaria gracilis* thallus that was subjected to 18 days of nitrogen deprivation, because it was determined from the previous chapter that the macroalga was greatly nitrogen limited following 18 days of nitrogen deprivation.

3.2.2.1 First Strand Synthesis

In a PCR tube, 800 ng of total RNA, 1 µl of 3' SMART CDS III Primer (10 µM) and 1 µl of SMART™ Oligonucleotide (10 µM) were mixed and made up to 5 µl with distilled water. The RNA-oligo mixture was incubated for 2 min at 72°C, chilled on ice and added to a reverse transcription mix (Table 3.1).

Table 3.1 Composition of the reaction mix used for First strand synthesis

Reaction Constituents	Final concentration
First strand buffer	1X
Dithiothreitol	2 mM
dNTP	1 mM (of each)
PowerScript ™ Reverse Transcriptase	1 µl

The reaction mix (10 µl in total) was incubated for 60 min at 42°C and stored at –20°C.

3.2.2.2 Second Strand Synthesis

One microlitre of cDNA was added to a PCR reaction mixture (Table 3.2) and made up to 50 µl with distilled water. Second strand synthesis was performed using cycle profile 1 (Appendix C.1). An aliquot (5 µl) of the amplified product was electrophoresed through a 1.1% agarose gel at 70 V in 1 X TAE running buffer to verify the presence of DNA.

Table 3.2 Composition of the reaction mix used for Second strand synthesis

Reaction Constituents	Final concentration
First strand cDNA	1 µl
Advantage 2 PCR buffer	1X
dNTP	0.2 mM (of each)
5' PCR primer	0.2 µM
SMART CDS III Primer	0.2 µM
Advantage 2 polymerase	1 µl

3.2.2.3 Purification of amplimers

Amplified DNA was purified using the High Pure PCR Product Purification Kit (Roche, Germany). Two hundred and fifty microlitres binding buffer was added to a 50 µl PCR reaction and mixed well. The High Pure filter and collection tubes were combined and the sample pipetted into the upper reservoir. The sample was centrifuged for 30 s at 15 800 x *g* in a microcentrifuge. The flow through was discarded and the filter tube

combined again with the same collection tube. Five hundred microlitres wash buffer was added to the upper reservoir and centrifuged for 30 s. The flow through was discarded and the filter tube again combined with the collection tube. Two hundred microlitres of wash buffer was added and the sample centrifuged and recovered as described above. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. DNA was eluted using 50 µl distilled water and stored at 4°C.

3.2.3 Restriction endonuclease digestion of cDNA

Purified amplified cDNA was digested with *Sfi*I restriction endonuclease. The reaction (50 µl) contained 1 µg DNA, 1 X restriction buffer and 20 U restriction endonuclease. The reaction mixture was incubated for 2 hrs at 50°C. Restriction digests were electrophoresed through a 1.1% agarose gel, visualised on a 365 nm uv transilluminator and DNA fragments in the 0.5-1.2 kb range were excised with sterile razor blade. DNA from the excised agarose gel slice was purified using the High Pure Purification Kit (Roche, Germany). Binding buffer (300 µl for every 100 mg gel) was added to the gel slices. The sample was incubated for 10 min at 56°C, with brief vortexing every 2-3 min to dissolve the gel slice in order to release the DNA. Isopropanol (150 µl for every 100 mg gel slice) was added to the sample. The filter and collection tubes were combined and the sample pipetted into the upper reservoir. The sample was centrifuged for 30 s at 15 800 x *g* in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Five hundred microlitres wash buffer was added to the upper reservoir and centrifuged for 30 s. The flow through was discarded and the filter tube again combined with the collection tube. Two hundred microlitres of

wash buffer was added and the sample centrifuged and recovered as described above. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. DNA was eluted using 30 µl distilled water and stored at 4°C.

3.2.4 Cloning of cDNA fragments

DNA fragments were ligated into *Sfi*I-digested, dephosphorylated pDNR-LIB vector (CLONTECH). The ligation reaction (20 µl) contained DNA (200 ng), pDNR-LIB (100 ng), T4 DNA ligase (2U) (Roche, Germany) and 1 X ligation buffer. The reaction mix was incubated overnight at 15°C. Ligated products were transformed into *Escherichia coli* XL1 Blue cells made competent with $CaCl_2$ using the method described by Draper *et al.* (1988) (Appendix A.4; D.2). Ten microlitres of ligation mix was added to 100 µl competent cells and incubated for 10 min on ice. The cells were heat shocked for 5 min at 37°C, followed by 5 min incubation on ice. Nine hundred microlitres LB broth was added to the transformed cells and incubated for 30 min at 37°C. Cells were plated on LA supplemented with 30 µg/ml chloramphenicol (Roche Diagnostics Gmbh, USA). The transformed cells were incubated overnight at 37°C.

3.2.5 Colony PCR

Colonies representing transformants were randomly picked with sterile toothpicks and inoculated into PCR reaction mixtures (final volume of 50 µl) (Table 3.3). Recombinant plasmids were amplified using M13 primers (Appendix B.2) and cycle profile 2 (Appendix C.2). PCR products were stored at 4°C.

Table 3.3 Composition of the reaction mix used for colony PCR

Reaction Constituents	Final concentration
Primers (Appendix B.2)	0.3 μM
DNTP	0.1 μM (of each)
$MgCl_2$	2.5 μM
Taq polymerase buffer	1X
Supertherm Taq polymerase	1 U

3.2.6 Blotting and hybridisation

Colony PCR amplimers were resolved on a 1.1% agarose gel at 60 V and transferred to Hybond-N^+ membrane (Amersham pharmacia biotech, England) using an AE-6680 Genopirator apparatus (ATTO Corporation) according to the manufacturer's instructions (Appendix D.3). Hybridisation was performed using amplified cDNA samples from (T = 0, 7 and 18) as probes against the prepared blotted day 18 cDNA clones. Probes were prepared by labeling the 5' PCR primer (Appendix D.4) with [γ-^{32}P] dCTP. First strand cDNA was synthesised as previously described (3.2.2.1) followed by second strand cDNA synthesis (as described in section 3.2.2.2) using radio-labeled 5' PCR primer. Hybridisation was performed overnight at 65°C (Appendix A.5; D.5). The same blot was hybridised with each of the three probes and this was accomplished by removing bound probe after each hybridisation using stripping buffer (Appendix A.5; D.6).

3.3 RESULTS

3.3.1 RNA isolation

Total RNA was extracted from *G. gracilis* thalli that had been cultured in nitrogen-limited media for various time periods (T = 0, 2, 4, 7, 11 and 18). The concentration of the extracted RNA was observed to be highly variable among the different samples (Fig. 3.1). In addition, the isolated RNA was found to be highly viscous. RNA obtained from day 18 *Gracilaria gracilis* thallus (T =18) was used to synthesise full-length cDNA which was subsequently cloned and blotted, while radio-labeled cDNA probe was generated from RNA isolated from *Gracilaria gracilis* subjected to 0, 7 and 18 days of nitrogen starvation (T = 0, 7 and 18). The RNA remaining from these samples, and the RNA obtained from the other *G. gracilis* thalli (T = 2, 4 and 11), was used for northern blot analysis (refer to section 4.2.3).

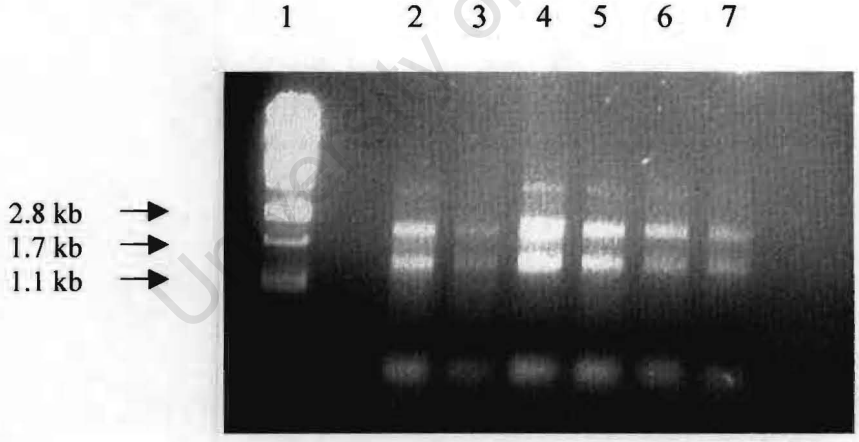

Fig. 3.1 Resolution of RNA isolated from *G. gracilis* exposed to different periods of nitrogen deprivation. Lane 1, λ DNA marker digested with *Pst*I; lanes 2 to 7 RNA samples T = 0, 2, 4, 7, 11 and 18 respectively.

3.3.2 Full-length cDNA synthesis

Full-length cDNA synthesised from *G. gracilis* RNA (T = 18) and digested using the restriction endonuclease *SfiI* is shown in Fig. 3.2. A moderately strong smear with some distinguishable bright bands was observed.

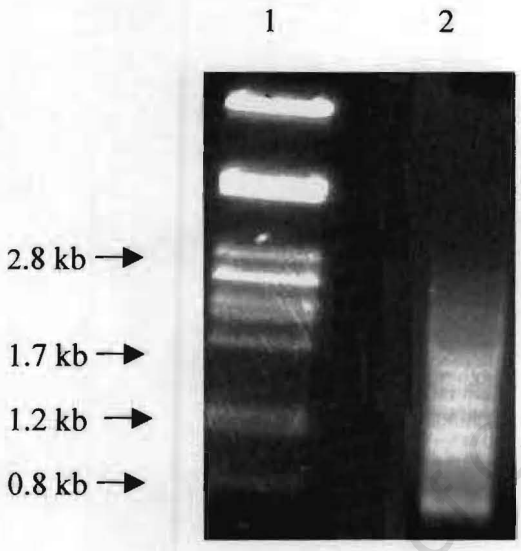

Fig. 3.2 Full-length cDNA synthesised from *G. gracilis* RNA obtained after18 days of nitrogen deprivation (lane 2). Lane 1 contains λ DNA marker digested with *Pst*I.

3.3.3 Cloning of cDNA fragments

cDNA restriction fragments (0.5-1.2 kb) excised from a 1.1% agarose gel were cloned into the plasmid pDNR-LIB vector and transformed into competent *E. coli* XL1 Blue cells. A total of about 13 000 positive transformants were obtained.

The presence of insert DNA was confirmed in plasmids isolated from 9 randomly selected clones by performing colony PCR. Eight of the nine tested clones were successfully amplified (Fig. 3.3). Colony PCR was then performed on 192 randomly selected clones and 98% of the clones were successfully amplified (Fig. 3.4). The size of the cloned *G. gracilis* DNA ranged from 0.5-1.2 kb.

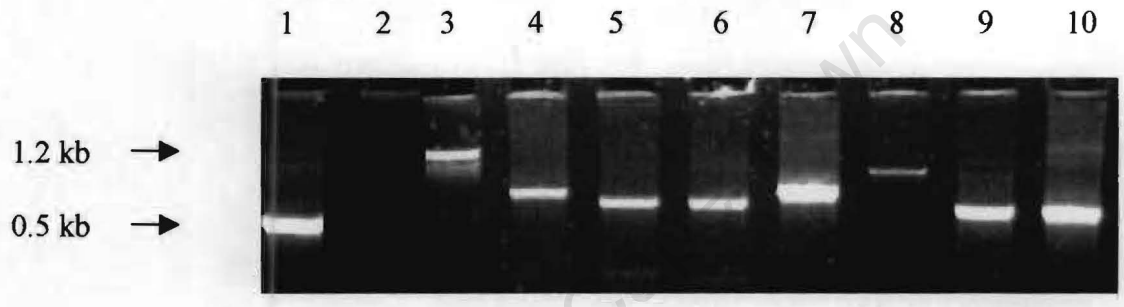

Fig. 3.3 Resolution of randomly selected colony PCR products by electrophoresis (lanes 2 to 10) through a 1.1% agarose gel. Lane 1 contains λ DNA marker digested with *Pst*I.

