INVESTIGATION OF THE *GRACILARIA GRACILIS* PROTEOME RESPONSE TO NITROGEN LIMITATION

By

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List of Abbreviations

α	alpha
β	beta
°C	degrees Celsius
µg	microgram(s)
µl	microlitre(s)
µm	micromole(s)
µmol	micromolar

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ALD	Fructose 1, 6, biphosphate aldolase
AS	Asparagine synthetase
ASB 14	Amidosulfobetaine-14
Asn	Asparagine
Asp	Aspartate
AspAT	Aspartate aminotransferase
ASW	Artificial seawater

BLAST	Basic local alignment search tool
BSA	Bovine Serum Albumin

C	Carbon
CO_2	Carbon dioxide
CBB	Calvin Benson Basham
CBB	Coomassie Brilliant Blue
cyt *b6/f*	cytochrome *b6/f*

DNPH	2,4 dinitrophenylhydrazine
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EF tu	Elongation factor tu
ES	Enriched seawater
ESI	Electrospray ionisation
ESTs	Expressed sequence tags

FAO	Food and Agriculture Organization (of the United Nations)
FBP	Fructose biphosphate aldolase
Fd	Ferredoxin
FNR	Ferredoxin NADP reductase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
Glu	Glutamate
Gln	Glutamine
GS	Glutamine synthetase
GOGAT	Glutamate synthase
I	Initial
ICAT	Isotope-Coded Affinity Tags
IEF	Isoelectric focussing
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantification
K	Potassium
kDa	Kilodalton(s)
L	Litre(s)
LC	Liquid chromatography
LEA	Late Embryogenesis abundant
MALDI	Matrix-assisted laser desorption/ionisation
mg	milligram(s)
Mg	Magnesium
min	minute(s)
ml	millilitre(s)
mRNA	Messenger RNA
MS	Mass spectrometry
MW	molecular weight
N	Nitrogen
NAD-ME	NAD Malic acid
NCBI	National Centre for Biotechnology Information
NCBInr	National Centre for Biotechnology Information non-redundant
NH_4^+	Ammonium
NiR	Nitrite reductase
NO_2	Nitrite
NO_3^-	Nitrate
NR	Nitrate reductase

NRA	Nitrate reductase activity
2-OG	Two oxo-glutarate
OPP	Oxidative Pentose Phosphate
P	Phosphorus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phycobilisome
PC	Phycocyanin
PE	Phycoerythrin
PEP	Phosphoenolpyruvate
PEPc	Phosphoenolpyruvate carboxylase
PES	Provasoli's enriched seawater
PGK	Phosphoglycerate kinase
PK	Pyruvate kinase
PR	Pathogenesis-related
PS I	Photosystem I
PS II	Photosystem II
PTMs	Post-translational modifications
R	Recovery
RNA	Ribonucleic acid
rpm	revolutions per minute
Rubisco	Ribulose biphosphate carboxylase
S	Sustained
SAB	Sample Application Buffer
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide dismutase
2D	Two-dimensional
2-DE	Two dimensional gel electrophoresis
TCA	Tricarboxylic acid cycle
TIFF	Tagged Image File Format
Tris	tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
u	Unit
v	Volts

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ABSTRACT

Gracilaria gracilis, a commercially important red macroalgal species found on the west coast of South Africa, is one of two *Gracilaria* species used in the production of hydrocolloid agar. In the past, commercial quantities of *G. gracilis* were harvested from Saldanha Bay until population collapses of the natural resource necessitated the need for research into alternative aquaculture programs for *G. gracilis* cultivation. One of the reasons for the *G. gracilis* population collapse was attributed to adverse conditions during summer which led to thermal stratification of the water column and subsequent nutrient limiting conditions. Inorganic nitrogen has been identified as the major nutrient factor limiting growth and production of *G. gracilis* populations in South Africa. Although the physiological mechanisms implemented by *G. gracilis* which allow adaption to low nitrogen environments have been investigated, not much is known about the molecular mechanisms which underlie these adaptions. Thus, it is necessary to elucidate the molecular basis of these adaptions in *G. gracilis* to complement the existing physiological data.

In this study, a differential proteomics approach employing two-dimensional gel electrophoresis and liquid chromatography tandem mass spectrometry was used to investigate *G. gracilis* proteome changes in response to nitrogen limitation and recovery. Putative identities of 22 proteins that were changed significantly ($P<0.05$) in their abundance in response to nitrogen limitation and recovery were obtained. The identified proteins functioned in a range of biological processes including glycolysis, photosynthesis, ATP synthesis, galactose metabolism, protein-refolding and biosynthesis, nitrogen metabolism and cytoskeleton remodelling. A detailed discussion proposing the possible role(s) of each putative *G. gracilis* protein, along with the associated metabolic pathways activated or repressed in response to N stress, is provided.

Fructose 1, 6 biphosphate aldolase and glutamine synthetase were selected for further validation and characterisation studies. The identities of these enzymes were confirmed by western blot analysis. Additionally, the decreased abundance of fructose 1, 6 biphosphate aldolase during nitrogen limitation observed with two-dimensional gel electrophoresis analysis was validated by enzymatic and western blot analysis. The over-expression of fructose 1, 6 biphosphate aldolase and glutamine synthetase determined with two-dimensional gel elctrophoresis during nitrogen recovery was not validated by western blot analysis. Re-supply of nitrogen to nitrogen limited *G. gracilis* elicited small changes in protein abundance that were not significant ($P<0.05$) due to the large degree of biological variation between seaweed samples. The effect of nitrogen limitation and recovery on the enzyme activity of pyruvate kinase was investigated. The enzyme activity of pyruvate kinase, a key enzyme integrating carbon and nitrogen metabolic pathways, did not respond to nitrogen limitation and recovery. The effect of nitrogen limitation on *G. gracilis* carbon allocation patterns was briefly investigated. Intracellular *G. gracilis* starch levels were observed to markedly increase during nitrogen limitation. This increase, combined with the decreased abundance of a number of glycolytic proteins, suggests that a change in carbon allocation patterns occurs in *G. gracilis* in response to nitrogen limitation.

To the best of our knowledge this study provides the first characterisation of *G. gracilis* proteome changes in response to nitrogen limitation and subsequent recovery. The identification of key proteins and pathways involved in the *G. gracilis* nitrogen stress response has provided an insight into the adaptions implemented by *G. gracilis* to cope with nitrogen limitation. The results of this study reinforce the relationship between carbon and nitrogen metabolism since the majority of the identified proteins are involved in glycolytic and photosynthetic processes.

CHAPTER 1

General Introduction

CONTENTS

1.1 Seaweed Mariculture

Aquaculture is the aquatic counterpart of agriculture and has grown substantially in the last thirty years to become one of the leading food industries producing fish, molluscs, crustaceans and seaweeds (Kautsky *et al.*, 2001; Titlyanov & Titlyanova, 2010). Seaweed mariculture, which originated in Japan, constitutes a large sector of this market and currently produces more than 92% of the market demand (Chopin & Sawhney, 2009). Mariculture of a particular species develops when the supply obtained from natural stock populations is no longer able to support the increasing demand for that species. Consequently, the increased demand leads to overexploitation of natural populations and mariculture of the appropriate species provides a solution to this problem (Chopin & Sawhney, 2009). The major goal of any successful mariculture system would be to optimise production, by improving the conditions of the natural environment from which the species being cultivated was obtained. This can be accomplished by improving the quality of the algal material and by creating an artificial environment which promotes optimal growth (Lobban & Harrison, 1997). Presently, the seaweed aquaculture sector represents 45.9% of the biomass and 24.2% of the value of the world mariculture production. In 2004, the seaweed aquaculture industry was estimated at approximately 11.3 million tonnes with an approximate value of US$ 5.7 billion (Chopin & Sawhney, 2009). Seaweed aquaculture production is conducted predominantly in Asian countries and these countries contribute 99.7% of the value of the industry. Brown seaweeds account for approximately 63.8% of production while red and green seaweeds represent 36% and 0.2%, respectively. Worldwide there are approximately 220 species of seaweed currently cultivated. However, only six genera, namely *Laminaria, Undaria, Porphyra, Eucheuma/Kappaphycus* and *Gracilaria,* represent the vast majority (94.8%) of cultivated seaweed production (Chopin & Sawhney, 2009).

Seaweed cultivation research studies have been focussed mostly on the characterisation of the life cycle of these algae and the impact of environmental stressors on their growth and cultivation (Oliveira & Plastino, 1994; Wilson & Critchley, 1997; Smit & Bolton, 1999). However, just as successful agriculture practices require an understanding of vascular-plant ecology and physiology, successful mariculture depends on an extensive basic knowledge of the biology and physiology of the seaweeds under cultivation (Lobban & Harrison, 1994, Phillips, 2009). Furthermore, seaweed production, contributes a major component to the mariculture industry and it is important to determine how factors important to seaweed growth can be manipulated to improve yields (Lobban & Harrison, 1994).

1.2 The Genus *Gracilaria*

Seaweeds can be broadly classified into three groups on the basis of their colour, namely Phaeophyceae (Brown), Rhodophyceae (Red) and Chlorophyceae (Green). Brown seaweeds are usually large and range in size from giant kelps (up to 20 m in length) to thick, leather-like seaweeds (2-4 m in length) and to smaller species (30 – 60 cm in length). Red seaweeds are usually smaller, generally ranging from a few centimetres to about a metre in length and sometimes they can be purple or brownish red in colour. Green seaweeds are in a similar size range to the red seaweeds.

The Rhodophyta are a large, morphologically diverse group of macroalgae comprised of more than 700 genera and 600 species (Chapman *et al.*, 1998). *Gracilaria* are classified as red macroalgae within the phylum Rhodophyta, class Florideophyceae, order Gracilariales and family Gracilariaceae. They are generally described as macroscopic algae ranging between 0.1 and 5 m in length. Morphologically they consist of highly branched filaments, with a cylindrical to flattened sometimes leafy, solid thallus of pseudoparenchyma (Fig. 1.1) (Oliveira *et al.,* 2000). They are generally reddish brown in colour but can manifest in yellow, green and black forms due to the presence of the photosynthetic pigments phycoerythrin, phycocyanin and allophycocyanin.

Gracilaria has become an important commercial agarophyte contributing approximately 70% of the raw materials required for the production of hydrocolloid agar (McHugh, 2002). *Gracilaria* is the main alga used in the production of agar as it can be cultivated successfully. The two main *Gracilaria* species of commercial interest, based on agar yield and quality, are *Gracilaria chilensis* and *Gracilaria gracilis* (Oliveira *et al.*, 2000).

Figure 1.1 *Gracilaria gracilis* indicating the distinctive red colour and highly branched morphology of the thalli indicative of a *Gracilaria* species of seaweed (*G. gracilis* was obtained from "Irvin & Johnson Abalone Culture Division", Danger Point, Gansbaai in the Western Cape, South Africa).

The most widely known application of agar is as a medium to culture microorganisms. However, agar is also used extensively on a large scale in a variety of commercial applications. These applications of agar depend on its ability to gel, emulsify and stabilise in combination with other products and additives. Agar obtained from *Gracilaria* species is able to blend with sugars and not lose gel strength and is therefore highly sought after in the food production industry (Abbot, 1996). Furthermore, agar can be used as adhesives, in cosmetics, for textile dyeing and in paper coating. Within the pharmaceutical industry agar is utilised in laxatives, tablets and capsules, radiological solutions as a suspending agent and as anti-coagulants (Lewis *et al.*, 1988). Worldwide, there are 30 known agar producers and it is estimated that there may be 20 other small producers (FAO, 2003). However, the on-going development of the agar industry is relatively slow and the estimated growth rate has been 1–2% per annum for the last thirty years (FAO, 2003). Furthermore, the increasing demand for agar has resulted in overexploitation and depletion of natural stocks in Taiwan, Chile, Brazil, the Phillipines and Thailand (Armisen, 1995). Cultivation of *Gracilaria* species is therefore the only means of meeting the growing demand for agar (Oliveira *et al.*, 2000).

Presently, gracilaroids are also cultivated as a human food source (mostly in salads and soups), as bioremediation agents for waste water treatment (Fralick *et al.*, 1981) and for biomass production to produce energy (Ryther *et al.*, 1979; Hanisak & Ryther, 1986; Flowers

& Bird, 1990). The use of gracilaroids as abalone feed is on the increase in South Africa. Abalone farmers have begun using a mixed diet of kelp, *Ulva* and gracilaroids as this combination results in better growth rates for the abalone (Naidoo *et al.*, 2006; Troell *et al.*, 2006).

1.3 Habitat and distribution of *Gracilaria*

The genus *Gracilaria* is widely distributed geographically and the majority of species are reported to be found in warm-water, tropical regions (Oliveira *et al.*, 2000). However, a few species such as *Gracilaria chilensis* and *Gracilaria gracilis* expand their distribution into temperate waters (Oliveira & Plastino, 1994). *Gracilaria* species grow optimally at temperatures ranging from 20 to 25°C (Critchley, 1993, Oliveira & Plastino, 1994), while certain *Gracilaria* species survive temperatures from 35°C (Yokoya & Oliveira, 1992) down to freezing, and can withstand being frozen for a few months (Titlyanov *et al.*, 1995). Most gracilaroid algae are found in the lower intertidal zone in areas not subject to strong surf conditions (Oliveira *et al.*, 2000). *G. gracilis* populations have been identified in Europe, Canada, Argentina, Namibia and South Africa (Oliveira *et al.*, 2000).

In South Africa red algae are found from mid tide to below the low tide mark (Chapman, 1970). In South Africa natural populations of the 'stringy', terete members of the Gracilariaceae have been found to occur within Saldanha Bay and the Langebaan lagoon system, Hout Bay, the Olifants, Keurbooms and Swartkops river mouths and along the Western and Southern coasts of St. Helena Bay (Isaac, 1956; Simons, 1977; Stegenga *et al.*, 1997; Iyer *et al.*, 2004). These terete gracilarioids can be found loosely attached in sand and mud substrata (Isaac, 1956; Simons, 1977) which is consistent with conditions found in Canada (Mclachlan & Bird, 1986), Chile (Santelices & Ugarte, 1987) and Namibia (Molloy & Bolton, 1995).

The Saldanha Bay and Langebaan lagoon system (Fig. 1.2) and St. Helena Bay are the only two areas which have in the past yielded commercial quantities of Gracilaroids. However, commercial quantities are no longer harvested here due to the collapse of natural stock populations. Currently, these are protected areas and harvesting is no longer permitted to protect natural stocks of gracilaroids within these systems (Rothman *et al.*, 2009).

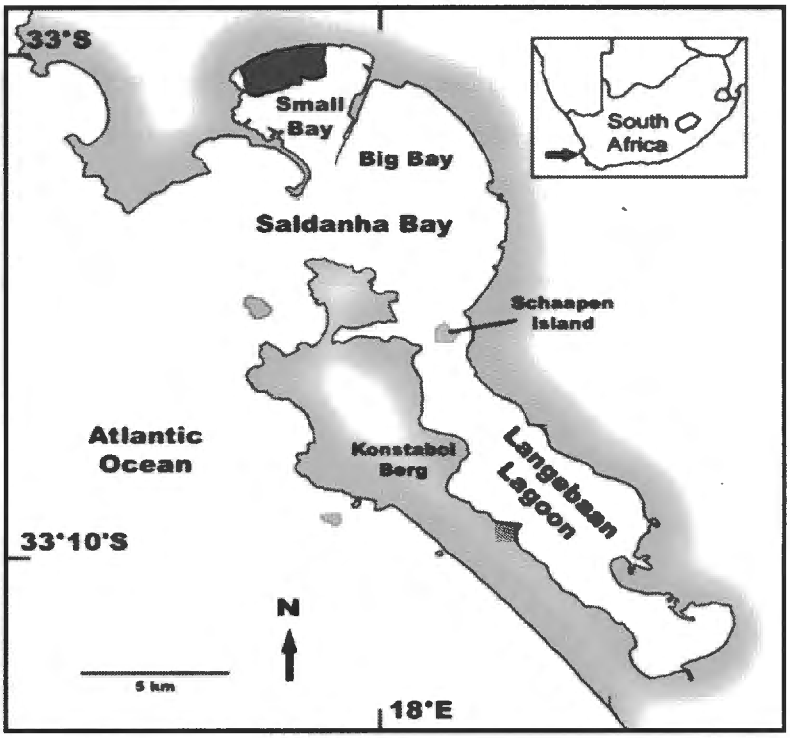

Figure 1.2 A map of the Saldanha Bay and the Langebaan lagoon system on the West coast of South Africa. Small Bay is enclosed by a breakwater and ore-jetty. The dark patch in Small Bay represents the distribution of gracilarioid algae within the Saldanha Bay and the Langebaan lagoon system (Rothman *et al.*, 2009).

1.4 *Gracilaria* cultivation in South Africa

Cultivation of *Gracilaria* species is an attractive option as they are some of the most useful algae in the world due mainly to their ability to anchor their thalli in soft substrata, fast growth rate, ease of vegetative reproduction and good agar yields (Buschmann *et al.,* 1995; Kain & Destombe, 1995). Furthermore, *Gracilaria* cultivation requires relatively simple equipment and production technologies which make farming of *Gracilaria* a commercially viable option (Phillips, 2009).

The commercial interest in gracilaroids in South Africa started during World War II when agar supplies from Japan became unavailable in Britain (Anderson *et al.,* 1989). The coast of South Africa has the potential to support the growth of a wide range of commercial seaweeds and commercial quantities of *G. gracilis* were harvested in Saldanha Bay, prior to the construction of an ore jetty in 1974 (Fig. 1.3). *Gracilaria* harvests in South Africa were mainly based on the collection of beach-cast material (Anderson *et al.,* 1989; Rotmann, 1990) while very small amounts were harvested from attached natural populations (Anderson *et al.*, 1989).

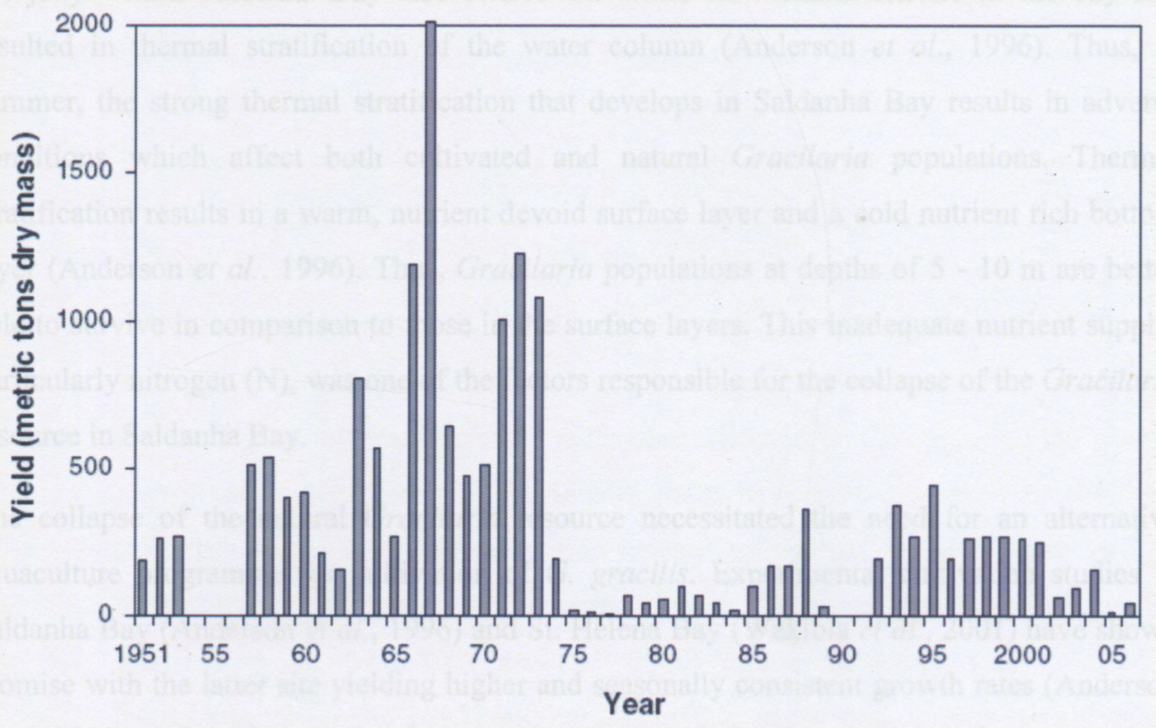

Figure 1.3 Commercial yields of *Gracilaria* harvested from Saldanha Bay 1951 – 2006 showing the large population collapses after construction of the ore jetty in 1974 (Rothman *et al.*, 2009).

The first trial production involving the extraction of agar from *Gracilaria* in South Africa was conducted in 1951 and resulted in the production of approximately 20 tonnes of agar (Anderson *et al.*, 1989). These yields were sustained by two factories in the area until 1974 when huge *G. gracilis* population collapses were experienced (Anderson *et al.*, 1989, Rotmann, 1990). *Gracilaria* was processed for agar up until 1974 but the subsequent collapse of the population and unreliable yields prevented the development of a sustainable industry (Anderson *et al.*, 1996). Prior to this, yields of over 1,000 tonnes dry weight were obtained (Anderson *et al.*, 1989). In the 1980s, beach-cast yields of *G. gracilis* began to recover, but in 1989 the natural *G. gracilis* resource collapsed again. As a result beach casts were not observed in 1990 and 1991, and underwater surveys suggested that *Gracilaria* had almost disappeared from Saldanha Bay and that only fragments of thalli remained (Anderson *et al.*, 2003). It was postulated that the *G. gracilis* population collapse was related to the presence of oligotrophic water in summer, the presence of numerous grazers and possibly degradation of the thalli by superficial bacteria (Jaffray *et al.*, 1997). The construction of the breakwater and ore jetty within Saldanha Bay also altered the water flow characteristics in the bay and resulted in thermal stratification of the water column (Anderson *et al.*, 1996). Thus, in summer, the strong thermal stratification that develops in Saldanha Bay results in adverse conditions which affect both cultivated and natural *Gracilaria* populations. Thermal stratification results in a warm, nutrient devoid surface layer and a cold nutrient rich bottom layer (Anderson *et al.*, 1996). Thus, *Gracilaria* populations at depths of 5 - 10 m are better able to survive in comparison to those in the surface layers. This inadequate nutrient supply, particularly nitrogen (N), was one of the factors responsible for the collapse of the *Gracilaria* resource in Saldanha Bay.

The collapse of the natural *Gracilaria* resource necessitated the need for an alternative aquaculture programme for cultivation of *G. gracilis*. Experimental cultivation studies in Saldanha Bay (Anderson *et al.*, 1996) and St. Helena Bay (Wakibia *et al.*, 2001) have shown promise with the latter site yielding higher and seasonally consistent growth rates (Anderson *et al*, 2003). Originally gracilaroids were harvested, dried and exported to foreign markets before being processed for agar; however the success of this industry relies on local processing and regular export. This is no longer viable on a commercial basis due mainly to unreliable yields of *G. gracilis*. Research into large-scale *Gracilaria* cultivation is on-going, as the development of a successful aquaculture industry would be commercially profitable and could lead to the development of a sustainable agar production industry in South Africa. However, a better understanding of the factors which have resulted in the collapse of this natural resource and the molecular responses of the seaweed to these factors is required, in order to prevent similar collapses within commercial aquaculture facilities.

1.5 Abiotic and biotic factors controlling *Gracilaria* cultivation

There are a number of abiotic and biotic factors which influence cultivation of *Gracilaria* and these have been comprehensively reviewed by Oliveira *et al.* (2000). These factors need to be monitored and controlled if *Gracilaria* cultivation is to be successful. The most important factors are as follows:

i) The presence of epiphytic algae in *G. gracilis* cultivation systems poses a huge problem. Epiphytic algae reduce the growth of their macroalgal hosts by competing for light, nutrients and increasing the mechanical drag on plants (Oliveira *et al.*, 2000). The most common epiphytes on gracilaroids are green algal species including *Cladaphora, Enteromorpha* and *Ulva*. More specifically, red algal epiphytism is generally associated with species belonging to the genera *Polisiphonia* and *Ceramium.* Various methods have been employed successfully to control epiphyte infestations within seaweed cultivation systems. These include treatment with copper chloride, immersion in freshwater for 5-10 min before planting out the thalli (Oliveira *et al.*, 2000), the use of grazers such as isopods (Shacklock & Doyle, 1983) and control of the supply of nutrients to cultivation tanks (Hanisak, 1987).

ii) The grazing of seaweed thalli by fish and invertebrates has been shown to cause damage in natural and cultivated beds of *Gracilaria.* It was one of the factors which contributed to the collapse of the natural *Gracilaria* population in Saldahna Bay. The resulting damage was attributed to grazing by herbivorous fish, keyhole limpets and urchins (Anderson *et al.*, 1993). However, this can be overcome in seaweed mariculture systems through raft and pond cultivation strategies (Oliveira *et al.*, 2000).

iii) Disease outbreaks are frequent occurrences in seaweed populations. The problem is intensified by environmental stressors which make *Gracilaria* more susceptible to disease. Typical symptoms of disease include whitening and necrosis of the thalli. These are associated with both natural and farmed populations of

Gracilaria (Santelices & Doty 1989; Anderson *et al.*, 1996) and could be a result of environmental stress and/or pathogens (Oliveira *et al.*, 2000).

iv) Nutrient availability has a crucial impact on cultivation practices as environmental changes in nutrient regimes have detrimental effects on cultivation processes associated with seaweeds. All seaweeds require various macro- and micro-nutrients as well as a range of trace elements (DeBoer, 1981). N is usually considered to be the limiting nutrient in seawater but phosphorus, iron and trace elements may in some cases also limit algal growth.

N availability is the most significant environmental factor affecting *G. gracilis* cultivation as it has the most detrimental effect on algal growth (Lobban *et al.*, 1985; Oliveira *et al.*, 2000). The 5 major sources of N in the environment are (Lobban *et al.*, 1985):

1. N fixation by bacteria and blue green algae;
2. Ammonium release from bacterial decomposition;
3. Deep water nitrates brought to the surface by physical advection;
4. Atmospheric input of ammonia through rain;
5. N input from sewage and fertilisers to coastal waters.

In the marine environment, N is available to algae in three major forms: as inorganic nitrate (NO_3^-), inorganic ammonium (NH_4^+) and organic urea (Phillips & Hurd, 2004). However, nitrate and ammonium are the two forms of N that can be assimilated by most algal species. Previous studies have reported that most gracilaroid algae demonstrate a preference for ammonium (D'Elia & DeBoer, 1978, Lapointe & Ryther, 1979, Lobban & Harrison 1997). Furthermore, it has also been previously reported that ammonium yielded higher biomass production than nitrate in some *Gracilaria* species (Deboer *et al.*, 1978).

The uptake rate of N by seaweed is influenced by environmental conditions such as light, temperature, water motion and desiccation (Hanisak, 1990). *Gracilaria* species have the capacity to take up and store N in excess of immediate requirements and use it during subsequent periods of nutrient deficiency to sustain growth for up to 3 weeks (Lapointe & Ryther, 1979; Ryther *et al.*, 1981). Storage can be in the form of inorganic N (Chapman & Craigie, 1977) and/or metabolites such as proteins and pigments (Conover, 1975; Wheeler & North, 1980; Lapointe, 1985; Vergara *et al.*, 1995; Lewis & Hanisak, 1996).

In many macroalgal species growth rates are reduced when the total N content of the thallus falls below 2% (Hanisak, 1979). Smit *et al.* (1997) reported that *G. gracilis* is considered N replete at 2.3% and N limited at 1.7% total N, respectively (Smit *et al.*, 1997). A relationship between thallus colour and N content has also been established. Seaweed that is dark brown or reddish-brown in colour usually has sufficient N available whereas N deficient seaweed usually appears straw coloured. Thus, thallus colour is an important indicator of the N status of a plant (Lapointe & Ryther, 1979; Oliveira *et al.*, 2000; Robertson-Andersson *et al.*, 2009).

1.6 Inorganic nitrogen assimilation in plants and algae

N is an indispensable element which plays an important role in plant and algal growth and development, as it is incorporated into important structural and functional molecules such as proteins and nucleic acids. On average, proteins and nucleic acids consist of approximately 15% and 13% N respectively (Inokuchi *et al.*, 2002). Thus, N assimilation is an important process and deficiencies of N have marked effects on plant productivity, biomass and crop yield (Hageman & Lambert, 1988, Lawlor *et al.*, 1989, Delgado *et al.*, 1994). A large number of biochemical and physiological studies have been conducted on N assimilatory enzymes in land plants and algae (Sivasankar & Oaks, 1996; Oliveira *et al.*, 1997; Ireland & Lea, 1999; Lancien *et al.*, 2000). The main enzymes involved in N assimilation are nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT), asparagine synthetase (AS) and phosphoenolpyruvate carboxylase (PEPc) (Inokuchi *et al.*, 2002). These enzymes are responsible for the bio-synthesis of the amino acids glutamine, glutamate, asparagine and aspartate, which serve as important N carriers in plants and algae (Lam *et al.*, 1996).

NR is involved in the first step of N assimilation and appears to be the rate limiting step in most plants and algae. NR is found in the cytosol of land plants and algae (Inokuchi *et al.*, 2002). The reaction is as follows:

$$\mathbf{NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P) + H_2O\ (1)}$$

Nitrate is reduced in the cytosol by NR to nitrite (NO_2^-) **(1)** which enters the chloroplasts where it is further reduced to ammonium by NiR **(2)** in the second step of the reaction.

$$\mathbf{NO_2^- + 8H^+ + 6e^- \longrightarrow NH_4^+ + 2H_2O\ (2)}$$

Ammonium is then assimilated by the GS/GOGAT cycle. Previously, GDH was considered to be the key enzyme governing this process but this concept was revised in the 1970s with the discovery of the enzymes GS and GOGAT (Temple *et al.*, 1998; Inokuchi *et al.*, 2002). GS catalyses the first reaction in the cycle, with the amination of glutamate to yield glutamine in an ATP dependent reaction **(3)**:

$$\mathbf{Glutamate + NH_4^+ + ATP \longrightarrow Glutamine + ADP + Pi + H_2O\ (3)}$$

The amide group of the glutamine molecule is then transferred to α-ketoglutarate (2-oxoglutarate (2-OG)) in a reaction catalysed by GOGAT **(4a and b)**, to yield two molecules of glutamate: GOGAT usually occurs as two isozymes in plant cells: Fd-GOGAT **(4a)** and NADH GOGAT **(4b)**. Ferredoxin NADPH or NADH are the cofactors used in these reactions.

$$\mathbf{Glutamine + 2\text{-}OG + 2\ ferredoxin_{reduced} \longrightarrow 2\ Glutamate + 2\ ferredoxin_{oxidised}\ (4a)}$$

$$\mathbf{Glutamine + 2\text{-}OG + NAD(P)H \longrightarrow 2\ Glutamate + NAD(P)^+\ (4b)}$$

The synthesised glutamate is then able to donate its amino group to form other N containing compounds or it is reduced to replenish the pool of glutamate for subsequent GS catalysis. Transfer of the amino group is catalysed by the enzyme AspAT and requires oxaloacetate obtained from the tricarboxylic acid (TCA) cycle in order to form aspartate **(5)**.

$$\mathbf{Glutamate + oxaloacetate \longleftrightarrow aspartate + 2\text{-}OG\ (5)}$$

The aspartate produced is transferred to mitochondria of bundle sheath cells and re-transaminated to oxaloacetate or alternatively it can be used together with glutamine and ATP to form asparagine in a reaction catalysed by AS **(6)**.

$$\mathbf{Glutamine + Aspartate + ATP \longleftrightarrow Glutamate + Asparagine + AMP + Pi\ (6)}$$

Glutamine, glutamate and aspartate therefore provide the starting points for the synthesis of organic N compounds such as amino acids, nucleotides, chlorophylls, polyamines and alkaloids (Inokuchi *et al.*, 2002). The TCA cycle provides the intermediates, 2-OG, oxaloacetate and ATP which are required in order for the GS/GOGAT cycle to function. This arrangement illustrates the tight co-ordination which exists between carbon (C) and N metabolism in plants and algae (Fig 1.4). Furthermore, it is estimated that up to 50 - 55 % of net plant C is committed to N assimilation (Vanlerberghe *et al.*, 1990; Inokuchi *et al.*, 2002,).

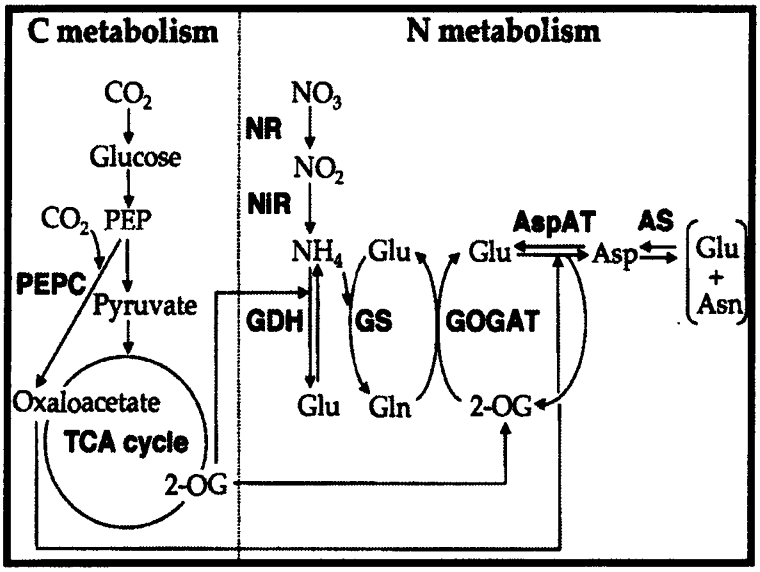

Figure 1.4 A simplified scheme showing carbon and nitrogen flow in a cell and the co-ordination which exists between these two metabolic pathways (Inokuchi *et al*, 2002). CO_2-carbon dioxide, NO_3-nitrate, NO_2-nitrite, NH_4-ammonium PEPC-phosphoenolpyruvate carboxylase, NR-nitrate reductase, NiR-nitrite reductase, GDH-glutamate dehydrogenase, GS-glutamine synthetase, GOGAT-glutamate synthase, AspAT-aspartate aminotransferase, AS-asparagine synthetase, PEP-phophoenolpyruvate, 2-OG-two oxo-glutarate, Gln-glutamine, Glu-glutamate, Asp-aspartate, Asn-asparagine, TCA-tricarboxylic acid

1.7 Co-ordination of Carbon and Nitrogen metabolism

Numerous interactions between C and N metabolism have been extensively documented over the last 30 years. These studies have shown that extensive cross-talk exists between these metabolic pathways and have revealed that changes in C and N status influences organ physiology and root/shoot relationships in plants (Rufty *et al.*, 1988, Foyer *et al.*, 2003). Two of the major metabolic check-points co-ordinating primary N and C assimilation are NR, which controls flux into N assimilation, and phosphoenolpyruvate (PEPc) which regulates organic acid production (Foyer *et al.*, 2003). Pyruvate kinase (PK) also plays a central role in integrating C and N metabolism and together with PEPc integrates glycolysis and N metabolism by providing glycolytic C for amino acid biosynthesis (Huppe & Turpin, 1994;

Whittaker *et al.,* 2007). These enzymes also replenish TCA intermediates which are withdrawn for the synthesis of organic acid precursors such as 2-OG, a key molecule involved in N signalling (Whittaker *et al.,* 2007). Furthermore, glycolysis, the TCA cycle and the pentose phosphate pathway are closely linked, and provide the substrates required for amino acid synthesis (Fig. 1.5).

An abundance of C and N stimulates glycolysis and promotes amino acid synthesis in plants. The energy for this process is provided by carbohydrate metabolism in the form of the reducing equivalents, NADH and Fd (Lian *et al.*, 2006). However, when N is deficient C skeletons are re-allocated to carbohydrate and starch synthesis and this causes profound changes in C metabolism. Subsequently, organic acid synthesis, which is regulated by nitrate and N metabolism, decreases along with amino acid precursors such as malate, citrate and 2-OG. Consequently, intermediates involved in glycolysis, such as glycerate-3-phosphate and glucose-6-phosphate increase (Paul & Stitt, 1993; Scheible *et al.*, 1997 and 2004). The resultant increase in glycolytic intermediates upsets the C:N balance. In higher plants there are four distinct N and- /or C sensing systems which monitor the accumulation of different molecules at crucial check-points in metabolism (Foyer *et al.*, 2003). Glucose sensors such as hexokinase modulate carbohydrate metabolism and balance supply and demand in carbohydrate-producing and consuming cells over a range of abiotic conditions (Coruzzi *et al.*, 2001; Foyer *et al.*, 2003). N sensing systems, which have been comprehensively reviewed by Lam *et al.* (2006), include a PII-like element that acts as a C:N sensor (Hsieh *et al.*, 1998), glutamate receptors and two-component regulatory systems' or 'multi-step His-Asp phophorelay' signalling systems (Coruzzi *et al.*, 2001; Foyer *et al.*, 2003). These C:N sensing mechanisms enable plants to activate genes involved in N assimilation when C skeletons are abundant and internal levels of organic N are low. Alternatively, N assimilation is halted when levels of photosynthate are low and internal levels of organic N are high. Together these systems monitor C and N flux and allow plants to both efficiently utilise or preserve the energy required to drive the transport and metabolism of C and N, and in this way regulates plant growth and development (Coruzzi *et al.*, 2001).

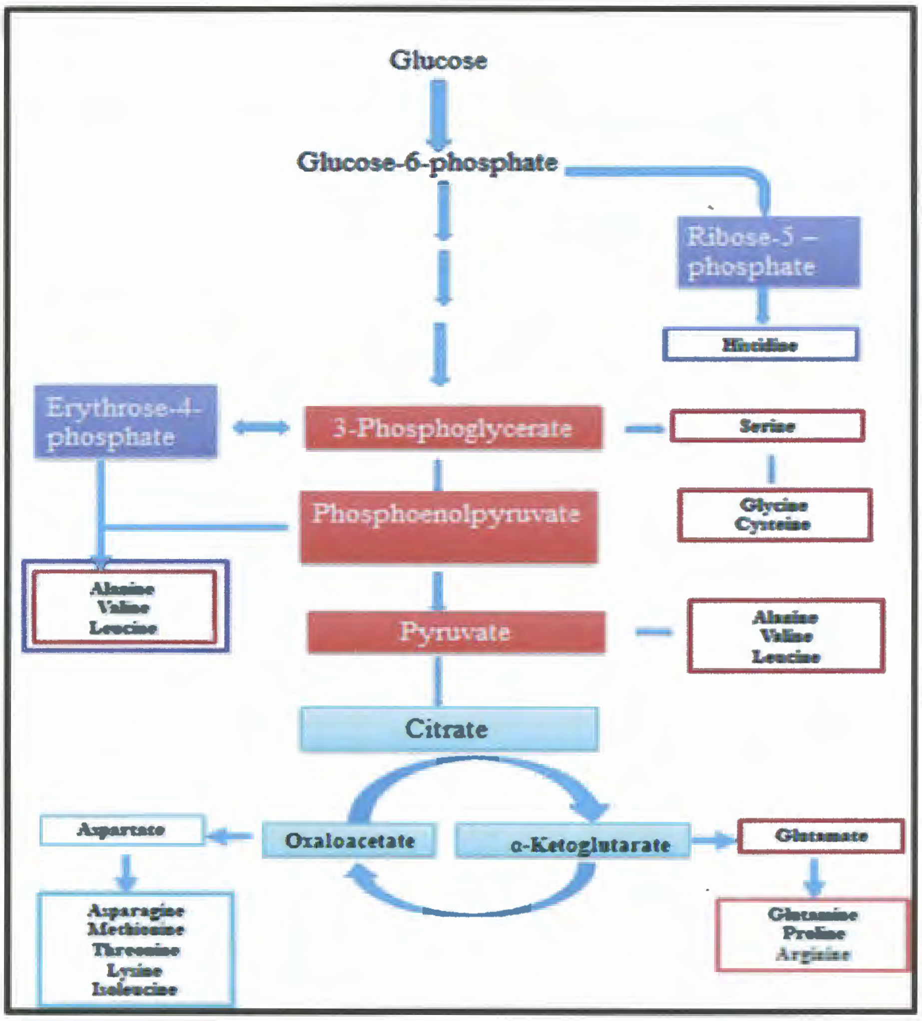

Figure 1.5 A simplified scheme illustrating the pathways of glycolysis, TCA cycle and the pentose phosphate pathway and their involvement in the synthesis of amino acids. Precursors from glycolysis (red), the citric acid cycle (blue), and the pentose phosphate pathway (purple) are shaded, and the amino acids derived from them are boxed in the corresponding colours. (adapted from http://www.bioinfo.org.cn/book/biochemistry/chapt21/sim3.htm)

1.8 The biological effects of nitrogen stress in plants and algae

Abiotic stresses such as N limitation, which adversely affect growth and productivity, offset a series of morphological, physiological, biochemical and molecular changes in both plants and algae (Bhatnagar-Mathur *et al.*, 2008). N deficiency is therefore detrimental to plants and algae as N is a major constituent of proteins, nucleic acids, chlorophyll, several cofactors, secondary metabolites and several plant hormones (Scheible *et al.*, 2004; Lian *et al.*, 2006; Kusano *et al.*, 2011). N deficiency has been shown to result in decreases in the levels of photosynthetic structural components such as chlorophyll and ribulose biphosphate carboxylase (Rubisco) in plants. Subsequently, photosynthetic capacity and carboxylation efficiency are reduced (Lam *et al.*, 1996). Similar effects are observed in algae, with a decrease in photosynthesis, Rubisco levels, N/C ratios, N, protein levels and fluorescence yield (Turpin, 1991). The most obvious effect of algal N-deficiency is the decline in the intracellular levels of nitrogenous photosynthetic pigments (chlorophylls and phycobilins) (Turpin, 1991).

Plant studies have shown that low N stress and N starvation elicit distinctive adaptive responses. Low N stress conditions are usually not severe enough to cause immediate N shortages (Lian *et al.*, 2006). Lian *et al.* (2006) used microarray analysis to investigate the early response of rice seedlings to low N stress (0.24 mM N) at three time points and reported that genes involved in photosynthesis and energy metabolism were rapidly down-regulated. Genes involved in an early response to biotic and abiotic stresses, such as pathogenesis related proteins and those involved in oxidative stress responses, were in turn up-regulated. Regulatory genes, including transcription factors and those involved in signal transduction, showed both up- and down-regulation, while genes involved in N uptake and assimilation were essentially unaffected by low N stress (Lian *et al.,* 2006).

Prolonged N limitation in higher plants results in reduced demands for C skeletons in sink tissues and organs that would normally support the uptake and assimilation of ammonium and nitrate. Subsequently, a decline in the uptake and assimilation rates of ammonium and nitrate occurs and as a result, the demand for anaplerotically derived C skeletons is decreased. A resultant decline in the activities of important glycolytic enzymes such as PEPc and PK is observed and fewer C skeletons are available to support the synthesis of organic acids in the TCA cycle. Although, organic acids are an essential requirement for amino acid synthesis, source leaves and tissues that remain metabolically active during N limitation use a higher percentage of C skeletons for starch and sucrose synthesis rather than channelling C metabolites into amino acid and protein synthesis (Robinson, 1997). Subsequently, an increase in foliar levels of glucose, sucrose and starch is observed in many species of green plants (Robinson, 1997). In red algae, carbohydrates, starch and floridoside are the main storage forms of C. It is thought that floridoside plays a similar role to sucrose in higher plants. Similar to plants, an increase in floridoside levels, agar and floridean starch is observed in red algae under conditions of N limitation (Macler, 1986; Bird, 1988, Ekman *et al.*, 1991; Collén *et al.*, 2004). Although the reasons for accumulation of starch and sugars under conditions of N limitation are not well understood, starch and sugars are thought to act as easily mobilisable energy stores that would be available should the plant become healthy again.