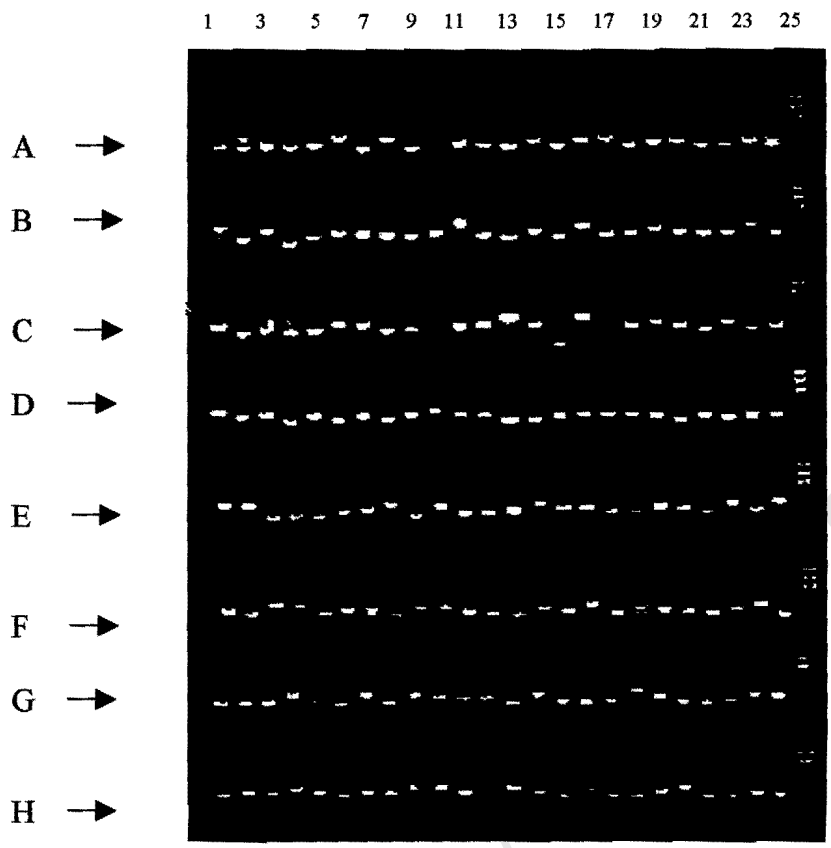

Fig. 3.4 Colony PCR products of cloned *G. gracilis* cDNA fragments synthesised from mRNA from nitrogen deprived thallus (day 18) following separation through a 1.1% agarose gel. Last lane contains λ DNA marker digested with *Pst*I and top three bands seen in the last lane of most rows represent restriction fragments of 14.0, 4.5 and 1.7 kbs in size respectively. Every other column has been labelled due to space constraints.

3.3.4 Blotting and hybridisation

G. gracilis cDNA clones (192 in total) generated from nitrogen deprived thallus (day 18) (Fig. 3.4) were probed with radio-labelled full length cDNA synthesised from RNA isolated from thalli on day 0, 7, and 18 of the growth experiment. Even though it is difficult to visualise each spot in Fig. 3.5, about 95% of the total cDNA fragments showed some degree of hybridisation to all three probes. The level of hybridisation, as indicated by the level of the intensity of individual bands, was observed to be variable between the cDNA fragments at each of the three time points (T = 0, 7 and 18).

Comparison of the autoradiographs showed that 27 of the 192 clones exhibited an enhanced hybridization signal when probed with the T = 18 probe. These cDNA clones are A9, A22, B1, B2, B9, B13, B24, C3, C5, C21, C23, D6, D13, D14, D23, D24, E15, F2, F5, F7, F12, G13, G16, H15, H17, H18 and H21 (Fig. 3.5). All 27 clones exhibited stronger hybridisation signals when probed with the T = 7 probe when compared to day 0. Some cDNA clones were also observed which may represent genes that were down regulated as nitrogen deprivation progressed over the 18 day period. These are A1, A3, A17, E14, G2, G6, G10 and H8.

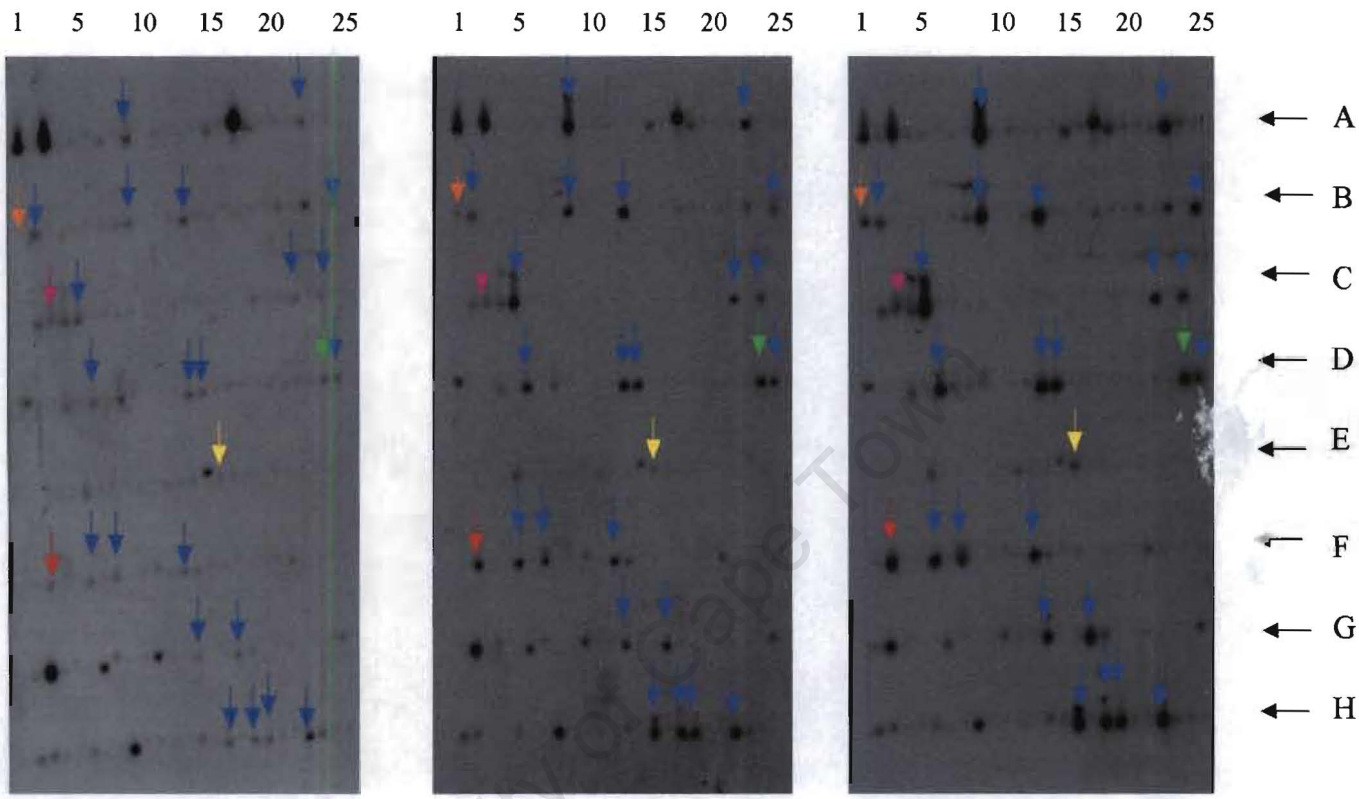

Fig. 3.5 Differential screening analysis of day 18 cDNA probed with radio-labelled cDNA from: a) day 0 b) day 7 and c) day 18 *G. gracilis* cultured in nitrogen depleted media. cDNA fragments that possibly represent up regulated genes on days 7 and 18 are marked by ↓. Every fifth column has been labelled due to space constraints. Arrows of the same colour mark identical cDNA fragments (refer to section 4.3.1).

3.4 DISCUSSION

Differential screening analysis was performed to determine whether *G. gracilis* specifically expresses genes in response to nitrogen deprivation. The cloned cDNA fragments that were probed with cDNA prepared from thalli sampled at different time points (T = 0, 7 and 18), were generated from *G. gracilis* starved of nitrogen for 18 days. Day 18 was selected because internal nitrogen in the macroalgal tissue, which was the only source of nitrogen from day 4 onwards, was extremely low at that time point. In addition, death of some *G. gracilis* thalli was observed after day 18.

The concentration of the *G. gracilis* RNA samples isolated at the different time points was highly variable. The RNA extract was highly viscous and at times very difficult to pipette. *G. gracilis* contains a considerable amount of gummy polysaccharides such as agarose and it is possible that these polymers interfere with the RNA isolation procedure (Zeng and Yang, 2002). Recently, the RNA to DNA ratio in marine organisms has been found to be correlated with the nutritional status of the organism (Okumura *et al.*, 2002). Since the quantity of DNA per cell is considered to be constant (Okumura *et al.*, 2002), the amount of RNA is likely to be influenced by the nutritional status of the organism. Since the nutritional condition of *G. gracilis* at the different time points in this study was variable, this may partially explain the variation in the concentration of RNA observed. However cDNA synthesis was performed using the same amount of RNA in each reverse transcription reaction.

Differential screening of the day 18 cDNA clones showed that 27 of the 192 exhibited darker bands when probed with cDNA from 18 day old thalli than with the cDNA from day 0 and 7. Since the level of nitrogen present in the culture medium was the only difference between the three time points, the darker cDNA bands possibly represent *G. gracilis* genes that were up-regulated in response to nitrogen deprivation. Very little is known about the effect of nitrogen availability on gene expression in marine plants. However, recent studies on terrestrial plants indicate that several genes respond to changes in nitrogen levels (Wang *et al.*, 2001; Cooke *et al.*, 2003). Hildebrand and Dahlin (2000) have cloned and characterised the first nitrate transporter genes from marine organisms. They have shown that increased mRNA levels of these genes occur in the diatom *Cylindrotheca fusiformis* when subjected to nitrogen limited conditions. Similarly, high expression of *nbl*, a key gene in nitrogen assimilation in blue green algae (cyanobacteria), was observed in response to nitrogen stress (Luque *et al.*, 2001).

In the present study only a very few cDNA clones (8 out of 192 clones) represented down-regulated genes when probed with day 18 cDNA. This was expected because all the cDNA clones were generated from nitrogen deprived (day 18) *G. gracilis* and it is highly likely that clones representing up-regulated genes dominate the randomly selected cDNA clones generated from RNA isolated on day 18. Conversely, a blot of cDNA from *G. gracilis* grown on a nutrient enriched media would be dominated by a set of genes that would be down-regulated when probed with day 18 cDNA than when probed with day 0 cDNA. Clones exhibiting down-regulated gene expression may be associated with reduced photosynthetic activity, enzyme activity or general cell metabolism that resulted

from nitrogen limitation. Some blue green algae have exhibited reduced activity of the enzyme glutamine synthetase coupled with reduced photosynthetic capacity under nitrogen stress (Lindell *et al.*, 2002).

Jaffray and Coyne (1998) employed a cDNA-AFLP protocol to identify nine differentially expressed gene fragments from cDNA isolated from diseased *G. gracilis*. Both the cDNA-AFLP and the differential screening method employed in the present study are based on the principle that a complex starting cDNA is fractionated into smaller subsets, PCR amplified and separated on gels. In both techniques the relative difference in the levels of expression is analysed by the difference in the intensity of bands observed. However, the differential screening method employed in the present study is relatively easier and more efficient in that no recovery of cDNA fragments from the gels is required for re-amplification and sequencing.

One of the major drawbacks of many techniques used to identify differentially expressed genes is that they restrict the analysis of differential expression to differences at the 3' end of cDNAs and that differences at the 5' portion are not detected (von Stein *et al.*, 1997). The smart cDNA synthesis method used in the present study uses modified oligo (dt) primer, an oligonuceotide (SMART IV oligonucleotide) and reverse transcriptase designed to produce full length single-stranded cDNA that contains the complete 5' end of the mRNA as well as sequences that are complementary to the smart oligonucleotide used. This anchor sequence, together with the poly A tail, serve as universal priming sites for end to end cDNA amplification. It is likely that the use of the smart cDNA synthesis

method in the present study has resulted in a more representative analysis of the mRNA present in *G. gracilis* at different stages of nitrogen limitation.

CHAPTER 4

ANALYSIS OF GENES EXPRESSED IN RESPONSE TO NITROGEN DEPRIVATION

CONTENTS

Chapter 4

ANALYSIS OF GENES EXPRESSED IN RESPONSE TO NITROGEN DEPRIVATION

4.1 INTRODUCTION

Plant cells respond to environmental stress by the expression of genes that may function in the signalling network or may play roles in actual stress tolerance. Genes expressed in response to stress may encode for specific stress proteins with possible protective functions. Several regulatory and signalling genes are involved in linking plant nutrient status to nutrient assimilation or acquisition (Wang *et al.*, 2001). Several studies have investigated the molecular responses of unicellular algae and identified genes that are differentially expressed under nitrogen deprivation (Lee *et al.*, 1999; Sauer *et al.*, 2000; Muro-Pastor *et al.*, 2001; Lindell *et al.*, 2002). However, despite the commercial importance of macroalgae, there is not enough information regarding the molecular responses of these organisms to environmental stresses.

Having observed differential gene expression in nitrogen deprived *G. gracilis* (section 3.3.4), northern blot analyses were performed to confirm that the cDNA clones truly represent upregulated genes expressed in response to nitrogen deprivation. The upregulated cDNA clones were sequenced and the sequences analysed for homology to sequences in the GenBank databases in order to assign a putative function to each cDNA fragment.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of plasmids containing differentially expressed cDNA fragments

Transformants containing recombinant plasmid harbouring differentially expressed cDNA fragments (section 3.2.5) were picked with sterile toothpicks and inoculated into 2 ml Luria broth (Appendix A.1) and incubated overnight at 37°C. Plasmid DNA was extracted using a High Pure Plasmid Isolation Kit (Roche, Germany) according to the manufacturer's instructions. Bacterial cells were centrifuged for 30 s at 9 000 x *g* and the pellet was suspended in 250 µl of suspension buffer. Lysis buffer (250 µl) was added and the tube was incubated for 5 min at room temperature. Chilled binding buffer (350 µl) was added and the sample incubated for 5 min on ice. The sample was centrifuged for 10 min at 15 800 x *g*. The High Pure filter and collection tubes were combined and the sample supernatant pipetted into the upper reservoir. The sample was centrifuged for 30 s at 15 800 x *g* in a microcentrifuge. The flow through was discarded and the filter tube combined again with the same collection tube. Seven hundred microlitres wash buffer were added to the upper reservoir and centrifuged for 30 s. The collection tube was discarded and the filter tube inserted into a clean 1.5 ml reaction tube. DNA was eluted using 100 µl elution buffer. The elution buffer was added to the filter tube and centrifuged for 30 s. Eluted samples were electrophoresed on a 1.1 % agarose gel.

4.2.2 Nucleotide sequencing of upregulated cDNA fragments

Cycle sequencing was performed using the DYEnamic ET Dye Terminator Cycle Sequencing kit for MegabACE (Amersham Biosciences). All reactions were performed according to the manufacturer's instructions. Samples were sequenced using a GeneAmp PCR System 9700, Perkin

Elmer (Applied Biosystems) and M13 primers (Appendix B.2). Both strands were sequenced for all samples.

4.2.3 Data analysis

Sequence data from individual samples was edited using DNASIS (version 2.1; Hitachi Software engineering). Nucleotide and amino acid sequence searches of the GenBank and European Bioinformatics Institute databases were performed using the tBlastX search protocol to determine whether the cDNA fragment sequences were homologous to any of the sequences included in the databases.

4.2.3 Northern hybridisation

Northern blots were prepared as described by Sambrook *et al.* (1989) using RNA isolated from *G. gracilis* (section 3.3.1). The RNA from these different time points (T = 0, 2, 4, 7, 11 and 18) was separated on a 1.1% agarose gel in 1 X TBE running buffer (Appendix A.3) and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, England) using an AE-6680 Genopirator apparatus (ATTO Corporation) according to the manufacturer's instructions (Appendix D.3). Spectrophotometric quantitation of RNA was performed (Appendix D.1) and about 3 μg of each RNA sample was loaded. DNA probes were radio-labelled with [α-^{32}P]dCTP using PCR labelling (Appendix D.8). Unincorporated nucleotides were separated from radio-labelled cDNA on a Sephadex G-50 spin column (Appendix D.9). RNA-DNA hybridisation was carried out at 65°C overnight on the transferred RNA using three radio labelled fragments (E15, C5 and C3) as probes (Appendix D.10). The same blot (Fig. 4.3a) was used for each of the three probes by removing the bound probe between hybridisations using stripping buffer (Appendix A.1; D.8).

4.3 RESULTS

4.3.1 Nucleotide sequencing of upregulated cDNA fragments

The cDNA fragments that were observed to be upregulated were sequenced in order to determine their identity. The origin of the fragments was confirmed by the observation that the nucleotide sequence at the ends of the fragments was that of the plasmid vector pDNR-LIB, which had been ligated to the cDNA fragments. The nucleotide sequences of the *G. gracilis* cDNA are shown in Fig. 4.1. Twenty-two upregulated cDNA fragments were found to be identical. These are A9, A22, B2, B9, B13, B24, C5, C21, C23, D6, D13, D14, D24, F5, F7, F12, G13, G16, H15, H17, H18 and H21 (Fig. 3.5). Identical sequences were excluded from further investigation except for a single representative sequence (C5).

The nucleotide sequences of the *G. gracilis* cDNA fragments were compared with sequences in the GenBank and European Bioinformatics Institute databases using the tBlastX search protocol in order to determine their identity (Fig. 4.2). The cDNA fragment C5 was significantly identical (90%) to the amino acid sequence of an expressed sequence tag (EST) from *G. gracilis* that Lluisma and Ragan (1997) reported. However, Lluisma and Ragan (1997) were unable to assign a function to the EST. E15 displayed 34.6% similarity to the amino acid sequence of the enzyme aspartate aminotransferase from *Lactococcus lactis*. Fragment B1 showed 88% amino acid similarity to an EST from the marine red alga *Porphyra yezoensis*. Fragment F2 displayed high levels of similarity (89%) to the amino acid sequence of a hypothetical protein from the red alga *Chondrus crispus*. C3 displayed 72% similarity to a cDNA clone from a soybean species obtained following bacterial infection and 84% similarity to a cDNA clone from the tsetse fly *Glossina morsitans* generated following infection by the parasite *Trypanosoma brucei*. Fragment D23

showed 62% identity to a cDNA clone from *Arabidopsis thaliana* that had been subjected to dehydration and cold stress and 59% similarity to a *Zea mays* (maize) cDNA clone that had been obtained from a plant that had been grown under drought conditions. The sequence analyses of all the upregulated cDNA clones are summarised in Table 4.1.

C5

```
1       GGCCATTACG GCCGGGGCTG GATATCCGTA CAGTCAATCA TCTAAACTTC
51      TTCAACCCTT ATCCTTCACA CCGTGTCAAC TCTCTCACCT TGTACTCCAA
101     AGCAACAATG GTCTCCACAG CTTTCGTCGC CGCCACCCCG GTCCTCTCCC
151     GCACTACAAC CTCTGTTGGC TCTACATCTT TCACATCCCG CCGCACTTGC
201     GTTAACCCAC CTCGTTGTTC GGCCACGCTC ATGATGGCCG ATAAAGAGAA
251     GATCCCTCAA GGCTTCTCAG CCTTCTCTGA AGTTCTCAAC GGTCGTGCCG
301     CTATGCTCGG ACTCGTACTT GCAATCACCA CCGAGGCCAT CACCGGAAAG
351     GGCATCATTG GCCAACTTGC TGCCCCTTGG GGATATCTCC GCCATTACGC
401     ACGCATTGGG ACTTTGGGCC TGACTGCCAC GCTGTGTATG CTACGTAGTG
451     AATCTAAATT GTATAAGTAA ATAAGCTTTC CTATACGAAA AAAAAAAAAA
501     AAAAAAAA
```

E15

```
1       GGGGTTGATC GACCAGGTGA CCAAGATTAA CGCCAACGAC GAGGCTTGCA
51      CCACGCACTT TGTGCAGGAG GCGGGTGTGA CGGCACTAAC CAACCCTGCT
101     GCCGCCAAGT TCACCAAGGA CATGGTGGCG CAACTGGAGG AACGTCGCGA
151     CGTGTTGTAC AAAGTGTTGA ACCAAGTACC TGGCTTCAAG GCCATCAAGC
201     CAAAGGCTAC GTTCTACATG ATGTGCAACG TGACGGAGGC TATGAGCAAG
251     ATGGGTATCA CTAACATCGA AGAGTTCAGA TCAAAGGTGT TGCAAAGTAC
301     CGGCGTGTCG TTCTGTACGA GAGCGCACTT TGGAACGCCT ATTCCGGGCG
351     AGACGGAGAT GTACGTGCGG TTTGCGTTCT CGGGGGTGAC GGTGGAGCAA
401     ATTGAGGCGG CTGGGAAGGC GCTCAAGCCG TTCCTTTCGC AGTTCTTCTA
451     GACGCTTAAT TTTAGTGTTG TGTACGTGTA GGTGATTTGA TGCCGTGCC
```

B1

```
1       GGCCATTACG GCCGGGGGGA GACGTTACCG TCGGTACCAC CGGTAGATAA
51      AACATGTTCG CGTTCGCCGC GTCGTTCACC CCATCACAAC TGCGTCGCAC
101     TGCGCTTGCG CCACCGACGT GCGCAGGCGC GCTGCGCCGT CATGGCTCGC
151     CGTTGCTCAC AACACAGCGC ACACGGCTTG TGCAACACGC CGCGCATGGG
201     CGTGATTCCG CCCGAGCCTT TCGCCGCTCT GCTGCTGAGC GCACGTGTCC
251     AGGCGTGAAC CGCAATCTCT TTTTCGCCAC CGCCTTTGTT GTTCTTTCCT
301     CACCTTCTGG GGCGGCATTT CCTTCGTCAA GGGCTCCACA AAGCCGCGAA
351     TCACGCAGGC GTCCTTCACA ACTCAGCCTG CCCTCCGCCG ACATTGCCAC
401     AGAAGGACCG CGCGCTACCT CATGGAGCAG CTCGTTCGTC GGCCGACCCG
451     CAAAAGGACT CCCGAACCGG CGTCATGACC TTCAGCGGCA AGGTGCGCGC
501     CAGCTCCAGT GTGGCTAGGC ATCTTGAGTG GCTGTGGCAA CCCAAGATGG
551     GCCTGATGGT CCTTGACGGT ACATTCTCCA ATTCATACTG CACACACACA
601     TTTCAAAGCC CAATATAGGG G
```

Fig. 4.1 Nucleotide sequences of six upregulated cDNA fragments designated C5, E15, B1, F2, C3 and D23. The designated name of each sequence corresponds to the coordinates marking the position of the clone in Fig. 3.4.