N limitation elicits a range of responses and has been shown to result in extensive cross talk between the response pathways of low N stress, pathogen attack and other abiotic stresses (Lian *et al.*, 2006). Thus, it is important to note that it is difficult to resolve cause and effect relationships in plants subjected to N limitation because N stress initiates many complex physiological responses which may vary over time and with the degree of stress (Rufty *et al.*, 1988). However, it has been suggested that genes up-regulated in response to N limitation may confer a protective function, while the down-regulation of stress responsive genes may make the plant more vulnerable to other stressors (Lian *et al.*, 2006).

The ability of plants and algae to recover following exposure to N limiting conditions has been reported for *Arabidopsis thaliana* (Wang *et al.*, 2000 and 2003, Scheible *et al.*, 2004), *Fucus serratus* and *Fucus vesicolus* (Young *et al.*, 2009). N is acquired mainly as nitrate in higher plants, and fluctuations of this mineral ion often limit growth and yield (Krouk *et al.*, 2010). Similarly, algae are often exposed to temporally variable N conditions with low N levels occurring during the summer months (Anderson *et al.*, 1996; Young *et al.*, 2007). Thus it is important to understand plant and algal adaption to a changing N environment. Numerous studies have investigated transcriptome changes in response to nitrate in higher plants (Wang *et al.*, 2000 & 2003; Scheible *et al.*, 2004; Krouk *et al.*, 2010) and nitrate has been shown to have a two-fold function. It serves as an important nutrient source and signals rapid changes in metabolism. These include the induction of genes involved in N assimilatory processes, as well as those responsible for the shift from starch biosynthesis to organic acid production (Stitt, 1999; Foyer *et al.*, 2003, Wang *et al.*, 2003). Metabolic processes stimulated by nitrate include nitrate uptake and reduction, ammonium assimilation, organic acid synthesis, ferredoxin reduction and glucose phosphate oxidation. Nitrate also suppresses starch synthesis to help mobilise C into organic acids (Wang *et al.*, 2000). These responses indicate that nitrate stimulates intricate regulatory networks and induces a range of metabolic and developmental pathways (Wang *et al.*, 2000).

The addition of nitrate to plants that are N limited has been investigated at the transcriptional level in *Arabidopsis thaliana* (Wang *et al.*, 2000, Wang *et al.*, 2003, Scheible *et al.*, 2004; Krouk *et al.*; 2010). Wang *et al.* (2000, 2003) investigated the response of *Arabidopsis* to both high and low concentrations of nitrate using microarray technology. Most of the induced genes were nitrate regulated genes such as NR, nitrate transporter genes and GS. Novel nitrate induced genes, such as AS and a MYB transcription factor, were also identified (Wang *et al.*, 2000). A similar study conducted by Scheible *et al.* (2004) reported transcriptional repression of genes involved in photosynthesis, chlorophyll synthesis and plastid protein synthesis in *Arabidopsis* plants subjected to two days of N deprivation. The addition of nitrate resulted in rapid responses in the expression of many genes potentially involved in trehalose metabolism, hormone metabolism, protein kinases and phosphatases, receptor kinases and transcription factors (Scheible *et al.*, 2004).

Proteomic studies investigating protein changes in response to nitrate and ammonium availability have been reported for barley roots, *Zea mays* and Triticale varieties (Prinsi *et al.*, 2009; Castillejo *et al.*, 2010; Møller *et al.*, 2011). These studies have reinforced the strict relationship which exists between C and N metabolism as evidenced by the identification of proteins involved in N assimilation, such as NiR and GSII, and C metabolism, such as phosphoglycerate mutase and glucose-6 phosphate dehydrogenase (Prinsi *et al.*, 2009, Møller *et al.*, 2011). However, studies of this nature are limited and have not been conducted in red macroalgae. Furthermore, an investigation of the whole-plant N response is essential if the regulation of N use efficiency is to be elucidated. This data could provide key target selection criteria for breeding programs (Castillejo *et al.*, 2010).

1.9 **Proteomics**

Proteins are the final products manufactured in living cells according to the 'blueprint' contained in the genome (Beranova-Giorgianni, 2003). They are important structural and functional molecules and therefore characterisation of proteomes is essential for a complete understanding of biological systems (Beranova-Giorgianni, 2003). The proteome of an organism represents the array of proteins that are expressed in a biological compartment (cell, tissue or organ) at a particular time, under a particular set of conditions (Beranova-Giorgianni, 2003). Thus, examination of the proteome of a cell provides a 'snapshot' of the protein environment at any given time. Proteomics is the term used to describe the large scale study of proteins and proteomics serves to determine how proteins change in abundance, form, location or activity during the biological state being investigated (Pandey *et al.*, 2000; Beranova-Giorgianni, 2003; Peck, 2005). Therefore, the goal of proteomics is to obtain a global and integrated view of biology by studying all the proteins in a cell rather than each one individually (Graves & Haystead, 2002). Identification and quantification of proteins, analysis of changes in protein expression (normal *versus* diseased/treated), characterisation of post-translational modifications (PTMs) and protein-protein interactions are a few of the most common applications of proteomics (Beranova-Giorgianni, 2003).

Genomics and transcriptomics have played crucial roles in elucidating cell function. Genomic data, however, only provides a static state of the inherited information (Wittmann-Liebold *et al.*, 2006). Proteins are the functional units of a cell and therefore a biological response cannot be determined from genes alone. Proteomics offers certain advantages over transcriptomics in several ways. Firstly, proteins are more evolutionary conserved which enables high throughput identification of gene products from non-model organisms to orthologous proteins (Carpentier *et al.*, 2008). Secondly, PTMs, which result in different isoforms, are not apparent from DNA sequences. It is estimated that up to 200 different types of PTMs exist and various protein isoforms can be identified using proteomic approaches (Pandey & Mann, 2000). Thirdly, proteins and not genes are responsible for the phenotype of cells and it is impossible to elucidate mechanisms of disease, aging and effects of the environment solely by studying the genome. Moreover, the analysis of mRNA is not a direct reflection of the protein content in the cell and consequently, many studies have shown a poor correlation between mRNA and protein expression levels (Pandey *et al.*, 2000; Graves & Haystead, 2002). The formation of mRNA is only the first step in a long sequence of events resulting in the synthesis of a protein. Additionally, mRNA is subject to post-transcriptional control in the form of alternative splicing, polyadenylation and mRNA editing which may result in the generation of different protein isoforms from a single gene. Lastly, proteins have been shown to be regulated by compartmentalisation and proteolysis. As a result certain biological fluids, such as serum and urine, have no mRNA source and therefore cannot be studied by mRNA analysis. Proteomic studies therefore provide a significant advantage over genomic studies when investigating environmental stress responses.

Differential expression proteomics is the quantitative study of protein expression between two samples in different physiological states and is the approach employed in this study. It involves comparative analysis of protein profile patterns in stressed versus non-stressed samples and allows the detection of specific changes in the proteome (Nesatyy & Suter, 2007). The identification of proteins that are either induced or suppressed in response to environmental stress conditions allows one to draw conclusions regarding the molecular mechanisms of a stress response (Nesatyy & Suter, 2007). The function of a protein can also be inferred from its differential presence in specific genotypes (i.e wild-type, mutant, transgenic), cell type or developmental stage and can be influenced by external conditions (Jorrin-Novo *et al.*, 2009). Two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has been the standard method for quantitative analysis of protein mixtures for many years (Chelius & Bondarenko, 2002). This approach is ideal when investigating global analysis of metabolic processes such as protein synthesis, glycolysis, gluconeogenesis, nucleotide biosynthesis, amino acid biosynthesis, lipid metabolism and stress responses (Futcher *et al.*, 1999; Patton *et al.*, 2002). Furthermore, information gained from this approach can identify novel proteins involved in signal transduction while also differentiating between changes arising from PTMs and those due to protein expression and degradation (Lewis *et al.*, 2000; Patton *et al.*, 2002). A typical 2-DE proteomics experiment follows the general strategy outlined below (Beranova-Giorgianni, 2003) (Fig. 1.6):

(i) solubilisation of proteins from the sample (e.g tissue)
(ii) separation of the proteins by 2-DE
(iii) digitization of 2D gels and computer-assisted analysis of protein spot patterns for different treatments
(iv) determination of specific attributes (peptide mass fingerprint or peptides) of the proteins of interest by MS
(v) database searches with these attributes to identify proteins

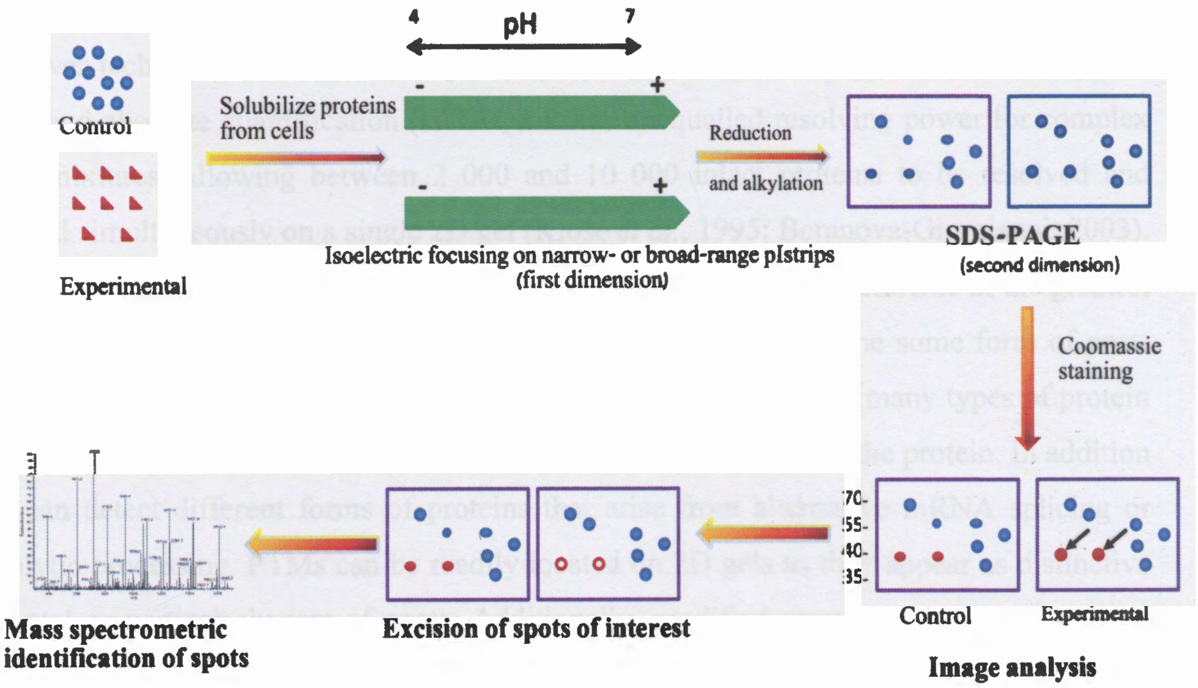

Figure 1.6 General strategy for proteome analysis by 2-DE, MS and database searching for the identification of differentially expressed proteins in response to nitrogen limitation (adapted from Pandey *et al.*, 2000).

1.9.1 Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) based proteomics is still thought to be the most powerful option for non-model organisms (Carpentier *et al.*, 2008). Two-DE separates complex protein mixtures on the basis of isoelectric point (p*I*) in the first dimension and molecular weight (MW) in the second dimension (Beranova-Giorgianni, 2003; Wittmann-Liebold *et al.*, 2006). Two-dimensional gel electrophoresis was first introduced in the mid-70s (MacGillivray & Rickwood, 1974; Klose, 1975, O'Farrell, 1975) and since then a number of improvements have increased the reproducibility and resolution of 2-DE as well as the sensitivity of protein detection (Gygi *et al.*, 2000; Patton *et al.*, 2002; Beranova-Giorgianni, 2003; Rabilloud *et al.*, 2010). These include the introduction of immobilized pH gradients, the use of fluorescent dyes (such as SYPRO Ruby protein gel stain) and specialized pH gradients (Patton *et al.*, 2002; Beranova-Giorgianni, 2003; Rabilloud *et al.*, 2010).

Despite being one of the older proteomic technologies, 2-DE still offers certain advantages over newer technologies such as Isotope-Coded Affinity Tags (ICAT) and Isobaric tags for relative and absolute quantification (iTRAQ). It has unequalled resolving power for complex protein mixtures, allowing between 2 000 and 10 000 intact proteins to be resolved and visualised simultaneously on a single 2D gel (Klose *et al.*, 1995; Beranova-Giorgianni, 2003). The MW, p*I* and relative quantity for each protein can also be determined. One of the greatest strengths of 2-DE is the ability to resolve proteins that have undergone some form of post-translational modification. This resolution is possible in 2-DE because many types of protein PTMs confer a difference in charge as well as a change in the mass of the protein. In addition 2-DE can detect different forms of proteins that arise from alternative mRNA splicing or proteolytic processing. PTMs can be readily located on 2D gels as they appear as distinctive horizontal or vertical clusters of spots. Additionally, modified proteins can be revealed by MS analysis when multiple spots are identified as the same protein (Beranova-Giorgianni, 2003). Two-DE has also been used in cell map proteomics to map proteins from microorganisms, cellular organelles and protein complexes and to characterize sub-proteomes (Graves & Haystead, 2002).

A distinct advantage of 2-DE technology is that the individual steps of a typical 2-DE experiment (2-DE, imaging, MS, database searching) can be separated in space and time. For instance, 2-DE and data analysis of spot patterns can be performed in an investigator's laboratory and proteins of interest can be analysed in a service facility at a later date. Furthermore, dried 2-DE gels allow long term archiving of proteins as thousands of proteins can be stored at room temperature. Rare protein samples can therefore be preserved for months or even years and still be identified by MS. Finally, 2-DE is well suited to academic research as equipment is relatively inexpensive and access to other essential and more expensive components such as mass spectrometers and bioinformatics resources, can be obtained through other academic institutions that provide fee for service facilities (Beranova-Giorgianni, 2003).

One of the big challenges facing proteomics research is the inherent complex nature of cellular proteomes. Protein expression in a biological system changes depending on the state of development, environmental stimuli or progression of disease to name a few. Therefore the number of proteins in a proteome is very large, and in fact, it has been estimated that up to 50 000 protein species may be simultaneously present in an eukaryotic cell (Beranova-Giorgianni, 2003). Thus the major drawback of 2-DE based proteomics is that it is not possible to analyse the entire proteome. Complex protein mixtures are not completely resolved on 2-DE gels as low abundant, large or very small, hydrophobic (those of extreme acidity or basicity) and membrane proteins are usually not well resolved. Therefore, the bias is towards the identification of highly abundant proteins (Chelius & Bondarenko, 2002; Patton *et al.*, 2002; Rabilloud, 2002; Beranova-Giorgianni, 2003; Domon & Aebersold, 2006; Rabilloud *et al.*, 2010). Some of these problems can be overcome with the use of different solubilisation techniques and enrichment protocols (Gianazza *et al.*, 1987; Rabilloud *et al.*, 1997). Secondly, 2-DE is a time consuming and labour intensive process which usually takes a few days to run and only one experiment can be analysed at a time. The low throughput of 2-DE is thus more suited to basic research studies and would be problematic when a large number of samples would need to be screened (e.g. clinical studies).

In recent years there has been a shift to gel free strategies such as ICAT and iTRAQ (Patton *et al.*, 2002, Rabilloud, 2002). However, these LC MS/MS methods rarely identify more than a few peptides per protein at a time which makes the detection of PTMs or truncated forms of proteins highly unlikely. In a detailed study of rice proteins, LC MS/MS identified 2363 proteins from different tissues. However, it failed to identify 165 out of 556 proteins identified from the same samples subjected to 2D gel electrophoresis (Koller *et al.*, 2002). Furthermore, Chevallet *et al.* (2007) analysed the secretome of myeloid cells (macrophages, dendritic cells) and demonstrated that with adequate sample preparation low-abundant secreted proteins such as cytokines can be detected with 2-DE. Gel-based and gel-free (LC MS/MS) approaches therefore complement each other and together provide the most complete coverage of a given proteome. The limitations of 2-DE, have been investigated extensively, as opposed to the more recent gel-free set-ups (Rabilloud *et al.*, 2010). Most importantly, 2-DE, the 'work horse' of proteomics, has been shown to work successfully for non-model organisms and has resulted in the identification of large numbers of proteins.

1.9.2 LC MS/MS for novel protein identification

The most significant breakthrough in proteomics has been mass spectrometric identification of gel-separated proteins (Shevchenko *et al.*, 1997; Shevchenko *et al.*, 2001; Liska & Shevchenko, 2003; Liska *et al.*, 2005). The defining feature of a mass spectrometer is its high sensitivity and ability to identify a protein with the highest level of confidence (Shevchenko *et al.*, 1997; Graves & Haystead, 2003). Additionally, newer mass spectrometers are packaged with software that facilitates protein identification and structural analysis by providing a bridge between mass spectra and public sequence databases (Loboda *et al.*, 2000; Shevchenko *et al.*, 2001; Aebersold & Mann, 2003; Ferguson & Smith, 2003; Lin *et al.*, 2003; Mann *et al.*, 2001; Rose *et al.*, 2004; Liska *et al.*, 2005). A typical mass spectrometer consists of an ion source, a mass analyser (measures the mass-to-charge ratio (*m/z*) of the ionised analytes) and a detector that registers the number of ions at each *m/z* value (Aebersold & Mann, 2003). Electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) are the two most commonly used techniques for mass spectrometric analysis. During ESI the analyte is eluted from a reversed-phase column to separate the peptides by hydrophobicity, ionized and transferred with high efficiency into the mass spectrometer for analysis. MALDI uses a different approach and sublimates and ionizes samples out of a dry-crystalline matrix via laser pulses. MALDI-MS is used mostly to analyse simple peptide mixtures while integrated liquid chromatography ESI-MS (LC-MS) methods are preferred for the analysis of complex samples (Aebersold & Mann, 2003). Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is a powerful technique for the analysis of peptides and proteins (Mann *et al.*, 2001). This technique allows for the analysis of complex protein samples even when concentration levels of different proteins vary by orders of magnitude (Mann *et al.*, 2001). LC MS/MS can be used on its own or in combination with 1-D, 2-D gel electrophoresis, immunoprecipitation or other protein purification techniques. The development of ESI has transformed LC MS/MS technology into a routine laboratory procedure sensitive enough to analyse peptides and proteins at levels which enable researchers to answer complex biological problems (Mann *et al.*, 2001). Presently, LC MS/MS is a popular technique used in the identification of proteins from non-model organisms (Carpentier *et al.*, 2008; Jorrin-Novo *et al.*, 2009). The main advantage of using this technique is that it generates sequence information derived from several peptides and is therefore much more specific with regard to protein identification than a peptide mass fingerprint. There are a number of sequential steps which need to be followed before sequence information can be obtained. First, proteins are separated using 2-DE and then digested with trypsin. The resulting peptides are gel extracted, separated by LC and ionised via electrospray into the mass spectrometer for characterisation by mass analysis (Bell *et al.*, 2009). The generated fragmentation data can be used to search protein sequence, nucleotide (such as expressed sequence tag (ESTs)) and more recently raw (unannotated) genomic sequence databases (Pandey *et al.*, 2000). ESI MS/MS also provides more informative MS/MS spectra of unmodified tryptic peptides than MALDI-TOF MS/MS (Carpentier *et al.*,

2008) as doubly charged peptide ions tend to fragment more equally across a given sequence than do singly charged ion species. Singly charged ions also require higher activation energies and often yield relatively low quality collision induced dissociation mass spectra. This results in poorer scoring with classical search algorithms (such as those used in Mascot) especially for cross-species identification (Carpentier *et al.*, 2008). The resulting MS/MS spectra can be analyzed with various software packages (e.g., Mascot, Paragon and ProGroup Algorithms, SEQUEST, X!Tandem) to obtain a protein identity (Rose *et al.*, 2004). Finally, ESI MS/MS offers a number of advantages over MALDI-TOF MS/MS. Firstly, it is the most specific method for protein identification and proteins can be identified with high confidence. Secondly, it provides a higher sensitivity than MALDI, allowing for the identification of low-copy proteins (Shevchenko *et al.*, 1997; Graves & Haystead, 2003). Finally, and perhaps most importantly, MS/MS is less affected by protein mixtures than peptide mass fingerprinting (Graves & Haystead, 2003). This is critically important because protein "bands or spots" isolated from polyacrylamide gels frequently contain more than one protein (Graves & Haystead, 2003).

1.10 Proteomics of algae

Proteomic studies of algae are currently limited relative to higher plants and most algal proteomic studies have focussed on the freshwater chlorophyte *Chlamydomonas reinhardtii,* an important model organism used to study photosynthesis. A comprehensive review on *C. reinhardtii* proteomics has been published by Rolland *et al.* (2009). This review discusses recent proteomic progress with respect to subcellular proteomics, redox signalling and eyespot apparatus analyses in *C. reinhardtii* (Rolland *et al.*, 2009). Proteomics can also be used for the elucidation of certain metabolic pathways and this has been demonstrated with various comparative studies conducted on microalgae. Forster *et al.* (2006) established a partial 2-DE reference map for wild type *C. reinhardtii,* primarily representing the soluble sub-proteome (Forster *et al.*, 2006). This reference map was subsequently used to investigate high light-induced changes between wild type and two very high light resistant (VHL^R) mutants (Forster *et al.*, 2006). Similarly, Naumann *et al.* (2007) used a comparative quantitative proteomics approach to investigate the remodelling of bioenergetic pathways in *C. reinhardtii* in response to iron deficiency. It was found that iron deficiency lead to the induction of stress responsive proteins such as peroxiredoxin and a stress induced light-harvesting protein, as well as a number of proteins of unknown function (Naumann *et al.*, 2007). Other studies investigating the effects of cadmium exposure on *C. reinhardtii* (Kim *et al.*, 2005) and the marine alga *Nannochloropsis oculata* (Gillet *et al.*, 2006) have also successfully identified key proteins involved in photosynthetic and oxidative stress responses. In *Haematococcus pluvialis* a proteomic approach was employed to investigate the alga's response to oxidative stress (Wang *et al.*, 2004a, b) and in the halotolerant alga *Dunaliella salina* the molecular basis of salinity tolerance was clarified by proteomics (Liska *et al.*, 2004). These studies provide an indication of how proteomics can be applied to elucidate mechanisms of abiotic stress in algae and show the wealth of information that can be obtained. However, proteomic progress in macroalgae has been slow as studies have focussed on the optimisation of 2-DE methods. Macroalgal proteomic studies have been complicated as algal protein extracts have been previously shown to contain high levels of polysaccharides, pigments, polyphenols and salts which can interfere with the 2-DE process. The first successful protein extraction method applicable for 2-DE and MS analysis of proteins from red alga was demonstrated for *Gracilaria changii* by Wong *et al.* (2006). The identified proteins included Rubisco, photosynthetic pigment proteins, metabolic enzymes and ion transporters and provided the first red seaweed model proteome for use in comparisons and differentiations between closely related species (Wong *et al.*, 2006).

While comparative proteomic studies are limited for red macroalgae a few have been conducted in brown macroalgal species. A comparative study investigating the copper tolerance of two strains of *E. siliculosus,* originating from habitats with contrasting levels of copper, enabled identification of several pathways involved in copper tolerance and led to the conclusion that photosynthesis-related proteins seem to be crucial for copper tolerance (Ritter *et al.*, 2010). Recently, protein expression changes due to seasonal variation were investigated in the Japanese kelp *Saccharina* (Yotsukara *et al.*, 2010). However, despite these studies, algal proteomics still lags behind plant proteomics and a lot remains to be determined in terms of protein responses to environmental stressors. To our knowledge no comparative proteomic studies investigating N stress in *Gracilaria* species have been conducted, which is the focus of this study.

1.11 Concluding remarks and research objectives of this study

N nutrition is an important environmental factor controlling the growth and survival of intertidal macroalgal populations. It is therefore important when developing a cultivation system, to have a good knowledge of the physiology and molecular mechanisms which allow for optimal growth and survival. Hanisak (1990) states that 'Mariculture is ultimately an attempt to manage a series of biological processes and that these processes need to be understood before they can be manipulated'. Thus, this study will focus on understanding how *G. gracilis* responds to N limitation and N recovery with a specific focus on the proteins involved in these responses. The identification of proteins which are differentially regulated in response to N limitation would aid in the selection of biomarkers which could potentially be used to monitor the health status of macroalgal populations in commercial farm settings. Furthermore, characterisation of the response of *G. gracilis* to N recovery would enable the identification of putative proteins which play important roles in N assimilation.

In this study, a 2-DE proteomics approach will be employed to evaluate the response of *G. gracilis* to N limitation and recovery. 2-DE has been shown to be successful at identifying proteins and pathways involved in various environmental stress responses (Ingle *et al.*, 2007; Thornton *et al.*, 2007., Prinsi *et al.*, 2009; Castillejo *et al.*, 2010; Møller *et al.*, 2011). Thus, this study aims to provide insight into the adaptive and regulatory mechanisms employed by *G. gracilis* in response to N stress. The proteomic data generated here, together with existing EST data, could lead to the identification of candidate genes involved in N metabolism in *G. gracilis*. The manipulation of these genes using targeted genetic engineering would enable one to breed *G. gracilis* strains which are able to use N more efficiently and could possibly allow adaption to low N environments.

The major objective of this study is to investigate global protein expression changes in response to N availability in the commercially important agarophyte *Gracilaria gracilis*. More specifically, the effects of N limitation and N recovery will be investigated. To achieve this objective this study has been divided into two parts:

I. In this study, differential expression proteomics was used to identify important proteins and pathways involved in the N limitation and recovery stress response in *G. gracilis*. In order to understand the molecular mechanisms employed by *G. gracilis* in response to N limitation, the initial and sustained responses of *G. gracilis* to N limitation were evaluated using 2-DE. Similarly, the N recovery response of N-limited *G. gracilis* was evaluated using a similar methodology. Proteins which were differentially regulated in response to N limitation and recovery were identified with LC MS/MS sequencing and discussed in context to N limitation and recovery in *G. gracilis*.

II. Differential expression proteomics has already been shown to be feasible for the identification of differentially regulated proteins in other plant and algal species. However, it is important to independently verify the data obtained using an alternative means. In this study, the identities of two selected proteins, fructose 1, 6 biphosphate aldolase (ALD) and glutamine synthetase (GS), were confirmed and the expression pattern, determined using 2-DE, validated. Subsequently, the effect of N limitation on ALD protein levels and activity in *G. gracilis* were investigated to evaluate the potential of this protein to monitor the health status of N-limited *G. gracilis*. Similarly, the effect of N recovery on *G. gracilis* ALD protein levels and activity as well as *G. gracilis* GS protein levels were investigated to determine if these proteins can be used to measure the recovery response of *G. gracilis* following N limitation. Furthermore, the activity of PK, an important enzyme integrating C and N metabolism was assessed for *G. gracilis* exposed to N limitation and recovery. Finally, starch accumulation in *G. gracilis* during N limitation was briefly assessed to determine whether a change in C allocation has occurred.

CHAPTER 2

The effect of nitrogen limitation and subsequent recovery on global protein expression in *Gracilaria gracilis*

CONTENTS

2.1 Introduction

Intertidal marine macroalgae live in a harsh environment and are subjected to repeated periods of immersion and emersion due to tidal fluctuations (Burrit *et al.*, 2002). As a result, macroalgal species, such as *G. gracilis*, are continuously exposed to unfavourable environmental conditions. These include nutrient limitation, high light, high and low temperatures and osmotic stress (Davison & Pearson, 1996). In intertidal environments the availability of inorganic nitrogen (N), an essential mineral element required by all living organisms, is considered to be a critical factor in the growth and survival of algae (Fernandez & Galvan, 2007). It has been suggested that N may be one of the most important limiting factors affecting intertidal macroalgal communities (Thomas *et al.*, 1987., Smit *et al.*, 1997; Smit, 2002). Thus, in order to survive organisms need to use N efficiently and adapt to fluctuating N levels (Fernandez & Galvan, 2007).

Algae have developed various adaptive and defensive responses which enable survival under these conditions (Treshow, 1970). One such mechanism is the phenomenon of 'luxury consumption' which is implemented by macroalgae such as *G. gracilis* to cope with N limitation. Southern African *G. gracilis* seaweed populations are predominantly found in Saldanha Bay, an area subject to oligotrophic conditions during summer (Smit, 2002). As a result, the N environment within Saldanha Bay is temporally variable. Nitrate concentrations can range from undetectable levels to values as high as 30 – 40 μM in some areas. Ammonium levels are much lower and seldom exceed 4 μm (Smit, 2002). A consequence of these highly variable environmental N states is nutrient limiting conditions, which appear to be responsible for the low growth rates of *G. gracilis* observed at certain times of the year (Anderson, 1996; Smit, 2002). The 'luxury consumption' of available nutrients enables *G. gracilis* to survive and adapt to these N limiting conditions (Smit *et al.*, 1997). The physiological mechanisms underlying these responses have been investigated by examining N uptake kinetics, N storage patterns and utilisation of N in relation to shore position and season (Smit *et al.*, 1997; Smit, 2002) and it was observed that *G. gracilis* is capable of growing rapidly for extended periods of time on internally stored N (Smit, 2002). Thus, *G. gracilis* has the ability to adapt to low N environments and it is therefore necessary to elucidate the molecular basis of these adaptions in *G. gracilis* to complement the existing physiological data.

Previous studies investigating N nutrition have primarily focussed on the effects of nitrate additions (Wang *et al.*, 2000 & 2003; Phillips & Hurd, 2003; Phillips & Hurd, 2004; Scheible *et al.*, 2004; Young *et al.*, 2009). However, in recent years there has been an increase in the number of studies investigating plant responses to N limitation (Lian *et al.*, 2006; Bi *et al.*, 2007; Peng *et al.*, 2007; Tschoep *et al.*, 2009; Krouk *et al.*, 2010; Patterson *et al.*, 2010; Prinsi *et al.*, 2010; Møller *et al.*, 2011). Recently, the responses of barley root and shoot proteomes to short-term N starvation, long-term N deficiency and usage of ammonium as the sole N source were investigated (Møller *et al.*, 2011). A total of 67 and 49 differentially expressed proteins were identified in roots and shoots, respectively. This corresponded to 62 functions and over 80 gene products, and helped to considerably advance the knowledge of N responses in barley plants (Møller *et al.*, 2011).

Recent evidence suggests that the nutrient signalling response in plants may occur in two distinct phases (Schachtman and Shin, 2007). These signalling responses constitute an early response, which can occur in less than six hours, and a much later response which may take days or weeks (Schachtman and Shin, 2007). Therefore, it has been proposed that the early signals in response to nutrient deprivation may occur when external concentrations of nutrients are reduced while the later responses may be initiated when the internal stores of nutrients are reduced, below a critical level (Schachtman and Shin, 2007). This has been demonstrated with studies investigating short-term responses to phosphorus (P) limitation in *Arabidopsis* (Hammond *et al.*, 2003; Misson *et al.*, 2005). It was observed that short-term P withdrawal resulted in transient changes in gene expression that were highly variable and resulted in the identification of mainly non-specific, shock-induced genes whereas the long-term responses resulted in the identification of genes specifically involved in P metabolism (Hammond *et al.*, 2005). Recently, Krouk *et al.* (2010) used predictive network modelling to propose a temporal working model for nitrate-driven gene networks in *Arabidopsis* (Krouk *et al.*, 2010). Their observations suggest that the early nitrate response involves mechanisms needed to prepare the plant to respond to nitrate, rather than mechanisms that relate directly to N. Genes that are involved in more specific N responses, such as those involved in nitrate transport and amino acid metabolism, were regulated later on in the time series (Krouk *et al.*, 2010). Short- and long-term sensing and signalling events in response to N limitation in macroalgae is therefore an area of interest, as definitive conclusions cannot be drawn regarding their importance in the stress response (Schachtman and Shin, 2007). Thus, in this study the N limitation response was further dissected and the effects of an initial and sustained period of N limitation in *G. gracilis* were assessed.

The goal of any successful cultivation system is to improve growth rates and biomass yields, and this involves improving our knowledge of N assimilation in *G. gracilis*. The identification of proteins involved in N assimilation has proved useful in plants and numerous studies have used this information to improve N use efficiency in plants. These studies have resulted in the identification of proteins such as glutamine synthetase (GS), glutamate synthase (GOGAT) and alanine aminotransferase which have been genetically engineered to improve the efficiency of N use in grain, rice and *Arabidopsis* plant species (Shrawat *et al.*, 2008). The over-expression of specific enzymes, such as GS, has been shown to increase grain yield significantly (Habash *et al.*, 2001; Tabuchi *et al.*, 2005; Martin *et al.*, 2006; Hirel *et al.*, 2007; Lea and Azevedo, 2007). In rice, several transgenic lines over-producing NADH-GOGAT under the control of a NADH-GOGAT promoter showed an increase in grain weight, indicating that NADH-GOGAT is a key step for N utilization and grain filling in rice (Yamaya *et al.*, 2002). The physiological mechanisms involved in N acquisition and assimilation has been investigated in a number of intertidal seaweed species including *Gracilaria, Fucus* and *Laminaria* species (Smit, 2002; Phillips & Hurd, 2003; Young *et al.*, 2009). However, very little work has been done on characterising the molecular mechanisms involved in N acquisition and assimilation in macroalgae. These mechanisms have, however, been extensively investigated in green plants and it has been shown that a highly co-ordinated system exists which balances the demand for various N-containing compounds with the availability of C-skeletons, energy and reductants for the assimilatory pathway (Nunes-Nesi *et al.*, 2010). To the best of our knowledge this is the first study investigating the effect of N re-supply to N deprived *G. gracilis* algal samples and the resulting effect on global protein expression. Given that N is an essential requirement for optimal growth of marine macroalgae, an understanding of these responses is crucial if *G. gracilis* is to be successfully cultivated.

In order to develop adequate cultivation systems we need to improve our understanding of the biology of *G. gracilis,* and more specifically the response of *G. gracilis* to N limitation and N recovery. Various proteomic approaches can be used to investigate *G. gracilis* proteome changes in response to N stress. These approaches can be gel-based or gel-free, each of which has advantages and disadvantages relating to protein coverage, resolution, protein loss, reproducibility, data analyses and cost (Beranova-Giorgianni, 2003; Carpentier *et al.*, 2008). One of the main challenges associated with any proteomic approach is the successful identification of proteins using mass spectrometry and then data interrogation. Model organisms with their sequenced genomes have a significant advantage over non-model organisms as protein identification can be obtained via MALDI-TOF and MS methods whereas non-model organisms, such as *G. gracilis,* rely on MS/MS for protein identification (Haynes & Roberts, 2007). Furthermore, annotated genomes of similar species for data comparison are usually not available for non-model organisms. Two-DE is a well-established gel-based strategy which gives fair protein coverage, good resolution and where costs can be controlled (Beranova-Giorgianni, 2003, Carpentier *et al.*, 2008). Thus, the 2-DE approach is well-suited to investigate the proteome of non-model organisms such as *G. gracilis,* whose genome has been poorly characterised (Lluisma & Ragan, 1997). Moreover, it is a protein based separation technique where the connectivity between protein derived peptides is preserved with high confidence whereas this connectivity is often lost in gel-free approaches (Carpentier *et al.*, 2008). Cross-species identification is the only option for protein identification when a genome is poorly characterised (Wilkins & Williams, 1997; Lester & Hubbard, 2002; Mathesius *et al.*, 2002; Liska & Shevchenko, 2003; Witters *et al.*, 2003; Samyn, 2007), and will be used to assign putative functions to *G. gracilis* proteins. Thus, this study used 2-DE and liquid chromatography tandem mass spectrometry (LC MS/MS) analyses to characterise changes in the *G. gracilis* proteome that occur in response to N limitation and recovery. The results generated in this study provide new insight into N metabolic and assimilatory mechanisms in *G. gracilis* and help to improve our understanding of N stress adaptions in *G. gracilis.*

2.1.1 Aim of this chapter

The aim of this chapter is to evaluate the effect of N limitation and recovery on global protein expression in *G. gracilis* by identifying proteins that are differentially regulated in response to these conditions. In order to accurately assess the effect of N limitation, *G. gracilis* will be exposed to an initial and sustained N limitation period. Similarly, N-limited *G. gracilis* will be re-supplied with N and the recovery response evaluated. The N status of *G. gracilis* thallus samples exposed to N limitation and recovery will be evaluated using physiological measurements of intracellular carbon (C) and N. Differentially regulated proteins identified using 2-DE will be selected based on significance values and sequenced using LC MS/MS. Putative protein identities will be determined on the basis of homology to proteins archived in databases. Furthermore, the functions of the identified proteins will be examined, in context to N limitation and recovery. The latter will be used to identify key pathways which are activated or repressed in response to N limitation and recovery, and which could potentially provide a starting point for the characterisation of the N stress response of *G. gracilis*.

2.2. Materials and methods

All of the growth media and solutions used in this study are listed in Appendix A

2.2.1 Algal material and cultivation conditions

G. gracilis algal samples were sourced from Irvin and Johnson (I&J), Abalone Culture Division, Danger Point, Gansbaai, Western Cape, South Africa. Algal epiphytes and sand were removed by successive washing with sterile distilled water. *G. gracilis* samples were grown in 500 ml Erlenmeyer flasks at a temperature of 18°C, with a light intensity of 45 µmol photons/m^2/s and a 16:8 hour (light:dark) photoperiod (Fig. 2.1). Each flask contained 1.0 g of seaweed in 300 ml of artificial seawater (ASW, Appendix A.1.1) enriched according to the method outlined by Provasoli (1968). All algal cultures were acclimatised for one week in ASW enriched with 1/3 strength Provasoli's enriched seawater (PES) medium (Appendix A.1.4) at the above mentioned conditions before any experiments were initiated. Water movement and aeration within the *G. gracilis* culture flasks was achieved by pumping compressed air into each flask.

Figure 2.1 The *G. gracilis* cultivation system. *G. gracilis* samples were cultivated in Erlenmeyer flasks supplied with constant aeration, maintained at 18°C and a 16:8 hour light:dark photoperiod.

2.2.2 Response of *G. gracilis* to an initial and sustained period of nitrogen limitation

Two separate experiments were conducted to investigate the effect of an initial and sustained period of N limitation on *G. gracilis* protein profiles. N-replete (control) and N-limited (experimental) samples were grown for each experiment and were cultured separately. N-replete samples were cultured in ASW (Appendix A.1.1) enriched with PES (Appendix A.1.4) and N-limited samples were cultured in ASW (Appendix A.1.1) supplemented with PES lacking $NaNO_3$ (Appendix A.1.5) The ammonium supply, $Fe(NH_4)_2SO_4$, was replaced with $Fe_2(SO_4)$ in the experimental flasks. Seaweed samples for the initial and sustained nitrogen response experiments were collected during the autumn season (May in the Southern Hemisphere) and during spring (October in the Southern Hemisphere) respectively. The growth media was replaced every 2 days for both N-replete and N-limited cultures to prevent depletion of essential nutrients required for growth.

2.2.2.1 Initial nitrogen response experiment

Following the initial acclimation period, N-replete samples cultured under control conditions were sampled at time 0. N-limited samples cultured under experimental conditions in media lacking N (as described in section 2.2.2) were collected after 6 hours. Samples (0.5 g) were collected in triplicate from each flask for protein isolation (as described in section 2.2.5). Following protein isolation, triplicate samples were pooled, flash frozen in liquid N and stored at -80°C for later 2-DE analysis (as described in section 2.2.6). Samples were also collected for pigment, N and C content analyses. Three biological replicate experiments were performed.

2.2.2.2 Sustained nitrogen response experiment

Following the initial acclimation period, N-replete samples cultured under control conditions were sampled at day 0. N-limited samples cultured under experimental conditions in media lacking N (as described in section 2.2.2) were sampled after 14 days. Samples (0.5 g) were collected in triplicate from each flask for protein isolation (as described in section 2.2.5). Following protein isolation, triplicate samples were pooled, flash frozen in liquid N and stored at -80°C for later 2-DE analysis (as described in section 2.2.6). Samples were also collected to determine cellular pigment, N and C content. Three biological replicate experiments were set up.

2.2.3 Responses of nitrogen limited *G. gracilis* to nitrogen re-supply

A separate experiment was conducted to investigate the recovery response of N-limited *G. gracilis* samples to a re-supply of N. The experimental design for this experiment differed to that employed for the N limitation experiments as here the control samples were cultured under N-limiting conditions and the experimental samples were cultured under N-replete conditions. All *G. gracilis* cultures were acclimatised for one week in ASW (Appendix A.1.1) enriched with PES (Appendix A.1.4) before any experiments were initiated. The growth media was replaced every two days for both control and experimental cultures to prevent depletion of essential nutrients required for growth.

Following the initial acclimatisation period, *G. gracilis* samples were cultured in growth media lacking N for 7 days. At day 7, N was re-supplied to each flask in the form of ASW (Appendix A.1.1) enriched with PES (Appendix A.1.4) containing nitrate (NO_3^-) and ammonium (NH_4^+). Control flasks were sampled at time 0 and experimental flasks were sampled 6 hours after the addition of growth media containing N. *G. gracilis* samples (0.5 g) were collected in triplicate from each flask for protein isolation (as described in section 2.2.5). Following protein isolation, triplicate samples were pooled, flash frozen in liquid N and stored at -80°C for later 2-DE analysis (as described in section 2.2.6). Samples were also collected for N, C and pigment analyses. Three biological replicate experiments were set up.

2.2.4 Physiological indicators of nitrogen limitation and recovery in *G. gracilis*

2.2.4.1 Total thallus nitrogen and carbon content of G. gracilis samples

G gracilis algal material (0.1 g) was dried at 80°C for 2 days and then homogenised by grinding with a mortar and pestle in liquid N to a fine powder. C and N measurements were determined for each of the three biological repeats. A Thermo Flash EA 1112 series elemental analyzer was used to conduct CHNS analysis on all *G. gracilis* thallus samples (Conducted by Piero Benincasa, Department of Chemistry, University of Cape Town). A combustion analyzer was used where the sample is combusted in a stream of helium with a small amount of oxygen, producing N, carbon dioxide and water vapour. A GC column was used to separate and channel them into a thermal conductivity detector which measures the quantity of each element.

2.2.4.2 Pigment content of G. gracilis samples

Phycocyanin (PC) and phycoerythrin (PE) concentrations in crude *G. gracilis* extracts were determined as described by Beer and Eschel (1985). Briefly, triplicate, 0.1 g *G. gracilis* samples were ground to a fine powder in liquid N with a mortar and pestle. The ground algal tissue was added to 1 ml of 0.1 M phosphate buffer (pH 6.8; Appendix A.2.1), gently mixed and the cell debris removed by centrifugation (14,000 rpm for 5 min at 22°C). The supernatant fraction was retained and the absorption spectra of crude algal extracts for PE and PC determined by absorbance readings at 564, 592, 455, 618 and 645 nm (DU 530, Beckman). PE has three absorption peaks at 495, 595 and 564 nm while PC has one smaller absorption peak at 618 nm. Two equations were used to determine PE and PC concentrations (Beer and Eschel, 1985).

PE concentrations (ml pigment/g tissue) were calculated using the formula (Beer and Eschel, 1985):

$$\mathrm{PE} = \{[(A_{564} - A_{592}) - (A_{455} - A_{592}) \times 0.2] \times 0.12\}$$

where 0.12 is the absorption coefficient for PE derived from specific absorbance values (O'Carra, 1965).

PC concentrations (ml pigment/g tissue) were calculated using the formula (Beer and Eschel, 1985):

$$\mathrm{PC} = \{[(A_{618} - A_{645}) - (A_{592} - A_{645}) \times 0.51] \times 0.15\}$$

where 0.15 is the absorption coefficient for PC derived from specific absorbance values by Oh'Eocha (1965).

2.2.4.3 Relative growth rate

Relative growth rate (μ; the percentage increase in fresh weight per day) was calculated using the equation:

$$\mu\ (\%\ \text{wet mass increase day}^{-1}) = \frac{\left[100\left(\left(\ln\left(\frac{N_t}{N_0}\right)\right)\right)\right]}{t}$$

where μ is the relative growth rate, t is the time in days, N_o is the initial weight of the *G. gracilis* thallus fragment at day 0, and N_t is the weight of the same thallus after t days (Smit and Bolton, 1999).