F2

```
1       GGCCATTACG GCCGGGGTAA TACTTCTGAG ATACCACTCA TTAAGCTTAG
51      TTTTTTACTT AATTTATAAA TAATATTATA TAAGACCGTT TAAAAGAGAA
101     AGTTTACTTG GGACGAGTGC CTCCTAAATA GTAACGGAGG TGTACAAAGG
151     TAAGTAACAG TTATTTATAA TAAATAATGC AGAATATAAT GATATAAACT
201     TGCTTGACAA TAAGATTTAT AAATCGAATT GCGACGAAAG TCAGTCATAG
251     TGACCCGATA TTAAAGCGTG GAATTGATAT CGTTTAACAG ATAAAAGGAA
301     CTCTAGGGAT AACAGATTCA TCGTGATTAA GAGTTCGTAT TGACGTCACG
351     GTTTGATACC TCGATGTCGA CTCATCTTAT CCTGAAATTG AAGTAGATTT
401     CAAGGGTCTA GTTGTTCGCT AGTGAAAAAG GTACGTGAGT TGGGTTCAGA
451     ACGTCGTGAG ACAGTTCGGT CCCTATCTAC TACAAAAAAA AAAAAAAAAA
501     AAAAAAAAAA AAA
```

C3

```
1       GGCCATTACG GCCGGGGGCT GAAAAAATAA TTTTCTAGAG TTTAAGAAAA
51      GATTATGGAA TTTTAATTGT AACAGTAAAA TGTTTTGATA TTTAAAAGAA
101     CCTCAATGGT GTAAACGATA ATCAGGAATA AAACTGACAC TGAGATATTA
151     AAGCGTGGGT AGCAAAAGGG ATTAGATACC CCTGTAGTCC ATGCCCTGAA
201     CGATGAGTGT TAATTTTTGA GTAATCAGAA ATAAAGCTAA CGCATTAAAC
251     ACTCCGCCTG GGAACTACGA TCGCAAGATT AAAACTCAAA GGAATTGACG
301     GGAACCCGCA CAAGCAGTGG AGCATGTGGT TTAAATCGAT AATACGCGCG
351     AAATCTTACC ATCTTTGAAT AGTGACAGGT GTTGCATGGC TGTCGTCAGT
401     CCGTGCTGTG AAGCGTTTGG TTCATTCCAC TAAACGGACA AA
```

D23

```
1       GGCCATTACG GCCGGGGAGA CCACTCGTTC ATCTCTTCGA CAAATCGACA
51      TCATCCACCT TGAACCACAA GTAGATACAG TTGTCAATTG ATCGAGTACG
101     AGATCAACAT GAGCTGGCGA GCAAGGCTCT CCACTAGCCT TAACGAACTG
151     CGGGTCGTTT ATTGCGCTAA CTCGCCGTCA AGCGTCGGTA CACGAGAGTT
201     TCTGAAGAAC AACTATGCAG ACCTTAAAGC TTTAAATCCC GGTCTCCCTA
251     TCTATGCCAG ACCCGCAGAT GGTGTTGAGC CCCATGTTGC GGCCCGATAC
301     GCTCGCGGGG TGTACGATGT AAAACGAACG TCGAATATGA CCGCAGACCA
351     AGTTTTGGGC ATTTTGAAAG AGTTCGAGGC CGCTGCCACT GTCGTGAATA
401     GCAGGGTTGG AAGTGGAACT TGGCGGGGAT GCCTTTAAAG CTCGCAGATG
451     TCGTTTAAGT GATCAAGGCT ATCTTAATTC TACGGAAGAG GTCGTGTTAG
501     TATGCTTTGC TCTGCGAAGG ATCCTGTCTG TACTTACATC GAATCCCTGT
551     TTTCCATGCT ACTCACCCCT TCCGCGTTTT TCTTGAAAGG TTATGAAATG
601     TAGGAATCAA TTCAGTACGC AGAAGTGGGC AGAACCACGG AGCACTTTTG
651     CAACGTTGAT ACTCCCATAT TCAAGTGCAT TAATCCGTAC TGAAAGGATG
701     AATTGGAAAA AAAAAAAAAA
```

Fig. 4.1 (cont'd).

```
C5              233     EKIPQGFSAFSEVLNGRAAMLGLVLAITTEAITGKGIIGQLAA   361
                        EKIPQGF  FSEVLNGRAAM G  LA  TEAITGKGI GQ
G. gracilis             EKIPQGFTGFSEVLNGRAAMMGFLLAVITEAITGKGILGQVGS


E15             8 LIDQVTKINANDEACTTHFVQEAGVTALTNPAAAKFTKDMVAQLEERRDVLYKVLNQVPG 187
                    I   TKI        T   Q A   A       K    M A  EER    Y  L  VPG
AspAT             AIAAMTKIASQTTSNPTAVAQYAAIEAFEE-INDKSFEKMHAAFEERLNKIYLQLSEVPG


B1              296   FLTFWGGISFVKGSTKPRITQASFT  370
                      FLT WGG SF KGS K R TQASFT
P. yezoensis          FLTAWGGLSFIKGSVKDRLTQASFT


F2              316 TCLTIRFINRIATKVSHSDPILKRGIDIV*QIKGTLGITDSS*LRVRIDVTV*YLDVDSS 495
                     CLTIR IN I T VSH DPI K GI IV QIKGTLGITDSS*LRVRIDVTV*YLDVDSS
C.crispus           SCLTIRLINQIGTQVSHNDPIFKCGINIVQQIKGTLGITDSS*LRVRIDVTV*YLDVDSS


C3              365     KMVRFRAYYRFKPHAPLLVRVPVNSFEF*SCDRSSQAECLMR*LY  231
                          VRF AYYR KPHAP LV  PVNSFEF SCD   QAECL R*L
soybean                 ELVRFCAYYRIKPHAPPLV*TPVNSFEFQSCDYTFQAECLTR*LL


C3              169     ANALNTPPGNYDRKIKTQRN*REPAQAVEHVV*IDNTREILPSLN  303
                        ANALN PPG Y RK KTQ N*R PAQAVEHVV* D TR  LP L
G. morsitans            ANALNRPPGEYNRKVKTQ N*RGPAQAVEHVV*FDATRT LPTLD


D23             109 MSWRARLSTSLNELRVVYCANSPSSVGTREFLKNNYADLKALNPGLPIYARPADGVEP  282
                    M WR   S S  ELR   C  SP S  TR F   NY DLK LNP LPI  R   GV P
A. thaliana         MAWRGSISKSMKELRILLCQSSPASAPTRTFVEKNYKDLKSLNPKLPILIRECSGVQP


D23             109  MSWRARLSTSLNELRVVYCANSPSSVGTREFLKNNYADLKALNPGLPIYARPADGVEP  282
                     M WRA LS SL E RV  C  SP S   REF K NYD  KA NP LP   R   GV P
Z. mays               MAWRASLSRSLKEIRVLFCQ SPASAAAREFVK NYGDIKARNPSLPLLVRECSGVQP
```

Fig. 4.2 tBlastX search protocol results of the upregulated cDNA fragments in the GenBank (C5, B1, F2, C3 and D23) and European Bioinformatics Institute databases (E15). Identical amino acids are depicted between the query sequence (top) and target sequence (bottom). Numbering refers to nucleotide positions within the DNA sequences presented in Fig. 4.1.

Table 4.1 Summary of the homology search of the GenBank and European Bioinformatics Institute databases.

Sequence name	Size (bp)	Homology to genes from	% identity
C5	508	*G. gracilis*	90
E15	499	AspAT from *Lactococcus lactis*	34.6
B1	621	*P. yezoensis*	88
F2	513	*C.crispus*	89
C3	442	soybean	72
C3	442	*Glossina morsitans*	84
D23	720	*A. thaliana*	62
D23	720	*Zea mays*	59

4.3.2 Northern hybridisation

Gracilaria gracilis RNA extracted from thallus fragments obtained at different time points during cultivation in nitrogen limited media (T = 0, 2, 4, 7, 11 and 18; section 3.3.1) were probed with radio labelled fragments E15, C5 and C3 in order to confirm that the cloned cDNA fragments represent mRNA transcribed from differentially expressed genes. The RNA gel that was used in the hybridisation experiments is shown in Fig. 4.3a. The amount of RNA loaded in each lane showed slight variation between the lanes. All three probes hybridised only to mRNA isolated from *G. gracilis* deprived of nitrogen for 18 days (T = 18), producing a positive hybridisation signal. No hybridisation signal was detected at T = 0, 2, 4, 7 and 11 (Figs. 4.3b; 4.3c and 4.3d). The remaining three cDNA clones (B1, F2 and D23) were not used as probes because stripping of the probe from

the membrane following the third hybridisation either damaged or removed the RNA samples from the membrane.