The relative growth rate of *G. gracilis* thallus fragments was determined for both control and experimental samples. Samples were weighed on days 0, 5, 7, 12 and 14 and the relative growth rates determined over the course of the experiment.

2.2.5 Protein isolation

G. gracilis sample tissue (0.5 g) was ground to a fine powder using a mortar and pestle in liquid N. Protein was isolated from three 0.5 g samples, each collected from the same control and experimental flasks. Total protein was isolated using a phenol extraction protocol (Ingle *et al.*, 2005). Briefly, protein was extracted in 1 ml of extraction buffer (Appendix A.2.2) and cell debris removed by centrifugation (14,000 rpm for 5 min at 4°C). The supernatant was transferred to a fresh microcentrifuge tube and an equal volume of ice-cold 0.5 M Tris-saturated phenol (pH 8.0) was added. The samples were thoroughly mixed for 30 s, before being centrifuged at 14,000 rpm for 1 min at 4°C. Approximately 80% of the aqueous phase was discarded and the organic phase re-extracted with ice-cold extraction buffer. Once again approximately 80% of the aqueous phase was removed and discarded. Five volumes of 0.1 M ammonium acetate in methanol (Appendix A.2.2) was added and the samples incubated at -20°C overnight. Thereafter, the samples were centrifuged (14,000 rpm for 5 min at 4°C), the supernatant discarded, and the protein pellet washed with 1 ml of 0.1 M ammonium acetate in methanol followed by a wash with 80% (v/v) acetone. *G. gracilis* proteins samples were re-suspended in urea lysis buffer (ULB), (Appendix A.2.2). The three *G. gracilis* protein isolations from each flask were subsequently pooled and used for 2-DE. The protein concentration of each sample was determined using the Bio-Rad protein assay (Bio-Rad, Germany) based on the method of Bradford (Bradford, 1976) with one modification: 0.1 M HCl was included to counteract the presence of urea in the ULB. A dilution series of bovine serum albumin (BSA, Pierce, USA) in ULB was used to construct a protein standard curve. The quality and integrity of all protein samples was determined using SDS-PAGE. Approximately 40 µg of high quality and intact *G. gracilis* total protein was separated on a 12% SDS-PAGE gel using the Bio-Rad Mini-Protean protein apparatus (Bio-Rad, Germany) prior to 2-DE.

2.2.6 Two – Dimensional Gel Electrophoresis (2-DE)

Two-dimensional gel electrophoresis separates proteins on the basis of isoelectric point (p*I*) in the first dimension and molecular weight (MW) in the second dimension. For the first dimension 7cm immobilized pH 4-7 gradient (IPG) strips (Bio-Rad) were rehydrated for approximately 13 hours with 140 µl rehydration solution (Appendix A.2.6) containing 250 µg protein. Isoelectric focussing (IEF) was conducted using a Bio-Rad Protean IEF Cell (Bio-Rad, USA). IEF was performed at 20°C using the following parameters: 250 V for 20 min (Linear), 4 000 V for 2 Vhrs (Linear), 4 000 V for 20 000 Vhrs (Linear). Following IEF, the IPG strips were equilibrated in equilibration buffer I (Appendix A.2.6) followed by equilibration buffer II (Appendix A.2.6), for 10 min at 22°C with shaking. The gel strips were rinsed in SDS running buffer and blotted dry. The gel strip was positioned on top of a vertical 12% polyacrylamide gel, held in place with 0.5% agarose (Appendix A.2.6) and electrophoresed at a constant voltage of 100 V for 2 hours. PageRuler™ Plus Prestained Protein Ladder (Fermentas) was loaded beside the strip before overlaying with 0.5% agarose (Appendix A.2.6). Gels were stained with Coomassie Brilliant Blue R-250 (CBB) (Appendix A.2.5) for 1 hour at 37°C and destained with Coomassie destaining solution overnight (O/N) (Appendix A.2.5) at 22°C with shaking. Two-DE was conducted on three biological repeats and three technical repeats were assessed for each biological repeat, therefore a total of 18 gels were used for each 2-DE analysis.

2.2.7 Statistical analysis of 2-DE SDS-PAGE gels and physiological indicators

Gels were scanned at a resolution of 300 dots per inch on a flat-bed Epson perfection V750 colour scanner (Epson, Germany). The scanned gel images were saved as black and white TIFF images. Acquired images were analysed using Melanie 7.0 software (Swiss Institute of Bioinformatics, Genevea, Switzerland). All images were cropped to a similar size and landmarks added to aid in alignment of all gels. Spots in all images were quantified and matched across all gels. Spot quantification was based on spot volume (integration of spot density over spot area) as a percentage of the total spot volumes of the gel. This was done in order to normalize for potential staining differences between gels. Gel annotation and the efficiency of gel matching was confirmed manually to eliminate potential matching errors generated by the software. Two classes, control and experimental, were included in the analysis. These were compared to each other to determine differential protein expression in response to N limitation and recovery. The one-way ANOVA data generated by Melanie for each spot was checked by conducting an independent one-way ANOVA statistical analysis on the same data using SigmaStat for Windows (Version 3.10). Statistically significant differences were established at critical levels of $P<0.05$. Only spots present on all replicate gels for both the control and experimental samples were included in the analysis.

N, C and pigment contents were assessed for statistical differences by one-way analysis of variance (ANOVA, $P<0.05$) with post-hoc tests using SigmaStat for Windows (Version 3.10.0). Data sets that were not normally distributed or that exhibited unequal variances were transformed to meet the requirements for parametric statistical testing. Where data could not be sufficiently transformed, a non-parametric ANOVA on ranks (Kruskal-Wallis) test was used instead.

2.2.8 LC MS/MS identification of proteins

LC MS/MS protein sequencing was conducted as a service by the W. M. Keck biotechnology resource laboratory at Yale University using the following procedure. Protein spots were manually excised from coomassie brilliant blue stained polyacrylamide gels. *In situ* enzymatic digestion was performed and the digested peptides analysed by LC MS/MS as follows. The gel plugs were washed with 250 µl 50% (v/v) acetonitrile/50% (v/v) sterile distilled water for 5 minutes followed by 250 µl of 50 mM ammonium bicarbonate/50% (v/v) acetonitrile/50% (v/v) sterile distilled water for 30 min. One final wash was performed using 10 mM ammonium bicarbonate/50% acetonitrile/50% sterile distilled water for 30 min. After washing, the gel plugs were dried in a Speedvac and rehydrated with 0.1 µg of either modified trypsin (Promega), endopeptidase Lys-C (Wako), -C (Roche) or chymotrypsin (Roche) per (approximately) 15 mm^3 of gel in 15 µl 10 mM ammonium bicarbonate. Samples were digested at 37°C for 16 hrs. Ten microlitres of sample was removed and placed in an injection vial with 5 µl injected.

LC MS/MS was conducted using an LTQ Orbitrap. The LTQ Orbitrap was equipped with a Waters nanoAcquity UPLC system, and used a Waters Symmetry® C18 180µm x 20mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35°C) for peptide separation. Trapping was done at 15 µl/min, using 99% Buffer A (100% water, 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nl/min with Buffer A (100% water, 0.1% formic acid) and Buffer B (100% CH_3CN, 0.075% formic acid). A linear gradient (51 minutes) was run with 5% buffer B at initial conditions, 50% B at 50 minutes, and 85% B at 51 minutes. MS was acquired in the Orbitrap part of the instrument (400-2000 m/z) using 1 microscan, and a maximum injection time of 900 ms in the LTQ part of the instrument followed by four data dependant MS^2 acquisitions of the four highest intensity precursor ions in the ion trap. An additional MS^3 fragment spectrum was obtained if any of the phosphopeptide neutral loss ions (-98.0, -49.0, -32.7, and -24.5 amu) were detected. Alternatively, Multistage Activation (which is a combination of MS/MS and MS^3 spectra) was utilized. In this approach, the MS^2 fragment spectra were acquired for the 6 highest precursor ions with multistage activation being triggered on the -98.0, -49.0, and -32.7 amu neutral loss fragment ions which results in further fragment ion detection in the same MS^2 spectrum. Hence, the data dependent MS^3 neutral loss stage fragment spectra were not acquitted as a state spectra. The data were searched using Mascot Distiller and the Mascot search algorithm.

2.2.9 Database searches

Peptides detected using LC MS/MS were searched against the NCBI public database. Additionally, the Swiss-Prot (expasy.org/sprot/) and NCBI (www.ncbi.nlm.nih.gov) green plants databases were also searched. Database searches were conducted by the W. M. Keck biotechnology resource laboratory at Yale University. All MS/MS spectra were searched using the Mascot algorithm (version 2.2.0) for un-interpreted MS/MS spectra after using the Mascot Distiller program to generate Mascot compatible files. The Mascot Distiller program combined sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 are preferentially located with a signal to noise ratio of 1.2 or greater and a peak list was generated for database searching.

Using the Mascot database search algorithm, the Keck Facility considered a protein identified when Mascot listed it as significant ($P<0.05$) and more than 2 peptides matched the same protein. The database searched was typically the NCBInr chosen over genome specific databases since a match to the correct species had more significance in the larger database. Mascot significance score match was based on a MOWSE score and relied on multiple matches to more than one peptide from the same protein. Typical parameters used for searching included partial methionine oxidation and carboxamidomethylated cysteine, a peptide tolerance of ±20 ppm, MS/MS fragment tolerance of ±0.6 Da, and peptide charges of +2 or +3. In addition, normal and decoy database searches were conducted. The criteria for considering a *G. gracilis* protein successfully identified were a match of at least two peptides to a homolog in the NCBI database and a significant ($P<0.05$) overall MOWSE score. The observed MW and p*I* of the *G. gracilis* proteins were determined from 2-DE polyacrylamide gels using p*I* (Bio-Rad) and MW protein ladders (Fermentas). The ExPASy-Compute p*I*/MW tool was used to determine the predicted MW and p*I* of the closest NCBI homolog in the database.

2.3 Results

2.3.1. Physiological indicators of nitrogen limitation and recovery

2.3.1.1 Carbon and nitrogen content of G. gracilis samples in response to nitrogen limitation and recovery

G. gracilis intracellular C and N levels were determined to evaluate the N status of each N-replete and N-limited sample using elemental CHNS analysis. C:N ratios were also determined as this is considered an additional measure of the N status of the alga (Hanisak *et al.*, 1990; Smit *et al.*, 1997). The C content of the *G. gracilis* tissue samples remained relatively constant in response to all three N treatments (Fig. 2.2A). The N content and C:N ratios of the N-limited *G. gracilis* tissue samples were similar to the N-replete samples over the first 6 hours of N limitation (Figs. 2.2B and C). The N content decreased slightly from 2.3± 0.09% at day 0 to 2.23 ± 0.08% six hours after the onset of N limitation. The C:N ratios were similar with values of 12.2 ± 0.43 and 12.7 ± 0.49 determined for the N-replete and N-limited samples, respectively (Fig. 2.2C). Similarly, intracellular C and N levels were determined in *G. gracilis* exposed to 14 days of N limitation and the N-replete control. The N content decreased from 1.96 ± 0.05% to 1.04 ± 0.16% (Fig. 2.2B) and the C:N ratios increased after 14 days of N limitation from 14 ± 0.16 at day 0 to 29 ± 4.1 at day 14 (Fig. 2.2C).

The experimental design for the N recovery experiment differed to that employed for the N limitation experiments, as the N-limited *G. gracilis* samples were the control and the N-replete samples were the experimental. The N content increased from 0.45 ± 0.13% in N-limited *G. gracilis* to 0.85 ± 0.13% in N-replete *G. gracilis* following 6 hours of N recovery (Fig. 2.2B). The C:N ratios decreased from 85 ± 34 at time 0 to 29 ± 6.17 following 6 hours of N recovery (Fig. 2.2C). Statistically significant ($P<0.05$) changes in N content or C:N ratios were not detected between the N-replete and N-limited *G. gracilis* samples for any of the N treatments.

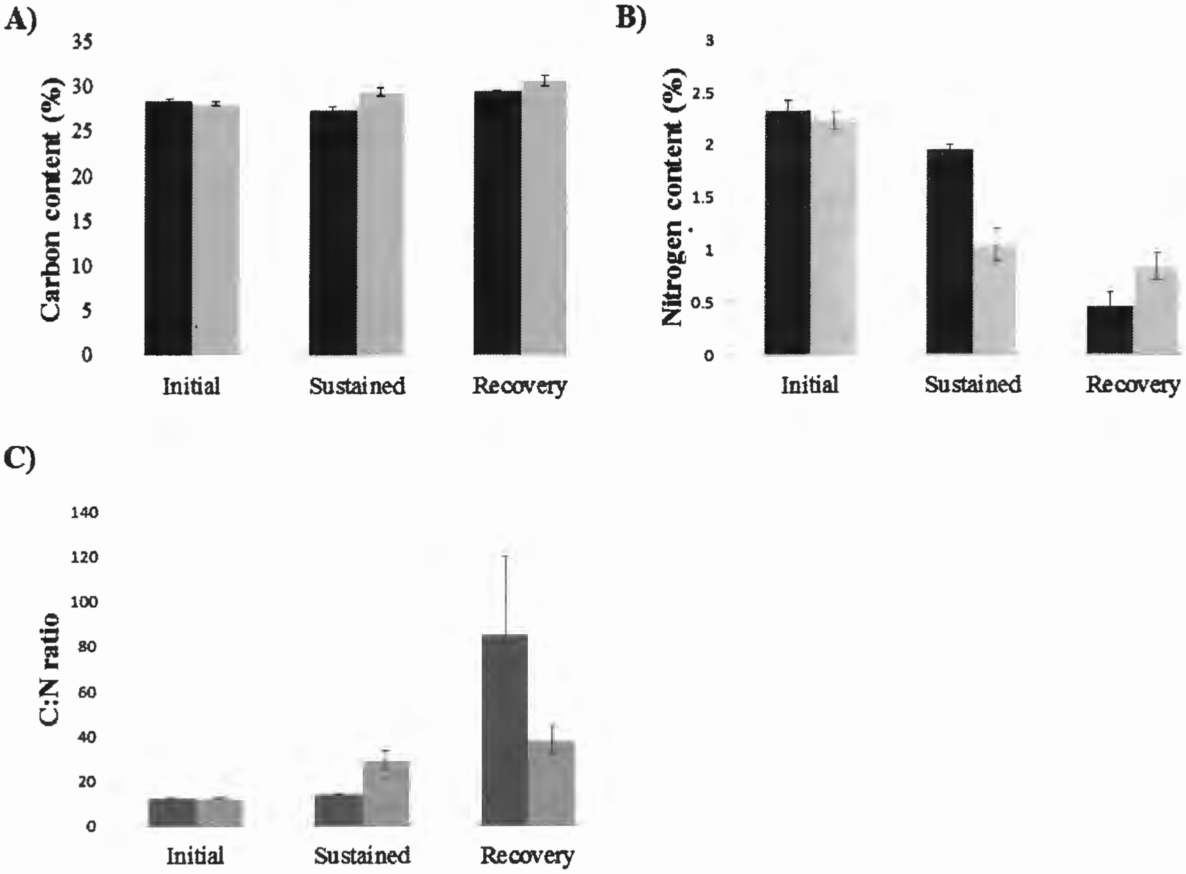

Figure 2.2 Carbon content (A), nitrogen content (B) and C:N ratios (C) of *G. gracilis* that had been nitrogen limited for 6 hours (Initial), nitrogen limited for 14 days (Sustained) and nitrogen recovered for 6 hours (Recovery). Each bar represents either a control (■) or experimental (■) *G. gracilis* sample. Data represents the mean ± standard error (*n*=3).

2.3.1.2 Pigment content of nitrogen limited and nitrogen recovered G. gracilis

Thallus colour is an important indicator of the N status of algae (Oliveira *et al.*, 2000) and indeed previous studies have shown that pigment contents of *Gracilaria* species are closely correlated with N content of the tissue (Lapointe & Ryther, 1979; Robertson-Andersson *et al.*, 2009). In this study, PE and PC pigmentation were found to decrease as the N content of *G. gracilis* tissue declined. PE and PC assayed at time 0 and over the first 6 hours of N limitation were 0.35 ± 0.007 and 0.066 ± 0.002 ml pigment/g tissue for the N-replete samples, while PE and PC contents for the N-limited samples were determined to be 0.34 ± 0.005 and 0.065 ± 0.002 ml pigment/g tissue, respectively (Figs. 2.3, 2.4).

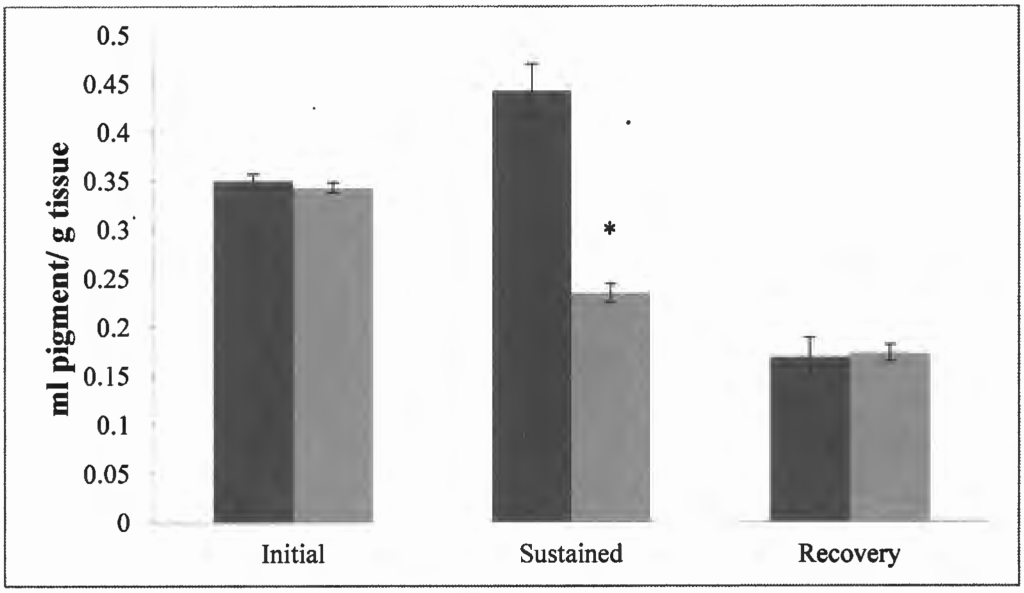

Figure 2.3 PE pigment content in *G. gracilis* that had been nitrogen limited for 6 hours (Initial); nitrogen limited for 14 days (Sustained) and nitrogen recovered for 6 hours (Recovery). Each bar represents either a control (■) or experimental (■) *G. gracilis* sample. Data represents the mean ± standard error (n=3). * ($P<0.05$; Holm-Sidak test) represents a significant difference between the pigment content of N-replete and N-limited *G. gracilis* following exposure to a sustained period of nitrogen limitation.

PE and PC content of the N-replete and N-limited samples differed significantly ($P<0.05$) following 14 days of N limitation. PE content was determined to be 0.44 ± 0.03 and 0.24 ± 0.009 ml pigment/g tissue for the N-replete and N-limited samples, (Fig. 2.3), while PC levels were 0.071 ± 0.001 and 0.040 ± 0.003 ml pigment/g tissue, respectively (Fig. 2.4).

PE and PC were similar for both the N-limited and N-replete samples over the 6 hours of N recovery. PE levels were determined to be 0.19 ± 0.03 and 0.17 ± 0.03 ml pigment/g tissue (Fig. 2.3) and PC pigment levels were 0.03 ± 0.003 and 0.029 ± 0.001 ml pigment/g tissue for the N-limited and N-replete samples, respectively (Fig. 2.4).

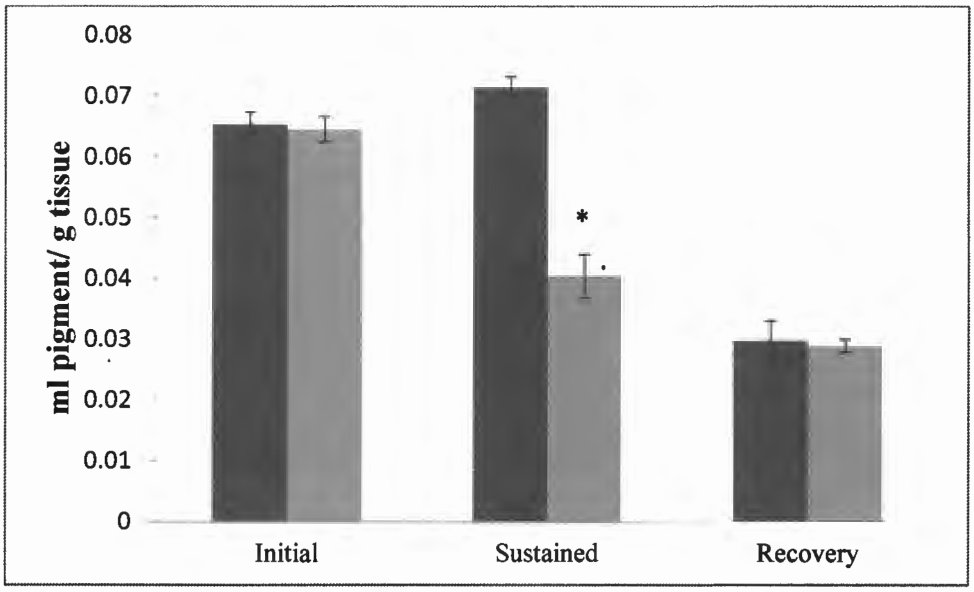

Figure 2.4 PC pigment content in *G. gracilis* that had been nitrogen limited for 6 hours (Initial); nitrogen limited for 14 days (Sustained) and nitrogen recovered for 6 hours (Recovery). Each bar represents either a control (■) or experimental (■) *G. gracilis* sample. Data represents the mean ± standard error (n=3). * (P<0.05; Holm-Sidak test) represents a significant difference between the pigment contents of the N-replete and N-limited *G. gracilis* following exposure to a sustained period of nitrogen limitation.

2.3.1.3 Effect of nitrogen limitation on the relative growth rate of G. gracilis

The relative growth rate (μ) of each *G. gracilis* thallus sample was determined to assess the effect of N limiting conditions on growth (Fig. 2.5). The μ decreased for both the N-replete and N-limited samples over the course of the experiment. However, the decline in μ was greater for the N-limited thallus samples (Fig. 2.5). A statistically significant (P<0.05) difference in μ between the N-replete and N-limited samples was observed at days 7, 12 and 14 (Fig. 2.5).

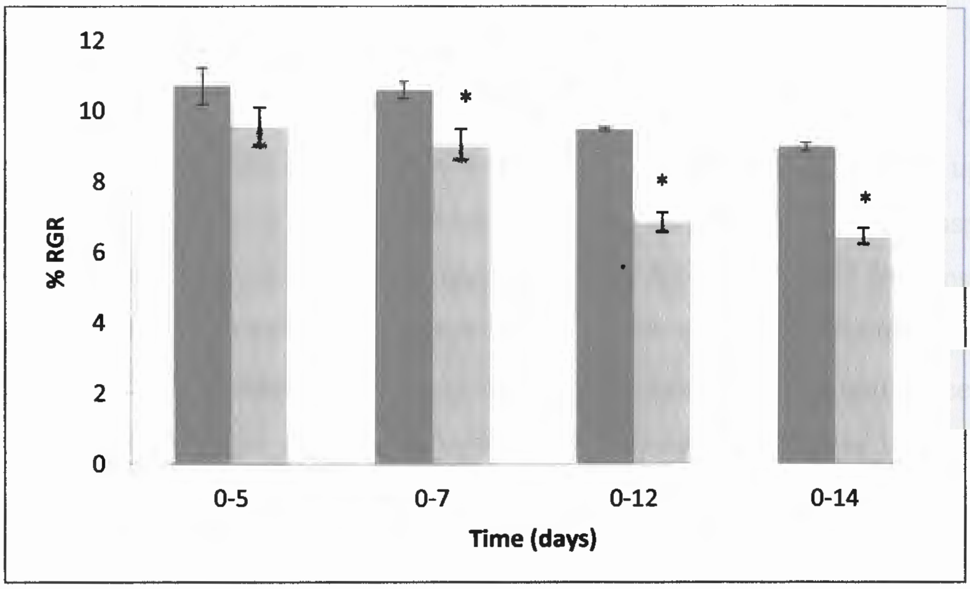

Figure 2.5 Relative growth rate (% RGR) of nitrogen-replete (■) and nitrogen-limited (■) *G. gracilis* samples on days 5, 7, 12 and 14. Data represents the mean ± standard error (n=3). * (P<0.05; t-test) denote significant differences between the nitrogen-replete and nitrogen-limited thalli at each time point.

2.3.2 Analysis of *G. gracilis* proteome response to varying levels of nitrogen stress

Protein integrity and quality was assessed for each N limitation experiment and the N recovery experiment using 1D SDS-PAGE and Coomassie Brilliant Blue (CBB) staining prior to the commencement of any 2-DE analyses. Protein quality was determined to be optimal for all N treatments as no protein degradation was observed; a representative CBB SDS-PAGE gel is displayed below (Fig. 2.6). Protein samples from each N limitation experiment were subjected to IEF and 2D SDS PAGE after which each data set was analysed individually using Melanie 2-DE analysis software. All analysed gels were normalised using Melanie 2-DE analysis software to minimise staining variations across gels. Selection of protein spots from each of the N experiments for LC MS/MS sequencing was based on the rank of each protein as determined by significance values (P<0.05) and whether the protein spots were well defined, and clearly visible. These were excised from the 2-DE gels with an automated spot picker for MS/MS identification. *De novo* proteins were not detected in response to N limitation or recovery in *G. gracilis*. However, it is possible that *de novo* proteins may be present in low concentrations and a more sensitive staining system may be required for their detection.

2.3.2.1 Proteome response of *G. gracilis* to initial nitrogen limitation

A total of 41 protein spots were determined to be differentially regulated ($P<0.05$) in response to an initial period of N limitation. Twenty-six of these proteins were up-regulated and 15 were down-regulated following 6 hours of N limitation. After manual inspection and editing of the data, it was decided to sequence eight of the up-regulated proteins (Fig. 2.7). While down-regulated proteins were observed in response to initial N limitation, these were not selected for further sequencing as they were ranked lower in the significance table. The observed fold changes were small. These were measured as spot intensity values and ranged from +0.02 to +0.1 (Table 2.1). The observed and predicted MW and p*I* were similar for all selected proteins except for spot 298, which had an increased MW (Table 2.1).

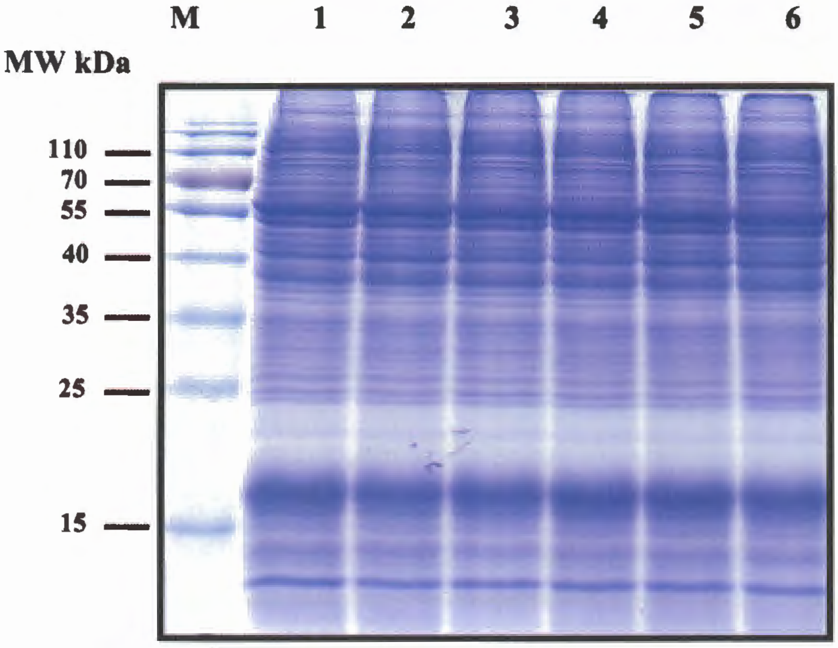

Figure 2.6 A representative CBB stained protein gel showing the quality and integrity of total protein extracted from *G. gracilis* following 14 days of nitrogen limitation. Thirty micrograms of total protein was mixed with 5x sample application buffer, boiled for 5 min and separated on a 12% SDS-PAGE gel. Lanes 1, 2 and 3 represent pooled nitrogen-replete *G. gracilis*, while lanes 4, 5 and 6 represent pooled nitrogen-limited *G. gracilis* from biological repeats 1, 2 and 3. Lane M represents the pre-stained molecular weight ladder (Fermentas).

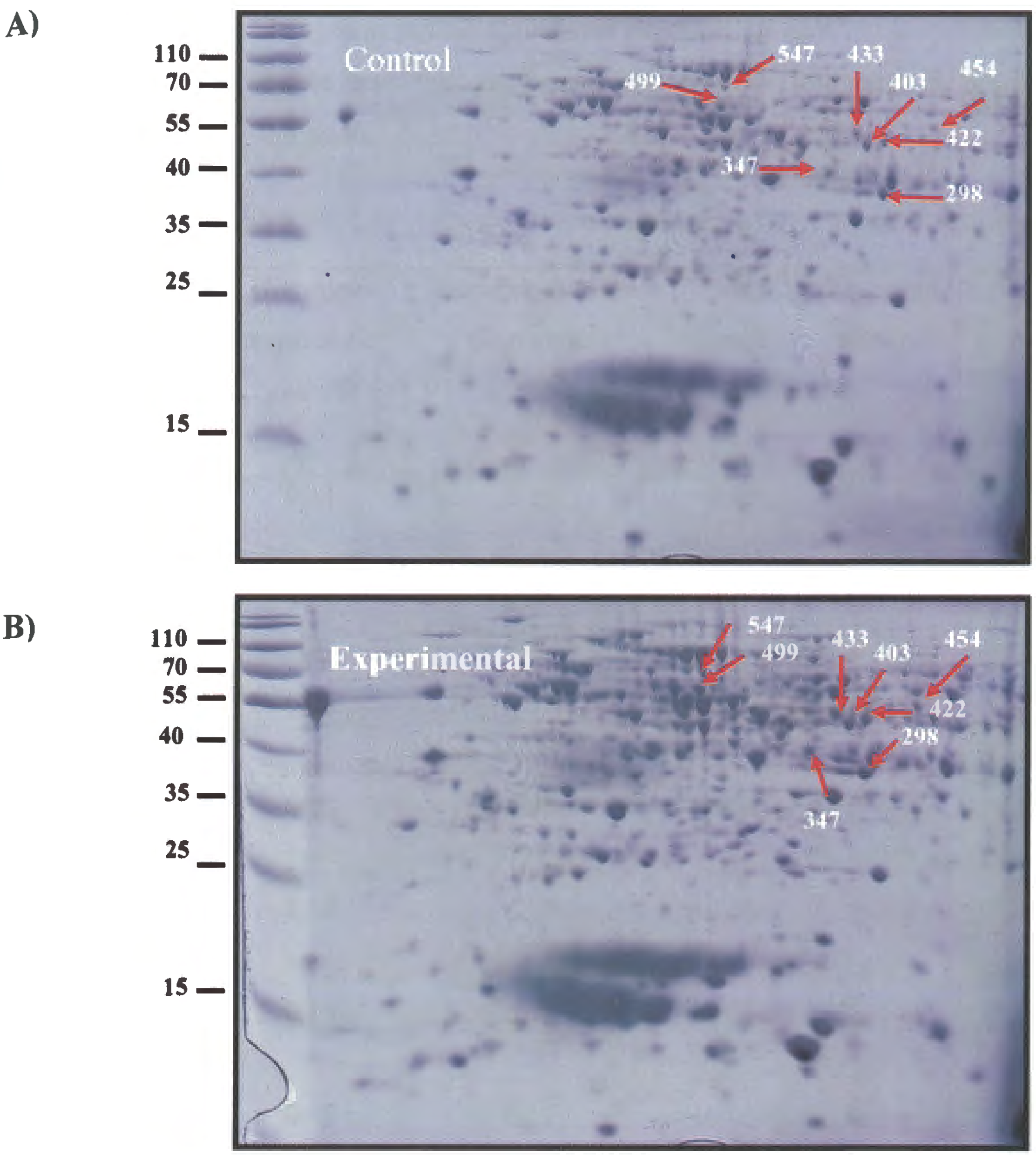

Figure 2.7 Two-dimensional gel of changes in the abundance of protein isolated from *G. gracilis* cultured in nitrogen-replete (control) (A) and nitrogen-limited (experimental) (B) media for 6 hours. Three replicate gels were run for each biological repeat. Nitrogen-responsive proteins that were successfully identified by tandem mass spectrometry (MS/MS) analysis are highlighted by red arrows. All proteins selected had increased levels in the nitrogen-limited samples relative to the control. A pre-stained molecular weight marker (Fermentas), on the left side of each gel image, represents approximate molecular weights in kDa to enable size estimation for each protein spot.

Table 2.1 Changes in protein abundance expressed as spot intensity values for nitrogen-replete and nitrogen-limited *G. gracilis* following 6 hours of nitrogen limitation.

Spot no.	**Nitrogen-replete *G. gracilis***	**Nitrogen-limited *G. gracilis***	**Fold change**	**Observed MW/p*I***	**Predicted MW/p*I***	***P* value**
433	0.11	0.19	+0.09	42/6.1	36/6.53	6.22E-04
403	0.21	0.31	+0.1	40/6.1	41/5.42	0.002442
298	0.25	0.32	+0.07	37/6.2	17/5.4	0.00365
454	0.06	0.08	+0.02	45/6.5	42/6.10	0.008013
422	0.16	0.22	+0.06	40/6.3	41/5.42	0.010973
499	0.13	0.19	+0.06	56/5.0	56/5.8	0.013535
347	0.12	0.17	+0.05	37/5.8	36/5.86	0.019443
547	0.09	0.12	+0.03	60/5.0	51/5.06	0.047777

Relative fold-changes and significance values ($P<0.05$) are indicated. Approximate molecular weight (MW) (kDa) and isoelectric point (p*I*) values as estimated from gels are also displayed, along with the predicted values of the closest homolog in the NCBI database as predicted using the ExPASy-Compute p*I*/Mw tool.

2.3.2.2 Proteome response of *G. gracilis* to sustained nitrogen limitation

A total of 44 protein spots were determined to be differentially expressed (*P*<0.05) in response to 14 days of N limitation. Twelve protein spots were up-regulated, 27 were down-regulated and 5 protein spots were deemed to be artefacts and were not analysed further. After manual inspection and editing of the data, it was decided to sequence 2 of the up-regulated and 6 of the down-regulated proteins (Fig. 2.8). The observed fold changes, measured as spot intensity changes, were small. They ranged from 0.05 to 0.13 for the down-regulated proteins (Table 2.2), while an increase in fold change of 0.5 and 0.13 was observed for spot 57 and 237, respectively. The observed and predicted MW values were similar for all selected proteins, however the observed and predicted p*I* varied slightly for some proteins (Table 2.2).

2.3.2.3 Proteome response of *G. gracilis* to nitrogen recovery

A total of 66 protein spots were determined to be differentially expressed (*P*<0.05) in response to the re-supply of N to N-limited *G. gracilis* samples. Of these 50 were up-regulated, 8 were down-regulated and 8 protein spots were artefacts and were discarded from the analysis. After manual inspection and editing of the data it was decided to sequence 6 of the up-regulated proteins (Fig. 2.9.). The fold changes, measured as spot intensity increases were small and ranged from +0.05 to +0.14 (Table 2.3). The observed and predicted MW and p*I* were calculated for each protein spot. The observed and predicted MW and p*I* were similar for all selected proteins except for spot 464, which had an increased MW (Table 2.3).

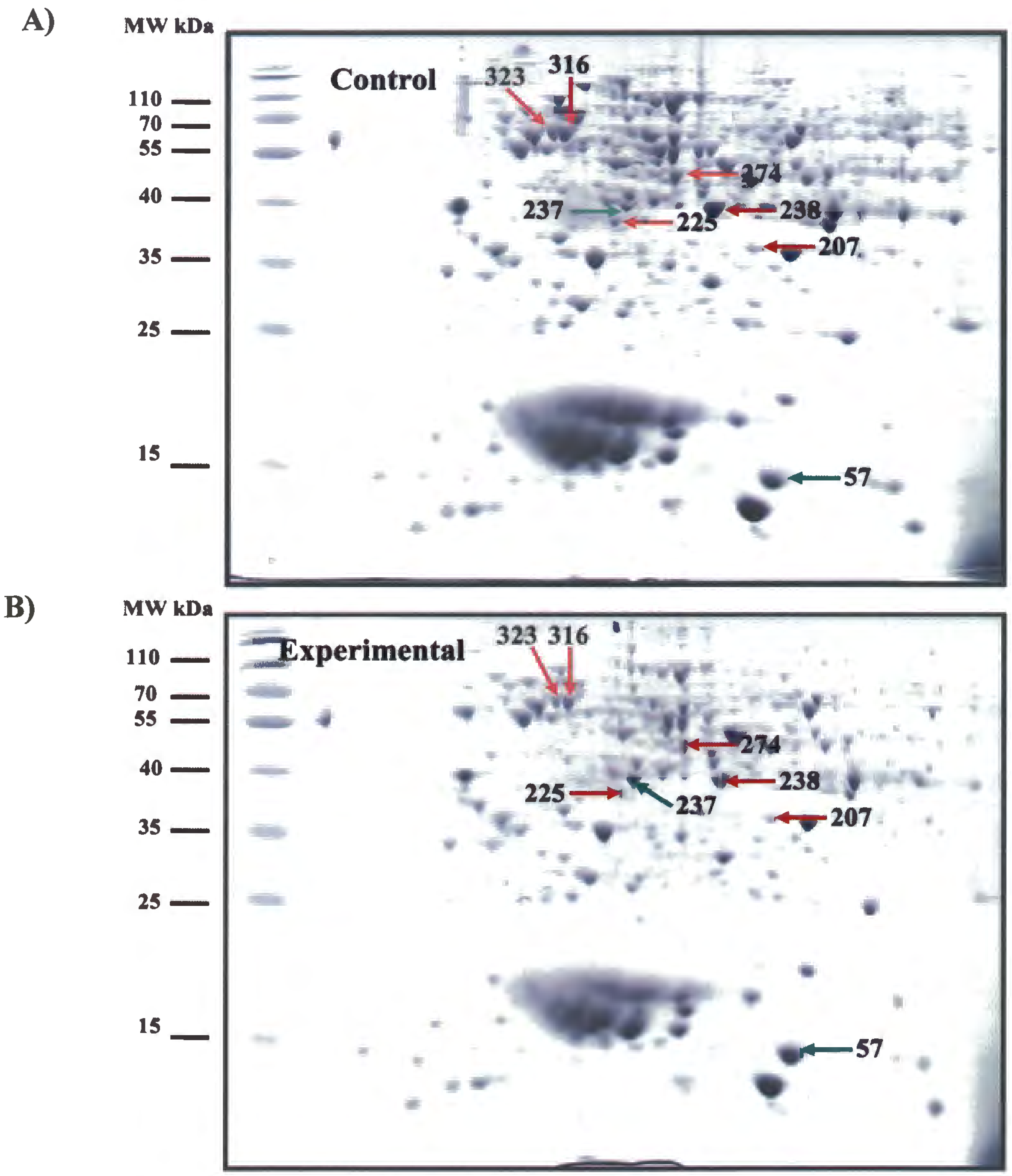

Figure 2.8 Two-dimensional gel of changes in the abundance of nitrogen-replete (A) and nitrogen-limited (B) *G. gracilis* proteins following 14 days of nitrogen limitation. Three replicate gels were run for each biological repeat. Nitrogen-responsive proteins that were successfully identified by tandem mass spectrometry (MS/MS) analysis are highlighted by red (down-regulated) and green (up-regulated) arrows. A pre-stained molecular weight marker (Fermentas), on the left side of each gel image represents approximate molecular weights in kDa to enable size estimation for each protein spot.

Table 2.2 Changes in protein abundance expressed as spot intensity values for N-replete and N-limited *G. gracilis* which had been N-limited for 14 days.

Spot no.	**Nitrogen-replete *G. gracilis***	**Nitrogen-limited *G. gracilis***	**Fold change**	**Observed MW/pI**	**Predicted MW/pI**	***P* value**
57	0.7	1.2	+0.5	15/6.0	17/6.57	1.13E-05
237	0.28	0.45	+0.13	38/4.8	25/4.6	2.71E-05
323	0.41	0.30	-0.11	60/4.5	54/4.79	4.43E-05
225	0.30	0.17	-0.13	38/5.4	45/8.75	8.67E-05
274	0.46	0.33	-0.13	45/5.1	47/6.06	9.86E-05
207	0.21	0.16	-0.05	36/6.0	40/8.36	2.44E-04
238	0.68	0.55	-0.13	38/5.3	38/5.96	0.001864
316	0.52	0.40	-0.12	60/4.5	54/4.79	0.008825

Relative fold-changes and significance values ($P<0.05$) are indicated. Approximate molecular weight (MW) (kDa) and isoelectric point (p*I*) values as estimated from gels are also displayed, along with the predicted values of the closest homolog in the NCBI database as predicted using the ExPASy-Compute p*I*/Mw tool.

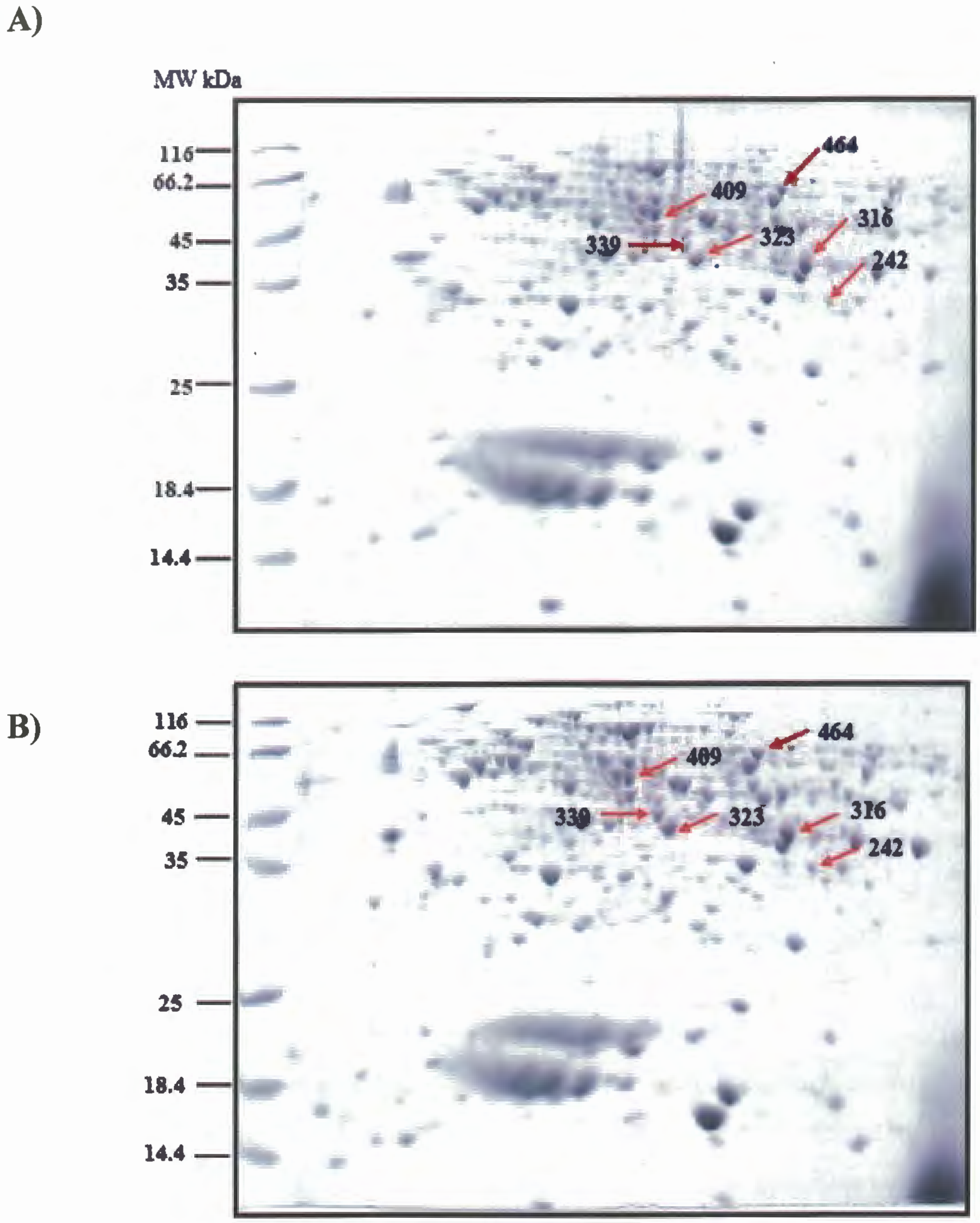

Figure 2.9 Two-dimensional gel of changes in the protein abundance of *G. gracilis* for nitrogen-limited (A) and nitrogen-replete (B) thallus samples. Nitrogen-responsive proteins that were successfully identified by tandem mass spectrometry (MS/MS) analysis are highlighted on representative gels by red arrows. All proteins selected were shown to have increased levels of protein in the experimental sample relative to the control sample. A pre-stained molecular weight marker (Fermentas), on the left side of each gel image, represents approximate molecular weights in kDa to enable size estimation for each protein spot.

Table 2.3 Changes in protein abundance expressed as spot intensity values for nitrogen-limited and nitrogen-recovered *G. gracilis* following six hours of nitrogen recovery.

Spot no.	N-limited *G. gracilis*	N-replete *G. gracilis*	Fold change	Observed MW/pI	Predicted MW/pI	*P* value
339	0.28	0.41	+0.1	40/6.0	39/5.36	7.73E-06
316	0.74	0.88	+0.14	37/7.0	35/8.57	1.20E-04
464	0.32	0.42	+0.1	60/6.8	11/4.54	2.72E-04
323	0.61	0.72	+0.11	38/5.4	45/8.75	0.002234
409	0.32	0.42	+0.1	50/5.5	51/5.06	0.002275
242	0.11	0.16	+0.05	35/6.5	35/6.94	0.005279

Relative fold-changes and significance values ($P<0.05$) are indicated. Approximate molecular weight (MW) (kDa) and isoelectric point (p*I*) values as estimated from gels are also displayed, along with the predicted values of the closest homolog in the NCBI database as predicted using the ExPASy-Compute p*I*/Mw tool.

2.3.3 Putative identity and function of differentially regulated *G. gracilis* proteins in response to nitrogen stress

ESI LC MS/MS protein sequencing was employed to obtain sequence information which enabled the putative identification of eight proteins which responded to initial N limitation, eight proteins which responded to sustained N limitation and six proteins which responded to N recovery (Table 2.4). Peptides, of each protein were searched against the NCBI and SwissProt databases and putative *G. gracilis* protein identities obtained. Protein spots 433, 347, 454, 547, 298, 403, 499 and 422 were observed to increase in abundance in response to initial N limitation in *G. gracilis*. Three spots, 433, 347 and 454 were putatively identified as enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and galactose-1-phosphate uridyltransferase (GALT) based on NCBI homology database searches, and are proposed to be involved in carbohydrate metabolic processes, glycolysis and galactose metabolism, respectively (Table 2.4). Spots 547 and 298 are proposed to be involved in ATP synthesis and photosynthesis based on their identity to ATP synthase α sub-unit and PE α sub-unit, respectively (Table 2.4). Two actin containing spots (403 and 422) and one chaperonin protein (499) are thought to be involved in cytoskeleton re-modelling and protein re-folding processes, respectively. Similarly, the putative identities of eight proteins differentially regulated in response to 14 days of N limitation (Table 2.4) were also obtained. Spots 57 and 237 were putatively identified as PC α sub-unit and elongation factor Tu based on NCBI homology database searches, respectively. These proteins were observed to increase in abundance in response to sustained N limitation in *G. gracilis*, and were involved in photosynthesis and protein biosynthesis processes. Six proteins observed to decrease in abundance in response to a sustained period of N limitation in *G. gracilis* were identified. Protein spots 225, 274 and 238 were putatively identified as GAPDH, phosphoglycerate kinase (PGK) and fructose 1, 6 biphosphate aldolase (ALD), key enzymes involved in glycolysis. Protein spots 323, 316 and 207 are thought to be involved in photosynthesis and energy generation due to their homology to ATP synthase α sub-unit and ferredoxin NADP reductase (FNR). Six proteins spots which increased in abundance in response to N recovery in *G. gracilis* were identified (Table 2.4). Spot 339, which was putatively identified as glutamine synthetase (GS), increased its abundance in response to N recovery. A putative CbbX protein (spot 242) and ATP synthase β sub-unit protein (spot 409) are proposed to be involved in the generation of energy during N recovery. Spots 316 (tRNA-dihydrouridine synthase B), 323 (ALD) and 464 (thioredoxin) are thought to be involved in transcriptional regulation, glycolysis and redox processes, respectively. The biological processes activated or repressed in response to N limitation and recovery were determined using bioinformatics analysis (www.blast2go.org) and are summarised below (Fig. 2.10).

Table 2.4 Putative identity of *G. gracilis* proteins which were differentially regulated in response to nitrogen limitation and recovery

Spot no.	Putative protein identity	Mascot Score^{a}	Sequence coverage (%)	Accession number^{b}	Biological Process
Proteins spots increasing in intensity in response to an initial period of N limitation					
403	Actin (*Chondrus crispus*)	371	19.6	gi|1703152	Cytoskeleton
422	Actin 1 (*Hildenbrandia rubra*)	271	13.4	gi|7655617	Cytoskeleton
433	α-enolase (*Homo sapiens*)	118	4.2	gi|2661039	Glycolysis
347	GAPDH (*Gracilaria gracilis*)	70	7.8	gi|1730194	Glycolysis
454	Galactose-1-phosphate uridyltransferase (*Gracilaria gracilis*)	57	13.3	gi|2674193	Galactose metabolism
547	ATP synthase α sub-unit (*Lactuca sativa)*	424	19.1	gi|8117624	ATP synthesis
499	Chaperonin GroEL (*Gracilaria tenuistipitata*)	348	14.8	gi|5120988	Protein re-folding
298	Phycoerythrin α sub-unit (*Porphyra yeozensis*)	109	9.8	gi|2317690	Photosynthesis
Proteins spots increasing in intensity in response to a sustained period of N limitation					
57	Phycocyanin α sub-unit (*Gracilaria tenuistipitata*)	288	24.7	gi|51210030	Photosynthesis,
237	Elongation factor Tu (*Gracilaria lemaneiformis*)	174	18.7	gi|1706610	Protein biosynthesis

Proteins spots decreasing in intensity in response to a sustained period of N limitation

323	ATP synthase α sub-unit (*Gracilaria tenuistipitata*)	959	37.6	gi|51209988	ATP synthesis
225	Glyceraldehyde-3-phosphate dehydrogenase (*Homo sapiens*)	130	8.4	gi|31645	Glycolysis
274	Phosphoglycerate kinase (*Chondrus Crispus*)	262	9.2	gi|27446627	Glycolysis
207	Ferredoxin NADP Reductase (*Cyanophora paradoxa*)	161	7.7	gi|399485	Photosynthesis
238	Fructose 1,6 biphosphate aldolase (*Galdieria sulphuraria*)	129	5	gi|6746596	Glycolysis
316	ATP synthase α sub-unit (*Gracilaria tenuistipitata*)	1009	33.6	gi|51209988	ATP synthesis

Proteins spots increasing in intensity in response to N recovery

339	Glutamine synthase 11 (*Gelidium crinale*)	95	5	gi|17223660	Nitrogen metabolism
242	CbbX protein (*Gracilaria tenuistipitata*)	128	7.5	gi|51210008	ATP synthesis
316	tRNA-dihydrouridine synthase B (*Dickeya dadantii*)	48	2.2	gi|24224106	Transcriptional regulation
323	Fructose 1,6, biphosphate aldolase (*Galdieria sulphuraria*)	140	5.0	gi|6746596	Glycolysis
464	Thioredoxin (*Campylobacter coli*)	157	27.9	gi|57168680	Redox
409	ATP synthase β sub-unit (*Gracilaria tenuistipitata*)	1777	54.5	gi|51209972	ATP synthesis

[a]: Mascot threshold score

[b]: Accession numbers refer to the NCBInr database.

Identified peptides are displayed in Appendix B

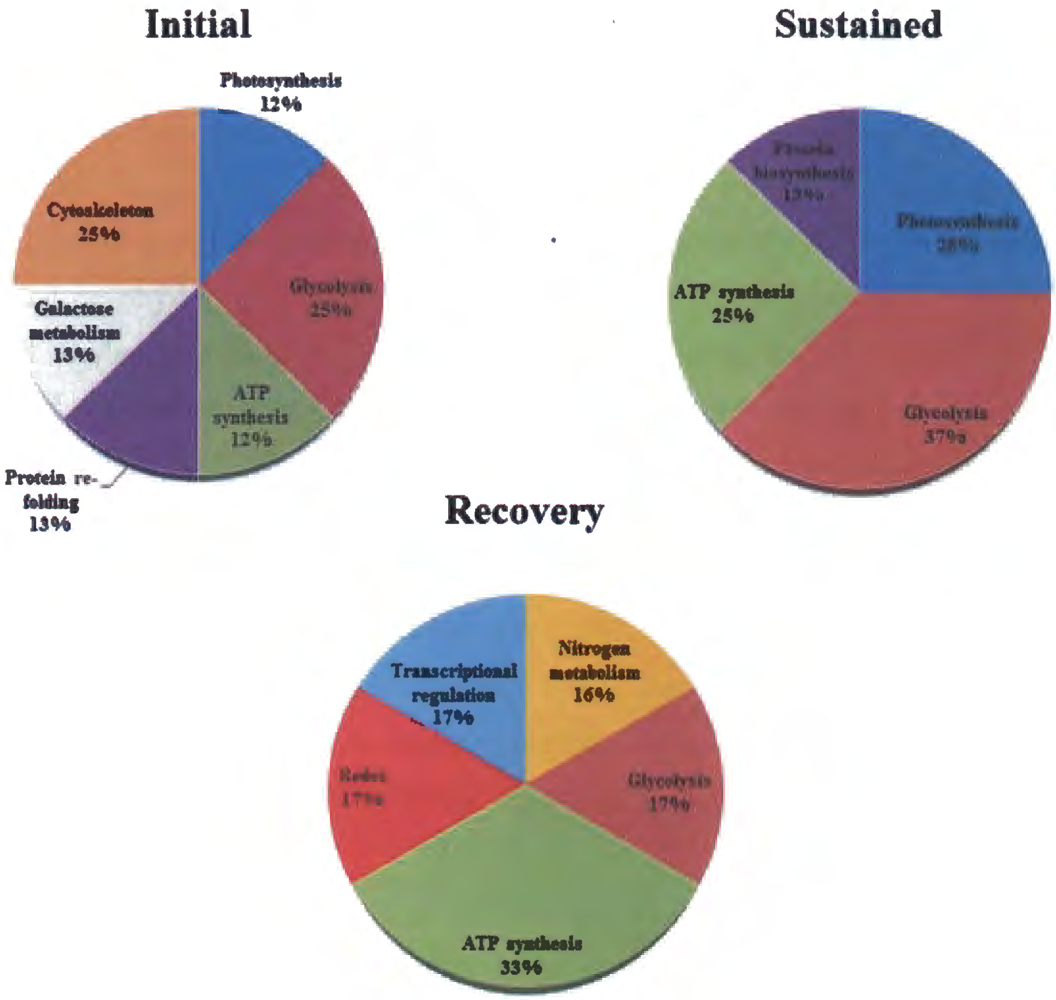

Figure 2.10 Pie chart representing the distribution of biological processes activated or repressed in response to an initial and sustained period of nitrogen limitation, and nitrogen recovery in *G. gracilis.* The identified proteins were classified into different ontologies using Blast2GO annotation.

2.4 Discussion

In natural ecosystems N is often in short supply and organisms such as bacteria, algae and plants must tightly couple growth to its availability. Research studies investigating the mechanisms involved in N limitation and recovery in macroalgae such as *G. gracilis* have mainly focussed on physiological responses such as N uptake kinetics, storage of N and utilisation of N in relation to shore position and season (Rico & Fernandez, 1996; Smit, 2002; Phillips & Hurd, 2003; Young *et al.*, 2009). However, it is important to gain an understanding of the molecular mechanisms involved as N signals are responsible for regulating genes and proteins involved in N metabolism, photosynthesis and C metabolism. Moreover, N stress elicits a large amount of cross-talk between the signalling pathways of N uptake, C metabolism and light response pathways (Hsieh *et al.*, 1998; Lam *et al.*, 1998; Lejay *et al.*, 1999; Moorhead *et al.*, 1999; Oliveira & Coruzzi, 1999; Sugden *et al.*, 1999). Thus, an investigation of global protein expression changes in response to N limitation and recovery in *G. gracilis* was undertaken in this study to further advance our knowledge of the N responses in *G. gracilis*.

Previous studies investigating proteome changes in response to N have been conducted in wheat, triticale, barley and maize plants (Bahrman *et al.*, 2004; Prinsi *et al.*, 2009; Castillejo *et al.*, 2010; Møller *et al.*, 2011). These studies have enabled detailed analysis of comparative proteome changes occurring in roots and shoots in response to various N treatments (Bahrman *et al.*, 2004; Prinsi *et al.*, 2009; Castillejo *et al.*, 2010; Møller *et al.*, 2011). Although proteins which have important functions in C and N metabolism, as well as photosynthesis, were identified, very little overlap was found among the individual proteins identified in these studies (Møller *et al.*, 2011). Thus, analysis of proteome changes in response to N limitation and recovery would allow a comparative analysis of N responses in *G. gracilis*. Furthermore, changes in protein abundance allow one to generate hypotheses regarding the molecular mechanisms used by *G. gracilis* to overcome N stress (Møller *et al.*, 2011).

In this study the response of *G. gracilis* to both an initial and sustained period of N limitation, as well as N recovery was investigated. While each N treatment elicited similar metabolic responses, the pattern of protein regulation was distinct for each treatment. The majority of the identified proteins were up-regulated in response to an initial period of N limitation. These *G. gracilis* proteins are proposed to be involved in a range of biological processes which included glycolysis, photosynthesis, protein re-folding and ATP synthesis and binding. However, no proteins directly involved in N metabolism were identified. To the best of our knowledge the effect of N limitation on global protein expression at such an early time-point has not been investigated in either plants or algae. However, the effects of short-term P deprivation were investigated in *Arabidopsis* by Hammond *et al.* (2003) using microarray technology. Here, the withdrawal of P for a short time period (4 hours) resulted in transient changes in gene expression that were highly variable and resulted in the identification of mainly non-specific, shock-induced genes. A long-term response to P limitation (100 hours) resulted in the differential expression of a new group of 61 genes and many of these 'late' responsive genes were specifically involved in P metabolism (Hammond *et al.*, 2003). Misson *et al.* (2005) also investigated the short-term effects of P limitation in *Arabidopsis* using Affymetrix gene chip (ATH1) technology and found that most of the genes differentially regulated in response to short-term P stress were up-regulated. Seventy-two genes showed an increase in expression and only 4 genes were suppressed (Misson *et al.*, 2005). Bi *et al.* (2007) conducted a comprehensive study which investigated the effect of varying levels of N stress on *Arabidopsis* plants using a whole genome array and their results suggested there is a possible interaction between general and N stress responses. However, it is difficult to draw direct comparisons as firstly Bi *et al.* (2007) investigated transcriptional changes in response to N limitation and secondly the experimental design differed to that employed in this study. Bi *et al.* (2007) used differing amounts of nitrate in their treatments while in this study, external N was removed from all treatment flasks for the limitation studies. However, a number of interesting observations were made. They investigated the effect of three different nitrate levels (treatments) on transcript levels with 3 mM nitrate defined as sufficient, 1 mM nitrate as a mild N-limiting condition and 0.3 mM nitrate as the severe N-limiting condition. It was observed that a greater number of genes were regulated in response to severe chronic N stress than mild N stress. Moreover, the majority of the proteins were up-regulated in response to mild N stress, i.e. 51 were found to be up-regulated and 1 down-regulated. Bi *et al.* (2007) observed a more pronounced effect in response to severe chronic N stress with a much larger number of genes found to be responsive. A total of 461 genes were identified as being differentially expressed with 261 genes reported to be up-regulated and 190 down-regulated. Thus, the change in expression pattern is much smaller for mild N stress than with severe N stress. It was also observed that under conditions of mild N-limitation, significant changes in gene expression were not observed for those genes directly involved in nitrate assimilation (Bi *et al.*, 2007). However, under conditions of severe N limitation, primary metabolic genes, including those involved in N assimilation, were found to be significantly down-regulated (Bi *et al.*, 2007). The identified genes were involved in a wide variety of biological processes including N metabolism, C metabolism, photosynthesis, oxidative stress responses and starch biosynthesis. Furthermore, it was observed that there was a 50% overlap between mild N-limitation and severe N-limitation with some genes showing increased levels of expression under severe N-limitation. These included genes involved in the abiotic and biotic stress response such as glutathione-S-transferases (GSTs), peroxidases and genes involved in anthocyanin synthesis. Together, these studies strongly indicate that the degree of nutrient limitation is an important factor to consider as the responses are not universal and may vary with the degree of stress imposed. Similar effects were observed in this study with *G. gracilis* samples grown in media with no N for six hours. Although, direct comparisons cannot be made with the results obtained for short-term P limitation or mild N limitation in *Arabidopsis,* it is clear that plants and macroalgal responses to different levels of N stress vary. In fact Bi *et al.* (2007) suggested that plants respond to N stress with a'unique scaled response system' where there is a gradual activation of genes from mild to severe N stress. Therefore, it is possible that the responses to short-term N limitation may serve as an initial indicator of stress. Although no protein changes directly linked to N sensing and signalling pathways were identified in this study, it is evident that short-term N limitation impacts metabolic functions in *G. gracilis*. Alternatively, and more likely, these changes could also be transient responses implemented by *G. gracilis* as it adjusts to changing environmental conditions as was observed by Hammond *et al.* (2003) with respect to P deprivation in *Arabidopsis*. Thus it is necessary to monitor the effects of an initial period of N limitation over a time-course period to determine if these are transient or maintained responses.

The pattern of protein expression in response to a sustained period of N limitation (14 days) differed to that determined for the initial period of N limitation, resulting in the identification of mainly down-regulated proteins. However, the identified proteins were involved in similar biological processes which included photosynthesis, electron transport, glycolysis, protein biosynthesis and ATP binding. The down-regulation of genes involved in primary N metabolism has been observed in response to N limitation in *Arabidopsis* (Bi *et al.*, 2007;

Peng *et al.*, 2007). While the down-regulation of proteins in response to sustained N limitation were observed in this study, proteins directly involved in primary N metabolic processes were not identified.

There was no co-ordinated induction of proteins involved in N assimilatory processes in response to either the initial or sustained period of N limitation in *G. gracilis*. This was unexpected as several gene expression studies have reported significant down-regulation of primary metabolic N proteins in response to N limitation (Bi *et al*; 2007; Peng *et al*; 2007). However, the absence of proteins involved in primary N metabolism such as NR, GS and GOGAT could be due to a number of reasons. For instance, it is unlikely that a protein such as GOGAT would be identified using 2-DE as it is a large protein with a molecular weight ranging from 145-168 kDa for fd-GOGAT and a 190-240 kDa for NADH-GOGAT in terrestrial plants and algae (Inokuchi *et al.*, 2002). NR in particular has not been identified in the present study or in any previous studies investigating proteomic responses to N (Bahrman *et al.*, 2004; Prinsi *et al.*, 2009; Castillejo *et al.*, 2010; Møller *et al.*, 2011). Possible reasons for this include its large size (~98 kDa), low abundance and neutral pH (~ 6.5) which place it at the borderline of effective resolution under standard 2-DE conditions (Møller *et al.*, 2011). Alternatively, it is possible that these proteins are differentially expressed in response to N limitation but were unfortunately not sequenced in this study. Only a few of the differentially expressed proteins identified using Melanie 2-DE software were sequenced in this study. However, it is unlikely that proteins directly involved in N metabolic processes are regulated in response to the initial period of N limitation as N metabolic genes have not been identified in response to low or mild N stress in *Arabidopsis* and *rice* plants (Lian *et al.*, 2006; Bi *et al.*, 2007). Additionally, there were no visual symptoms of N deficiency or significant changes ($P<0.05$) in physiological measurements of N content, C:N ratios, and pigment content (PE and PC) between the N-replete and N-limited samples (6 hour exposure). Thus, it is possible that the initial period of N limitation is perceived as a general stress response rather than a N deficiency stress response. This has been observed in *Lycopersicon esculentum* with the rapid induction of stress responsive genes which code for pathogenesis-related (PR) proteins, late embryogenesis abundant (LEA) proteins, trypsin inhibitors, hin1 and glutathione-*S*-transferase in response to nitrate re-supply (Wang *et al.*, 2001).

The *G. gracilis* physiological response to a sustained period of N limitation differed to the responses observed with the initial response and resulted in profound visual changes in thallus colour, with the thallus colour changing from reddish-brown to greenish-brown. Thallus colour is an important indicator of the N status of algae (Oliveira *et al.*, 2000) and indeed, previous studies have shown that pigment levels of *Gracilaria* species are closely correlated with N content (Lapointe & Ryther, 1979; Robertson-Andersson *et al.*, 2009). This phenomenon was observed in this study with pigment content between the N-replete and N-limited *G. gracilis* samples differing significantly ($P<0.05$) from each other in response to a sustained period of N limitation. The second physiological variable measured was N content. It has been previously reported that a N content of between 1.5 and 1.7% in *G. gracilis* (Smit *et al.*, 1997) and C:N values above the proposed critical limit of 10 is indicative of N limitation (D'Elia & DeBoer, 1978; Harrison & Druehl, 1982). C:N values, however, may vary between species as a value of 13.5 was determined to be the critical value for *Gracilaria tikvahiae* (Hanisak, 1987). Furthermore, N content measurements are preferable over C:N ratios as changes in this ratio can be attributed to a change in C metabolism as well (Hanisak, 1990). In this study, the N content of N-replete and N-limited *G. gracilis* samples did not differ significantly ($P>0.05$) from each other in response to a sustained period of N limitation. This was attributed to the large degree of variation observed between biological repeat experimental samples, and in future studies it may be necessary to increase the number of biological repeats. Cumming *et al.* (2007) suggests that one could decrease the biological variation by increasing the number of biological repeats, as this results in narrower inferential error bars, and therefore more precise estimates of the true population values can be achieved. Despite this, N measurements indicated that *G. gracilis* samples were N-limited after 14 days. Furthermore, the N content, C:N ratios and pigment content data demonstrate that *G. gracilis* samples were sufficiently limited after 14 days when compared to the N-replete control samples. Thus, given these changes in total N content, the up-regulation of primary N metabolic proteins was expected. However, in a separate study investigating transcriptional and translational regulation of *G. gracilis* it was observed that GS transcript and protein levels did not show a significant ($P>0.05$) increase in response to a sustained period of N limitation in *G. gracilis* (Reddy, unpublished data). Instead, significant ($P<0.05$) increases in GS enzyme activity was observed, indicating that the protein may be regulated at the post-translational level (Reddy, unpublished data). Møller *et al.* (2011) also investigated the response of barley roots and shoots to long-term N deficiency and short-term N starvation. Although GS and NR were not regulated in response to 5 days of N starvation, two proteins involved in N metabolism, glutamate dehydrogenase and cyanate hydratase, were found to decrease in abundance (Møller *et al.*, 2011). In the same study, nitrite reductase (NiR) was the only protein involved in N metabolism which increased in abundance in response to long-term (33 days) low nitrate conditions. Finally, the possibility exists that the changes in protein abundance may be occurring at an earlier time-point. The time-points investigated in this study cannot be directly compared to other published studies as our study investigated changes in protein abundance in response to no N and not low N. Thus, it may be necessary to conduct these experiments over a range of different time-points to determine whether proteins involved in N metabolism show increased levels of expression over a period of 6 hours to 14 days.

A number of studies have investigated changes in transcriptional expression patterns in response to nitrate and ammonium in *Arabidopsis* (Wang *et al.*, 2000 & 2003; Scheible *et al.*, 2004; Krouk *et al.*, 2010; Patterson *et al.*, 2010). These studies have all demonstrated that the response to nitrate is rapid and changes in gene expression levels have been observed as early as three minutes after nitrate addition (Krouk *et al.*, 2010). It has been further determined that the biological functions induced earliest after nitrate addition do not involve N directly. Instead ribosomal proteins and those involved in the oxidative pentose-phosphate (OPP) pathway are among the first proteins induced in response to nitrate (Krouk *et al.*, 2010). Ribosomal proteins may be involved in the synthesis of new proteins required for N acquisition and the OPP pathway may be activated to provide reductants which are necessary for nitrate assimilation. Thus, these changes suggest that the early nitrate response involves mechanisms required to prepare the plant for N assimilation rather than mechanisms directly related to N (Krouk *et al.*, 2010). The proteomics of N acquisition has also been investigated in green plants, although not as extensively as the transcriptomics. Møller *et al.* (2011) investigated the effect of a re-supply of ammonium to barley shoots using 2-DE. Barley shoots which had been grown under control conditions (i.e an adequate N supply) were exposed to ammonium nutrient solution for 5 days and the effect on global protein expression was assessed. Prinsi *et al.* (2009) used 2-DE analysis to evaluate the response of starved maize plants to nitrate addition. A total of 20 and 18 proteins were observed to change significantly in abundance by at least two folds in roots and leaves, respectively. In roots, changes in protein levels of enzymes involved in nitrate assimilation and metabolic pathways implicated in the balance of the energy and redox status of the cell, such as the pentose phosphate pathway, were observed. In leaves, most of the characterized proteins were involved in the regulation of photosynthesis (Prinsi *et al.* 2009). These studies have helped to further underline the strict relationship which exists between C and N metabolism and have advanced the knowledge regarding N responses considerably in these plant species.

Although, proteomics studies of this nature are limited in macroalgae, numerous physiological studies have investigated the effect of a re-supply of nitrate and ammonium to N deprived intertidal red and brown macroalgae (Smit, 2002; Phillips & Hurd, 2003; Young *et al.*, 2009). Smit *et al.* (2002) investigated the N uptake ecophysiology of *G. gracilis* in response to a temporally variable N environment and found that the use of rate-unsaturated kinetics for ammonium uptake and a high affinity system for nitrate uptake allow *G. gracilis* to use dissolved inorganic N over a wide concentration range. Young *et al.* (2009) examined the effects of long-term N deprivation, recovery following N re-supply, and the effects of elevated ammonium and nitrate exposure on N acquisition in intertidal algae using manipulations of N supply in tank culture. The effect of these treatments were evaluated by monitoring changes in internal nitrate$^-$ and ammonium levels, total N and nitrate reductase activity (NRA) changes (Young *et al.*, 2009). Studies of this nature have demonstrated that the ability to rapidly take up nitrate and store high concentrations, and to maintain constitutive and excess NRA, are some of the physiological adaptions implemented by intertidal macroalgae to sequester inorganic N efficiently (Phillips & Hurd, 2004; Young *et al.*, 2009). However, with the exception of NR, the molecular mechanisms underlying these adaptions have not been investigated in macroalgae such as *G. gracilis*. Thus, in this study the effect of N recovery on global protein expression in *G. gracilis* was evaluated to provide some insight as to molecular mechanisms involved in this response. It was found that the vast majority of proteins (i.e 50 of 66) were up-regulated in response to N recovery in *G. gracilis*. The physiological responses of *G. gracilis* to N recovery were determined prior to the commencement of proteomics characterisation. Visual changes in thallus colour were not observed in response to N recovery as the recovery period only lasted for 6 hours. The N content, C:N ratios and pigment levels did not differ significantly ($P>0.05$) between N-limited and N-recovered *G. gracilis* samples. While increases in N content were observed in response to N recovery, the variation was too large and as a result no statistically significant changes were observed. Furthermore, the levels of N determined for these samples were exceedingly low considering they had only been limited for 7 days. This may be due to the nutritional state of the seaweed which is influenced by seasonal variations as this experiment and the N limitation experiments were conducted at different times of the year and this may have been a contributing factor. This is an important consideration which should be factored into any experimental design in future studies.

In summary a total of 22 proteins observed to either increase or decrease in abundance in response to N stress were identified in this study. Eight of these proteins responded to 6 hours of N limitation, eight to 14 days of N limitation and six to 6 hours of N recovery. The role(s) of these proteins and the biological processes which are either activated or repressed will be discussed in the following sections and their importance in the N stress response of *G. gracilis* evaluated.

Nitrogen Metabolism

GS was the only primary N metabolic protein identified in this study and it was observed to be significantly increased in abundance ($P<0.05$) in response to N recovery in *G. gracilis* (Fig. 2.11). GS functions as the major assimilatory enzyme for ammonia produced from N fixation, and nitrate or ammonia nutrition. It also re-assimilates ammonia released as a result of photorespiration, the breakdown of proteins and N transport compounds. GS functions in two essential biochemical reactions: ammonium assimilation and glutamine (Gln) biosynthesis. It catalyzes the formation of Gln from ammonium and glutamate in the presence of ATP and magnesium (Mg) (Fig. 2.11) and is involved in the biosynthesis of essentially all cellular nitrogenous compounds (Chen *et al.*, 1996). GS belongs to the GSII gene family and is distributed in different subcellular locations, tissues and organs in plants. The chloroplastic and cytosolic forms each play specific roles in N assimilation. The chloroplastic form of GS is involved in the assimilation of ammonia generated through photorespiration and nitrite reduction, while the cytosolic GS isoforms are thought to play a role in N uptake, translocation and mobilisation (Fuentes *et al.*, 2001). It is not possible to determine which isoform of GS is responding to the re-supply of N, as the identity of the up-regulated protein species was based on the identification of two peptides which are found within conserved GS protein domains. Furthermore, only the cytosolic form of GS has been identified in red algae (Freshwater *et al.*, 2002; Terashita *et al.*, 2006). However, there is evidence for a chloroplastic form of GS in *G. gracilis* (Reddy, 2011; pers. comm.).

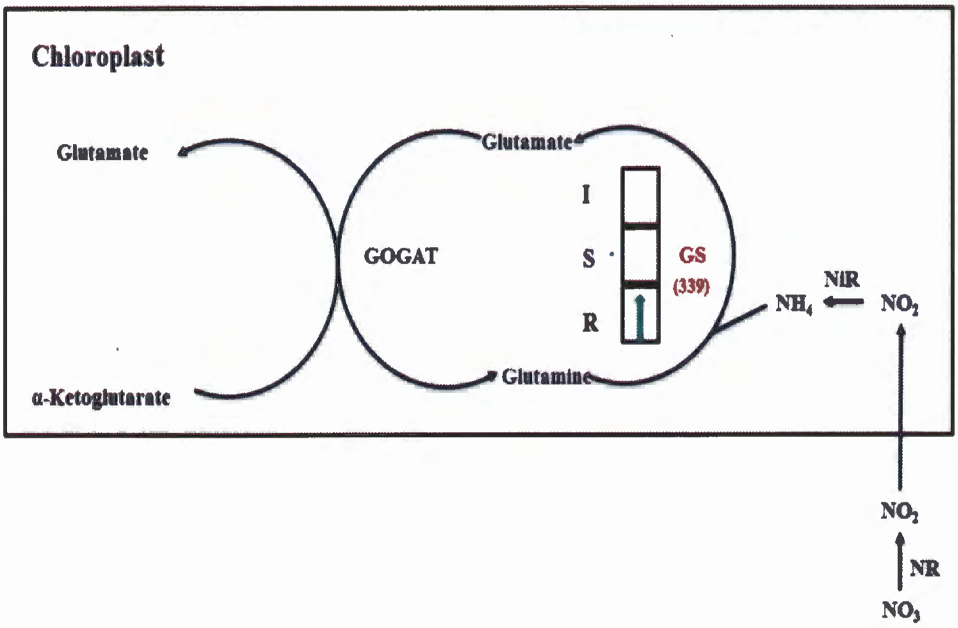

Figure 2.11 A simplified scheme showing the assimilation of ammonium by the GS/GOGAT pathway and the production of α-ketoglutarate. The intensity changes in two dimensional (2D) gel spots are represented by boxes for each of the treatments I, S and R. I-Initial nitrogen limitation, S-Sustained nitrogen limitation and R-Nitrogen recovery. Arrows indicate increased or decreased intensity with respect to the control. An empty box indicates that the protein was not identified for that particular nitrogen treatment. Spot numbers are displayed in red in brackets. NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthase; GOGAT, glutamate synthase; NO_2, nitrite; NO_3, nitrate. Adapted from Bahrman *et al.*, 2004.

GS synthesis and activity are tightly regulated by cellular metabolism and environmental signals and in higher plants its expression is governed by environmental signals such as light, nitrate and ammonium, as well as the metabolic status of the cell (Chen *et al.*, 1996). However, the response of *gs* expression and activity to ammonium or nitrate varies depending on the specific member of the gene family and the plant species (Hirel *et al.*, 1987; Kozaki *et al.*, 1991; Hoelzle *et al.*, 1992; Stanford *et al.*, 1993; Sukanya *et al.*, 1994). Generally, the presence of ammonium and nitrate results in increased *gs* gene expression and/or GS activity (Chen *et al.*, 1996). Early increases in *gs* mRNA transcript levels within seedlings and roots of *Arabidopsis* have been observed in response to nitrate re-supply at 2 hours and 20 minutes, respectively (Wang *et al.*, 2000 and 2003). GS activity has also been observed to increase with increasing ambient N concentration (Touchette and Burkholder, 2000) and Li *et al.* (2006) observed increases in GS activity in rice in response to a re-supply of N sources. Prinsi *et al.* (2009) found that both the chloroplastic and cytosolic GS protein isoforms increased in abundance in maize roots in response to nitrate addition. Møller *et al.* (2011) investigated the effect of ammonium addition to N replete (control) barley shoots and observed a decrease in abundance of chloroplastic GS. This contrasts with the observed increases in *G. gracilis* GS protein levels in this study. However, the studies cannot be directly compared as firstly a combined nitrate and ammonium source as opposed to an ammonium only N source was added to N-starved *G. gracilis* and not N-replete *G. gracilis* as was conducted with barley shoots (Møller *et al.*, 2011). Secondly, comparative analysis is further complicated as *G. gracilis* does not have a root and shoot proteome as is present in green plants. However, it is clear that N addition, either in the form of nitrate, ammonium or both, affects GS at the gene and protein level (Wang *et al.*, 2001; Li *et al.*, 2006, Prinsi *et al.*, 2009, Møller *et al.*, 2011). Thus, the increased abundance of GS protein levels in response to a short period (6 hours) of N re-supply in *G. gracilis* suggests that macroalgal responses are similar to those observed at the transcriptional and translational level in green plants. Furthermore, the response appears to be rapid and it is clear that primary N metabolic proteins are activated at an early stage to assimilate the newly available N.

Carbohydrate metabolism

Glycolysis

The majority of the proteins found to be differentially regulated in *G. gracilis* in response to N stress are proposed to be involved in carbohydrate metabolic processes. As previously discussed in Chapter 1, primary N assimilation and carbohydrate metabolism are integrally linked. Primary N assimilation and the subsequent biosynthesis of nitrogenous molecules is an energy consuming process which requires abundant amounts of energy, reducing equivalents and C intermediates (Werf *et al.*, 1988; Lian *et al.*, 2006; Peng *et al.*, 2007). These compounds are generated via photosynthesis, the tri-carboxylic acid (TCA) pathway and the pentose phosphate pathway (Peng *et al.*, 2007). The energy required for all these processes is provided by carbohydrate metabolism in the form of the reducing equivalents, Fd and NADH, and the organic acid 2-oxoglutarate (Kaiser *et al.*, 2000). In this study, a number of glycolytic enzymes were found to be differentially regulated in response to N limitation and recovery (Fig. 2.12). Plaxton (1996) suggested that cells increase glycolysis rates to compensate for the reduced energy levels brought about by different stresses (Plaxton, 1996). Moreover, it is thought that the cytosolic network of glycolytic enzymes may enable plants and algae to adapt and acclimate to environmental stress conditions (Minhas *et al.*, 1999). Two enzymes, enolase and GAPDH involved in glycolysis, were up-regulated in response to an initial period of N limitation in *G. gracilis*.

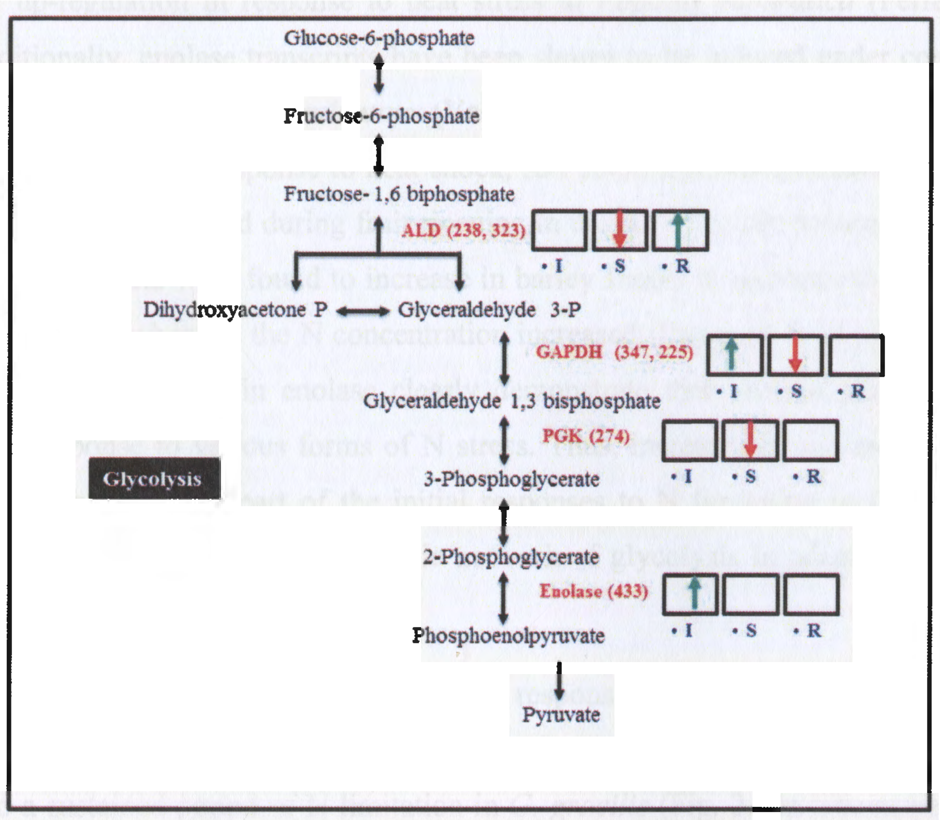

Figure 2.12 A summary of glycolytic proteins affected by different N treatments in *G. gracilis*. The intensity changes in two dimensional (2D) gel spots are represented by boxes for each of the treatments I, S and R. I-Initial nitrogen limitation, S-Sustained nitrogen limitation and R-Nitrogen recovery. Arrows indicate increased or decreased intensity with respect to control. An empty box indicates that the protein was not identified for that particular nitrogen treatment. Spot numbers are displayed in red in brackets. ALD, fructose 1,6 biphosphate aldolase, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, PGK, phosphoglycerate kinase. Adapted from Bahrman *et al.*, 2004, and Møller *et al.*, 2011.

Enolase and GAPDH are both highly conserved enzymes found in every living organism. These enzymes have been reported to be regulated in response to a wide variety of abiotic stresses in plants at the transcriptional level. GAPDH transcript levels increased in response to dehydration stress and ABA treatment in *Craterostigma plantagineum*, while in rice seedlings transcript levels were increased in response to salt stress (Velasco *et al.*, 1994). Enolase transcripts have been shown to be up-regulated in response to oxidative stress in the green alga *Haemotococcus pluvialis* (Wang *et al.*, 2004a) and both these enzymes showed short term up-regulation in response to heat stress in *Populus euphratica* (Ferreira *et al.*, 2006). Additionally, enolase transcripts have been shown to be induced under conditions of water stress in *Lycopersicon esculentum* (Van der Straeten *et al.*, 1991) and *Zea mays* (Riccardi *et al.*, 1998), in response to heat shock, salt stress and ABA treatment in ice plant (Forsthoefel *et al.*, 1995), and during fruit ripening in tomato (Van der Straeten *et al.*, 1991). Enolase proteins levels were found to increase in barley shoots in response to long-term low nitrate stress and in wheat as the N concentration increased (Bahrman *et al.*, 2004, Møller *et al.*, 2011). These increases in enolase clearly demonstrate that enolase protein levels are regulated in response to various forms of N stress. Thus, increases in enolase and GAPDH protein levels appear to be part of the initial responses to N limitation in *G. gracilis* and reinforce the important role of the cytosolic network of glycolysis in adaption responses to abiotic stress conditions.

In contrast to the pattern of regulation observed in response to an initial period of N limitation the protein levels of three glycolytic enzymes, ALD, GAPDH and PGK were decreased in response to a sustained period of N limitation in *G. gracilis* (Fig. 2.12). The major effect of prolonged N limitation is a decline in the uptake and assimilation rates of ammonium and nitrate. Thus, fewer C skeletons are available to support the synthesis of organic acids in the TCA cycle and the resultant effect is a decrease in glycolytic enzyme activities. Numerous studies have demonstrated decreases in transcript levels of genes encoding glycolytic enzymes in response to N limitation. Peng *et al.* (2007) demonstrated that genes involved in the synthesis of nitrogenous molecules were significantly repressed in response to N limitation in *Arabidopsis*. Consequently, a reduction in the consumption of energy, reductants and organic C intermediates for nitrate assimilation and the anabolism of nitrogenous macromolecules was observed as evidenced by the decreased transcript levels of genes involved in photosynthesis, the TCA cycle and the pentose phosphate pathway (Peng *et al.*, 2007). These included a decrease in the expression of the chloroplastic forms of GAPDH and

PGK in response to N limitation (Peng *et al.*, 2007). The genes encoding the glycolytic enzymes GAPDH, ALD and fructose-6-phosphate-2-kinase were also shown to be repressed in response to low N stress in rice plants (Lian *et al.*, 2006). ALD protein levels have been observed to increase in response to long-term nitrate conditions in barley shoots along with a number of enzymes involved in glycolysis (Møller *et al.*, 2011). Although, this contrasts with down-regulation of ALD protein levels observed in this study, it suggests that the mechanisms implemented to cope with a long-term low N source differ to the mechanisms employed when a N source is absent.