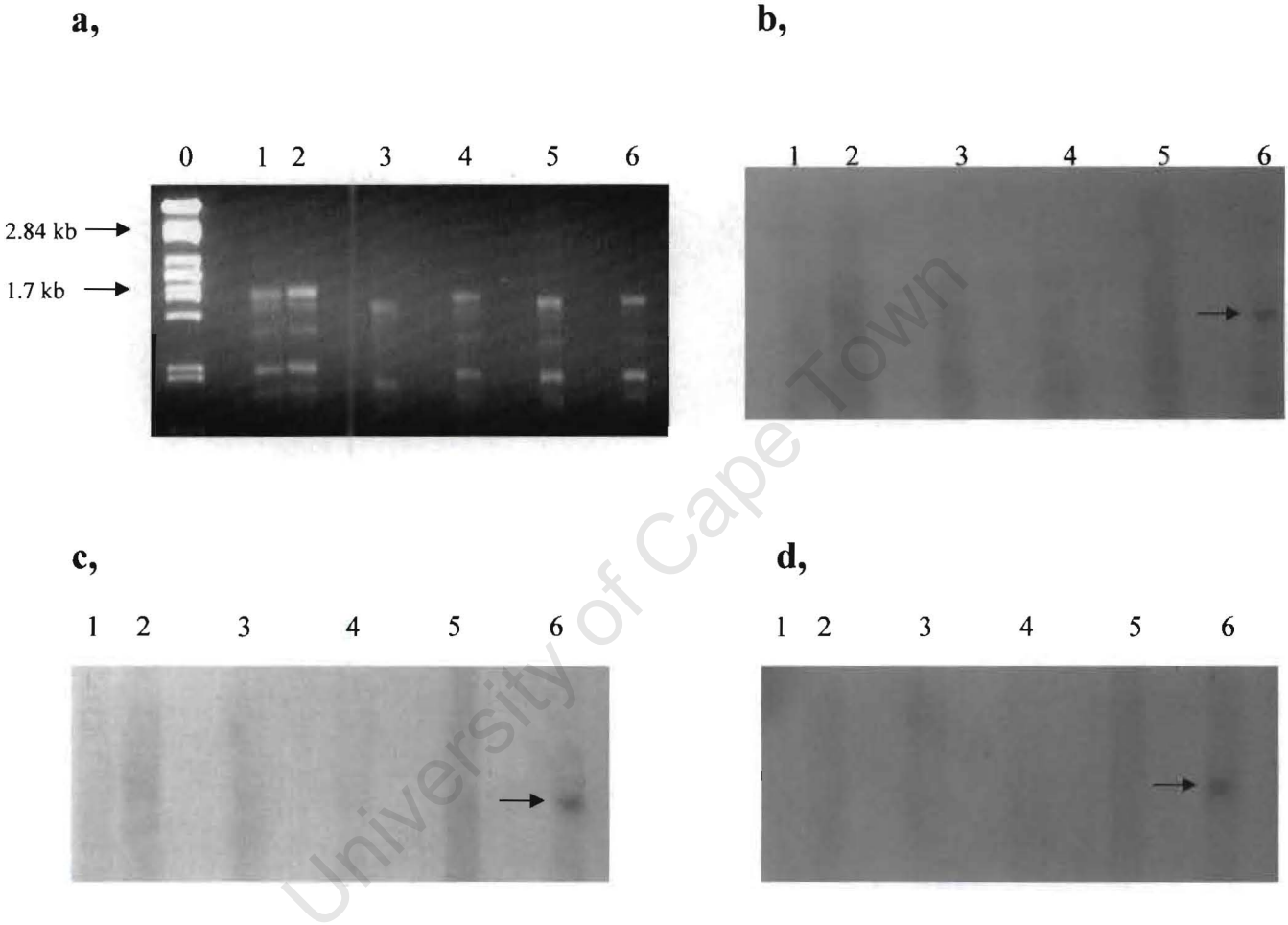

Fig. 4.3 Northern blot analysis of *G. gracilis* deprived of nitrogen for 0, 2, 4, 7, 11 and 18 days (lanes 1, 2, 3, 4, 5 and 6 respectively), RNA gel (a), membrane probed with cDNA fragments E15 (b), C5 (c) and C3 (d). Lane 0 contains λ DNA marker digested with *Pst*I. The arrows show positive hybridisation signals that are about 0.5kb in size.

4.4 DISCUSSION

A number of cDNA fragments were cloned and sequenced to determine whether they display any similarity to sequences in GenBank databases. The fact that twenty two of the sequenced cDNA fragments were identical indicates that that particular cDNA represents an especially abundant mRNA in nitrogen deprived *G. gracilis*. The deduced amino acid sequence of cDNA fragment E15 showed some homology to aspartate aminotransferase, a universally distributed enzyme critical for carbon and nitrogen metabolism. Aspartate aminotransferase (AspAT) catalyses the transamination reaction that produces aspartate, an important nitrogen transport compound in plants. Aspartate aminotransferase in plants is present in multiple isoenzymes. Taniguchi *et al.* (1995) have shown that while mRNA levels of two isoenzymes (cAspAT and mAspAT) decreased during nitrogen stress, mRNA levels of the isoenzyme pAspAT were higher in nitrogen stressed corn plants (*Panicum miliaceum*) than in healthy plants.

Fragment C3 was highly homologous to a cDNA clone generated from diseased soybean tissue following 48 hours of infection by the pathogen *Phytophthora sojae* (Qutob *et al.*, 2000). Even though the molecular characterisation of the gene that the soybean CDNA clone represents is not complete, Qutob *et al.* (2000) have indicated that it might be one of over 300 cDNA clones expressed in response to pathogen infection that encode pathogenesis-related or other defence-related proteins. Fragment C3 also showed 84% similarity to a cDNA clone from the tsetse fly *Glossina morsitans* following infection by the parasite *Trypanosoma brucei* that may represent an immune-related gene (Lehane *et al.*, 2003). Fragment D23 showed some amino acid homology to a cDNA clone from *Arabidopsis thaliana* that had been subjected to dehydration and cold stress (Asamizu *et al.*, 2000). Fragment D23 also showed some similarity to a *Zea mays* (maize) cDNA clone generated from tissue from a plant that had been grown under drought conditions. Living

organisms can show common responses to different types of stress (Morgan, 1979). It is possible that these cDNA fragments represent genes expressed in response to various abiotic stresses including nitrogen starvation.

Recently, EST (expressed sequence tag) analysis has become a commonly used approach to identify genes involved in stress and disease tolerance in marine organisms (Gross *et al.*, 2001; Jenny *et al.*, 2002; Gueguen *et al.*, 2003). Fragment C5 showed 90% identity to the amino acid sequence of an EST found in a small database for *Gracilaria gracilis* that Lluisma and Ragan (1997) generated. This database mostly consists of genes involved in cell wall biosynthesis and carbohydrate metabolism. However, since Inokuchi *et al.* (2002) have shown that nitrogen and carbon metabolic processes are closely co-ordinated, it is possible that the function of the gene represented by cDNA fragment C5 may indeed be relevant to nitrogen deprivation in *G. gracilis*.

Fragment B1 was homologous to an EST from the red alga *Porphyra yezoensis*. Most ESTs in the databases are functionally unassigned. However, since the GenBank databases are by no means complete, ESTs can be used as a start to further characterise and identify genes of unknown function. The cDNA clone F2 showed 89% similarity to a cDNA clone that represents a hypothetical protein from another red alga *Chondrus crispus* that Leblanc *et al.* (1995) found while performing an analysis of mitochondrial DNA sequences of the red algae.

Northern hybridisation was performed to confirm that the cDNA fragments were truly representative of mRNA that are relatively highly transcribed in nitrogen-deprived thalli. All the cDNA fragments tested (E15, C5 and C3) were found to hybridise to mRNA extracted from *G. gracilis* that was deprived of nitrogen for 18 days, showing that they indeed are differentially

regulated in response to nitrogen starvation. No hybridisation was observed to mRNA isolated from *G. gracilis* that had been deprived of nitrogen for up to 11 days. The tissue nitrogen content of *G. gracilis* at this time point was greater than 1.5% and dropped to less than 1% after 18 days of nitrogen starvation (Fig. 2.7). It is possible that these cDNA fragments represent a special set of genes that are upregulated when the plant is subjected to extreme levels of nitrogen stress.

It was shown in the previous chapter that cDNA clones E15, C5 and C3 showed some degree of hybridisation when probed with cDNA obtained from *G. gracilis* deprived of nitrogen for 7 days and even to cDNA obtained from nitrogen rich, day 0 thallus albeit to a lesser extent (Fig. 3.5). This, together with the negative hybridisation of the cDNA fragments to day 0 and day 7 RNAs (Fig. 4.3), suggests that the level of expression of the genes represented by cDNA fragments E15, C5 and C3 was very low until about 18 days of nitrogen deprivation.

The remaining three cDNA clones (B1, F2 and D23) were not used as probes because stripping of the probe following the third hybridisation either damaged or removed the RNA samples from the membrane. Even though northern analysis still has to be carried out in order to confirm that clones B1, F2 and D23 are indeed differentially expressed, the fact that all of the other three cDNA clones identified by differential screening (Fig. 3.5) hybridised to T = 18 RNA suggests it is highly likely that B1, F2 and D23 represent genes that are up-regulated in response to nitrogen deprivation stress in *G. gracilis*.

Chapter 5

GENERAL DISCUSSION

Nitrogen is an essential mineral comprising about 16% of total proteins in plants (Frink *et al.*, 1999) and its availability is a major limiting factor for growth and productivity. Survival of marine organisms in a changing environment relies on their capacity to modulate cell metabolism according to the external conditions and the ability and efficiency of the cell to utilise a particular nitrogen source. Seaweed species vary in their capacity to take up and store excess nitrogen. The red algae *Ceramium rubrum,* for example, has a nitrogen storage capacity of up to four times the concentration that is necessary to sustain a maximum growth rate (Lyngby, 1990). In the present study, *Gracilaria gracilis* was able to sustain growth on stored tissue nitrogen for the entire study period. This capacity to store nitrogen enables *G. gracilis* to remain productive in environments such as Saldanha Bay where there is seasonal variability in the nitrogen content of the water column. In fact Smit (2002) has suggested that the hydrodynamics of Saldanha Bay is such that the periodicity of nitrogen availability is well within the storage capacity of *G. gracilis* in that upwelling results in a nitrogen rich water column every 6-8 days.

The nitrogen storage capacity of *G. gracilis* also has implications for mariculture. Nitrogen supply is an important aquacultural investment worldwide. Plants absorb only about two-thirds of the applied nitrogen as fertliser and the unabsorbed nitrogen contaminates water supplies and leads to pollution of the environment (Frink *et al.*,

1999). A better understanding of the molecular and physiological basis of nitrogen uptake, storage and metabolism in *G. gracilis* may help develop strategies that reduce pollution without affecting *G. gracilis* growth and productivity. One strategy would be to introduce pulse feeding. Two important variables that regulate nutrient loading in a pulse fed culture system, namely concentration of nutrients during the pulse and frequency of nutrient pulses (Lapointe, 1985), should be determined prior to introducing pulse feeding. Smit *et al.* (1997) has shown that *G. gracilis* grows at non-nitrogen limiting rates at one pulse per week with 1200 μM ammonium nitrogen. Another important implication of the ability of *G. gracilis* to grow in a nitrogen-limited environment for mariculture is avoidance of competition. Pulse feeding limits the growth of competitive species that are unable to store nitrogen for extended periods of time.

Nitrogen in *G. gracilis* is mainly stored in pigments. During periods of nitrogen starvation de-pigmentation provides the nitrogen necessary for amino acid and protein synthesis in macroalgae. The loss of the pigments, which are primarily light harvesting organelles, also has an additional function in that excessive absorption of excitation energy is avoided in conditions in which cell metabolism is slow due to nitrogen limitation (Luque *et al.*, 2001).

The C:N value can be used as a useful indicator of past nitrogen levels in a water column (Lapointe and Ryther, 1979) and has generally been found to be more suitable for monitoring and detecting long term trends in nutrient availability and general changes in nutrient levels in coastal waters than water quality data (Lyngby, 1990). In the present

study, the C:N ratio started to increase as soon as the nitrate in the culture medium dropped to very low levels. However, the C:N value may not always be an accurate indicator of past nutritional history. A change in C:N ratio could at times be due solely to a shift in carbohydrate metabolism (Hanisak, 1979).

Recently, Lindell and Post (2001) developed a more sensitive protocol to determine the nitrogen status of the microalgal *Synechococcus* species. This protocol compares the transcription levels of n*tcA* as an indicator of nitrogen status. *NtcA* is a differentially expressed stress response gene that is involved in the response of a cell to changing nitrogen conditions in all blue green algae (Herrero *et al.*, 2001). In a similar way, analysis of the transcriptional levels of the genes representing the differentially expressed cDNA fragments identified in this study could be used in the future to determine the nitrogen status of *G. gracilis* in Saldanha Bay.