The addition of N to N starved plants results in major changes in C metabolism which includes decreased starch biosynthesis and increased synthesis of organic acids (Scheible, 1997). Organic acids are required for the incorporation of ammonium into amino acids and for the maintenance of cellular pH as nitrate reduction generates hydroxide ions (Wang *et al.*, 2000). Therefore, nitrate re-supply results in the co-ordinated induction of a number of metabolic pathways, including glycolysis and the TCA cycle (Wang *et al.*, 2002 & 2003; Scheible *et al.*, 2004). Thus, glycolytic enzyme activities are increased in response to N recovery. In this study, ALD, a glycolytic enzyme, was shown to be up-regulated in response to N recovery. Numerous studies conducted with *Arabidopsis* have shown that genes involved in the latter part of glycolysis are rapidly induced upon addition of nitrate to N-starved seedlings or plants (Wang *et al.*, 2000 and 2003; Scheible *et al.*, 2004). These include the genes for phosphoglycerate mutase, phosphoglucose isomerase and glucose-6-phosphate isomerase. Scheible *et al.* (1997) used Nia30(145) transformants, which resemble nitrate-limited wild-type plants with respect to growth rate and protein and amino acid content, to investigate whether growth on nitrate supplemented media leads to widespread changes in the expression of key genes in N and C metabolic pathways. They observed increased transcript levels and activity for a number of important enzymes involved in N metabolism as well as the transcript levels for phosphoenolpyruvate carboxylase and cytosolic pyruvate kinase (PK). PK is the end product of glycolysis and therefore an increase in transcript levels of glycolytic enzymes, such as ALD, may be expected. Although, ALD was not specifically induced in response to nitrate addition in these studies, it is clear that nitrate addition affects genes involved in the glycolytic pathway. Thus, it is likely that similar effects would be observed with glycolytic enzymes, as was observed in this study

Galactose metabolism

Red algae produce and utilize D-galactose extensively (Lluisma *et al.*, 1998). Galactans such as carrageenans which are essentially polymers of D-galactose, and agarans which are polymers of D- and L- galactose, are the most abundant components of red algal cell walls (Lluisma *et al.*, 1998). D-galactose is a constituent of floridoside, an important photosynthetic product in red algae, which can function as a short-term C reserve and is involved in osmoregulation (Kremer, 1978; Kirst, 1980; Kremer & Kirst, 1981; Reed, 1990; Ekman *et al.*, 1991). One of the key enzymes in galactose metabolism is galactose-1-phosphate-uridyl-transferase (GALT) which catalyzes the reversible transfer of the uridine 5' phosphoryl moiety from uridine 5'-diphosphate glucose (UDP Glc) to galactose-1-phosphate (Gal1P) to produce uridine 5'-diphosphate galactose (UDP Gal) and glucose-1-phosphate (Glc1P) ((Fig. 2.13), (Frey, 1996)). The main function of GALT is it allows the utilisation and re-utilisation of D-galactose, which is derived from the hydrolysis of floridoside. The floridoside pool can vary depending on time of day, N content and salinity and a change in the floridoside pool would affect GALT activity. For instance a reduction in the floridoside pool would require an increase in GALT activity to enable D-galactose to re-enter the metabolic pathway (Lluisma *et al.*, 1998). However, it is unlikely that the increase in GALT activity observed is due to a reduction in the floridoside pool. Floridoside levels are known to increase under N limitation but not at such an early stage. D-galactose can also originate from other sources in red algae and it has been reported that heterotrophic organisms such as *Galderia sulphuraria* can utilize D-galactose as the sole carbon source (Gross & Schnarrenberger, 1995). Additionally, in red algae GALT may also participate in salvaging D-Galactose derived from the degradation of certain metabolites such as glycoproteins and low-molecular weight carbohydrates. Collén *et al.* (2004) investigated the effect of nutrient stress on UDP-Gal levels in *Gracilaria tenuistipitata* for 3 weeks. UDP-Gal levels were found to increase for the first 6 days in response to nutrient limitation without a concomitant increase in UDPGlc 4 epimerase (the enzyme catalysing the conversion of UDP-Glc to UDP- Gal). The authors suggest that the increased UDP-Glc and UDP-Gals concentrations observed during nutrient limitation could be attributed to the increased C flux into starch and floridoside synthesis, and that this accounts for the absence of increased activity of their biosynthetic enzymes (Collén *et al.*, 2004). It is possible that the observed increases in UDP-Gal levels could be due to increases in GALT activity, but without further investigation it is not possible to confirm this hypothesis. This enzyme has also been suggested to play an important role in agar biosynthesis. UDP-Gal is an important substrate for agar synthesis in red algae and could potentially be used to screen for strains with high agar content (Li *et al.*, 2010). In addition, agar content has been shown to increase in response to N limitation, but again at a much later stage of N limitation (Macler, 1986; Bird, 1988; Ekman *et al.*, 1991). Thus, the increase in GALT activity could be attributed to an increase in agar synthesis although this is unlikely at such an early stage and would also need to be investigated further. Given these findings the increase in GALT levels may therefore be an early adaption to N limitation as the algae adapts to the removal of an external N source but requires further investigation as the role of GALT is poorly understood in red algae (Lluisma *et al.*, 1998; Li *et al.*, 2010).

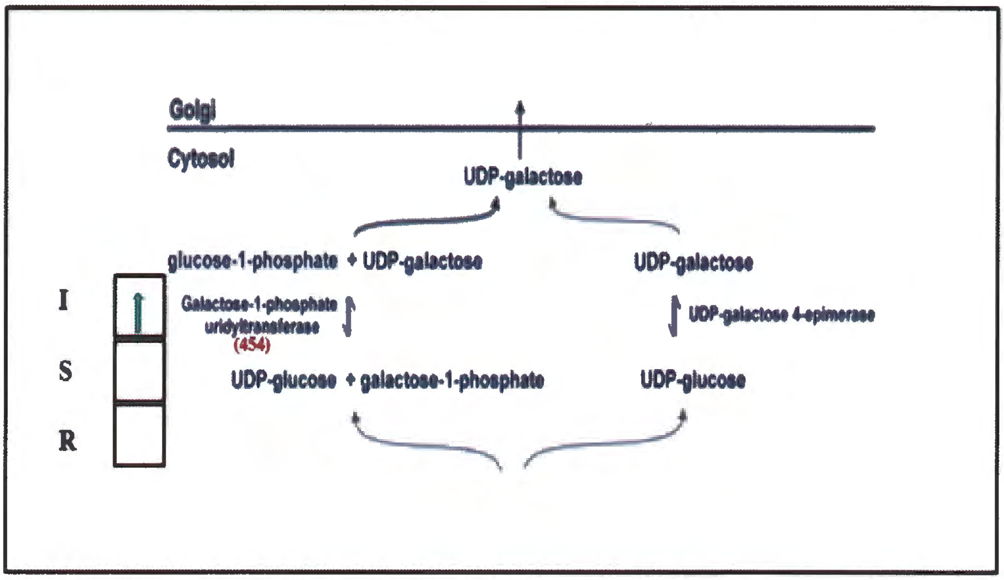

Figure 2.13 A simplified scheme showing the involvement of galactose-1-phosphate uridyl transferase in the metabolism of Galactose. The intensity changes in two dimensional (2D) gel spots are represented by boxes for each of the treatments I, S and R. I-Initial nitrogen limitation, S-Sustained nitrogen limitation and R-Nitrogen recovery. Arrows indicate increased or decreased intensity with respect to the control. An empty box indicates that the protein was not identified for that particular nitrogen treatment. Spot numbers are displayed in red in brackets. Adapted from Lamerz *et al.*, 2006.

Cytoskeleton changes

Cytoskeleton changes were only observed in response to an initial period of N limitation in *G. gracilis*. The protein levels of actin and actin 1 increased significantly ($P<0.05$) following 6 hours of N limitation in *G. gracilis*. The actin cytoskeleton exists as a highly organised, dynamic network in plants and algae. Actin exists as tight bundles of longitudinal filaments linked to one another and to the cell membrane, as polymeric actin or as monomeric (unpolymerized) actin. The actin cytoskeleton is rapidly remodelled upon exposure to various endogenous and external stimuli (Jiang *et al.*, 2007; Kulikova *et al.*, 2009). Additionally, actin plays an important role in cellular homeostasis in plants and is involved in a large majority of the processes which occur in living cells (Jiang *et al.*, 2007; Kulikova *et al.*, 2009). These include roles in both cytoplasmic and nuclear events such as the establishment of cell polarity (La Carbona *et al.*, 2006), cell motility (Rafelski and Theriot, 2004), cytoplasmic streaming in plants (Shimmen, 2007), endocytosis (Carreno *et al.*, 2004) and transcription (Pederson & Aebi, 2005). Environmental stress often results in changes in the organization of the actin filaments as well as changes in the proportion of polymeric and monomeric forms of actin (Kulikova *et al.*, 2009). These changes are regulated by actin-binding proteins that interact with either the monomeric or polymeric actin (Song *et al.*, 2011). Therefore, it has been suggested that the actin cytoskeleton may be involved in the initial adaption of plants and algae to stress factors as they function in a range of biological processes. These include their involvement in signal transduction and perception, development, defensive and regulatory mechanisms. Research studies investigating the effects of environmental stressors such as low temperature, aluminium and NaCl stress have been shown to cause rearrangements in cytoskeletal structure (Abdrakhamanova *et al.*, 2003; Dhonukshe *et al.*, 2003; Sivaguru *et al.*, 2003). More recently, a study conducted on rape plants (*Brassica napus* L.), investigating the effects of copper stress, has shown that actin is involved in early plant responses to heavy metal stress. The presence of excess copper, which is a potent inducer of reactive oxygen species and oxidative stress, led to a considerable increase in the amount of polymeric actin. Moreover, an increase in the amount of the molecular chaperones HSP 60 and 70 associating with the actin filaments was also observed. Therefore it was proposed that this interaction protects the cytoskeleton from damage from the stressor (Kulikova *et al.*, 2009). Thus, an increase in actin protein abundance may be one of many initial signalling mechanisms employed by *G. gracilis* to signal a change in the external N environment. However, further investigation would be required to determine the relative proportions of polymeric and monomeric actin in *G. gracilis* and the mechanisms involved in modulating these levels in response to N limitation.

Molecular chaperones

Molecular chaperones play important roles in plants and algae contributing to cellular homeostasis in cells under both optimal and adverse growth conditions (Timperio *et al.*, 2008, Wang *et al.*, 2004c). They are responsible for protein folding, assembly, translocation and degradation in cellular processes. The protein GroEL belongs to the family of chaperonins and is involved in protein folding and assists in the refolding of proteins under stress conditions (Wang *et al.*, 2004c) and was found to be up-regulated in response to an initial period of N limitation in *G. gracilis*. In eukaryotes GroEL is structurally and functionally similar to HSP 60 (Wang *et al.*, 2004c). HSPs/chaperones are not only expressed under conditions of high temperature but have also been shown to increase under a wide variety of abiotic stress conditions. These include water, salinity, oxidative, osmotic and cold stress (Wang *et al.*, 2004c). It has also been reported that N availability affects the accumulation of HSPs in *Zea mays* suggesting that HSPs are involved in the N stress response (Heckathorn *et al.*, 1996). Previous studies have also observed changes in superoxide dismutase (SOD) and HSP proteins and transcripts in rice and wheat under different levels of N nutrients (Konishi *et al.*, 2001; Beatty *et al.*, 2009; Grove *et al.*, 2009). Furthermore, the interaction of actin and HSPs are of particular interest in this study as it has been reported that the levels of actin increased together with an increase in the levels of HSP 60 and 70 in response to short-term heavy metal stress in rape plants (Kulikova *et al.*, 2009). Therefore, the increases observed in HSP 60 and actin protein levels in this study may be correlated as these proteins have been shown to associate together to protect the cytoskeleton from damage under stressful conditions (Kulikova *et al.*, 2009). However, further studies involving the extraction of actin and the use of immunoblot analysis with both actin and HSP antibodies would need to be conducted to determine increases in actin content, and its possible association with the HSPs in *G. gracilis*.

Protein levels of elongation factor Tu (EF-tu) were shown to be increased in response to a sustained period of N limitation in this study. EF-tu is a member of a highly conserved, nuclear encoded multi-gene family. It is a heat responsive protein that acts as a molecular chaperone, while also being involved in polypeptide elongation, and protects chloroplast proteins from thermal aggregation and inactivation (Rao *et al.*, 2004; Singh *et al.*, 2004). EF-tu interacts with aminoacyl tRNA and transports the codon specific tRNA to the aminoacyl site on the ribosome during the translation elongation step. Several gene expression studies have shown elongation factors to be differentially expressed in response to abiotic stress conditions (Guo *et al.*, 2002; Rausell *et al.*, 2003; Singh *et al.*, 2004; Lebi, 2006). Guo *et al.* (2002) reported the involvement of translation elongation factor 2 in *Arabidopsis* in low temperature induced cold acclimation. Similarly, Rausell *et al.* (2003) showed that the overexpression of sugar beet translation initiation factor increased salt tolerance in both yeast and *Arabidopsis,* and also improves *in vitro* protein synthesis under salt stress conditions. Singh *et al.* (2004) investigated the response of EF-tu to salinity stress, low temperature stress, salicyclic acid treatment and ABA treatment in pea plants at both an mRNA transcript and protein level. The response of EF-tu to each particular stress factor differed with a decrease in expression observed in response to high salt and ABA treatment, and an increase in expression in response to light, salicyclic acid treatment and low temperature (Singh *et al.*, 2004). In a previous *G. gracilis* microarray study, in which N-replete samples were compared to 18 day N-limited samples, an increase in expression levels of elongation factor 3 homolog and elongation factor 1 α were observed (Lebi, 2006). These studies suggest that EF-tu plays a role in the adaption of plants to stressful conditions. EF-tu has not been identified in any proteomic studies investigating N nutrition. However, Møller *et al.* (2011) observed increased protein levels of the chaperonin Cpn 10 in response to long-term nitrate deficiency and ammonium addition in barley shoots indicating a possible increase in transcriptional/translational activity in the chloroplasts in response to these treatments. Furthermore, the phycobilisome (PBS) is degraded in algae under conditions of sustained N limitation and this degradation could provide the cell with amino acids which could be used for the synthesis of new proteins required for acclimation to N limitation (Grossman *et al.*, 1993). Thus, one could hypothesise that an increase in EF-tu protein levels under N limiting conditions would support the biosynthesis of proteins that enable *G. gracilis* to survive and adapt to N limitation.

Energy production and conversion

Plants and algae use light energy to drive electron and proton transport across the thylakoid membrane, resulting in the synthesis of NADPH and adenosine triphosphate (ATP) (Maiwald *et al.*, 2003). These processes involve photosystems I (PSI) and II (PSII), cytochrome *b6/f* (cyt *b6/f*), and the chloroplast ATP synthase (Fig. 2.14) (Maiwald *et al.*, 2003). ATP is the cell's main energy currency and tight control over its production and hydrolysis is essential to ensure the survival of all living organisms (Bunney *et al.,* 2001). ATP synthase is present in mitochondria, chloroplasts and (photosynthetic) bacteria (Bunney *et al.,* 2001). ATP synthase is responsible for the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate and the energy required for this process is supplied in the form of protons moving down an electrochemical gradient, either from the lumen into the stroma of chloroplasts or from the inter-membrane space into the matrix in mitochondria. Chloroplastic ATP synthase α sub-unit protein levels were significantly increased ($P<0.05$) in response to an initial period of N limitation and significantly decreased ($P<0.05$) under conditions of sustained N limitation in *G. gracilis* (Fig. 2.14).

ATP synthase is a complex enzyme and the effect of thermal denaturation on the CF1 portion of ATP synthase has provided some insight into the workings of this enzyme. It has been shown that it is the interaction between α, β and γ ATP synthase subunits that provide the necessary force to stabilize CF1 under thermal denaturation (Wang *et al.*, 1993). Thus, it was proposed that the CF1 α-subunit has an organizing function in the assembly of the multi-subunit enzyme ATP synthase, as it contains non-catalytic sites where ATP can bind and stabilize ATP synthase CF1 (Wang *et al.*, 1993). Additionally, the CF1 α–β complex is the minimum catalytic core of ATP synthase (Avital *et al.*, 1991). Ferreira *et al.* (2006) suggested that the up-regulation of ATP synthase α and β sub-units in *Populus euphratica* under conditions of heat stress is related to their central role in the enzyme complex. In *G. gracilis* the removal of an external N source may lead to the activation of proteins involved in N signalling and sensing pathways and would possibly require energy. Thus, increased levels of ATP synthase CF1 α sub-unit protein levels suggest that it may provide the energy required for the initial transient responses to N limitation in *G. gracilis.*

It has been well established that N deficiency results in a reduction in photosynthetic capacity in plants (Verhoeven *et al.*, 1997; Bi *et al.*, 2007, Peng *et al.*, 2007) and that ATP synthase protein abundance is affected by N stress conditions (Bahrman *et al.*, 2004; Prinsi *et al.*, 2009). Prolonged N limitation also elicits a number of stress responses and studies with plants under N-deficient conditions have not only shown that chlorophyll and Rubisco content decreases per unit leaf area, but that the ratio of Rubisco to chlorophyll decreases as well (Wong, 1979; Ferrar & Osmond, 1986; Evans & Terashima, 1987; Seemann *et al.*, 1987). Therefore, a key effect of N limitation would be a decreased capacity for C assimilation due to a decrease in the synthesis of the Calvin cycle enzymes (Terashima & Evans, 1988; Sugiharto *et al.*, 1990). Consequently, a given light intensity is potentially in greater excess under conditions of N deficiency and could potentially mimic a situation where light is in excess (Verhoeven *et al.*, 1997). Thus, the ATP synthase response under conditions of prolonged N limitation may be similar to that observed in response to light stress. Proteomic analysis of the response of chloroplast proteins to high light stress has been investigated in *Arabidopsis* (Phee *et al.*, 2004). Many photosynthetic proteins, including ATP synthase were observed to be down-regulated in response to varying degrees of light stress (Phee *et al.*, 2004). N deficiency may also activate a range of genes and proteins involved in oxidative stress responses which have been shown to be induced upon nutrient stress (Logan *et al.*, 1999; Tewari *et al.*, 2007). Moreover, chloroplasts, the site of photosynthesis, are the major generation sites of reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) which are formed under stress conditions during electron transfer along the electron transport chain. Chloroplasts are therefore susceptible to oxidative stress. Proteomic analysis of the effect of oxidative stress in the green alga *Haematococcus pluvialis* found that the chloroplast β-ATP synthase subunit was repressed under these conditions, however, the mitochondrial β-ATPase subunit was transiently up-regulated with the highest protein levels being detected after 48 hrs of stress induction. While these studies did not investigate N limitation specifically, oxidative and light stress are two major consequences of prolonged N limitation (Logan *et al.*, 1999; Tewari *et al.*, 2007). Given that N limitation results in extensive cross-talk between the oxidative and light stress pathways, it is likely that a combination of all these effects results in the repression of ATP synthase in response to sustained N limitation in *G. gracilis*.

The assimilation of N into protein requires both energy and organic C skeletons and as a result, major interactions between N assimilation and photosynthetic metabolism occurs

(Turpin *et al.*, 1988). One of the major effects of N limitation is a decrease in photosynthetic capacity due to a restriction of C skeletons which are required for N assimilation and growth. Consequently, Rubisco levels have been shown to decline under N limiting conditions (Levy & Gantt, 1990; Paul & Driscoll, 1997; Young & Beardall, 2003). Therefore, the addition of N to N-starved algal samples would be expected to activate a range of processes including photosynthesis and possibly lead to the up-regulation of enzymes such as Rubisco (Levy & Gantt, 1990; Wang *et al.*, 2002 & 2000; Young & Beardall, 2003). N re-supply resulted in the increased abundance of a protein, CbbX, which is linked to Rubisco and suggests an increase in photosynthetic capacity. At the transcriptional level the *cbbx* gene is predicted to be involved in Rubisco expression, and in red algae is thought to be a transcriptional regulator of the Rubisco sub-unit genes, *rbcL* and *rbsS* (Fujita *et al.*, 2008). In the unicellular rhodophyte, *Cyanidioschyzon merolae,* the plastid genome contains an operon consisting of the genes *rbcL, rbsS* and *cbbX.* The *cbbX* gene is also one of 8 genes presumed to be involved in the Calvin Benson Basham (CBB) cycle (Caldwell *et al.*, 2007). Furthermore, *cbbX* encodes a protein of unknown function that is thought to be involved with some form of post-translational activation of Rubisco (Li & Tabita, 1997). The gene *cbbX* has been found to be transcribed in both the nucleus and the plastid of *Cyanidioschyzon merolae* and functional analysis studies revealed that the Rubisco operon may be negatively regulated by the plastid *cbbX* repressor, but not by the nuclear *cbbX* homologue (Fujita *et al.*, 2008). The increased abundance of CbbX protein could possibly be correlated to changes in protein levels of Rubisco in response to either increased or decreased photosynthetic rates. It is important, however, to determine whether the identified CbbX protein is located in the plastid or nucleus and functional studies such as conducted by Fujita *et al* (2008) may help to elucidate how increased *cbbX* levels affect Rubisco expression in *G. gracilis.*

ATP synthase β sub-unit protein levels were increased in response to N recovery in *G. gracilis* (Fig. 2.14). N addition stimulates a wide range of cellular metabolic processes as previously described. Thus, an increased ATP supply would be required to drive these processes. GS for instance, requires ATP to synthesize Gln (Chen *et al.*, 1996). ATP synthase has also been identified as a target of thioredoxin, an antioxidant protein, which was also observed to be up-regulated in response to N recovery in *G. gracilis*. Furthermore, these results correlate well with those observed during late stage N limitation where it was seen that ATP synthase α sub-unit protein levels were down-regulated and upon N re-supply, these levels of ATP synthase β sub-unit were shown to be increased.

Photosynthesis

Three proteins involved in photosynthetic responses were observed to change in abundance in response to N limitation in *G. gracilis*. PE protein levels were significantly increased ($P<0.05$) in response to an initial period of N limitation, PC protein levels were significantly increased ($P<0.05$) in response to a sustained period of N limitation and FNR protein levels were significantly decreased ($P<0.05$) in response to a sustained period of N limitation in *G. gracilis* (Fig. 2.14). In red algae such as *G. gracilis*, the pigments chlorophyll *a* and phycobilipigments are the major photosynthetic pigments. These pigments complex to form the PBS whose primary function is the absorption of light and the transfer of excitation energy to the reaction centres of PS I and PS II (Kursar & Alberte, 1983). The photosynthetic accessory pigments, namely PE, PC and allophycocyanin are arranged concentrically, with PE as the outermost of the three pigments. PE is the most sensitive to external changes in both light and nutrient availability (Kursar & Alberte, 1983), and has been reported to have a storage function in *Porphyra* (Kim *et al.*, 2007) and *Gracilaria* species (Smit *et al.*, 1997). The increase in PE α sub-unit protein levels in *G. gracilis* following 6 hours of N limitation was unexpected. In red algae, PE levels are known to decrease in response to prolonged N limitation as it is an important storage compound in red algae and serves as a N source when N is limited. However, PE responses to an initial period of N limitation in red macroalgae have not been investigated. Thus, one could hypothesise that this increase in PE α sub-unit protein levels is associated with the light harvesting function of the PBS. Furthermore, the increase in PE α sub-unit observed in this study may be linked to the observed increase in ATP synthase α sub-unit protein levels as both these proteins have specific functions in the light reactions of photosynthesis. These responses are most likely transient responses as *G. gracilis* adapts to the change in the external N environment. However, the exact reason for the observed increase in PE α sub-unit protein levels in response to an initial period of N limitation is unclear and needs to be investigated further. It should also be noted that the identified protein has a molecular mass larger than that expected for PE. This could be due to some form of post-translational modification or the pigment could be complexed to a protein which results in the increased molecular weight. However, further experiments would need to be conducted to determine and validate the identity of *G. gracilis* PE.

Degradation of the PBS occurs under N-limiting conditions, and consequently the concentrations of the pigments PE and PC are known to decrease (Grossman *et al.*, 1993;

Richaud *et al.*, 2001). This degradation of the PBS may provide the cell with amino acids which could be used for the synthesis of new proteins which are important for the acclimation process (Grossman *et al.*, 1993). In this study, PC α sub-unit protein levels increased in response to N limitation which conflicts with what has been described in the literature for macroalgae (Grossman *et al.*, 1993). It also contrasts with the *G. gracilis* PC pigment decreases observed in response to 14 days of N limitation in this study. However, an additional antioxidant role has been proposed in *Spirulina platensis*, a planktonic blue-green algae (Estrada *et al.*, 2001). It has been reported that low N availability significantly reduces a plant's photosynthetic capacity and this could make plants more susceptible to excessive light induced oxidative damage (Diaz *et al.*, 2006; Lea *et al.*, 2007; Peng *et al.*, 2007). However, plants have implemented an adaptive strategy to cope with this phenomenon. For instance, anthocyanin synthesis is increased in plants grown under N limiting conditions as anthocyanin functions as a ''sunscreen'' and scavenger of reactive oxygen to protect plants from photodamage (Bongue-Bartelsman & Phillips, 1995; Chalker-Scott, 1999; Diaz *et al.*, 2006). Genes involved in this pathway have been shown to be up-regulated in response to N limitation. This was demonstrated by Peng *et al.* (2007) as transcriptional levels of major genes involved in anthocyanin synthesis were strongly induced in response to N limitation in *Arabidopsis*. Although red algae do not contain anthocyanin, the increase in PC levels could possibly represent a similar survival mechanism to counteract and/or prevent photodamage. Furthermore, recent evidence suggests that phycobiliproteins have the ability to act as antioxidants. Although this has not been demonstrated in macroalgae, phycobiliproteins have been shown to act as antioxidants in mice exposed to mercury treatment (Cano-Europa *et al.*, 2010). They are able to prevent oxidative stress because of their nucleophilic ability to neutralize reactive species derived from oxygen and N, avoiding oxidative biomolecule damage (Cano-Europa *et al.*, 2010). N, P and K limitation have been shown to elicit oxidative stress responses in Mulberry plants (Tewari *et al.*, 2007). Logan *et al.* (1999) also reported an increase in the activities of antioxidant enzymes in N-limited plants. Therefore, the observed increase in PC α sub-unit protein levels may represent a possible antioxidant-type role in N-limited *G. gracilis*. However, no definitive conclusions can be drawn without further investigation.

Ferredoxin NADP reductase (FNR) is involved in two important processes in plants, namely photosynthesis and N assimilation. One of the effects of N limitation in plants is a decrease in the photosynthetic rate due to a decline in the rate of CO_2 assimilation. This decrease in CO_2

assimilation is attributed to a decline in the expression of several key enzymes in the CBB cycle (Evans & Terashima, 1987; Sage & Pearcy, 1987; Terashima & Evans, 1988; Sugiharto *et al.*, 1990). However, despite this decrease in CO_2 assimilation photosynthetic electron transport usually remains active (Evans and Terashima, 1987). This was demonstrated by Antal *et al.* (2010) when they investigated the acclimation of photosynthesis to N deficiency in *Phaseolus vulgaris*. They showed that N deficiency resulted in a decrease in photosynthetic capacity due mainly to a decline in the number of chloroplasts and thylakoids. A decrease in Rubisco and FNR content was also observed and attributed to the reduced consumption of photosynthates under conditions of N deficiency (Antal *et al.*, 2010). FNR, however, is not only involved in photosynthesis. The involvement of ferredoxin (Fd) and FNR in N assimilation in plants has been well documented (Hanke *et al.*, 2005). Most studies have focused on the effect of nitrate or ammonium supply on the action of these proteins (Wang *et al.*, 2000 & 2003; Hanke *et al.*, 2005). Fd is required as a reductant in both nitrite assimilation and ammonium assimilation by Fd-GOGAT (Lian *et al.*, 2006). Furthermore, plants possess both a leaf and root FNR, with the algal FNR suggested to be more similar to the root type FNR. Phylogenetic analysis conducted by Arakaki *et al.* (1997) on FNR showed an unexpected relationship between the algal and root FNR groups. Their results were not conclusive due to the small number of sequences considered which precluded a definitive explanation for the clustering. However, they suggested that an ancient duplication event of the FNR gene may have occurred prior to the separation of the algal and plant groups, followed by differential loss of the leaf-type gene in the algal lineage. Comparative analysis between plants and algae is further complicated by the fact that plants have defined root and shoot systems. Thus, most assimilated N comes from nitrate and ammonium uptake by roots in plants. However, given the proposed similarity to plant root FNR it is possible that the algal FNR is involved in primary N assimilation. The possible involvement of FNR in N assimilation is a complex process. In root cells nitrate is reduced to nitrite in the cytosol which is toxic and consequently, it is immediately imported to the plastid where Fd–dependent Nitrite Reductase (NiR) reduces it to ammonium. FNR reduces Fd to provide the energy required for this process. Ammonium fixation into amino acids is subsequently performed by NADH–dependent GOGAT which also requires Fd as a reductant. Nitrate and ammonium are then transported via the vasculature to the leaves. Here, the malate–oxaloacetate shuttle transfers photosynthetic reducing power to the cytosol for nitrate reduction. Nitrite is subsequently imported into the chloroplast and reduced by Fd–dependent NiR to ammonium which is in turn fixed by Fd-dependent GOGAT (Hanke *et al.*, 2005). It is therefore evident that FNR plays a key role in N assimilation in plants. The decreased protein levels of *G. gracilis* FNR observed in response to a sustained period of N limitation (Fig. 2.14) suggests that N is not being actively assimilated from the environment and enzymes such as FNR, which are involved in the assimilation of N, would likely be repressed.

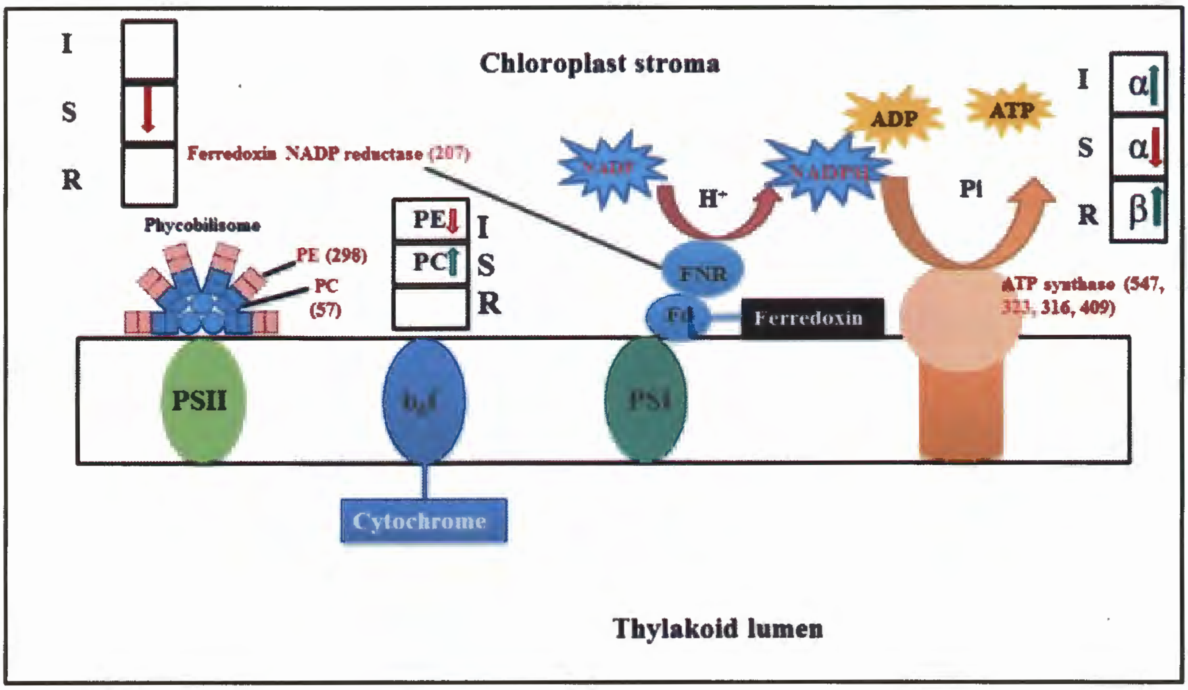

Figure 2.14 A simplified scheme depicting the light reactions of photosynthesis at the thylakoid membrane and the *G. gracilis* photosynthetic proteins which respond to changes in nitrogen nutrition. The intensity changes two dimensional (2D) gel spots are represented by boxes for each of the treatments I, S and R. I-Initial nitrogen limitation, S-Sustained nitrogen limitation and R-Nitrogen recovery. Arrows indicate increased or decreased intensity with respect to control. An empty box indicates that the protein was not identified for that particular nitrogen treatment. Spot numbers are displayed in red brackets. ATP, adenosine triphosphate, ADP, adenosine diphosphate, FNR, ferredoxin NADP reductase, Fd, ferredoxin, PE, phycoerythrin, PC, phycocyanin, PSI, photosystem I, PSII-photosystem II. Adapted from Bahrman *et al.*, 2004.

Redox processes

Thioredoxins (Trx) are generally small ubiquitous proteins involved in redox regulation which act as antioxidants by facilitating the reduction of other proteins by cysteine-thiol-disulfide exchange. However, they can vary considerably and range in size from 12 to 65 kDa in *Arabidopsis* and other plants (Meyer *et al.*, 2005). We identified and characterised a large molecular weight protein spot (~ 60 kDa) as thioredoxin in the N recovery experiment. The identity of this protein spot was based on two peptides with scores greater than the identity score, as well as NCBI blast analysis. Both of these peptides are found within conserved thioredoxin domains and, therefore, it is difficult to determine which type of thioredoxin was identified in this study without further investigation. However, thioredoxins are thought to play a central role in N metabolism as several enzymes of N metabolism have been identified as potential Trx targets, including GS, alanine and aspartate aminotransferase, branched-chain keto acid decarboxylase, glutamate, ATP synthase, fructose biphosphate aldolase-like proteins, HSPs 60 and 70, and elongation factor Tu (Balmer *et al.*, 2004; Meyer *et al.*, 2005). A comprehensive list of thioredoxin protein targets as identified in plants was reviewed by Meyer *et al.* (2005).

Thioredoxin proteins have been shown to be regulated in response to abiotic stress conditions as they participate in a wide range of cellular functions. These include redox regulation where they act as electron donors to several enzymes involved in protection against oxidative stress such as peroxiredoxin, methionine sulfoxide reductase, and glutathione reductase. *Arabidopsis* seedlings have been observed to respond to a re-supply of nitrate with small changes in the expression of specific genes involved in redox regulation. These include thioredoxins, genes involved in ascorbate and glutathione metabolism, peroxiredoxins, catalases and dismutases which are activated 3 hours after the addition of nitrate (Scheible *et al.*, 2004). Thioredoxins have also been shown to be regulated at the protein level in response to chilling stress in rice and heat stress in rice and *Populus* leaves (Ferreira *et al.*, 2006; Lee *et al.*, 2007). In *G. gracilis*, thioredoxin has been reported to be regulated at the transcriptional level in response to disease elicitors (Ealand, 2011), as well as high salinity, oxidative stress and abscisic acid treatment in *Arabidopsis* cell cultures (Takahashi *et al.*, 2004).

The increase in protein levels of thioredoxin observed in response to N recovery may be correlated to increased protein levels of GS. It may further indicate that the identified GS protein is the chloroplastic form (GS2) as it is this isoform which is a target of thioredoxin. The ferredoxin/thioredoxin system in plants has been shown to be involved in the regulation of CO_2 fixation and storage of reduced C. However, its role in the assimilation of inorganic nitrate to organic amino acids in leaves is not well understood (Geigenberger *et al.*, 2005). The initial step of nitrate assimilation is catalysed by NR in the cytosol. The subsequent reactions of NiR, GS and ferredoxin-dependent glutamine:oxoglutarate amino transferase (Fd:GOGAT) occur in the chloroplast (Geigenberger *et al.*, 2005). Additionally, the ATP-dependent conversion of ammonium and glutamate to glutamine, and the conversion of glutamine and 2-oxoglutarate to glutamate, respectively, also occur in the chloroplast. Previous work conducted on Fd-GOGAT found that the enzyme was significantly stimulated by DTT and thioredoxins when isolated from spinach chloroplasts (Lichter & Haberlein, 1998). In the same study, NiR and GS from spinach revealed no thioredoxin-dependent activation. In contrast to this, Choi *et al.* (1999) demonstrated that the chloroplast isoform of glutamine synthetase (GS2) from *Canavalia lineata* is activated by DTT. Site-directed mutagenesis studies demonstrated that the redox-sensitivity of GS2 is due to two conserved cysteine residues which are not present in the redox-insensitive cytosolic isoform GS1 (Choi *et al.*, 1999). Furthermore, GS has been identified as a protein target of thioredoxin using resin-bound mutant thioredoxins to capture target proteins (Motohashi *et al.*, 2001; Balmer *et al.*, 2004). Nevertheless, while these studies do provide evidence that the two enzymes responsible for the assimilation of ammonium in the chloroplast, Fd-GOGAT and GS2 are subject to thioredoxin-dependent redox regulation, this finding is not conclusive and requires further investigation (Geigenberger *et al.*, 2005). Whilst it is clear that important enzymes in N assimilation such as GS and GOGAT are targets of thioredoxin, further investigation is needed to establish which thioredoxin isoform is present in *G. gracilis*. In order for this to be accomplished, the full-length gene sequence of the identified protein would need to be determined to clarify which isoform of thioredoxin responds to N recovery in *G. gracilis* or alternatively, given the large size of this protein, it may indicate that this is a protein which has a strong similarity to thioredoxin.

Transcriptional regulation

tRNA-dihydrouridine synthase protein levels were increased in response to N re-supply in *G. gracilis*. tRNA-dihydrouridine synthase is involved in transcriptional regulation and catalyzes the synthesis of dihydrouridine, a modified base found in the D-loop of most tRNAs (Bishop *et al.*, 2002). Thus, it is most likely required for the correct folding of tRNAs and indirectly involved in the synthesis of proteins since tRNAs transfer specific amino acids to growing polypeptide chains during translation. Furthermore, the field of tRNA biology is a relatively new field and the involvement of tRNA and tRNA fragments in signalling pathways and stress response pathways needs to be elucidated. This will undoubtedly lead to new links between tRNA function and other global cellular response systems which will improve the understanding of tRNA processing, trafficking and function (Phizicky & Hopper, 2010). Transcriptional regulators and transcription factors have been implicated in the nitrate response in *Arabidopsis* using microarray and Affymetrix ATH1 studies (Wang *et al.*, 2003; Scheible *et al.*, 2004; Peng *et al.*, 2007). Despite the increased abundance of this protein in response to N re-supply in *G. gracilis*, its role remains unclear. However, the increased abundance suggests that it may be involved in the synthesis of new proteins, which are either directly or indirectly involved in N assimilation.

The analysis of proteome changes in response to N limitation and recovery enabled the identification of key pathways involved in the N stress response in *G. gracilis* (Fig. 2.15). The identified proteins reinforce the strict relationship which exists between C and N metabolism as the majority of the identified proteins were involved in these processes. This relationship has already been observed in proteomics studies conducted in green plants by Bahrman *et al.* (2004), Prinsi *et al.* (2009), Castillejo *et al.* (2010) and Møller *et al.* (2011). Despite the relatively small number of proteins sequenced in this study it can be seen that N stress, whether it be N limitation or recovery, for the most part impacts similar pathways. However, the pattern of protein regulation differed for the three N treatments. It is interesting to note that *G. gracilis* responds to an initial period of N limitation and 6 hours of N recovery in a similar manner, with most proteins up-regulated indicating that the early responses to N stress are there to prime the plant for the later more specific N responses. While bearing in mind that the identification of a protein in a 2-DE gel spot does not prove that the protein is present in an active form, changes in the abundance of proteins can be used to generate hypotheses about the molecular mechanisms employed by macroalgae, such as *G. gracilis* to overcome N stress. These changes in the protein profile provide us with an indication of which biological processes are activated or repressed in response to N availability (Fig. 2.15) and based on these responses we can attempt to generate hypotheses about what metabolic responses may be occurring in *G. gracilis*. In this study, proteins which either increase or decrease in abundance in response to an initial period of N limitation may be involved in the initial adaptive responses implemented by *G. gracilis* to adjust to the changing N environment. For instance the observed increased abundance of actin protein levels suggest that this may be the case for *G. gracilis* as actin has been implicated in early stress responses in plants (Kulikova *et al.*, 2009). It is also likely that these responses are transient and thus it may be necessary to sample a range of time-points from as early as 30 minutes to ascertain whether these are transient or maintained responses. The majority of proteins were down-regulated in *G. gracilis* in response to a sustained period of N limitation. The increased abundance of proteins involved in protein biosynthesis such as EF-tu may indicate that new proteins are being synthesised to enable *G. gracilis* to cope with the stress being experienced. The absence of proteins involved in N metabolism has already been discussed. However, it is possible that these proteins may be regulated earlier than 14 days and thus future studies should focus on determining what happens between 6 hours and 14 days of N limitation. The vast majority of proteins showed increases in protein abundance in response to N recovery. This has been observed with gene expression studies in *Arabidopsis* investigating nitrate addition and proteomics studies investigating ammonium addition in barley shoots (Krouk *et al.*, 2010; Møller *et al.*, 2011). In order to further elucidate the molecular mechanisms of N assimilation in *G. gracilis* future studies should investigate the response of *G. gracilis,* to nitrate and ammonium individually to determine whether the responses are similar to that obtained with the combined nitrate and ammonium source. An experimental plan similar to that employed by Li *et al.* (2006) could be employed. Li *et al.* (2006) investigated the response of N-starved rice plants to a re-supply of different N sources in the form of nitrate$^-$, ammonium and a combined nitrate/ammonium source. The authors found that a combined N supply of nitrate and ammonium resulted in higher biomass of both roots and shoots and a higher total N uptake rate (Li *et al.*, 2006). Finally, as mentioned with the N limitation experiments, it may be necessary to sample at earlier time-points after N addition to provide a more comprehensive overview of N assimilation in *G. gracilis*. It may be necessary to sample time-points as early as 20 or 30 minutes as was previously reported for *Arabidopsis* in gene expression studies (Wang *et al.*, 2000 & 2003; Scheible *et al.*, 2004). Krouk *et al.* (2010)

reported that the transcriptome response to nitrate in *Arabidopsis* was rapid, with the first changes being observed as early as 3 minutes after nitrate addition.

Further studies should attempt to sequence the un-sequenced proteins which were observed to be differentially regulated in response to N limitation and recovery as only a few of the identified proteins were sequenced. The use of a complementary gel-free proteomics strategy such as isobaric tags for relative and absolute quantitation (iTRAQ) to further investigate the effects of N limitation and recovery in *G. gracilis* should also be considered. A common problem with 2-DE is that it only identifies the most abundant proteins and membrane proteins are notoriously under-represented. A strategy such as iTRAQ would also circumvent the problem of being able to analyse only one time-point at a time as it enables one to conduct multiplex experiments where a total of 8 samples can be analyzed concurrently under different experimental conditions (Pierce *et al.*, 2007). This would allow the analysis of different time-points concurrently and would provide both relative and absolute quantification of proteins. Most importantly, iTRAQ allows the detection of low abundant, very acidic, very basic, high and low molecular weight proteins (Ross *et al.*, 2004; Lin *et al.*, 2005; Shadforth *et al.*, 2005; Chong *et al.*, 2006; Gan, 2007) which are not identified using conventional 2-DE approaches.

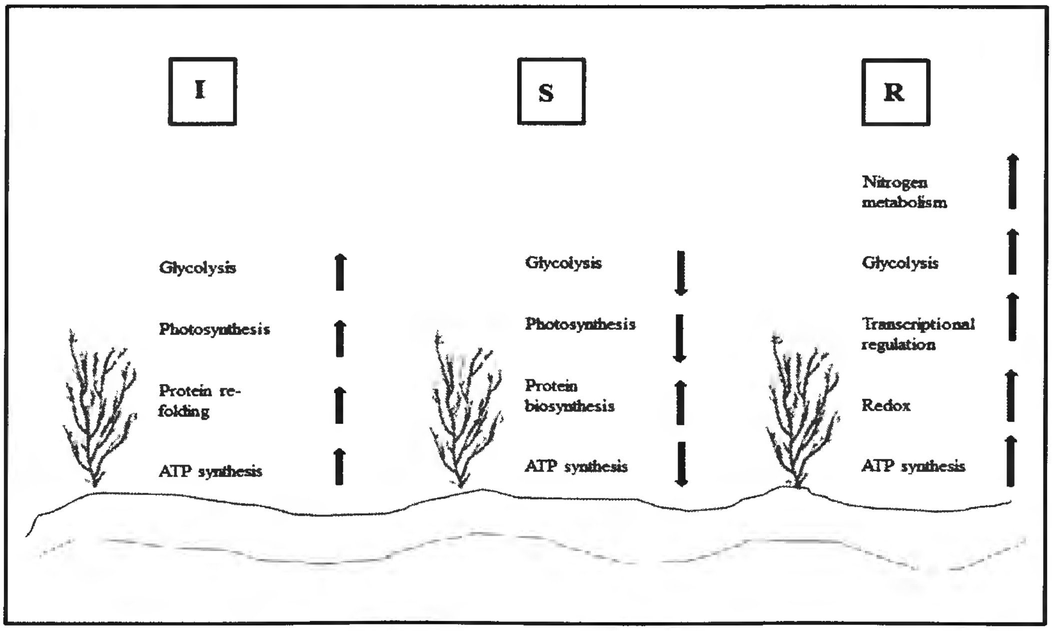

Figure 2.15 Summary of the proposed *G. gracilis* proteome responses to different N treatments in comparison to the control I-Initial nitrogen limitation, S-Sustained nitrogen limitation and R-Nitrogen recovery.

Finally, an understanding *G. gracilis* proteome changes in response to varying degrees of N limitation could lead to the identification of biomarkers. Biomarkers would enable monitoring of the N status of *G. gracilis* populations, particularly in cultivation systems where populations are often exposed to nutrient limiting conditions. Moreover, the identified proteins have allowed us to identify important pathways involved in the N stress response in *G. gracilis* and the response of specific groups of proteins to N limitation and recovery could be monitored. Future work should attempt to characterise the proteins identified in this chapter and their response to additional stress conditions including disease and temperature should be evaluated. In time, this information may help to develop a biomarker system enabling one to monitor the health status of *G. gracilis* in commercial cultivation systems, and in so doing improve growth and biomass yields. In the long-term this may have a positive impact on commercial *G. gracilis* farming and may lead to the creation of a sustainable agar production industry in South Africa.

CHAPTER 3

Characterisation of fructose 1,6 biphosphate aldolase, glutamine synthetase and pyruvate kinase in response to nitrogen limitation and recovery in *Gracilaria gracilis* and changes in starch patterns during nitrogen limitation.

CONTENTS

3.1 Introduction

Two-dimensional gel electrophoresis (2-DE) coupled to liquid chromatography mass spectrometry (LC MS/MS) was used to identify proteins which are differentially regulated in response to nitrogen (N) limitation and recovery in *G. gracilis*. A protein can be positively identified using tandem mass spectrometry if it has at least two tandem MS sequenced peptides or a single high-quality tandem MS spectrum (Veenstra *et al.*, 2004). However, it is important to validate the expression and identity of the protein obtained using 2-DE and mass spectrometry (MS) analyses. For global proteomic studies, triplicate MS analyses are often impractical and therefore targeted techniques such as western blotting or ELISA are used to confirm the expression and characterise interesting and novel proteins (Veenstra *et al.*, 2004). In this study, fructose 1, 6 biphosphate aldolase (ALD) and glutamine synthetase (GS) were selected for further characterisation. These proteins were selected as they were shown to be differentially regulated during N limitation and recovery in *G. gracilis*. ALD protein abundance was significantly decreased ($P<0.05$) in response to N limitation and significantly increased ($P<0.05$) during N recovery. GS was the only primary N metabolic protein identified in this study and was observed to significantly increase ($P<0.05$) in abundance during N recovery.

ALD and GS each have specific roles in metabolic pathways in plants and algae. ALD is an essential enzyme involved in glycolysis, fermentation and photosynthesis. It catalyses the reversible cleavage of fructose 1, 6 biphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Rutter, 1964). ALD has been purified from the red alga *Gracilaria chorda* (Kakita *et al.*, 2006), but has not been well characterised in other red macroalgal species. Furthermore, ALD has been found to be differentially expressed in response to a wide range of abiotic stressors at the transcriptional level (Minhas & Grover, 1999, Purev *et al.*, 2008) and at the protein level in response to short-term N starvation in barley roots (Møller *et al.*, 2011). ALD was also the only glycolytic protein observed to be differentially regulated in response to N limitation and recovery in *G. gracilis* and was thus selected for further characterisation studies.

GS plays an important role in N metabolism and is responsible for the initial step at which inorganic N is brought into cellular metabolism (Freshwater *et al.*, 2002). It functions in two essential biochemical reactions, ammonium assimilation and glutamine (Gln) biosynthesis.

GS catalyzes the formation of Gln from ammonium and glutamate in the presence of adenosine 5' triphosphate (ATP) and magnesium (Mg). Subsequently, Gln is used to produce glutamate in a reaction catalysed by glutamate synthase (GOGAT). Glutamate is a central molecule as it is involved in the biosynthesis of essentially all cellular nitrogenous compounds. A large amount of research has been conducted on GS. Biochemical studies have shown that distinct isoenzymes of GS are located in the cytosol (GS1) and chloroplast (GS2) of numerous plant species (Hirel & Gadal, 1980). In all higher plants examined to date, there is a single nuclear gene for chloroplastic GS2 and multiple homologous but distinct genes for cytosolic GS1 (Tingey & Coruzzi, 1987; Tingey *et al.*, 1987; Sakamoto *et al.*, 1990; Cock *et al.*, 1991; Peterman & Goodman, 1991; Sakakibara *et al.*, 1992; Li *et al.*, 1993; Oliveira *et al.*, 1997; Oliveira & Coruzzi, 1999). The chloroplastic and cytosolic GS isoenzymes seem to serve distinct roles, based on their organ and cell-specific expression patterns (Edwards *et al.*, 1990; Carvalho *et al.*, 1992; Kamachi *et al.*, 1992). Cytosolic and chloroplastic GS genes have been isolated and characterized from the green algae *Chlamydomonas reinhardtii* (Chen & Silflow, 1996) and *Dunaliella tertiolecta* (Robertson *et al.*, 1999), and the gene for chloroplast GS has been described from the diatom *Skeletonema costatum* (Robertson *et al.*, 1999).

The fact that C and N metabolism are integrally linked has been discussed extensively in Chapters 1 and 2 of this thesis. Moreover, N is an important regulator of C flow in higher plants and algae (Smith *et al.*, 1989) and these processes are integrally linked by two enzymes, phosphoenolpyruvate carboxylase (PEPc) and pyruvate kinase (PK) through the provision of glycolytic carbon for amino acid biosynthesis (Fig. 3.1), (Lunn & Furbank, 1999; Stitt *et al.*, 2002; Foyer *et al.*, 2003). Plant PK is of particular interest as there is considerable evidence to suggest that it is the primary site of control of glycolytic flux for pyruvate (Plaxton, 1996). PK catalyses the irreversible transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP yielding one molecule of pyruvate and one molecule of ATP (Smith *et al.*, 2000). The assimilation of N into amino acids, proteins and other macromolecules result in changes in the levels of key regulatory metabolites. Consequently enzymes involved in the later stages of glycolysis such as PK are activated to provide C for amino acid synthesis (Bassham *et al.*, 1981; Elrifi & Turpin, 1987; Lin *et al.*, 1989; Turpin *et al.*, 1990; Turpin, 1991). In the green alga, *Selenastrum minutum,* starch degradation, PEPc and PK are all activated within seconds following ammonium addition to N limited samples

(Smith *et al.*, 1989). In contrast, the enzyme activities of important glycolytic enzymes such as PEPc and PK have been observed to decrease during N limitation (Champigny & Foyer 1992; Huber *et al.*, 1994; Huppe & Turpin 1994). Although PK was not found to be up- or down-regulated in response to either N limitation or recovery in this study, *G. gracilis* PK activity levels were investigated because of its crucial role in integrating C and N metabolism. Furthermore, a number of proteins involved in glycolysis were found to be differentially regulated during N limitation and recovery in *G. gracilis,* suggesting possible changes in the expression and activity levels of PK (Fig. 3.1).

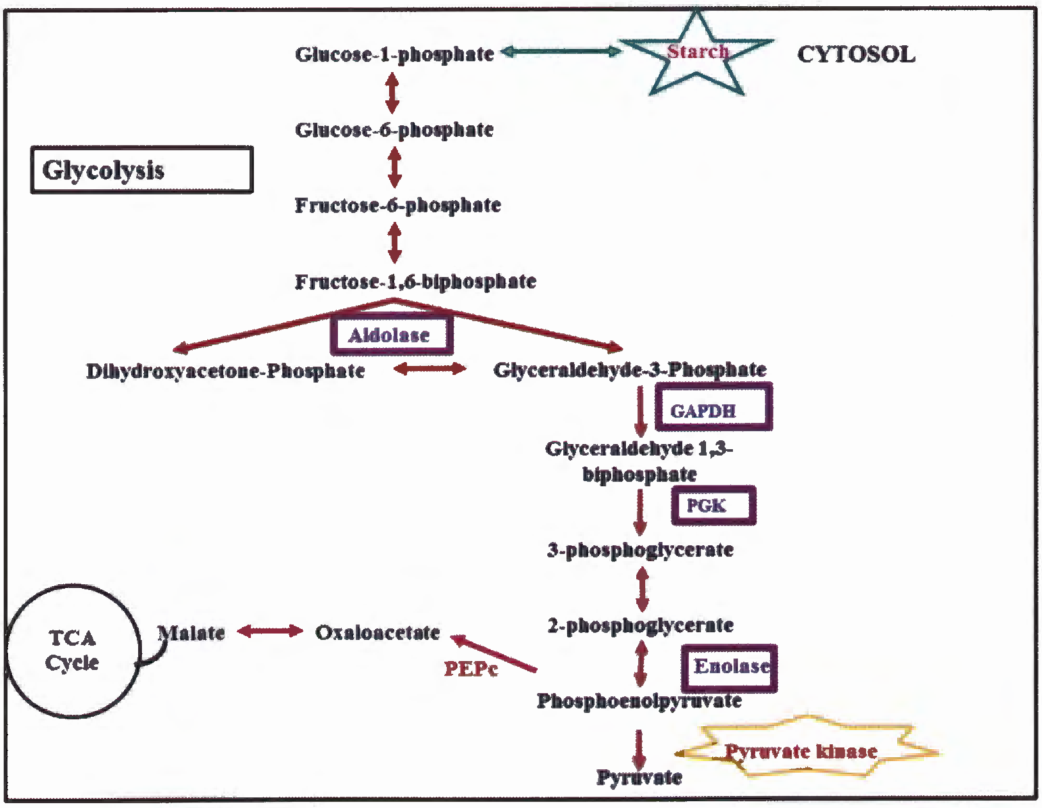

Figure 3.1 A simplified scheme illustrating the links between glycolysis, the TCA cycle and starch synthesis. Putative *G. gracilis* proteins identified to be differentially regulated during N limitation and recovery are boxed in purple (□). Starch is synthesized from glucose-1-phosphate in the cytosol of red algae and pyruvate kinase catalyses the final reaction in the glycolytic pathway. PEPc, phosphoenolpyruvate carboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; TCA, tricarboxylic.

One of the consequences of N limitation in red algae is a change in C partitioning as fewer C skeletons are available to support amino acid and protein synthesis. Instead the available C skeletons are used for the synthesis of storage compounds with a resultant increase in the content of floridoside, floridean starch and agar (Macler, 1986; Bird 1988; Ekman *et al.*, 1991). Changes in the allocation of C between different carbohydrate pools in response to nutrient limitation and re-supply has been investigated in the red alga *Gracilaria tenuistipitata* and consequently increased levels of floridean starch and floridoside was observed under nutrient limiting conditions in *G. tenuistipitata* (Collén *et al.*, 2004). Conversely, nutrient addition resulted in a decrease in the levels of floridean starch and floridoside. This demonstrated that N availability affects C allocation patterns in *G. tenuistipitata.* The decreased protein levels of ALD, phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during N limitation suggests that changes in C allocation have occurred in *G. gracilis* in response to N limitation. Thus, the effect of 14 days of N limitation on starch accumulation was assessed in this study.

3.1.1 Aims of this chapter

It is important to use an alternative method to validate the identity and expression pattern of a protein determined by 2-DE and tandem MS. However, as it is not possible to validate every identified protein in this study, the identities of two selected proteins, ALD and GS, were confirmed. Moreover, the expression pattern of these proteins in response to N limitation and recovery was validated. Western blot analysis and enzymatic assays were employed to determine the effect of N limitation on ALD protein levels and activity in *G. gracilis.* Similarly, the effect of N recovery on *G. gracilis* ALD protein levels and activity, as well as on *G. gracilis* GS protein levels, was quantitated using western blot analysis to determine whether these proteins can be used to measure the recovery response of *G. gracilis* following N limitation. For each time-course experiment, the N status of *G. gracilis* thallus samples exposed to either N limitation or N recovery were evaluated using physiological measurements of intracellular C and N levels. The strict relationship which exists between the metabolic pathways of C and N metabolism has been extensively discussed and reinforced in this study by the identification of a number of proteins involved in C metabolism. Thus, the activity of PK, an enzyme which integrates C and N metabolism, was measured in response to N limitation and recovery in *G. gracilis.* Finally the accumulation of starch was measured to determine whether starch levels increase as glycolytic protein abundance decreases, resulting in a shift in C allocation patterns in *G. gracilis* during N limitation.

3.2 Materials and Methods

All of the growth media and solutions used in this study are listed in Appendix A

3.2.1 *G. gracilis* material and cultivation conditions

G. gracilis algal samples were sourced from Irvin and Johnson (I&J), Abalone Culture Division, Danger Point, Gansbaai, Western Cape, South Africa. Algal epiphytes and sand were removed by successive washing with sterile distilled water. Cultivation conditions were as described in Chapter 2, section 2.2.1.

3.2.2 Responses of *G. gracilis* to nitrogen limitation and recovery

These experiments were set up as described in section 2.2.2 and 2.2.3 of this thesis.

3.2.2.1 Nitrogen limitation time-course experiment

Following the initial acclimatisation stage, *G. gracilis* samples were grown in N-lacking media and samples were collected at 0, 3, 6, 10 and 14 days. *G. gracilis* samples collected at day 0 served as the N-sufficient time-course control. *G. gracilis* samples (0.5 g) were collected in triplicate from each flask for protein isolations (as described in section 3.2.4.2). Following protein isolation, triplicate samples were pooled, flash frozen in liquid N and stored at -80°C for western blot analysis. Samples were also collected for intracellular C and N level analysis, pigment analysis, PK and ALD activity assays, and starch analysis. All samples were flash frozen in liquid N and stored at -80°C for later use. Four biological replicate experiments were conducted.

3.2.2.2 Nitrogen recovery time-course experiment

Following the initial acclimatisation period, N-sufficient *G. gracilis* samples were sampled at day 0. Thereafter, N-limited *G. gracilis* samples were collected following seven days growth in N-lacking media. N recovery was initiated by transferring the algae into fresh media containing a N source. Thereafter, *G. gracilis* samples were collected at 6, 12, 24, 72 and 168 hours and the effect of N recovery assessed. *G. gracilis* samples (0.5 g) were collected in triplicate from each flask for protein isolations (as described in section 3.2.4.2). Following protein isolation, triplicate samples were pooled, flash frozen in liquid N and stored at -80°C for western blot analysis. Samples were also collected for intracellular C and N analysis, pigment analysis, PK and ALD activity assays. All samples were flash frozen in liquid N and stored at -80°C for later use. Three biological replicate experiments were conducted.

3.2.3 Physiological indicators of nitrogen limitation and recovery in *G. gracilis*

3.2.3.1 Total thallus nitrogen and carbon content of G. gracilis samples

The intracellular C and N levels of *G. gracilis* samples from both the N limitation and recovery experiments were determined as described in Chapter 2, section 2.2.4.1.

3.2.3.2 Pigment analysis of G. gracilis samples

PC and PE content of *G. gracilis* samples collected during N limitation and recovery were determined as described in Chapter 2, section 2.2.4.2.

3.2.4 Validation of protein identity and expression pattern using western blot analysis

3.2.4.1 Antibodies

Polyclonal antibodies against ALD and GS were purchased from Agrisera antibodies (Sweden). ALD antibodies were raised to the overexpressed cytosolic ALD sequence from *Arabidopsis thaliana* and a 38 kDa band was expected as a positive signal. GS antibodies were raised to a KLH-conjugated synthetic peptide derived from a wide range of available sequences including all *Arabidopsis thaliana* GS isoforms. GS antibodies were able to detect both the cytosolic (GS1) and chloroplastic (GS2) isoforms and the expected molecular weight (MW) for GS1 was 39-40 kDa and GS2 was 44-45 kDa. These antibodies were used to validate the identity and expression pattern of ALD and GS determined using 2-DE and MS analysis.

3.2.4.2 Protein isolation and 2-DE

Protein was isolated from *G. gracilis* samples collected during the N limitation and N recovery time-course experiments as described in Chapter 2, section 2.2.5. Two-DE was conducted as described in Chapter 2, section 2.2.6.

3.2.4.3 SDS-PAGE

Total protein extracted from each *G. gracilis* sample collected during the N limitation and recovery experiments was separated according to size by SDS-PAGE (in duplicate). Stacking gels were prepared as follows: 4% (w/v) acrylamide, Tris-Cl/SDS pH 6.8, 0.05% (w/v) ammonium persulphate (AMPS,), 0.01% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED) while resolving gels were prepared as follows: 12% (w/v) acrylamide, Tris-Cl/SDS pH 8.8, 0.2% (w/v) ammonium persulphate (AMPS, Appendix A.2.4), 0.04% (v/v) TEMED. Acrylamide solution (40% (w/v) (Sigma)) was used as the acrylamide stock solution. Tris-Cl/SDS buffer composition can be found in Appendix A.2.4. All protein samples for SDS-PAGE were prepared by mixing 5 X sample application buffer (Appendix A.2.4) with the appropriate volumes of protein. Thirty micrograms of protein sample was loaded equally, as determined by the Bradford assay for 1D SDS PAGE. SDS PAGE was also conducted with protein samples which had been subjected to isoelectric focussing (IEF). Protein samples were electrophoresed at 100 V until the dye front was eluted. One protein gel was stained with Coomassie solution (Appendix A.2.5) while the other was used for western blot analysis.

3.2.4.4 Western blot analysis

Protein electrophoresis was performed using a Mini-PROTEAN Tetra cell system (Bio-Rad. Germany) set to a 100 V constant current until the dye front was eluted. SDS-PAGE gels were equilibrated in Towbin buffer (Appendix A.2.7) following electrophoresis. The separated proteins were transferred onto nitrocellulose membrane (S&S Protan, PerkinElmer Life Sciences) at 4°C using a Mini Trans-Blot cell (Bio-Rad, Germany) for 1 hour at 100 V. Transfer efficiency and equal loading of the samples was determined visually by Ponceau S

(Appendix A.2.7) staining which was subsequently removed by 2 successive wash steps with TBS (Appendix A.2.7). Membranes were blocked for 1 hour at RT in blocking buffer (Appendix A.2.7) with shaking. Primary and secondary antibody dilutions were prepared in blocking buffer. Primary and secondary antibodies for ALD detection were diluted at 1:5000 and 1:10000, respectively. Similarly, the primary and secondary antibodies for GS detection were diluted 1:10000 and 1:20000, respectively. Membranes were incubated individually with primary antibody for ALD and GS at 4°C O/N with shaking. The membranes were washed 4 times with blocking buffer (Appendix A.2.7) and then incubated for 3 hours with secondary antibody at RT for 3 hours. Four washes with TBST (Appendix A.2.7) and a final TBS (Appendix A.2.7) wash step were conducted prior to chemi-luminescent visualisation with the Immun-Star^{TM} WesternC^{TM} Chemiluminescent Kit (Bio-Rad, Germany) as per the manufacturer's instructions. Chemi-luminescent signals were visualised using the Molecular Imager ChemiDoc XRS+ system (Bio-Rad, Germany). All western blot visualisations were conducted with chemi-luminescence, with the exception of the GS 2D western blot. GS 2D western blots were visualised colorimetrically using 500 µl of a freshly prepared developing substrate solution (TMB Membrane Peroxidase substrate; KPL) which was added to the membrane and colour development was allowed to proceed for a few minutes. Reactions were stopped simultaneously by washing each membrane in water.

3.2.4.5 Densitometry analysis of western blots

Quantity One Software (Version 4.5.2 (Bio-Rad, Germany)) operating a ChemiDoc XR molecular imager (Version 4.6.9 (Bio-Rad, Germany)) was used to determine the densities of each chemi-luminescent signal. Only the band with the correct molecular weight was assessed. The volumetric analysis tool was selected to calculate the signal intensity of each band. The area of interest was defined by drawing a rectangle around the selected band. The density of each band was calculated by dividing the signal intensity by the area of the rectangle.

3.2.5 Enzyme assays

3.2.5.1 Fructose 1, 6 biphosphate aldolase (ALD) assay

G. gracilis samples (0.1 g) collected during N limitation and recovery were ground in liquid N using a mortar and pestle. The samples were resuspended in buffer consisting of 50 mM KH_2PO_4, 4 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 10% glycerol and 5 mM DTT. This was followed by centrifugation at 14,000 rpm at 4°C. The resultant crude extract was assayed for ALD activity. ALD activity was determined in a coupled reaction using glycerol-3-phosphate dehydrogenase and triose phosphate isomerase in the forward direction (glycolytic) at 22°C by continuously monitoring NADH utilisation at 340 nm using a DU®530 UV/Vis spectrophotometer. The reaction mixture for the assay contained (in a final volume of 1.0 ml): 50 mM HEPES (pH 7.2); 1.0 mM EDTA (pH 8.0) 4 mM NADH; 1 unit glycerol-3-phosphate dehydrogenase, 10 units triose-phosphate isomerase and 50 ul crude protein extract. The reaction was initiated by the addition of 4 mM fructose 1, 6 biphosphate (FBP) and was corrected for NADH oxidase activity by omitting FBP from the reaction. One unit of activity is defined as the amount of enzyme resulting in the cleavage of 1 µmol of substrate/min at 22°C or the oxidation of 2 µmol NADH.

Enzyme activity was determined with the following formula:

$$\text{Enzyme activity} = \frac{(slope)*(final\ vol.in\ cuvette)}{(vol.sample\ in\ cuvette)*(extinction\ coefficient\ of\ NADH)}$$

$$= \frac{(slope)*(1000\ ul)}{(100\ ul)*(6.22)}$$

where the slope is the change of absorbance over time.

The protein concentration of crude extract samples was determined using the Bio-Rad protein assay based on the method of Bradford (Bradford, 1976) and using BSA as the standard protein. Specific activity is expressed as enzyme U/ mg protein.

3.2.5.2 Pyruvate kinase (PK) assay

G. gracilis samples (0.5 g) collected during N limitation and recovery were ground in liquid N using a mortar and pestle. These samples were resuspended in buffer consisting of 50 mM Tris-Cl (pH 7.5), 5 mM $MgSO_4$, 1mM Na_2EDTA, 2 mM ascorbic acid, 2 mM 2-mercaptoethanol, 0.1% Tween 80 (an essential requirement for optimal enzyme activity), 20% glycerol and 10% PVP. This was followed by centrifugation at 14,000 rpm at 4°C. The resultant crude extract was assayed for PK activity using a colorimetric assay based on the determination of pyruvate by 2,4 dinitrophenylhydrazine as described by Kachmar & Bayer, (1953) and Miller & Evans, (1957). The assay reaction mixture contained (in a final volume of 0.5 ml): 50 mM HEPES (pH 7.0); 50 mM KCl; 10 mM $MgSO_4$; 1.0 mM cyclohexylammonium ADP and 5mM cyclohexylammonium PEP. Differing amounts of protein were tested in preliminary assays to determine the protein concentration required for optimal detection of PK activity in crude extracts of *G. gracilis* samples (data not shown). This was determined to be 30 µg of protein. The reaction was initiated by the addition of 30 µg crude protein extract and incubated at 25°C for 15 minutes. The reaction was terminated by the addition of 0. 25 ml 0.025% 2,4 dinitrophenylhydrazine (DNPH) in 2 M HCl. After 10 min at RT, 0.25 ml of 3 M NaOH and 1.0 ml of distilled water were added, and the solution centrifuged at 14,000 rpm for 5 minutes to pellet debris. The brown coloration due to pyruvate was measured in 1.0-cm path-length cuvettes at 510 nm 15 min after the addition of the NaOH. The pyruvate production was quantified according to a standard curve of known quantities of sodium pyruvate (0.05 – 4 mM) (Davison, 1987).

3.2.6 Starch assay

A starch assay kit (Biovision) was used according to the manufacturer's instructions to determine the intracellular levels of starch in N-sufficient and N-limited *G. gracilis* samples. Starch was extracted using the soluble starch extraction method outlined in the kit protocol with a few modifications: Starch was extracted from 0.1 g of *G. gracilis* sample tissue ground in liquid N. The samples were washed with 90% ethanol, warmed at 60°C for 5 min and centrifuged at 14,000 rpm for 2 min as described in the kit protocol. The washes were conducted until the supernatant was clear and not just thrice as described in the kit protocol. Starch was extracted with 1 ml distilled water in a boiling water bath for 15 minutes. The samples were centrifuged at 14,000 rpm for 2 min to pellet insoluble material and the supernatant containing soluble starch was collected. The fluorometric assay method was used to determine the concentration of soluble starch as described in the kit protocol. A starch standard curve was constructed using pure starch (Biovision) in concentrations ranging from 0.03 to 0.2 μg.

3.2.7 Statistical analyses

Densitometry readings as determined by western blot analysis (which corresponded to relative amount of a specific protein present in each sample), N and C content, ALD and PK activity, and concentrations of starch were assessed for statistical differences by one-way analysis of variance (ANOVA, $P<0.05$) with post-hoc tests using SigmaStat for Windows (Version 3.10.0). Data sets that were not normally distributed or that exhibited unequal variances were transformed to meet the requirements for parametric statistical testing. Where data could not be sufficiently transformed, a non-parametric ANOVA on ranks (Kruskal-Wallis) test was used instead.

3.3 Results

3.3.1 Evaluation of the carbon and nitrogen status of *G. gracilis* in response to N limitation and recovery

G. gracilis was grown in N-limiting media for 14 days and samples were collected at day 0, 3, 6, 10 and 14. The intracellular C and N levels of N-sufficient *G. gracilis* collected at day 0 and N-limited *G. gracilis* collected at days 3, 6, 10 and 14 were determined to assess the C and N status of the alga. The C content of *G. gracilis* tissue sampled over the 14 day experimental period remained constant (Fig. 3.2A). The intracellular N levels decreased significantly ($P<0.05$) from 4.4 ± 0.18% at day 0 to 2.1 ± 0.26% following 14 days of N limitation, and was also significantly lower ($P<0.05$) than the intracellular N levels of *G. gracilis* sampled at day 3 (3.4 ± 0.22%) and 6 (3.5 ± 0.44%) (Fig. 3.2B). The C:N ratios increased significantly ($P<0.05$) following 14 days of N limitation from 6.7 ± 0.17 to 14.6 ± 1.2 (Fig. 3.2C). Additionally, the C:N ratio of *G. gracilis* at day 14 was found to be significantly higher ($P<0.05$) than the C:N ratios determined for *G. gracilis* at days 0, 3, 6 and 10.

The ability of N-limited *G. gracilis* to recover following re-supply of N was evaluated using time-course analysis measurements. Intracellular C and N levels, as well as C:N ratios, were determined to evaluate the C and N status of N-sufficient *G. gracilis* collected at day 0, *G. gracilis* collected following 7 days of N limitation and N-recovered *G. gracilis* collected following the re-supply of N. N recovery was assessed 6, 12, 24,72 and 168 hours after the introduction of N. Intracellular C levels of G. *gracilis* samples remained constant over the course of the experiment (Fig. 3.3A). Intracellular N levels of N-sufficient *G. gracilis* decreased significantly ($P<0.05$) from 2.87±0.17% to 1.26±0.14% after exposure to N-limiting conditions (Fig. 3.3B). The N levels increased gradually following the addition of N to 1.72±0.2%, after 72 hours of recovery. A decline in N level to 1.6±0.29% was observed after 168 hours of N recovery. Despite the observed increase at 72 hours, intracellular N levels failed to return to that observed in *G. gracilis* sampled at day 0. An increase in the C:N ratio from 9.5 ± 0.52 at day 0 to 23 ± 2.75 was observed following 7 days of N limitation (Fig. 3.3C). This increase however, was not statistically significant. A statistical difference was observed between the C:N ratios of N-sufficient *G. gracilis* collected at day 0 and N-recovered *G. gracilis* collected 12 hours after recovery (Fig. 3.3C). A decrease in the C:N ratio was observed following 24, 72 and 168 hours of N recovery when compared to the C:N ratio of *G. gracilis* samples collected 12 hours after N recovery, however these changes were not significant.

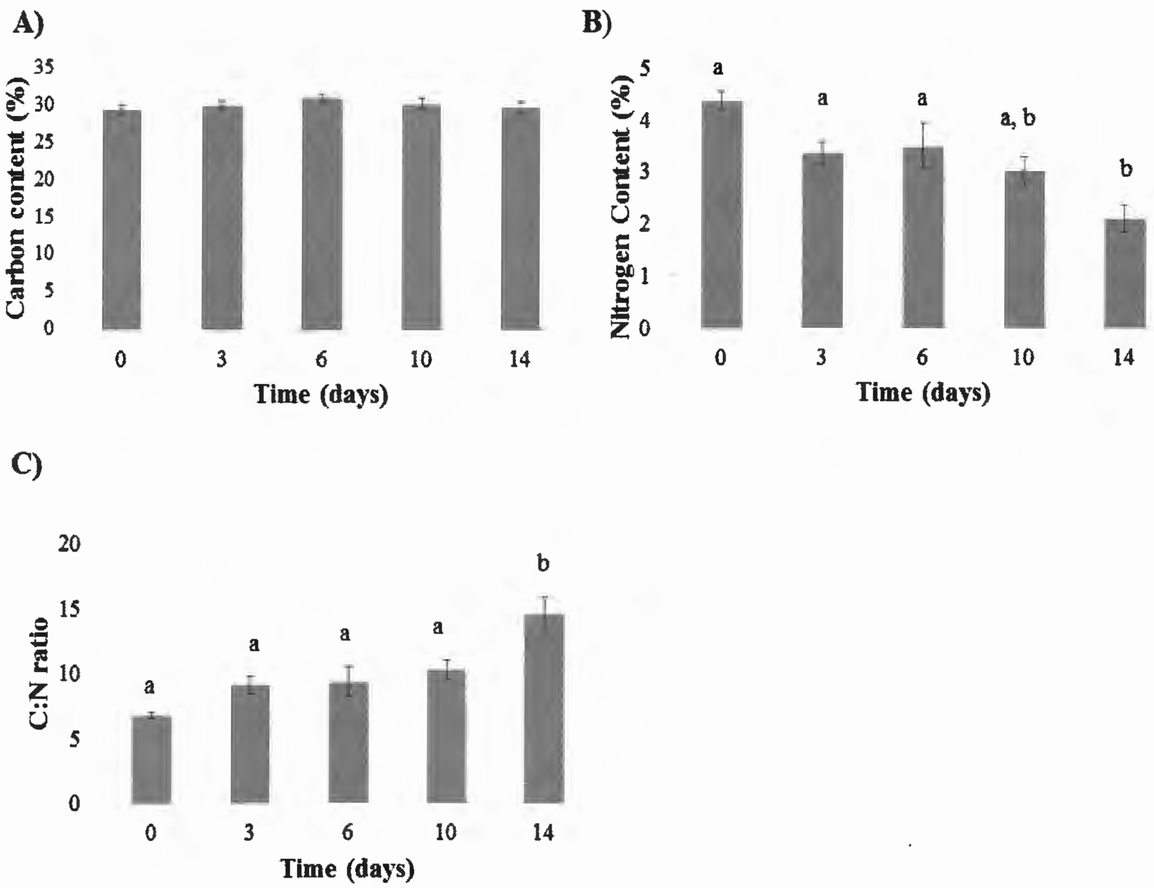

Figure 3.2 Intracellular carbon levels (A), nitrogen levels (B) and C:N ratios (C) of nitrogen-limited *G. gracilis* sampled over a 14 day period. Data represents the mean ± standard error (n=4). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in N levels and C:N ratios of the *G. gracilis* samples.

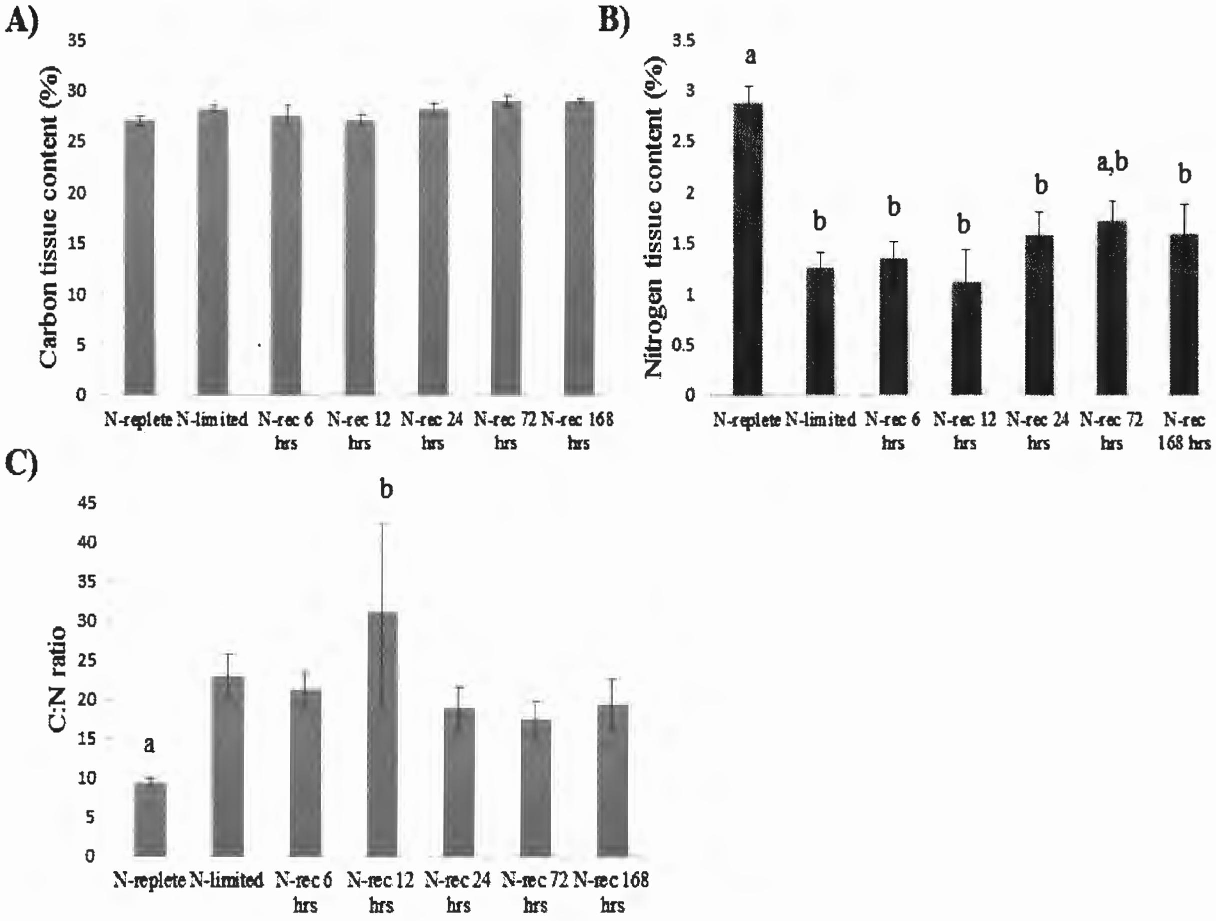

Figure 3.3 Intracellular carbon levels (A), nitrogen levels (B) and C:N ratios (C) of *G. gracilis* that had been nitrogen limited for 7 days followed by nitrogen recovery for 7 days. Data represents the mean ± standard error (*n*=3). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in N levels and C:N ratios of the *G. gracilis* samples.

3.3.2 Pigment content of nitrogen limited and nitrogen recovered *G. gracilis*

Thallus colour is considered an important indicator of the N status of algae (Oliveira *et al.*, 2000) as studies with *Gracilaria* species have shown that pigment levels are closely correlated with N content (Lapointe & Ryther, 1979). Profound visual changes in *G. gracilis* thallus colour were observed following 14 days of N limitation (Fig. 3.4). Thus, PE and PC levels were determined to further evaluate the N status of each *G. gracilis* sample. PE levels decreased significantly ($P<0.05$) from 0.58 ± 0.044 ml pigment/g tissue in N-sufficient *G. gracilis* at day 0 to 0.31 ± 0.037 ml pigment/g tissue in N-limited *G. gracilis* following 14 days of N limitation (Fig. 3.5). Similarly, PC levels also decreased significantly ($P<0.05$) from 0.08 ± 0.007 ml pigment/g tissue at day 0 to 0.05 ± 0.005 ml pigment/g tissue at day 14 (Fig. 3.6).

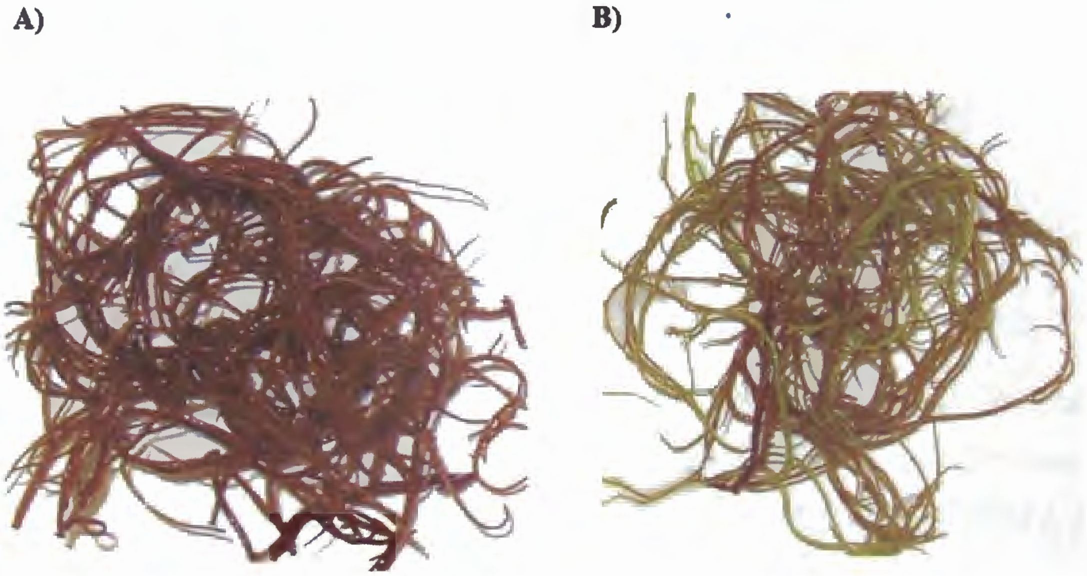

Figure 3.4 Profound visual changes in *G. gracilis* thallus colour following N limitation. Nitrogen sufficient (A) and nitrogen limited (B) *G. gracilis*.

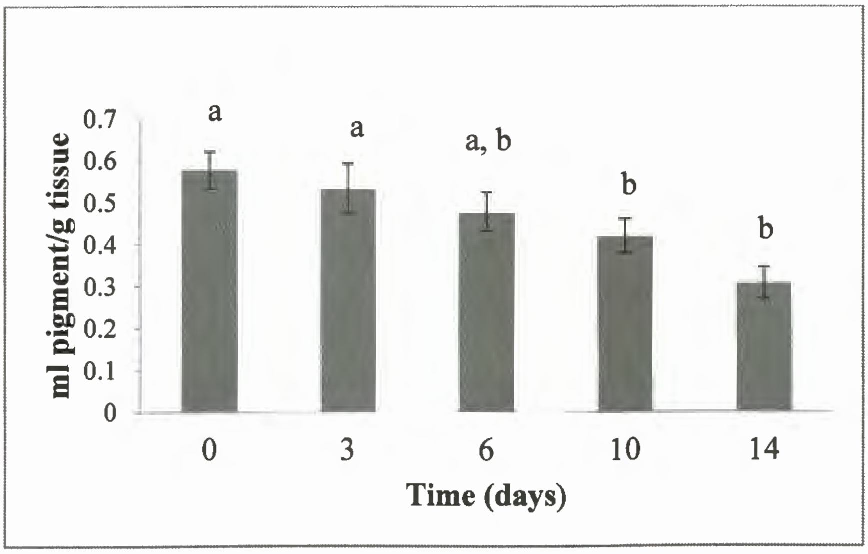

Figure 3.5 *G. gracilis* PE levels following exposure to N limiting growth conditions for 14 days. Data represents the mean ± standard error (n=4). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in PE level of the *G. gracilis* samples

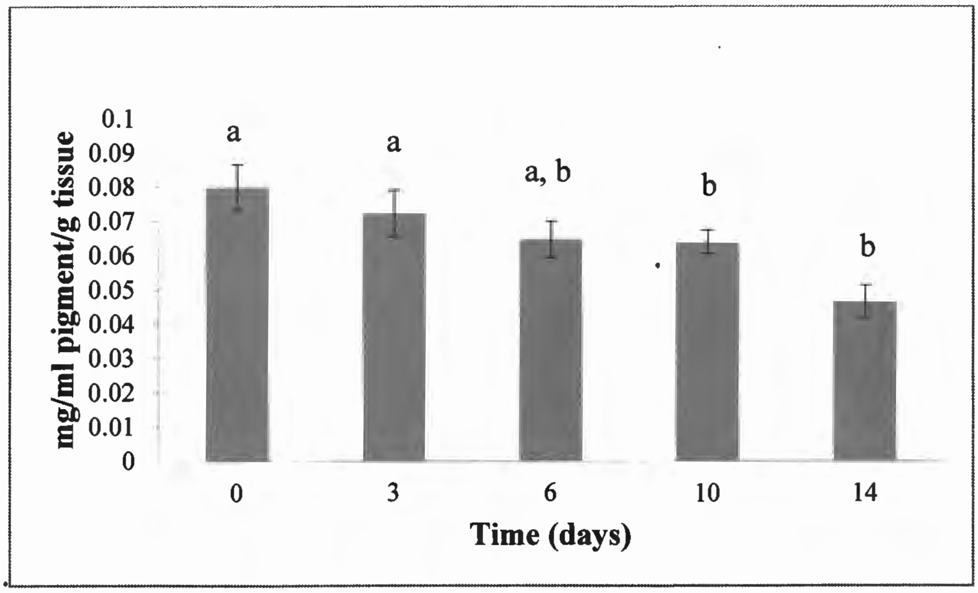

Figure 3.6 *G. gracilis* PC levels following exposure to N limiting growth conditions for 14 days. Data represents the mean ± standard error (n=4). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in PC levels of the *G. gracilis* samples

The changes in *G. gracilis* pigment in response to N recovery were smaller than those observed in response to N limitation, and profound visual changes in thallus colour were not observed. PE levels decreased from 0.41 ± 0.01 ml pigment/g tissue in N-sufficient *G. gracilis* to 0.35 ± 0.002 ml pigment/g tissue following 7 days exposure to N-limiting growth conditions. However, this decrease was not significant ($P>0.05$). Despite the re-supply of N in the form N-sufficient growth media, significant decreases ($P<0.05$) in PE levels were still observed following 6 and 12 hours of N recovery, indicating a possible lag period before PE levels were replenished (Fig. 3.7). PE levels increased gradually following 24 hours of N recovery until a concentration of 0.34 ± 0.02 ml pigment/g tissue was attained following 168 hours of N recovery. PC levels decreased significantly ($P<0.05$) from 0.08 ± 0.004 ml pigment/g tissue in N-sufficient *G. gracilis* to 0.06 ± 0.006 ml pigment/g tissue after 7 days of N limitation (Fig. 3.8). PC levels then gradually increased following N recovery reaching a concentration of 0.06 ± 0.001 ml pigment/ g tissue 7 days after N was re-supplied to *G. gracilis*.