Various epiphytes use *G. gracilis* thalli as a suitable attachment surface. These epiphytes include bacteria capable of secreting enzymes such as agarase that are potentially harmful to *G. gracilis*. Agarolytic strains of bacteria isolated from *G. gracilis* from Saldanha Bay caused disease symptoms in a laboratory *G. gracilis* culture (Jaffray and Coyne, 1996). A number of die-offs of the natural populations of *G. gracilis* have occurred in Saldanha Bay during the summer months when the water column becomes oligotrophic and the water temperature increases (Jaffray *et al.*, 1997). However, in the present study, no disease symptoms were observed even though the macroalga was subjected to extreme levels of nitrogen deprivation. It is likely that multiple physical factors such as

temperature, nitrogen and other nutrients play a role in disruption of the equilibrium between *G. gracilis* and its bacterial epiphytes. The effect of all the above parameters would have to be fully tested on *G. gracilis* in order to gain a better understanding of the relationship of the macroalga and its epiphytic bacteria.

Very little is known about the effect of nitrogen availability on gene expression in macroalgae and how nitrogen induced changes in gene expression mediate changes in growth. Recent studies of nitrate responsive gene expression patterns in terrestrial plants show that many genes that encode for proteins involved in several cellular processes respond rapidly to changes in nitrogen (Wang *et al.*, 2000; Wang *et al.*, 2001). The cellular processes include metabolism and signal transduction. In the present study, genes that were up-regulated or down-regulated in response to nitrogen deprivation can be considered to be a subset of the nitrate responsive genes in *G. gracilis*. It is possible that some of the genes that are represented by the differentially expressed cDNA fragments are directly involved in the perception of nitrogen, while others are likely to be part of an overall stress signal transduction network. In addition, nitrate uptake in plants is highly regulated and coordinated with other transport and metabolic pathways (Crawford, 1995). Thus, while some genes may be directly responsive to nitrogen deprivation, others are likely to be indirectly responsive. In the future it will be crucial to characterise and assign the specific role each gene represented by the differentially expressed cDNA fragments plays. It would be more appropriate to start with the particular cDNA that was especially abundant during nitrogen deprivation in the present study and investigate the molecular mechanisms of the regulation of expression of the gene it represents. In addition,

differential screening of other cDNA clones that were not investigated in the present study is necessary to identify additional nitrogen stress related genes and to gain a better understanding of the effect of nitrogen deprivation on *G. gracilis* at the molecular level.

Recent strategies to improve nitrogen efficiency of plants involve developing transgenic plants. In this respect Vincent *et al.* (1997) successfully transferred cytosolic glutamine synthetase gene from soybeans to the plant *Lotus corniculatus* that resulted in early flowering.

Once the nitrogen deprivation stress resistance mechanisms in *G. gracilis* are well understood, the next step would be to attempt to genetically engineer commercially important macroalgae for increased resistance to stress. This may be particularly desirable in environments such as Saldanha Bay in that it may be possible to improve the stability of the *Gracilaria* harvest and avoid the total collapse of the population during the summer months.

APPENDIX

A. Media and solutions

A.1 Media

Artificial Sea Water (ASW)

NaCl	24.7 g
$MgCl_2.6H_2O$	4.7 g
KCl	0.66 g
$CaCl_2.2H_2O$	1.9 g
$MgSO_4.7H_2O$	6.3 g
$NaHCO_3$	0.18 g
Water to	1L

Autoclave

Fe-solution

$Fe(NH_4)_2(SO_4)_2.6H_2O$	702 mg
Na_2 EDTA	600 mg
Water to	1L

PII metal solution

Na_2 EDTA	100 mg
H_3BO_3	114 mg
$FeCl_3.6H_2O$	4.9 mg
$MnSO_4$	16.4 mg
$ZnSO_4.7H_2O$	2.2 mg
$CoSO_4.7H_2O$	0.48 mg
Water to	100 ml

PES-enriched seawater medium (1/3 strength) (Provasoli, 1968)

$NaNO_3$	350 mg
Na_2 glycerophosphate $5H_2O$	50 mg
Fe solution	25 ml
PII	25 ml
Vitamin B_{12}	10 µg
Thiamine	0.5 mg
Biotin	5 µg
Tris buffer (Sigma Co.)	500 mg
Water to	100 ml

Adjust pH to 7.8, autoclave and store at 10°C.
Add 6.6 ml to 1L ASW.

PES-N (ES medium lacking nitrogen)

Prepared exactly the same as ES medium except no $NaNO_3$ is added and $Fe_2(SO_4)$ instead of $Fe(NH_4)_2(SO_4)_2.6H_2O$ when making Fe-solution.

Luria broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Water to	1L

Autoclave

Luria agar

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15g
Water to	1L

Autoclave

Ψ broth

Tryptone	20 g
Yeast extract	5 g
MgSO4.7H2O	4 g
KCl	0.75 g
Water to	1 L
Autoclave	

A.2 Antibiotic cocktail for generating axenic *G. gracilis*

Antibiotic stock solutions were prepared in water, filter-sterilised through a 0.2 um filter (Millex, Millipore) and stored at 4°C. The following volumes were added to 500 ml ASW.

100 mg/ml streptomycin sulphate (Boehringer Mannheim)	2.5 ml
100 mg/ml penicillin G (Sigma)	2.5 ml
100 mg/ml kanamycin sulphate (Sigma)	2.5 ml
0.1 mg/ml nalidixic acid solution (Sigma)	3.0 ml

A.3 Buffers

Tris-Acetate-EDTA (TAE) buffer (50X stock)

Tris base (Roche)	242 g
Glacial acetic acid (Saarchem)	57.1 g
0.5 M EDTA (pH 8)	100 ml
Water to	1 L

Tris-Borate-EDTA (TBE) buffer (10X stock)

Tris base (Roche)	108 g
Glacial acetic acid (Saarchem)	55 g
0.5 M EDTA (pH 8)	20 ml
Water to	1 L

A.4 Solutions for Rubidium Chloride (RbCl) competent cell method

TFB 1

RbCl	1.21 g
$MnCl_2.4H_2O$	0.99 g
KOAc	0.294 g
$CaCl_2.2H_2O$	147 mg
50% Glycerol	30 ml

Adjust to pH 5.8 with Glacial Acetic acid, make to volume of 100 ml with dH_2O and filter sterilise.

TFB 2

MOPS	0.21 g
RbCl	0.121 g
$CaCl_2.2H_2O$	1.1 g
50% Glycerol	30 ml

Make to volume of 100 ml with dH_2O and filter sterilise.

A.5 Solutions for blotting and hybridisations

STE buffer

1 M Tris-HCl (PH 8)	1 ml
1 M Nacl	1 ml
0.5 M EDTA (PH 8)	200 µl
Water to	100 ml

Sephadex G-50

Sephadex G-50 (medium)	30 g
STE buffer	250 ml
Autoclave.	

Tracking dye

Blue dextran	3% in 50 mM NaCl
Orange G	1%

20 X SSC

NaCl	175 g
$C_6H_5Na_3O_7 \cdot 2H_2O$	88.2 g
Water to	90 ml

Adjust PH to 7.4 and make upto 1 L, add 2 ml DEPC and autoclave.

10% BSA

BSA	10 g
Water to	100 ml

Filter sterilise and freeze.

PB stock

$Na_2HPO_4.2H_2O$	89 g
85% H_3PO_4	4 ml
Water to	1 L

25% SDS

SDS	25 g
Water to	100 ml

Pre-hybridisation buffer

BSA (10 %)	10 ml
0.5 M EDTA	0.2 ml
1 M PB stock	50 ml
25% SDS	28 ml
Water to	100 ml

Wash buffer A

20 X SSC	10 ml
25% SDS	2 ml
Water to	100 ml

Wash buffer B

20 X SSC	2.5 ml
25% SDS	0.4 ml
Water to	100 ml

Stripping buffer

NaOH	8 g
SDS	10 g
Water to	1 L

B PRIMERS

B.1 Primers and oligonucleotides used in full length cDNA synthesis

SMART IV oligo	5'	AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG 3'
5' PCR primer	5'	AAG CAG TGG TAT CAA CGC AGA GT 3'
CDS III	5'	AAT CTA GAG GCC GAG GCG GCC GAC ATG (T)30 N-1 N 3'

B.2 Primers used in colony PCR and sequencing

M13(-21)-F	5'	TGT AAA ACG ACG GCC AG	3'
M13(-21)-R	5'	CAG CTA TGA CCA TGT TCA C	3'

B.3 Primers used in PCR labeling of DNA probes for northern blot analysis

Lib-F	5'	AAC GAC CGA GCG CAG CGA GTC	3'
Lib-R	5'	AGT CAT ACC AGG ATC TCC TAG	3'

C. PCR cycle profiles

C.1 PCR cycle profiles (for second strand synthesis)

1 cycle	denaturation	95°C	20 s
20 cycles	denaturation	95°C	5 s
	annealing	68°C	6 min

C.2 PCR cycle profiles (for colony PCR)

1 cycle	denaturation	96°C	5 min
25 cycles	denaturation	94°C	30 s
	annealing	61°C	30 s
	extension	95°C	1 min
1 cycle	extension	72°C	7 min

C.3 PCR labeling (for northern analysis)

1 cycle	denaturation	95°C	3 min
30 cycles	denaturation	95°C	1 min
	annealing	55°C	1 min
	extension	72°C	7 min
1 cycle	extension	72°C	10 min

D Standard Methods

D.1 Spectrophotometric quantitation of DNA and RNA

1. Perform A DNA or RNA scan of the DNA/RNA solution between 310 and 220 nm to determine the UV light absorbance of the sample.
2. The absorbance peak at 260 nm allows the calculation of the concentration of the DNA since 1 OD unit at 260 nm is equivalent to 50 µg/ml for double strand DNA and 40 µg /ml for single strand DNA or RNA.

D.2 Preparation of *E. coli* competent cells

1. Streak *E.coli* XL1 Blue onto a minimal media plate and incubte at 37°C, O/N.
2. Using a sterile loop, pick a single colony from the freshly streaked bacterial plate and resuspend in 5 ml Ψ broth (Appendix A.1).
3. Incubate at 37°C, O/N, as well as an uninoculated 200 ml Ψ broth.
4. Inoculate the starter culture (i.e. 5 ml O/N culture) into 200 ml pre-warmed Ψ broth.
5. Incubate at 37°C UNTIL OD_{550} reaches 0.35 (i.e. log phase and contains 3.5-4X10^7 cells/ml.
6. Chill the culture on ice for 15 min.
7. Centrifuge at 2 500 x g for 10 min at 4°C.
8. Resuspend cells in 21 ml ice cold TFB 1 (Appendix A.4) and incubate on ice for 90 min.
9. Centrifuge at 2 500 x g for 10 min at 4°C.
10. Resuspend cells in 4.5 ml ice cold TFB 2 (Appendix A.4).
11. Aliquot 100 µl of cells into 1.5 ml eppendorf tubes.
12. Cells may either be used immediately for transformation, or may be frozen for many months at –70°C.

D.3 cDNA blotting

(AE-6680 Genopirator apparatus, ATTO Corporation)

1. Immerse the gel in 0.25 N HCl solution in a clean tray for 15 to 20 min.
2. Discard the solution, pour denaturation solution (0.5 M NaOH, 1.0 M NaCl) and allow 20 to 25 min.
3. Overlay a porous sheet (Whatman paper) on the vacuum chamber.
4. Layer membrane that is cut according to the gel size, centering it on the vacuum seal.
5. Connect Genopirator pump to the vacuum chamber, gently pour 20 X SSC (Appendix A.5) and allow transfer for 40 to 60 min.

D.4 End labeling of 5' PCR primer

1. To a clean eppendorf tube, add 1 µg of 5' PCR primer (CLONTECH), 10 µl of 10 X T4 PNK buffer, 6 U T4 PNK enzyme.
2. Add 400 µCi [γ-^{32}P] dCTP and add nuclease-free water to a final volume of 100 µl.
3. Incubate the reaction mix at 37°c for 30 min, 65°c for 15 min and 4°c for 1 min.
4. Precipitate the labeled DNA using Ammonium acetate preciptation.

D.5 Prehybridisation, hybridisation and washing of Southern blots

1. Place cDNA blot in plastic container and add pre-hybridisation buffer (Appendix A.5)
2. Incubate at 65°C for three hours with agitation.
3. Denature DNA probe at 95°C for 5 min, place immediately on ice and add to the pre-hybridisation buffer.
4. Hybridise overnight at 65°C with agitation.
5. Wash membrane with wash buffer A (WBA) and wash buffer B (WBB) (Appendix A.5) and monitor the radioactivity between each wash on the membrane using a Geiger counter
6. Seal washed membranes in a new plastic bag and place in an X-ray cassette.
7. Expose the membrane to X-ray film and develop.

D.6 Stripping of Southern blots

Wash membrane using stripping buffer (Appendix A.5) for 30 min, monitor radioactivity using a Geiger counter and wash again if necessary.

D.7 Northern blotting

Done the same as cDNA blotting (Appendix D.3)

D.8 PCR labeling of DNA probes for northern blot analysis

Reaction Constituents	Final concentraton
Primers (Appendix B.3)	0.1 µM
DNTP mix (ATP, GTP and TTP)	2.5 mM
$MgCl_2$	1.5 mM
Taq polymerase buffer	1X
Template DNA	2 ng/µl
Supertherm Taq polymerase	1 U
[γ-^{32}P] dCTP	50 µCi

Adjust final volume to 50 µl with water and PCR (Appendix C.3)

D.9 Separation of unincorporated nucleotide from radiolabelled DNA

1. Plug the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool.
2. In the syringe prepare a column (0.9 ml bed volume) of Sephadex G-50 equilibrated in STE buffer (Appendix A.5) by adding Sephadex to the top of the syringe and then placing the syringe in a disposable bench top centrifuge tube in a bench top centrifuge.
3. Centrifuge the tube for 1 min at 1 600 x g.
4. Repeat steps 2 and 3 until a column bed volume of 0.9 ml is obtained.
5. Add 0.1 ml of STE to the column and re-centrifuge at the same speed as in step 3.
6. Prepare labeled DNA sample by adding 10 µl tracking dye (Appendix A.5) and 40 µl STE buffer.
7. Place an eppendorf tube (with cup removed) at bottom of bench top centrifuge tube so that the syringe will empty into the eppendorf tube.
8. Load the DNA to the column and centrifuge for 4 min. The tracking dye will move with the samples as follows: the blue Dextran will move with the DNA probe, while the Orange G will migrate with the unincorporated nucleotides on the column.
9. Determine the specific activity of the labeled DNA by counting 1 µl of probe in 2 ml of scintillation fluid.

D.10 Prehybridisation, hybridisation and washing of Northern blots.

Done the same as given on Appendix D.5.

D.11 Stripping of Northern blots

Done the same as Stripping of Southern blots (Appendix D.6).

REFERENCES

Abbott, I.A. 1988. Food and food products from seaweeds. In: Algae and Human Affairs (Lembi, C.A. and Waaland, J.R., eds.), pp. 136-147, Cambridge University Press, New York.

Abbot, I.A. 1996. Ethnobotany of seaweeds: clues to the use of seaweeds. *Hydrobiologia* **326/327**: 15-20.

Anderson, R.J., Simons, R.H. and Jarman, N.G. 1989. Commercial seaweeds in southern Africa: A review of utilisation and research. *South African Journal of Marine Science* **8**:277-299.

Anderson, R.J., Levitt, G.J., Keats, D.W. and Simons, R.H. 1993. The role of herbivores in the collapse of the Gracilaria resource at Saldanha Bay, South Africa. *Hydrobiologia* **260/261**: 285-290.

Anderson, R.J., Monteiro, P.M.S. and Levitt, G.J. 1996a. The effect of localised eutrophication on competition between Ulva lactuca and a commercial resource of Gracilaria verrucosa (Gracilariaceae, Rhodophyta). *Hydrobiologia* **326/327**: 291-296.

Anderson, R.J., Levitt, G.J. and Share, A. 1996b. Experimental investigations for the mariculture of Gracilaria in Saldanha Bay, South Africa. *Journal of applied Phycology* **8**: 421-430.

Andria, J.R., Vergara, J.J. and Perez-Llorens, J.L. 1999. Biochemical responses and photosynthetic performance of Gracilaria sp. (Rhodophyta) from Cadiz, Spain, cultured under different inorganic carbon and nitrogen levels. *European Journal of Phycology* **34**: 497-504.

Asamizu, E., Nakamura, Y., Sato, S. and Tabata, S. 2000. A large scale analysis of cDNA in Arabidopsis thaliana: Generation of 12,028 non-reduntant expressed sequence tags from normalized and size-slected cDNA libraries. *DNA Research* **7**: 175-180.

Bird, N.L., Chen, C.M. and McLachlan, J. 1979. Effects of temperature, light and salinity on growth in culture of *Chondrus crispus, Furcellaria lumbricalis, Gracilaria tikvahiae* (Gigartinales, Rhodophyta), and *Fucus serratus* (Fucales, Phaeophyta). *Botanica Marina* **22**: 521-527.

Bird K.T., Habig C. and DeBusk, T. 1982. Nitrogen allocation and storage patterns in Gracilaria tikvahiae (Rhodophyta). *Journal of Phycology* **18**: 344-348.

Bird, C.J. 1995. A review of recent taxonomic concepts and developments in the Gracilariaceae (Rhodophyta). *Journal of Applied Phycology* **7:** 255–267.

Bird, C.J. and Kain, J.M. 1995. Recommended names of included species of Gracilariaceae. *Journal of Applied Phycology* **7**: 335-338.

Bold, H.C. and Wynne, M.J. 1985. Introduction to the Algae. Prentice-Hall, New Jersey.

Breyne, P. and Zabeau, M. 2001. Genome-wide expression analysis of plant cell cycle modulated genes. *Current Opinions in Plant Biology* **4:** 136–142.

Chapman, V.J. 1970. Seaweeds and their uses. The Camelot Press Ltd., London.

Chapman, V.J. and Chapman, D.J. 1980. Seaweeds and their uses. Chapman and Hall, London.

Cooke, J.E.K., Brown, K.A. Wu, R. and Davis, J.M. 2003. Gene expression associated with N-induced shifts in resource allocation in poplar. *Plant, Cell and Environment* **26**: 757-770.

Coruzzi, G. and Bush, D.R. 2001. Nitrogen and Carbon Nutrient and Metabolite Signaling in Plants. *Plant Physiology* **125**: 61-64.

Crawford, N.M. 1995. Nitrate: nutrient and signal for plant growth. *Plant Cell* **7:** 859-868.

Darley, W.M. 1982. Algal Biology: A physiological Approach. Blackwell Scientific Publications, London.

DeBoer, J.A., Guigli, H.J., Israel, T.L. and D'elia, C.F. 1978. Nutritional studies of two red algae. Growth rate as a function of nitrogen source and concentration. *Journal of Phycology*. **14**: 261-266.

Draper, J., Scott, R., Armitage, P. and Walden, R. 1988. Vectors of the transformation of plant cell using agarobacterium, pp. 47. In: Plant genetic transformation and gene expression. A laboratory manual, Blackwell Scientific Publications.

Dring, M.J. 1982. The Biology of Marine Plants. Cambridge University Press, New York.

Frink, C.R., Waggoner, P.E. and Ausubel, J.H. 1999. Nitrogen fertilizer: retrospect and prospect. *Proceedings of the National Academy of Sciences* **96:** 1175-1180.

Fujita, R.M., Wheeler, P.A. and Edwards, R.L. 1988. Meatbolic regulation of ammonium uptake by Ulva rigida (Chlorophyta) a compartmental analysis of the rate-limiting step for uptake. *Journal of Phycology* **24**: 560-566.

Gantt, E. 1990. Pigmentation and Photoacclimation. In: Biology of the Red Algae (Cole, K.M. and Sheath, R.G., eds.) pp. 203-219, Cambridge University Press, New York.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapmann, R.W. and Warr, G.W. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Developmental and Comparative Immunology* **25**: 565-577.

Gueguen, Y., Cadoret, J.P., Flament, D., Barreau-Roumiguiere, C., Girardot, A.L., Garnier, J., Hoareau, A., Bachere, E. and Escoubas, J.M. 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. *Gene* **303**: 139-145.

Hanisak, M.D. 1979. Nitrogen limitation of Codium fragile ssp. Tomenntosoides as determined by tissue analysis. *Marine Biology* **50**: 333-337.

Happe, T. and Kaminski, A. 2002. Differential regulation of the Fe-hydrogenase during anaerobic adaptation in the green alga Chlamydomonas reinhardtii. *European Journal of Biochemistry* **269**: 1022-1032.

Harlin, M.M. and Darley, W.M. 1988. The algae: an overview. In: Algae and Human Affairs (Lembi, C.A. and Waaland, J.R., eds.), pp. 3-83, Cambridge University Press, New York.

Herrero, A., Muro-Pastor, A.M. and Flores, E. 2001. Nitrogen control in cyanobacteria. *Journal of Bacteriology* **183**: 411-25.

Hewitt, E.J. and Smith, T.A. 1974. Plant mineral nutrition. The English Universities Press Ltd, London.

Hildebrand, M. and Dahlin, K. 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (BACILLARIOPHYCEAE): mRNA levels controlled by nitrogen source and by the cell cycle. *Journal of Phycology* **36**: 702-713.

Hoek, C.Van der, Mann, D.G. and Jahns, H.M. 1995. Algae An introduction to Phycology. Cambridge University Press, New York.

Holmström, C., Egan, S., Franks, A., McCloy, S. and Kjelleberg, S. 2002. Antifouling activities expressed by marine surface associated Pseudoalteromonas species. *FEMS Microbiology Ecology* **41**: 47-58.

Inokuchi, R.I., Kuma, K., Miyata, T. and Okada, M. 2002. Nitrogen assimilating enzymes in land plants and algae: phylogenetic and physiological perspectives. *Physiologia Plantarum* **116**: 1-11.

Isaac, W.E. 1956.The ecology of *Gracilaria confervoids* (L.) Grev. In South Africa with special reference to its ecology in the Saldanha-Langebaan Lagoon. *Proclamation of International Seaweed Symposium* **2**: 173-185.

Jaffray, A.E. and Coyne, V.E. 1996. Development of an *in situ* assay to detect bacterial pathogens of the red alga Gracilaria gracilis (Stackhouse) Steentoft, Irvine et Fanham. *Journal of Applied Phycology* **8**: 409-414.

Jaffray, A.E., Anderson, R.J. and Coyne, V.E. 1997. Investigation of bacterial epiphytes of the agar producing red seaweed *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham from Saldanha Bay, South Africa and Luderitz, Namibia. *Botanica Marina* **40**: 569-576.

Jaffray, A.E. and Coyne, V.E. 1998. The identification and characterization of *Gracilaria gracilis* defense genes expressed in response to a bacterial infection. *In*: New

Developments in Marine Biotechnology (Gal, L. and Halvorson eds.), pp. 217-219, New York, USA.

Jaffray, A.E. 1998. The investigation of bacterial pathogens of the red macroalga *Gracilaria gracilis* and its response to bacterial infection. PhD Thesis. University of Cape Town, South Africa.