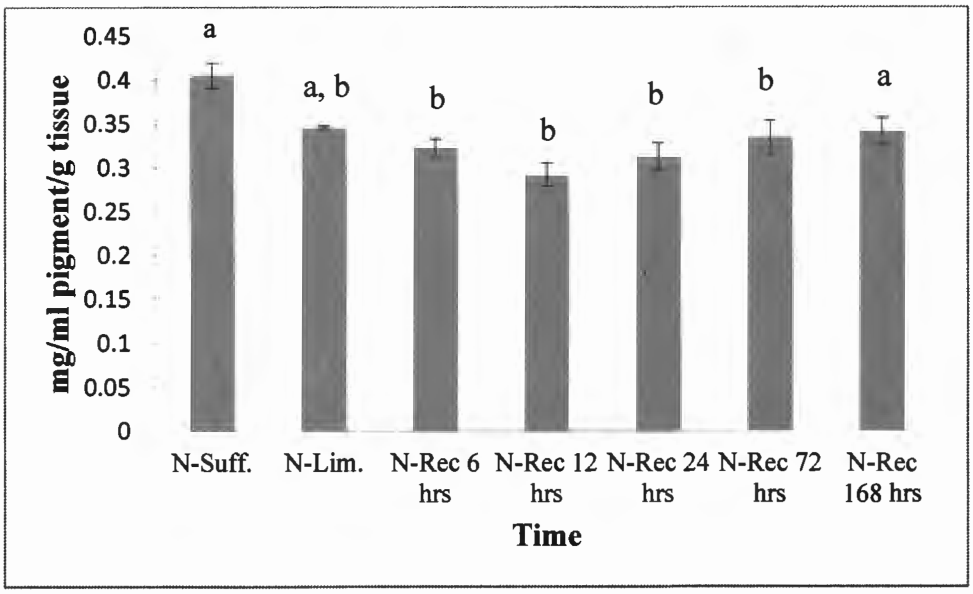

Figure 3.7 PE levels as determined for nitrogen-sufficient, nitrogen limited and nitrogen recovered *G. gracilis*. Data represents the mean ± standard error (*n*=3). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in PE levels of the *G. gracilis* samples.

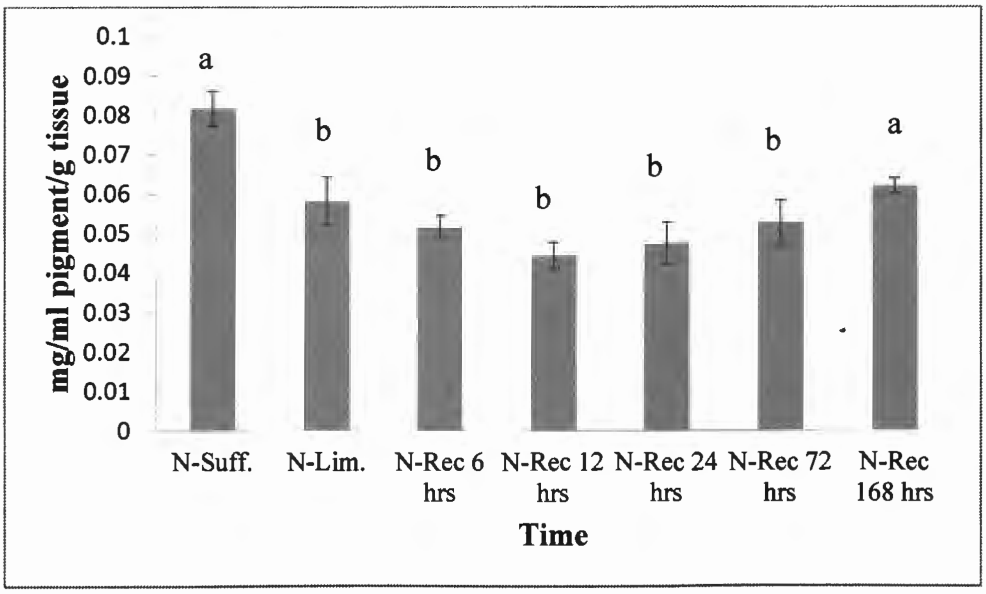

Figure 3.8 PC levels as determined for *G. gracilis* in response to 7 days of nitrogen recovery. Data represents the mean ± standard error (*n*=3). Different letters above the bars indicate significant differences ($P<0.05$, Holm-Sidak test) in PC levels of the *G. gracilis* samples

3.3.3 Validation of fructose 1,6 biphosphate aldolase (ALD) and glutamine synthetase (GS) protein identities

Western blot analysis was performed to validate the identity of ALD and GS, which were differentially regulated in response to N limitation and recovery in *G. gracilis*. Polyclonal antibodies raised to ALD and GS confirmed the identities of *G. gracilis* ALD and GS as identified by MS (Figs. 3.9 and 3.10). The expected MW for the ALD protein was 38 kDa. Thus, based on this information it was confirmed that the band indicated by the arrow is the correct band as evidenced by similar molecular weights for this band and protein spot 238 identified using LC MS/MS (Fig. 3.9A and B). The GS antibody detects two isoforms of GS, the chloroplastic and the cytosolic isoforms. The expected MW for GS1 (cytosolic isoform) is 39-40 kDa and for GS2 (chloroplastic isoform) is 44-45 kDa. Western blot analysis was conducted using a N-sufficient and N-limited *G. gracilis* sample following 1D SDS PAGE and a N-limited sample following 2D SDS PAGE (Fig 3.10A and B). GS was detected at the same MW on both the 1D and the 2D western blot images (Fig. 3.10A and B), with two bands evident in the N-limited *G. gracilis*. Although it is difficult to size the bands accurately, it is possible that these two bands represent the cytosolic and chloroplastic forms of GS. Only one GS band was detected in the N-sufficient *G. gracilis*. A 'train' of spots was detected with the 2D western of the N-limited *G. gracilis* sample. This usually indicates some form of post-translational modification as often 'trains' of spots are formed by multiple versions of the same protein that differ in isoelectric point (p*I*) due to increasing numbers of post-translational modifications such as phosphorylation or deamidation (Halligan, 2009).

3.3.4 ALD western blot analysis of ALD expression following nitrogen limitation and recovery in *G. gracilis*.

Prior to western blot analysis, total protein extracts from all *G. gracilis* samples obtained during the N limitation and recovery experiments (four and three biological repeats for N limitation and recovery, respectively) were assessed for protein integrity using SDS-PAGE. Isolated protein was of excellent quality with no signs of degradation (Figs. 3.11A and 3.13A) which ensured that the results observed in subsequent western blot analysis would not be compromised. The Coomassie stained protein gels (Figs. 3.11A and 3.13A) are representative of one biological repeat, however all biological repeat samples displayed the same protein quality and integrity. A polyclonal anti-ALD antibody was used to validate the expression pattern observed with 2-DE analysis during N limitation and recovery in *G. gracilis*. Following the protein quality check, 30 μg of *G. gracilis* protein from each time-point sample from the N limitation experiment was separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes for western blot analysis of the native *G. gracilis* ALD protein. Densitometry and statistical analysis was used to determine whether the amount of ALD protein changed significantly as a result of exposure to N-limiting conditions as well as N-recovery. ALD protein levels were observed to significantly decrease ($P<0.05$) following 14 days of N limitation (Fig. 3.11B and 3.12), validating the expression pattern observed previously using 2-DE analysis. The ALD protein level observed at day 14 was also significantly lower ($P<0.05$) than ALD protein levels at days 0, 3, 6 and 10 (Fig. 3.12).

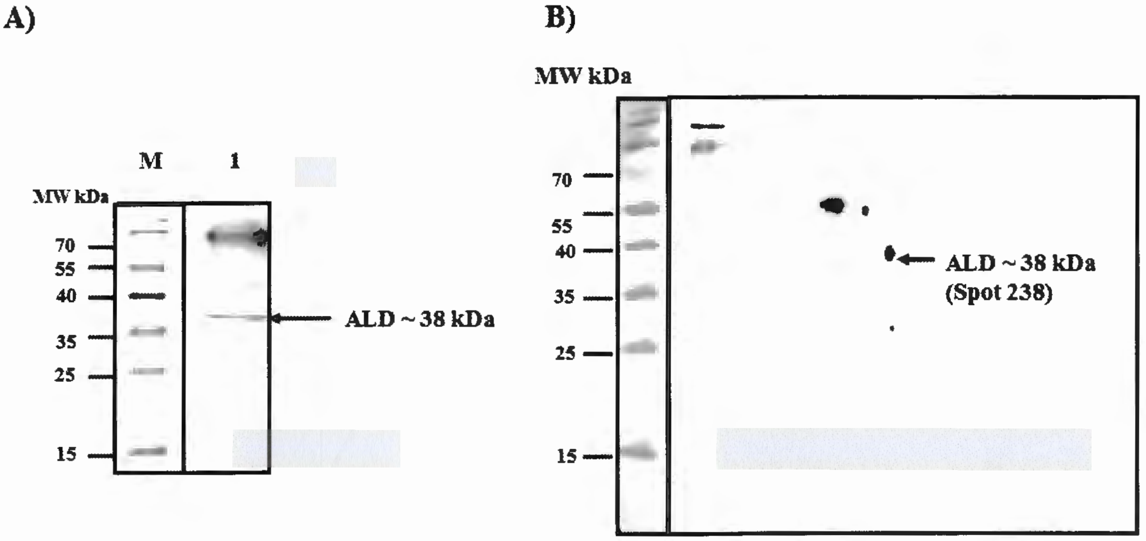

Figure 3.9 Validation of protein identity using western blot analysis with polyclonal ALD antibodies. Western blot results are shown exhibiting specific staining with anti-ALD antibodies following 1D SDS PAGE (A) and 2D SDS PAGE (B). A nitrogen sufficient *G. gracilis* sample was used for the 1D and 2D SDS PAGE analyses. Lane M represents the pre-stained protein molecular weight marker in both western blot images (Fermentas). The ALD protein and approximate molecular weight is indicated by the arrow.

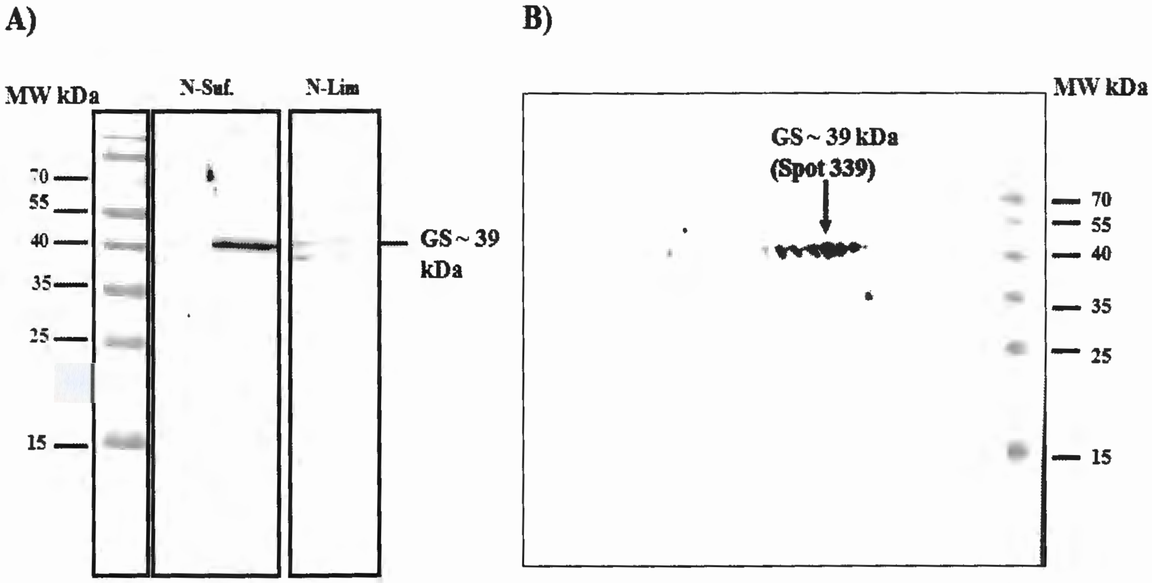

Figure 3.10 Validation of protein identity using western blot analysis with polyclonal GS antibodies by Western blot analysis. Western blot results are shown exhibiting specific staining with anti-GS antibodies following 1D SDS PAGE (A) and 2D SDS PAGE (B). A nitrogen-sufficient and nitrogen-limited *G. gracilis* sample was used for 1D SDS PAGE analysis. 2D SDS PAGE was conducted with nitrogen-limited *G. gracilis*. Lane M represents the pre-stained protein molecular weight marker in both western blot images (Fermentas). The GS protein and approximate molecular weight is indicated by the arrow.

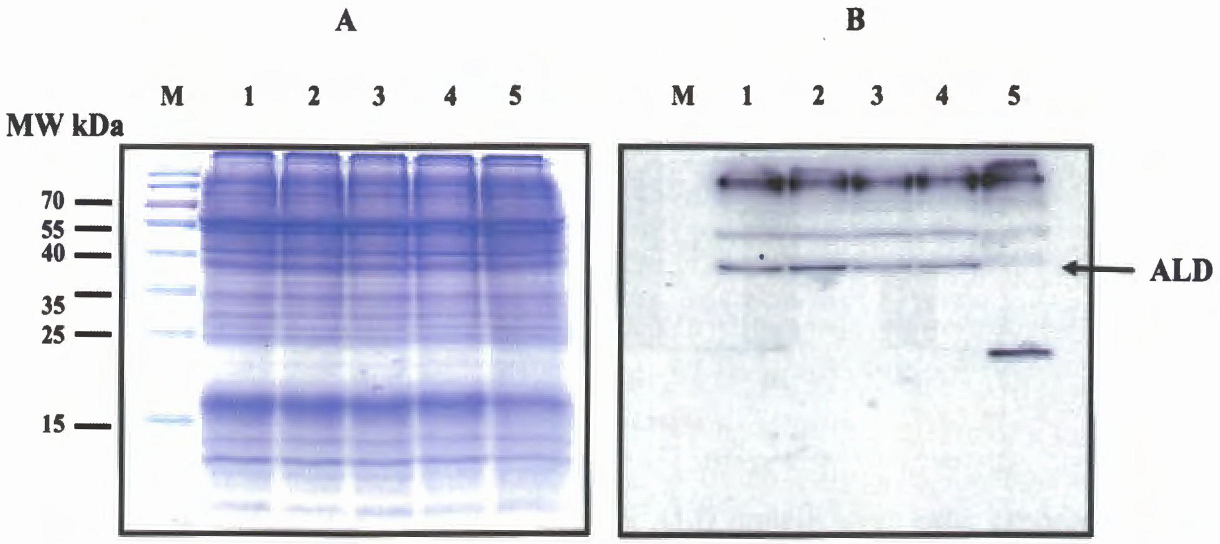

Figure 3.11 Coomassie stained protein gel (A) to establish the quality and integrity of *G. gracilis* total protein extracts from *G. gracilis* samples obtained during N-limitation. Thirty micrograms of total protein was mixed with 5X sample application buffer, boiled for 5 min, loaded into each lane and separated on a 12% SDS-PAGE. Lanes 1-5 represent the loading order of the total protein extracts obtained from *G. gracilis* samples collected at days 0, 3, 6, 10 and 14, respectively. Western blot analysis (B) of *G. gracilis* ALD protein levels following 14 days growth in N-limiting media. The loading order of *G. gracilis* samples was identical to that described for Panel A and a 1:5000 dilution of anti-ALD was used to detect the native *G. gracilis* ALD protein. Proteins were sized with a pre-stained molecular weight marker (Lane M, not visible with chemi-luminiscence).

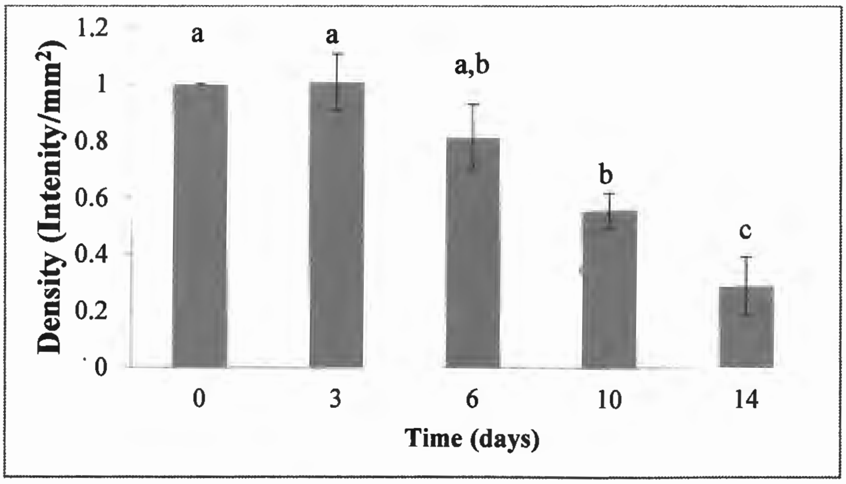

Figure 3.12 Quantitative analysis of *G. gracilis* ALD protein levels after exposure to N-limiting conditions, displayed as a mean densitometry reading. Data represents the mean ± standard error (*n*=4). Different letters above the bars indicate significant differences ($P<0.05$, Student Neumann Keuls test) in ALD protein levels of the *G. gracilis* samples during nitrogen limitation.

Western blotting was also used to investigate the effect of N recovery on ALD protein levels in *G. gracilis*. *G. gracilis* ALD protein levels had been observed to increase significantly ($P<0.05$) 6 hours after N was re-supplied to N-limited *G. gracilis* using 2-DE analysis (Chapter 2). However, the increase in ALD protein levels observed 6 hours after N recovery by western blot analysis were not significantly greater than the N-limited sample ($P>0.05$) following western blot analysis (Fig. 3.13B and 3.14). This lack of statistical significance could possibly be attributed to the variation observed between the biological repeats and since the change in expression is small, a larger number of biological repeats may be required before statistically significant differences become evident.

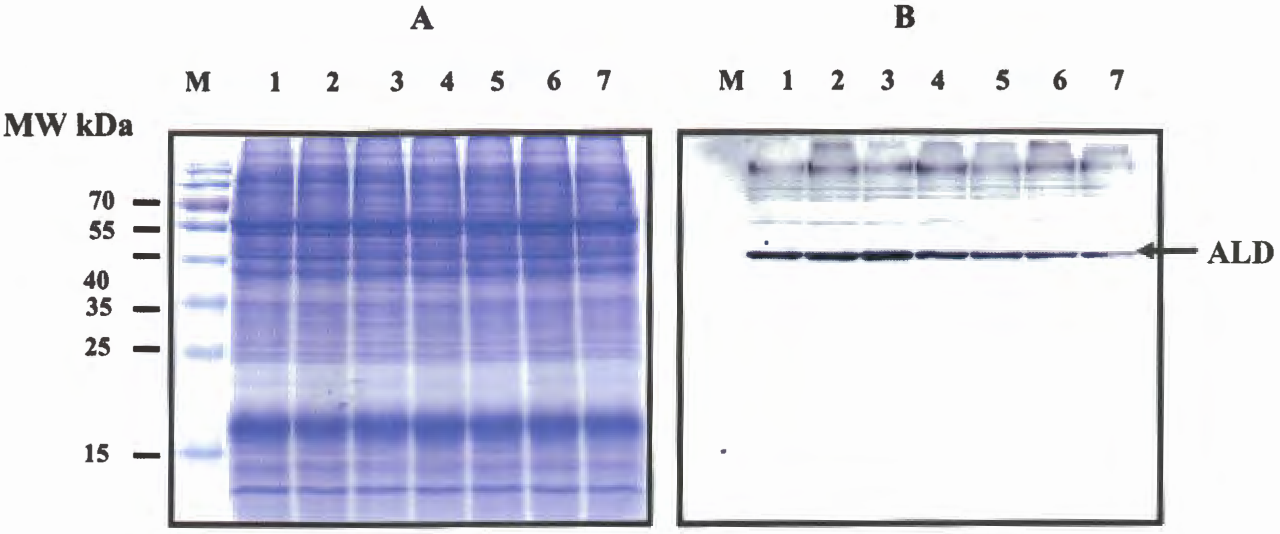

Figure 3.13 Coomassie stained protein gel (A) to establish the quality and integrity of *G. gracilis* total protein extracts from *G. gracilis* samples obtained during N-recovery. Thirty micrograms of total protein was mixed with 5X sample application buffer, boiled for 5 min, loaded into each lane and separated on a 12% SDS-PAGE. Lanes 1-7 represent the loading order of the total protein extracts obtained from *G. gracilis* samples. Lane 1 represents N-sufficient *G. gracilis* at day 0, Lane 2 represents N-limited *G. gracilis* and lanes 3-7 represent N-recovered *G. gracilis* at times 6, 12, 24, 72 and 168 hours, respectively. Western blot analysis (B) showing changes in *G. gracilis* ALD protein levels in N-sufficient, N-limited and N-recovered *G. gracilis*. The loading order of *G. gracilis* samples was identical to that described for panel. A. and a 1:5000 dilution of anti-ALD was used to detect the native *G. gracilis* ALD protein. Proteins were sized with a pre-stained molecular weight marker (Lane M, not visible with chemi-luminiscence).

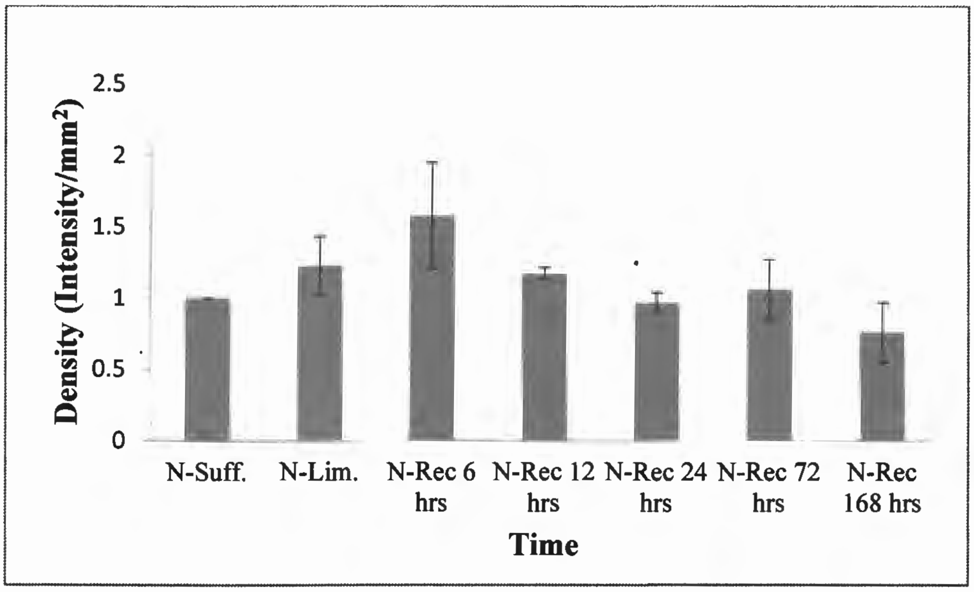

Figure 3.14 Quantitative analysis of *G. gracilis* ALD protein levels in response to N recovery, displayed as a mean densitometry reading Data represents the mean ± standard error (*n*=3).

3.3.5 Western blot analysis of GS expression following *G. gracilis* recovery from nitrogen limitation

A commercially available global GS antibody was used to validate the GS expression pattern observed by 2-DE (Chapter 2). The integrity and quality of the protein was deemed optimal and coomassie staining was used to demonstrate equal loading of protein samples (Fig. 3.15A). Western blot analysis revealed an increase in GS protein levels after 6 hours, however, as with ALD, this increase was not statistically significant (Fig 3.15B, 3.16).

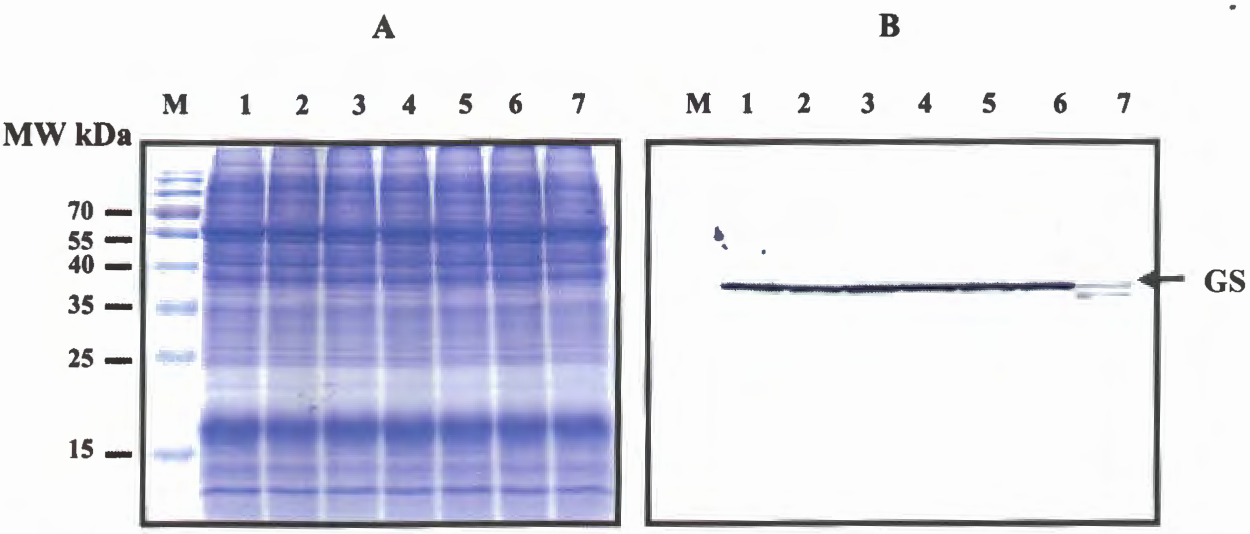

Figure 3.15 Coomassie stained protein gel (A) to establish the quality and integrity of *G. gracilis* total protein extracts from *G. gracilis* samples obtained during N-recovery. Thirty micrograms of total protein was mixed with 5X sample application buffer, boiled for 5 min, loaded into each lane and separated on a 12% SDS-PAGE. Lanes 1-7 represent the loading order of the total protein extracts obtained from *G. gracilis* samples. Lane 1 represents N-sufficient *G. gracilis* at day 0, Lane 2 represents N-limited *G. gracilis* and lanes 3-7 represent N-recovered *G. gracilis* at times 6, 12, 24, 72 and 168 hours, respectively. Western blot analysis (B) showing changes in *G. gracilis* GS protein levels in N-sufficient, N-limited and N-recovered *G. gracilis*. The loading order of *G. gracilis* samples was identical to that described for panel A and a 1:10000 dilution of anti-GS was used to detect the native *G. gracilis* GS protein. Proteins were sized with a pre-stained molecular weight marker (Lane M, not visible with chemi-luminiscence).

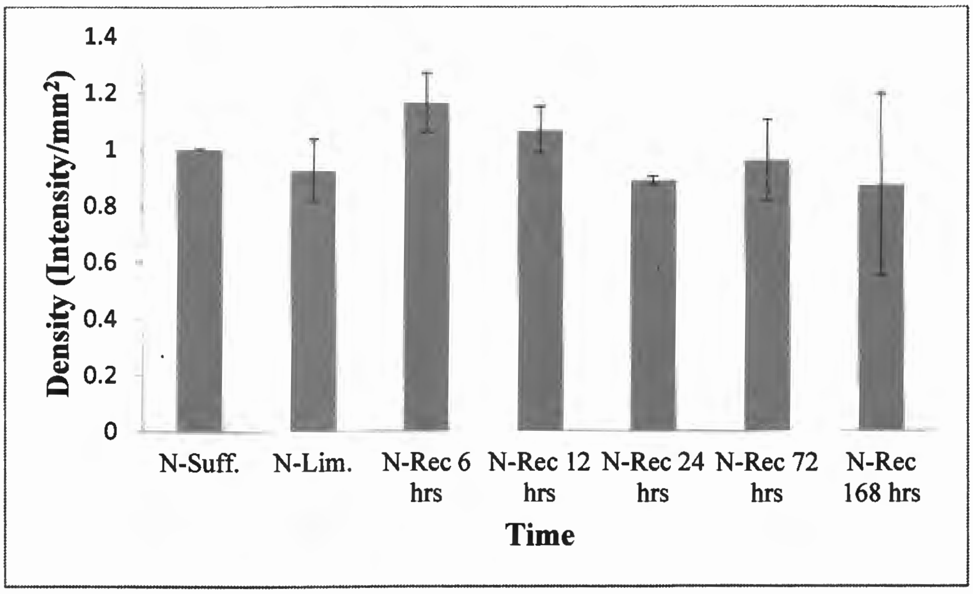

Figure 3.16 Quantitative analysis of *G. gracilis* GS protein levels in response to N recovery, displayed as a mean densitometry reading Data represents the mean ± standard error (*n*=3).

3.3.6 Fructose 1, 6 biphosphate aldolase (ALD) activity assays

In order to complement the western blot data, ALD enzyme activity in *G. gracilis* crude extracts obtained from samples collected during N limitation and recovery were assayed. ALD activity was measured in *G. gracilis* samples grown in N-lacking media for 14 days. ALD activity was observed to decrease significantly ($P<0.05$) in *G. gracilis* following 14 days of N limitation (Fig. 3.17). The decreased ALD activity observed following 14 days of N limitation was also significantly lower than the ALD activity levels observed at days 0, 3, 6 and 10 (Fig. 3.17). This data correlated well with what was observed with western blot and 2-DE analysis of N limitation in *G. gracilis*.

Similarly, *G. gracilis* ALD enzyme activity in response to N recovery was evaluated. It was observed that the ALD activity assays followed a similar trend as determined with western blot analysis, however the observed increase in ALD activity was not statistically significant (Fig. 3.18). The biological patterns determined using western blot and enzymatic analysis were virtually identical and may suggest that N recovery elicits small changes in ALD expression in *G. gracilis* and thus a greater number of biological replicates may be required before statistically significant changes are apparent.

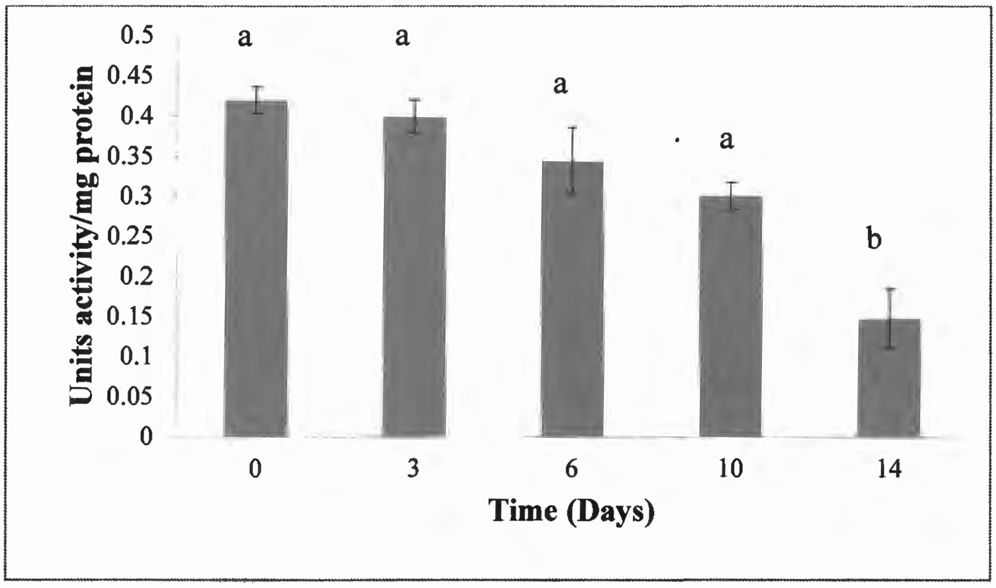

Figure 3.17 Changes in ALD activity in *G. gracilis* grown under N-limiting conditions for 14 days. Data represents the mean ± standard error (*n*=3). Different letters above the bars indicate significant differences (P<0.05; Holm-Sidak test) in ALD activity levels of the *G. gracilis* samples during nitrogen limitation.

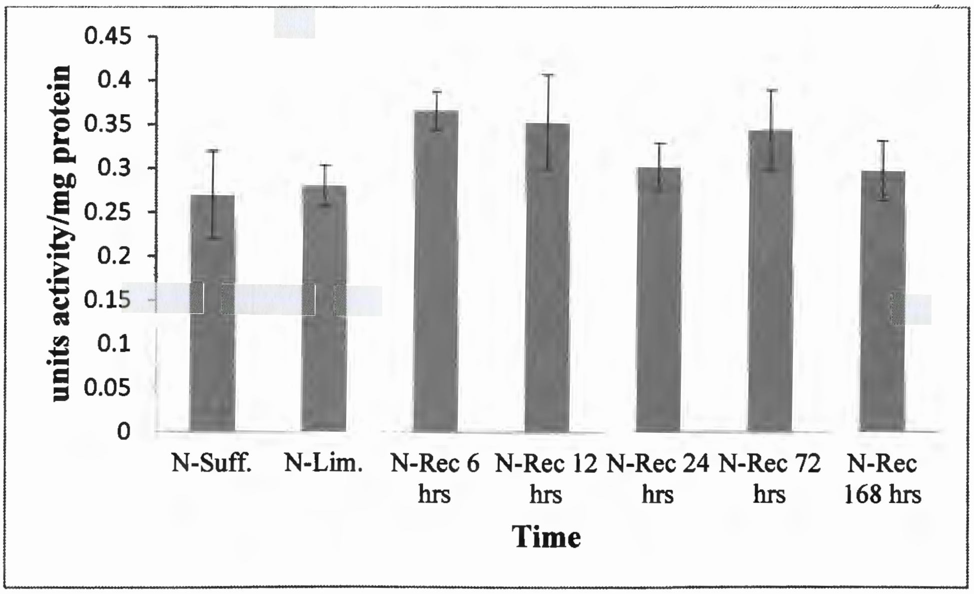

Figure 3.18 Quantitative changes in ALD activity levels of *G. gracilis* samples following N recovery. Data represents the mean ± standard error (*n*=3).

3.3.7 Pyruvate kinase (PK) activity

Since PK is an important enzyme that integrates C and N metabolism, the effect of N limitation and recovery on PK activity was assessed in *G. gracilis*. G. *gracilis* PK activity levels displayed no significant changes (P>0.05) in response to N limitation (Fig. 3.19). Although, there was a slight increase in PK activity at day 3 from 0.49 ± 0.07 to 0.63 ± 0.03 mM pyruvate/mg protein/min, this change was not significant (P>0.05).

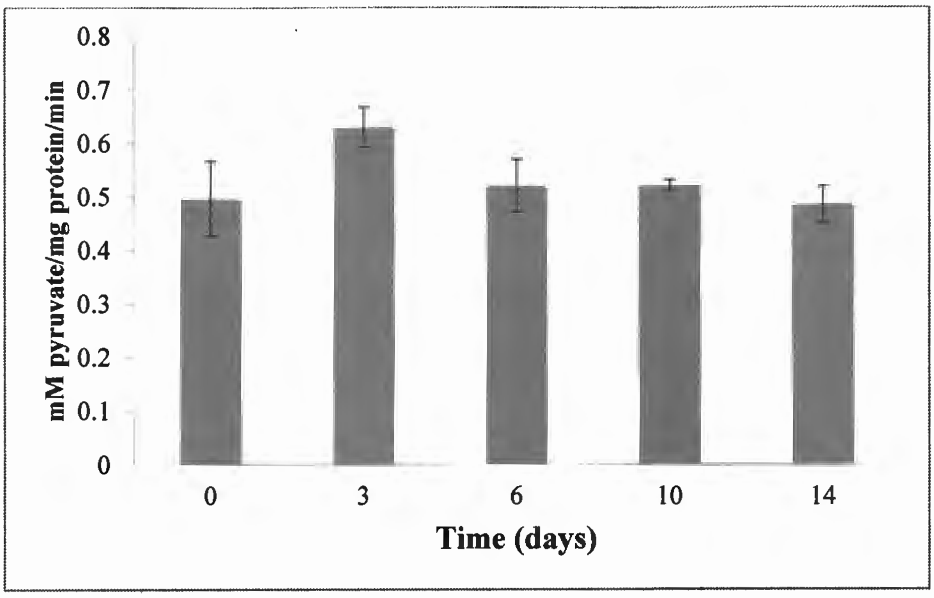

Figure 3.19 *G. gracilis* PK activity changes in response to 14 days of nitrogen limitation. Data represents the mean ± standard error (n=3).

PK activity levels did not change significantly (P>0.05) in response to 6 hours of N recovery in *G. gracilis* (Fig. 3.20). PK activity decreased from 0.56 ± 0.06 at day 0 to 0.44 ± 0.03 mM pyruvate/mg protein/min after 12 hours of recovery and then increased to a maximum value of 0.62 ± 0.03 pyruvate/mg protein/min 72 hours after recovery (Fig. 3.20). The trend does show a slight decrease in response to N limitation with a subsequent increase in activity being observed upon the addition of N. This increase is only observed 24 hours, 3 days and 7 days post recovery.

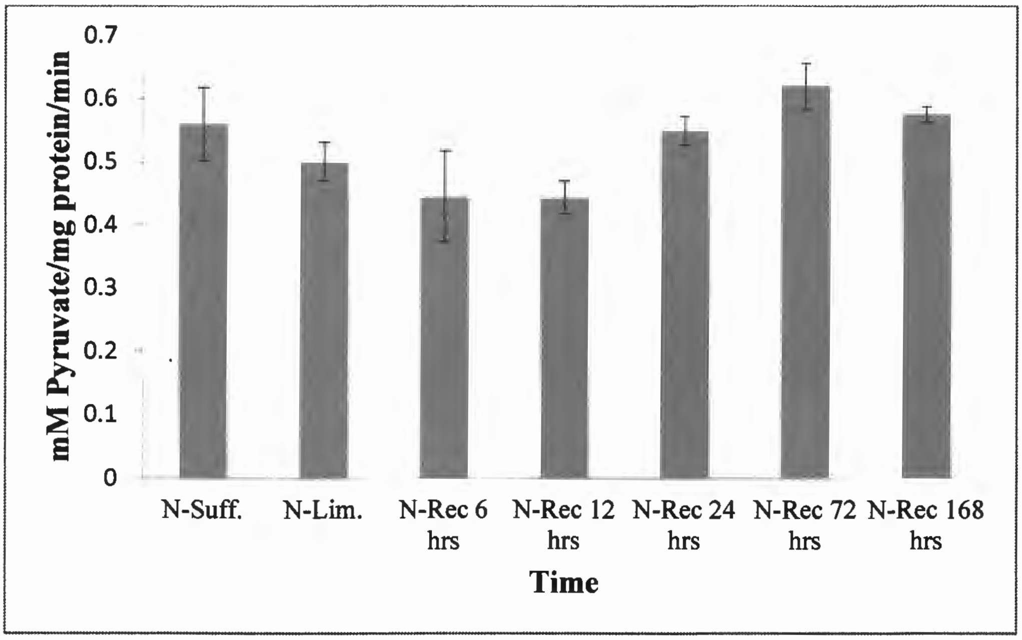

Figure 3.20 *G. gracilis* PK activity changes in response to 7 days of nitrogen limitation and 7 days of nitrogen recovery Data represents the mean ± standard error (*n*=3).

3.3.8 Intracellular starch accumulation in *G. gracilis* following nitrogen limitation

Starch accumulation in *G. gracilis* was investigated under N limiting conditions as starch is known to accumulate in algae experiencing prolonged N limitation. Increased starch levels were observed in the N-limited *G. gracilis* as compared to the N-sufficient *G. gracilis* (Fig. 3.21). However, this difference was not statistically significant due to the large degree of variability between the biological repeats. Despite the lack of statistical significance, there is a marked increase in starch levels (~ 7 fold) at day 14 (Fig. 3.21).

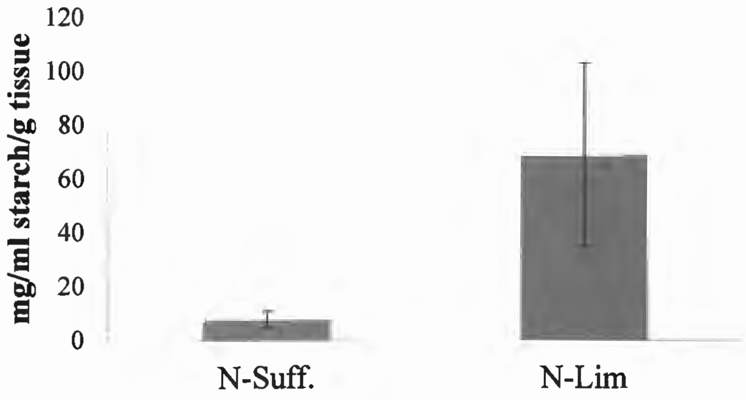

Figure 3.21 Intracellular starch level in nitrogen-sufficient *G. gracilis* and nitrogen-limited *G. gracilis* following 14 days of nitrogen limitation. Data represents the mean ± standard error (n=3)

3.4 Discussion

Proteomics was employed in this study to investigate changes in protein abundance in *G. gracilis* during N limitation and recovery. Moreover, proteomic approaches using 2-DE and MS technologies have been successful in generating quantitative protein expression data on a scale and sensitivity comparable to that achieved at the genetic level (Wu *et al.*, 2002). However, it is important to validate the expression pattern of the identified proteins using an alternative technique such as western blots (Wu *et al.*, 2002; Kageyama *et al.*, 2004; Ingle *et al.*, 2007). Western blots have been used to successfully validate 2-DE and MS results in cancer, cardiovascular, Alzheimer's and nephrolithiasis studies in humans (Chutipongtanate *et al.*, 2005; Wang *et al.*, 2005; Shen *et al.*, 2006; Eisenhardt *et al.*, 2011). Thus, western blot analysis was used to validate the identities of ALD and GS detected by 2-DE and MS (Chapter 2). Following validation the proteins were characterised further using a combination of western blotting and enzymatic analysis to evaluate their response to N limitation and recovery over time.

The N status of *G. gracilis* samples collected during the N limitation experiment was assessed by measuring the intracellular C and N levels as well as C:N ratios. These physiological measurements indicated that *G. gracilis* samples were N-limited following 14 days of culture in media lacking N when compared to the N-sufficient control samples at day 0. The intracellular N levels were significantly decreased ($P<0.05$) after 14 days of N limitation. However, the N content (2.1%) of the N-limited samples was not as low as was previously observed during the 2-DE N limitation experiments (Section 2.3.1.1). This reinforces the fact that significant biological variation occurs between different batches of seaweed and thus the nutritional status of the seaweed is a variable factor that is influenced by culture conditions at the Irvin & Johnson Abalone Culture Division. Therefore, each batch of seaweed was acclimatised for two weeks prior to the initiation of experiments to partly overcome this problem; however, it is clear that the nutritional status of each batch of seaweed may still vary considerably. Samples taken at day 18 showed that the intracellular N levels had decreased to 1.7% (data not shown). However, for the purposes of consistency and comparison to 2-DE data, the conditions and sampling points were kept the same. The C:N ratio was also significantly increased ($P<0.05$) following 14 days of N limitation when compared to the N-sufficient time-course control and correlated well with the N data.

As with the 2-DE N limitation experiments, profound visual changes in *G. gracilis* thallus colour was observed with the thallus colour changing from reddish-brown to greenish-brown. Changes in thallus colour in response to changes in N content of cultured seaweeds have been firmly established for a range of seaweed species. These include *Gracilaria spp.* (Lapointe & Ryther, 1979; Wilson, 1999), *Chondrus crispus*(Linnaeus) J. Stackhouse (Haxo and Strout, 1950; Neish & Shacklock, 1971; Harvey & McLachlan, 1973; Mackenzie, 2003) and *Ulva rigida* C. Agardh (Lahaye *et al.*, 1995). Recently, the relationship between seaweed N content and thallus colour in *G. gracilis* was successfully quantified (Robertson-Andersson *et al.*, 2009). Thus, decreases in the concentration of PE and PC are indicative of N limitation in seaweed species. Significant declines ($P<0.05$) in *G. gracilis* PE and PC pigment levels were observed following 14 days of growth in N-lacking media. This data, combined with the N content and C:N ratios demonstrate that *G. gracilis* was sufficiently N-limited at day 14 when compared to the N-sufficient time-course control.