Jenny, M.J., Ringwood, A.H., Lacy, E.R., Lewitus, A.J., Kempton, J.W., Gross, P.S., Warr, G.W. and Chapmann, R.W. 2002. Potential indicators of stress response identified by expressed sequence tag analysis of hemocytes and embryos from the American oyster, *Crassostrea virginica. Marine Biotechnology* **4**: 81-93.

Jones, A.B., Preston, N.P. and Dennison, W.C. 2002. The efficiency and condition of oysters and macroalgae used as biological filters of shrimp pond effluent. *Aquaculture Research* **33:** 1-19.

Lapointe, B.E. and Ryther, J.H. 1979. The effects of nitrogen and seawater flow rate on the growth and biochemical composition of Gracilaria folifera var. angustisima in mass outdoor cultures. *Botanica Marina* **22**: 59-537.

Lapointe B.E. and Duke C.S. 1984. Biochemical strategies for growth of Gracilaria tikvahiae (Rhodophyta) in relation to light intensity and nitrogen availability. *Journal of Phycology* **20**: 488-495.

Lapointe, B.E. 1985. Strategies for pulsed nutrient supply to Gracilaria cultures in the Florida Keys: interactions between concentration and frequency of nutrient pulses. Journal of Experimental Marine *Biology and Ecology* **93**: 211-222.

Largo, D.B., Fukami, K., Nishijima, T. and Ohno, M. 1995. Laboratory induced development of the ice-ice disease of the farmed red algae Kappaphycus alvarezii and Eucheuma denticulatum (Solieriaceae, Gigartinales, Rhodophyta). *Journal of Applied Phycology* **7**: 539-543.

Leblanc, C., Boyen, C., Richard, O., Bonnard, G., Grienenberger, J. and Kloareg, B. 1995. Complete sequence of the Mitochondrial DNA of the Rhodophyte *Chondrus crispus* (Gigartinales). Gene content and genome organization. *Journal of Molecular Biology* **250**: 484-495.

Lee, H.M., Vazquez-Bermudez, M.F. and de Marsac, N.T. 1999. The global nitrogen regulator ntcA regulates transcription of the signal transducer PII (GlnB) and influences its phosphorylation level in response to nitrogen and carbon supplies in the *Cyanobacterium Synechococcus* sp. Strain PCC 7942. *Journal of Bacteriology* **181**: 2697-2702.

Lehane, M.J., Aksoy, S., Gibson, W., Kerhornou, A., Berriman, M., Hamilton, J., Soares, M.B., Bonaldo, M.F., Lehane, S. and Hall, N. 2003. Adult midgut expressed sequence tags from the tsetse fly *Glossina morsitans morsitans* and expression analysis of putative immune response genes. *Genome Biology* **4**: 63.1-63.10.

Levitt, J. 1980. Responses of plants to environmental stresses. Academic press, inc., New York.

Lewis, J.G., Stanley, N.F. and Guist, G.G. 1988. Commercial production and applications of algal hydrocolloids. In: Algae and Human Affairs (Lembi, C.A. and Waaland, J.R., eds.), pp. 206-236, Cambridge University Press, New York.

Liang, P. and Pardee, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*: **257**:967-971.

Lignell, A. and Pedersen, M. 1987. Nitrogen metabolism in Gracilaria secundata Harv. *Hydrobiologia* **151/152**: 431-441.

Lindell, D. and Post, A.F. 2001. Ecological aspects of *ntcA* gene expression and its use as an indicator of the nitrogen status of marine *Synechococcus*. *Applied and Environmental Microbiology* **67**: 3340-9.

Lindell, D., Erdner, D., Marie, D., Prasil, O., Koblizek, M., Gall, F.L., Rippka, R., Partensky, F., Scanlan, D.J. and Post, A.F. 2002. Nitrogen stress response of Prochlorococcus strain PCC 9511 (OXYPHOTOBACTERIA) involves contrasting regulation of ntcA and amt1. *Journal of Phycology* **38**: 1113-1124.

Lluisma, A.O. and Ragan, M.A. 1997. Expressed sequence tags (ESTs) from the marine red alga Gracilaria gracilis. *Journal of Applied Phycology* **9**: 287-293.

Lobban, C.S., Harrison, P.J. and Duncan, M.J. 1985. The physiological ecology of Seaweeds. Cambridge University Press, New York.

Luque, I., Zabulon, G., Contreras, A. and Houmard, J. 2001. Convergence of two global transcriptional regulators on nitrogen induction of the stress-acclimation gene nblA in the cyanobacterium Synechococcus sp. PCC 7942. *Molecular Microbiology* **41**: 937-947.

Lyngby, J.E., 1990. Monitoring of nutrient availability and limitation using the marine macro alga *Ceramium rubrum* (Huds.) C. Ag. *Aquatic Botany* **38**: 153-161.

Morgan, P.W. 1979. Hormone Systems. In: Stress responses in plants: Adaptation and acclimation mechanisms (Alscher, R.G. and Cumming, J.R. eds), pp. 128-138, Wiley-Liss, New York.

Muro-Pastor, M.I., Reyes, J.C. and Florencio, J.F. 2001. Cyanobacteria percieve nitrogen status by sensing intracellular 2-Oxoglutarate levels. *The Journal of Biological Chemistry* **276**: 38320-38328.

Naldi, M. and Wheeler, P.A 1999. Changes in nitrogen pools in Ulva fenestrata (Chlorophyta) and gracilaria pacifica (Rhodophyta) under nitrate and ammonium enrichment. *Journal of Phycology* **35:**70-77.

Naldi, M. and Wheeler, P.A. 2002. ^{15}N measurments of ammonium and nitrate uptake by Ulva fenestrata (Chlorophyta) and gracilaria pacifica (Rhodophyta): comparison f net nutrient disappearance, release of ammonium and nitrate, and ^{15}N accumulation in algal tissue. *Journal of Phycology* **38**: 135-144.

Okumura, T., Nagasawa, T., Hayashi, I. and Sato, Y. 2002. Effects of starvation on RNA : DNA ratio, glycogen content, and C : N ratio in columellar muscle of the Japanese turban shell *Turbo* (*Batillus*) *cornutus* (Gastropoda). *Fisheries Science* **68**: 306-312.

Oliveira, de, E.C. and Plastino, E.M. 1994. Gracilariaceae. In: Biology of Economic algae (Akatsuka, I., ed.), pp. 185-226, SPB Academic Publishing, The Hague.

Pearson, G., Serrao, E.A. and Cancela, M.L. 2001. Suppression subtractive hybridisation for studying gene expression during aerial exposure and desiccation in fucoid algae. *European Journal of phycology* **36**: 359-366.

Provasoli, L. 1968. Media and prospects for the cultivation of marine algae. In: Cultures and collections of algae (Watanabe, A. and Hattori, A. eds.), pp. 63-75, Proc. U.S.-Japan Conf., Hakone.

Provasoli, L. and Pintner, I.J. 1980. Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of Phycology* **16:** 196–201.

Qutob, D., Hraber, P.T., Sobral, B.W.S. and Gijzen M. 2000. Comparative analysis of expressed sequences in *Phytophthora sojae. Plant Physiology* **123**: 243-253.

Rosenberg, G. and Ramus, J. 1982. Ecological growth strategies in the seaweeds Gracilaria folifera (Rhodophyceae) and Ulva sp. (Chlorophyceae): soluble nitrogen and reserve carbohydrates. *Marine Biology* **66**: 251-259.

Rotmann, K.W.G. 1990. Saldanha Bay, South Africa: recovery of gracilaria verrucosa (Gracilaiales, Rhodophyta). *Hydrobiologia* **204/205**: 325-330.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning. A laboratory manual, 2nd edition, Cold Harbor Laboratory Press. Cold Spring Harbor, New York.

Sauer, J., Dirmeier, U. and Forchhammer, K. 2000. The *Synechococcus* strain PCC 7942 glnN product (Glutamine Synthetase III) helps recovery from prolonged nitrogen chlorosis. *Journal of Bacteriology* **182**: 5615-5619.

Schroeder, D.C., Jaffer, M.A. and Coyne, V.E. 2003. Investigation of the role of a β(1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. *Microbiology* **149**: 2919-2929.

Simons, R.H. 1996/1997. The algal flora of Saldanha Bay. *Transactions of the Royal Society of South Africa* **42**: 461-482.

Smit, A.J., Robertson, B.L. and du Preez D.R. 1997. Influence of ammonium-N pulse concentrations and frequency, tank condition and nitrogen starvation on growth rate and biochemical composition of Gracilaria gracilis. *Journal of Applied Phycology* **8**: 473-481.

Smit, A.J. 2002. Nitrogen uptake by Gracilaria gracilis (Rhodophyta): Adaptations to a temporally variable nitrogen environment. *Botanica Marina* **45**: 196-209.

Taniguchi, M., Kobe, A., Kato, M. and Sugiyama, T. 1995. Aspartate aminotransferase in *Panicum miliaceum* L., an NAD-malic enzyme type C_4 plant: comparison of enzymatic

properties, primary structures, and expression patterns. *Archives of Biochemistry and Biophysics* **318**: 295-306.

Thomas, T.E. and Harrison, P.J.1985. Effect of nitrogen supply on nitrogen uptake, accumulation and assimilation in Porphyra perforata (Rhodophyta). *Marine biology* **85**: 269-278.

Troell, M., Halling, C., Nilsson, A., Buschmann, A.H., Kautsky, N. and Kautsky, L. 1997. Integrated marine cultivation of Gracilaria chilensis (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. *Aquaculture* **156**: 45-61.

Uyenco, F.R., Saniel, L.S. and Jacinto, G.S. 1981. The 'ice-ice' problem in seaweed farming. In: Proc. Tenth Int. Seaweed Symp. (Levring, L., Ed.) pp. 625-630, Walter de Gruyter and co., Berlin.

Vincent, R., Fraisier, V., Chaillou, S., Limami, M.A., Deleens, E., Philipson, B., Douat, C., Boutin, J.P. and Hirel, B. 1997. Overexpression of a soybean gene encoding cytosolic glutamine synthetase in shoots of transgenic *Lotus corniculatus* L. plants triggers changes in ammonium and plant development. *Planta* **201:** 424–433.

Von Stein, O.D., Thies, W. and Hofmann, M. 1997. A high throughput screening for rarely transcribed differentially expressed genes. *Nucleic Acids Research* **25**: 2598-2602.

Vos, P., Hogers, R., Bleeker, M., Reijans, M. van de L.T., Horness, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Wakibia, J.G., Anderson, R.J. and Keats, D.W. 2001. Growth rates and agar properties of three gracilarioids in suspended open-water cultivation in St. Helena Bay, South Africa. *Journal of Applied Phycology* **13**:195-207.

Wang, R., Guegler, K., LaBrie, S.T. and Crawford, N.M. 2000. Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *Plant Cell* **12**: 1491-1509.

Wang, Y., Garvin, D.F. and Kochian, L.V. 2001. Nitrate-induced genes in tomato roots. array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology* **127**: 345-359.

Wilson, J. 1994. The role of bioindicators in estuarine management. *Estuaries* **17**: 94-101.

Zeng, Y. and Yang, T. 2002. RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Molecular Biology Reporter* **20**: 417a-417e.

Zhang, H. and Forde, B.G. 1998. An Arabidopsis MADS box*Science* **9:** 407-409.
Gene that controls nutrient induced changes in root architecture.

Zhu, J.K. and Xiong, L. 2001. Abiotic stress signal transduction in plants: Molecular and genetic perspectives. *Physiologia Plantarum* **112**: 152-166.