In this study a cytosolic ALD, a key glycolytic enzyme, was shown be to be down-regulated in response to N limitation in *G. gracilis*. The effect of abiotic stressors such as O_2 deprivation, salt, desiccation, low temperature and high temperature stress on glycolytic enzymes has been well studied at the transcriptional level in rice (*Oryza sativa*) (Minhas & Grover, 1999). These studies have demonstrated that changes in gene expression patterns of glycolytic enzymes enable plants to adjust to the increased energy demand and supply of intermediates for acclimation to environmental stress conditions (Plaxton, 1996; Minhas & Grover, 1999, Purev *et al.*, 2008). In this study, *G. gracilis* ALD protein has been implicated in the N limitation stress response. Western blot analysis using polyclonal ALD antibodies confirmed the putative identity of the *G. gracilis* ALD protein as identified by MS. Polyclonal antibodies were subsequently used in western blot analysis to quantitate the levels of native ALD proteins in complex *G. gracilis* protein samples in response to N limitation. Western blot analysis showed that *G. gracilis* ALD protein levels were significantly decreased ($P<0.05$) following 10 and 14 days of N limitation when compared to N-sufficient control samples. Similarly, *G. gracilis* ALD activity was significantly decreased ($P<0.05$) following 14 days of N limitation compared to *G. gracilis* ALD activity at days 0, 3, 6 and 10. Furthermore, these results validate the protein expression pattern determined for *G. gracilis* ALD using 2-DE analysis.

The observed changes in ALD protein and activity levels in response to N limitation appear to be part of the stress adaption process in *G. gracilis.* Additionally, decreases in PGK and GAPDH protein levels were also detected by 2-DE studies in *G. gracilis* during N limitation (Chapter 2). While ALD may appear to be an unremarkable protein as it has no known regulatory properties or discernible effect on growth and metabolism, it is known to be regulated in response to a wide variety of abiotic stressors. ALD has been shown to respond to anoxia, oxidative, hyperosmotic, light, dark, wounding and chilling stress in *Codonopsis lanceolata* (Purev *et al.*, 2008). Moreover, both the cytosolic and chloroplastic isoforms play important roles in carbohydrate metabolism, the production of triose phosphates and signal transduction (Schaeffer *et al.*, 1997). Konishi *et al.* (2004) demonstrated the important role ALD plays in root elongation in rice plants and suggested that changes in protein and activation states of ALD may play a role in plant growth mainly via stimulation of the glycolytic pathway with a subsequent net gain of ATP (Konishi *et al.*, 2004). In rice roots treated with gibberellins, a regulator of cell elongation, ALD levels were shown to increase at both the mRNA and protein levels. This accumulation of ALD suggests that it is involved in tissue growth and differentiation triggered by gibberellins. This was further confirmed with ALD-antisense transgenic rice plants which had reduced ALD levels and showed a significant decrease in root length (half the length of the controls root length) (Konishi *et al.*, 2004). A similar result was obtained with ALD activity studies (Konishi *et al.*, 2004). These findings propose a role for ALD in root growth and demonstrate the importance of this enzyme in enhancing the metabolic rate of glycolysis. Given these findings and the decrease in ALD protein and activity levels observed in response to N limitation in *G. gracilis*, it may be possible to use ALD to monitor the health status of *G. gracilis* in commercial farm settings. A decline in ALD protein and activity levels could possibly indicate a decrease in growth rate, photosynthetic capacity and intracellular N levels. However, since significant declines ($P<0.05$) in ALD protein levels are only observed after 10 days of N limitation, it may be necessary to identify a biomarker which responds earlier to N stress. Alternatively, it may be necessary to use a suite of biomarkers. For instance it may be that a combination of ALD, GAPDH and PGK may prove effective for monitoring the nutritional status of *G. gracilis*. However, these proposals would need to be investigated further with western blot studies conducted on a range of glycolytic enzymes, and the ALD gene would need to be cloned and sequenced.

N recovery of N-limited *G. gracilis* was evaluated over a 7 day period prior to western blot analysis of ALD and GS. Intracellular C and N levels, as well as C:N ratios, were determined to assess the N status of N-sufficient, N-limited and N-recovered *G. gracilis*. *G. gracilis* samples were observed to be N-limited relative to the N-sufficient control following growth in N-lacking media for seven days. In concordance with this, the N percentage decreased significantly ($P<0.05$) from 2.87±0.17% to 1.26±0.14% after 7 days of N-limitation. N-re-supply resulted in a gradual increase in N levels, with a maximum N level of 1.72% attained at 72 hours post recovery. The same trend was observed with the C:N ratios. The increased C:N ratio following 7 days of N limitation indicated that *G. gracilis* was N limited at this point. Although the C:N ratio decreased following N recovery after 24, 72 and 168 hours, *G. gracilis* did not completely recover as N levels did not return to those observed in the N-sufficient control.

The PE and PC pigment levels changed significantly ($P<0.05$) in response to N recovery. However, these did not follow the same trend as the N and C:N ratio data. Instead, an initial decline in PE levels was observed, with the greatest decrease occurring at 12 hours post-recovery. PE levels then increased gradually until 7 days post recovery, when they reached a level that was comparable to pigment levels observed at day 0. PC levels decreased significantly ($P<0.05$) after 7 days of N limitation. Again, the biggest decrease in PC levels was observed 12 hours post recovery, followed by a gradual increase up to 7 days post-recovery when PC levels returned to those observed at day 0. It should be mentioned that differences in the nutritional status of the seaweed were observed between seaweed samples included in this experiment and those used for the N limitation experiment. Intracellular N content of *G. gracilis* samples at the start of the N recovery experiment was 2.87% as opposed to 4.4% for the N limitation experiment samples, resulting in the algae becoming N limited at a faster rate. Although the experimental set-up and conditions for each experiment were the same, we had limited control over the starting quality of the algae which is an important factor to consider when interpreting the data. Therefore, given that the seaweed became N limited after 7 days, the seaweed never recovered completely over the course of the experiment, and thus a longer recovery period may have been required.

The effect of N-recovery on *G. gracilis* ALD and GS protein levels was investigated using western blot analysis. Significant increases ($P<0.05$) in GS and ALD protein levels were observed in *G. gracilis* after 6 hours of N recovery. Western blot analysis was employed to validate the observed protein expression pattern. However, the associated changes were small and no significant (P>0.05) changes in ALD or GS protein levels were observed following 6 hours of N recovery in *G. gracilis*. A similar result was obtained with the ALD activity assay with a non-significant (P>0.05) increase in activity being observed. These results were unexpected in light of the large decrease in N content after 7 days of N limitation; indeed, a larger response was expected upon the re-supply of N. Furthermore, these results do not correlate with the significant changes in ALD and GS protein abundance observed with 2-DE studies. It is, however, possible that these proteins responded earlier than expected to a re-supply of N. As previously discussed, gene expression changes in response to nitrate re-supply occur as early as 30 minutes after nitrate addition in *Arabidopsis* (Wang *et al.*, 2000, 2003; Scheible *et al.*, 2004). Scheible *et al.* (2004) observed induction of a set of genes, including enzymes such as PK and enolase that function in the latter part of glycolysis and organic acid metabolism, as early as 30 minutes after nitrate-re-supply. Similar results were obtained with a previous study conducted on *Arabidopsis* shoots and roots using microarray analysis. Here, the response to the addition of nitrate to plants with an exhausted N supply was investigated after 20 min (Wang *et al.*, 2003). Similarly, induction of genes involved in glycolysis, such as phosphoglycerate mutase and Glc-6-phosphate isomerise, was also observed. Thus, it is clear that glycolytic enzymes in plants respond rapidly at the transcriptional level to a re-supply of nitrate. Similarly, differential changes in the abundance of glycolytic enzymes, such as PGK and ALD, have been observed in response to ammonium in barley shoot proteomes (Møller *et al.*, 2011).

Genes involved in nitrate assimilation and transport, such as NR, are strongly induced at the transcriptional level in response to nitrate. However, genes involved in ammonium assimilation, such as GS and GOGAT, are often induced at much lower levels (Wang *et al.*, 2000 & 2003). Li *et al.* (2006) demonstrated this by investigating the effect of N recovery in rice and observed that regulated *gs* gene expression in N-deprived rice seedlings was unaffected by nitrate, ammonium and a combination of nitrate and ammonium treatments. However, GS activity was observed to increase in response to all three treatments. Furthermore, a significant increase in GS activity was observed for 4 days following re-supply of nitrate and ammonium (Li *et al.*, 2006). Similar results were obtained with GS activity studies in *G. gracilis* exposed to nitrate, ammonium and combined nitrate and ammonium treatments (Reddy, unpublished data). While GS enzyme activity was found to increase in response to these treatments, statistically significant changes in transcript levels were not observed (Reddy, unpublished data). These studies therefore suggest that N re-supply (in the form NO_3^-, NH_4^+ or NH_4NO_3) in plants and algae elicits small changes in *gs* gene expression. Although, mRNA and protein studies are often not well correlated, it appears that N recovery in *G. gracilis* elicits small changes in GS protein abundance. These small changes in protein abundance combined with the fact that N recovery has been observed to elicit small changes in *gs* expression may indicate that GS is post-translationally regulated at the activity level. For instance, GS activity is known to be regulated by phosphorylation and 14-3-3 proteins in plants (Finnemann & Schjoerring, 2000; Man & Kaiser, 2001; Riedel *et al.*, 2001) and the green alga *Chlamydomonas reihardtii* (Pozuelo *et al.*, 2001). Furthermore, the 'train' of protein spots observed on the GS 2D western blot may be indicative of protein phosphorylation; however this cannot be confirmed without further investigation.

The small changes in ALD and GS protein abundance in response to N recovery appear to be insignificant. The biological importance of small changes in the expression of individual genes is often not apparent. However, when these genes are grouped into functional categories these changes gain significance (Scheible *et al.*, 2004). This was well demonstrated in *Arabidopsis* with nitrate re-supply and subsequent data analysis with MapMan (Scheible *et al.*, 2004). The individual changes in gene expression patterns for genes involved in amino acid synthesis and breakdown, and protein synthesis were relatively small, however these changes became meaningful when grouped into functional categories (Scheible *et al.*, 2004). While this study was conducted at the transcriptional level, it is possible that similar effects would be observed with protein studies. Functional characterisation studies could not be conducted in this study due to the small number of proteins identified. However, if further studies are conducted with glycolytic enzymes, such as GAPDH and PGK (identified during this study), and small changes are consistently observed, then it may be that it is the combined action of these enzymes which allow metabolic adaption to N recovery.

A number of glycolytic proteins were observed to significantly change in abundance in *G. gracilis* during N limitation and recovery. Thus, it was postulated that N limitation and recovery may have an effect on PK, an important enzyme integrating C and N metabolism. There were no suitable commercially available antibodies to test this theory and therefore enzyme activity was assayed. *G. gracilis* PK activity was mostly unaffected by N limitation over a period of 14 days. The observed changes in enzyme activity were small and non-significant ($P>0.05$). Since, the *G. gracilis* samples were not fully N limited at day 14 (N content: 2.1%), it is possible that PK activity levels may only decrease significantly ($P<0.05$) when the algae reaches the critical N content of 1.7% or lower. Previous published studies have found that PK activity and transcript levels may remain unchanged in response to environmental stress conditions. PK transcript levels remained unchanged in *Chlamydomonas reinhardtii* during N deprivation (Miller *et al.*, 2010). Similarly, PK activity in bean plants deficient in P remained constant, despite an accumulation of pyruvate. Instead, an increase in the activities of PEPc, PEP (phosphoenolpyruvate) phosphatase and NAD malic enzyme (NAD-ME) (Juszczuk & Rychter, 2002) were observed. Furthermore, PK is expected to remain active at the beginning of leaf senescence when other proteins such as PEPc are already being degraded (Streit & Fuller, 1983). This may be due to the involvement of PK in the synthesis of amino acids and proteins required for adaption to adverse stress conditions. Thus, it may be important to investigate the activities of PEPc, PEP phosphatase and NAD-ME in *G. gracilis* during N limitation before accurate conclusions regarding the involvement of PK in the N limitation stress response can be made.

The PK activity of *G. gracilis* did not change significantly ($P>0.05$) during N recovery. PK activity changes followed a similar biological pattern to that observed with PE and PC, but differed to the biological pattern determined for ALD activity. PK activity decreased gradually until 12 hours post-recovery, where after activity levels increased steadily to levels comparable to those observed at day 0. As previously discussed, glycolytic enzymes exhibit increased expression in response to a re-supply of nitrate, and PK has been shown be induced in *Arabidopsis* in response to nitrate addition (Scheible *et al.*, 2004). Furthermore, the re-supply of N to N-limited algae is known to activate a range of processes leading to increased synthesis of organic acids in the TCA cycle. Subsequently, these organic acids are used in amino acid and protein synthesis and as PK is found at the end-point of glycolysis, increased activity levels were expected. Although accurate conclusions cannot be drawn regarding the regulation of PK, due to the lack of statistically significant changes in activity, it is of interest that the PK pattern of enzyme regulation differs to that determined for ALD. This may suggest that PK responds differently to N recovery when compared to other glycolytic enzymes. However, as mentioned previously PK is not the only enzyme involved in the synthesis of organic acids and the small changes in activity observed here may implicate the involvement of other enzymes, such as PEPc or NAD-ME, in the synthesis of organic acids (Juszczuk & Rychter, 2002). PEPc in particular is involved in the regulation of organic acid synthesis and thus N recovery may have more pronounced effects on PEPc enzyme activity. Furthermore, PK was not detected with 2-DE in either the N limitation or recovery experiments. However, not all of the proteins that were differentially expressed were sequenced and thus it is possible that PK may be amongst one of these proteins. Finally, it may be useful to conduct a metabolite analysis to measure the increase or decrease of pyruvate as well as PEP in response to N recovery and N limitation. A decrease in PEP levels with unchanged PK activity would implicate the involvement of other enzymes, such as PEPc, in N assimilation.

The effect of N limitation on C partitioning in *G. gracilis* was briefly investigated by measuring starch accumulation. It has been well established that N limitation causes shifts in C metabolism which favour carbohydrate accumulation (Robinson, 1997). Specifically, cells use a higher percentage of C skeletons for the synthesis of starch as opposed to amino acid and protein synthesis (Robinson, 1997). The decreased protein levels of enzymes involved in glycolysis suggest that a shift in C partitioning has occurred in *G. gracilis* in response to N limitation. Although starch levels increased sharply (~7 fold) in *G. gracilis* during N limitation, these increases were not statistically significant (P>0.05). This lack of statistical significance is likely due to the large degree of biological variation observed. However, the trends for each individual biological repeat (data not shown) were consistent and marked increases in starch levels in response to N limitation were observed with each individual biological repeat data set. This increase in starch levels is of biological significance as starch is thought to function as a C storage compound under unfavourable stress conditions on both a short- and long-term basis (Collén *et al.*, 2004). Short-term storage provides plants with a store of carbohydrates that can be used to maintain metabolic functions when photosynthesis is inactive (Plaxton & McNamus, 2006). Longer term storage usually occurs in tubers and seeds to support reproductive tissue development (Plaxton & McNamus, 2006). Although, the significance of starch accumulation and the regulation of starch synthesis and degradation have not been well investigated in red algae (Turpin, 1991; Huppe & Turpin, 1994), it is evident that starch accumulation functions as a store of carbohydrates in *G. gracilis* during N limitation.

In conclusion, the identity of *G. gracilis* ALD and GS and the expression pattern determined for ALD during N limitation using 2-DE and MS were validated. *G. gracilis* ALD protein and activity levels decreased significantly ($P<0.05$) in response to N limitation. Moreover, the ALD western blot and activity assays correlate well for the N limitation experiment, and combined with starch data, suggests that a shift in C partitioning has occurred in *G. gracilis* in response to N limitation. This observation is further strengthened when combined with the decreases in GAPDH and PGK protein levels determined with 2-DE analysis (Chapter 2) and reinforces the fact that the cytosolic network of glycolytic enzymes plays an important role in stress adaption processes. This, combined with the fact that these proteins are often multifunctional, and thus involved in processes other than carbohydrate metabolism, make these proteins extremely useful in predicting and monitoring stress responses in *G. gracilis* (Plaxton, 1996). Thus, the use of ALD in combination with other glycolytic enzymes could be useful in monitoring the nutritional status of *G. gracilis* in a commercial farm setting. Although, *G. gracilis* ALD and GS protein levels were slightly increased, but not statistically significant, in response to N recovery the observed biological trends were consistent and similar to the results obtained with ALD activity assays suggesting that N recovery in *G. gracilis* elicits small changes in protein and activity levels. PK enzyme activities did not change significantly ($P>0.05$) during N recovery, suggesting that the PK route of pyruvate synthesis may be bypassed during the initial stages of N assimilation in *G. gracilis*. Finally, while the effect of N limitation on C allocation patterns was briefly assessed in this study, future work should investigate the changes in floridoside and agar levels in response to N limitation in *G. gracilis*. This would complement the existing data and provide a more comprehensive overview of C partitioning in *G. gracilis* in response to N limitation.

CHAPTER 4

Conclusions and Future prospects

4.1 Conclusions and Future Prospects

Intertidal macroalgae such as *G. gracilis* exist in unfavourable marine environments and are routinely exposed to potentially stressful environmental conditions such as nutrient limitation, high light, temperature stress, desiccation and osmotic stress (Davison and Pearson, 1996). Nutrient limitation was of particular interest in this study, and more specifically nitrogen (N) limitation, as it is a major stress factor affecting South African *G. gracilis* seaweed populations. The physiological mechanisms underlying the adaption of *G. gracilis* to temporally variable N environments have been investigated; however the molecular basis of these adaptions has not been well studied in *G. gracilis*. Thus, a proteomics approach was chosen to investigate the molecular mechanisms which underlie *G. gracilis* adaptions to N stress. The field of differential expression proteomics has advanced rapidly in recent years and has been used to investigate stress responses in a range of important plant species including *Oryza sativa*, *Arabidopsis thaliana* and *Zea mays* (Jorrin-Novo *et al.*, 2009). For this study, a differential proteomics approach involving the use of two-dimensional gel electrophoresis (2-DE) and tandem mass spectrometry (MS/MS) was employed to investigate global protein expression changes in response to N availability. While the effect of N limitation and recovery has been extensively investigated in plants, very little is understood regarding the molecular mechanisms employed by macroalgae under these conditions. Thus, this study aimed to identify proteins and the associated pathways which are activated or repressed in response to N limitation and subsequent recovery in *G. gracilis*.

In Chapter 2, 2-DE analysis was employed to detect differential changes in protein abundance in response to N limitation and recovery in *G. gracilis*. Given that the nutrient signalling response has been suggested to occur in two distinct phases, an early and late response, the responses of *G. gracilis* to N limitation was further dissected and the effect of an initial and a sustained period of N limitation on *G. gracilis* protein profiles was assessed. Following 2-DE, Melanie 2D analysis software was used to identify proteins differentially regulated in response to N limitation and recovery in *G. gracilis*. A total of 41 proteins displayed differential changes in protein abundance in response to an initial period of N limitation in *G. gracilis* with 26 proteins increasing and 15 proteins decreasing significantly ($P<0.05$) in abundance. A similar number of proteins were found to be differentially regulated in response to a sustained period of N limitation with the majority of these proteins displaying decreased abundance as compared to the initial response where the majority of proteins were up-regulated. A total of 44 proteins were differentially regulated in response to a sustained period of N limitation with 12 proteins increasing and 27 proteins decreasing significantly ($P<0.05$) in abundance. In contrast, the *G. gracilis* proteome reacted significantly to N recovery with a total of 66 proteins differentially regulated in response to N recovery with 50 proteins increasing and 8 proteins decreasing significantly ($P<0.05$) in abundance. Following 2-DE analysis, eight proteins from each of the N limitation experiments and six proteins from the N recovery experiment, were sequenced using tandem MS. Thus, a total of 22 *G. gracilis* proteins which responded to changes in N availability were successfully identified using LC MS/MS protein sequencing in this study. The identified proteins functioned in a range of biological processes including glycolysis, photosynthesis, ATP synthesis, galactose metabolism, protein-refolding and biosynthesis, N metabolism and cytoskeleton remodelling. Although there was a large amount of overlap between the biological processes activated or repressed in response to the three N treatments, only glycolysis and ATP synthesis responded to all three N treatments. ATP synthase was the only protein found to respond to all three N treatments, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose 1,6 biphosphate aldolase (ALD) each responded to two N treatments. Due to the prohibitive costs associated with LC MS/MS protein sequencing only a sub-set of differentially regulated proteins was sequenced for this study. However, it is necessary to sequence the un-sequenced proteins as it would provide a more comprehensive overview of N stress responses in *G. gracilis*. Only one protein involved in N metabolism, namely GS, was found to be regulated in response to N recovery. The lack of key N metabolic proteins was discussed extensively in Chapter 2 and possible reasons were provided for their absence. A detailed discussion proposing the possible role(s) of each putative *G. gracilis* protein, along with the associated pathways activated or repressed in response to N stress, was provided.

In Chapter 3, the identities of the *G. gracilis* ALD and GS proteins were validated using commercially available polyclonal antibodies and western blot analysis. Western blot analysis was also used to quantify changes in the protein levels of ALD during N limitation and recovery in *G. gracilis*. ALD protein and activity levels followed a similar pattern and were significantly decreased ($P<0.05$) following 14 days of growth under N-limiting conditions. These results successfully validated the expression pattern determined for ALD during N limitation using 2-DE analysis. The effect of N recovery on *G. gracilis* ALD and GS protein levels was also investigated. However, significant increases ($P<0.05$) in *G. gracilis* ALD and GS protein levels were not observed following 6 hours of N recovery. Thus, the significant increases observed in response to N recovery by 2-DE were not validated by western blot analysis. A large amount of biological variation was observed for this experiment and this may partly account for the lack of statistical significance. However, it appears that N recovery exerts very little effect on ALD and GS protein levels and these small changes in protein abundance may gain significance when a number of proteins are grouped into functional categories as was observed with transcriptional studies conducted by Scheible *et al.* (2004) using MapMan. Finally, the increase in intracellular *G. gracilis* starch levels together with the decreased protein abundance of glycolytic proteins ALD, PGK and GAPH suggest that a change in C allocation patterns has occurred in *G. gracilis* during N limitation. However, future work should monitor changes in floridoside and agar levels during N limitation. Furthermore, changes in the activities of key enzymes involved in the synthesis of these carbohydrate compounds need to be assessed to provide further insight into changes in C partitioning in *G. gracilis* during N limitation.

Although 2-DE coupled to MS is by far the most commonly used approach for differential expression proteomics (Jorrin-Novo *et al.*, 2009), and was used successfully to identify proteins regulated in response to N limitation and recovery in *G. gracilis*, the approach has a number of limitations. These include the lack of reproducibility, failure to resolve large proteins and the inability to separate most membrane proteins. Thus, the biggest disadvantage of 2-DE is that it allows the analysis and detection of only a sub-set of relatively abundant and soluble proteins. This issue was clearly demonstrated in this study as the majority of the identified proteins are abundant proteins which function in a range of general stress responses with only one protein directly involved in N metabolism being identified. One possible approach to increase the number of proteins that can be studied by 2-DE is pre-fractionation of the proteome prior to 2-DE separation (Beranova-Giorgianni, 2003; Ingle *et al.*, 2007). Pre-fractionation of complex protein mixtures reduces the complexity of the sample, allowing analysis of low abundant proteins which are usually masked by high abundant proteins such as house-keeping and structural proteins (Stasyk & Huber, 2004). Generally, low abundant proteins such as kinases, phosphatases, or GTPases can only be detected after applying additional fractionation technologies such as subcellular fractionation (Dreger, 2003; Huber *et al.*, 2003), protein and peptide affinity purification (Lee & Lee, 2004), chromatographic protein pre-fractionation (Lescuyer *et al.*, 2004), the use of narrow pH range gels for 2-DE (Görg *et al.*, 2002), or multi-dimensional peptide separations (Righetti *et al.*, 2003; Stasyk & Huber, 2004). The proteomics approach employed in this study provided the first characterisation of *G. gracilis* proteome responses to N stress. This concept can now be expanded upon using the optimised protocols in this study to investigate *G. gracilis* subcellular proteomics. This would add another dimension to the data generated in this study and may lead to the identification of novel proteins as pre-fractionation allows access to intracellular organelles and multi-protein complexes, and enriches for low-abundant proteins and signalling complexes. Thus, pre-fractionation of complex *G. gracilis* protein samples prior to 2-DE would provide deeper proteome coverage and possibly more sensitive proteome analysis.

Alternatively, a complementary gel-free strategy such as isobaric tags for relative and absolute quantitation (iTRAQ) could be employed to greatly increase the number of proteins that can be analysed, with the added benefit that hydrophobic proteins that are lost during isoelectric focusing (IEF) can be studied (Suzuki *et al.*, 2006). The iTRAQ system allows multiplexing of samples simultaneously, circumventing the problem of conducting multiple 2-DE experiments to analyse *G. gracilis* responses to N stress over a long time-period. It also allows one to perform biological replicates in a single analysis thus allowing the generation of statistically relevant data in a single experiment. However, while the use of gel-free strategies such as iTRAQ has a significant advantage over 2-DE, it rarely detects PTMs, isoforms or truncated forms of proteins. Moreover, LC-MS/MS may have uncharacterized biases of its own. An in depth study of rice proteins demonstrated that although LC-MS/MS identified 2,363 proteins from different tissues, it failed to identify 165 of the 556 proteins identified from the same samples run on 2-D gels (Koller *et al.*, 2002). The majority of the proteins identified by 2-DE were relatively abundant, yet they escaped detection by LC-MS/MS for unknown reasons. Therefore, any attempt to use proteomics to characterize whole-cell changes in protein accumulation regardless of the technology employed is likely to only detect a limited sub-set of the proteome. Thus, gel-based and gel-free LC-MS/MS strategies complement each other and together would provide the most complete coverage of the *G. gracilis* proteome.

A major aim of any proteomics study investigating N stress responses is to identify candidate protein biomarkers for use in aquaculture settings. These biomarkers could potentially be used to monitor the nutritional status of *G. gracilis* subjected to various abiotic (and biotic) stresses which negatively impact seaweed production. However, the identification of proteins involved in a particular stress response is only the first step in biomarker discovery. Once a candidate protein has been identified the next step would be to clone and sequence the gene. The third step would be to study the expression of these candidate genes at the RNA or protein level in an extended set of situations including different N levels and genotypes.

These candidate genes could also be manipulated using targeted genetic engineering approaches to improve the efficiency of N use and thus allow adaption to low N environments. Shrawat *et al.* (2008) demonstrated that manipulation of alanine aminotransferase can increase both the percentage N and the plant biomass by improving N uptake efficiency in *Oryza sativa*. Similar studies conducted with GS were mentioned in Chapter 2 (Habash *et al.*, 2001; Tabuchi *et al.*, 2005; Martin *et al.*, 2006; Hirel *et al.*, 2007; Lea & Azevedo, 2007). Finally, the metabolomics of N limitation and recovery in *G. gracilis* should also be explored. Tschoep *et al.* (2009) used metabolome studies to determine the metabolite phenotype of *Arabidopsis* plants exposed to low N conditions and concluded that *Arabidopsis* responds to low N stress by decreasing the rate of growth, while maintaining overall protein content and either maintaining or increasing the level of amino acids (Tschoep *et al.*, 2009). Thus, metabolite analysis would provide another level of understanding of the effects of N stress in *G. gracilis*.

In conclusion, this study has used proteomics to identify key proteins involved in the N stress response system in the commercially important agarophyte *G. gracilis*. The study provided insight into some of the proteins which are regulated in *G. gracilis* in response to N limitation and recovery. Following 2-DE analysis only two proteins were validated and characterized at the translational level using western blot analysis, highlighting the need for extending this analysis to the other proteins putatively involved in the *G. gracilis* N stress response. To the best of our knowledge, this is the first study using a differential expression proteomics approach to investigate global protein expression changes in response to N nutrition in a red macroalgal species. Further studies are required to obtain a complete understanding of the functions of the identified proteins and the molecular involvement of these proteins in the *G. gracilis* response to N limitation and subsequent recovery. While each protein can be studied individually, a systems biology approach combining proteomics, metabolomics and transcriptomics will lead to a better understanding of the molecular mechanisms implemented by macroalgae to tolerate N stress.

APPENDIX A

Media and solutions

CONTENTS

APPENDIX A

MEDIA AND SOLUTIONS

All media were autoclaved at 121 °C for 20 min prior to use, unless otherwise specified.

Water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system. Ultrapure water used was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system.

A.1 MEDIA

A.1.1 Artificial Sea Water (ASW)

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

Autoclave

A.1.2 Fe-solution

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

A.1.3 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

A.1.4 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 1 L ASW.

A.1.5 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.

A.2 GENERAL SOLUTIONS

A.2.1 General stock solutions

- **1 N NaOH**

NaOH (Saarchem)	4 g
water to	100 ml

Store in a plastic bottle.

- **1 M HCl**

37% HCl (Saarchem)	8.4 ml
water to	100 ml

Store in a foil covered glass bottle.

- **0.5 M EDTA**

Na_2EDTA (Saarchem)	186.1 g
water to	800 ml

The pH was adjusted to 8.0 with NaOH pellets prior to making the volume to 1 l with water and autoclaving.

- **1 M Tris-HCl**

Tris base	121.5 g
water	800 ml

The pH was adjusted to 8.0 with HCl prior to making the volume to 1 l with water and autoclaving.

- **25% (w/v) Sodium dodecyl sulphate (SDS)**

SDS	50 g
sterile water	200 ml

Stir on warm plate to dissolve. Do not autoclave. The concentration of the SDS solution can be altered by altering the amount of SDS added.

- **70% (v/v) Ethanol (EtOH)**

Absolute ethanol	70 ml
water to	100 ml

Do not autoclave.

- **50% (v/v) Glycerol**

glycerol	25 ml
water to	50 ml

- **2 M Sodium phosphate, mono-sodium (NaH_2PO_4)**

$NaH_2PO_4.H_2O$	55.2 g
water to	200 ml

- **2 M Sodium phosphate, di-sodium (Na_2HPO_4)**

$Na_2HPO_4.2H_2O$	71.2 g
water to	200 ml

- **1 M Phosphate buffer (pH 6.8)**

$NaH_2PO_4.H_2O$ (2 M)	39 ml
$Na_2HPO_4.2H_2O$ (2 M)	61 ml
water to	200 ml

- **0.1 M Phosphate buffer**

Phosphate buffer (1 M, pH 6.8)	10 ml
Water to	100 ml

- **DTT (30%)**

0.3 g DTT added to 1 ml distilled water
50 µl aliquots were made and stored at -20°C

- **ASB 14 (10%)**

0.1 g ASB 14 added to 1 ml distilled water
50 µl aliquots were made and stored at -20°C

A.2.2 Solutions for protein extractions

- **Protein extraction buffer**

Tris (0.5 M) pH 7.5	5 ml
EDTA (0.5 m)	200 µl
Triton	1%
β-mercaptoethanol	2%
water to	10 ml

- **0.1 M ammonium acetate in methanol (w/v)**

3.85 g ammonium acetate added to 500 ml of methanol

- **80% acetone (v/v)**

80ml of acetone added to 20ml of water

- **Urea Lysis Buffer (ULB)**

Urea (8 M)	24.0 g

Dissolve in 25 ml water by stirring and then add:

CHAPS (2 %) (Calbiochem)	0.25 g
Thiourea (Sigma-Aldrich)	7.6 g

Make up to 50 ml with distilled water and filter sterilize.

A.2.3 Solutions for the modified Bradford assay for determining protein concentration

- **Bovine serum albumin (BSA) (1 mg/ml^{-1})**

BSA (Roche)	0.01 g
ULB to	10 ml

- **Bradfords reagent**

Dye Reagent Concentrate (Bio-Rad)	1 ml
sterile water to	5 ml

Make up fresh before every use

- **0.1 M HCl**

HCl	10 ml
Water to	100 ml

A.2.4 Solutions for SDS-PAGE

- **Stacking gel**

4X Tris-Cl / SDS solution (pH 6.8)

Tris base	6.05 g
SDS	0.4 g

Adjust pH to 6.8 with HCl
Add water to100 ml
Filter-sterilize and store at room temperature

Separating gel

4X Tris-Cl / SDS solution (pH 8.8)

Tris base (1.25 M)	91.0 g
SDS	2.0 g

Adjust pH to 8.8 with 1 M HCl
Add water to 500 ml
Filter-sterilize and store at room temperature

- **10 x SDS-PAGE running buffer**

Glycine	150 g
Tris	30 g
SDS	20 g
water to	1000 ml

- **1x SDS PAGE running buffer**

Dilute 10 x SDS-PAGE running buffer 1:10 with water before use

- **10% (w/v) Ammonium persulfate (AMPS)**

AMPS	1 g
Water to	10 ml

Do not autoclave. Filter sterilize before aliquoting into sterile microfuge tubes and store at -20°C.

- **5x SDS-PAGE Sample Application Buffer (SAB)**

Tris-Cl (pH 6.8)	250 mM
DTT (Promega)	500 mM
SDS (Sigma)	10%
Bromophenol blue (Saarchem)	0.5%
Glycerol	10 ml
water to	5 ml

A.2.5 Solutions for Coomassie staining of polyacrylamide gels

- **Coomassie SDS-PAGE gel stain**

Coomassie brilliant blue R250 (Sigma)	1 g
Methanol (50%)	225 ml
Glacial acetic acid (10%)	50 ml
water to	

Dissolve the Coomassie Brilliant Blue R in methanol before adding acetic acid and water 500 ml

- **Coomassie SDS-PAGE destain**

Methanol	50 ml
Glacial acetic acid	70 ml
water to	1000 ml

- **SDS-PAGE Gel storage solution**

Glacial acetic acid	7 ml
Water to	100 ml

A.2.6 Solutions for 2-DE

- **Rehydration solution**

ULB	X ml
DTT	2%
ASB 14	0.4%
Biolyte 3-10 ampholytes (20%)	1%
Trace amount of bromophenol blue	
Protein sample (250 μg)	Y μl

Made in a total volume of 140 μl.

- **Equilibration buffer I (EQB I)**

Urea (6M)	72.1 g
Tris-HCl (pH 8.8, 0.375 M)	50 ml
Glycerol	20%
SDS	2%
Bromophenol blue	0.0005%

Made up to 200 ml with distilled water, aliquoted into 10 ml volumes and stored at -20°C.

0.1 g DTT was added to 10 ml of EQB I prior to the equilibration step II.

- **Equilibration buffer II (EQB II)**

Made up as for EQB I except that 0.48g of iodoacetamide was added to EQB II prior to equilibration step I.

- **0.5% Agarose**

0.5 g agarose in 1 X SDS running buffer with a trace amount of bromophenol blue.

A.2.7 Western Blot solutions

- **Ponceau S**

Ponceau S powder	0.1%
Ethanol	5%
Made in distilled water	

This stain is re-usuable but due to its sensitivity to light needs to be covered in foil and stored and used in the dark.

- **10X TBS Stock**

50 mM Tris	30.25g
150 Mm NaCl	43.8g

Distilled water to 300 ml
Adjust pH to 7.4 with HCl
Distilled water to 500ml
Autoclave

- **Blocking buffer (5 % skim milk)**

Skim-milk (Elite)	5 g
10 X TBS (1 X)	10 ml
Water to	100 ml

- **TBST (0.1 % Tween in 1X TBS)**

Tween 20 (Saarchem)	0.1 ml
10X TBS (1 X)	100 ml
Water to	1000 ml

Make up fresh
Do not autoclave

- **Towbin Buffer**

Tris base	3.03 g
Glycine (Merck)	14.42 g
Methanol (20%) (Merck)	200 ml
Water to	1000 ml

APPENDIX B

Peptide Identifications

APPENDIX B: Peptide identifications

Table 1: Peptide identifications obtained using ESI LC MS/MS for each protein identified in this study

Spot no.	**Protein name**	**Protein score**	**E-value**	**Peptides**
Proteins spots increasing in intensity in response to an initial period of N limitation				
403	Actin (*Chondrus Crispus*)	371	6.7E-31	K.EYELPDGQVITVEAER.F K.EIETLAPPSMK.I + Oxidation (M) K.EIETLAPPSMK.I R.VDPQEHPVLLTEAPLNPK.A K.GIMVGTGQK.D K.GIMVGTGQKDEYVGDAAMAR.R + 2 Oxidation (M) K.DEYVGDAAMAR.R + Oxidation (M) K.GIMVGTGQKDEYVGDAAMAR.R + Oxidation (M) K.GIMVGTGQK.D + Oxidation (M) K.GIMVGTGQKDEYVGDAAMAR.R K.DEYVGDAAMAR.R + Oxidation (M) R.DLTGWMAK.L + Oxidation (M) R.DLTGWMAK.L
422	Actin 1 (*Hildenbrandia rubra*)	271	8E-21	K.DEYVGDAAMAR.R K.GIMVGTGQKDEYVGDAAMAR.R + 2 Oxidation (M) K.GIMVGTGQK.D + Oxidation (M) R.VDPQEHPVLLTEAPLNPK.A K.GIMVGTGQK.D K.GIMVGTGQKDEYVGDAAMAR.R -.VVIDNGSGR.C K.DEYVGDAAMAR.R + Oxidation (M)

433	α-enolase (*Homo sapiens*)	118	0.000016	K.VVIGMDVAASEFFR.S
347	GAPDH (*Gracilaria gracilis*)	70	0.85	R.LATETSYDDIK.A R.GAGANIIPSSTGAAK.A
454	Galactose-1-phosphate uridyltransferase (*Gracilaria gracilis*)	57	20	R.YDNLFTTLFPYSMGIHQSPTTNGTDPAK.H + Oxidation (M) K.RPWQGSVEDLPPDERPEYDPK.D
547	ATP synthase α sub-unit (*Lactuca sativa)*	424	3.9E-36	K.IVNTGTVLQVGDGIAR.I K.TAVATDTILNQQGK.N K.ASSVAQVVTNFQER.G K.TFTEEAEAILK.E R.IAQIPVSEAYLGR.V R.FILQEQAA.- R.SVYEPLQTGLIAIDSMIPIGR.G + Oxidation (M)
499	Chaperonin GroEL (*Gracilaria tenuistipitata*)	348	1.6E-28	K.GMDTLVEAVAITLGPK.G + Oxidation (M) K.TGRPLLIIAEDIEKEALATIIVNK.L K.VGAATETEMR.D + Oxidation (M) K.ITLIQQELLPILEKVTK.T
298	Phycoerythrin α sub-unit (*Porphyra yeozensis*)	109	0.00013	R.FPSSSDLESVQGNIQR.A
Proteins spots increasing in intensity in response to a sustained period of N limitation				
57	Phycocyanin α sub-unit (*Gracilaria tenuistipitata*)	288	1.7E-22	K.TPITEAIASADSQGR.F R.FLSNGELQSINGR.Y R.LITGAAQSVYTK.F

237	Elongation factor Tu (*Gracilaria lemaneiformis*)	174	4.5E-11	K.TLDEGMAGDNIGILLR.G K.TFLMAVEDVFSITGR.G + Oxidation (M) K.QVGVPNIVVFLNK.Q

Proteins spots decreasing in intensity in response to a sustained period of N limitation

323	ATP synthase α sub-unit (*Gracilaria tenuistipitata*)	959	1.2E-89	K.LELAQFAELEAFSQFASDLDK.A K.TAVALDTIINQK.G K.ATLVVYDDLTK.Q R.EAYPGDVFYLHSR.L -.MLNIRPDEISNVIR.Q R.NSKPEFGESIR.T M.LNIRPDEISNVIR.Q K.QSIEDVK.Q -.MLNIRPDEISNVIR.Q + Oxidation (M) K.ATQNQLSR.G R.NILEGSSVK.A R.QMSLLLR.R R.VVDPLARPIDAK.G R.ELIIGDR.Q R.QMSLLLR.R + Oxidation (M) R.VGSAAQIK.A R.QQIDKYEQEIQVANIGTVLQVGDGIAR.V R.QSVCEPLQTGITAIDSMIPIGR.G + Oxidation (M)
225	Glyceraldehyde-3-phosphate dehydrogenase (*Homo sapiens*)	130	0.0000011	K.LISWYDNEFGYSNR.V K.LVINGNPITIFQER.D

274	Phosphoglycerate kinase (*Chondrus Crispus*)	262	5.9E-20	K.MSHISTGGGASLELLEGK.V + Oxidation (M) K.LIIGGGMVFTFLK.A + Oxidation (M) K.LIIGGGMVFTFLK.A K.VLPGVAALDDN.- K.MSHISTGGGASLELLEGK.V
207	Ferredoxin NADP Reductase (*Cyanophora paradoxa*)	161	8.2E-10	R.LYSIASTR.H R.LDYAISR.E K.MYIQNR.I K.MYIQNR.I + Oxidation (M) K.TVSLSVK.R
238	Fructose 1,6 biphosphate aldolase (*Galdieria sulphuraria*)	129	0.0000013	K.GILAVDESTK.T R.GPWSLSFSYGR.A
316	ATP synthase α sub-unit (*Gracilaria tenuistipitata*)	1009	1.3E-94	K.LELAQFAELEAFSQFASDLDK.A K.TAVALDTIINQK.G K.ATLVVYDDLTK.Q M.LNIRPDEISNVIR.Q K.LKLELAQFAELEAFSQFASDLDK.A R.EAYPGDVFYLHSR.L -.MLNIRPDEISNVIR.Q -.MLNIRPDEISNVIR.Q + Oxidation (M) R.NSKPEFGESIR.T K.QSIEDVK.Q R.QMSLLLR.R R.NILEGSSVK.A K.ATQNQLSR.G R.VVDPLARPIDAK.G R.EAYPGDVFYLHSR.L R.ELIIGDR.Q

				R.QMSLLLR.R + Oxidation (M) M.LNIRPDEISNVIR.Q R.VGSAAQIK.A R.VVDPLARPIDAK.G

Proteins spots increasing in intensity in response to N recovery

339	Glutamine synthase 11 (*Gelidium crinale*)	95		L.TGKHETASIEQF.K G.YGYFED.R
242	CbbX protein (*Gracilaria tenuiistipitata*)	128	0.0000013	R.DDLVGQYIGHTAPK.T K.LMLEEQQYR.F K.LMLEEQQYR.F + Oxidation (M)
316	tRNA-dihydrouridine synthase B (*Dickeya dadantii)*	48	0.12	R.NCVEIAR.L
323	Fructose 1,6, biphosphate aldolase (*Galdieria sulphuraria)*	140	9.6E-8	K.GILAVDESTK.T R.GPWSLSFSYGR.A
464	Thioredoxin (*Campylobacter coli)*	157	1.7E-9	K.VNTDEQGDLAAEFGVR.S K.YIELTSDNFAQAK.E
409	ATP synthase β sub-unit (*Gracilaria tenuistipitata)*	1777	0	R.ITSTTDGSITSIQAVYVPADDLTDPAPATTFAHLDATTVLSR.S K.ELQDIIAILGLDELSEDDRLTVAR.A K.ELQDIIAILGLDELSEDDR.L K.TVLIMELINNIAK.A + Oxidation (M) R.FLSQPFFVAEVFTGSPGK.Y R.FVQAGSEVSALLGR.M R.MPSAVGYQPTLATEMGTLQER.I + Oxidation (M)

	K.VALVYGQMNEPPGAR.M + Oxidation (M)
	R.MPSAVGYQPTLATEMGTLQER.I + 2 Oxidation (M)
	K.QDVLLFIDNIFR.F
	R.MPSAVGYQPTLATEMGTLQER.I
	K.VALVYGQMNEPPGAR.M
	K.TVLIMELINNIAK.A
	R.VGLTALTMAEYFR.D
	K.AHGGVSVFGGVGER.T
	R.VGLTALTMAEYFR.D + Oxidation (M)
	R.GIEVTDTGAPITVPVGIPTLGR.I
	R.DINKQDVLLFIDNIFR.F
	R.AVAMSSTEGLK.R + Oxidation (M)
	K.IGLFGGAGVGK.T
	R.AVAMSSTEGLK.R
	R.EGNDLYEEMK.E
	R.EGNDLYEEMK.E + Oxidation (M)
	R.FLSQPFFVAEVFTGSPGK.Y
	K.VVDLLAPYR.K
	K.TVLIMELINNIAK.A + Oxidation (M)
	R.EGNDLYEEMK.E + Oxidation (M)
	R.AVAMSSTEGLKR.G + Oxidation (M)
	R.MPSAVGYQPTLATEMGTLQER.I
	R.EGNDLYEEMKESK.V + Oxidation (M)
	R.YKELQDIIAILGLDELSEDDR.L

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